Natural Products and Drug Discovery

From Pharmacochemistry to Pharmacological Approaches

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Presentation

This book addresses chemical, biological and pharmacological aspects related to natural products. Specialists in different fields were invited to contribute relevant information of chemical compounds, isolation and identification, biological activities (anti-inflammatory, antimicrobina, antidepressive, etc.), synthesis, derivatization, QSAR analysis and chromatographic. In addition, it brings the histological aspect and its importance to the scientific world community of the Postgraduate in Natural and Synthetic Bioactive Products, of the Federal University of Paraíba (PgPNSB - UFPB).

The book begins by describing the review of silico techniques that aid in the natural products drug discovery, bringing advantages and disadvantages of using this technique. The authors also provide an overview of the most common methods described in the literature for the extraction, purification, identification, and elucidation of natural products, always highlighting the most recently developed methods.

In addition, field experts provide an in-depth discussion of antimicrobial, anti-inflammatory, antidepressant, anxiolytic, and cardiovascular assays in various animal models. Finally, the book brings a retrospective view of the PgPNSB-UFPB, showing its importance to the scientific community, with a large group of researchers recognized worldwide.

The book is intended for the scientific community that works with natural products, both those that investigate specific groups of natural compounds, and those who wish to further explore their potential as new medicinal products for medical purposes.

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Chapter 1

In-silico Approaches to Natural Products Drug Discovery: A Review of the Recent Literature

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1. Introduction

Natural products have been and continue to be excellent sources of medicinal agents, not only the structures themselves, but as templates for synthetic modification, optimization of selectivity and bioavailability. *In-silico* screening methodologies such as quantitative structure activity relationships (QSAR), molecular docking, and pharmacophore modeling, have proven to be beneficial complements to high-throughput screening, tissue culture, and animal studies.

Several reviews have recently appeared summarizing *in silico* methods for the drug discovery process [1,2], including natural products drug discovery [3,4]. In this chapter, we review the recent literature that has focused on *in-silico* approaches to natural product drug discovery. In particular, we have concentrated on computational methodologies using natural products from plant sources. We present a survey of current medicinal problems, what computational resources are available, both commercial and freeware, and include advantages and disadvantages of the *in-silico* methods.

2. Representative Diseases and their Targets

Target selection is the first step in *in-silico* drug discovery. For a given disease state, there are often numerous potential targets to be considered, and with genomics, many potential protein targets are becoming accessible. These include human protein targets as well as targets of numerous pathogenic organisms. There are web-based sources for biomolecular targets. The Potential Drug Target Database (PDTD) is a web-accessible protein database for *in silico* target identification [5] (http://www.dddc.ac.cn/pdtd/). PharmMapper server is a web server that uses a pharmacophore mapping approach to identify potential drug targets [6] (http://59.78.96.61/pharmmapper/). The ChEMBL database contains compound bioactivity data against drug targets and currently includes more

than 1.6 million compounds and more than 11,000 drug targets (https://www.ebi.ac.uk/chembl/). BindingDB is a web-accessible database of binding affinities of drug-like compounds with protein drug targets, and currently contains more than 6,000 protein targets (https://www.bindingdb.org/bind/index.jsp).

2.1 Alzheimer's Disease

There are several potential drug targets for treatment of Alzheimer's Disease [7–9]: Acetylcholinesterase (AChE) and butyrlcholinesterase (BuChE), γ-aminobutyric acid receptors ($GABA_AR$ and $GABA_BR$), amyloid precursor protein (APP), catechol *O*-methyl transferase (COMT), cyclin-dependent kinase 5 (CDK5), glycogen synthase kinase 3 beta (GSK3β), histamine receptor 3 (H_3R), 5-hydroxytryptamine receptors ($5\text{-}HT_{1A}R$, $5\text{-}HT_4R$, $5\text{-}HT_6R$), ionotropic glutamate receptor AMPA (GRIA1 and GRIA2), *N*-methyl-d-aspartate receptor (NMDAR), monoamine oxidase B (MAOB), phosphodiesterases (PDE4A and PDE4B) and β-secretase 1 (BACE1). Of these, the more popular targets for investigation have include the cholinesterases (AChE and BChE) and β-secretase.

2.2 Antibacterial Targets

A number of potential protein targets for antibacterial chemotherapy investigations have been identified [10–17]. Protein targets subjected to *in-silico* screening have included α-l-arabinofuranosidase, cytochrome P450 CYP121, cytochrome P450 CYP51 (sterol 14α-demethylase), DNA gyrase / topoisomerase IV, α-fucosidase, glucosamine-6-phosphate synthase (GLMS), glucosyltransferase, β-hydroxyacyl-acyl carrier protein dehydratase (FabZ), β-ketoacyl-acyl carrier protein reductase (MabA, also named FabG1), β-ketoacylacyl carrier protein synthase I (KAS I), β-lactamase, long-chain enoyl-acyl carrier protein reductase (InhA), NAD+-dependent DNA ligase, nitric oxide reductase, pantothenate kinase (PanK), peptide deformylase, phospho-Mur-NAc-pentapeptide translocase (MraY), protein tyrosine phosphatase, and UDP-galactopyranose mutase.

2.3 Antifungal Targets

Genomics has led to identification of a number of potential targets for development of antifungals, including H^+-ATPase, chitin synthase, 1,3-β-glucan synthase, β-hydroxymethylglutarate reductase, inositol phosphoceramide (IPC) synthase, lanosterol 14α demethylase, *N*-myristoyl transferase, 2,3-oxidosqualene-lanosterol cyclase, protein-farnesyltransferase (β-subunit homolog), squalene epoxidase, topoisomerase I, and translation elongation factors (EF2 and EF3) [18–22].

2.4 Cancer

There are numerous drug targets for cancer chemotherapy [23–27]. Some of the popular targets include the androgen receptor (AR), aromatase, casein kinase 2 (CK2), cycli-dependent kinase 2 (CDK2), cyclooxygenase 2 (COX-2), DNA (both groove binding and intercalation), DNA methyltransferase (DNMT) epidermal growth factor receptor (EGFR), estrogen receptor (ERα and ERβ), heat shock protein 90 (Hsp90), insulin-like growth factor 1 (IGF1) receptor kinase, insulin-like growth factor 1 receptor (IGF1R), lipoxygenase (5-LOX), mitogen-activated protein kinase kinase 1 (MEK1), Myc-Max complex, nuclear factor kappa-light chain-enhancer of activated B cells (NF-κB), p90 ribosomal S6 kinase 2 (RSK2), P-glycoprotein (ATP-dependent efflux pump), phosphoinositide 3-kinase (PI3K), sodium/potassium ATPase, topoisomerases I and II, tubulin, vascular endothelial growth factor receptor (VEGFR), and xanthine oxidase (XO).

2.5 Diabetes

Drug targets for diabetes include aldose reductase (ALR), α-amylase, fructose-1,6-bisphosphatase, glucokinase, α-glucosidase, 11β-hydroxysteroid dehydrogenase, peroxisome proliferator-activated receptor-γ (PPAR-γ), and protein tyrosine phosphatase 1B (PTP1B) [28,29].

2.6 Inflammation

Potential targets for the treatment of inflammation have included cyclooxygenases (COX-1 and COX-2), c-Jun terminal-NH2 kinase (JNK), inducible nitric oxide synthase (i-NOS), IκB kinase β (IKKβ), lipoxygenase (LOX), microsomal prostaglandin E2 synthase (mPGES)-1, myeloid differentiation protein 2 (MD-2), myeloperoxidase (MPO), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), p38 mitogen-activated protein kinase (p38 MAPK), peroxisome proliferator-activated receptor γ (PPAR-γ), phosphoinositide 3-kinase (PI3K), phospholipase A2 (PLA2), prostacyclin synthase, and type 4 cAMP-specific phosphodiesterase (PDE4) [30–34].

2.7 Antiviral Targets

Several virus proteins have emerged as important chemotherapeutic targets. An important Alphavirus (*e.g.*, Barmah Forest virus, Chikungunya virus, O'nyong'nyong virus, and Venezuelan equine encephalitis virus) target is the NSP2 cysteine protease [35]. Flavivirus (*e.g.*, dengue virus, West Nile virus, yellow fever virus, and Zika virus) targets that have been identified include the envelope protein, the NS2B-NS3 serine protease, the NS3 RNA helicase, the NS5 methyl transferase, and the NS5 RNA-dependent RNA polymerase [36–38].

Human immunodeficiency virus (HIV) targets are reverse transcriptase [39], HIV integrase [40], and protease [41]. Hepatitis B virus targets include the HBV core protein and DNA polymerase [42], while targets for hepatitis C chemotherapy have included the NS3 polymerase, the NS3 RNA helicase, and the NS5B RNA-dependent RNA polymerase [43]. The primary targets for influenza A viruses have been neuraminidases [44] while the primary herpesvirus targets have been proteases [45].

2.8 Parasitic Protozoa

There are numerous protein targets with three-dimensional structures available from the Protein Data Bank (PDB) from *Leishmania donovani*, *L. infantum*, *L. major*, *L. mexicana*, *Plasmodium falciparum*, *Trypanosoma brucei*, and *T. cruzi* [46,47]. These include cysteine proteases, deoxyuridine triphosphate nucleotidohydrolase, dihydroorotate dehydrogenase, farnesyl diphosphate synthase, glyceraldehyde 3-phosphate dehydrogenase, nucleoside diphosphate kinase B, pteridine reductase, pyruvate kinase, sterol 14α-demethylase, triosephosphate isomerase, and trypanothione reductase.

Additional parasitic protozoa and their targets include *Entamoeba histolytica* *O*-acetyl-L-serine sulfhydrylase, serine acetyltransferase, phosphofructo-1-kinase, triosephosphate isomerase, and alcohol dehydrogenase 2; *Giardia lamblia* carbamate kinase, pyruvate ferredoxin oxidoreductase, and protein disulfide isomerases; *Cryptosporidium parvum* phosphofructokinase and inosine-5′-monophosphate dehydrogenase [48]; *Trichomonas vaginalis* cysteine proteases, triosephosphate isomerase, lactate dehydrogenase, methionine gamma-lyase, thioredoxin reductase, and purine nucleoside phosphorylase [49].

3. Molecular Docking

Molecular docking is an *in-silico* method for placing small molecule ligands into active sites or binding sites of macromolecular biochemical targets. Starting from a known target, which can be obtained from structural biology (X-ray diffraction, neutron diffraction, NMR) or homology modeling, *in-silico* investigations are carried out to identify suitable ligands (Fig. 1). In the context of this review, biomolecular targets include enzymes, receptors, and polynucleotides; ligands are plant-derived natural products. There are several natural product databases available, including the ZINC natural products database [50], the *Dictionary of Natural Products* [51], Duke's Phytochemical and Ethnobotanical Databases [52], and NAPRALERT [53].

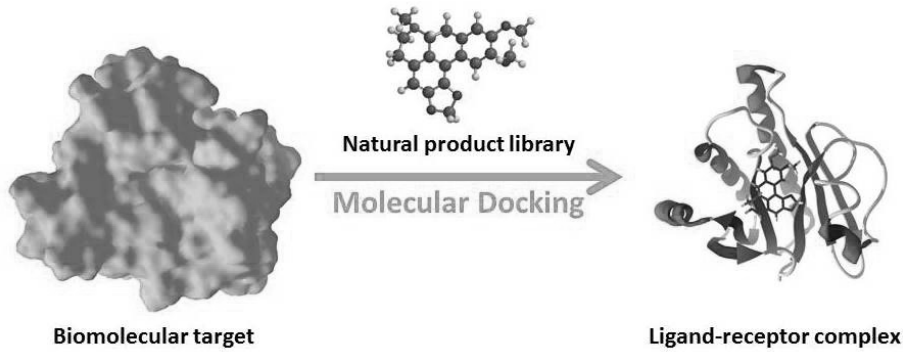

Figure 1. Scheme of molecular docking. The three-dimension structure of the biomolecular target is obtained either from the protein data bank (PDB) or by homology modeling. A library of small-molecule ligands (natural products) is virtually screened and evaluated. The ligand-receptor complex obtained can be used as a basis for experimental evaluation or as a scaffold for synthetic optimization.

There are several recent reviews on molecular docking [54–57], so the principles of docking methodology will not be covered here. While molecular docking is computationally fast and large libraries of small molecules can be virtually screened rapidly, there are several important limitations: (1) desolvation of the protein and the ligand are poorly handled, if at all; (2) entropy effects, therefore, are poorly modeled; (3) scoring functions are inherently inaccurate and often do not correlate with experimental binding affinities; (4) protonation states of the target binding site can be inaccurately predicted; (5) ionization and/or tautomerization of the ligand can change during binding; (6) although ligand flexibility is incorporated in most docking programs, flexibility of the macromolecule is often ignored or poorly modeled; (7) docking of large, highly flexible ligands often gives poor results. Nevertheless, molecular docking has led to numerous promising natural product lead structures which can serve as starting points for optimization and drug development.

Table 1 lists popular molecular docking software recently used for virtual screening of natural product libraries. The list is not meant as an endorsement, but does reflect the current availability of molecular docking software. There are several other molecular docking packages available.

Table 1. Popular molecular docking programs used for virtual screening of natural product libraries.

Docking Program	Source
AutoDock	Scripps Research Institute, http://autodock.scripps.edu/
Molegro Virtual Docker	Molegr ApS (no longer available)
GLIDE	Schrödinger, https://www.schrodinger.com/Glide/
AutoDock Vina	Scripps Research Institute, http://vina.scripps.edu/
Molecular Operating Environment (MOE)	Chemical Computing Group, http://www.chemcomp.com/MOE-Molecular_Operating_Environment.htm
CDOCKER (Discovery Studio)	Dassault Systèmes BIOVIA, http://accelrys.com/products/collaborative-science/biovia-discovery-studio/
ArgusLab	http://www.arguslab.com/arguslab.com/ArgusLab.html
iGemDock	National Chiao Tung University, http://gemdock.life.nctu.edu.tw/dock/download.php
Surflex-Dock	Certara USA, Inc., https://www.certara.com/
GOLD	Cambridge Crystallographic Data Centre (CCDC), http://www.ccdc.cam.ac.uk/solutions/csd-discovery/components/gold/
FlexX	BioSolveIT, http://www.biosolveit.de/FlexX/

Natural product libraries have been used to investigate a number of disease targets, including cancer, diabetes, neurodegenerative diseases (Alzheimer's disease, Parkinson's disease), bacterial infections, viral infections, and parasitic infections. A summary of some recent docking studies with phytochemical ligands is presented in Tables 2-9. Molecular docking has served to identify potential phytochemical ligands that may serve as inhibitors or structural scaffolds for drug design, to identify potential biomolecular targets of biologically active phytochemicals, or to provide insight into the binding mode of known inhibitors with the target protein. Molecular dynamics (MD) calculations are sometimes carried out subsequent to initial docking to provide improved docking scores [58].

Table 2. Recent molecular docking investigations of natural products with Alzheimer's disease targets.

Biomolecular Target	Comments	Ref.
Acetylcholinesterase / Butyrylcholinesteraase	Molecular docking (MOE) of the diterpenoid alkaloids ajaconine and delectinine with human AChE and BChE indicated ajaconine to dock into the catalytic active site of the enzymes while delectinine docked into the peripheral anionic sites.	[59]
	Vasicine from *Adhatoda vasica* showed *in vitro* AChE inhibitory activity. Molecular docking (MOE) showed binding similarity to both galantamine and tacrine in the catalytic site of *Tetronarce californica* AChE.	[60]
	A molecular docking study (AutoDock 4.2) of 12 ginger phytochemicals with 13 protein targets revealed AChE to be the most promising target. Zerumbone was found to be the most strongly docking ligand with AChE.	[61]
	Molecular docking (AutoDock 4.2) of three compounds (loganin, morroniside, and 7-O-galloyl-D-sedoheptulose) from *Cornus officinalis* with AChE, BChE, and BACE1, revealed loganin to be the strongest docking ligand for AChE and better than the known inhibitor tacrine.	[62]
	Steroidal alkaloids (mokluangins A-C, antidysentericine) from the bark of *Holarrhena pubescens* demonstrated anticholinesterase activity. Molecular docking (AutoDock 4.2) showed that all four compounds docked into the hydrophobic cavity of *Electrophorus electricus* AChE.	[63]
	Rosmarinic acid from *Salvia trichoclada* showed *in vitro* and *ex vivo* AChE inhibitory activity. Molecular docking (AutoDock Vina) showed high affinity to human AChE.	[64]
	Seven indole alkaloids from *Rauvolfia reflexa* were screened for anticholinesterase activity. Rescinnamine was found to be the most active against both AChE and BChE. Molecular docking showed different binding modes with the compound interacting with the peripheral anionic site in AChE, but binding at the active site of BChE.	[65]

Biomolecular Target	Comments	Ref.
	Molecular docking studies (BioMedCAChe) of saffron phytochemicals revealed that safranal interacted only with the binding site of the AChE, but crocetin and dimethylcrocetin docked simultaneously to the catalytic and peripheral anionic sites.	[66]
	Molecular docking (AutoDock 4) of γ-solanine with human AChE showed the compound to dock more strongly to the protein than the known inhibitor donepezil.	[67]
	In vitro AChE screening of phytochemicals from *Garcinia hombroniana*, along with molecular docking indicated garcihombronane N to dock well to AChE.	[68]
	Molecular docking (AutoDock 3.0.5) of conypododiol with torpedo AChE indicated the ligand to interact with both the active (catalytic triad) site as well as the peripheral anionic site of the protein.	[69]
	Xanthones with C8-prenyl and C7-hydroxyl substituents were found to be good AChE and BChE inhibitors. Molecular docking (AutoDock 3.0.5) studies revealed that α-mangostin, γ-mangostin and garcinone C interact differently with the five important regions of AChE and BChE. The nature of protein–ligand interactions was described as mainly hydrophobic and hydrogen bonding.	[70]
	Nine isoquinoline alkaloids from *Cryptocarya* species were screened for anticholinesterase activity. 2-Methoxyatherosperminine showed the most potent activity against BChE. Molecular docking (AutoDock 3.0.5) showed the compound to interact with the enzyme at both the active (catalytic triad) site as well as the choline binding site.	[71]
	Seven phenolic abietane diterpenoids from *Salvia officinalis* were screened for in vitro AChE activity. Two of the compounds (7α-methoxyrosmanol and isorosmanol) showed anticholinesterase activity. Molecular docking (Molegro) energies were consistent with the observed AChE inhibitory activities.	[72]

Biomolecular Target	Comments	Ref.
	The furanocoumarins imperatorin, xanthotoxin, and bergapten, from *Angelica officinalis*, showed strong anti-BChE activity. Molecular docking (Internal Coordinate Mechanics Dock) confirmed potent interactions between the furanocoumarins and human BChE.	[73]
	Quercetin-7-*O*-β-D-glucoside showed in vitro BChE inhibitory activity and patuletin-7-*O*-β-D-glucoside showed AChE inhibitory activity. Molecular docking (Surflex-Dock) showed both compounds occupying the active sites of their respective enzyme targets.	[74]
	Seven compounds from *Myristica cinnamomea* were screened for anticholinesterase activity. Malabaricones B and C were found to be active against both AChE and BChE. Molecular docking showed both compounds docked to AChE and BChE more strongly than the reference compound physostigmine.	[75]
Monoamine oxidase	Four flavonoids from *Sideritis* were docked into the active site of human monoamine oxidase isoforms (hMAO-A and hMAO-B). Salvigenin and xanthomicrol were found to be promising hMAO-A inhibitors.	[76]
β-site amyloid precursor protein cleaving enzyme 1 (β-secretase 1, BACE1)	The coumarins umbelliferone-6-carboxylic acid, esculetin, and daphnetic, from *Angelica decursiva*, showed BACE inhibitory activity. Molecular docking (AutoDock 4.2) showed umbelliferone-6-carboxylic acid and esculetin to dock into the binding site of the co-crystallized ligand, while daphnetin docked preferentially in a remote site of the enzyme.	[77]
	A molecular docking study (iGEMDOCK) of 15 phytochemical ligands with BACE1 revealed three compounds, azadirachtin, cardiofolioside, and kutkin, to be strongly docking compounds compared to the standard drug triethylphosphonobutyrate.	[78]
	A molecular docking study (FlexX) of a library of 200 phytochemicals with BACE1 revealed hesperidin to the the most promising compound. The compound docks close to the catalytic site, blocking the cavity opening. Hesperidin has shown in-vitro BACE1 inhibitory activity.	[79]

Biomolecular Target	Comments	Ref.
	The ginsenosides (Rb1, Rb2, Rc, Re, Rg1, and Rg3) from *Panax ginseng* were screened for BACE1 inhibitory activity. All compounds were modestly active compared to the positive control. Molecular docking (AutoDock 4.2.6) showed ginsenosides Rb1 and Rb2 to show particular good binding affinities.	[80]
	In vitro screening of constituents from *Cassia obtusifolia* for anti-BACE1 activity, showed emodin, alaternin, and cassiaside to be better inhibitors than the positive control quercetin. Molecular docking (AutoDock 4.2) showed these compounds to dock into the active site of BACE1.	[81]
	The phlorotannins eckol, phlorofurofucoeckol-A, and dieckol, from *Eisenia bicyclis*, showed BACE1 inhibitory activity. Molecular docking (AutoDock 4.0, FRED 2.0) indicated these compounds to bind to the active site of BACE1.	[82]
	A molecular docking study (AutoDock 4) of seven phytochemical ligands with BACE1 revealed four compounds (valeranone, γ-gurjunene, jatamansin, and β-sitosterol) to have docking energies comparable to the known inhibitors galantamine and nicardipine.	[83]
	A molecular docking study (AutoDock Vina) of 12 ginsenosides from *Panax ginseng* with BACE1 revealed four ginsenosides (CK, F1, Rh1, and Rh2) to be potential inhibitors.	[84]
	γ-Linolenic acid was found to be an inhbitor of BACE1. Molecular docking (AutoDock Vina) of the ligand with BACE1 showed the compound to dock preferentially in an allosteric site of the enzyme.	[85]
	The major polymethoxyflavones from *Kaempferia parviflora* were screened for BACE1 inhibitory activity. 5,7-Dimethoxyflavone, 5,4',7-trimethoxyflavone, and 3,3',4',5,7-pentamethoxyflavone all showed strong BACE1 inhibitory activity. Molecular docking (AutoDock Vina) revealed all three compounds to preferentially dock to an allosteric site of the enzyme.	[86]

Biomolecular Target	Comments	Ref.
	Biochanin A, an isoflavonoid from red clover, was found to show *in vitro* inhibitory effects on BACE1. Molecular docking (AutoDock Vina) showed the compound to dock preferentially to an allosteric site of the enzyme.	[86]

Table 3. Recent molecular docking investigations of phytochemical ligands with bacterial biomolecular targets.

Biomolecular Target	Comments	Ref.
α-L-Arabinofuranosidase	A molecular docking study (AutoDock Vina) of 272 xanthones with *Geobacillus stearothermophilus* was carried out. Two structurally dissimilar prenylated xanthones showed strong docking to the enzyme, due primarily to hydrophobic interactions in one case and hydrogen bonding in the other.	[87]
Cytochrome P450 CYP121	Molecular docking (MolDock) of 561 antibacterial phytochemicals with *M. tuberculosis* CYP121 showed only two phytochemical ligands (ε-viniferin and 3''''-(2-hydroxybenzyl)isouvarinol) had docking scores comparable a synthetic MtCYP121 inhibitor.	[17]
Cytochrome P450 CYP51 sterol 14α-demethylase	A molecular docking study (AutoDock Vina) of 272 xanthones with *Mycobacterium tuberculosis* CYP 51 was carried out. Three prenylated xanthones showed strong docking to MtCYP51, due primarily to hydrophobic interactions.	[87]
DNA Gyrase / Topoisomerase IV	Molecular docking (InsightII v. 97) of quercetin with EcGyrB suggested a binding orientation of the ligand with the enzyme.	[88]
	A molecular docking analysis (MolDock) of 561 antibacterial phytochemicals was carried out. Angusticornin B, kanzonol C, and mulberrofuran D docked well with both EcTopoIV and MtGyrB; piperaduncin B, garcinoic acid, and cochinchinenene B docked to MtGyrB; rhinacanthin H and mulberrofuran Y docked to EcTopoIV.	[17]

Biomolecular Target	Comments	Ref.
α-Fucosidase	A molecular docking study (AutoDock Vina) of 272 xanthones with *Thermotoga maritima* fucosidase was carried out. Two prenylated xanthones showed strong docking to the enzyme, due primarily to hydrophobic interactions.	[87]
Glucosamine-6-phosphate synthase (GLMS)	The oleanane triterpenoid entagenic acid showed antibacterial activity against *Bacillus cereus* and *B. subtilis*. The compound showed good docking (AutoDock 3.0) properties to *E. coli* GLMS.	[89]
Glucosyltransferase	A docking study (Discovery Studio 4.0 CDOCKER) of *Mycobacterium avium* glucosyltransferase with 14 antibacterial phytochemicals was carried out. Gingerol was identified as the best compound based on docking score, binding affinity, and ADMET analysis.	[90]
β-Hydroxyacyl-acyl carrier protein dehydratase (FabZ)	Molecular docking (Glide) of *Crocus sativus* compounds crocin and safranal suggested that *Heliobacter pylori* FabZ is a likely antibacterial target.	[91]
β-Ketoacyl-acyl carrier protein reductase (MabA, also named FabG1)	Molecular docking (AutoDock Vina) of ellagic acid derivatives with *Mycobacterium tuberculosis* MabA showed pteleoellagic acid to dock with MabA with docking energies comparable to the known inhibitor isonicotinic-acyl-NADH.	[92]
β-Ketoacylacyl carrier protein synthase I (KAS I)	A docking study (iGemdock) of *E. coli* KAS 1 with 50 flavonoid ligands was carried out. Of the 50 flavonoids, genistein and isorhamnetin showed superior docking energies while also satisfying drug likeness conditions.	[93]
β-Lactamase	An in-silico investigation (iGEMDOCK and Accelrysligandfit) of 300 antibacterial phytochemicals with New Delhi metallo-β-lactamase-1 (NDM-1) was carried out. Six compounds (keivitone, gossypetin, taxifolin, galaturinic acid, kaempferol, and eriodictyol) showed better docking properties than the known inhibitor captopril.	[94]

Biomolecular Target	Comments	Ref.
Long-chain enoyl-acyl carrier protein reductase (InhA)	Molecular docking (AutoDock Vina) of two ellagic acid derivatives with *Mycobacterium tuberculosis* InhA showed that neither docked to the enzyme as well as the known inhibitor, isonicotinic-acyl-NADH.	[92]
NAD^+-Dependent DNA ligase	A molecular docking analysis (MolDock) of 561 antibacterial phytochemicals revealed that several phytochemical ligands showed selective docking to bacterial DNA ligase (*E. coli* LigA or *S. aureus* LigA).	[17]
Nitric oxide reductase	A molecular docking study (AutoDock Vina) of 272 xanthones with *Geobacillus stearothermophilus* NO reductase was carried out. Two prenylated xanthones showed strong docking to the enzyme, due primarily to hydrophobic interactions.	[87]
Pantothenate kinase (PanK)	Molecular docking (AutoDock Vina) of ellagic acid derivatives with *Mycobacterium tuberculosis* PanK showed 3,8-di-*O*-methyl-2-*O*-α-L-rhamnosylellagic acid to dock to the enzyme more strongly than the known triazole inhibitor, 2-chloro-*N*-[1-(5-{[2-(4-fluorophenoxy)ethyl]sulfanyl}-4-methyl-4*H*-1,2,4-triazol-3-yl)ethyl]-benzamide.	[92]
Peptide deformylase	Molecular docking (Glide) of *Crocus sativus* compounds crocin and safranal suggested that *Heliobacter pylori* PDF is a likely antibacterial target.	[91]
	Molecular docking (iGEMDOCK, AutoDock Vina) of 452 phytochemicals found betulinic acid, carpaine, cycloartenol, ginkgolide A, glycyrrhetic acid, gossypol, nimbidin, oleanolic acid, procyanidins, quercetin, tomatidine, and ursolic acid to be strongly docking ligands.	[95]
	A molecular docking analysis (MolDock) of 561 antibacterial phytochemicals revealed one alkaloid, seven chalcones, four flavonoids, seven stilbenoids, and four phenolic compounds showed excellent docking properties to one or more bacterial PDF structures.	[17]

Biomolecular Target	Comments	Ref.
Phospho-Mur-NAc-pentapeptide translocase (MraY)	The bis-naphthylisoquinoline alkaloid michellamine B was found to inhibit *E. coli* MraY and *B. subtilis* MraY and showed antibacterial activity against *B. subtilis*. Molecular docking (AutoDock Vina) indicated michellamine B to bind to a hydrophobic groove formed between two transmembrane helices.	[96]
Protein tyrosine phosphatase	Molecular docking (MolDock) of 561 antibacterial phytochemicals with *M. tuberculosis* protein tyrosine phosphatase showed only two phytochemical ligands (angusticornin B and garcinoic acid) had docking scores comparable to synthetic MtPtp inhibitors.	[17]
UDP-Galactopyranose mutase	Three phenolic ligands, drummondin D, drummondin E, and hyperbrasilol C, showed strong, selective docking (MolDock) to *M. tuberculosis* UGM.	[17]

Table 4. Recent molecular docking investigations of natural products with fungal biomolecular targets.

Biomolecular Target	Comments	Ref.
Lanosterol 14α demethylase	Two coumarins, robustic acid and thonningine-C from *Millettia thonningii*, showed promising activity against *Candida albicans*. Molecular docking (GOLD 5.4) against lanosterol 14α-demethylase (CYP51) revealed a plausible binding mode in which the hydroxyl group of the coumarin binds with a methionine backbone carboxylic group blocking access to the iron catalytic site.	[97]
N-myristoyl transferase	Autodock/Vina was used to dock 33 antifungal natural products with *Candida albicans* NMT (CaNMT). Isopiscerythrone and berberine exhibited the best docking scores.	[98]
	A set of 272 phytochemical xanthones were submitted to molecular docking (AutoDock Vina) calculations with *Candida albicans* NMT. Prenylated xanthones were shown to be the best docking ligands, with tovophyllin A as the overall best.	[87]

Table 5. Representative molecular docking studies with cancer relevant biomacromolecular targets.

Biomolecular Target	Comments	Ref.
Androgen receptor (AR)	Phytochemicals from *Hymenodictyon excelsum* were docked (MolDock) to the androgen receptor. Esculin showed superior docking properties with AR compared to dihydrotestosterone.	[99]
	Molecular docking (PLANTS, GOLD, and MOE) of a library of 39,000 compounds was carried out.	[100]
Aromatase	Cytotoxic prenylated chalcones showed preferential docking (MolDock and ArgusDock) to aromatase compared to isoflavonoids or non-prenylated flavonoids.	[101]
	A molecular docking (AutoDock Vina) analysis of 10 acridone alkaloids from *Zanthoxylum zanthoxyloides* and *Z. leprieurii* fruits has predicted 3-hydroxy-1,5,6-trimethoxy-9-acridone, 4-methoxyzanthacridone, 4-hydroxyzanthacridone, and 4-hydroxyzanthacridone 2,4'-oxide, to be aromatase inhibitors.	[102]
Casein kinase 2 (CK2)	Coumestrol, a tumor cell proliferation inhibitor and reversible ATP competitive CK2 inhibitor, docked (Discovery Studio) to the ATP site of CK2.	[103]
Cyclin-dependent kinase 2 (CDK2)	A molecular docking (AutoDock 4.2) analysis of 15 cytotoxic Himalayan plant-derived compounds revealed 12β-hydroxycalotropin and asclepin to show good docking properties to CDK2.	[26]
	Twenty-five phytochemical ligands were docked (AutoDock 4) to CDK2. The best docking ligands were cannabinol, lutein, neurosporene, oleanolic acid, and zeaxanthin.	[104]
Cyclin-dependent kinase 4 (CDK4)	Molecular docking (AutoDock Vina) revealed that catechin and wedelactones docked strongly to CDK4.	[105]
Cyclin-dependent kinase 6 (CDK6)	A molecular docking (AutoDock 4.2) analysis of 15 cytotoxic Himalayan plant-derived compounds revealed 12β-hydroxycalotropin and asclepin to show good docking properties to CDK6.	[26]
Cyclooxygenase 2 (COX-2)	Isoeugenol docked (MolDock) well to COX-2.	[106]

Biomolecular Target	Comments	Ref.
	Molecular docking (FlexX and ArgusDock) of 12 flavonoids with COX-2 revealed that all 12 showed favorable docking energies.	[107]
	A virtal library of 2092 flavonoids was docked (iGEMDOCK) to murine COX2. Genistein and the flavone glycosides isoquercitrin, rutin, and hyperoside exhibited much better docking energies than the substrate arachidonic acid.	[108]
DNA (groove binding)	Anthocyanins (anthocyanidin glycosides) were found to have a great affinity for the minor groove of poly $(dA\text{-}dT)_{12}$ or poly $(dG\text{-}dC)_{12}$ DNA.	[109]
	Shikonin was found (Surflex) to be a DNA minor-groove-binding agent, but not a DNA intercalator.	[110]
DNA (intercalation)	Ten quinoline alkaloids were docked with DNA using the Molegro (MolDock) software. A total of four alkaloids showed intercalation with DNA.	[111]
DNA methyltransferase (DNMT)	A library of 447 natural products was docked (AutoDock Vina and Surflex-Dock) to human DNA methyltransferases. A total of six compounds were identified as virtual hits.	[112]
Epidermal growth factor receptor (EGFR)	A library of 93 steroidal saponins, 43 phenylpropanoid, 134 flavonoids, and 34 carbohydrates was docked (CHARMM) with human epidermal growth factor receptor-2 (HER2).	[113]
	Shikonin was found (AutoDock 4.2) to dock into the catalytic site of EGFR.	[114]
	A docking (AutoDock 4.2) analysis of four pentacyclic triterpenoids (ursolic acid, 18α-glycyrrhetinic acid, carbenoxolone, and dimethyl melaleucate) revealed these compounds to preferentially dock with the tyrosine kinase domain of EGFR.	[115]
	A library of 67 phytochemical ligands was docked with EGFR using GOLD. Hesperidin showed notable docking properties to EGFR.	[116]
	Molecular docking (GLIDE) of a library of cytotoxic natural products revealed the flavonoids quercetin and luteolin to have good docking scores to the kinase domain of EGFR.	[117]

Biomolecular Target	Comments	Ref.
Estrogen receptor	A library of 4209 phytochemicals were docked to ERα using the MOE software. A total of 10 compounds showed promising binding compared to tamoxifen.	[118]
	A library of 568 phytochemicals from 17 of the most popular herbal supplements were docked (MolDock) with ERα and ERβ. In some of the most popular herbal supplements, there were numerous compounds that docked strongly with the estrogen receptor: Licorice (26), wild yam (15), black cohosh (11), muira puama (8). red clover (8), damiana (3), and dong quai (3).	[119]
	Five flavonoids from *Erythrina crista-galli* were docked with human Erα using Discovery Studio (GOLD). Apigenin and luteolin both showed docking energies more exothermic than 17β-estradiol.	[120]
	A docking study (AutoDock) of phytochemical polyphenolics revealed that the isoflavonoids daidzein and genistein and the flavonoid quercetin showed promising docking properties to ERα.	[121]
	A library of 27,418 traditional Chinese medicine (TCM) compounds was docked (DOCK 6.3) with the ligand-binding domain of human ERα. The docking revealed 79 compounds to TCM compunds that might serve as Erα modulators.	[122]
Heat shock protein 90 (Hsp90)	Molecular docking (MolDock) of dietary flavonoids with the ATP binding site of Hsp90 has revealed that quercetin, myricetin, and kaempferol are promising inhibitors of Hsp90.	[123]
	Molecular docking (GLIDE) of 16 limonoids with Hsp90 was carried out. The limonoids docked in the C-terminal domain of the dimeric protein.	[124]
	Virtual screening (LIGANDFIT) of a library of 248 cytotoxic phytochemicals with Hsp90 indicated 41 compounds to have better docking scores than the standard drug. Epigallocatechin gallate and isoquercitrin showed the best docking scores to Hsp90.	[125]

Biomolecular Target	Comments	Ref.
Insulin-like growth factor 1 (IGF1) receptor kinase	A library of 50,000 natural products was docked (GLIDE) to IGF1 receptor kinase. Epigallocatechin gallate (EGCG) exhibited excellent docking properties and was further evaluated in cell based assays.	[126]
Insulin-like growth factor 1 receptor (IGF1R)	AutoDock 4.2 was used to carry out molecular docking of dietary phytochemicals (curcumin, genistein, apigenin, lycopene, silibinin, and luteolin) with human IGF1R. The best docking ligand was apigenin.	[127]
Lipoxygenase (5-LOX)	α-Amyrin docked (AutoDock 4.2) well to 5-LOX.	[128]
	Isoeugenol docked (MolDock) well to 5-LOX.	[106]
Mitogen-activated protein kinase kinase 1 (MEK1)	A molecular docking study (AutoDock 4) with phytochemicals from *Tarconanthus camphoratus* predicted parthenolide to target MEK1.	[129]
	An *in-silico* investigation of 2,620 flavonoids revealed the MEK1 inhibitory flavonoid isorhamnetin to dock (GLIDE) to the ATP-noncompetitive pocket of MEK1.	[130]
Myc-Max complex	Shikonin was found (AutoDock 4.2) to dock into the DNA binding region of the Myc-Max complex.	[114]
Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)	Ursolic acid and pomolic acid docked (AutoDock4) strongly to DNA-bound NF-κB and docked to the DNA binding site of free NF-κB, suggesting that these compounds inhibit DNA binding by NF-κB.	[131]
	Coclaurine, haiderine, and hirsutine, phytoconstituents of *Cocculus hirsutus*, showed good docking (GLIDE) properties with NF-κB.	[132]
	NF-κB was suggested to be a molecular target for the cytotoxic prenylated chalcone paratocarpin E based on molecular docking (DOCK 6.5) studies.	[133]
p90 ribosomal S6 kinase 2 (RSK2)	A library of 2,620 flavonoids was virtually screened against RSK2 using molecular docking (GLIDE). Six compounds (pedalitin, quercetin, quercetin 3-sulfate, 5-hydroxy-4'-methoxy-7-methylflavone, kaempferol, and 3,3'-di-*O*-ethylquercetin, showed good docking profiles with RSK2 with docking scores better than a well-known synthetic RSK2 inhibitor.	[130]

Biomolecular Target	Comments	Ref.
P-Glycoprotein (ATP-dependent efflux pump)	Molecular docking (GLIDE) of 40 dietary phytochemicals revealed that flavonoids, particularly quercetin and rutin, exhibited promising interactions with P-gp.	[134]
	An investigation of cardiotonic steroids has shown that six P-glycoprotein modulating compounds docked (AutoDock 4.2) well to both the transmembrane domain (TMD) and the nucleotide binding domain (NBD) of human P-glycoprotein.	[135]
Phosphoinositide 3-kinase (PI3K)	A library of 80 antiinflammatory phytochemicals was examined in a molecular docking study (MolDock) with PI3K. Seven compounds (berberine, chelerythrine, isaindigotone, malvidin, isovitexin, vitexin, and cucurbitacin B) were found to dock strongly and selectively with PI3K.	[136]
	A library of 51 natural products was docked (GLIDE) with PI3K signaling components (PI3K, PDK1, Akt, and mTOR). The flavonoids myricetin, quercetin, morin, and luteolin, and the anthraquinone emodin were found to be promising molecules docking to PI3K.	[137]
Sodium/Potassium-ATPase	Molecular docking (AutoDock 4) of cytotoxic cardiotonic steroids (cardenolides and bufadienolides) with Na^+/K^+-ATPase did not reveal correlation between docking energies and cytotoxicities, suggesting additional molecular targets may be involved.	[138]
Topoisomerase	Curcumin and curcumin derivatives docked (AutoDock) at the DNA cleavage site of DNA in the topo I and topo II / DNA complexes.	[139]
	A molecular docking (AutoDock 4.2) analysis of 15 cytotoxic Himalayan plant-derived compounds revealed 12β-hydroxycalotropin to show good docking properties to topoisomerase I and topoisomerase II.	[26]
	Cytotoxic norhopene triterpenoids selectively docked (MolDock) to the DNA binding site of topoisomerase II.	[140]

Biomolecular Target	Comments	Ref.
	The cytotoxic dammarane triterpenoid aglaitriol selectively docked (MolDock) to the DNA binding site of topoisomerase II.	[141]
	Ten acylated triterpenoid saponins that had been shown to inhibit human topoisomerase I were docked (GOLD) with the topoisomerase I - DNA complex. The saponins docked into the same cavity as camptothecin, at the DNA clevage site.	[142]
Tubulin	Docking studies (Schrödinger-Maestro) showed that two flavnoids, artemetin and chrysosplenetin, bind to the colchicine binding site of tubulin, in agreement with their modulation of microtubule depolymerization.	[143]
	Cembrene diterpenoids docked (MolDock) into the colchicine binding site of tubulin with docking energies comparable to colchicine.	[144]
	East Indian sandalwood oil and its major components, α-santalol and β-santalol, were shown to be cytotoxic and microtubule disruptors. Molecular docking (Rhodium protein docking simulation program) predicted the binding site of santalols to be the colchicine site rather than the vinblastine site of tubulin.	[145]
Vascular endothelial growth factor receptor (VEGFR)	Puerarone and tuberostan (from *Pueraria tuberosa*) were found to dock well (AutoDock) with VEGFR-1 and VEGFR-2.	[146]
	Five phytochemicals from *Indigofera aspalathoides* were docked (AutoDock) to VEGFR2. Indigocarpan showed stronger docking energy compared to the positive control drug sorafenib.	[147]
	Ellagic acid preferentially docked (Discovery Studio/ CHARMm) to the ATP binding site rather than the catalytic domain of VEGFR-2.	[148]
	A library of 50,000 natural products was docked (GLIDE) to VEGFR-2. Epigallocatechin gallate (EGCG) exhibited excellent docking properties and was further evaluated in cell based assays.	[126]

Biomolecular Target	Comments	Ref.
	Coclaurine, lirioresinol, and haiderine, phytoconstituents of *Cocculus hirsutus*, showed good docking (GLIDE) properties with VEGFR.	[132]
	Virtual screening (LIGANDFIT) of a library of 248 cytotoxic phytochemicals with VEGFR indicated 10 compounds (quercetin, kaempferol, rhamnetin, resveratrol, luteolin, apigenin, caffeic acid, salicylic acid, ferulic acid, and malic acid) to have better docking scores that the standard drug.	[125]
Xanthine oxidase (XO)	Cytotoxic prenylated chalcones showed preferential docking (MolDock and ArgusDock) to XO compared to isoflavonoids or non-prenylated flavonoids.	[101]
	A virtal library of 2092 flavonoids was docked (iGEMDOCK) to bovine XO. Luteolin, apigenin, sculellarin, quercetin, myricetin, and genistein exhibited much better docking energies than the substrate xanthine.	[108]
	Molecular docking (AutoDock 4.2) revealed the XO-inhibitor luteolin to dock at the hydrophobic pocket (molybdopterin domain) of XO.	[149]
	Molecular docking (AutoDock Vina) revealed the XO-inhibitors galangin and pinobanksin to dock at the hydrophobic pocket (molybdopterin domain) of XO.	[150]
	The XO inhibitor, 6-(3-methylbut-1-enyl)-5,7-dimethoxy-4'-hydroxy flavone, docked (AutoDock) preferentially in the hydrophobic pocket (molybdopterin domain) of XO.	[151]
	A docking (AutoDock 4.0) study of dietary flavonoids (chrysin, apigenin, galangin, quercetin, rutin, genistein) all docked in the molybdopterin domain of XO.	[152]

Table 6. Recent molecular docking studies of natural product ligands with biomolecular targets relevant to diabetes.

Biomolecular Target	Comments	Ref.
Aldose reductase (ALR)	A molecular docking (GLIDE) study of nine phytochemicals revealed gingerenones A, B, and C, laricіresinol, quercetin, and calebin A, to dock well to ALR.	[58]
	Molecular docking (AutoDock 4.2) of *Scrophularia* polyphenolics with human ALR2 indicated the flavonoid acacetin to dock most favorably with the enzyme.	[153]
	Molecular docking (GLIDE) of (4*Z*,12*Z*)-cyclopentadeca-4,12-dienone from *Grewia hirsuta* with several molecular targets related to diabetes showed the compound to dock well with ALR.	[29]
α-Amylase	A molecular docking (AutoDock 4.2.6) analysis of 22 phytoconstituents from *Syzygium cumini* with α-amylase showed the best docking compounds to be triterpenoids friedelin, epifriedelinol, and betulinic acid.	[154]
Fructose-1,6-bisphophatase	Molecular docking (GLIDE) of 1,2,8-trihydroxy-6-methoxyxanthone with several molecular targets related to diabetes showed the compound to dock most strongly with fructose-1,6-bisphosphatase.	[28]
α-Glucosidase	(*S*)-3',4',5-Trihydroxy-7-prenyloxyflavanone, isolated from *Arcytophyllum thymifolium*, showed potent α-glucosidase inhibitory activity. Molecular docking (AutoDock) indicated the ligand binds to a hydrophobic cavity within the enzyme catalytic site.	[155]
11β-Hydroxysteroid dehydrogenase	Molecular docking (GLIDE) of 1,2-dihydroxy-6-methoxyxanthone-8-*O*-β-D-xylopyranosyl with several molecular targets related to diabetes showed the compound to dock most strongly with 11β-hydroxysteroid dehydrogenase.	[28]
Peroxisone proliferator-activated receptor-γ (PPAR-γ)	Molecular docking (GLIDE) of (4*Z*,12*Z*)-cyclopentadeca-4,12-dienone from *Grewia hirsuta* with several molecular targets related to diabetes showed the compound to dock well with PPAR-γ.	[29]

Biomolecular Target	Comments	Ref.
Protein tyrosine phosphatase 1B (PTP1B)	The isoquinoline alkaloids berberine, epiberberine, magnoflorine, and coptisine, from *Coptis chinensis*, showed remarkable inhibitory activities against PTP1B. Molecular docking (AutoDock 4.0, Fred 2.0) indicated all four ligands to dock into the active site of PTP1B, but with different orientations.	[156]

Table 7. Recent molecular docking studies of natural product ligands with biomolecular targets relevant to inflammation.

Biomolecular Target	Comments	Ref.
Cyclooxgenase (COX-1, COX-2)	Molecular docking (Glide) of compounds isolated from *Alpinia officinarum* with murine COX-2 revealed galangin and 5-hydroxy-7-(4-hydroxy-3-methoxyphenyl)-1-phenyl-3-heptanone to be the strongest docking phytochemical ligands, in agreement with experimental anti-inflammatory screening.	[157]
	Stilbenoids isolated from licorice (*Glycyrrhiza glabra*) leaves were evaluated using molecular docking (AutoDock 4.2) with COX-1 and COX-2. The study showed the stilbenoids docked preferentially to COX-2 over COX-1, in agreement with experimental selectivity.	[158]
	Thirty-five compounds from *Erythrina variegata* were studied using molecular docking (GOLD 4.12) with COX-1 and COX-2. The docking study revealed phaseollin to have the best docking scores against both COX-1 and COX-2.	[159]
	A docking study (AutoDock 4.2) of seven compounds from *Jatropha* spp. indicated palmarumycin CP_1 to have the best affinity for COX-2.	[160]
	A molecular docking study (PatchDock) of phytochemicals from *Delonix regia* bark showed that β-elemene was the best docking ligand to COX-2.	[161]

Biomolecular Target	Comments	Ref.
	A molecular docking study (AutoDock 4.2) was carried out with the essential oil components eugenol, myristicin, and limonene, with COX-1 and COX-2. Eugenol and myristicin were more selective for COX-1 over COX-2, while limonene was more selective for COX-2. None of these ligands had docking energies comparable to known COX inhibitors flurbiprofen (COX-1) or celecoxib (COX-2).	[162]
	Molecular docking (GOLD) of nepodin and chrysophanol with COX-1 and COX-2 showed chrysophanol to have a better GOLD fitness score for COX-2 over COX-1, in agreement with experimental COX selectivity. Nepodin had very similar GOLD fitness scores for COX-1 and COX-2, but lower than those for chrysophanol, in contrast to experimental COX inhibitory activities.	[163]
	A molecular docking (GOLD) study of 12 phytochemicals from *Mimosa pudica* with COX-1 and COX-2 revealed the best docking ligand to be vitexin, which also showed docking selectivity for COX-2.	[164]
	A collection of 24 phytochemicals from *Pseudarthria viscida* were used as ligands for molecular docking (CDOCKER) analysis. From these, the isoflavonoid dalbergioidin gave the best docking score with COX-2.	[165]
	Molecular docking (AutoDock 4.0) of five alkaloids from *Rhizophora mucronata* with COX-2 showed the best docking compound to be the indole alkaloid serpentine, which had a better docking energy than the standard drug ibuprofen.	[166]
	A library of 2092 flavonoids and isoflavonoids was evaluated in silico for COX-2 binding using iGEMDOCK. The docking analysis revealed 21 flavonoids with docking energies better than the co-crystalized ligand arachidonic acid.	[108]
	Molecular docking (GOLD 4.2) of six phytochemicals from Alangium salvifolium revealed salviifoside A to have the best interaction with COX-2.	[167]

Biomolecular Target	Comments	Ref.
	Molecular docking (FlexX) of five compounds (caryophyllene oxide, cedrane, menthol, methyl acetate, daucol, and α-terpinyl acetate) from *Erygium foetidum* showed that caryophyllene oxide was the best docking ligand of the group.	[168]
	Molecular docking (AutoDock 4.2) of six compounds (asarone, luteolin, quercetin, resveratrol, piceatannol, and kampferol) from *Cissus quadrangularis* with COX-2 showed the flavonoids luteolin and quercetin to be the strongest docking.	[169]
	An in-silico screening (GLIDE) of 17 phytochemicals against PLA2, 5-LOX, and COX-2 showed epigallocatechin gallate, β-boswellic acid, celastrol, rutin and scalaradial gave the best docking scores.	[33]
Lipoxygenase (LOX)	Molecular docking (AutoDock 3.0.5) of the steroid sieboldogenin with soybean LOX showed the compound to dock deeply within the catalytic site.	[170]
	A molecular docking (AutoDock 4.2) analysis of *Spatholobus suberectus* flavonoids with 5-LOX revealed butin, 3',4',7-trihydroxyflavone, plathymenin, and gallocatechin to be the most strongly binding ligands.	[171]
	Molecular docking (MolDock 6.0.1) of the dammarane triterpenoid aglaitriol with 5-LOX showed stronger docking than the known LOX inhibitor ursolic acid.	[141]
	Molecular docking (C-Docker) of five compounds (6,10,17-trimethyl-2-octadecanone, sitosterol-3-*O*-glucoside, ipolamiide, and lamiide) isolated from *Phlomis thapsoides* with 5-LOX showed the strongest docking ligand to be sitosterol-3-*O*-glucoside.	[172]
	Molecular docking (AutoDock 4.2) of α-amyrin showed docking preference for 5-LOX over COX-2.	[128]
	An in-silico screening of 17 phytochemicals against PLA2, 5-LOX, and COX-2 showed the flavonoid karanjin to be the best docking ligand to 5-LOX.	[33]

Biomolecular Target	Comments	Ref.
myeloid differentiation protein 2 (MD-2)	Molecular docking (Glide)followed by molecular dynamics (MD) simulations revealed xanthohumol to imbed into the hydrophobic pocked of MD-2 and form stable hydrogen bonds with Arg90 and Tyr102.	[173]
	Molecular docking (Glide) and molecular dynamics (MD) simulation studies showed that curcumin could be embedded into the hydrophobic pocket of MD-2 and form stable hydrogen bonding interactions with residues Arg90 and Tyr102 of MD-2.	[174]
IκB kinase β (IKKβ)	Molecular docking (Glide) of 80 pentacyclic triterpenoids with IKKβ revealed 45 compounds with drug-like properties (Lipinski's rule of five) that docked favorably with IKKβ.	[175]
p38α Mitogen-activated protein kinase (p38α MAPK)	A molelcular docking (MolDock) analysis of 22 phytochemicals from *Morinda citrifolia* with p38α MAPK showed the best docking ligand to be the lignan isoprincepin.	[176]
Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)	Five flavonoids from Ziziphus spina-christi were evaluated using molecular docking (AutoDock 4) with NF-κB and IκK. Gallocatechin and epigallocatechin showed good docking to NF-κB pathway proteins.	[34]
Phopholipase A_2 (PLA2)	An in-silico screening of 17 phytochemicals against PLA2, 5-LOX, and COX-2 showed epigallocatechin gallate and celastrol to have significantly higher docking scores than the other ligands.	[33]
Phosphoinositide 3-kinase (PI3K)	An *in-silico* screening (MolDock 6.0) of 80 anti-inflammatory phytochemicals with three isoforms of PI3K allowed identification of seven natural products (the alkaloids berberine, chelerythrine, and isaindigotone; the flavonoids malvidin, isovitexin, and vitexin; and the triterpenoid cucurbitacin B) with drug-like properties that docked strongly and selectively with PI3K.	[136]

Table 8. Recent molecular docking studies of antiviral target proteins with natural product ligands.

Biomolecular Target	Comments	Ref.
Chikungunya virus (CHIKV) NSP2 cysteine protease	A library of 2147 plant-derived natural products was virtually screened (MolDock) against 10 alphavirus protease targets. The alkaloid drodrenin showed docking selectivity for CHIKV protease over human caspase 3 as well as over other alphavirus proteases. The docking energy for drodrenin was not as good as those for synthetic cysteine protease inhibitors, however.	[177]
	In silico screening (GLIDE) of 22 phytochemicals based on Indian traditional medicine was carried out on CHIKV NSP2 protease. Six compounds (andrographoside, deoxyandrographoside, neoandrographolide, 14-deoxy-11-oxoandrographolide, butoxone and oleanolic acid) showed docking scores better than the reference compound chloroquine.	[178]
Dengue virus (DENV) envelope protein	The envelope proteins of DENV2-Thai and DENV2-Malaysia (homology model) were docked (AutoDock Vina) with nine flavonoids (baicalin, baicalein, EGCG, fisetin, glabranine, hyperoside, ladanein, quercetin, and flavone). Of these, baicalein showed the best docking properties to the envelope proteins.	[179]
	In silico screening (AutoDock Vina) of seven anti-dengue flavonoids (baicalein, fisetin, hesperetin, naringenin, naringin, quercetin, and rutin) with the enveolope protein of dengue virus showed quercetin to the the only effective compound.	[180]
	A phytochemical library of 2194 compounds was virually screened (MolDock) against DENV EP. The best docking ligands were cannflavin A (a geranylated flavonoid from *Cannabis sativa*) and paratocarpin L (a bis-prenylated flavonoid from *Artocarpus venenosa*). Both of these ligands completely occupied the channel of the envelope protein.	[181]

Biomolecular Target	Comments	Ref.
Dengue virus (DENV) NS2B-NS3 serine protease	*In vitro* protease inhibition (DENV NS2B-NS3 protease) showed agathisflavone and myricetin to be inhbitors. Molecular docking (FlexX) showed the compounds to preferentially dock into an allosteric binding site of the protein.	[182]
	A molecular docking study (AutoDock 4.2) of five triterpenoids from *Azadirachta indica* (nimbin, desacetylnimbin, desacetylsalannin, azadirachtin, and salannin) against DENV NS2B-NS3 protease was carried out. Azadirachtin and salannin did not show exothermic docking scores, but nimbin, desacetylnimbin and desacetylsalannin did, with nimbing the best docking ligand.	[183]
	Molecular docking (MOE) of panduratin A and 4-hydroxypanduratin A, isolated from the rhizome of *Boesenbergia rotunda*, as well as semisynthetic derivatives, was carried out with the active site of DENV NS2B-NS3 protease. The docking revealed the two compounds to be viable templates for synthetic modification.	[184]
	Molecular docking (AutoDock 3.0.5) of three flavanones and three chalcones from the rhizome of *Boesenbergia rotunda* with DENV NS2B-NS3 protease revealed the compounds to dock preferentially to an allosteric site of the target protein rather than the active site.	[185]

Biomolecular Target	Comments	Ref.
	A phytochemical library of 2194 compounds was virtually screened (MolDock) against DENV NS2B-NS3 protease. Prenylated stilbenoids (3,3,4,5-tetrahydroxy-5-prenylbibenzyl, 3,3,5-trihydroxy-4-methoxy-5-prenylbibenzyl, 3-acetoxy-4,5-dihydroxy-3-prenyldihydrostilbene, licobenzofuran) and isoflavonoids (glycyrrhisoflavone, 4-*O*-methylglycyrrhisoflavone) chalcones (kanzonol Y) from *Glycyrrhiza glabra* demonstrated outstanding docking properties to DENV protease. Balsacones A-C, dihydrochalcones from *Populus balsamifera*, exhibited excellent docking properties to DENV NS2B-NS3 protease. Two diprenylated flavanones, euchrestaflavanone A from *Macaranga pleiostemona* and flemiflavanone D from *Flemingia stricta* also showed notably strong docking with DENV NS2B-NS3 protease.	[181]
	Seven phenolic compounds from the leaf extract of *Carica papaya* were docked (SurFlex Dock) with DENV NS2B-NS3 protease. The flavonoid quercetin showed the best docking properties with the active site of the protease.	[186]
	A molecular docking survey (MOE) of 1300 antiviral alkaloids with DENV NS2B-NS3 protease revealed five alkaloids (6′-desmethylthalifaboramin, 3,5-dihydroxythalifaboramin, betanin, reserpic acid, and tubulosine) showed potential interactions with the catalytic triad of the active site.	[187]
	A docking analysis (MOE) with DENV NS2B-NS3 protease using a library of 940 phytochemicals was carried out. *Garcinia* phytochemicals were found to be the best hits, including gossypol, mangostenone C, garcidepsidone A, and dimethylcalabaxanthone.	[188]
	Molecular docking (AutoDock 4.2) of phytochemicals from *Murraya koenigii* were carried out. The bis-indole alkaloid bismurrayafoline E was concluded to be a promising lead compound for futher investigation.	[189]

Biomolecular Target	Comments	Ref.
Dengue virus (DENV) NS3 RNA helicase	A phytochemical library of 2194 compounds was virtually screened (MolDock) against DENV NS3 helicase. The phytochemical ligands showed docking preference for the ATP site of DENV helicase over the RNA binding site. The best docking compounds were curcumin II, caffeoyl-*p*-coumaroyltartaric acid, balsacone C, curcumin I, and angusticornin B. The common structural features of these compounds are two phenolic groups with a flexible linker between them.	[181]
Dengue virus NS5 methyltransferase (DENV MTase)	A phytochemical library of 2194 compounds was virtually screened (MolDock) against DENV NS5 MTase. The strongest docking ligands all showed docking preference for the SAM site of the enzyme over the GTP site. Five compounds (isoborreverine, diplacone, styracifolin B, neosilyhermin A, and 8β-[4-hydroxy-5-(5-hydroxytigloyloxy)tigloyl]-santamarin) showed docking energies more exothermic than SAM for DENV MTase and more exothermic for DENV MTase than for human MTase. These compounds, therefore, are predicted to show inhibition of DENV MTase by competition with SAM and to selectively inhibit viral MTase over human MTase.	[181]
Dengue virus NS5 RNA-dependent RNA polymerase (DENV RdRp)	A phytochemical library of 2194 compounds was virtually screened (MolDock) against DENV RdRp. Four ligands (dimethylisoborreverine, drummondin D, flinderole B, and pungiolide A) docked well to the GTP site of DENV RdRp , while other ligands (flinderole A, neosilyhermin B, and kanzonol Y) docked well to hydrophobic allosteric sites.	[181]
Hepatitis B virus (HBV) DNA polymerase	Molecular docking (GLIDE) of a homology model of HBV DNA polymerase with repensine and bentysrepinine, dipeptides from *Dichondra repens*, was carried out. Both ligands showed better docking energies than thymidine triphosphate or lamivudine.	[190]

Biomolecular Target	Comments	Ref.
	A set of 35 compounds from *Phyllanthus niruri*, a medicinal herb used in traditional Indian medicine for the treatment of hepatitis B, was docked (CDOCKER) with a homology-modeled structure of HBV DNA polymerase. The strongest docking ligand was quercitrin.	[191]
Hepatitis C virus (HCV) NS3 RNA helicase	Molecular docking (MolDock) of six antiviral phytochemicals (quercetin, β-carotene, resveratrol, catechin, lycopene, and lutein) with a homology model of Pakistani HCV NS3 helicase was carried out. The best docking energies were observed for quercetin and catechin followed by resveratrol and lutein.	[192]
	Molecular docking (MOE) of six phytochemicals from *Amelanchier alnifolia* against HCV NS3/4A helicase was carried out. The best docking ligand was cyanidin-3-*O*[β-D-glucouronopyranosyl-(1→2)-6-O-malonyl-β-D-glucopyranoside.	[193]
Hepatitis C virus (HCV) NS3 serine protease	Molecular docking (MOE) of phytochemicals from *Azadirachta indica* with HCV NS3 protease indicated 3-deacetyl-3-cinnamoylazadirachtin possesses good binding properties.	[194]
	Molecular docking (GLIDE) of phytochemicals from *Boerhavia diffusa* with a homology modeled structure of HCV NS3 protease revealed liriodendrin to be the most promising compound.	[195]
	Molecular docking (MOE) of six phytochemicals from *Amelanchier alnifolia* against HCV NS3/4A protease was carried out. The best docking ligand was isohyperoside.	[193]
	In vitro protease inhibition (HCV NS3 protease) with ursolic acid, taraxerol, β-sitosterol, and berberine, showed that ursolic acid was the most active, followed by berberine. Molecular docking (FlexX with homology model of HCV NS3 protease) showed these two compounds also had the best docking scores.	[196]

Biomolecular Target	Comments	Ref.
	A library of 75 phytochemicals from medicinal plants was screened using GLIDE against HCV protease. Rutin showed good binding characteristics with the target protein.	[197]
	Virtual screening (iGEMDOCK) of 40 phytochemicals with HCV NS3/4A protease was carried out. Three compounds, berberine, berlambine, and quercetin, showed comparable docking energies to the known inhibitor simeprevir.	[198]
Hepatitis C virus (HCV) NS5B RNA-dependent RNA polymerase (RdRp)	Twelve compounds isolated from the antiviral extract of *Taraxacum officinale* were investigated using molecular docking (MOE) with HCV NS5B polymerase. The flavonoids quercetin, luteolin, and luteolin-7-glucoside showed more exothermic docking energies than the standard antiviral agent sofosbuvir.	[199]
Herpes simplex virus type II (HSV II) protease	A library of 75 phytochemicals from medicinal plants was screened using GLIDE against HSV II protease. Bilobetin was the best docking ligand with HSV II protease.	[197]
Human immunodeficiency virus (HIV) integrase	*In-vitro* and *in-silico* (AutoDock 4.2) screening of compounds from *Dioscorea bulbifera* bulbs with HIV-1 integrase showed the most active compound to be myrecetin, which also had the lowest docking score.	[200]
	The different geometrical isomers of 3,5-dicaffeoylquinic acid were docked (AutoDock Vina) with HIV-1 integrase. Although all four isomers effectively docked to the protein, there were important differences in their binding interactions.	[201]
	Catechin and protocatechuic acid, isolated from *Albizia procera* bark, were docked (AutoDock 4.2) with HIV-1 integrase.	[202]
Human immunodeficiency virus (HIV) protease	Dolichin A and dolichin B, pterocarpans from *Macrotyloma uniflorum*, were docked (Glide) with HIV reverse transcriptase, HIV protease, and HIV integrase. The compounds showed selective docking to HIV protease.	[203]

Biomolecular Target	**Comments**	**Ref.**
Human immunodeficiency virus (HIV) reverse transcriptase (RT)	A molecular docking analysis (AutoDock Vina) of a library of 272 xanthones was carried out on several antimicrobial and antiviral protein targets. In general, mono-oxygenated xanthones showed excellent docking selectivity for HIV reverse transcriptase over the other protein targets; prenylation generally resulted in strong docking energies to the target enzymes; pyranojacareubin and gerontoxanthone B were identified as potentially potent HIV reverse transcriptase inhibitors.	[87]
	Twelve alkaloids from the medicinal plant *Toddalia asiatica* were screened (AutoDock 4.0) against HIV-1 RT. The docking results suggested toddanol, toddanone, and toddalenone to be potent inhibitors of HIV-1-RT.	[204]
Influenza H1N1 neuraminidase (NA)	A set of 14 known H1N1 inhibitors was docked (Glide v. 6.1) with H1N1 neuraminidase. The most promising compounds were rutin and aloe emodin, which had better docking energies than the known antiviral agents zanamvir and laminavir.	[205]
	The homology modeled structure of influenza A H1N1 NA was docked with 13 phytochemicals using AutoDock 4.2. The green tea component, theaflavin, was found to inhibit H1N1 NA and showed the lowest docking energy.	[206]
Influenza H5N1 neuraminidase (NA)	A library (Malaysian-Plants Natural-Product, NADI, database) of 3000 compounds was docked (AutoDock 3.0.5) against H5N1 neuraminidase. Four of the top 100 compounds from the virtual screen (α-mangostin, rubraxanthone, garcinone C, and gartanin) showed neuraminidase inhibitory activity. It was noted, however, that the correlation between predicted free energies and experimentally measured potencies was poor.	[207]
	A library of 75 phytochemicals from medicinal plants was screened using GLIDE against H5N1 NA. Oleanolic acid exhibited the best docking characteristics with the target protein.	[197]

Biomolecular Target	Comments	Ref.
Semliki Forest virus (SFV) NSP2 cysteine protease	A library of 2147 plant-derived natural products was virtually screened (MolDock) against 10 alphavirus protease targets. The lignans hibalactone and hinokinin, as well as the phenolic compound cimicifugic acid F, showed selective docking to SFV NSP2 protease over the other cysteine proteases examined, including human caspase 3. The docking energies for these phytochemical ligands were not as good as those for synthetic cysteine protease inhibitors, however.	[177]
Sindbis virus (SINV) NSP2 cysteine protease	A library of 2147 plant-derived natural products was virtually screened (MolDock) against 10 alphavirus protease targets. The indole alkaloid flinderole B, the chalcone angusticornin B, and the sesquiterpenoid 8β-[4-hydroxy-5-(5-hydroxytigloyloxy)tigloyl]-santamarin, showed docking selectivity for SINV protease over the other alphavirus proteases examined as well as over human caspase 3. The docking energies for these phytochemical ligands were not as good as those for synthetic cysteine protease inhibitors, however.	[177]
	A library of 75 phytochemicals from medicinal plants was screened using GLIDE against a homology model of SINV NSP2 protease. Juglone showed good binding characteristics with the target protein.	[197]
Varicella zoster virus (VZV) serine protease	A library of 1500 randomly selected phytochemicals was screened for drug-likeness and bioavailability, reducing the library to 330. Molecular docking (iGEMDOCK) of the set with VZV protease revealed the most promising compound to be erysenegalensein E.	[208]
Zika virus (ZIKV) NS2B-NS3 serine protease	Molecular docking (MolDock) of a library of 2263 phytochemicals with a homology modeled structure of ZIKV NS2B-NS3 protease was carried out. Two bis-indole alkaloids (flinderole A and flinderole B) and two chalcones (angusticornin B and kuraridin) showed promising docking properties with ZIKV NS2B-NS3 protease.	[209]

Biomolecular Target	Comments	Ref.
Zika virus (ZIKV) NS3 RNA helicase	Molecular docking (MolDock) of a library of 2263 phytochemicals with ZIKV NS3 helicase was carried out. The isoquinoline alkaloid cassiarin D showed strong and selective docking for the ATP site, while the bis-indole alkaloid 4,4'-dimethylisoborreverine was selective for the RNA site of ZIKV NS3 helicase.	[209]
Zika virus NS5 methyltransferase (ZIKV NS5 MTase)	Molecular docking (MolDock) of a library of 2263 phytochemicals with a homology modeled structure of ZIKV NS5 MTase was carried out. The phytochemical ligands all showed docking preference for the GTP site of the enzyme rather than the SAM site. The best docking ligands were polyphenolic compounds with at least two phenolic groups connected with a flexible linker; cimiphenol, cimiracemate B, and rosemarinic acid showed strong docking energies. Prenylation also improved docking scores; prenylated chalcones kanzonol Y and kuraridin showed excellent docking properties.	[209]

Table 9. Recent molecular docking studies of phytochemicals with parasitic protozoal protein targets.

Biomolecular Target	Comments	Ref.
Leishmania protein targets	Molecular docking of phytochemical agents to molecular targets in *Leishmania* spp. has been recently reviewed and will not be included here.	[47]
Naegleria fowleri heat shock protein 70 (NfHSP70)	A set of 11 amebicidal phytochemicals were screened *in silico* (MOE) against NfHSP70. The best compounds from this study were bergenin and epigallocatechin gallate (EGCG).	[210]
Plasmodium protein targets	Molecular docking of phytochemical agents to molecular targets in *Plasmodium* spp. has been recently reviewed and will not be included here.	[47]

Biomolecular Target	Comments	Ref.
Trichomonas vaginalis methionine gamma-lyase (TvMGL)	An *in silico* screening (MolDock) of a library of 952 antiprotozoal phytochemicals was carried out against TvMGL. Of the compounds examined, eight showed remarkable docking properties to TvMGL: the aurone 6-benzoyl-2-[oxomethylpheny]- 3-hydroxy-benzofurane, the lignans eupomatenoid-5, eupomatenoid-6, and eupomatenoid-7, the cannabinoids 5-acetyl-4-hydroxycannabigerol and cannabigerolic acid, and the stilbenoids *trans*-4-(3-methyl-*E*-but-1-enyl)-3,5,2',4'-tetrahydroxystilbene and *trans*-4-isopentenyl-3,5,2',4'-tetrahydroxystilbene.	[49]
Trichomonas vaginalis purine nucleoside phosphorylase (TvPNP)	An *in silico* screening (MolDock) of a library of 952 antiprotozoal phytochemicals was carried out against TvPNP. Several phytochemicals showed selective docking to TvPNP over their human homologous isozymes. The best docking ligands were 2',4,4'-trihydroxy-3,3'-diprenylchalcone, curcumin, and piperine.	[49]
Trichomonas vaginalis triosephosphate isomerase (TvTPI)	An *in silico* screening (MolDock) of a library of 952 antiprotozoal phytochemicals was carried out against TvTPI. Only the chalcone 2',4,4'-trihydroxy-3',5'-diprenylchalcone showed promising docking to TvTPI.	[49]
Trypanosoma protein targets	Molecular docking of phytochemical agents to molecular targets in *Trypanosoma* spp. has been recently reviewed and will not be included here.	[47]

4. Reverse Docking

Reverse docking is a computational method used to determine potential protein targets for an active natural product. In this method, the phytochemical of interest is docked to all of the three-dimensional protein structures in a database. Potential drug targets are then evaluated based on the relative docking scores (Fig. 2). Reverse docking is often used to identify a mechanism of activity of a bioactive natural product [211].

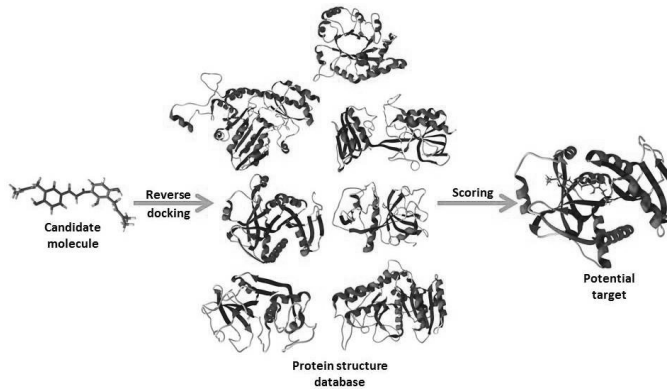

Figure 2. Scheme of reverse docking. A small molecule potential drug (e.g., a phytochemical) is used to probe a protein database for potential binding sites of macromolecular targets. Potential drug targets are ranked based on docking scores.

Genomic technologies have led to the identification of numerous potential targets for diseases and infectious agents. A protein drug target database is prepared using known three-dimensional protein structures (e.g., from the Protein Data bank, PDB). Alternatively, protein structures can be prepared using homology modeling based on proteins of known structure. There are several protein drug target databases currently available, including:

- DrugBank [https://www.drugbank.ca/],
- Therapeutic Targets Database (TTD [http://bidd.nus.edu.sg/group/cjttd/]),
- Potential Drug Target Database (PDTD [http://www.dddc.ac.cn/pdtd/]),
- TarFisDock (which utilizes PDTD [http://www.dddc.ac.cn/tarfisdock/]), and
- sc-PDB [http://bioinfo-pharma.u-strasbg.fr/scPDB/].

Because reverse docking is just a molecular docking method, it suffers from the same drawbacks that molecular docking suffers from (see above). Additionally, because many protein structures are likely to be sampled, the computational time can be very high. Recent examples of reverse docking in natural products drug discovery are summarized in Table 10.

Table 10. Recent reverse docking studies of biologically active phytochemicals.

Active Phytochemical	Target(s) Identified Using Reverse Docking	Ref.
Berberine (epigenetic modulation)	Lysine-*N*-methyltransferase	[212]

Active Phytochemical	Target(s) Identified Using Reverse Docking	Ref.
N-trans-Caffeoyltyramine (antibacterial)	Helicobacter pylori peptide deformylase	[213]
Curcumin (cancer)	Cyclin-dependent kinase 2 (CDK2)	[214]
Dioscin (anti-tumor, anti-hyperlipidemic, anti-fungal, antiviral, hepatoprotective)	Cyclin A2, calmodulin, hemoglobin subunitβ, DNA topoisomerase I, DNA polymerase λ, nitric oxide synthase, UDP-*N*-acetylhexosamine pyrophosphorylase	[215]
Epicatechin (antineoplastic)	CYP450, histone deacetylase, dihydrofolate reductase	[216]
Epicatechin gallate (antineoplastic)	Leukotriene A4 hydrolase, mammalian PCB-binding protein, CYP450, histone deacetylase, dihydrofolate reductase	[216]
Epigallocatechin (antineoplastic)	CYP450, dihydrofolate reductase	[216]
Epigallocatechin gallate (antineoplastic)	Leukotriene A4 hydrolase, farnesyl protein transferase, mammalian PCB-binding protein, CYP450, histone deacetylase, dihydrofolate reductase, hepatopoietic prostaglandin synthase, TGF-β receptor type I	[216]
Esculentoside A (anti-inflammatory)	Casein kinase 2 (CK2)	[217]
[6]-Gingerol (cancer)	Androgen receptor (AR)	[218]
Ginsenoside Rf Ginsenoside Rh4	Dual specificity mitogen-activated protein kinase 1 (MEK1)	[219]
Ginsenoside R2	Epidermal growth factor receptor (EGFR)	[219]
Glycopentalone (cancer)	Cyclin-dependent kinase 2 (CDK2)	[220]
Pyrrolizidine alkaloids (hepatotoxic)	Glutathione *S*-transferase A1 (GSTA1) and glutathione peroxidase 1 (GPX1)	[221]
Rhodomyrtone	Methicillin-resistant Staphylococcus aureus (MRSA) dihydrofolate reductase (DHFR), MRSA filamenting temperature-sensitive (FtsZ) protein	[222]
Triptonide (anti-inflammatory, immune-suppressive, anti-tumor)	Human estrogen receptor α (ERα)	[223]

5. Quantitative Structure-Activity Relationships

Non-structure-based *in silico* modeling techniques such as conventional quantitative structure-activity, 3D-QSAR and pharmacophore modeling have been increasingly relegated to adjunct methods within natural products drug design in recent years, and represent smaller and smaller fractions of the computational efforts reported in literature, compared to structure-based techniques. Computational researchers don't need to be focused solely on natural products to find leads, so they tend not to. Nevertheless, there is a well-founded assumption that, since many of the drugs currently in use today are based on structures found in nature, natural products represent a rich source of privileged structures [224] that avoid some of the drawbacks to which synthetic molecules are prone in bringing a drug to market, such as toxicity and side effects. So, there are still examples to be found in the literature.

QSAR, as applied to natural products – especially in the arena of drug design and medicinal chemistry [225] – presents a special (but not unique) problem for modeling, as the chemical diversity is so large when considering the breadth and depth of scaffolds represented in the plant and animal kingdoms, as represented in the ~150k compounds in the CRC *Dictionary of Natural Products*. Simple structure-activity relationships tend not to yield models with reliable predictivity because they must span so large a chemical space. Typically, combinatorial chemistry is more focused and does not span this diversity of chemical space, where a simple QSAR can reliably give good predictivity for the activity of a set of chemically-similar congeners. The points in chemical space where QSAR's consistently fail to predict biological activity *in vitro* or *in vivo* are referred to broadly as "activity cliffs" [226,227], and a great deal of time and effort has been spent investigating the chemical spaces where failures occur.

5.1 Conventional QSAR

In principle, quantitative structure-activity relationships are a way of describing the changes in activity that result from changes in chemical topology and are, in principle, best described by simple linear responses in the fewest number of variables needed to relate chemical structure to observed activity (see Table 11 for software packages that include QSAR). Ideally, this approach allows for a straightforward calculation and a straightforward interpretation. QSAR in practice, however, often involves a complex relation that uses many chemically-related variables, resulting in a description that is often nebulous and vague, even though the Pearson coefficient with activity may be very nearly 1.00. For QSAR's that are considered to be widely applicable, derived from a large training set of (relatively) diverse chemical structures, an $r^2 \geq 0.9$ is often, and unfortunately, a

good indication of model overfitting. And this is usually borne out in significant prediction errors in the test set. Since the QSAR model is intended to generalize, an r^2 of approximately 0.7 is a perfectly respectable value, provided the test set predictions are within the standard error of the training set. And there are those QSAR expressions that are refreshingly short and sweet, involving only one or two terms, and with respectable r^2 statistics, but are composed of curious terms such as the HOMO-LUMO gap for structures that are non-covalent inhibitors.

Table 11. Some software packages used in QSAR.

Program	Source
RDKit	http://www.rdkit.org/docs/index.html
Chemaxon/JChem	https://www.chemaxon.com/
DRAGON	http://www.talete.mi.it/products/dragon_description.htm
VolSurf+	http://www.moldiscovery.com/software/vsplus/
KNIME	https://www.knime.org/
Molecular Operating Environment	https://www.chemcomp.com/MOE-Molecular_Operating_Environment.htm
Schrodinger Suite	https://www.schrodinger.com/
MayaChemTools	http://www.mayachemtools.org/
ROCS	https://www.eyesopen.com/rocs
Various cheminformatics tools	http://silicos-it.be.s3-website-eu-west-1.amazonaws.com/software/software.html
OpenBabel	http://openbabel.org/wiki/Main_Page
Open3DQSAR	http://open3dqsar.sourceforge.net/
ChemGPS-NP	http://chemgps.bmc.uu.se/batchelor/queue.php?show=submit
DataWarrior	http://www.openmolecules.org/datawarrior/
SpotFire	https://spotfire.tibco.com/
Statistica	http://statistica.io/products/
WEKA	http://www.cs.waikato.ac.nz/ml/weka/

Chemical space analysis is a method of projecting vector descriptions of molecules into an *n*-dimensional space to evaluate similarities that may not be apparent from structure alone. Dimensionality reduction methods such as principal component analysis (PCA) or singular value decomposition (SVD) are then used to render (sometimes large sets of) conventional QSAR descriptors

of compound sets into a reduced orthogonal space. Euclidean distances are then calculated for pairs of compounds to determine similarities. Since the latent factor analysis methods return factors that are composites of input variables in order of the fraction of the variance in the input space, oftentimes only the first few output variables are needed to describe the reduced chemical space. This is handy for viewing the space in three dimensional plots, provided an acceptable percentage of the variance is explained with the first three factors. Care must be given not too place too much emphasis on the distance metric in these cases, however. Since the original descriptor space was more than likely non-Euclidean (*e.g.*, how many oxygen atoms equal one logP unit?), the transformed space is also non-Euclidean. A standardized chemical space using only natural products from the *Dictionary of Natural Products* has recently been made into a web-accessible analysis tool, ChemGPS-NP [228], and appears in several recent articles. Some recent studies using conventional QSAR methods are summarized in Table 12.

Table 12. Recent works with conventional QSAR components.

Target/Activity	Comments	Ref.
Human acetylcholinesterase	A brief review of studies using flavonoids, alkaloids and xanthones against targets associated with neurogenerative diseases, followed by cross-validated CADD of a set of 469 alkaloids from the dogbane family (Apocynaceae) with known activity in human AChE using random forest training with VolSurf+ descriptors and Molegro docking results. Nine alkaloids presented as hits.	[229]
Trypanosoma brucei	A set of 85 alkaloid structures with associated activities in *T. brucei* from the ChEMBL database were examined with both docking (target: trypanothione reductase) and QSAR methodologies; random forest models from VolSurf+ descriptors.	[230]
Methicillin-resistant *Staphylococcus aureus*	Synthesis and QSAR study of phenazine derivatives of endophenazine G as anti-MRSA agents	[231]
Human myeloperoxidase	Synthesis and QSAR analysis of xanthone derivatives as free radical scavengers and prooxidant enzyme myeloperoxidase inhibitors.	[232]
Anti-leishmanial	Conventional QSAR of piperine alkaloid derivatives was modeled using in vitro activity against *L. donovani*.	[233]

Target/Activity	Comments	Ref.
Anti-leishmanial	Conventional QSAR of sesquiterpene lactones was modeled using *in vitro* activity against promastigotes of *Leishmania amazonensis* and *Leishmania braziliensis*.	[234]
Human monoamine oxidase B	Virtual screening of 108 caulerpin alkaloid analogs using – including docking – drug-likeness scoring with VolSurf+ descriptors and KNIME.	[235]
Anti-leishmanial	A chemical space analysis of *Leishmania* metabolites using ChemGPS-NP; one compound (ixoside) isolated from the stem bark of *Tecoma mollis* identified as likely to target nucleoside diphospate kinase.	[236]
mitogen-activated protein kinase P38, MAP kinase-activated protein kinase 2	A chemical space analysis of nine known HSP27 phosphorylation inhibitors using ChemGPS-NP was performed; 37 natural product structures identified and molecular docking followed.	[237]
Various targets in neglected diseases: *Trypanosoma*, *Leishmania*, and *Plasmodium*	Review of studies of natural products used in drug discovery that include neural networks.	[230]

5.2 "Bleeding Edge" QSAR

QSAR descriptors such as molecular weight and logP, typically used by groups in the West (the "Western School"), tend toward a more straightforward calculation and interpretation, while the current trend coming out of Asia (the "Eastern School") is toward more exotic, quantum-mechanically-derived values with dubious theoretical underpinning (*e.g.*, HOMO and LUMO energies, HOMO-LUMO gaps and some forms of condensed Fukui functions [238]2p). It is somewhat surprising to see these values used so often as QSAR descriptors, given the (often large) sources of unsystematic error introduced by the methods employed to calculate them – regardless of how well, or how poorly, they may correlate with activity. Inevitably these energies are calculated in the gas phase for the ground state molecule with density functional theory using the B3LYP functional and the big 6-311G Pople split-valence basis set with polarization and diffuse functions. It is commonly accepted in theoretical chemistry that accurate LUMO energies must be calculated for the excited state [239] – and that generalized gradient approximation (GGA) functionals are exceptionally bad at calculating LUMO energies from the ground state [240]. And while B3LYP is not

prone to wild errors stemming from basis set incompleteness error, correlation-consistent (Dunning) basis sets that minimize this error from first principles would seem to be a more logical choice. And that is to say nothing of the physical interpretation of such descriptors as applied to non-covalent interactions in biological systems. Works using newer QSAR descriptors are listed in Table 13.

Somewhat ironically, where the "Eastern School" typically uses more straightforward statistical analytical techniques to derive a QSAR expression (*e.g.*, linear regression, multiple linear regression, partial least squares regression, *etc.*), the "Western School" seems to favor far more intensive, non-linear machine learning methods such as Bayesian networks, support vector machines and even swarm intelligence in determining a relationship to activity. While the process of adding or removing terms to evaluate the goodness of fit may remain the same, if there is no substantial change in the metric, how does one properly evaluate the robustness of such models? So, at the risk of making a sweeping generalization, QSAR in Asia tends to get troublesome in the descriptor end of the pool, while QSAR in Europe and North America tend to get troublesome in the training algorithm end.

Table 13. Recent works using newer descriptors or modeling schemes.

Target/Use	Comments	Ref.
Cucumis trigonus and *Cucumis sativus* fruit extracts	Density functional theory QSAR relating quantum chemical properties to biological activity for a set of phytochemicals.	[241]
Molecular docking	Free energies of binding were calculated using QSAR-derived parameters from ~600 ligand-receptor complexes with associated IC_{50} data as corrections to docking scores.	[242]
QSAR model validation	Test cases evaluating QSAR models using the metric	[243]

In an effort to standardize best practices in QSAR, a joint workshop by the European Chemical Industry Council (CEFIC) and the International Congress and Convention Association (ICCA) held in Setubal, Portugal in 2002, established a set of standards that were then submitted to the U.S. Organisation for Economic Co-operation and Development (OECD). Referred to as The Setúbal Principles, the group agreed that, under best practices, QSAR models should have:

1) A defined end point
2) An unambiguous algorithm
3) A defined domain of applicability

4) Appropriate measures of goodness-of-fit, robustness and predictivity
5) A mechanistic interpretation (if possible)

Depending on whether the emphasis is mechanistic or statistical, these may be prioritized in a different order. And noting that *all* of the principles cannot, in practice, be satisfied simultaneously, a compromise between interpretability and predictivity must always be struck, *i.e.*, one can construct a model that predicts well, but is not easily interpreted *or* one may have a model that is readily interpretable, but does not always predict well – *generally speaking*, one cannot have both. Studies that do not adhere closely with these principles are abundant and a healthy skepticism should be enjoined when the QSAR appears either too byzantine or is poorly described. As with all things, *caveat emptor*.

So what makes for a "good" QSAR approach? In terms of a set of descriptors, one might look at those that figure most prominently in factor analyses of chemical diversity. For instance, Larsson *et al.*[228] found that 77% of the variance in natural products structures is captured by the first four principal components of the ChemGPS-NP method. The first of these encodes normalized descriptors of size, shape and polarizability; the second, aromatic rings and conjugated bonds; the third, lipophilicity and H-bond donor/acceptors; and the fourth, flexibility. And this is typical of the results one gets when one performs PCA on any large data set – even when one includes novel descriptors. It could, of course, be the case that a descriptor that contributes little to the variance of a data set nevertheless correlates well with a local trend in activity. The task then becomes one of justifying the correlation in light of an absence of one in the larger population, and this, it seems (to these authors), to strain the bounds of more than one of the tenets set forth in The Principles. Likewise, the pursuit of ever more elaborate mathematical formulations to divine activity correlations that defy ready interpretation seems counterproductive to the goals of a standardized QSAR protocol.

5.3 Molecular Fingerprints

A useful method for screening large sets of molecules quite rapidly is to represent them as molecular fingerprints. These strings of numbers or characters capture generalized chemical features across various structures in either two or three dimensions and are used with clustering methods or similarity searching to rank similar structures, usually with a metric like a Tanimoto or Tversky coefficient. These values are then used in comparison with activity values to establish a structure-activity relationship. Fingerprints such as MACCS keys [244] encode various chemical structural features (number of sulfurs, number of multiple bonds, *etc.*) as a string of integers, or as longer stretches of bits. See Table 14 for recent studies using molecular fingerprints.

Pharmacophores are frequently represented by components such as H-bond donors and acceptors, hydrophobic and ring structures as points, or volumes, in space. When screening against a database of compounds, this allows for a fast, "fuzzy" selection scheme whereby ligand structures may be quickly rendered as pharmacophores and aligned to and compared with a template pharmacophore. The pharmacophore may be generated from a known active, a set of aligned actives, or from complementarity with pharmacophoric features in the binding site of a receptor molecule. Scaffold hopping is a trending technique whereby a chemical structure is represented by either a set of pharmacophoric features in three dimensions, two-dimensional substructure or a fingerprint, which is then used to query similar structures. This allows for the identification of distinct, but structurally similar, molecular fragments that may function as common pharmacophoric features in ligand-receptor complexes.

Table 14. Recent works utilizing molecular fingerprints.

Target/Activity	Comments	Ref.
Various targets: drug-likeness	A set of 6320 compounds from herbal databases (TCMID, TCM-ID, KTKP and KAMPO) with some reported (traditional) use against diabetes, asthma or hypertension were screened for OpenBabel FP2 fingerprint similarity with known actives in G-protein-coupled receptors, ion channels, enzymes, receptors, transporters, and other proteins in the DrugBank database.	[245]
Anti-malarial	A statistical/QSAR analysis of of 1040 natural products with reported *in vitro* anti-plasmodial activity was performed to generate "cheminformatic profiles" of anti-malarials; structural and chemical similarity analysis with current anti-malarial drugs was performed using MOE QSAR descriptors; activity cliff and ChemGPS-NP chemical space analyses were also performed using DataWarrior and KNIME.	[246]
Various: drug-likeness	A variety of fingerprints, such as MACCS keys, were used to compare the structures of known drugs and metabolites (Recon2 [247]) by similarity scoring with structures in The Dictionary of Natural Products and The Universal Natural Products Database using KNIME.	[248]

Target/Activity	Comments	Ref.
Anti-cancer	MACCS keys for 21,334 compounds in the Traditional Chinese Medicine (TCM) database were compared with known actives in the NCI database.	[249]
Human cyclooxygenases	Fingerprint QSAR modeling of *cis*-stilbene derivatives as COX-1 and COX-2 inhibitors	[250]
Human monoamine oxidase B and cyclooxygenase 1	Ligand-target fingerprints were used to construct models to screen structures, including natural products, from the ChemBL database as MAO-B and COX-1 inhibitors; natural product hits	[251]
Trypanosoma brucei glyceraldehyde-3-phosphate dehydrogenase	A database of 700 natural products was screened as G3P dehydrogenase inhibitors using pharmacophore scoring in MOE along with molecular docking.	[252]
Methicillin-resistant *Staphylococcus aureus*	Part of this work involves scaffold hopping using 2D descriptors and MACCS substructure keys to screen the ZINC database for compounds similar to the natural product hamamelitannin.	[253]

5.4 3D/4D-QSAR

So called 3D-QSAR uses some form of a particular conformation of a known active as a template for aligning query structures to find common physical and chemical topologies. What is most often used is some variation of Comparative Molecular Field Analysis (CoMFA). In the age of structure-based methods, CoMFA is still useful in teasing out a QSAR (and in 3D, no less!), but is subject to a substantial amount of unsystematic noise. It is well known by now that rotating or translating the electrostatic and steric grids result in relatively large variations in the r^2 and q^2 of the model. And in a model where a "good" q^2 is often somewhere between 0.4 and 0.6, this can be disheartening to learn. Modifications to this general method give us the 4D (or even *n*D) methods that include some form of conformational analysis in the alignment of the template structure, as well as that of the query structures. Recent works using multidimensional QSAR are summarized in Table 15.

Table 15. Recent works using nD QSAR.

Target/Activity	Comments	Ref.
Glycogen phosphorylase inhibitors	From a book on natural product antidiabetic agents. In Chapter 2, sections 3 and 4, there is a description of a number of naturally-occurring compounds analyzed using 3D-QSAR and compared with allosteric inhibition data for GP. These studies also include docking, molecular dynamics and QM/MM components.	[254]
Anti-trypanosomals	Eighty-four anti-trypanosomal natural products were evaluated for endocrine and metabolic disruption in humans using the 4D-QSAR tool *VirtualToxLab*.	[255]
Anti-Chagas	A CoMFA analysis of 13 coumarins relate anti-trypanosomal activity to three-dimensional structure.	[256]

6. Conclusions

In silico methods have provided opportunities for rapidly screening large libraries of natural products for drug likeness, target binding and selectivity, and pharmacophore development in drug design. And drawing on the many instances of drugs developed from natural products, drug design, on the whole, has kept a close association with the natural products community, albeit not always an exclusive one. And computational researchers in drug research have followed suit, developing methods and databases specializing in natural products in pursuit of not just a pharmaceutical windfall, but also a mechanistic picture of drug-receptor interactions. The computational methods highlighted in this review represent the state of the art in the ongoing development of a refined encoding of chemical intuition and insight into the small molecule modulation of disease states. Each has a valuable part to play in drug design and each has inherent limitations, and must be applied with an eye toward value added to the process as a whole.

Conflicts of Interest

The authors declare no conflict of interest.

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Chapter 2

Topics on metabolomics

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1. Introduction

Metabolomics is an emerging science, aimed at the comprehensive and integrated study of a wide range of low-molecular-weight metabolites, including global screening approaches and quantitative and/or qualitative analysis of the entire metabolome present in a biological system, such as a specific cell, tissue, or organism [1]. Metabolomics studies focused on plants are rapidly increasing in number, and different applications are currently available in the literature.

Metabolomics studies have been classified mainly into two groups: targeted and untargeted analysis. The choice of the most suitable metabolomics approach will determine the strategy of data analysis. Targeted metabolomics studies focus on the identification and/or quantification of one, or a set, of compounds, and typically require a high level of purification and selective extraction of the metabolites [2]. Untargeted metabolomics studies focus on the detection of as many metabolites as possible, through comprehensive analysis of the entire metabolome in complex matrices, obtaining fingerprints, but not necessarily requiring identification nor quantification of specific compounds [3,4]. By comparison, traditional phytochemical methods generally focus on the isolation and quantification of major compounds in a given plant matrix, usually evaluating their potential biological activities. Such an approach represents a challenge to studying interactions between major and minor compounds in complex matrices, and screening significant compounds responsible for a particular biological inquiry.

The overall goal of metabolomics is, therefore, to identify chemical features against a large and complex background matrix that uniquely defines a biological system [5]. Qualitative and quantitative analysis of the entire metabolome of a complex matrix is a very ambitious goal, however, and represents a challenge due to several limitations. The metabolome represents a complex and vast array of compounds, with a very broad spectrum of physicochemical properties (polarity, solubility, etc.). These compounds belong to a wide range of chemical classes,

from simple chemical structures, such as amino acids, lipids, organic acids, and nucleotides, to more complex ones, such as alkaloids, terpenoids, phenolics, etc. The metabolome composition is very dynamic, and may vary depending on several conditions to which the matrix is exposed. A multifaceted and fully integrated strategy must be well defined for sampling, optimum metabolite extraction, data acquisition, processing, and analysis, thus ensuring that the generated information is enough to answer the initial biological question.

This holistic approach provided by metabolomics is driven primarily by recent advances in separation (e.g., gas chromatography [GC], ultra-high-performance liquid chromatography [UHPLC], capillary electrophoresis [CE]) and detection (e.g., mass spectrometry [MS], nuclear magnetic resonance [NMR], diode array detector [DAD], flame-ionization detector [FID]) techniques [6], and bioinformatics development. In metabolomics, the most common techniques used for metabolite profiling and metabolic fingerprinting are GC coupled with MS (GC–MS), liquid chromatography coupled with MS (LC–MS), and NMR [7]. Many other techniques have been reported in metabolomics, including thin-layer chromatography (TLC), diode array detector (DAD), Fourier-transform infrared spectroscopy (FTIR), and Raman spectroscopy [1]. None of these techniques are able to simultaneously monitor the entire metabolite range of a given matrix, however. Consequently, there is no single method that is able to reach the ultimate goal of analyzing all metabolites in a given matrix. The choice of separation and detection methods for metabolomics studies is case specific, depending on the purpose of the metabolomics analysis [4].

Besides advances in analytical techniques, the constant development and improvement of bioinformatics/chemoinformatics also plays a very important role in the establishment of metabolomics as having real potential in several fields of research. In order to understand the huge amount of experimental data obtained from metabolomics, bioinformatics is being combined with chemoinformatics with the aim of generating a basic computational infrastructure for metabolomics data processing and analysis [1]. Interpretation of the acquired information requires the use of appropriate algorithms and mathematical models to manipulate and process the large raw datasets, and to provide workable and understandable biological interpretations. The most popular unsupervised methods used for metabolomics data analysis are principal component analysis (PCA) and hierarchical cluster analysis (HCA). The most common supervised methods currently used are partial least squares discriminant analysis (PLS–DA), orthogonal projections to latent structures discriminant analysis (OPLS–DA), and artificial neural networks (ANNs) [8].

In summary, metabolomics may come up with more accurate and sensitive methodologies, enabling a quick and efficient acquisition of data, and the processing of huge and complex datasets. Therefore, metabolomics plays a very important role in system biology research, providing a real alternative for faster and more accurate discoveries of new bioactive compounds, carrying out quality control of herbal medicines, studying food plants, food and dietary supplements, searching for disease markers, and for chemotaxonomic studies and the study of plant interactions with the environment, among many other possibilities [4,9].

This chapter will focus on the discussion of key points that are involved in plant metabolomics.

2. Experimental topics

2.1 Biological questions to be answered, or clarified, by metabolomics studies

The first question to be raised in order to initiate the setup of a protocol for metabolomics studies is the intended use/application of the study. Metabolomics studies represent a powerful tool for contributing to clarifying and understanding the biological system, and it is essential that its goals are very well assigned and properly defined before planning the entire study. In order to precisely set up the several experiments, and organize all of the steps involved, for a metabolomics study, answering some simple questions may contribute to finding the one that really needs to be answered upon concluding the study (Figura 1).

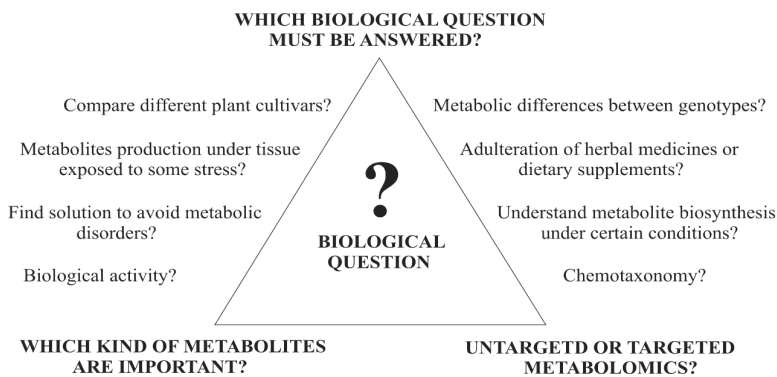

Figure 1. Examples of types of biological question to be answered by metabolomics studies.

In general, this procedure is underestimated and, frequently, not enough information is used to support the chosen metabolomics approach and the intended biological question to be answered. This scenario may represent a great source of error that may appear in the subsequent steps, and be an issue in planning the experiments in a rational and effective way. Consequently, such errors may lead to the lack of important information that should have been included in a robust metabolomics study, ending with an erroneous interpretation of the acquired data. Thus, the initial question will guide the effective planning of the subsequent experiments, and may be refined, based on the study outcomes.

2.2 Sample preparation

2.2.1 Sampling

Sample preparation plays an important role in metabolomics, since it represents the primary source of non-biological variability [10]. An initial step in metabolomics is usually to carry out cell quenching immediately after sampling the tissues. As soon as the tissues have been harvested, the cellular enzymes should immediately be inactivated, and the metabolic pathways halted, in order to minimize metabolite degradation and metabolite composition alteration, ensuring that the naturally occurring metabolite composition represents the normal living cells within a minimum external variability [11]. Quenching is not always mandatory in metabolomics studies [12,13], but it is a critical procedure, depending on the application of the study. There is a lack of studies comparing metabolites from frozen and fresh (*in natura*) plant material. Indeed, studies that aim to identify active compounds against a pharmacological target (within a drug discovery framework) do not necessarily need to use frozen plant material, since such a procedure could be a challenge in terms of pharmacological bioprospecting. The use of quenching protocols depends on the research goal, and many different protocols have been described. The most common procedure for animal and plant cells is the use of liquid nitrogen, or even the use of cold methanol, to keep the sample temperature below -20°C [14]; however, the use of these processes to stop enzymatic activity may be a challenge, depending on the circumstances, such as plant collection in the wild.

The concentration of metabolites and the metabolic profiles are totally dependent on the growth stage of the plant and its environmental conditions. An interesting strategy to accommodate this is to standardize harvest time (e.g., day and/or season), and also to sample different tissues at different vegetative stages to standardize the height of the harvest point from the apex, in order to provide a more realistic and representative sample set for a metabolomics study [15].

Since each tissue analysis represents a single fingerprint of the sample, enough tissue replicates (biological replicates) and sample preparation replicates (technical replicates) from the same batch of tissue should be analyzed in order to contribute to the estimation of biological variation in the sample [16]. The biological replicates contribute to accessing variability in the population of study, and the technical replicates contribute to accessing the variability in sample preparation and methodology.

Another important aspect related to the sampling step is that the use of biological and technical replicates depends on the study goals [16]. The biological replicates aim to verify the occurrence of metabolite profile differences within, or between, populations of the same species, or to verify the metabolite profile differences of a species during different periods (circadian or seasonal). One of the most important points to be considered is the standardization and reporting of the harvesting conditions of the plant material, not only the presence or absence of biological replicates in the metabolomics analysis. Technical replicates aim to ensure quality control of the extraction and data acquisition of the metabolites, being a tool for monitoring the reproducibility of certain features (such as chromatographic retention times), and the detection of certain metabolites.

The use of replicates in metabolomics may greatly increase the amount of samples to be analyzed and, consequently, increase the time taken for data acquisition, as well as the cost of the analysis. One strategy is to combine portions of available samples (make a pool) and prepare technical replicates [17]. Another possible strategy is to randomly choose a sample and make technical replicates only of that [12]. PCA score plots can be used to ensure quality control of that extractions and data acquisition [17].

2.2.2 Metabolite extraction

In general, biological tissues represent a complex matrix containing a wide number of metabolites from different classes of compounds and, consequently, the physicochemical properties of such compounds are highly variable [18]. Thus, metabolite extraction is often a rate-limiting step [11], representing one of the challenges in metabolomics.

For untargeted metabolomics, the number and amount of metabolites extracted should be maximized in order to contribute to the goal of acquiring a realistic snapshot of the whole metabolome of a given tissue. The use of optimization protocols for extraction and sample preparation is extremely important, so as to enable the detection of as many metabolites as possible [11]. These protocols should provide a given number of studies to justify the best conditions for extraction and sample preparation, including reproducibility and

replicability studies. The conditions of extraction should be validated, in addition to the analytical method, to acquire a relevant and reproducible metabolic profile that can be used in the subsequent steps of the study.

After harvesting (and quenching/drying, when necessary), tissues are usually homogenized into smaller particles, in order to increase the surface area, to facilitate metabolite extraction [14]. Different protocols have been used to homogenize tissues, such as grinding with a mortar and pestle and liquid nitrogen, milling in vibration mills [11], and using ball mills or analytical mills [16].

In general, the homogenized biological tissues are mixed with organic solvents and aqueous solutions at different ratios, at low or high temperatures [19]. Some additives may be used in order to change the pH of the extraction medium to enhance the extraction of a certain class of metabolite, or even to avoid degradation of specific compounds expected to be present in the matrix; for instance, adding butylated hydroxytoluene (BHT) to the extraction solvent for tissues containing oxygen-sensitive compounds, such as carotenoids and polyunsaturated fatty acids. Ultrasound-assisted extraction is one of the most common strategies used for metabolite extraction; however, alternative extraction techniques include microwave-assisted extraction (MAE), solid-phase extraction (SPE) [20], supercritical fluid extraction (SFE), subcritical water extraction (SWE) [21], pressurized liquid extraction, and extraction by orbital shaker [12].

In a recent study, Bijttebier *et al*. compared different extraction protocols for plant untargeted metabolomics. Extraction protocols were tested by analyzing two plant matrices by UHPLC–UV–MS, covering a wide range of compound properties––meadowsweet (*Filipendula ulmaria* (L.) Maxim., Rosaceae) and spicy paprika powder (*Capsicum*, Solanaceae). Meadowsweet contains high amounts of polar and moderately polar compounds, such as phenolic acids, glycosylated flavonoids and its aglycones, and hydroxycynnamic acids, and is very rich in tannins. On the other hand, spicy paprika contains high amounts of non-polar compounds, such as lipid-soluble vitamins, carotenoids, capsaicinoids, and phytosterols [22]. Based on their outcomes, the protocol using only ethyl acetate as a solvent has been shown to extract only a small fraction of the polar compounds, in comparison with other extraction methods that used water and allowed extraction of the more polar compounds.

A strategy, combining ethyl acetate and water, has also been tested that led to a significant increase in the extent of extraction of polar compounds, such as some phenolic compounds; however, it extracted less tannins, but represents an interesting approach to be studied in the extraction protocol development phase in metabolomics studies. Bijttebier *et al*. also observed better extraction of some phenolic compounds using ethyl acetate:water protocols, instead of using

only polar methods [22], such as those developed by De Paepe *et al*. [23], using water:methanol 4:1, 40 mM ammonium formate buffer, and ultrasound-assisted extraction. The protocol used by Bijttebier *et al*., using chloroform:methanol:water, has also been revealed to be a good strategy for extracting more polar compounds (tannins and phenolic compounds), compared to the ethyl acetate extraction protocol; however, it showed low recovery results for those compounds [22]. In contrast to the results obtained by Bijttebier *et al*. from using ethyl acetate protocols, Halabalaki *et al*. investigated the variation in metabolite composition of *Vitis* (Vitaceae) wood cultivars, using only ethyl acetate as solvent, enabling the extraction of a significant range of secondary metabolites, and also efficiently extracting moderately polar compounds, not only less polar compounds [24].

Bijttebier *et al*. also extracted chilli pepper powder by continuous extraction, using accelerated solvent extraction (ASE). The sample was loaded into a SPE column and extracted under a continuous solvent flow, using an increasing solvent polarity elution sequence (*n*-hexane, acetone, methanol, and water), resulting in 39 fractions. The resulting 25 most non-polar fractions were combined and analyzed, and the content of the most polar compounds was compared to the results provided by a non-polar reference method, which used acetone:methanol 7:3 (v/v) and BHT 0.1% as extraction solvents, with the addition of NaCl aqueous solution in back-extraction with *n*-hexane. The most polar compounds in chilli pepper (capsaicinoids and glycosylated phytosterols) were detected in high relative abundances by the ASE method; however, using the non-polar reference method, the extraction of the most polar compounds was not favored [22]. Another study used a mixture of methanol and acidified aqueous solution, at a final concentration of 75% methanol (v/v) and 0.1% formic acid (v/v), as the most suitable solvent for the extraction of secondary metabolites from different *Arabidopsis* Heynh. (Brassicaceae) species, using ultrasound-assisted extraction, producing optimum conditions for metabolomics studies [16].

In summary, no extraction protocol is able to exhaustively extract the wide range of metabolites present in a given matrix. Moreover, metabolite extraction is highly dependent on the sample matrix under evaluation [22]. Since metabolomics involves the metabolite profile of a given complex matrix [14], which significantly differs from that of every other organism (different species, varieties, harvesting seasonality, quenching protocol, etc.), the selection of an optimum extraction procedure should be evaluated for each biological matrix, and the existing extraction protocols available in the literature should not be directly applied to a new metabolomics study before the extraction conditions are studied and validated for the new application. The extraction protocol should be developed and validated, in addition to the chosen analytical methodology, before

being applied to metabolomics studies, by assigning a few suitable acceptance criteria, such as extraction efficiency evaluation, matrix effect on analytical signal acquisition, recovery and repeatability evaluation [22]. Moreover, the choice of extraction protocol should be considered to be a compromise between providing a good extraction efficiency and replicability, robustness, time-consumption and less complex sample preparation methods, considering that, in general, a large number of samples are analyzed in metabolomics studies.

A simple approach to determining the best conditions for metabolite extraction is the use of design-of-experiments (DOE) procedures, instead of one-factor-at-a-time methods. DOE procedures assist in the rapid assignment of experimental variables and interactions, which may impact significantly on the results (e.g., metabolite extraction). The results can be measured in one, or in several, ways [25].

2.2.3 DOE procedures applied to the optimization of metabolite extraction conditions

The approach used by experimental design is so that multivariate data can be fitted to an empirical function (linear or quadratic, with interaction terms), which can be used to provide information about the system (variable values to generate maximum and minimum response, and trends in response) [26].

In other words, experimental design evaluates the influences of experimental variables and interactions, and how they effect on the responses [19]. A full factorial design can estimate the main effects, and all interaction effects, by setting up L^K experiments, K being the number of variables, and L the levels to be studied, where the minimum number of levels is two. As the number of variables increases, the number of required experiments will also increase. For instance, a two-level full factorial design, evaluating seven variables, would need to set up a matrix of 128 experiments (2^7 = 128 experiments). Fractional factorial design is a subset of full factorial design, and may be set up when the number of identified variables is relative high (e.g., more than five factors). Other experimental methods include the Plackett–Burman, central composite, Box–Behnken, Doehlert, D–optimal, G–optimal, and mixture designs [26].

A very common strategy used is the setup of an initial screening study, using one of the experimental designs mentioned above, in order to evaluate the variable effects and their interaction with the measured response(s). As soon as the primary significant variable effects and their interactions are evaluated, a second set of experiments may be set up in order to refine and optimize the model, by excluding the variables that were shown not to be significant to the model [25]. In general, two different strategies for optimization are used––

simplex optimization and response surface methodology. The response surface methodology determines an optimum for the measured response, and the simplex optimization only encircles the optimum region for the measured response [25].

In order to apply this strategy to optimize metabolite extraction conditions, the variables (factors) to be studied should be chosen based on the target compounds to be analyzed, and on the analytical methodology used. Therefore, first of all, the metabolomics approach (e.g., targeted or untargeted metabolomics) must be assigned.

The most common variables studied, using experimental design for metabolite extraction optimizations, are: type of organic solvent; proportion of organic solvent and aqueous solution mixture; solvent:sample ratio; time of extraction; number of extraction cycles used; temperature of extraction; type of procedure to assist extraction (ultrasound, MAE, SFE, SWE, etc.); addition of additives to enhance extraction; and reagents for the derivatization reaction prior to analysis by a given analytical technique. It is highly recommended that the identification of the suitable variables to be evaluated in the screening study, as well as the variable values assigned for the optimization of the model, are made case-by-case, and based on the prime goal of the metabolomics study, analytical technique, and nature of the matrix studied. The method for extraction should also be reproducible, and recovery studies of prime interest metabolites should be conducted. The experiments should be performed in a random order to eliminate the effects of uncontrolled variables that might vary over the experiments (time-dependent changes) [16].

2.3 LC–MS data acquisition

As already mentioned in the introduction, many techniques should be used to obtain the biological matrix fingerprint and metabolic profile in metabolomics. The technique should be chosen based on the biological question to be answered, its applicability, and the limitations of analysis. This section will focus only on metabolome fingerprint acquisition by LC–MS.

The analytical method can be developed, using experimental design methods, to optimize variable values related to the chromatographic method to improve metabolite separation, metabolite ionization, and detection by MS. This strategy may shorten the method development in such a way that a maximum of information can be gained from a minimum number of experiments.

In untargeted metabolomics, the goal of the LC method is usually to provide a chromatographic profile with a minimum number of metabolite co-elutions as possible, narrower peak widths, and higher resolution between peaks. The goal of the MS method in untargeted metabolomics is to ionize the largest number

of metabolites as possible, maintaining low in-source decay and the highest sensitivity possible (enough ion intensity).

Metabolite identification is usually included in the metabolomics protocol, and must rely on a minimum of MS data features and strategies of acquisition to provide robust support to the identification proposal. Therefore, the acquisition of MS data, with high mass accuracy of observed ionic species (deprotonated/protonated ions, adducts, fragment ions, and isotopic ionic species), as well as abundance of isotopic ionic species, is very important in metabolomics. This particular aspect explains the wide use of HRMS analyzers in metabolomics, such as time-of-flight (TOF), FTIR, FT ion cyclotron resonance (FT–ICR), and Orbitrap mass analyzers [7].

Before starting the analysis, the MS system must be tuned and calibrated over an appropriate mass range, by using a solution with known compounds. Exploratory runs may be evaluated in order to set up the best conditions for the analysis. Lock mass is also highly suggested to be used during sample analysis by MS in metabolomics studies.

2.3.1 LC–UV–MS analysis protocol

A sequence of valuable procedures for a LC–MS-based metabolomics protocol is suggested below:

1. before starting the analysis, first tune and calibrate the MS system, using a solution composed of known compounds (recalibrate the system whenever needed during the analyses);
2. set up the best LC–MS parameters for the study, developed based on previous discussion. Some MS parameters that can be studied are as follows: ionization mode (atmospheric pressure chemical ionization, electrospray ionization [ESI], atmospheric pressure photoionization, etc.), polarity (positive or negative mode analysis), skimmer/focusing lens voltages (e.g., capillary voltage, etc.), gas flow (e.g., nebulization gas, cone gas, source temperature, spray voltage, desolvation temperature);
3. run the analysis using independent data analysis, if possible, acquiring full-scan MS spectra and MS/MS (or MS^n) spectra. Set up an appropriate scan time, mass range, and parameters for ionic species filtering for MSn. The collision energy can be set up with different energies, in both ionization modes (e.g., 5, 25, 40 eV).

Note: when using a mass analyzer, such as Orbitrap and TOF, in tandem with a quadrupole mass analyzer, target ion parameters for MS^n spectrum acquisition must be assigned, such as the abundance of ions, number of target precursor ions over an assigned scan time range, etc. A mass exclusion

list, containing abundant eluent mass-to-charge ratio (*m/z*) signals, can also be assigned in order to prevent switching to MS/MS (or MS^n) mode for these background compounds;
4. inject sample set and lock mass solution during the sample analysis.

2.4 Handling MS data in metabolomics

The handling of data generated by LC–MS for metabolomics studies can be divided into three steps as follows: raw data format converted to open-source format, data processing, and data analysis (Figure 2).

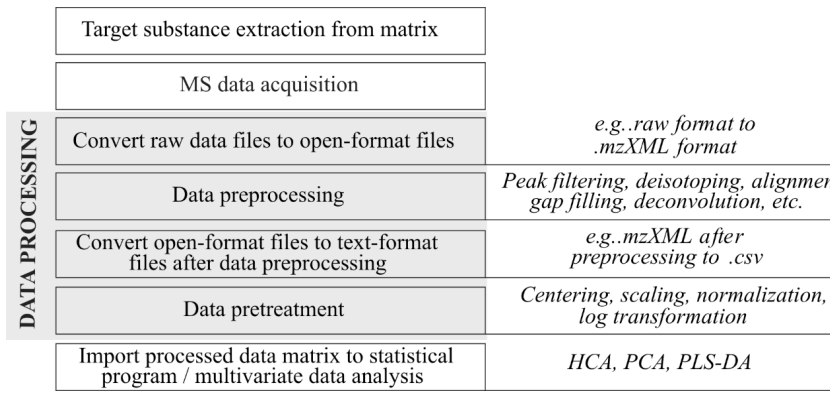

Figure 2. Suggested flowchart for LC–MS metabolomics.

2.4.1 Conversion of LC–MS raw data files to open-format files

Different kinds of MS systems, from different vendors, have been used for acquiring LC–MS raw data for metabolomics studies. Depending on the MS system used, the user must deal with different MS output files, with different formats, as follows: *.d* (Agilent), *.raw* (Thermo Scientific), *.baf*, *.fid*, *.yep*, *.xms*, and *.sms* (Bruker), *.wiff* (AB Sciex – with the exception of the TOF–TOF instrument), *.qgd* and *.spc* (Shimadzu), and *.raw* (Waters) [27]. These files are able to be read only by specific software from the vendor, which is not freely available (they are proprietary), and they usually contain extensive metadata for acquisition parameters (voltage, pressure, temperature, etc.) [28].

The raw data vendor-specific format files are, however, able to be encoded/converted into open-format files, and important information about the MS run (ion mass-to-charge ratio, ion intensity, retention time, etc.) can be accessed

using open-source software for data processing and subsequent data analysis. Usually, the open-format files used are: *.mzXML*, *.mzData*, *.mzML*, *.mz5*, etc. [27]. Some packages can be used to convert a raw data format file to an open-format file, such as ProteoWizard [28], which can subsequently be read by other data-processing programs.

2.4.2 MS data processing – data preprocessing and pretreatment

Many sources of non-biological variability may be introduced into the data matrix through the whole metabolomics workflow, which may lead to incorrect interpretation of the real biological information. The non-biological variability may occur in all of the steps, starting at the very beginning with sampling, sample preparation, and analysis, as well as in the subsequent steps of processing the data matrix, data analysis, and interpretation.

The total variability observed in the metabolome data is a result of biological and non-biological variability. A few sources may represent non-biological variability, as follows:

a. depending on a metabolite's properties, it may show a broad variation in concentration, even when analyzed under identical conditions. Generally, this characterizes a non-robust analytical method/metabolite extraction procedure for the metabolite analysis, and will be a barrier to reaching similar results when samples are prepared and analyzed in different batches;
b. tests for the evaluation of system suitability are not run before starting data acquisition by an analytical method, which may raise the possibility of analytical error being considered to be biological variability;
c. no robust metabolite extraction from the study matrix;
d. the standard deviation of a metabolite concentration very often is not constant for all metabolites present in the metabolome that is being analyzed. This issue characterizes the heteroscedasticity of the data, which should be considered when choosing a pretreatment method;
e. some metabolites are present in higher concentrations than others in the metabolome. This does not necessarily characterize those highly-concentrated metabolites as more important than those present in low concentrations [29].

For raw data processing, several methods can be used in order to reduce the non-biological variability, which could mask the real biological information. Prior to data pretreatment, the raw data files must be transformed into open-format files, including important and relevant characteristics of each observed ionic species, which may be easily used in the subsequent data analysis [30]. Consequently, the data processing step plays a significant role in the data interpretation quality in metabolomics studies.

2.4.2.1 Data preprocessing methods

In metabolomics studies, the snapshot of the acquired metabolome usually represents a specific condition of the matrix (plant, animal, microorganism), such as phenotype, cellular state under specific experimental conditions, etc. [29]. The MS-based profiling methods (e.g., LC–MS, GC–MS, CE–MS) can detect a huge amount of metabolites, resulting in a complex dataset, requiring extensive preprocessing, followed by pretreatment of the data. The most important algorithms involved in the preprocessing pipeline are noise filtering, peak detection, deconvolution, alignment, gap filling, and deisotoping (Table 1), which are covered by several freely available packages [31]. With each available package update, new or updated algorithms may be included in order to provide a wider range of preprocessing possibilities.

A large number of preprocessing programs are available for MS data treatment, which can cover several data processing steps, or a specific step in the data processing workflow [31]. Among several programs, MZmine [32], MetAlign [33], and XCMS [34] are some examples of robust open-source software that offer several tools for MS data processing for metabolomics [30].

Table 1. Aspects of the most common algorithms used for data preprocessing.

Preprocessing Method	Purpose	Ref.
Noise filtering	To distinguish the metabolite signal from the background signal.	[27]
Peak detection/ deconvolution	To find the peaks corresponding to the compounds (e.g., *m/z*), and estimate their intensity. To avoid detection of false positive signals.	[26, 27, 31]
Alignment	To compare features between samples, and find matching peaks associated with a particular compound across all samples in the dataset.	[27, 31]
Deisotoping	To group the isotopic peaks with the corresponding monoisotopic peak.	[31]
Gap filling	To recover missing signals from the raw data that were not detected in the peak detection step, due their low intensity, poor-quality shape, or detection mistakes.	[27]

After MS data preprocessing, the data can be exported as a text file that encodes information about the detected features and their attributes, such as intensity of detected ionic species, mass-to-charge ratio values, and retention time. The text files are usually exported as spreadsheets in *.csv* format, or even as *.txt* files, which can be opened by Microsoft Excel, or other free spreadsheet software, and/or exported to statistical software or packages. Additional data pretreatment must be run in order to reduce the non-biological variability by several tools using different programs or packages, such as R, MATLAB, or even Microsoft Excel (or other free spreadsheet software). The programs MZmine (open-source) and the The Unscrambler (proprietary, from CAMO Software) can also be used to apply some pretreatment methods to the dataset; however, some methods may not be present in those programs, whilst R offers the possibility to create customized scripts.

Fusion of metabolomics data matrices, obtained from orthogonal analytical techniques (e.g., LC–MS and NMR), has been used in order to combine metabolome complementary information in a single data matrix. After fusion of the data matrices, an appropriate data processing method must be applied, before moving forward to data analysis.

2.4.2.2 Data pretreatment methods

Data pretreatment methods aim to minimize the non-biological variability of a metabolome, focusing on the relevant biological information. The pretreatment methods most used are scaling, centering, and transformations. Refer to the publication information [25] for further details on the aspects, goals, and advantages of each pretreatment method.

2.4.3 MS data analysis

The data analysis step typically includes multivariate data analysis (MVA) methods, and interpretation of the processed data. MVA is commonly used because a large amount of data is generated in metabolomics studies, and this enables interpretation of the complex metabolic information, and assignment of which (group of) metabolites are relevant for the biological question that is being addressed. In general, MVA focuses on clustering metabolic profiles, or pointing out significant differences between groups of samples, assigning important metabolites for identification [30,35].

Initially, analysis of the data matrices obtained by the analytical techniques are often performed by unsupervised MVA, such as PCA and HCA, which allow pattern recognition visualization among different groups. PCA is the MVA method most widely used for metabolic fingerprinting. While the unsupervised

analysis provides a means of achieving a reduction in unbiased dimensionality, its application only reveals group structure when within-group variation is significantly lower than between-group variation [12].

After this, usually the processed data matrices are analyzed by supervised MVA, such as PLS or OPLS, associated with a discriminant analysis (DA), which enables the setup of models to predict class membership of new samples, based on previously analyzed samples, and contributes to the compound/marker identification, which is responsible for class differences [5]. PLS–DA is one of the most well-known classification procedures in chemometrics, and this approach has also been extensively used in 'omics'-related fields [36]. Moreover, there are other MVA methods, such as self-organizing maps, ANNs, decision trees, and regression classification, among others, the choice of which will depend on the intended purpose of the study in question, as well as on the peculiarities of each of these algorithms.

MarkerView, Unmetrics SIMCA, The R Project for Statistical Computing, MATLAB, The Unscrambler and MZmine are some examples of statistical software and/or programs or packages that provide tools for supervised and unsupervised MVA methods for data analysis [5,36]. Combinations among different software and algorithms are also possible.

2.4.4 Best practices for metabolomics studies

The analysis protocol for metabolomics must be performed after considering the best practices for each step involved in the study, in order to reduce the non-biological variability present in the final dataset, enable reproducible results, and correct interpretations of the biological information. A minimum core set of necessary data that describe the experimental methods for the metabolomics studies must be clearly reported; for instance, the metadata and information describing the nature of the experiments, and how they were executed [37].

Relevant experimental aspects of sample processing and preparation must be reported and discussed, as well as sufficient metadata to enable sample preparation reproducibility. Specific acceptable practices for instrumental analysis setup must be adhered to for each analytical technique used. Parameters and procedures used for raw data acquisition must be reported, and must clearly show all necessary instrumental conditions in detail, in order to enable experimental replication, including robustness studies. This includes a clear report on data preprocessing and pretreatment methods, as well as statistical data analysis methods. Metabolite identification must rely on a minimum amount of metadata to support the identification proposal.

2.4.4.1 Protocol for data processing and analysis for metabolomics by LC–MS

This section details a suggested operational procedure for LC–MS data processing and analysis for metabolomics studies.

a. install .NET Framework (if it is not already installed), and then install the ProteoWizard software (http://proteowizard.sourceforge.net/);
b. convert raw data format files to open-format files (*.mzXML*) in order to choose one of the protocols for conversion of raw data format files to open-format files, referring to publication [28]);
   Note 1 – refer to vendor's specifications and format features;
   Note 2 – for Thermo Scientific systems, all MS output is encoded as *.raw*, including a single raw data file of information acquired in both modes (+ESI and -ESI), if the mode switching acquisition mode was set up by the user previously. If both modes were acquired in the same run, the polarity must also be separated by applying a polarity filter to the protocol;
   Note 3 – choose a suitable signal cut-off, which represents the background noise for the MS acquired data;
c. the open-format files *.mzXML* are saved for each run and can be exported to an open-source data processing program;
d. open the *.mzXML* files with MZmine (http://mzmine.github.io);
e. first, filter the noise by setting up the threshold level for the relevant signals, facilitating the next step, 'peak detection';
f. run 'peak detection', which should differentiate the true metabolite signals from the background signals. Choose a suitable algorithm for creating a list of unique *m/z* (chromatogram builder), and build a continuous chromatogram [31];
g. run 'FTMS (Fourier transform mass spectrometry) shoulder peak filtering' for those MS data acquired with FTMS systems (Orbitrap, FT–ICR) ;
h. run the 'deisotoping' algorithm, followed by 'peak deconvolution', by choosing a suitable algorithm;
i. run 'alignment', 'gap filling' and duplicate Pak filter'. Export the data matrix as a *.csv* file;
j. apply suitable statistical pretreatment methods, such as centering, scaling, and transformation, using Microsoft Excel (or other free spreadsheet software) tools, R, MATLAB, or other available statistical programs. Refer to publication [29] for further details on different aspects provided by each pretreatment and how to run them. Save the processed data matrix as a *.csv* file;
k. import the *.csv* or *.txt* processed matrix data file to a statistical software package. Run a suitable MVA method (HCA, PCA, PLS, etc.);

l. report very clearly a minimum set of information and details on sampling conditions, sample preparation conditions, instrumental parameters, and data processing and analysis methods, in order to allow the procedure/methodology used to be reproducible. Report enough metadata in order to support the metabolite identification, in case the metabolomics study requires metabolite identification to answer or clarify the biological question that is the basis for the study. Refer to publication [37] for further discussion on the harmonization of minimum reporting standards in metabolomics.

3. Applications

In this section, some selected applications of plant metabolomics are described, some of which have been carried out by our research group.

3.1 Plant chemotaxonomy and chemosystematics

Plant chemotaxonomy can be described as a research field concerned with the taxonomical organisation of plants, according to their biosynthesized chemical constituents (usually secondary metabolites) [38]. Up to the second half of the last century, plant chemotaxonomy was conducted on the basis of data surveys of isolated or detected compounds. Subsequent advances in computer technology and hyphenate techniques in the last few decades have enabled the use of metabolomics [42] as an important approach for chemotaxonomy.

For example, Spring and Schilling [39–41] classified the infrageneric levels of the genus *Helianthus* L. (Asteraceae), based on different distributions of structural subtypes of sesquiterpene lactones (STLs). Other studies have detected STLs, extracted from glandular trichomes by hyphenated techniques, in order to provide more comprehensive information on taxonomy and plant systematics [42–45].

Following the analytical advances provided by hyphenated techniques, improvements in computer science, and an increase in databases, has enabled the use of chemoinformatics and chemometrics tools in chemotaxonomy. A recent chemotaxonomy study was carried out on species of the family Asteraceae, using chemometrics to analyze the oxidation degrees of their secondary metabolites, as reported in the literature [46,47]. In addition, other studies, based on chemoinformatics and chemometrics tools, have contributed to a greater understanding of metabolites in the classification of some tribes of Asteraceae [48,49].

Ten species of *Vernonia* Schreb. (Asteraceae) were clustered into four groups, based on metabolic fingerprints acquired by LC–MS [50]. According to these

results, the authors suggested that the use of metabolomics in chemotaxonomy could facilitate, or assist, taxonomical classifications. Furthermore, a recent chemosystematics work was carried out, based on the LC–MS metabolites from 15 species of the subtribe Lychnophorinae (tribe Vernonieae) [51]. The phylogenetic tree of these species, combined with the chemical data, have indicated 12 putative chemical synapomorphies, such as trans-cinnamic acid and polyacetylenes, contributing to a better understanding of chemical relationships in the subtribe Lychnophorinae [51].

In another work, based on LC–MS data, 62 plant species from six different families (Asteraceae, Rosaceae, Fabaceae, Fagaceae, Betulaceae, and Aceraceae) were clustered by unsupervised statistical analysis, such as PCA and HCA, and distinguished according to their chemical constituents. Moreover, the authors also applied supervised analysis, such as PLS–DA and OPLS–DA, to achieve more robust and accurate results in chemotaxonomic classifications, as well as to distinguish the plants based on their bioactivities [52].

Another work has used targeted metabolomics, based on leaf extracts rinsed with acetone, and LC–UV–MS data for flavonoids identification and, using this approach, the authors distinguished four species and two subspecies of *Chrysanthemum* L. (Asteraceae) [53].

As discussed above, metabolomics can also be carried out by other techniques, not just MS, such as the study that applied chemometric analysis to high-resolution NMR and FTIR data to determine chemotaxonomic classifications of 11 species of lichens, from six different genera and two different families [54]. Another NMR-based metabolomics study (one- and two-dimensional NMR) clustered 11 *Ilex* L. species (Aquifoliaceae) through chemometrics approaches [54,55]. Other studies have employed GC–MS-based metabolomics for chemotaxonomy purposes, such as one that clustered 18 *Ferula* L. species (Apiaceae) into four main groups [56], thus characterizing them chemically. Two other two studies have used the GC–MS metabolomics approach to distinguish and identify the biomarkers of 27 *Salvia stenophylla* Burch. ex Benth. and 20 *S. runcinata* L. f. samples (Lamiaceae) [57].

In the study carried out by Gallon *et al.* [58], metabolic profiles of 154 samples of 79 species of the subtribes Vernoniinae, Lepidaploinae and Rolandrinae from the large tribe Vernonieae (Asteraceae) were acquired by LC-MS, and the data was processed by unsupervised and supervised multivariate statistical analysis (Figure 3).

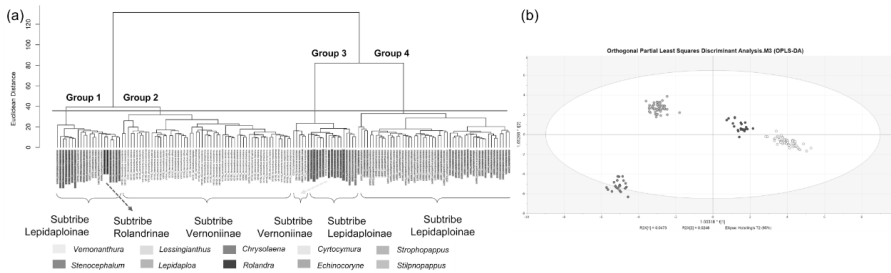

Figure 3. Chemometric analysis based on extracts of 79 species of the subtribes Vernoniinae, Lepidaploinae and Rolandrinae. **(a)** HCA: species are colored according to the genera proposed by Robinson; **(b)** OPLS–DA score plot: group 1 (purple), group 2 (yellow), group 3 (pink) and group 4 (green). Figure adapted from Gallon *et al.* [58].

The analysis of methanol:water (7:3) extracts from leaves by UHPLC–UV–HRMS has allowed the identification of several peaks, including 17 discriminants obtained in the OPLS–DA that corresponded to flavonoids and STLs. Also, the chemical characterization of the extracts has indicated several similarities and differences among the species, being possible to point that species of the groups 1 and 2 were characterized by the presence of a methylated flavonoid and two STLs, while the species of the groups 3 and 4 were chemically distinct and shared a flavonoid glycoside and a different STL. Chemometric analysis (HCA, PCA and OPLS–DA) has allowed the identification of four primary clusters correlated with the taxonomy, in accordance with the previous generic classification proposed by Robinson and with recent phylogenetic studies. Hence, this study has shown that untargeted metabolomic studies combined with multivariate statistical analysis, allowed the identification of potential chemotaxonomic markers in the tribe Vernonieae.

As previously mentioned, chemotaxonomy is a historic and consolidated field, contributing to taxonomy as well as the chemical characterization of a particular taxon. Thus, metabolomics analysis represents a new approach in performing chemotaxonomic studies, with the clustering of plants based on the set of all metabolites biosynthesized by them, thus allowing a better understanding of the relationships between plants, the identification of their metabolites, and the discovery of new compounds.

3.2 Environmental metabolomics

Metabolomics can also be used to investigate the effects of environmental factors on plant metabolism, based on a holistic approach. This can be suitable

for studies related to plant identification, chemotaxonomy, ecological studies, plant adaptability, cultivation, and so on. There are several environmental factors that may influence the metabolism of plants, such as rainfall, humidity, temperature, soil composition, luminosity, CO_2 availability, interaction with other living organisms, circadian rhythm, and seasonality [59–64]. In general, environmental metabolomics [65,66] can be divided into three main subjects, which often overlap––biogeography, cultivation, and ecology.

Metabolomics in biogeographical studies is useful for the taxonomic differentiation or characterization of herbal medicines, food plants and dietary supplements. Metabolomics for plant cultivation usually investigates plants under stress conditions, aiming to understand and characterize changes induced in the metabolome. Finally, metabolomics for ecological studies can be subdivided into research related to the temporal characterization of plant metabolism, such as circadian and seasonal studies, other studies related to intraspecific and interspecific (herbivory, tenancy, mutualism, for example) interactions, and ones related to phenology.

Metabolomics studies related to traditional Chinese medicine (TCM) have been performed mainly in order to characterize medicinal plants according to their geographical origin. For instance, Gong *et al*. [67] used GC–MS-based metabolomics to determine the chemical components of *Cinnamomi* cortex (Lauraceae) oils from different producing areas. Another study, based on near-infrared reflectance spectroscopy (NIR) and chemometrics, was able to discriminate between herbal medicines according to their geographical origins [68].

Two NIR-based metabolomics studies have been conducted, aimed at TCM discrimination according to their corresponding geographic regions. PCA and PLS–DA have been used in order to discriminate the mushroom *Ganoderma lucidum* (Curtis) P. Karst. (Ganodermataceae) [69] and, in another study, two-dimensional correlation spectra was applied to discriminate fructus lycii (Solanaceae) [70]. Moreover, a NMR-based metabolomics study, combined with PCA, discriminated ginseng roots from different sources, such as China and Korea [71]. Another two NIR-based metabolomics studies, on TCM combined with chemometrics, were conducted in order to geographically discriminate samples of roast green tea (*Camellia sinensis* (L.) Kuntze, Theaceae) and rhizoma corydalis (Papaveraceae) [72,73].

In relation to metabolomics studies for the geographic characterization of samples, those focused on beverage recognition should be pointed out. For example, a study based on direct analysis in a real-time ion source coupled to MS (DART–MS) was able to distinguish and identify putative metabolites of beers from different origins [74]. Another two metabolomics studies, which employed chemometrics with LC–MS data [75] and direct infusion MS data (ESI FT–MS)

[76], distinguished and classified several red wines, according to their varieties (geographic origin).

Metabolomics applied to biogeographic discrimination could also be used to aid in taxonomic classification. In this type of study, the goal is not just to distinguish the species taxonomically, but also to discuss the results in terms of biogeographic chemical characterization. For instance, a LC–MS-based metabolomics study, combined with PCA and PLS–DA, distinguished and characterized three *Vaccinium* L. (Ericaceae) species by means of metabolites, according to their localization and taxonomic positions [77].

Another recent study, based on UHPLC–UV–MS metabolomics from 120 plant samples of *Espeletia* (Asteraceae) was conducted with the purpose of revealing subtle biogeographical trends in the diversification of this genus (Figure 4) [78].

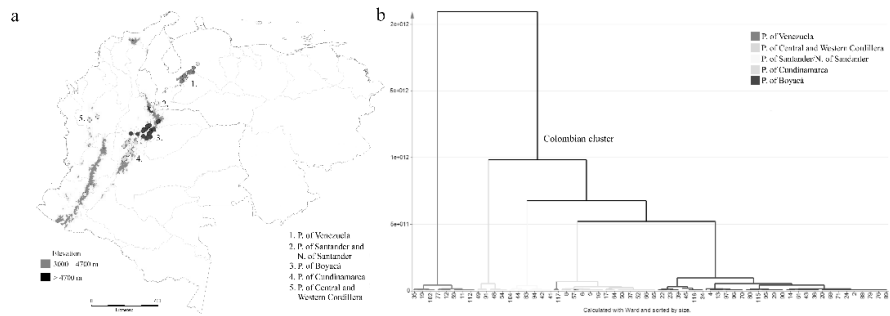

Figure 4. Clustering of *Espeletia* species, based on metabolic fingerprinting of 120 plant samples analyzed by UHPLC–UV–high-resolution MS, ESI (negative mode). **(a)** Map of Colombia and Venezuela, with species (represented as dots) colored according to their OPLS–DA groupings; **(b)** OPLS–DA dendrogram, showing clustering of species according to their páramo massifs of origin. Figure adapted from Padilla-González *et al.* [78].

The results demonstrated that *Espeletia* lineages can be distinguished, and correlated to their country of origin (Colombia and Venezuela) on a global scale, and to the páramo massif (a special Andean ecosystem) on a regional scale. In addition, a distinctive pattern in the accumulation of secondary metabolites was also identified, according to the main diversification centers of *Espeletia*. These findings demonstrate that the metabolic variation in lineages may reflect the biogeography of the genus.

Another GC–MS-based metabolomics and chemometrics study distinguished different populations of three species of pine trees (*Picea omorika* (Pančić) Purk.,

Pinus heldreichii Christ. and *Pinus peuce* Griseb., Pinaceae) [79], according to their taxonomic and geographic positions. Moreover, both GC–MS and LC–MS-based metabolomics, combined with HCA and PCA, were used to discriminate and identify metabolites of six *Nigella* L. species (Ranunculaceae), according to their genetic and geographic origins [80].

With regard to the metabolomics of cultivated plants, an interesting study related to crops of *Arabidopsis* as a model was carried out, aimed at understanding the integrated behavior of plant metabolites in response to temperature [81] or nutritional [82] stresses. Another work used *Arabidopsis* as a model to understand changes in the gene–protein–metabolite network in plants, caused by circadian regulation [83].

In addition, plants can be understood not only in relation to environmental stress, but also from the phenology point of view, as demonstrated by a LC–MS metabolomics study on *Hypericum origanifolium* Willd. (Hypericaceae) [84]. Likewise, metabolomics studies also play an important role in understanding the relationships between plants and other living beings, such as a targeted and untargeted metabolomics study that was carried out by GC–MS and LC–MS, which reported metabolite profiles combined with volcano plots to analyze plant defenses against herbivores mediated by phytohormones [85]. Additionally, allelopathy and seasonality play a significant role in plant metabolism, as demonstrated by a NMR-based metabolomics for the discrimination and chemical characterization of Mediterranean plant species [86,87].

NMR-based metabolomics, combined with chemometrics, were used to evaluate the relationship between the metabolic profiles and elemental analysis of leaf extracts of *Erica multiflora* L. (Ericaceae), according to seasonality, plant growth, and moderate stress conditions of drought and warming [88]. A holistic work, based on a NMR metabolomics approach combined with chemometrics, was used to analyze 26 pollen extracts, according to their seasonality and geographical positions, in order to classify them and identify the metabolites responsible for allergic disease [89].

Finally, a study focused not only on seasonality, but also on geographical origin and the influence of environmental abiotic factors, was able to discriminate *Tithonia diversifolia* (Hemsl.) A. Gray (Asteraceae), based on NMR and LC–MS fused (concatenated) data [13]. In that study, metabolomics was used to investigate the influence of different abiotic environmental factors on the metabolite profile of T. *diversifolia* by multivariate statistical analyses of spectral data, acquired by UHPLC–UV–MS and NMR methods (Figure 5).

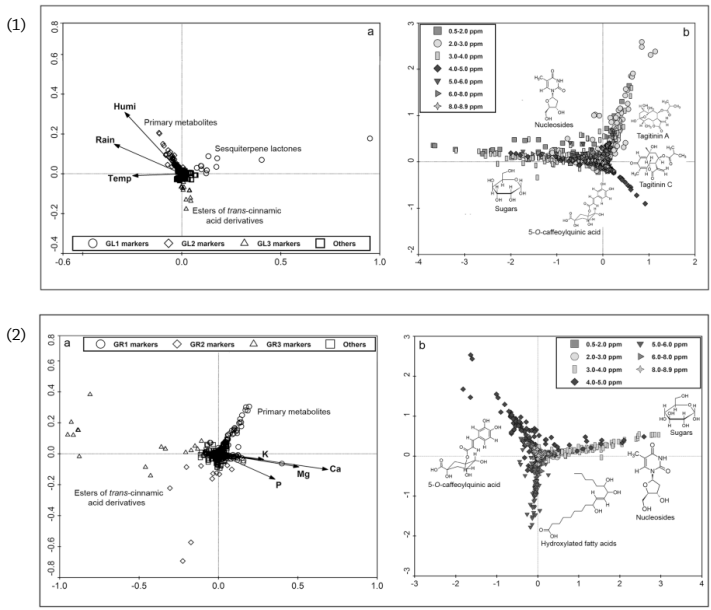

Figure 5. *T. diversifolia* leaf extracts used to correlate discriminant classes of metabolites with several environmental factors. **(a)** PCA loading plots of the concatenated data; **(b)** *J*-resolved chemical shift data. **(1)** Leaf samples; **(2)** root samples. Rain = rainfall, Humi = humidity, Temp = temperature. Minerals in soil: Ca = calcium, Mg = magnesium, P = phosphorus, K = potassium. Figure adapted from Sampaio *et al.* [13].

The results showed that a seasonal pattern was observed for sugars, STLs, and phenolics in the leaves and stems, which can be correlated to the amount of rainfall and changes in temperature. The distribution of the metabolites in the inflorescences and roots were mainly affected by variation of some soil nutrients, such as Ca, Mg, P, K, and Cu. Therefore, this study allowed a holistic understanding of the influence of abiotic environmental factors on the production of metabolites in various plant parts from two distinct regions in Brazil.

Studies using environmental metabolomics approaches have greatly contributed to the understanding of the relationships between living beings and the environment in an integrated or holistic way. As discussed in this section, several studies rely on metabolomics for environmental information, such that metabolomics applications are demonstrated as being widespread, sophisticated, and constantly under improvement.

3.3 Quality control of herbal medicines, food plants and dietary supplements

Herbal medicines, food plants and dietary supplements may present a high variability between batches, due to several variability factors arising from

different sources, including genetics, cultivation and geographic parameters, growth conditions and diseases, and harvesting and post-harvesting processes [90,91].

The quality control of herbal medicines can ensure that the final product variability does not influence the therapeutic effect. Health and regulation agencies, such as the United States Food and Drug Administration (FDA) and the Brazilian health regulatory agency (National Sanitary Surveillance Agency, ANVISA), demand several tests for identification of herbal drugs, such as: macroscopic and microscopic characterization purity tests; ashes or water content; presence of heavy metals; compounds qualitative and/or quantitative analysis; and biomarker evaluation. Quality control should be based on the quantitative and qualitative analysis of markers (actives or analytical) by a suitable analytical technique, such as chromatography, titration, and/or spectroscopy methods, etc.

Chemical markers have been used as qualitative and/or quantitative parameters to ensure safety and efficacy of herbal medicines. Suitable chemical markers for herbal medicines and dietary supplements should prove therapeutic activity or nutritional content, but not be limited to these. More features could also be demonstrated by evaluating other important compounds [92]. Key points about compounds/markers to be assigned for the quality control of herbal medicines and dietary supplements are as follows: compounds that are responsible for biological activity; compounds that contribute to the authenticity evaluation of one species/genus (a compound restricted to this species/genus, for instance); synergic and/or bioactive compounds, co-responsible for therapeutic effects; toxic compounds, such as pyrrolizidine alkaloids, which could indicate hepatotoxicity of herbal medicines; adulterant compounds added to herbal medicines or botanical raw material; the presence/absence of markers that, in different concentrations, may indicate authenticity, differentiation of species, traceability, time of harvesting, sample treatment process, stability or shelf life, and toxicity or therapeutic activity.

Unfortunately, at this moment, many herbal medicines and dietary supplements have no chemical/biological markers for suitable quality control and, frequently, very common or non-specific/widespread compounds found in plants are assigned as markers for many plant species, such as rutin, quercetin, ferulic acid, and chlorogenic acid [92,93].

Moreover, the increase in manufacturers of food and herbal medicines that are not regulated by appropriate agencies, or appropriate methodology/reference standards, leads to several issues that may impact directly on public health. Many steps involved in the production process may occur, such as adulteration of herbal medicines or dietary supplements, as well as botanical drug mislabelling (authenticity issues), which can result in the release of cheap or poor-quality products on to the market [94,95].

Food, dietary supplements, and herbal medicines are usually constituted from a different number of plant-derived products, and several compounds with different physicochemical properties, representing complex matrices. This scenario represents a challenge for the quality control of herbal medicines and dietary supplements. Standard quality control protocols usually demand quantification of one or more marker compounds, often related to the biological activity. For herbal medicines, usually the chemical marker is the compound that most accounts for their bioactivity; however, synergistic effects may influence the therapeutic efficacy [95], and should be also considered. Traditional Chinese medicine represents a big challenge regarding quality control analysis, since the remedies are usually composed of a mixture of different plant species extracts or powders. Therapeutic effects (and adverse effects) are very often related to different associations of plants and the synergy of their biological activities. Minor compounds, and interactions among them, may also lead to an increase/reduction of bioavailability, and pharmacological and toxic activities, and should not be neglected in quality and safety product evaluation [96].

Metabolomics represents a potential approach for the quality control of complex matrices, since it enables high-throughput analyses and a possible integrated view of all of the compounds/metabolites and their interactions. Therefore, targeted and untargeted metabolomics approaches could be used as methodologies for evaluating the authenticity of species and product adulteration, to improve the quality control of herbal medicines, food plants and dietary supplements, including determining minor, as well as major, compounds [97]. Another important feature of metabolomics approaches is the potential to find chemical markers, besides the major compounds.

Relevant analytical techniques support the potential of metabolomics studies in the quality control field, such as LC–MS, CG–MS, and NMR, thus enabling the identification of several compounds, or compound features, in a single analysis [98]. The applicability of metabolomics in this field is very wide. For instance, it may be applied to the certification of the geographic origin of products, identification of fraud and adulteration, evaluation of genetic modifications, comparison of the variability of product batches, detection of poor-quality samples, and management of manufacturing practices. The most used analytical techniques for quality control and authentication of products are GC–MS, LC–MS, or NMR spectroscopy. Other techniques are available, especially with an easier and faster sample preparation step, such as DART–MS, coupled or not with TLC, as demonstrated in a targeted metabolomics analysis of turmeric powder curcuminoids [99]. An interesting work on honey adulteration was also successfully carried out using a metabolomics approach by NIR [100].

A holistic quality control of samples is already used to study food and beverages, especially ones with economic importance. In Germany, analysis by ^{1}H NMR

spectroscopy of wines led to a prediction model that is able to differentiate wines by geographical origin, grape variety, and year of production. There is an increasing requirement for organic products due to their aggregated market value, but there is also a lack of robust and selective analytical methods that are able to distinguish these types of products. Therefore, metabolomics studies have been demonstrated as being an important and innovative approach in distinguishing agricultural systems, such as between organically- or traditionally-cultivated crops [101–103].

Another important potential for metabolomics in food quality control is the diagnosis of sick plants, as demonstrated in a study on mangoes during harvest and storage, by CG–MS volatile metabolites analysis, which differentiated between fungus-infected samples and healthy mangoes, even in the early stages of the infections. Therefore, metabolomics may support a risk-assessment plan, so that farmers and manufacturers can make decisions to avoid the production and release of poor-quality products, which could result in wasting an entire product by the farmer/manufacturer, or represent a risk to consumers. Another important finding was a few volatile metabolites present in healthy samples [104], whilst a similar study relied on targeted metabolomics to quantify naphthodianthrones and flavonoids by LC–UV, and an untargeted approach by CG–MS in essential oil analysis discovered patterns in infected samples of *H. perforatum* (Hypericaceae) [105].

Saffron (*Sassafras albidum* (Nutt.) Nees, Lauraceae) is one of the most expensive spices in the world, due to its limited and difficult production and handling processes, which led to adulteration for a long time with the purpose of increasing the final product weight. The standard quality control of saffron is based on marker quantification, which many times failed to detect adulteration with other plants or powders of the same color. A recent study analyzed saffron samples by ^{1}H NMR, and a reliable model for identification of adulteration in those samples was proposed [106]. The ^{1}H NMR fingerprint analysis of stored saffron demonstrated that signals of fatty acids (usually not investigated for this product, due to their low concentrations) could be due to the presence of aging markers, and consequently deterioration markers [107].

Quality control studies on ginseng are good examples of metabolomics approaches used in herbal medicine studies. UHPLC–quadrupole-TOF-based metabolomics have demonstrated a potential for classifying *Panax ginseng* L. (Araliaceae) samples with different cultivation ages, observing a greater difference in an older sample group [108]. A metabolomics study by UHPLC–QTOF–MS/MS was able to differentiate leaf extracts of *P. ginseng* from leaf extracts of *P. quinquefolius* L., indicating nine ginsenosides as possible markers of these species [109]. In another study, ^{1}H NMR analysis was applied as a quality control method in order to differentiate wild (more expensive) from (less expensive) cultivated

P. quinquefolius and cultivated *P. ginseng* (even cheaper). In addition, this study indicated the presence of sucrose, glucose, arginine, choline, 2-oxoglutarate, and malate as the metabolites responsible for different pattern profiles among the species [110]. In a similar way, species of *Curcuma* L. (Zingiberaceae) were differentiated by CG–MS data, based on essential oil analysis, by performing analyses with PCA in order to visualize class clusters, and PLS–DA to determine metabolites that could be markers [111]. Another study distinguished three species of the genus *Ephedra* L. (Ephedraceae) that are traditionally used as medicines, based on the differences in ephedrine-type alkaloids and benzoic acid analogues [112].

Targeted analysis can also be used to evaluate specific active compounds, such as iridoid oleuropein and its main degradation product in batches of a natural syrup [95]. Relying on a LC–MS-based metabolomics study, it was possible to propose multivariate data control charts, developing a product quality management tool to guide the entire product process, and evaluate the quality of a complex product online, to stay in compliance with quality requirements.

In another study, it was possible to evaluate PCA score plots that clustered samples by country of origin and herbal medicine quality (percentage of undesired plant parts) by ^{1}H NMR spectrum analysis of chamomile (*Matricaria recutita* L., Asteraceae) flowers extracts, obtained from Slovakia, Egypt, and Hungary. In that study, the Slovakian flower powders were spiked with stalks of the plant. The PCA loading plot indicated sample clustering due to differences in sugar, glutamate, and glutamine concentrations. A second model was set up to differentiate the metabolite extraction method used, and to compare extracts obtained with different solvents [113].

3.4 Drug discoveries from natural sources

Metabolomics allows a systematic study of complex matrices, which can be linked to observations obtained through biological systems analysis, without the need to isolate active compounds, as in classical phytochemical studies [9,91]. This section focuses on a few examples of metabolomics applications in several important fields related to drug discoveries from natural sources.

The metabolic profiling of *Zingiber* Mill. species (Zingiberaceae) has been performed by GC–MS, and related to anti-inflammatory activity data. The results showed that *Z. zerumbet* (L.) Roscoe ex Sm. had a similar anti-inflammatory activity as *Z. officinale* Roscoe; however, there was no correlation with gingerol content, a specific compound of *Z. officinale*, which was considered to be responsible for the anti-inflammatory activity. Therefore, it was concluded that other compounds contributed to this activity, and needed to be further identified and quantified in order to ensure the bioactivity of a particular *Zingiber* sample. Also, this study used a metabolomics approach as a tool for authentication of *Z. officinale*, using a phylogenetic tree [114].

In traditional Chinese medicine, chenpi (dried citrus peel) is used for the treatment of indigestion and inflammatory syndromes. In a targeted metabolomics study, carried out by HPLC–DAD, using dried citrus peels [115], it was possible to evaluate the effect of storage periods (one versus three years) and heat treatment (90 min versus 3 h at 120 °C) on the total phenolic content (TPC) related to the antioxidant activity of chenpi. It was found that the chenpi stored long-term had a higher TPC and higher levels of DPPH (2,2- diphenyl-1-picrylhydrazyl) radical-scavenging activity, when compared to short-term stored chenpi, and that heat treatment increased both TPC and bioactivity.

A study by Chagas-Paula *et al.* [12]but there is not much investigation reported in the literature for natural products. In this study, 57 leaf extracts (EtOH-H2O 7:3, v/v described the application of a LC–MS-based untargeted metabolomics method using leaf extracts (ethanol:water 7:3, v/v) of 57 species of Asteraceae to find dual inhibitors of cyclooxygenase (COX-1) and lipoxygenase (5-LOX) enzymes (Figure 6). The metabolic profiling of each extract was obtained by HPLC–ESI–HRMS. Each extract had been previously tested *in vitro* directly against each enzyme. The outcomes from OPLS–DA were able to detect mostly phenolics and STLs as the main active compounds. In addition, this study showed that the detected biomarkers were not the major compounds in the extracts, therefore clarifying and enforcing the importance of metabolomics approaches, rather than classical approaches, in natural products research to find biomarkers that are otherwise difficult and more time-consuming to determine.

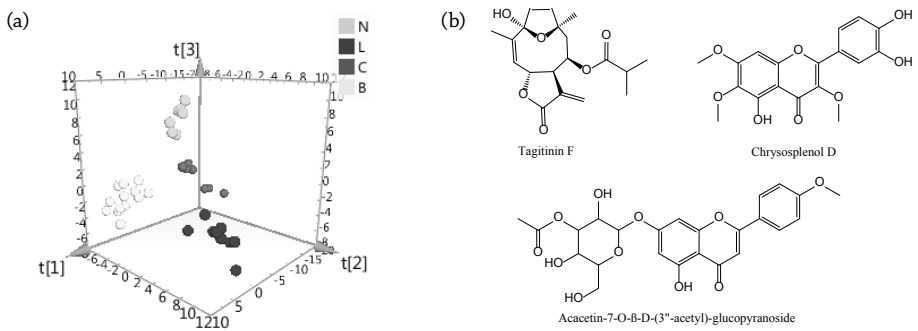

Figure 6. Three-dimensional OPLS–DA score plot of the HRMS data (in positive mode) for 57 Asteraceae leaf extracts, grouped according to their anti-inflammatory potential to inhibit COX-1 and/or 5-LOX. **(a)** Sample codes are colored as follows: dual inhibition = yellow (B); only COX-1 inhibition = red (C); only 5-LOX inhibition = blue (L); and no inhibition = green (N). **(b)** Some biomarkers for dual inhibition. Figure adapted from Chagas-Paula *et al.* [12]but there is not much investigation reported in the literature for natural products. In this study, 57 leaf extracts (EtOH-H2O 7:3, v/v.

In another case, NMR-based metabolomics was applied to find the metabolites responsible for the antitussive and expectorant activities of *Tussilago farfara* L. (Asteraceae). Different plant parts (roots, flower buds, and leaves) were analyzed, and an *in vivo* study revealed that leaves and flower buds had strong antitussive and expectorant effects. The result of multivariate analysis (PCA and OPLS–DA) showed that chlorogenic acid, 3,5-dicaffeoylquinic acid, and rutin might be closely related to the antitussive and expectorant activities [116].

GC–MS and multivariate analysis were used to identify antiplatelet agents from leaves of *Ardisia elliptica* Thunb. (Primulaceae), a Malaysian native plant traditionally used to treat chest pains. In that study, the methanolic extract was partitioned with *n*-hexane, *n*-butanol, and water. The data on the effect of the plant extracts on platelet aggregation *in vitro* were correlated to their chemical profiles obtained by GC–MS. The triterpene β-amyrin was one of the identified compounds with the highest correlation to the activity [117].

In a metabolomics study performed by Tang *et al.* [118], *Arctium lappa* L. (Asteraceae) extract, known as burdock, was tested against the bacteria *Salmonella typhimurium*. The crystal violet assay indicated that the ethanolic extract (1 mg/mL) of burdock leaves completely inhibited the biofilms. Then, the metabolic fingerprints of the burdock leaf fractions were analyzed by UHPLC–MS, PCA, and PLS–DA, before and after biofilm inhibition. The results revealed that 43 variables were screened as potential anti-biofilm ingredients, and among them, chlorogenic acid and quercetin were confirmed as potential anti-biofilm compounds in burdock leaves.

Rice bran has been shown to have a beneficial effect on chronic disease and, consequently, it is used in functional food and dietary supplements. Metabolites from *Saccharomyces boulardii* fermented rice bran were detected by GC–MS, and assessed for bioactivity (cell viability assay) and compared to non-fermented rice bran, in normal and malignant lymphocytes. The multivariate analysis results (PCA, PLS–DA and OPLS–DA) revealed a reduction in human B lymphomas by fermented rice bran extracts from all three rice varieties, compared to all other non-fermented controls. An altered metabolomic profile was found in the bioactive fermented varieties, where salicylic, *p*-coumaric, ferulic, and caffeic acids, as well as α-tocopherol and β-sitosterol, were found to be the major discriminating metabolites [119].

Another metabolomics approach was used in the study performed by Mhlongo *et al.* [120], where lipopolysaccharides (LPS) from the bacterium *Burkholderia cepacia* were used to induce defense responses in *Nicotiana tabacum* L. (Solanaceae) cell suspensions. Intracellular metabolites were extracted with methanol, and the metabolic profiles were analyzed using UHPLC–QTOF–MS/MS. The results of the untargeted metabolomics studies by multivariate analysis (PCA and OPLS–DA) showed that time-dependent dynamic changes and the accumulation of glycosylated

signaling molecules, specifically those of azelaic acid, salicylic acid, and methyl-salicylate, were contributors to the altered metabolomic state in LPS-treated cells. In addition, the results suggested that LPS treatment of tobacco cells induced changes in the phytohormones responsible for triggering defense responses, thereby leading to a primed state that is regulated in concert with azelaic and salicylic acids.

In addition to the compounds correlated with certain biological activities, it is also possible to use metabolomic approaches as prediction models; for example, as in the study by Chagas-Paula *et al*. [121]. In that work, hydroethanolic extracts from 57 Asteraceae species, with previously obtained IC_{50} values of *in vitro* dual inhibition of COX-1 and 5-LOX, were analyzed by UHPLC–MS and subjected to *in silico* studies, using machine learning tools (decision tree J48 classifier). A prediction model was built, using an ANN and the biomarker data. As a result, 11 compounds were identified as biomarkers responsible for dual inhibition of the enzymes, and a robust ANN model for predicting anti-inflammatory activity of natural compounds was obtained. Thus, this model can be useful in predicting potential extracts with anti-inflammatory activities, and identifying the responsible compounds for such activities, relying only on LC–MS data, therefore shortening the study before starting the subsequent *in vitro* tests.

The examples mentioned above demonstrate the ability to determine biomarkers using a metabolomics approach, associated with multivariate data analysis; however, it is common to select false-positive relevant 'biomarkers'. One of the main sources of assigning false-positive biomarkers is the size of the sample analyzed. In addition, it is important to prove that the biomarker is actually discriminant, and it is highly recommended to use another classifier algorithm to validate the chemometric analysis. Another common mistake is model overfitting, usually caused by failure to perform cross-validation. Furthermore, the identification of biomarkers is not the first step in data processing; often, it is included in one of the last steps, where discriminants, which are highly correlated with specific characteristics, must be identified [11]. This would be one of the most rational ways for metabolite identification in metabolomics, since the number of metabolites produced by plants is large, and the complete identification of all metabolites is neither necessary nor viable.

Therefore, as discussed in the experimental section of this chapter, despite the huge potential of metabolomics applicability, many critical points must be considered and clearly evaluated, since metabolomics studies usually work on highly complex matrix datasets, and many sources of errors may be involved. In this way, planning the sampling, experimental design, plant harvesting, data processing, and identifying the biomarkers with care will assure better-quality results.

4. Final remarks and future perspectives

Remarkable emerging developments in metabolite detection and characterization sciences, such as HRMS and high-field NMR, and the use of hyphenated techniques (LC–MS, GC–MS, and CE–MS), have outlined metabolomics as a highly efficient approach and strategy for comprehensive analysis of complex metabolite data matrices. Acquisition of the broadest metabolic composition of a certain tissue still represents a challenge, however. Thus, a multifaceted strategy, in addition to the establishment of acceptance criteria, must be applied for appropriate sampling, optimal metabolite extraction, development of analytical methodology, handling/processing of complex data matrices, and statistical data analysis. Metabolite identification is often included in metabolomics study workflows, but may represent a source of false-positive compound assignment if the identity proposal does not rely on enough, and proper, information. Therefore, the best practices for data acquisition and data processing must be considered before starting a metabolomics study. In addition, minimum features must be reported in order to support metabolite identifications.

Due to the high amount and complexity of the data acquired, besides the analytical technological advances, metabolomics science development is also supported by computational advances, such as in bioinformatics and chemometrics. Therefore, the development of methods for reducing data complexity and extracting the most important and significant raw data features represents a key point for further data analysis and interpretation of biological information.

Plant metabolomics is emerging as an alternative approach to existing and traditional phytochemical methods, which do not allow a comprehensive analysis of plant metabolic profiles. Such an approach has been used for quality control of herbal medicines, food plants and dietary supplements, significantly impacting on public health. Plant metabolomics may also be considered as a powerful strategy for novel bioactivities screening and discovery. In summary, metabolomics may herald a new era in phytochemical research, providing an integrative analysis of phytomedicines and plants, contributing to the complete metabolic profiling of herbs, and a global analysis of non-targeted compounds.

Metabolomics studies still have several issues that should be overcome in the future; however, it is a dynamically developing science that already plays a significant role in modern life and bioanalytical sciences. Metabolomics studies have been applied in several fields of studies, as emphasized in this chapter.

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Conflicts of Interest

The authors declare no conflict of interest.

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Chapter 3

NMR Strategies for Natural Products Identification

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1. Introduction

In modern times, nuclear magnetic resonance (NMR) is arguably the most versatile and informative analytical technique employed in the structure elucidation of organic compounds [1]. NMR spectrometer represents a unique investment for routine tasks in a natural products laboratory. There are several pulse sequences, which can achieve particular information of the structure evaluated [2]. Each NMR experiment contains parameters of the measurements that need an appropriate care for the success of the analyses. The combination of 1D and 2D NMR experiments provides a variety of tools to determine the skeletal structure, conformation and configuration in a scaffold. In addition, the quantitative NMR (qNMR) is extremely useful to evaluate the purity of components and to scale the amount of a substance in organic mixtures. It can also serve for monitoring the degradation of a substance over time [3].

The technology of NMR spectroscopy has evolved into high resolution and sensitive technique. The modern NMR equipment assists the students and professionals to easily operate the machine with possibility of recording data of several samples under fully automated conditions. Nowadays, data of with few milligrams of an isolated substance can easily be recorded within extremely short time if compared to previous NMR machine. The quality and the resolution of the spectra improve the performance and the yield of groups working on structure elucidation. This point is directly associated to the advent and evolution of capillary and microcryoprobe NMR [3].

The observation of the NMR signal requires a magnetically active nuclide that will have two or more allowed nuclear spin states. These nuclides have properties that require different care into NMR experiments. Among the active NMR nuclei, hydrogen (^{1}H) is the most important. The characteristics of ^{1}H, such as its natural abundance and the high sensitivity to structure modifications, make it immensely useful in NMR natural products analysis. However, nuclei

with low natural abundance can be employed effectively as alternative structure elucidation [4].

Most NMR investigations start with ^{1}H NMR analysis. The reason is because the ^{1}H NMR protocol provides essential information to the structure as chemical shifts, coupling constants and relative signal intensities. In some cases, only these data are sufficient to determine the structure accurately. Another reason is the information about the NMR sample acquired in the ^{1}H NMR spectrum such as concentration, resolution and purity. These parameters are important to expand for complex experiments and other nuclei. The next steps for NMR experiments depend on the information required to carry on and complete the structure elucidation. There are two main subjects for NMR structure elucidation. The first information is related with through-bond interactions, obtained by scalar (*J*) spin coupling via bonding electrons. The second information is related with through-space interactions, obtained by the nuclear Overhauser enhancement (nOe) mediated through dipole-dipole coupling and spin relaxation. These two principal points are technically represented by different experiments (Table 1) that can be optimized and adjusted regarding diverse situations such as size of the molecule, sample conditions, entropy of the molecule, chemical groups, poor presence of hydrogen atoms, instability of the sample, and skeletal structure.

Table 1. Typical NMR protocols for routine structure elucidation of natural products.

Protocol	Technique	Information
1D ^{1}H spectrum	1D	Chemical shifts, coupling constants, integrals
1D ^{13}C spectrum (with spectrum editing)	1D (DEPT 135°, DEPT 90°, APT)	Number of carbons and multiplicity determination
2D ^{1}H-^{1}H correlation	COSY	*J*-Coupling relationships
2D ^{1}H-^{1}H long range correlation	TOCSY	Remote protons assignments by *J*-coupling system
2D ^{1}H-^{13}C correlation	HMQC or HSQC (with editing)	Carbon assignments from proton signal. Carbon multiplicity from edited HSQC
2D ^{1}H-^{13}C long range correlation	HMBC	Carbon identified over two or three bonds
Through-space correlation	1D and 2D nOe	Configuration and conformation analysis

Usually, after obtaining the ^{1}H NMR spectrum, carbon nucleus is the next target. The possibility to edit ^{13}C spectrum due to the number of attached protons to each carbon a peak is decisive for the determination of the skeletal structure. In ^{13}C-edited experiments the one bond ^{13}C-1H coupling constant ($^{1}J_{C,H}$) should be verified by possible variations giving unreliable editing, especially when the coupling constant are large. Despite the high sensitivity of modern spectrometers, the 1D ^{13}C NMR experiments are limited by the obtained amount during the routine isolation process of natural product, due the low abundance of ^{13}C nucleus (1.1 %). However, this limitation can be overcome by 2D ^{1}H-^{13}C correlation experiments.

The 2D NMR experiments are widely used at present to determine the structure of the natural products. Homonuclear ^{1}H-^{1}H correlations NMR experiments are efficient tools to answer the remaining doubts about the natural product structure by pair or system of coupled protons. Nowadays, there are several variants of COSY (COSY 45°, DQ-COSY, Z-COSY and E-COSY) that can be used for specific situation of the analysis. TOCSY experiment is a protocol in which, the extent of magnetization transfer in a coupling system depends on both the duration of spin lock and the size of ^{1}H-^{1}H couplings. Thus, the TOCSY experiment should be optimized for a greater probability of success. one-bond ^{1}H-^{13}C correlations usually employ proton observation to aid sensitivity rather than the direct detection of the carbon nucleus. The ^{1}H-detected sequences require less time than 1D ^{13}C experiments because the sensitivity is increased around ten-fold. Moreover, there is the possibility to edit 2D NMR experiments to differentiate the multiplicity of the carbons. Quaternary carbons can be identified by ^{1}H-^{13}C long-range correlation experiments. The sequences are usually optimized for 8 Hz coupling constant, but this value can be changed (3 – 12 Hz) to improve the signal intensity or its possible to employ variant pulse sequences to obtain specific long-range correlations [5].

The structure of natural products, often far more difficult because is necessary to determine the configuration and/or the conformation of the molecule. In this sense, the through-space correlation experiments and coupling constants are used to evaluate the three-dimensional structure. Initially, the dihedral angle between a pair of vicinal protons can be used to obtain the three-dimensional information by the size of the coupling constant between them based on Karplus relationship. The vicinal ^{1}H-^{1}H coupling constant is useful to identify *cis* and *trans* configurations in C=C double bonds and to estimate the *anti* and *gauche* conformation in cyclic and rigid fragments. In some cases, it is helpful to obtain the geminal and vicinal ^{1}H-^{13}C to accurate proposes the three-dimensional structure, although the measurement of the coupling constant is far more difficult.

Another alternative to obtain three-dimensional information is to employ the nuclear Overhauser enhancement (nOe) experiments. The nOe effect is observed when one proton is irradiated and it occurs a change in the intensity of another proton that has r^{-6} distance dependence. Thus, it is possible to obtain information of pairs of protons, which are spatially close. There are two pathways to perform this effect. The first is the 2D NOESY experiment that uses a mixing time (without pulse) to build up nOes. The 2D NMR experiment spent more time, but show a full spatial correlation in the molecule. The second is the 1D nOe-Diff experiment. The 1D version is faster and selective for a specific proton. In addition, the 1D nOe-Diff affords a simpler procedure to measure the nOe effect by integration of the signals.

Obviously, the experiments reported in this chapter represent the routine tools for structure elucidation of natural products. Nevertheless, there are many improvements for acquiring and processing of NMR data, based on different pulse sequence, spectrometer devices (software, hardware) and methodologies. Among the perspectives to NMR techniques, the application of non-uniform sampling and pure-shift experiments show the greatest promise in the near future. Moreover, the advances in NMR spectrometer such as the automation utilizing sample changers has become a most valued tool for natural products research [6].

2. Materials and Methods

Compounds **1** – **7** used in the NMR experiments were obtained in the Chemical Biology Laboratory – UFABC compounds library, which were isolated from different natural sources. Compounds **1** and **3** were provided by Aldrich Chemical Company Inc. The NMR spectra were recorded with 5 mg of pure compounds dissolved in 600 mL of $CDCl_3$ at 25°C in tubes of 5 mm i.d. at 500/300 e 125/75 MHz to ^{1}H and ^{13}C nuclei, respectively, in a Varian Unity-plus 500 MHz or in a Ultrashield 300 Bruker Avance III spectrometers. Chemical shifts δ/ppm were recorded based in the observed signal of internal standard TMS (in $CDCl_3$).

3. Results and Discussion

To illustrate the use of NMR in the structural elucidation and differentiation of related compounds, this chapter initially describes the identification of structurally simple monoterpenes *p*-cymene (**1**), carvacrol (**2**), thymol (**3**) and menthol (**4**). In sequence, the structural elucidation ofmore complex biomolecules – one terpene (isopimara-7,15-diene-2a,3b-diol – **5**), one neolignan (dihydrodieugenol B – **6**) and one alkaloid (alchornedine – **7**) isolated from natural sources [7 – 9] is discussed.

3.1 Structural identification of p-cymene (*1*), carvacrol (*2*), thymol (*3*) and menthol (*4*)

The ^{1}H NMR spectrum of compound **1** showed a typical feature of an aromatic system due to signals observed at the range δ_H 7.09 – 7.13 (4H) attributed to hydrogens H-2/H-6 and H-3/H-5. Otherwise, the presence of two doublets atdH 7.00 (J = 8.2 Hz, H-5) and 6.60 (J = 1.6 Hz, H-2) as well as one double-doublet at dH 6.70 (J = 8.2 and 1.6 Hz, H-6) in the 1H NMR spectrum of compounds 2 indicated an 1,2,4-trisubstitutedaromatic system. A similar spin system was detected in compound 3 due to the signals at dH 7.07 (d, J = 7.8 Hz, H-6), 6.72 (dd, J = 7.8 and 0.9 Hz, H-5) and 6.55 (d, J = 0.9 Hz, H-3). The menthane skeleton was proposed to compounds 1 – 3 based on the presence of one singlet at approximately dH 2.3 (H-10) and one doublet at dH 1.2 (J ~ 7 Hz) assigned to the methyl groups H-8 and H-9. 13C NMR spectra of compounds 1 – 3 displayed ten signals among them, six resonated at the interval of dC 113 and 153 confirming the occurrence of related aromatic monoterpenes. The DEPT 135° NMR spectrum of 1 showed carbons atoms of five methyne groups (four aromatic), two quaternary (two aromatic) and three methyl groups. The proton bearing carbon signals were assigned by one bond heteronuclear data observed in the HMQC spectrum. Therefore, as HMBC spectrum showed cross peaks between carbons C-7 and H-2/H-6, between carbon C-7 and H-8/H-9 as well as between C-10 and H-3/H-5 (Figure 1) the proposed structure was 1-isopropyl-4-methylbenzene, knowledged as p-cymene.

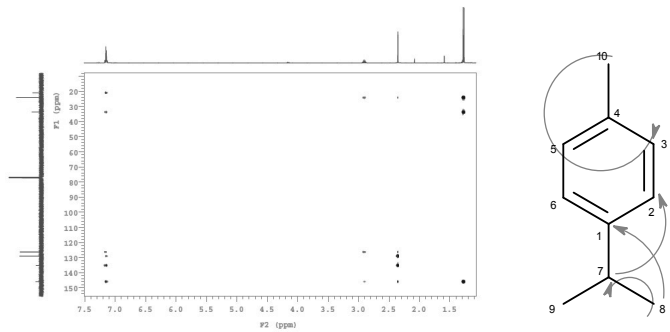

Figure 1. HMBC spectrum of **1** and important observed long-range correlations

In the case of compounds **2** and **3**, similar profile was observed in their 13C NMR spectra compared to those recorded for compound **1**, except by the presence of a carbinolic aromatic carbon at approximately δ_C 153. The position of

the additional hydroxyl group in C-3 of aromatic ring of compound 2 was proposed by using cross peaks between H-2, H-5 and H-10 with the signal attributed to C-3 in the HMBC spectrum (Figure 2).

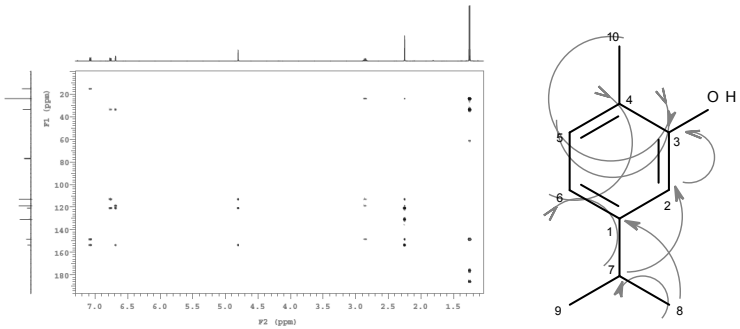

Figure 2. HMBC spectrum of **2** and important observed long-range correlations

Similarly, NMR data of compound **3** showed the shift of a carbon attached to an OH group which was positioned at C-2 since cross peaks between H-3, H-6 and H-7 were observed in the HMBC spectrum (Figure 4). Therefore, the structures of **2** and **3** were identified as carvacrol and thymol, respectively.

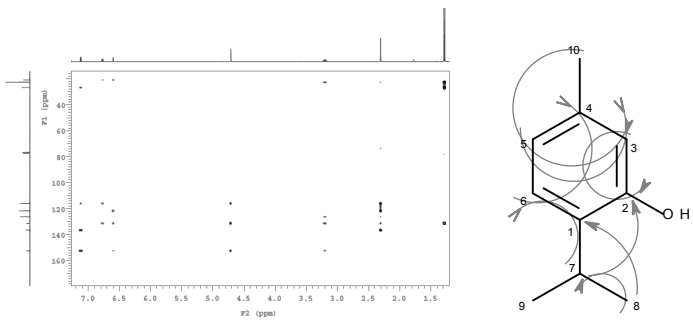

Figure 3. HMBC spectrum of **3** and important observed long-range correlations

The ^{13}C NMR spectrum of compound **4** showed ten peaks at range of δ_C 72 – 16 which were attributed to three methyls, three methylenes and four methines (one carbinolic) based in the DEPT 135° and 90° spectra. based on these data, a non-aromatic menthane skeleton was proposed for the monoterpene **4**. The ^{1}H NMR spectrum showed several peaks including three doublets assigned to

methyl groups at δ_H 0.80 (*J* = 6.9 Hz, H-8), 0.91 (*J* = 6.9 Hz, H-10) and 0.93 (*J* = 6.9 Hz, H-9) as well as to H-2 at δ_H3.40 (td, *J* = 10.5 and 4.3 Hz). These data suggested the positioning of hydroxyl group at C-2 which was confirmed by analysis of HMBC spectrum (Figure 4). This spectrum showed also additional cross-peaks between H-2 and C-4/C-6, H-3 and C-2, H-5 and C-3, H-6 and C-4, H-7 and C-2/C-6, H-8 and C-7 as well as between H-9 and C-1.

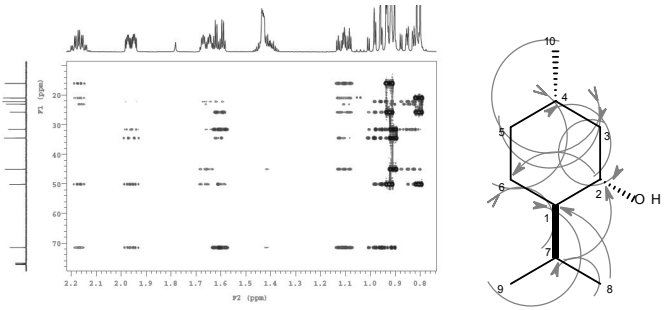

Figure 4. HMBC spectrum of **4** and important observed long-range correlations

Finally, the relative stereochemistry of C-1, C-2 and C-4 were determined based in analysis of NOESY spectrum, which showed, among others, cross peaks between H-2_{ax} and H-4_{ax}/H-6_{ax}/H-8 as well as between H-4_{ax} and H-6_{ax}, suggesting that these hydrogens are positioned at same size of the molecule. Therefore, hydroxyl and methyl group linked at C-2 and C-4, respectively, were positioned in equatorial as showed in figure 5. Therefore, the structure of **4** was identified as (1R*,2S*,4S*)-1-isopropyl-4-methyl-cycloexan-2-ol (menthol).

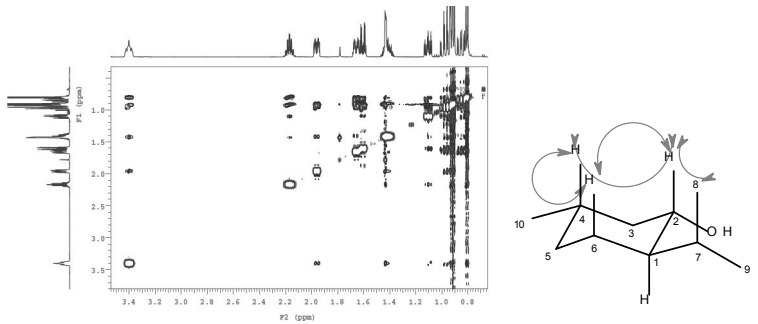

Figure 5. Important spatial correlations observed in the NOESY spectrum of **4**.

3.2 Structural identification of natural products isolated from Brazilian plants

3.2.1 Isopimara-7,15-diene-2α,3β-diol (5) from leaves of *Guarea macrophylla* (Meliaceae)

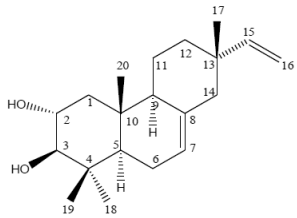

Figure 6. Structure of isopimara-7,15-diene-2α,3β-diol (**5**)

The ^{1}H NMR spectrum of **5** showed signals of hydrogens linked to sp^{2} carbons at δ_H 5.81 (dd, *J* = 17.5 and 10.8, H-15), 4.99 (dd, *J* = 17.5 and 1.4 Hz, H16a), 4.90 (dd, *J* = 10.8 and 1.4 Hz, H-16b) and 5.40 (br s, H-7) which in association to the singlets attributed to methyl groups at δH 0.87 (H-17), 0.88 (H-18), 0.95 (H-19) and 1.05 (H-20), indicating the occurrence of a isopimara-7,15-diene derivative. This spectrum also showed two coupled resonances at δ_H 3.71 (ddd, *J* = 11.7, 9.6 and 4.2 Hz) and 3.05 (d, *J* = 9.6 Hz) which could be assigned to H-3α and H-2β based in splitting patterns and coupling constants values. ^{13}C and DEPT 135° NMR spectral data confirmed this proposal based on signals of the sp^{2} carbons at δ_C 150.2 (C-15), 109.3 (C-16), 121.3 (C-7), and 135.2 (C-8) as well as the oxymethine carbons at δ_C 68.4 (C-2) and 83.7 (C-3). The hydrogen bearing carbon signals were assigned by analysis of the HSQC spectrum. As showed in figure 7, important long-range correlations were observed in the HMBC spectrum, including those between H-15 and C-13/C-14, between H-17 and C-15/C-14, between H-14 and C-7, between C-3 and H-2/H-4/H-20. These results confirmed the structure of **5** as isopimara-7,15-diene-2α,3β-diol.

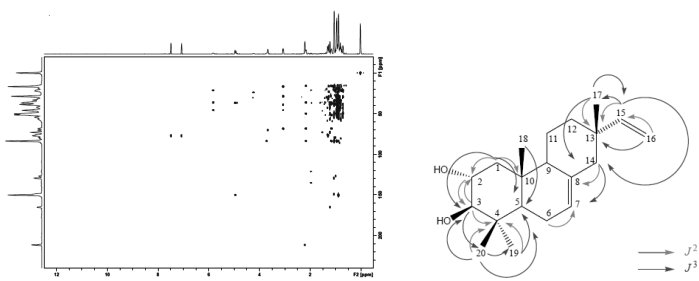

Figure 7. HMBC spectrum of **5** and important observed long-range correlations

3.2.2 Dihydrodieugenol B (6) from leaves of *Nectandra leucantha* (Lauraceae)

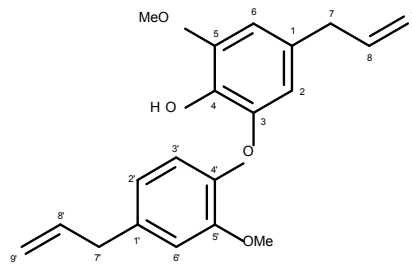

Figure 8. Structure of dihydrodieugenol B (**6**)

The ^{1}H NMR spectrum of **6** displayed characteristic signals of phenylpropanoid derivatives: two doublets involved in an aromatic AB spin system at δ_H 6.49 (J = 1.8 Hz, H-2) and 6.40 (J = 1.8 Hz, H-6) corresponding to one 1,3,4,5-tetrasubstituted aromatic ring as well as one doublet at δ_H 3.24 (J = 6.6 Hz, H-7) and two multiplets at δ_H 5.92 (H-8) and 5.06 (H-9). This spectrum showed also signals of an aromatic ABX spin system at δ_H 6.70 (dd, J = 8.1 and 2.0 Hz, H-2'), 6.79 (d, J = 2.0 Hz, H-6') and 6.89 (d, J = 8.1 Hz, H-3') indicative of a 1,2,4-trisubstituted aromatic ring. These signals associated to the presence of another doublet at δ_H 3.36 (d, J = 6.6 Hz, H-7') and the multiplets at δ_H 5.06 (H-9') and at δ_H 5.92 (H-8') suggested the presence of another phenylpropanoid unit in order to form a neolignan derivative. Additionally, two methoxyl singlets at δ_H 3.86 and 3.89 were observed. The ^{13}C and DEPT 135° NMR spectra displayed twelve signals of two aromatic ring at range δ_C 148 – 107 (C-1 to C-6 and C-1' to C-6') and four additional sp2 carbons at approximately δ_C 137 (C-8/C-8') and 116 (C-9/C-9') which, in association to the methylene carbon at δ_C 40, were assigned to the allyl side chains. Furthermore, the methoxyl groups were assigned to the peaks observed at δ_C 56.2 and 55.9. The hydrogen bearing carbon signals were assigned by analysis of the HSQC spectrum. As showed in figure 9, important correlations were observed in the HMBC spectrum, including those between H-9 and C-7, between H-8 and C-1, between H-7 and C-9/C-6/C-2, between C-4 and H-6 in the ring A and between H-3' and C-5'/C-1', between H-6' and C-4'/C-2'/C-7' and between H-7' and C-6'/C-9'/C-2'. Finally, the methoxyl groups were linked to C-5 and C-5'based in the HMBC correlations. These results confirmed the structure of **6** as 5-methoxy-3-[5'-methoxy-1'-(8'-propenyl)phenoxy]-1-(8-propenyl)benzene knowledged as dehydrodieugenol B.

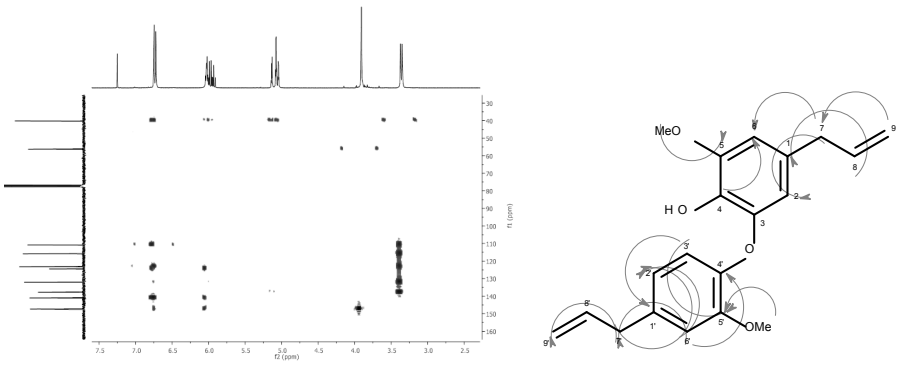

Figure 9. HMBCspectrum of **6** and important observed long-range correlations

3.2.3 Alchornedin (7) from leaves of *Alchornea glandulosa* (Euphorbiaceae)

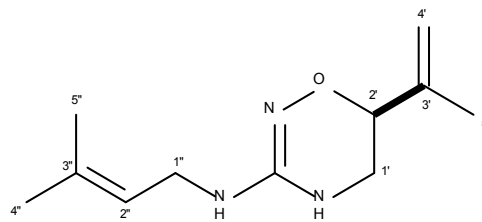

Figure 10. Structure of alchornedin (**7**)

^{1}H NMR spectrum of compound **7** showed two methyl signals at δ_H 1.77 (d, *J* = 1.2 Hz, H-4") and 1.73 (d, *J* = 1.2 Hz, H-5"), which associated to the peaks at δ_H 5.26 (triple-septet, *J* = 6.9 and 1.2 Hz, H-2'') and 3.88 (d, *J* = 6.9 Hz, H-1"), indicating the presence of an isoprene unit of a guanidine alkaloid. The presence of an oxymethine hydrogen at δ_H 4.35 (dd, *J* = 9.6 and 9.0 Hz, H-2'), coupled to two diasteriotopic hydrogens linked to nitrogen at δ_H 3.42 (dd, *J* = 9.9 and 9.6 Hz, H-1'a) and 3.76 (dd, *J* = 9.9 and 9.0 Hz, H-1'b), suggested the occurrence of a heterocyclic unit in the structure of alchornedine. Additional peaks at δ_H 5.13 (qd, *J* = 1.5 and 1.5 Hz, H-4'a), 5.19 (m, H-4'b), and 1.81 (dd, *J* = 1.5 and 0.9 Hz, H-5') indicated the presence of an isopropenyl unit. Coupling between the hydrogens was confirmed by analysis of ^{1}H-^{1}H COSY spectrum (Figure 11).^{13}C and DEPT 135° NMR spectral data confirmed the occurrence of a guanidine alkaloid due

to the peak at δ 163.2, assigned to C-2, and an isoprene unit due to the signals at δ_C 41.7 (C-1''), 119.1 (C-2''), 139.4 (C-3''), 25.9 (C-4''), and 18.2 (C-5''). These spectra also showed two sp^2 carbon peaks at δ_C 118.2 (C-4') and δ 140.6 (C-3'), confirming the isopropenyl unit, as well as one carbinolic carbon at δ_C 71.3 (C-2'), in order to form a cyclic isoprene unit. The remaining peaks at δ_C 45.9 (CH2) and 17.5 (CH3) were assigned, respectively to C-1'' and C-5'. These assignments were confirmed by analysis of the HSQC spectrum. The position of carbon/hydrogen/oxygen/nitrogen atoms at the heterocyclic ring of alchornedine was fully elucidated by analysis of HMBC spectrum (Figure 11) due to the correlations between the peaks of C-2 with H-1', C-2' with H-1', H-4' and H-5', C-1' with H-2', C-3' with H-1', H-4' and H-5', C-4' with H-2' and C-5' with H-4'. Similarly, the correlations between the peaks of C-2 with H-1", C-2" with H-1", H-4" and H-5", C-4" with H-2" and H-5" as well as between the peak of C-5" with H-2" and H-4" confirmed the isoprene unit.

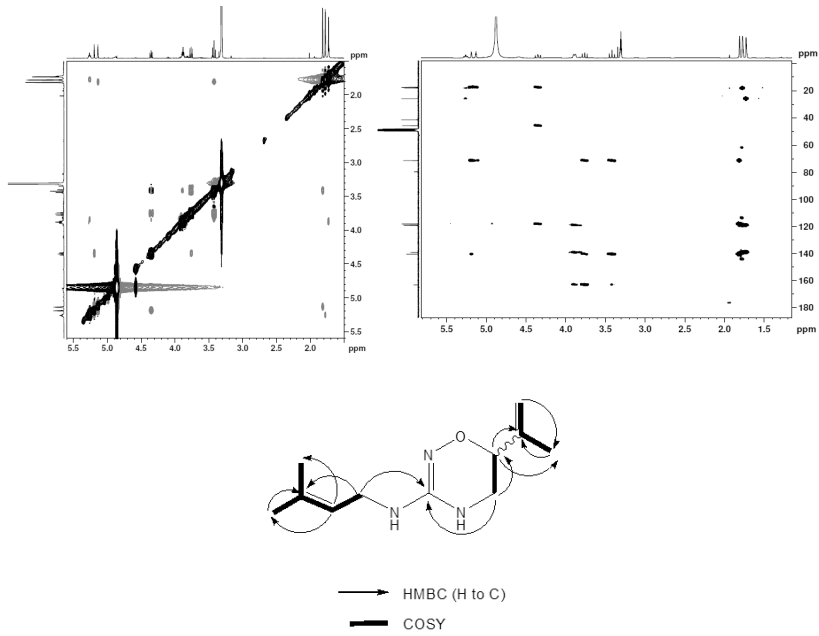

Figure 11. ^1H-^1H COSY (left) and HMBC (right) spectra of **7** and important observed long-range correlations

Finally, the NOESY spectrum (Figure 12) showed cross-peaks between the signals of H-1'a with H-1'b, H-5'), H-1'b with H-1'a/H-2', H-4'a with H-5', H-4'b with H-2' as well as H-5' with H-1'a/H-4'a, which suggest R configuration to C-2.

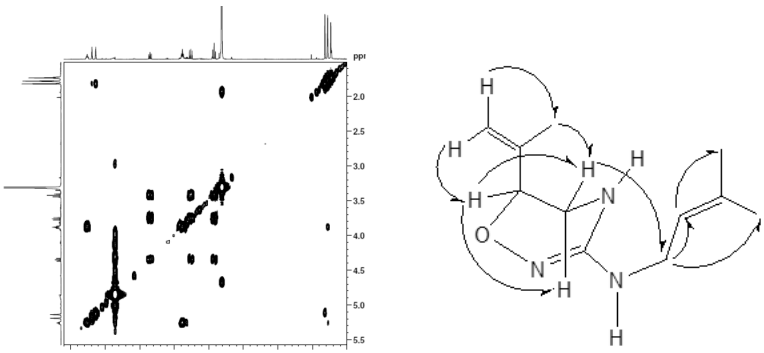

Figure 12. NOESY spectrum of **7** and important observed long-range correlations

As could be exemplified here, the use of different experiments of NMR allowed the chemical characterization of several natural products. However, the unmistakable characterization of these metabolites by NMR involves necessarily the purification of interest compounds (at least 98% of purity). Several authors describe the use of dereplication procedures to detection and identification of several natural products in a crude extract using ^{1}H NMR and chemometric approach [10]. However, the chemical characterization of compounds in reduced amounts or with new structures still consists of a challenge in the analysis of these complex matrices. To solve this problem, an important method of analysis of natural products involves the "hyphenated" techniques such as liquid-chromatography (LC-NMR). There are several methods to deliver the HPLC fraction to NMR probe including on-line or off-line, which are discussed in detailed in the literature [11,12]. However, the use of this hyphenated method is limited to high field spectrometer (700 MHz or superior frequency) and, consequently, large investments to acquisition and maintenance of these spectrometers are mandatory. This may be a limiting stage of the use of procedures as an experimental routine in Natural Products Chemistry laboratories.

Conclusions

In this chapter, we have tried to provide the reader with an overview of the routine NMR experiments for determination of simple or complex structures

of natural products. The interpretation of NMR data allows evaluate different aspects of these compounds such as purity, stability, molecular dynamics, isomerism and other features of organic compounds. The 2D NMR experiments presented are valuable tools for structural elucidation of natural products, with were mandatory to an unmistaken characterization. Other important topic involving 2D NMR experiments is associated to quantity of material necessary to record the spectra. Due to new technology involving inverse detection and field gradient associated to high frequency spectrometers (> 600 MHz) less than 1 mg of a pure natural product can be fully analyzed by NMR in approximately 24h. Additionally, some "hyphenated" methods have been developed in order to reduce the chromatographic steps necessary to obtain the pure compound. Using this approach, a crude extract, composed by more than hundreds ofmetabolites, could be chemically characterized. Therefore, based in the points discussed in this chapter was possible to conclude that the use of NMR (frequently in association to other techniques such as UV, IR, MS etc) consist in the more powerful method to structural elucidation of natural products.

Acknowledgments

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Conflicts of Interest

The authors declare no conflict of interest.

Supplementary material

NMR data of compounds **1** – **7** discussed in this chapter.

p-*Cymene* (**1**): ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 7.09-7.13 (m, H-2/H-6 and H-3/H-5), 2.87 (hept, *J* = 6.9 Hz, H-7), 2.31 (s, H-10), 1.23 (d, *J* = 6.9 Hz, H-8 and H-9). ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C 20.9 (C-10), 24.1 (C-8 and C-9), 33.7 (C-7), 126.3 (C-2 and C-6), 129.0 (C-3 and C-5), 135.1 (C-4), 145.8 (C-1).

Carvacrol (**2**): ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 7.00 (d, *J* = 8.2 Hz, H-5), 6.70 (dd, *J* = 8.2 and 1.6 Hz, H-6), 6.60 (d, *J* = 1.6 Hz, H-2), 4.75 (s, OH), 2.81 (hept, *J* = 6.9 Hz, H-7), 2.20 (s, H-10), 1.20 (d, *J* = 6.9 Hz, H-8 and H-9). ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C 15.2 (C-10), 23.9 (C-8 and C-9), 33.6 (C-7), 113.0 (C-2), 118.8 (C-6), 120.8 (C-4), 130.9 (C-5), 148.4 (C-1), 153.5 (C-3).

Tymol (**3**): ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 7.07 (d, *J* = 7.8 Hz, H-6), 6.72 (dd, *J* = 7.8 and 0.9 Hz, H-5), 6.55 (d, *J* = 0.9 Hz, H-3), 4.66 (s, OH), 3.15 (hept, *J* =

6.9 Hz, H-7), 2.26 (s, H-10), 1.23 (d, *J* = 6.9 Hz, H-8 and H-9). ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C 20.8 (C-10), 22.6 (C-8 and C-9), 26.7 (C-7), 116.0 (C-3), 121.7 (C-5), 126.2 (C-6), 131.3 (C-2), 136.6 (C-1), 152.5 (C-4).

Menthol (**4**): ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 3.40 (td, *J* = 10.5 and 4.3 Hz, H-2), 2.17 (dhept, *J* = 7.0 and 2.8 Hz, H-7), 1.95 (dsex, *J* = 12.0 and 2.1 Hz, H-3), 1.65 (dquint, *J* = 12.0 and 3.1 Hz, H-6), 1.61 (dq, *J* = 13.0 and 3.1 Hz, H-5), 1.37-1.47 (m, H-4 and OH), 1.10 (qt, *J* = 12.0, 10.0 and 3.0 Hz, H-1), 0.92-1.01 (m, H-3' and H-5'), 0.93 (d, *J* = 6.9 Hz, H-8), 0.91 (d, *J* = 6.9 Hz, H-9), 0.81-0.88 (m, H-6'), 0.80 (d, *J* = 6.9 Hz, H-10). ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C 16.0 (C-10), 21.0 (C-8), 22.2 (C-9), 23.1 (C-5), 25.8 (C-7), 31.6 (C-4), 34.5 (C-6), 45.0 (C-3), 50.1 (C-1), 71.5 (C-2).

Isopimara-7,15-diene-2α,3β-diol (5): ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 5.81 (dd, *J* = 17.5 and 10.8 Hz, H-15), 5.40 (br s, H-7), 4.99 (dd, *J* = 17.5 and 1.4 Hz, H-16a), 4.90 (dd, *J* = 10.8 and 1.4 Hz, H-16b), 3.71 (ddd, *J* = 11.7, 9.6 and 4.2 Hz, H-2), 3.05 (d, *J* = 9.6 Hz, H-3), 2.16 (m, H-14a), 2.15 (m, H-1a), 1.97 (m, H-6), 1.96 (m, H-14b), 1.73 (m, H-9), 1.56 (m, H-11), 1.49 (m, H-12a), 1.37 (m, H-12b), 1.25 (m, H-5), 1.15 (m, H-1a), 1.05 (s, H-20), 0.95 (s, H-19), 0.88 (s, H-18), 0.87 (s, H-17). ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C 150.2 (C-15), 135.2 (C-8), 121.3 (C-7), 109.3 (C-16), 83.7 (C-3), 68.4 (C-2), 51.8 (C-9), 49.9 (C-5), 45.8 (C-14), 45.1 (C-1), 38.9 (C-4), 36.8 (C-13), 36.5 (C-10), 35.9 (C-12), 28.8 (C-18), 23.1 (C-6), 21.5 (C-17), 20.1 (C-11), 16.8 (C-10), 15.6 (C-19).

Dihydrodieugenol B (**6**):^{1}H NMR ($CDCl_3$, 300 MHz): δ_H 6.89 (d, *J* = 8.1 Hz, H-3'), 6.79 (d, *J* = 2.0 Hz, H-2'), 6.70 (dd, *J* = 8.1 and 2.0 Hz, H-6'), 6.49 (*J* = 1.8 Hz, H-2), 6.40 (d, *J* = 1.8 Hz, H-6), 5.93 (m, H-8'), 5.92 (m, H-8), 5.06 (m, H-9a and H-9'), 3.89 (s, 5-OCH_3), 3.86 (s, 5'-OCH_3), 3.36 (d, *J* = 6.6 Hz, H-7'), 3.24 (d, *J* = 6.6 Hz, H-7). ^{13}C NMR ($CDCl_3$, 75 MHz): δ_C 150.4 (C-3'), 147.8 (C-5), 144.4 (C-3), 144.2 (C-4'), 137.4 (C-8'), 137.2 (C-8), 136.4 (C-1', 135.2 (C-4), 131.0 (C-1), 120.8 (C-6'), 119.5 (C-5'), 116.0 (C-9'), 115.7 (C-9), 112.9 (C-2'), 111.8 (C-6), 107.3 (C-2), 56.2 (5-OCH_3), 55.9 (5'-OCH_3), 40.0 (C-7), 39.9 (C-7').

Alchornedine (**7**):^{1}H NMR ($CDCl_3$, 300 MHz): δ_H 5.26 (t sept, *J* = 6.9 and 1.2 Hz, H-2"), 5.19 (m, H-4'b), 5.13 (qd, *J* = 1.5 and 1.5 Hz, H-4'a), 4.35 (dd, *J* = 9.6 and 9.0 Hz, H-2'), 3.88 (d, *J* = 6.9 Hz, H-1"), 3.76 (dd, *J* = 9.9 and 9.0 Hz, H-1'b), 3.42 (dd, *J* = 9.9 and 9.6 Hz, H-1'a), 1.81 (dd, *J* = 1.5 and 0.9 Hz, H-5'), 1.77 (d, *J* = 1.2 Hz, H-4"), 1.73 (d, *J* = 1.2, H-5"). ^{13}C NMR ($CDCl_3$, 75 MHz): δ_C 163.2 (C-2), 140.6 (C-3'), 139.4 (C-3"), 119.1 (C-2"), 118.2 (C-4'), 71.3 (C-2'), 45.9 (C-1'), 41.7 (C-1"), 25.9 (C-4"), 18.2 (C-5"), 17.5 (C-5').

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Chapter 4

Liquid chromatography coupled to mass spectrometry for quantitative analyses of medicinal plants and natural products

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1. Introduction

Reports show that medicinal plants have been used to heal people from ancient civilizations. Nowadays, plants are still used as alternative therapy. Medicinal plants have played a key role in world health. Despite the great advances observed in modern medicine, plants still make an important contribution to health care. Medicinal plants are distributed worldwide, and about 70-95% of the population in developing countries depends on plants for primary health care [1]. Natural remedies have gained popularity in developed countries and, as a result, the international herbal medicine market has grown in the last 15 years [2]. Furthermore, plants are essential for the development of new drugs [3-7].

Herbal medicines (or phytotherapics) are developed exclusively from active compounds of medicinal plants (extracts or fractions), which cannot contain the addition of pure active substances. They have complex compositions and their pharmacological actions can be due to one or several substances. The development of new technologies to produce these medicines, as well as the improvement of analytical tools, has led to advances in their quality control, efficacy, and safety.

To be marketed, phytotherapeutic agents need to be licensed by regulatory agencies of a country. Drug regulatory agencies across the globe have provided guidelines for manufacturing, quality control, safety, and efficacy of these

products. The Food and Drug Administration (FDA) has implemented stringent criteria for approval of any herbal product with specific therapeutic benefits [8,9]. The European medicine agency (EMEA) requires that an herbal medicinal product is identified and standardized in terms of therapeutic or analytical marker(s). It also requires that stability of the product should be determined by suitable analytical methods, in accordance with guidelines for stability testing[10]. In Brazil, *Agência Nacional de Vigilância Sanitária* (ANVISA) recommends some chemical tests for quality control, such as purity and integrity tests (humidity, ash, microbiological contaminants, foreign matter, and heavy metals), aflatoxin determination, chromatographic profiling or phytochemical prospecting, qualitative and quantitative analysis of markers, among other regulations, to license an herbal drug [11].

To comply with the rigorous requirements of regulatory guidelines, quality control and assurance have taken a front seat in the herbal product development process. The most acceptable and widely followed approach to guarantee quality is through the standardization of the herbal product with respect to its active and/or analytical markers [12]. The markers are a substance or a class of substances presented in plant extracts or herbal drugs that are used in quality control tests, including their quantification. It is important to note that the active ingredient and the marker constituent of herbal drugs are not always the same chemical. However, the marker should be related to the therapeutic effect [13-15].

The quantitative analysis of markers from extracts can be performed by spectrophotometric techniques, which rely on reactions between one of the chemical classes (alkaloids, saponins, flavonoids, and others) of the compounds present in the plant material with certain reagents. The results herein are expressed in relation to one of the substances, for example *Passiflora incarnata*, which is described as total flavonoids expressed in isovitexin or vitexin [16].

The spectrophotometric techniques present some undesirable characteristics such as lack of sensitivity, inability to quantify only a single substance, or nonexact quantification results due to the false-positive or negative results. One of the most reliable techniques for quantitative determination is the analytical chromatographic techniques, such as liquid chromatography [15].

Several chromatographic and electrophoretic techniques can be applied for quantitative and qualitative studies of plant extracts to separate the compounds present in these extracts. After separation, the compounds are detected so the chromatogram/electropherogram is produced after the response of the detector. The separation techniques include: gas chromatography (GC), high performance liquid chromatography (HPLC), ultra-high liquid chromatography (UHPLC or UPLC) and capillary electrophoresis (CE). Besides these, thin layer chromatography

(TLC) and high efficiency thin layer chromatography (HPTLC) are also used for quality control of plant extracts, mainly in qualitative studies [13-15].

Therefore, chromatographic techniques (HPLC, UPLC e GC) are widely used, since they are able to separate the components from complex mixtures. These components are detected after the elution from the chromatographic system. The commonly used detectors are flame ionization, electron capture, thermal conductivity, thermionic, mass spectrometry (MS), ultraviolet-visible (UV-Vis), refractive index, light scattering, fluorescence, electrochemical, electrical conductivity, circular dichroism, etc. The first four detectors cited are exclusively applied for GC technique.

This chapter highlights the analysis of medium to high polarity compounds through HPLC coupled to the mass spectrometer and the parameters that must be considered when validating analytical methods are used for quantitative studies of analytes from a complex matrix, such as a plant extract. It is important to note that mass spectrometry also determines structural information of compounds from the plant extracts, such as their molecular mass and fragmentation profile, which are valuable for the chemical identification of its components.

2. Liquid Chromatography (HPLC, UPLC)

Liquid chromatography (LC) is an important technique that enables the separation of components from complex mixtures. The separation takes place in a column, which contains a stationary phase. The mobile phase (solvent) transports a mixture of analytes to be resolved through column. The samples are separated by a selective distribution between the mobile and stationary phase and leave the column as narrow bands, subsequently, the eluted compounds are detected [17].

Basic theory played an important role in the development of HPLC, but its implementation was the result of new separation modes or techniques, a better understanding of how to vary conditions for a satisfactory separation and improved chromatographic columns, as well as the improvement of detectors [18].

The LC techniques can be applied in qualitative and quantitative analysis depending on the type of detector used. For these analyses a good resolution in the separation of the compounds is fundamental, and thus the optimization of several parameters is required.

The resolution is a measurement of the ability to separate two adjacent peaks in a chromatogram. In quantitative analysis, the resolution must be greater than 1.5, which impacts the robustness of the analytical method. Thus, the resolution (R_s) depends on the values of separation factor (α), retention factor (K), and efficiency (N). These parameters are commonly described in chromatography books [18-20]. In this chapter, only strategies for achieving better resolutions will be addressed.

The optimization of the chromatographic resolution can be evaluated by **Equation 1**. The first term of Equation is related to the efficiency, while the second term is related to selectivity, and the last term to the retention. It is important to understand these parameters, to change them when necessary and improve the resolution of chromatographic separations.

$$Rs = \frac{\sqrt{N}}{4} \times \frac{(\alpha - 1)}{\alpha} \times \frac{k}{k + 1} \qquad \textbf{Equation 1}$$

α: separation factor
K: retention factor
N: efficiency

The type and size of the chromatographic particles of stationary phases are key factors for the effectivity and stability of columns [21]. The details of particle properties have a decisive impact on the column bed structure and determine the efficiency and lifetime columns.

The efficiency of chromatographic columns improved considerably due to the development of new particles for the stationary phase. These new particles improved the selectivity and thermal, chemical, and mechanical stabilities of the stationary phases [22]. In addition, the time for the chromatographic analysis was greatly reduced, because of improvements of the particles. This has contributed to faster and lower cost studies.

The lack of efficiency in the chromatographic separation happens because of the broadening or asymmetry of the peaks. The band broadening leads to a reduction of the resolution since the resolution is directly proportional to the efficiency (**Equation 1**). The biggest contributor to band broadening is usually the column itself. The broadening of chromatographic peaks can be due to multiple paths, longitudinal diffusion and mass transfer [20].

The multiple paths (**Figure 1A**), also called Eddy´s diffusion, depends on the type of particle and the packing. In this situation the enlargement occurs due to the possibility of different path lengths that the analytes can go through. Thus, the same analytes will reach the detector at different speeds. Eddy diffusion can be minimized by using smaller and highly uniform packing materials, smaller diameter particles, smaller inner diameter columns, or stationary phases with small size distribution.

For longitudinal diffusion, molecules diffuse in the mobile phase during elution (**Figure 1B**). The longitudinal diffusion is inversely proportional to the mobile phase flow and directly proportional to the diffusion coefficient of the analyte in the mobile phase and to the retention time in the column. This effect can be solved by increasing the flow rate. However, the pressure of the chromatographic column can increase and damage it, consequently reducing its life time.

The mass transfer (**Figure 1C**) occurs when the analyte molecules enter the pores of the stationary phase particles. The molecules inside of deep pores remain in the column for a longer time, because they require longer time to exit of the column. This effect can be minimized by the reduction of the flow rate of the mobile phase or by using columns packed with smaller particles.

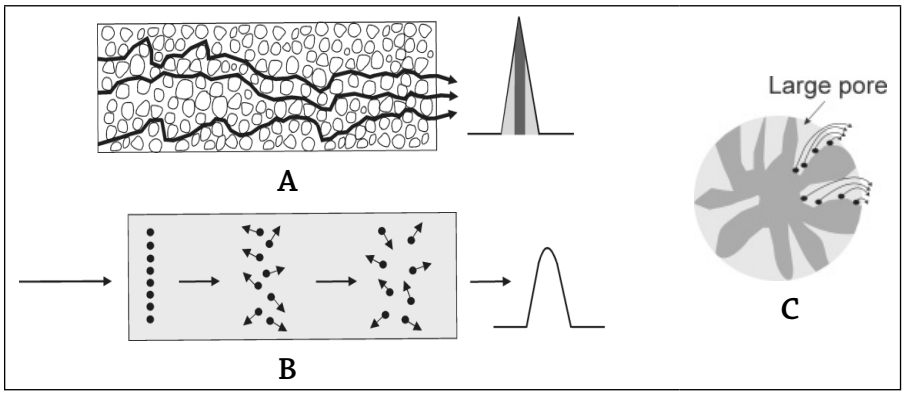

Figure 1. Effects responsible for the loss of efficiency: multiple paths (**A**), longitudinal diffusion (**B**) and mass transfer (**C**).

The selectivity factor (**Equation 1**) qualifies the separation of adjacent chromatographic peaks, and should be between 1.2 and 1.5. In analysis with non-selective stationary phases, separation of the compounds is more difficult. Therefore, for separations of complex mixtures, i.e. plant extracts, it is necessary to use stationary phases with high selectivity. For this reason, the selection of stationary phase is fundamental for studies that involve quality control of plant extracts. This issue will be discussed in section 2.3.

The retention factor is measured as the ratio of retention time of the analyte on the column and the retention time of a non-retained compound (dead time). The non-retained compound has no affinity for the stationary phase and

elutes with the solvent front at time t0, which is also known as the hold-up-time or dead time. The retention factor is recommended between 2 and 10 [22,23]. Small changes in the flow rate and the column dimension induce insignificant variations in the retention factor, but it is important to optimize this parameter.

Table 1 shows some alternatives for optimizing each parameter that influences the chromatographic resolution, which are useful for better resolutions. It is important to emphasize that before optimizing these parameters it is fundamental to select the type of stationary phase (such as normal or reversed phase). Stationary phase must be consistent with the polarity of analytes from the plant extract.

Table 1. Parameters that can be optimized to improve the efficiency, selectivity, and retention, and consequently the resolution.

Factor	Variation of Parameters
Efficiency	- Use particles with smaller diameter - Increase the column length - Optimize the flow rate of the mobile phase - Optimize the temperature of the chromatographic analysis
Selectivity	- Change the composition of the mobile phase - Change of the stationary phase type
Retention	- Change the elution force of mobile phase - Change the polarity of mobile phase

Normal stationary phases, such as silica, alumina, cyano and amino, should be used to separate non-polar compounds (eg. triterpenes, carotenoids, sesquiternepes and others). While reversed stationary phase, such as C18, C8, and phenyl, should be used to separate medium to high polarity compounds (flavonoids, saponins, tannins, phenolic acids, and others). In addition, to successfully separate the chromatographic analysis, the particle size, column dimensions, and the selected parameters of analyses, such as flow rate, temperature, etc., should be carefully chosen.

2.1. Particles for liquid chromatography

The first stationary phases used in liquid chromatography consisted of irregular particles with diameters between 100 to 200 μm. In the 1960´s pellicular particles were developed with diameters of 40-50 μm. These particles have a solid core with a stationary phase coating layer on them (**Figure 2**). However, these particles still had reduced sample capacity and low efficiency [24].

Afterwards, the spherical porous particles with 10 μm were developed. These particles were six times more efficient than the previously described pellicular particles. In the 80´s, spherical particles with diameter of 5 μm were produced, showing greater efficiency than the previously produced particles with 10 μm [22,25]. The reduction of particle diameter resulted in better chromatography resolution and efficiency, but higher pressure of systems was also observed.

Despite the development of the 5 μm spherical particles, the search for more efficient separations and shorter analysis times continued. Alternatives for reducing the analysis times include the reduction of column size and increased flow rate of the mobile phase. However, with the first strategy there is loss of efficiency in the separations and in the second there is a considerable increase of the system pressure [25,26]. Thus, one alternative was the development of chromatographic columns with smaller lengths, but packed with smaller size particles to compensate for the reduction of the column size.

In the mid-1990s, spherical particles with diameter of 3-3.5 μm were developed, but they did not represent a significant advance in resolution [27]. In 1996, the particles of 1.5 μm were prepared and they showed efficiency of 30,000 theoretical plates/15 cm, representing a meaningful increase in resolution. In 2004, columns packed with 1.7 μm particles were produced with similar efficiency as packed columns with 1.5 μm particles [24,28,29].

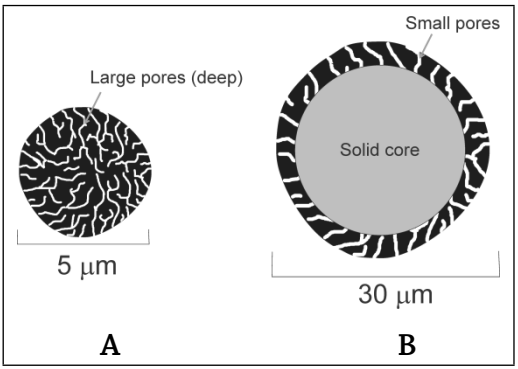

Figure 2. Porous (A) and pellicular particles (B).

Furthermore, the packed columns with sub-2 μm particles represent a significant increase in the separation efficiency, but a very large increase in system pressure is observed that is incompatible with HPLC systems. Thus, the instruments were modified to endure higher pressures, and are called ultra-

performance liquid chromatography (UPLC or UHPLC). These changes were carried out in the whole chromatographic system, such as columns, detectors, pumps, and others [30,31].

Pumps were changed to move the mobile phase more smoothly and increase reproducibility. Ovens were also included is the equipment to heat the column, keep a constant temperature, reduce the pressure in the columns, and increase the chromatographic resolutions. Due to the high linear velocities of the mobile phase, temperature variations can occur during the chromatographic analysis and can harm the reproducibility. The detectors coupled to the UPLC were also changed to operate with high sampling rates, and thus enough points were obtained for satisfactory chromatographic peaks. In addition, they could maintain the reproducibility, accuracy, and sensitivity [31].

The increment and evolution of UPLC was only possible through the development of the chromatographic supports of packing particles with higher resistance. The most commonly used support is silica. These supports allow the production of particles with higher mechanical, thermal, and chemical resistance [19,22]. These new chromatographic supports based on silica are used to manufacture of columns for both HPLC and UPLC, which are described in section 2.2. Thus, UPLC systems operate using packed columns with sub-2 μm particles and pressures higher than 100 MPa, meaning the average analysis time is about 1 to 5 min. The reduction of time analysis compared to the time of HPLC analysis results in higher productivity, lower costs, and lower consumption of organic solvents.

In addition, high temperatures (> 60 °C) can also be used in liquid chromatography to reduce the time of the chromatographic analyses. There is an increase in efficiency and mass transfer, but problems with the stability of the stationary phases can be observed, which is why it is important to check the temperature limit for each type of packed material. Another relevant point is the decrease of the mobile phase viscosity with higher temperature that allows the use of higher flow rates, without a significant increase in pressure [32,33].

2.2. Chromatographic supports to stationary phases: Stability and faster analyses

The chemically bonded stationary phases are most commonly used for separating organic compounds, mainly medium to high polarity compounds. Among them, the most used are phases C18 and C8, which are stationary phases containing 18 and 8 carbons, respectively, attached to a silica supports. For this kind of phase, known as the reversed phase, there is high retention of non-polar

compounds. Therefore, these phases are recommended for analysis and separation of polar compounds.

The advances in stationary phase supports followed two paths: higher stabilities (thermal and chemical) and faster analyses. The main advances occurred with silica support, which is the most widely used support to produce stationary phases. In the past, silica was obtained from the sodium silicate, but nowadays it is yielded from a sol-gel process, using tetraethoxysilane as the SiO_2 source. Hence the production of porous spherical particles with a small diameter is possible [30].

Despite the wide use of silica, it presents some disadvantages, such as a narrow pH range (2 < pH <8) and the presence of the residual silanols (-Si-OH) that produces losses of resolution due to undesirable interactions and losses of efficiency in basic compound analyses. These residual silanol groups show higher activity when metals (contaminants) are present [34,35]. To minimize the problem related to the presence of metal in silica (0.1 to 0.3%), a few additional steps were taken when producing silica, which can now be obtained with low metal content, denominated by high purity silica or type B (Unger *et al*., 2013). High purity silica increases the efficiency and selectivity of the chromatographic separations [34,36].

The disadvantages of the conventional silica support promoted the development of the hybrid silica, which presents higher stability (chemical and thermal), reduced undesirable interactions (steric impediment and/or reduced number of residual silanols), and greater mechanical resistance. The first hybrid silica particles (1st generation) were produced from the reaction between tetraethoxysilane and methyltriethoxysilane, and methyl groups were incorporated in the inorganic matrix (**Figure 3**) [22].

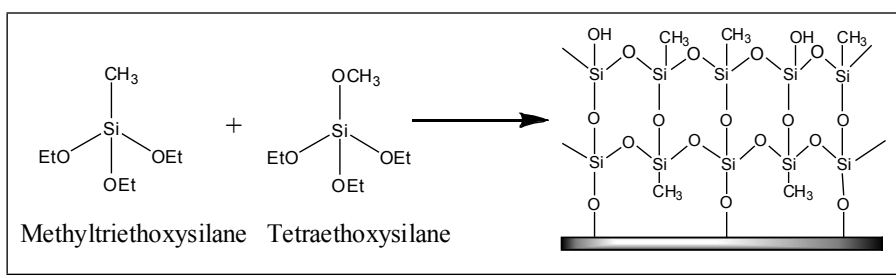

Figure 3. Synthesis reaction of the 1st generation hybrid silica particles produced by Waters (XTerra®).

Afterwards, other types of hybrid silica were developed with ethane bridges replaced by silane bridges and with an organosilane layer inserted on the surface of a silica core (**Figure 4 A, B**). Some of these commercially available particles and their specifications are shown in **Table 2**.

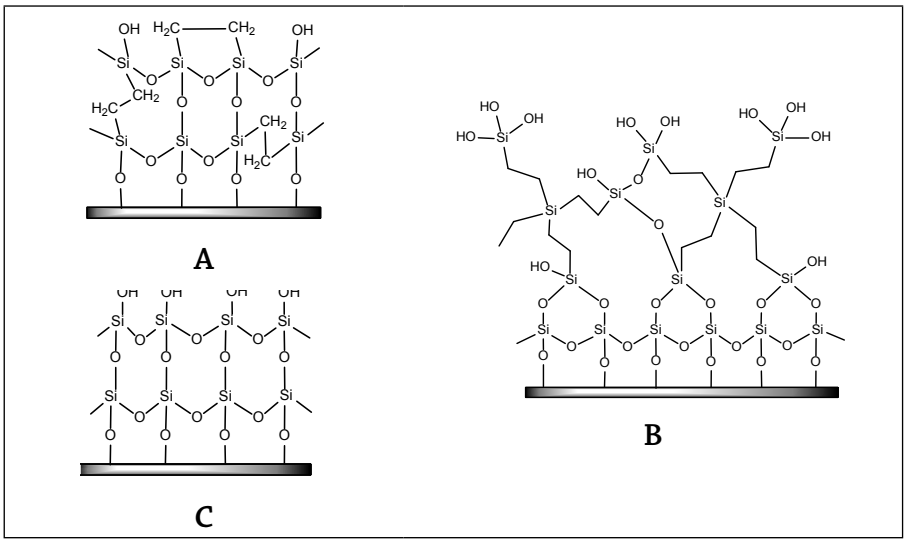

Figure 4. Hybrid (ethane bridging) (**A**) and polydentate (**B**) silica structures compared to conventional silica (**C**).

Table 2. Stationary phase particles produced by hybrid silica support which are commercially available for HPLC and / or UPLC and their analytical conditions.

Stationary Phase (manufacturer)	Type of Support	T_{max} (°C)	pH range	Bonded phases
Type-C (Microsolv)	Hybrid silica with Si-H bonds	80	1.5 – 10.0 2.0 – 8.2	C8, C18 UDC-Cholesterol
XTerra® (Waters Corp.)	Hybrid silica with methyl groups	60	2.0 – 12.0	C8, C18, phenyl
XBridge® (Waters Corp.)	Hybrid silica with ethane bridges	60 45 45 60	1.0 – 12.0 2.0 – 11.0 1.0 – 8.0 2.0 – 11.0	C8, C18, phenyl Protected C18 HILIC Amide

TWIN (Phenomenex)	Hybrid silica (organosilane layer containing methyl groups) implanted on the silica surface	60	1.0 – 12.0	C18, phenyl-hexyl
TWIN-NX (Phenomenex)	Hybrid silica (organosilane layer containing ethane bridge) implanted on the sílica surface	100	1.0 – 12.0	C18
Eternity (Kromasil, Akzonobel)	Hybrid silica (organosilane layer) implanted on the silica surface (inorganic-organic interface)	80	1.0 – 12.0	C18, phenyl-hexyl
$Blaze_{200}$ (Selerity Technologies)	Polydentate hybrid silica	200	1.0 – 12.0	C8, C18

T_{max}: maximum temperature recommended.

The packed columns with hybrid silica are stable over a wide pH range (1<pH<12) and show high mechanical resistance. These particles are stable even for bonded stationary phases, such as with long hydrocarbon chain linkages containing 18 carbons (C18 column - reversed phase) or other chains. In addition, particles with hybrid silica support are used to produce UPLC columns because they are stable at high pressures [22,24,28].

Since several particles do not allow the use of temperatures above 100°C, another generation of hybrid silica was developed, such as the $Blaze_{200}^{TM}$ particles, (Selerity Technology Inc.). This hybrid is produced from silica particles coated by polydentated silane containing silica binding sites (**Figure 4B**), revealing high efficiency, high hydrolytic resistance and high chemical stabilities (1<pH<12), and thermal stabilities (up to 200 °C) [37]. If the methodology for compound analysis of the plant extract requires a high pH of mobile phase (pH≥8), or a higher temperature, to have high efficiency and resolution in the separation, the analyst should select packed columns with particles of hybrid silica support, since these are more stable under extreme conditions and will not degrade during the chromatographic analyses.

Another strategy to increase the chemical, thermal, and mechanical stabilities of chromatographic supports are the metallic oxide supports (alumina, zirconia, titania and others), which act in different ways depending on the pH. They can be cationic, anionic, or amphoteric exchangers. These supports can be coated with polymers to separate the compounds through different mechanisms (reverse phase or ionic exchange), representing a different selectivity of the conventional

reversed phase [19,22,24]. The Zirchrom®-PBD column (polybutadiene-coated zirconia) is an example and is manufactured by Zirchrom. This is stable over a wide range of pH (1-14) and temperature (200°C), and is the most widely used among chromatographic columns with metal oxide support.

In addition to the chromatographic supports of hybrid silica, porous silica particles called fused core (solid core) were developed. These particles have a solid silica core and an external layer of porous silica coating (**Figure 5**). The particles have low resistance to mass transfer and their sample capacities are similar to the capacity of porous particles with 2 μm diameter. Furthermore, these particles have similar efficiency to sub-2 μm particles used in UPLC. Despite the high efficiency of fused core particles, they present lower pressures than the superficially porous silica particles and particles used in the UPLC. Thus, fused core particles are also compatible with HPLC systems, but it is important to note that the chromatographic separations using these particles (≤ 6 min) are performed at shorter times than those by conventional HPLC particles. Therefore, the stationary phases with silica support of fused core type have shown similar performance for UPLC analyses, with high efficiency and chromatographic resolution [22,30,33].

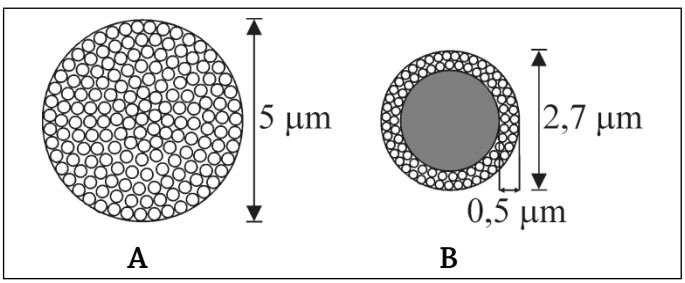

Figure 5. Spherical particles: 5 μm fully porous (**A**) and 2.7 μm superficially porous (fused Core) (**B**).

The fused core particles are named depending on the manufacturer, such as Poroshell, Halo, and others (**Table 3**). Although these particles represent efficient separations, they do not show significant increases in chemical and thermal stability. Generally, the recommended pH range is around 2 to 9 to avoid column damage.

Several types of chemically bonded phases on the surface porous silica particles are commercially available, such as C8, C18, phenyl-hexyl and others. Besides that, particles with different pore sizes can be found. For example, Poroshell 120 and 300 with pores of 120 and 300 Å (both with a thickness of the external porous layer of 0.5 μm). The phases are indicated by analysis of small molecules and macromolecules (proteins and peptides), respectively.

Table 3. Commercially available stationary phases with fused core particle supports.

Column	Manufacturer	Particle size (μm)	Pore Size (Å)	pH range	Bonded phases
Poroshell 120	Agilent	2.7 and 5.0	120	1.0 – 8.0 2.0 – 8.0	C18 C8*,C18*
Poroshell 300	Agilent	2.7	300	1.0 – 8.0 2.0 – 11.0	C3, C8, C18 C18*
Halo	Advanced	2.7	90	2.0 – 9.0 2.0 – 8.0	C8, C18, RP-Amide, phenyl-hexyl HILIC, PFP
Ascentis Express	Supelco	2.7	90	2.0 – 9.0 2.0 – 8.0	C8*, C18*, RP-Amide*, PFPP*, phenyl-hexyl* HILIC
Kinetex	Phenomenex	1.7 and 2.6	100	1.5 – 10.0 1.5 – 8.0 2.0 – 7.5	C8, C18, C18* PFP HILIC

RP: reverse phase; RP-amide: reversed phase embedded-polar stationary phase (amide); PFP: pentafluorophenyl; PFPP:pentafluorophenyl propyl; * end-capped.

To reduce the analysis time, monolithic silica support was also developed. This support reveals a significant decrease in analysis time without loss of resolution and efficiency [38-40]. Monolithic particle is a continuous bed of separation, which is a single (monolith) particle of silica with macropores (2 μm) and mesopores (13 nm). This particle is rod-shaped, which is packed inside a poly(ether-ether-ketone) (PEEK) tube. Due to pores (macropores and mesopores) of monolithic support, it has high permeability (low resistance) of mobile phase, allowing for high flow rates, but with low pressures. The efficiency of monolithic columns is like packed column with 3-5 μm porous particles. However, monolithic columns still present the disadvantage related to the activity from the residual silanols, but several manufacturers are yielding functionalized monolithic columns to reduce the activity of these groups.

In 2000, the first monolithic columns were made commercially available. They were produced by Merck and were named Chromolith®. Since then, several studies have been done with them, demonstrating their popularity [41]. Nowadays, these columns are also produced by Phenomenex, named Onyx. The commercially available columns can be found with various internal diameters (from capillary to preparative), lengths, and chemically bonded phases (end-capped C8 and C18).

The end-capping reactions, which blocked the residual silanol groups, are another strategy to increase the stability of stationary phases (chromatographic supports). Generally, these stationary phases are sterically protected or bidentate phases (**Figure 6**) to reduce the activity of residual silanols. In addition, they show a different selectivity, as well as chemical stability [36].

The end-capping reactions are performed by small organosilane reagents, such as trimethylsilyl chloride, to reduce the silanol groups. This kind of chromatographic support is widely used to produce chemically bonded stationary phases. They have greater selectivity and chemical stability, and normally they can be used in pH above 8 [42,43]. Zorbax Eclipse XDB, produced by Agilent, is an example from this chromatographic support. It is commercially available with the bonded C8, C18, phenyl and cyano (CN) phases, which can be applied to mobile phases with pH range of 2 to 9. However, CN phase is an exception, which is stable only in the pH range of 2 to 8.

The sterically protected and bidentate phases are produced by Agilent, and named Zorbax Stable Bond® and Zorbax Extend-C18®, respectively. The sterically protected phase can be used for analyses with pH lower than 2 and bidentate phases with pH above 8. To produce the sterically protected phases monofunctional silylating agents containing voluminous groups, such as isobutyl or isopropyl, are used and result in a steric barrier of the siloxane bonds. The confection of bidentate phases is performed by bidentate silylating agents (ethyl or propyl chains bonded to two silicon atoms), which reduce access to silanols. Thus, the chemical stability of the stationary phases is increased [44].

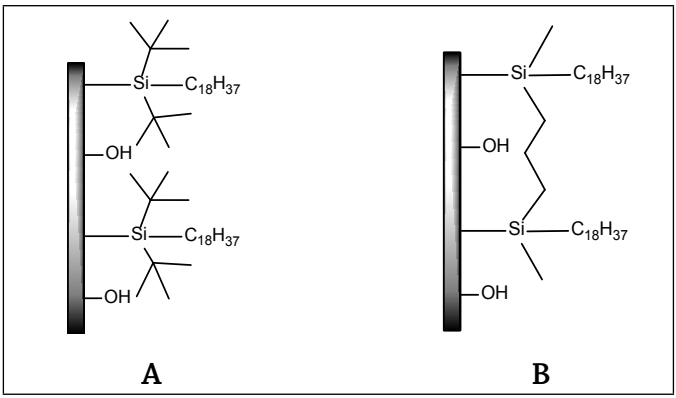

Figure 6. Sterically protected stationary phase (**A**) and bidentate stationary phase (**B**), both are bonded C18 (support: silica).

2.3. Stationary phases with different selectivities

Selectivity is a fundamental parameter in chromatographic analysis, which is directly related to chromatographic resolution (**Equation 1**). The low selectivity (no completely separated peaks) and low efficiency (broadening peaks) analyses have low chromatographic resolutions, besides the quantitative determinations are impaired. Thus, to develop a HPLC method for quality control of plant extracts, it is necessary to know about stationary phases and the criteria involved in chromatographic resolution, because plant extracts are complex matrices and the analyst should know this [45].

Selectivity can be optimized by changing some parameters, such as mobile phase composition, temperature, and pressure in the analyses, as well as by stationary phase types [46,47]. Besides that, the compounds nature that will be analyzed can influence separation selectivity[48], mainly in basic compounds analyses [49,50]. The mobile phase can be adequate to change the selectivity, as changes in pH, in mobile phase organic modifiers, and in organic solvent composition [51-55]. The selectivity triangles can be used to select the mobile phase composition [19,54].

The addition of buffer solutions to the mobile phase, to inhibit pH variation and stabilize analyte and silanols ionizations, is a common practice to improve the chromatographic resolution and reduction of tailing chromatographic peaks related to basic compounds, for example [50,52]. However, carefully cleaning the equipment and the chromatographic columns are necessary when buffers are used. In addition, it is important to know the pH range limit of the stationary phase and to verify if the buffer and detector of equipment are compatible, since several buffers are not compatible with mass spectrometers.

The selectivity can be modified by several factors, but in this chapter, we only approach the stationary phase, which is the most significant factor. Among the stationary phases with different selectivity, it is possible to find phases with embedded polar groups [56,57], fluorinated [58,59], metal oxides and mixed [60,61], phenyl [62,63], immobilized polymers[64], mixed-mode reversed-phase [65,66], hydrophilic interaction (HILIC) [67], and end-capped [42]. The end-capped packed columns were discussed in the previous section, as well as the increase of chemical stability [42,43].

Due to the difficult analyses for highly polar compounds and basic compounds, such as alkaloids, new stationary phases with higher selectivity were developed. The most important variables related to the selectivity of stationary phases are: the characteristics of the bonded phase; shape, size, and distribution of particles, and amount of residual silanol groups [46]. The end-capping reactions enable stationary phases to be more selective, because these reactions reduce the activity of the residual silanols [43]. The stationary phases with embedded polar groups have a polar group between alkyl chain and the silicon atom and the spacer is

generally a propyl group (**Figure 7**). These stationary phases are manufactured by several companies and the most polar groups used are: carbamate, ether-phenyl, amide, amine, urea, and ether [68]. The following packed columns are some of the commercially available ones: Supelcosil ABZ (amide and C16, Supelco), XTerra RP18 (carbamate and C18, Waters), Zorbax Bonus RP (amide and C14, Agilent), Symmetry Shield RP8 (carbamate and C8, Waters), Prims RP (urea and C12, Thermo), etc.

The main alkyl chains of stationary phases with embedded polar groups are constituted by 8 (C8) or 18 (C18) carbons. Due to embedded polar groups in these stationary phases, they show a different selectivity when compared to conventional C8 and C18 stationary phases. The polar groups can interact with the sample molecules by forming hydrogen bonds with the following groups: residual silanols, polar groups of stationary phases, water molecules and analyte molecules [57]. These interactions can reduce the activity of residual silanols and result in more selective separations of basic compounds [68,69]. Moreover, high proportions of water in mobile phases can be used without breaking down these stationary phases.

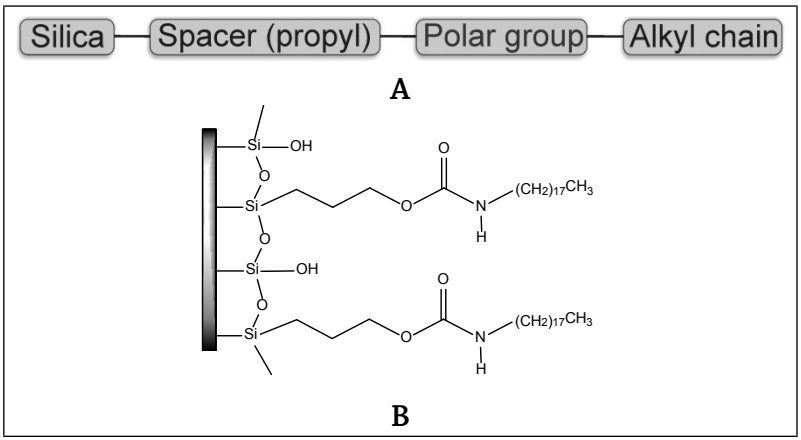

Figure 7. Scheme of the stationary phase with embedded polar group (**A**) and an example of an embedded amide group and alkyl chain of C18 stationary phase (**B**).

The fluorinated stationary phases have fluor atoms attached to the alkyl chain (alkyl fluorinated) or phenyl groups (phenyl fluorinated) (**Figure 8**). Theses stationary phases promote higher retention of basic compounds, esters, and ketones. In addition, they maintain or increase the retention of fluorinated compounds and reduce the retention of hydrocarbons[59]. The fluorinated phases have differentiated selectivity, since they have groups with C-F binding that increase the dipolar

character of stationary phase and, thus, promote additional interactions [70]. Nowadays several manufacturers produce fluorinated stationary phases, such as Discorevy F5 HS (Supelco), Fluorophase (Thermo), Fluoro-Sep-RP (ES Industries), etc.

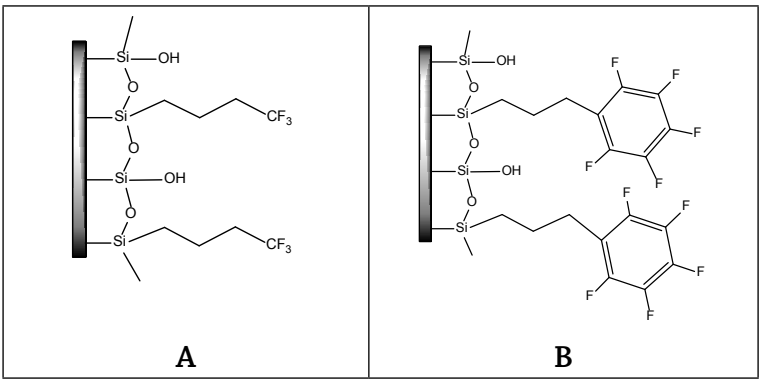

Figure 8. Alkyl fluorinated (A) and phenyl fluorinated (B) stationary phases.

The main advantage of fluorinated phases is their application in chromatographic separations of low to high polarity compounds. In this case, the separation depends on the organic solvent concentration in mobile phase; higher concentrations of organic solvent induce higher retention of polar and basic compounds [71]. These fluorinated phases can be applied for analyses of tocopherols [72], aromatic compounds [73], herbicides [74], lipidic compounds [75], alkaloids [76], phenols [77], fluorinated compounds [78] etc.

The stationary phases with phenyl groups have one or two aromatic rings attached to the silicon atom through a carbon chain that has 0 to 6 carbon atoms. In some phases there is a heteroatom, such as oxygen, between the aromatic ring and the alkyl chain used as a spacer [79]. Synergi Polar RP is an example of this kind of stationary phase that is commercialized by Phenomenex (**Figure 9**). These stationary phases are also commercially available in end-capped form: Zorbax Eclipse fenil-XDB (Agilent) and XTerra phenyl (Waters). The phases with phenyl groups have differentiated selectivity due to the possibility of interactions between the π electrons of the phenyl groups and other analyte molecules. Therefore, the separation (retention) of compounds containing π electrons depends on the π-π interactions and the alkyl chain type, since this chain influences the hydrophobicity of the stationary phase and may modify its selectivity [80].

The mixed mode stationary phases have ionic exchange groups inserted on the alkyl chain (**Figure 10 A, B**). These groups may be anionic or cationic

exchangers (**Figure 10 C, D**). The exchanger group may be inserted into the end portion of alkyl chain, such as Acclain columns (Dionex) or between the spacer and the alkyl chain, such as Primesep columns (SIELC Tech.).

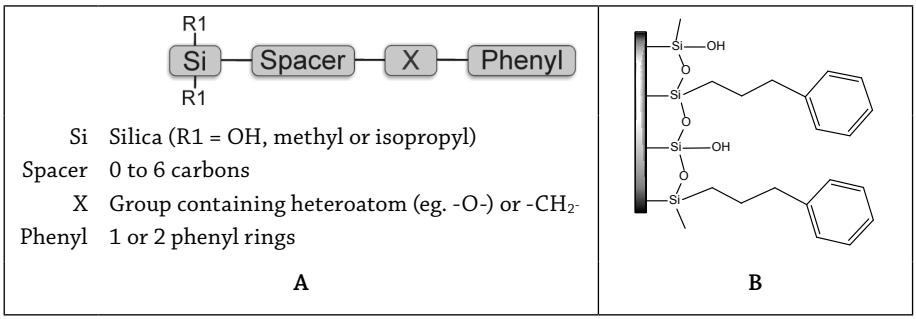

Figure 9. Scheme of phenyl stationary phase (**A**) and an example with one phenyl ring stationary phase (**B**).

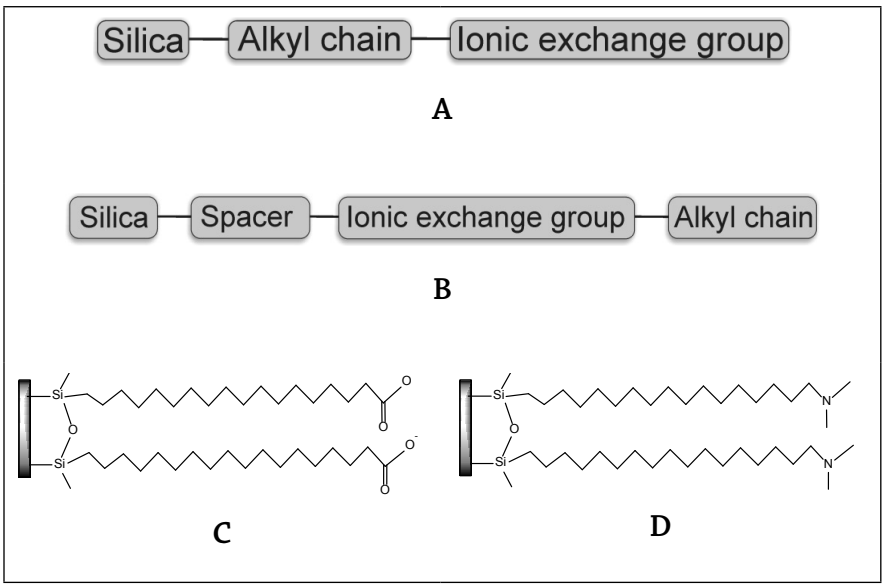

Figure 10. Scheme of the ionic exchange stationary phases (**A, B**). Examples of a cationic exchange (**C**) and an anionic exchange (**D**) stationary phases.

For the mixed mode stationary phases, separations occur through a combination of reversed phase and ionic exchange mechanisms [36,81]. These stationary phases are recommended to separate complex mixtures of polar, ionic, ionizable, and neutral compounds. The neutral substances may interact with reversed phase (alkyl chain). The ionic compounds may be separated by the ion exchange mechanisms, as well as ionic exclusion or hydrophilic interactions [66,82]. These stationary phases can be applied in analyses of complex mixtures, such as anthocyanins [83,84], alkaloids [65,85] and others [86-89].

Therefore, the selectivity of the analysis can be adapted in the mixed mode stationary phases by modifying some parameters related to the mobile phase (organic modifier, pH, temperature, and the type of buffer) which can activate or deactivate the ionic groups of the stationary phase [66,81]. The main advantages of the mixed mode phases are the possibility of separation of different compounds present in the matrix (including alkaloids and other ionizable substances), easily controlled selectivity, and different mechanisms of separation.

Another type of stationary phase, which is used as an alternative to the reversed phase, is HILIC (Hydrophillic Interaction Chromatography)[90]. Its application has been enlarged and highly polar, basic, and ionizable compounds can be analyzed with this stationary phase [67]. Currently, there are several types of commercially available HILIC stationary phases (**Figure 11**). These phases have silica chromatographic supports or others, as well as groups linked to alkyl chain, such as diol, polyol, sulfobetaine, cyano and others.

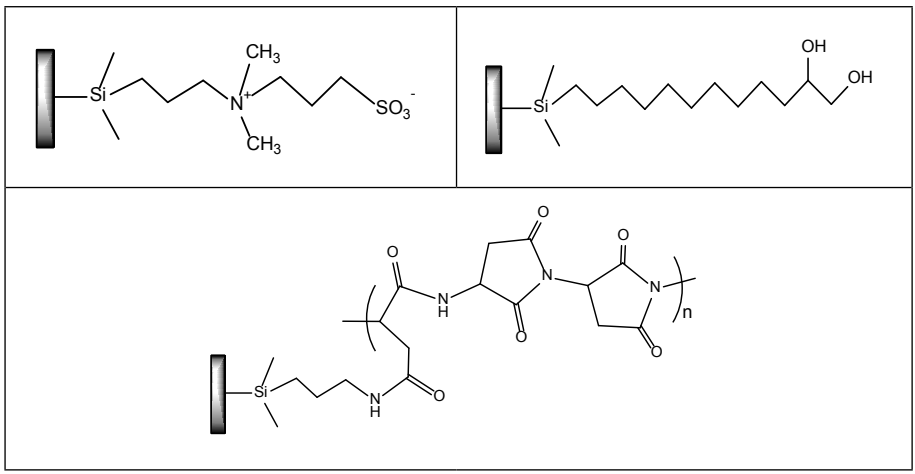

Figure 11. Some commercially available HILIC stationary phases.

For the hydrophilic interaction chromatography (HILIC), the stationary phase is polar, and the mobile phase is non-polar. However, unlike normal phase chromatography, the mobile phase consists of a high proportion of polar organic solvents (acetonitrile and methanol) and water is the strongest solvent. For this reason, this type of chromatography is also referred to as reversed-phase or normal-phase aqueous liquid chromatography [91]. The main mechanism of separation of HLIC is partition, where a small ratio of water in the mobile phase produces an aqueous layer on the polar stationary phase. Thus, the analytes establish a balance between the layer of water (more hydrophilic) and the mobile phase (more hydrophobic). Generally, the analytes have an inverse order elution when compared to reversed phase. In many cases analysis time is also reduced, since there is significant reduction in the viscosity of the mobile phase [67,90].

HILIC columns have some advantages when compared to normal phase, such as improvement of solubility of polar compounds, the possibility of coupling with the mass spectrometer with electrospray ionization source (ESI) improving the sensitivity [92,93], and analyses with high chromatographic resolutions of basic compounds like alkaloids [90,94]. This stationary phase can also be used in the separation of peptides, proteins, oligosaccharides [95], and drugs [96].

3. Mass spectrometry (MS)

Recently, several advances have been applied in mass spectrometry (MS), which is a technique that is widely used in research and developing areas, as well as for quality control of drugs and products [97]. Diverse and extensive applications of MS are related to its superior characteristics, such as sensitivity, reproducibility, and selectivity. Moreover, MS analyses can produce important structural information, like molecular weight, molecular formulae (when high resolution equipment is used), and fragmentation pathway. Thus, compound structures are proposed without isolation and the metabolites present in extracts and fractions from plants can be identified, which is also denominated as dereplication [98, 99].

MS has been efficiently coupled to chromatographic techniques to separate compounds, such as HPLC, UPLC, and gas chromatography (GC). So, higher efficiency for analysis of complex mixtures is possible, since the structural information, along with the separation of the components, can be obtained easily. Additionally, the selectivity and detection capacity of MS are essential for quantification studies, mainly for low concentration analytes presented in complex matrices [98-100].

Mass spectrometers are instruments that can ionize analytes, fragment the ions, and separate the ions according to their mass-to-charge ratio. MS

instruments are basically composed of three parts: ionization source, analyzer, and detector (**Figure 11**). The neutral molecules are transformed to ions (addition or removal of electron or hydrogen, as well as coordination reactions with metals) in the ionization source; subsequently, the yielded ions are separated based on their *m/z* and fragmented in the analyzer. After the separation of ions, they are registered by the detector and the data are converted to mass spectra [100].

The electron ionization (EI) is applied to analysis of volatile (non-polar) and small compounds (<600 Da). Firstly, the compounds are volatilized in the injection compartment and an electron beam is used to remove an electron from neutral molecules (M), yielding molecular ions with an odd number of electrons (M+•). This ionization process applies high energy (70 eV) and several dissociation reactions occur, yielding fragment ions. EI is a technique widely coupled to gas chromatography (GC), and is a simple technique which is applied to an inert gas as mobile phase [99-101]. GC-MS is a robust technique and several studies from plant extracts have been performed for volatile analyses[101], but the focus of this chapter is centered on LC-MS.

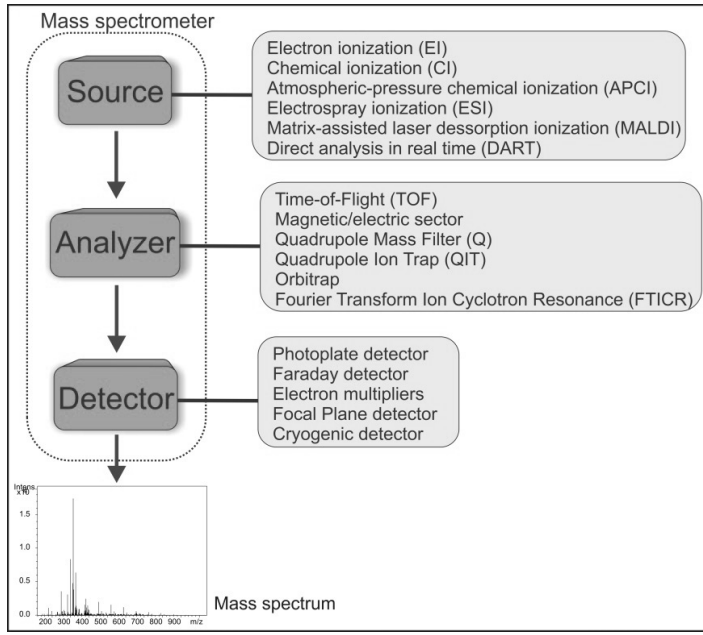

Figure 11. Scheme of the components of a mass spectrometer and types of ionization sources, analyzers, and detectors.

The main advances in the ionization sources of mass spectrometers were conducted for non-volatile, polar, and thermo unstable compounds analyses, since EI (generally coupled to GC) of the analytes must be volatile and thermostable. Thus, these limitations stimulated the development of new sources, which allow analysis of polar and thermo unstable compounds, such as atmospheric pressure ionization (API) techniques [electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI)]. These techniques could be coupled with HPLC / UPLC, and interface problems would be solved, including a mobile phase (liquid) that is vaporized and ions that are not easily eliminated by the vacuum system. The ionization process of these techniques involves solvent evaporation from spray model, allowing coupling with liquid chromatography [101,102].

The ionization sources most commonly used with HPLC/UPLC are ESI and APCI and are described in detail herein. Selecting the ionization source for analyses of complex mixtures is essential for accurate and satisfactory results, which depends on the polarity and molecular weight of the substances. Thus, this selection of ionization source and the optimization of the method are necessary for qualitative and quantitative analyses, since a precarious or absent ionization of compounds could result in sensitivity problems.

3.1. Electrospray ionization (ESI)

By the end of the 1500', William Gilbert observed the deformation of a water droplet through an electrically charged ambar slice. In 1882, Lord Rayleigh performed theoretical studies about the behavior of a droplet through an electric field and described the increase of charge density from the evaporation of droplet solvent. This charge density increases the critical value, where the charge repulsion is so high and overcomes the superficial tension of the droplet, producing smaller droplets. The maximum charge density that a droplet tolerates is known as the Rayleigh limits[103]. Ten years later, Rayleigh's prediction was confirmed and fine liquid jets were emitted when the droplets overcame the Rayleigh limits [104].

In 1914, a scientific study reported the behavior of liquid droplets in the glass capillary, which supported the evidence for electro-nebulization[105] and the researcher Geoffrey Ingram Taylor proposed the theoretical fundamentals of electro nebulization [106-108]. Thus, Taylor described the changes of the meniscus from a droplet produced by a cone due to electric field effect. This distortion of meniscus in the capillary with the application of an electric field is known as the Taylor cone, likewise to happen in ESI source (**Figure 12**).

In 1964, Dole and collaborators showed the ion production in the gas phase of a spray when a solution goes through an electrically charged capillary. Thus, when a small volume of a conductor liquid goes through an electric field, this

liquid has a distortion with a cone format and, at the same time, the superficial tension of liquid reveals a trend to maintain the non-deformed surface. From a determined voltage, the superficial tension is not preserved and a fine liquid filament is produced and extended from the Taylor cone, transmitting a jet of small droplets toward the counter electrode. As the droplets go towards the counter electrode, the solvent is evaporated, increasing the charge/volume ratio [109].

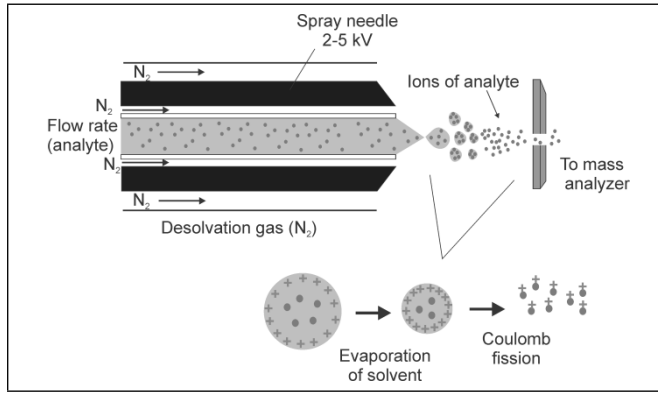

Figure 12. Representation of the electrospray ionization (ESI) source.

From the appointments described here, Fen and collaborators developed ESI source[110,111], which is one of the most commonly used soft ionization techniques for natural product analysis [99]. The ionization process in ESI begins with an electric potential applied in the capillary (2-4.5 kV) that contains the analyte solution, and charged droplets are formed. The evaporation solvent is promoted in the chamber by nebulizer gas that reduces the size of droplets and, consequently, the electrostatic repulsion increases the Rayleigh limit when the Coulomb fission explosion happens (**Figure 12**). The rupture of these small droplets produces ions of gas phase in a spray and, therefore the analytes (medium to high polar compounds) can be ionized [99-101].

Acid-base and coordination reactions with metals are the most common ionization reactions in the ESI, but redox (oxidation/reduction) reactions can occur in special cases. Thus, the main ions observed in positive ion mode are the ions $[M+H]^+$, $[M+Na]^+$, and $[M+K]^+$, while the ions $[M-H]^-$ and $[M+Cl]^-$ are the main ions in negative ion mode. Solvent, chemical structural properties, and the acquisition parameters influence the extension of the ionization reactions into the capillary. The substances with acid groups in the structure are preferentially

applied in the negative ion mode for the analysis, such as flavonoids, phenolic acids, phenylpropanoic acids, and others. In contrast, positive ion mode is applied to analyze compounds with basic groups, including ester, amines and amides, such as alkaloids, peptides, and others. The coordination reactions with metal occur in substances with lone electron pairs, requiring spatial disposition of atoms and a number of these electrons in polyethers [99,112,113]. For ESI ionization, the solvent and pH are important, and low and high pH are ideally applied for positive and negative ion modes, respectively [113].

Organic additives can be used in the composition of mobile phase, but limitations of them for the LC-MS have been described, such as the addition of low volatile salts and/or others that negatively influence the ionization, such as trifluoroacetic acid that suppresses the ionization. The most organic acids applied for LC-MS analyses are acetic and formic acid, while the most commonly used buffers for LC-MS are ammonium formate, ammonium acetate, and triethylammonium acetate [113,114].

Although LC-MS is highly sensitive, background ions can interfere negatively to this sensitivity and the contamination sources must be removed. The background can be general or specific ions. The general background commonly originates from hydrocarbons and chromatographic column bleed. In addition, it produces large signals on total ion chromatogram, which normally decreases the sensitivity [114]. The presence of background specific ions can interfere significantly from a single ion, as well as in the extracted ion chromatogram. Thus, the contaminants must be avoided and eliminated from the system when they are present. They can originate from the chromatographic system, sample or source/analyzer of mass spectrometer, and the measurements should be selected according to contamination source, such as the application of clean up techniques for the samples.

3.2. Atmospheric pressure chemical ionization (APCI)

In the 70's, Hornning and collaborators developed the atmospheric pressure chemical ionization (APCI) technique, initially using a ^{63}Ni slice (or leaf) and subsequently a corona discharge electrode [115]. Recently, this technique has been applied for analyses of triterpenes, carotenoids, lipids, fatty acids, and others [116,117].

Differing from ESI, the electric field in the APCI is not applied in the spray region, but it is applied on a needle that promotes discharge (corona discharge). Initially, the corona discharge reacts with N2 (used as nebulizer gas), which promotes consecutive reactions with water molecules and other molecules from the LC solvent. The water is the main molecule for ionization due to its proton

affinity, thus it suffers protonation producing H_3O^+ ions, which transfer the proton to vaporized analyte molecules that emerge from spray (**Figure 13**). The use of water in APCI is relevant to increase the sensitivity and a syringe pump can be used to increase its concentration. In negative ion mode, the process begins with the oxygen, which produces proton abstraction reactions and the OH- from water removes an hydrogen from analytes molecules. Thus, proton transfer, proton abstraction and charge exchange (redox) reactions can occur in APCI source.

APCI-MS is a limited technique for analyses of volatile compounds. For example, sugars (polar) cannot be detected, since they are thermally instable and show insufficient vapor pressure. Therefore, APCI is a recommended for analyses of non-polar compounds, with molecular weights between 100 and 2000 Da. Analyses of substances with molecular weight less than 100 Da are really complicated because of the background ions in this mass range. The ionization reactions are driven according to acidity and basicity of the analytes similar to the ESI, but differences between ESI and APCI are summarized on **Table 4**. ESI and APCI are complementary MS techniques and several articles have been published highlighting this [118,119].

The APCI shows lower influence on flow rate than ESI, besides APCI revealed higher tolerance in relation to salts including buffers, but the signals yielded in APCI are mass-dependent while the signals for ESI are concentration-dependent [99,118,119].

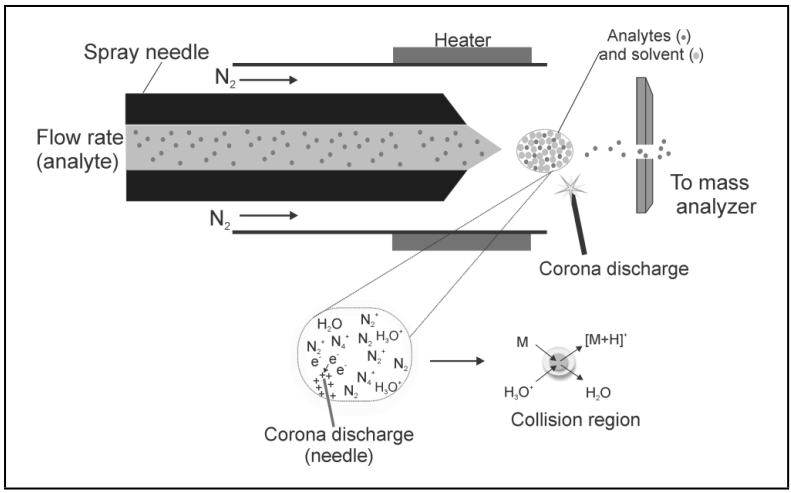

Figure 13. Representation of atmospheric-pressure chemical ionization (APCI) source.

Table 4. Important appointments about electrospray ionization (ESI) and atmospheric-pressure chemical ionization (APCI).

	ESI	APCI
Ionization	Liquid phase process; first the ionization happens (solvent) and subsequently the vaporization	Gas phase process; first the vaporization happens and subsequently the ionization
Process	A spray of charged droplets is produced;	Probe heater vaporizes the solvent;
	Solvent is evaporated from droplets;	Molecules are transferred to gas phase;
	Droplets yield smaller droplets;	Corona discharge needle yields ions;
	Coulombic fission transfers the ions to gas phase and charge to analytes.	Ions produced from solvent after corona discharge transfer the charge to analytes.
Sample	Small and large molecules, and polar compounds	Low molecular weight and (<2000 Da) and nonpolar compounds
Charge	Multicharged ions	Ions with one charge (commonly)
High voltage	Applied on capillary	Applied on corona needle
Flow (mL min^{-1})	0.001 to 1 (typical/ideal 0.3 to 0.5)	0.2 to 2 (typical/ideal 0.3 to 0.5)
Temperature	Direct infusion: ~80°C (source), ~120°C (desolvation) LC : ~120°C (source), ~350°C (desolvation)	~120 to 140°C (source)

3.3. Mass analyzers

The ions are produced in ionization source and are separated in the mass analyzer according to their *m/z* and based on the effect from electric and/or magnetic fields or lack of field, as time-of-flight (TOF) analyzer [99]. There are several types of analyzers, which should be selected depending of the application and the chemical-physics properties of analytes. The most applied analyzers are: quadrupole mass filter (Q), quadrupole ion trap (QIT), time-of-flight (TOF), Orbitrap, Fourier-transform ion cyclotron resonance (FT-ICR), electric and magnetic sector [99,120-122]. Beyond these, coupling with more analyzers is possible and commonly used, such as triple quadrupole (QqQ) or hybrid spectrometers as quadrupole time-of-flight (Q-TOF) [99,123].

The analyzers can be of low and high resolution, which is an important choice, especially for quantitative studies, since the analyzers showed different sensitivities. In addition, three characteristics of analyzers should also be considered, such as the mass limit, ion transmission, and the resolution power in mass. The resolution of an analyzer is related to the ability of separation for two ions with a determined ΔM, so the resolution of an analyzer is defined as R = M/ΔM (**Figure 13**). Thus, the low, high, and ultra-resolution analyzers can separate ions with differences in unit mass (quadrupole, quatrupole ion trap), 0.0001 (Q-TOF) and 0.00001 (FT-ICR) [124-126]. Mass accuracy is provided by analyses of high resolution analyzers and the molecular formula of analytes can be suggested in dereplication studies. Therefore, the equipment choice for a study should consider the following characteristics: resolution, mass range, mass accuracy, ion transmission rate, sensitivity, scanning speed, and the facility of coupling of the separation system, such as the liquid chromatography.

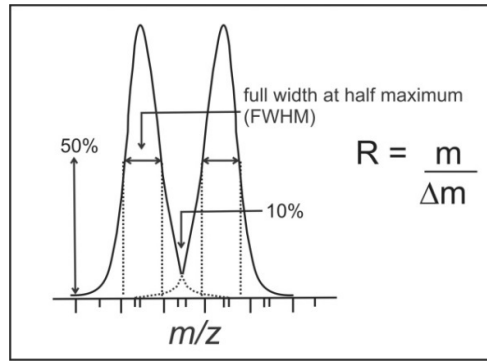

Figure 13. Scheme demonstrating the determination of the resolution.

The quadrupole *m/z* filters (Q) are widely used in mass spectrometers, mainly in GC-MS. It consists of four electric rods in parallel at a fixed distance with opposite poles, that are connected to radio frequency (RF) and direct current (dc), which are combined alternatively and produce an electric field. The interactions of the applied potentials stabilize the ions introduced in the center of the quadrupole according to *m/z*. So, the ions with different *m/z* can be analyzed by Q depending of the intensities and frequencies of applied voltages. The stabilized ions oscillate harmonically in a perpendicular movement of the rods and go through the quadrupole, while the non-stabilized ions produce random oscillation and collide with the rods, and cannot be detected (**Figure 14**).

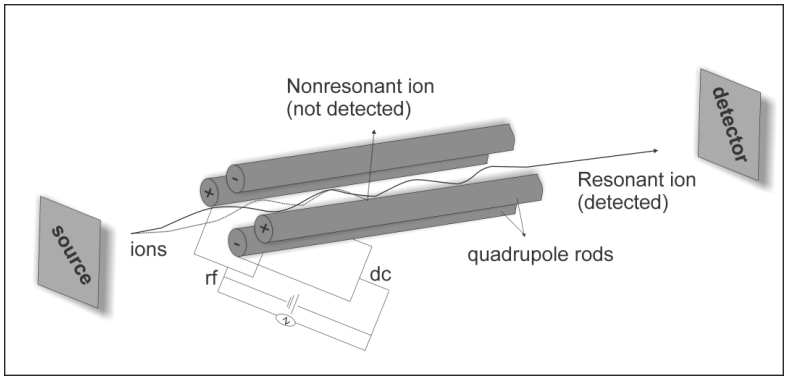

Figure 14. Schematic of a quadrupole analyzer (Q).

The quadrupole analyzer can be connected in series, such as the triple quadrupole (TQ) instrument, and the second quadrupole can be used as a collision cell to yield fragments from precursor ions (**Figure 15**). The precursor ions are isolated on the first Q and accelerated through the second Q, where an inert gas (such as N_2 and Argon, named of Collisional Induced Dissociation-CID) collides with these ions, increasing its potential energy and then the fragmentation occurs. These fragments or product ions are separated on the third Q, resulting in high selectivity and important structural information.

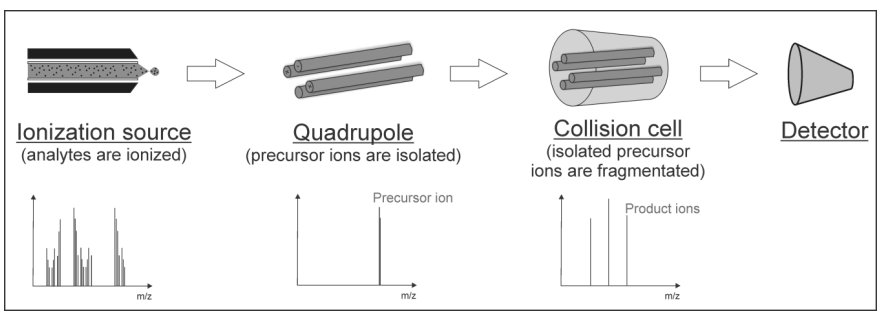

Figure 15. Scheme of a triple quadrupole (TQ) instrument.

The equipment as TQ can be operated in the following modes: full scan, single ion monitoring (SIM), neutral loss scan, product scan (daughter scan), and MRM (multiple reaction monitoring). The choice of this operation mode is essential for quantitative results, which makes it possible to develop high selectivity

and sensitivity methods for MRM. In addition, NMR reduces the noise and is commonly applied for pharmaceutical analyses (anti-inflammatory, β-blockers, antibiotics, and others), including monitoring of animal fluids, and pesticides [127]. This analyzer is most commonly used for quantitative studies. The detailed description of other analyzers can be found in books and articles [99,128,129].

4. Validation of analytical methods

Drugs and herbal products require quality control, including the control of the excipients and insume. For such quality control and research and development of drugs, quantitative analyses are frequently required and guidelines to validate the analytical methods should be used. International Conference on Harmonization (ICH) developed harmonized guidelines (ICH Q2B(R1)) for the European Union (EU), United States (US), and Japan for registration of pharmaceutical products. While guidelines for method validation of single laboratory was determined by AOAC International (AOAC) [130] and the International Union of Pure and Applied Chemists (IUPAC) [131], as well as the Food and Drug Administration (FDA) preconized guidelines for analytical and bioanalytical method validation [9]. There are many other validation guidelines, including specific guidelines, such as analyses of pesticide residue from food [132,133], and there are review articles in the literature about the validation for quantitative measures, mainly applying to LC-MS methods [132-136].

For example, if there is no methodology in official pharmacopeia, analytical validation should be performed to confirm that the methodology is appropriate for the aimed analysis, such as qualitative, quantitative, or semiquantitative analysis of drugs or insumes for industries (pharmaceutical, food and cosmetics). However, the method must have specific parameters for analytical validation, which can be performed as described in guidelines (ICH Q2B, AOAC, FDA, ANVISA) related to the interest. Thus, ion suppression, matrix effects, quantification, and detection limits, recovery, among others, may be required.

Reliable analytical methods, that apply international quantification guidelines have been used with higher frequency, which are required. Thus, analytical laboratories should implement roles and actions that ensure the quality of chemical analyses through reliable and traceable methods, since they need to show and prove the quality of results. Thus, the analyst can use one or more guidelines for quantification studies, which show a general discussion of validation parameters, calculations, and their interpretation. Therefore, important validation parameters for the method should be identified and validation protocol and acceptance criteria should be designed [132,133].

LC-MS methods for quantification have been widely applied, but many parameters should be optimized. The ionization (mainly for ESI and APCI) must be

evaluated, since the changes of in source parameters can represent losses or increases of efficiency and sensitivity. Another important point is the inferior repeatability of MS detector when compared to other detectors, such as UV, but MS detector shows higher sensitivity and is chosen for many quantification studies. The optimization of the ionization method, the evaluation of the suppression effects, and sample preparations are required to increase the data quality. In addition, sample replicates should also be analyzed in a short time, which improves the repeatability [132,133,135,137].

4.1. Analytical parameters

The validation process of a method must be clearly reported, and present adequate procedures, furthermore, studies must be performed on adequate instruments within the specifications, functioning properly with acceptable calibration and validation. In addition, the analyst should be an expert in this area, and have sufficient knowledge, making appropriate decisions when performing experiments and interpreting results.

The analytical parameters should be compatible with the aimed results and the method application. Generally, the evaluated parameters include linearity, precision, selectivity, robustness, limit of detection, limit of quantification, specificity, accuracy, and recovery [132,133].

4.1.1. Selectivity

The selectivity of an analytical method is its ability to determine the other sample components that can interfere in determining an analyte, so the extension of this interference can be evaluated. In addition, specificity is related to the final selectivity, thus, no detectable interferences are suggested. These interferences are normally substances related to analytes (chemistry), such as isomers, metabolism products, endogen substances, degradation products, impurities, as well as compounds with similar physical and chemical properties presented in the matrix. The interferents can be suppressed or enhanced by the analyte signal (in the sample preparation or/and in the detection), and the analyte signal cannot be distinguished [133].

Therefore, selectivity is a parameter to establish the effects of interferents on the analyte signal. It can be evaluated comparing a matrix without analytes and a matrix added with the analyte, and in this case any interference cannot elute together the analyte (in the same retention time), and a satisfactory resolution between the adjacent chromatographic peaks is required [138-141], as previously described in this chapter. The selectivity can also be determined by the UV and MS spectra, which are obtained when the chromatographic system is coupled to a

diode array detector and a mass spectrometer, comparing the spectra of analytes and the authentic standards to indicate the chromatographic peak purity [142-144].

Another possibility applies the method of analyte addition when it is not possible to acquire a blank matrix (without the analyte). For this method, a comparison between the calibration curves performed without the matrix and with the addition of analyte is accomplished [144,145]. If the curves are parallel, no matrix interference is determined for the analyte determination. In addition, selectivity can be determined through isolation of a chromatographic technique and, subsequently, analyzing by mass spectrometry (MS), nuclear magnetic resonance (NMR), and infrared spectroscopy [144].

The LC-MS analyses have been applied for quantification studies with validation of the methods. In this context, selectivity should be settled by chromatographic resolution (Rs), which should have a resolution of 1.5 or higher (preconized by AOAC) [130], or more than 2, as preconized by FDA [9]. The Rs calculation was previously described in this chapter. Some of the preconized values are rigorous and sometimes the selectivity of the detector is neglected, mainly for MS detector[132]. Furthermore, additional matrix effects should also be evaluated when LC-MS is used, as required by FDA guidelines [9].

4.1.1.1. Matrix effect

Matrix effect (ME) is an important measurement for developing and validating an analytical method, but sometimes analysts forget this relevant issue in their studies. ME occurs when substances of a matrix present the same retention time as the interest analyte (compound that will be quantified). This is a parameter of high interest for quantification studies applying HPLC coupled to detectors such as fluorescence, ultraviolet and visible (UV-Vis), and electrochemical and MS [146].

Regarding complex matrices, errors in LC-MS/MS commonly happen due to high selectivity and specificity, since the presence of non-monitoring substances that co-elute with the analyte can interfere in its detection, change the ionization efficiency, and result in suppression or enhancement ionization of the analyte [147-150]. In 1991, Tag and Kebarle described the matrix effects (ME) using organic bases for the first time. For the ESI-MS systems, these researchers confirmed that some organic bases monitored in a matrix lost the detector response when the concentration of other organic bases increased. These effects are a constant and recurrent problem for sources of atmospheric pressure ionization (API), and should be evaluated and determined [151-153].

The ME can happen during solution or gas phase of the ionization sources of MS, and the main reason for this is a change in the properties of droplets in the solution due to the presence of non-volatile compounds or less volatiles

modifying the efficiency production and/or evaporation of the droplets, affecting the number of charged ions in the gas phase that reaches the detector [153,154]. Besides, different mechanisms of ionization suppression have been proposed, and most of them are specific for the API, mainly for ESI [146,155].

The most commonly used API techniques for LC-MS are ESI and APCI, and Jessome and Volmer [155] revised the different theories about suppression and enhancement ionization for them. In this context, they defined and described that with higher concentration of the analytes, the linearity of the ESI response can sometimes be lost due to an excess of available charge on the surface or a saturation of droplets produced by electro nebulization with the analytes on their surfaces, inhibiting the ejection of the trapped ions inside the droplet. For example, endogenous compounds can compete with the analytes by charge presented on the surfaces of droplets [132,133,155-157].

Other theories consider the increased effects on the viscosity and superficial tension of the droplets resulting from interferent compounds, which reduces the evaporation of the solvent and the capacity of analyte to achieve the gas phase [155,156]. Besides, non-volatile compounds can decrease the efficiency of droplet production by co-precipitation of analytes or precluding the small droplets to achieve their critical radius necessary for emission of ions toward gas phase [153,155,156]. The ions from analytes can also be neutralized in the gas by deprotonation reaction with alkaline substances, suppressing the signal [132,133,155,156].

In some cases, lower ion suppression is observed for APCI than ESI, since ionization occurs elsewhere in these techniques, as previously described in this chapter. Conversely for ionization in ESI, there is no competition in APCI between the analytes in the gas phase, because the neutral analytes are transferred to the gas phase through liquid vaporization with a heated gas flow and, posteriorly, the charge is produced in the corona [155,156]. However, ion suppression can also occur in APCI, which is explained by the effect of sample composition for the efficiency of charge transfer between the needle discharge corona and the analyte. Other mechanism proposed for ion suppression in APCI is the solid production, such as a pure substance or a solid co-precipitated substance with other non-volatile sample components [155,158].

ME can be induced by any co-eluted compound that enters in the ion source (API) [159]. Some additives of mobile phase, such as trifluoroacetic acid, affect the responses of MS, reducing the ionization of the analytes [160]. However, some strategies to minimize the ion suppression induced by trifluoroacetic acid are reported, including the use of weak acids as ionic pairing agents [154], such as the post-column addition of a mixture of propionic acid and isopropanol [161] or the addition of acetic and propionic acids in the mobile phase acetic acid [162].

Moreover, ME can also be induced by exogenous materials, such as polymers from plastic tubes or tubes with lithium heparine, for anticoagulant effects [163].

The ME quantification provides important information for the validation of methods that apply to mass spectrometry. Viswanathan and collaborators [164] proposed that ME can be expressed by the matrix factor (MF), which is calculated from six samples of the same matrix from different sources, as demonstrated in **equation 2**. In this equation, the response of analyte can be the area, area ratio, or height of chromatographic peak ratio.

$$MF = \frac{\text{response of analyte in the presence of matrix}}{\text{response of analyte in the absence of matrix}}$$

Equation 2

When the MF value is 1, the matrix effect is not observed, but if the MF value is lower than 1 the matrix effect is observed, and the ion suppression is suggested. In addition, if the MF value is higher than 1, the enhancement of ionization is observed. The absolute MF value of circa 1 does not recognize that the developed method is totally reliable. On the other hand, variable MF values can be a problem related to the lack of precision, which should have a variation coefficient of less than 15%.

Basically, the ME can be evaluated through two methods: post-column infusion and post-extraction addition (**Figure 16**) developed by Bonfiglio and collaborators [137] and Matuszewski and collaborators [146], respectively. The post-column infusion method provides a qualitative evaluation, because the chromatographic regions that are more susceptible to the ME are identified. In this method, an infusion pump is used to produce a constant flow ratio of the analyte after the chromatographic column and before the ionization source of mass spectrometer. For the optimized conditions of the method, the extracted matrix (without the addition of the analyte) is injected and analyzed by chromatography. When the analyte is infused in MS source by a constant flow, a constant response is yielded in function with time. For this application, the multiple reaction monitoring (MRM) method should be selected to register the analyses [119,165]. During the analysis, any compound from the matrix that elutes from the chromatographic column and induces a variation in the response of the infused analyte is determined as a suppression or an enhancement of the response, characterizing the matrix effect (ME). The post-column infusion allows for verification of the influence of matrix in the analyte response. However, the concentration of infused analyte must be in the same concentration range to be

chromatographed. Otherwise, the ability of ionization source to yield ions can be hampered and questionable results can be obtained.

However, the post-column infusion method presents some disadvantages, as the quantitative results for some specific analytes cannot be obtained and the direct infusion of each separate analyte is necessary to evaluate the ME. While in post-extraction addition method, a quantitative analysis of ME is possible, since the responses, obtained from the samples whose analytes were added post-extraction, are compared with the solutions prepared in the mobile phase under the same conditions (**Figure 16B**). This analysis is considered static, because it only provides the information for ME in the elution time of the analyte, while the post-column infusion is considered dynamic for the determination of ME [132,133,151,159,166].

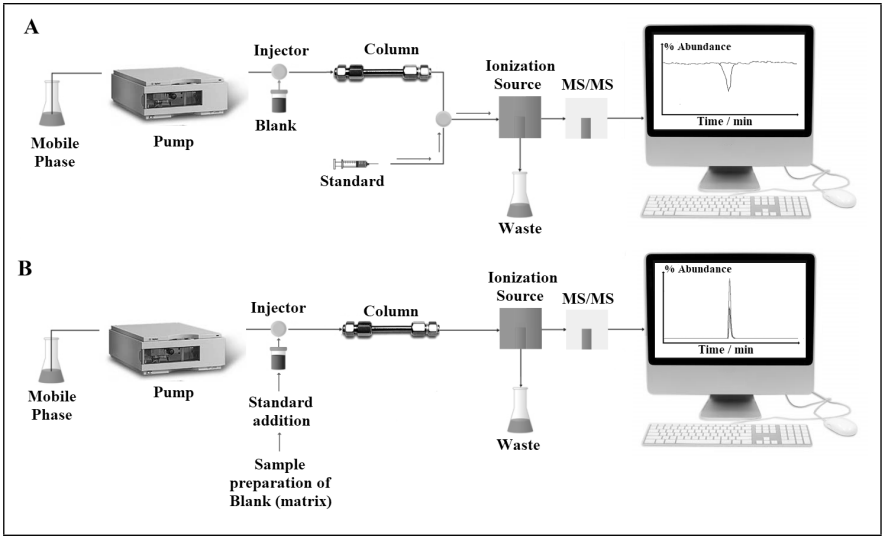

Figure 16. Scheme of methods to evaluate the matrix effects (ME): post-column infusion (**A**) and post-extraction addition (**B**).

For the ME reduction, the analyst can improve the chromatographic analysis by using more selective chromatographic methods (column and/or components of mobile phase) or ultra-high-performance liquid chromatography (UHPLC or UPLC), as well as sample dilution. These two methods have been used by many authors to reduce the ME [167,168]. The dilution methods presented satisfactory results, but quantification can be damaged if the analyte concentration is below the limit of quantification and, sometimes, the extrapolative dilution approach is useful [169].

4.1.2. Linearity evaluation and calibration curve

Linearity is determined through the analyses of the standard (analyte) solutions at different concentrations, including the concentration range for the interest in the study, thus the evaluated concentrations change according to the aim of the analysis and type of instrument used. The calibration curve corresponds to mathematic models that correlate instrumental responses, such as area or height of the chromatographic peak, and the concentration of analyte. If there are several analytes to quantify, it is necessary to elaborate a calibration curve for each analyte (standard) [9,170].

The calibration curve should be constructed with known concentrations (generally six to eight points/concentrations) of the analytes added in the same matrix (matrix overlay) for the optimized and developed method [171]. The selected concentrations should cover the expected variation range from quantification limit (QL) to 120% of maximum concentration to be analyzed [9, 170]. Besides, the analysis of the sample blank, the sample without the analyte and internal standard, should also be performed.

If the relation (concentration x response) is linear, results should be evaluated through appropriate statistical methods, for example, the calculation of least squares regression method [172]. In addition to the regression coefficients **a** and **b**, it is also possible to calculate, from the experimental points, the coefficient of Pearson correlation (r) or coefficient of determination (r^2) [173]. This parameter estimates the quality of the curve obtained, because the closer to 1,000, the lower the dispersion from the set of the experimental points and the lower the uncertainty of the estimated regression coefficients. To verify if the regression equation is statistically significant, the fit test for a linear model, regression validity, its efficiency, and the maximum efficiency must be performed [173,174].

4.1.3. Recovery

Recovery tests are used to evaluate the efficiency of the extraction sample method. The recovery studies provide the quantity of analyte that is recovered by an extraction procedure in relation to the real quantity presented in the sample [175].

The recovery method must be evaluated comparing the response obtained from the analyte added in the matrix and extracted with the response obtained from the extracted matrix and later added by the analyte. This procedure should be adopted to mimic the analysis of fortified samples before the extraction procedure, avoiding possible errors produced by injecting pure (only) standards.

The following three levels of concentrations should be used to determine the recovery: low, medium, and high, according to the calibration curve. Even though values closer to 100% are desirable, recovery is not necessary to reach this value, but it should be consistent, accurate, and reproducible [132,133,176].

The analyte extraction time, sample preparation steps (solid phase extraction, liquid/liquid extraction and others), solvent amount and composition, are variables that influence the recovery and quantification and detection of limits. The recommended extent of variation for recovery is up to 20% [132,133]. Besides, an internal standard (IS) can be used to correct the sample preparation, including the recovery. The IS must be chemically like the analyte (structural analogs) or be isotopically labeled by the analyte. It is added into the samples before the sample preparation steps, and the ratio of the analyte and IS signals are used to construct the calibration curve [177,178].

4.1.4. Precision

Precision is a measurement of random errors and represents the proximity of the measurements performed independently from multiple samplings of a homogeneous sample [179, 180]. The measurements can be evaluated simultaneously (repeatability or intra-assay precision), on different days (intermediate or inter-assay precision) and in different laboratories (interlaboratory precision or reproducibility). Precision is expressed as an absolute standard variation (S), variance (S_2), relative standard deviation (RSD), or coefficient of variation (CV). Thus, this parameter can be estimated by coefficient of variation (CV%) or the relative standard deviation (RSD%) both in percentages [181], according to the **equations 3** and **4**. For the LC-MS, precision should be determined at different concentrations, since the repeatability is concentration-dependent [182].

$$\mathrm{SD} = \sqrt{\frac{(x_i - x_o)^2}{n-1}} \qquad \textbf{Equation 3}$$

$$\mathrm{SDR\ or\ CV\ (\%)} = \frac{SD}{x_o} \mathrm{x}\ 100 \qquad \textbf{Equation 4}$$

x_i = individual value of a replicate
x_o = mean of the replicates
n = number of replicates
SD = standard deviation

4.1.5. Trueness and accuracy

Trueness is a measurement of systematics errors, which is determined from the average of diverse replicates and the true value of the quantity measured

(reference values). Thus, the trueness is the distance of the expected and obtained values and can calculated using **equation 5**.

The ICH and ANVISA agencies suggest that precision and trueness should be investigated by analyzing a minimum of nine replicates, including three different concentration levels. For example, triplicate assays with low, medium, and high concentration levels in relation to the concentrations of the calibration curve can be performed for quality control (QC). Furthermore, the most commonly used methods to evaluate trueness are: reference materials, comparison of methods, recovery assays, and standard addition.

The meaning of trueness described here is applied in the International Vocabulary of Metrology (VIM), while accuracy has been used with different meanings, which applies the measured value compared to the true value. Accuracy can be applied to an individual analysis, unlike trueness that is applied to several analyses. So, the accuracy is influenced by systematic and random error components, which are denominated bias and precision, respectively.

$$RE\% = \left(\frac{x_i - x}{x} \right) x\ 100 \qquad \textbf{Equation 5}$$

RE (%) = percentage of relative error from samples analyzed by replicates
x_i = measurement of concentration value (experimental)
x = nominal concentration value of analysis

4.1.6. Limit of detection (LoD)

The limit of detection (LoD) is the lowest concentration of an analyte that can be detected in a sample through analytical methods [132,133,135,184,185], and is not necessarily the concentration quantified. The LoD can be calculated by the visual method, method of signal-to-noise ratio (S/N), or the method based on the analytical parameters of the calibration curve. In addition, it is suggested to frequently re-determine the LoD, since it is a parameter with low reproducibility (day-to-day) [135,186].

The visual method is used to determine the LoD using the matrix along with known amounts of the analyte and thus, the lowest amount of analyte detectable is determined from the differentiation of noise and signal. This procedure can also be done using the instrument to detect parameters in the integration method [186].

The method of signal-to-noise ratio (S/N) can only be applied in analytical methods that show the baseline noise. The noise-signal relation is determined through comparison between the measurements of the sample signals at low concentration known for the analyte in the matrix and a sample blank (matrix without the analyte). The S/N ratios of 3:1 or 2:1 are commonly accepted as estimates of LoD. However, LC-MS/MS cannot be used for this method, since the MS/MS mode cannot show noise[132,133].

The data required to calculate the LoD based on the analytical curve can be obtained from the matrix added to the analyte in the concentration range close to LoD, and can be expressed by the **equation 6**.

$$\mathrm{LoD} = 3.3\ x\ \frac{s}{b} \qquad \textbf{Equation 6}$$

s = the standard (analyte) deviation of the signal (from 3 or more different ways)
b = calibration graph slope

4.1.7. Limit of quantification (LoQ)

The limit of quantification (LoQ) is the lowest concentration of the analyte that can be quantified with trueness and repeatability using analytical method, so precision and trueness should also be determined [132,133,135,184,185].

Similar methodology for LoD is applied to determine the LoQ, but the ratio used is 10:1. Thus, the LoQ can be calculated using the visual method, signal-to-noise ratio, (S/N) or the relationship between the estimate of the standard deviation of the response (*s*) (that can be an estimate of the standard deviation from blank, the regression line equation, or linear coefficient of equation) and the calibration graph slope (b), at concentration levels close to LoQ. The **equation 7** is applied for this calculation, and this approach is preconized by ICH. The more adequate method to determine LoQ is the ICH approach, which is recommended to be carried out 5 times over a longer period [132,133].

$$\mathrm{LoQ} = \mathbb{0}\quad x\ \frac{s}{b} \qquad \textbf{Equation 7}$$

s = the standard (analyte) deviation of the signal (from 3 or more different ways)
b = calibration graph slope

4.1.8. Robustness

Robustness is essential and important for the analysts to establish the factors to be controlled during the execution of analytical methods. Therefore, the robustness measures the susceptibility to minor variations that may occur during the analyses. The evaluation of robustness can be performed in the development phase and depends on the type of study. If susceptibility to these variations is observed, this should be controlled and precautions must be taken in the study.

Robustness can be evaluated by changes in several factors to determine if these modifications will affect the results. Thus, the variation of results can be determined by several changes, such as the changes in pH, additives concentration, organic solvent content, column temperature , flow rate, the column (batch and age), the parameters in the sample preparation, mass spectrometer parameters, and others[132,133]. The variations of the analyses conditions must produce results that have acceptable selectivity, trueness, and precision[9,132,133,187-189]. The recommended extension of variations for each parameter are the following: pH (± 5 units), concentration of the additives (± 10%, precautions: some additives can induce ion suppression or enhancement), and organic solvent content (± 2%) in the eluent, and column temperature (± 5 °C, the resolution and retention time are influenced)[132].

For sample preparation, the extraction time, amount, composition, and injection solvents can be evaluated. In addition, the matrix effect should be evaluated using at least six different blank matrices. Furthermore, the MS parameters can be evaluated in robustness such as drying gas temperature, nebulizer gas (pressure and flow rate), and ion source conditions (nebulizer aging, ion source contamination, and others).

4.1.9. Stability

Stability studies should be performed to determine the time (days, weeks, or months) and the conditions for the standards (analytes), samples, and reagents that are stable, allowing for reliable and reproducible results, and so the analyses must be executed before the degradation of the analytes[138]. Therefore, stability can influence the LOD, as well as the results, and should be determined before the validation of method [190].

The analytical methods with autoinjector application (the samples are stored for a long time in the chromatographic system) required highest stability of samples in solution. Some samples can be unstable with temperature (lower temperatures can avoid degradation), light, or other conditions, and in special cases the samples need to be prepared daily, and some analytes (substances) require stability evaluation to clarify storage time [23,191].

5. Final appointments and some application examples

System suitability should be performed to determine the conformity of the system, and to produce reliable and acceptable results. For these experiments, the resolution and repeatability of chromatographic system are satisfactory (adequate), but the whole system is verified. Generally, the evaluated parameters and their recommended limits are: retention factor (k >2), repeatability (RSD < 1%, n = 5), resolution (Rs > 2), tailing factor (TF ≤ 2), and the number of theoretical plates (N > 2000). At least two of these criteria are necessary for the system suitability[192].

Natural products are important for the development of new drugs, including phytotherapics[193,194]. Thus, analytical methods for analysis and quantification of natural products are widely required and are oftentimes not described in the literature or compendia for quality control, pharmacokinetic studies, or other literature.

The analytical methods for secondary metabolites analysis include chromatographic techniques coupled to detectors, while recently mass spectrometry has received more attention. This is likely related to electrospray ionization source, since it can be coupled to the high-performance liquid chromatography and allows the analyses of polar compounds that are not possible through gas chromatography. Thus, HPLC has been widely applied to phototherapy and natural products analysis. For quantification studies, one or a group of compounds (chemical marker) with similar structures can be determined, which is a common method for complex mixtures of secondary metabolites in plants where the active compounds or action of the mixtures are unknown[195].

Some researchers developed a method using LC-ESI-MS that made the identification and quantification of 32 bioactive compounds from flowers of *Lonicera japonica* with high selectivity, sensitivity, and precision[196]. While Du and collaborators[197] developed a quantitative and qualitative method by LC-ESI-MS to analyze and quantify 20 constituents from *Isodon nervosa*. However, this availability of reference standards is difficult and normally the quantification of some constituents is not possible. In this way, most methods for quantitative studies applying multi-constituents are normally performed at Universities and research laboratories for scientific research only. Recently, ultra-performance liquid chromatography has been applied for quality control analysis, which provides higher efficiency, resolution, and shorter analysis times, and has been used for analysis of flavonoids, aristolochic acids, and piperine and heterocyclic amines from *Trollius ledibouri,* phytotherapic formulations and black pepper, respectively[198-200]. **Table 5** summarizes several examples of the natural products analyzed by LC-MS.

Table 5. Examples of qualitative and quantitative analyses of quality control of plant extracts by LC-MS and UPLC-MS.

Analyzed compounds	Species	Reference
HPLC-MS		
	Qualitative analyses	
Ginsenosides	*Panax ginseng*	Cui et al.[201]
Alkaloids	*Tylophora atrofolliculata*	Cui et al.[202]
Phenolics	*Tagetes maxima*	Parejo et al.[203]
Lignans, flavonoids, quinic acid derivatives	*Cuscuta chinensis*	Ye et al.[204]
Kaempferol	*Crocus sativus*	Carmona et al.[205]
Saponins	*Salvia miltiorrhizae and Panax notoginseng*	Zhang & Cheng[206]
Diarylheptanoid	*Curcuma longa*	Jiang et al.[207]
Glycosylated flavanones	*Citrus aurantium*	Zhou et al.[208]
Antraquinones	*Polygonum multiflorum*	Yi et al.[209]
Flavonoids	*Citrus aurantium*	Zhou et al.[210]
Isoflavonoids, astragalosides	*Astragalus membranaceus Astragalus membranaceus var. mongholicus*	Zhang et al.[211]
	Quantitative analyses	
Nucleosides	*Cordyceps sinensis*	Huang et al.[212]
Phenolic acids, phthalides, glycosylated terpenes	Si-Wu-tang	Zhang et al.[213]
Saponins	*Panax notoginseng*	Lai et al.[214]
Flavonoids	*Citrus aurantium*	Lu et al.[215]
Coumarins	*Notopterygium incisum, Notopterygium forbesii*	Li et al.[216]
UPLC-MS		
Flavonoids	*Trollius ledibouri*	Li et al.[198]
Aristolochic acids	Phytotherapic formulations	Jacob et al.[199]
Alkaloid and heterocyclic amines	Black pepper	Zeng et al.[200]
Phenolic acid and Flavonoids	*Polygonum capitatum*	Huang et al.[217]

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Conflicts of Interest

The authors declare that they have no conflict of interest.

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Chapter 5

Mass spectrometry as a tool in the structure elucidation of rare natural products

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1. Introduction

Structure elucidation of natural products can sometimes be challenging although many techniques have been created to facilitate their identification. However, natural products are involved in many areas namely drug development [1], toxicology [2], forensic science [3], fragrance and flavor development [4], and crops protection [5] which require, an unambiguous structure determination. Usually, nuclear magnetic resonance (NMR) and mass spectrometry (MS) are used as routine tools for structure determination. Some other problems can also impact the structure elucidation of a given metabolite such as its quantity, its stability (inter-conversion between two metabolites, light degradation, and mobile and stationary phase sensibility) [6-8], and its structural scaffold (no functional group, similarity of the signals, and poor hydrogen bearing compounds) [9] leading to the difficulty of producing any useful information during the data acquisition. Thus, several hyphenated techniques such as LC-NMR, LC-MS, and GC-MS have been developed to overcome some of the problems associated to structure elucidation. Besides, computational tools have also been implemented as an alternative solution; among them quantum chemical calculation and computer-assisted structure elucidator (ACD Lab) have been widely used to match NMR resonances [9, 10] and more importantly mass databases (GNPS: Global Natural Products Social Molecular Network [11], ReSpect: RIKEN MS^n spectral database for phytochemicals [12]) have played a crucial role in metabolomics studies. Other analytical techniques including infrared absorption spectroscopy, ultra-violet absorption spectroscopy, optical rotation, circular dichroism and crystallography provide evidences of the presence of functional groups, reveal the right absolute configuration or to confirm the proposed structure.

The chapter will be intensively focused on MS application in structure determination. As well described in the literature, this technique generates

ions from a given analyte and separates them based on their mass/charge ratio (m/z) [13]. The molecule can be transformed into ions by thermal exposure, by entering an electric field or by impacting accelerated particles (matrix, electrons, or photons) [13]. MS techniques have increasingly been employed after the invention of soft ionization sources such as fast atom bombardment (FAB), matrix-assisted laser desorption ionization (MALDI), atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) [13]. More importantly, newly developed analyzers such as time of flight (TOF), Fourier transform ion cyclotron resonance (FTICR), triple quadrupole and ion trap improve the performance of this technique. So, among its applications, MS can simultaneously allow the identification of a tremendous number of components of a mixture when it is associated with chromatographic techniques [14]. Moreover, mass spectrometry analysis required a small sample quantity; for instance, UPLC-MS requires from 5-500 µg/mL while the concentration of a single pure compounds used in a direct injection is 1 ng/mL. These quantities are practical and efficient for the detection of new components. It can also be an upstream work before a routine phytochemical study. However, ionization requests the consideration of many backgrounds and preliminary information of the sample such as:

- Acidity and the basicity of the sample. These knowledges from chemotaxonomy data and previous chemical reports enable the choice of the ionization mode (positive or negative) and more importantly the ionization method (sources of ions).
- Presence of RNH, ROH, and sugar moieties. Soft ionization sources such as ESI, and APCI operate with a capillary energy which can go up to 5 kV; usually, high capillary energy induces for example the loss of NH_3 (17 Da), H_2O (18 Da), MeOH (32 Da), pentose (132 Da), Hexose (162 Da) and deoxyhexose (146 Da) which can lead to confuse the base peak formed to the peak of the molecular ion. So, different MS methods need to be developed in order to avoid any waste of information.
- Volatility of the constituents in a sample also helps for the choice of an ionization source. Volatile sample such as fatty acids and oily components can be detected by EI but sugars containing compounds and other thermal sensible samples won't be detectable by EI.

So, resulting ions in soft ionization techniques (ESI, ASAP, APPI, APCI positive mode) are usually bearing an adduct and are detected as $[M+H]^+$, $[M+Na]^+$, $[M+NH_4]^+$, and $[M+K]^+$. Besides, clusters species such as $[2M+H]^+$, $[2M+Na]^+$, and $[2M+K]^+$ can also be observed in the mass spectrum. Negative mode in turn, produces $[M-H]^-$, $[M+Cl]^-$, $[M+HCO_2]^-$, $[M+H_3CCO_2]^-$ and their clusters. Ionization by electrons impact (EI) provides radical ions and ions. Radical ion species can

also be found among the fragments produced by a hemolytic cleavage during the tandem mass analysis using soft ionization techniques. After the analytes ionization, the full structure identification is based on fragments obtained from the tandem mass analysis. In the case of EI, fragments are produced after the particles of the analyzed sample collided with electrons accelerated at 70 eV (electron induced fragmentation) [13]. This technique affords a lot of fragments and sometimes, a disappearance of the molecular peak ion. However, soft techniques require an inert gas (Argon) to promote fragmentation from the precursor ion. This reaction occurs in a collision cell where the inert gas collides the selected precursor with a kinetic energy usually < 70 eV. After the collision, the precursor ion has an internal energy (E_{int}) which is the sum of its internal energy (E^{o}_{int}) before the collision and the energy received during the impact. Therefore, when the internal energy (E_{int}) is sufficiently high, fragmentation occurs otherwise no fragments are observed [15]. Further, this energy also influences the abundance of the ion observed in the spectrum [15]. The methodology by which a precursor ion is selected and fragmented to produce daughter ions is termed tandem mass (MS/MS, MSn). In general, ion trap and quadrupole analyzers are mostly used for the ion selection while different techniques such as collision-induced-dissociation (CID), Electron-detachment dissociation (EDD) charge transfer dissociation (CTD), Electron transfer dissociation (ETD), Fourier transform ion cyclotron resonance (FTICR), and so forth are used for the fragmentation of different organic species.

2. Mass spectrometry as a tool in natural products structure elucidation

2.1 Identification of marine toxins by using MS^2 analysis

Marine algae and bacteria produce various natural products characterized by diverse skeletons. Among them, marine toxins are one of the extremely dangerous groups since it can contaminate seafood such as fish and shellfish. Once ingest by human, its intoxication can be manifested by hepatic, dermatologic, neurological, gastrointestinal, or cardiovascular problems sometimes, leading to death [16]. Since this group of metabolites represent an economical and human health concern, their identification by using mass spectrometry have been well documented [17]. Also called phycotoxins, they can be grouped in amino acid derivatives (domoic acid), purines derivatives (saxitoxin), cyclic imines (spirolides), linear polyethers (okadaic acid, azaspiracids) and cyclic polyethers (brevetoxins) [17].

2.2 Identification of cyclo-imines toxins. case of spirolides

Among other metabolites, two spirolides were identified *Alexandrium ostenfeldii* on the triple-quadrupole equipment using Collision-induced dissociation

(CID) [18]. Both compounds have the same mass value of m/z 708.5 with different elemental composition $C_{42}H_{62}NO_8$ (1) and $C_{43}H_{66}NO_7$ (2). Precursor 1 afforded fragments at m/z 690, m/z 672, and m/z 654 corresponding to the losses of one, two and three molecules of water respectively. It seems like the ionization occurred mainly on the allylic OH group of the macro-cycle. So, after the dehydration, the secondary carbocation formed can trigger the macrocyclic ring opening with the charge delocalization. The positive charge located on carbon C-2 of the spirofurane portion can be stabilized by the oxygen lone pair electrons. Cyclohexene ring in the 8-azaspiro[5.6]dodeca-2,7-diene moiety of each of these fragments opened in a Retro-Diels-Alder (RDA) manner (Fig. 1) and afforded the fragments m/z 460, m/z 442 and m/z 424, respectively. Similar fragmentation behavior was noted on a related spirolide (2) (Fig. 2) suggesting that these two mechanisms can be used as mass fragmentation feature to identify related spirolides.

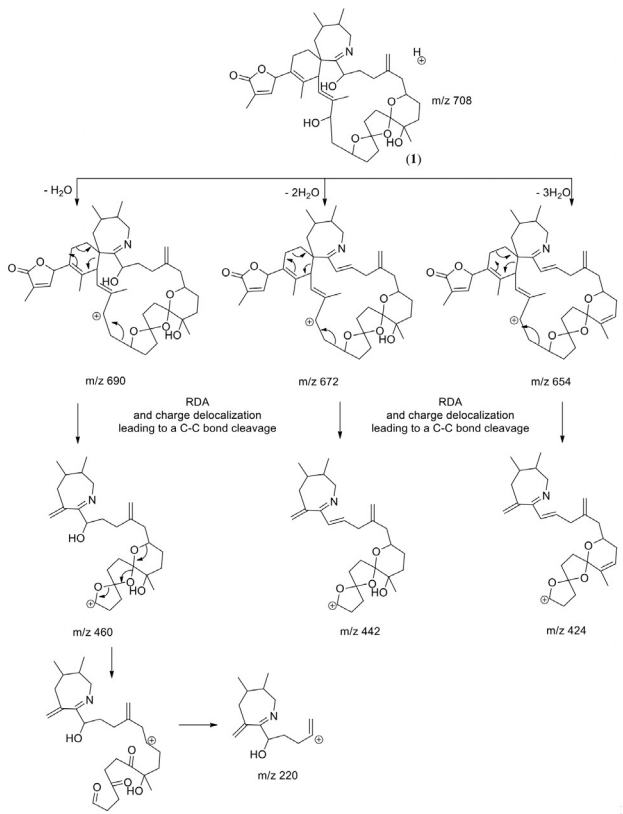

Figure 1. CID fragmentation of a spirolide at $C_{42}H_{62}NO_8$ (m/z 708.5 $[M+H]^+$)

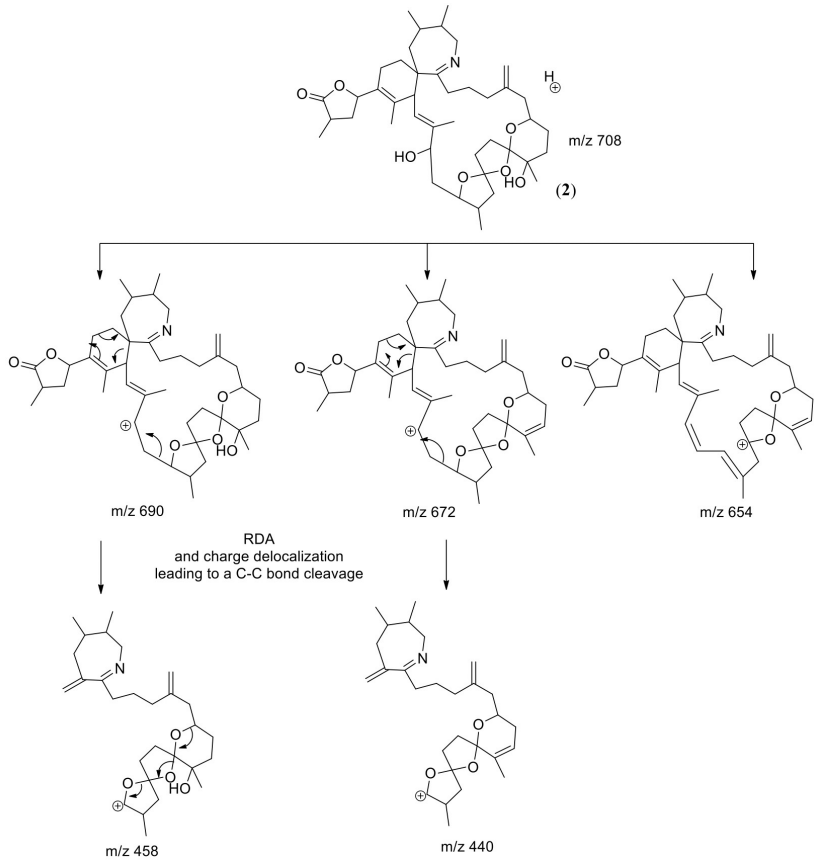

Figure 2. CID fragmentation of a spirolide at $C_{43}H_{66}NO_7$ (m/z 708.5 $[M+H]^+$)

2.3 Identification of polyether toxins. case of dinophysistoxins

The MS2 of three polyethers (**3**-**5**) were performed by CID on an ESI-Orbitrap machine. Their mass spectra displayed some similar features in the fragmentation. Among them, the base peak ion m/z 255.1233 resulted from a RDA rearrangement (Fig. 3) of the 4-methyl-1,7-dioxaspiro[5.5]undec-4-ene moiety [19]. A cleavage of a C-C bond bearing vicinal oxygenated functions was further observed in all the polyethers. These fragments might serve as a fingerprint to further characterize other related polyether metabolites while working in negative mode ionization.

2.4 Identification of guanidine containing toxins. case of saxitoxin

Fast atom bombardment analysis in positive mode ionization of the neurotoxin saxitoxin (**6**) produced ions mostly formed from the bond cleavage in the side chain (Fig. 4) [20]. Three fragments were observed in the tandem mass spectrum (Fig. 4) of 21-sulfo-11α-hydroxysaxitoxin sulfate (**7**) suggesting that some of those can be used as an information to guide the identification of guanidine containing toxins [20].

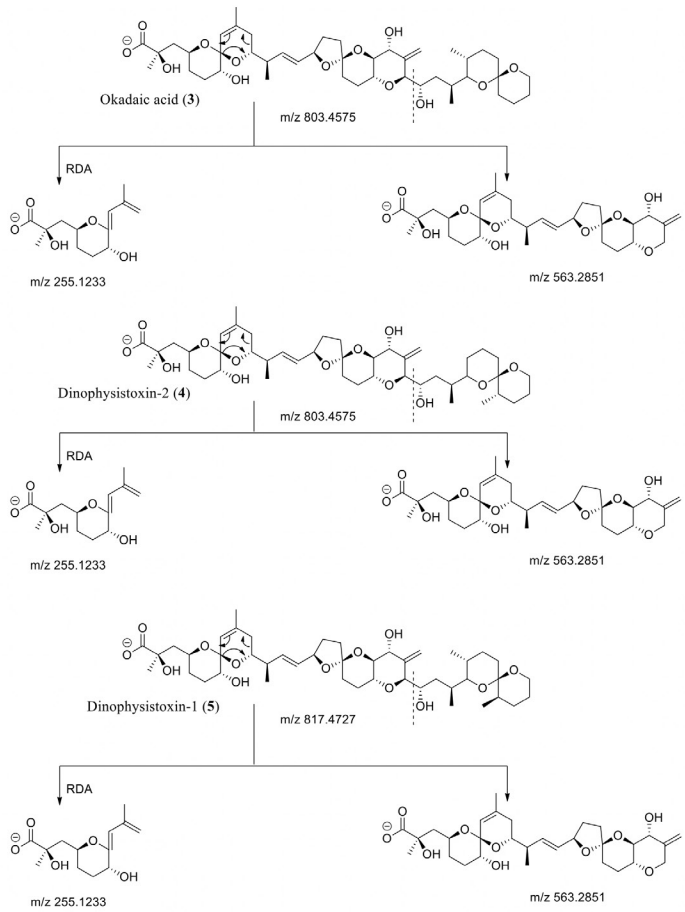

Figure 3. CID fragmentation of three polyethers (**3**-**5**)

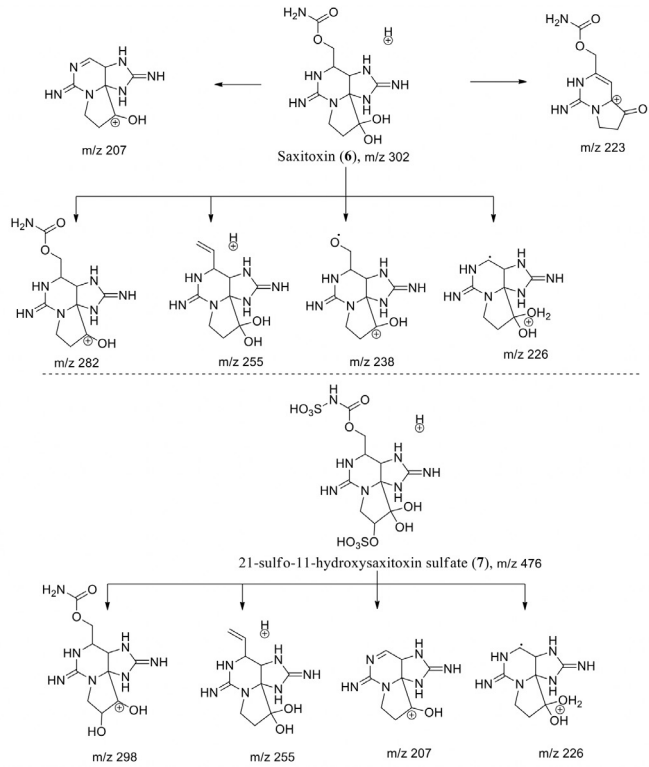

Figure 4. FAB-MS2 fragmentation of saxitoxin (**6**) and 21-sulfo-11α-hydroxysaxitoxin sulfate (**7**)

2.5 Identification of macrolides by using MS2 analysis

2.5.1 Identification of (5Z,13R)-tetradec-5-en-13-olide

Electron impact of (5Z,13R)-tetradec-5-en-13-olide (**8**), a macrolide identified from the African reed frog (*Hyperolius cinnamomeoventris*) provided interesting ions from the allylic cleavage and the McLafferty rearrangement (Fig. 5) [21]. These two mechanism pathways led to the ring opening at two positions, one on allylic C-C covalent bond and another on the ester function, respectively. The ion species formed in the first pathway lost a neutral entity (C_7H_{14} equivalent to 98 Da) to afford m/z 126; subsequently, it also lost two radicals species C_2H_5 and C_3H_7 to give m/z 195 and m/z 181, respectively. The ion resulting from the McLafferty rearrangement eliminated the neutral portion C_6H_{12} (64 Da) to yield m/z 140.

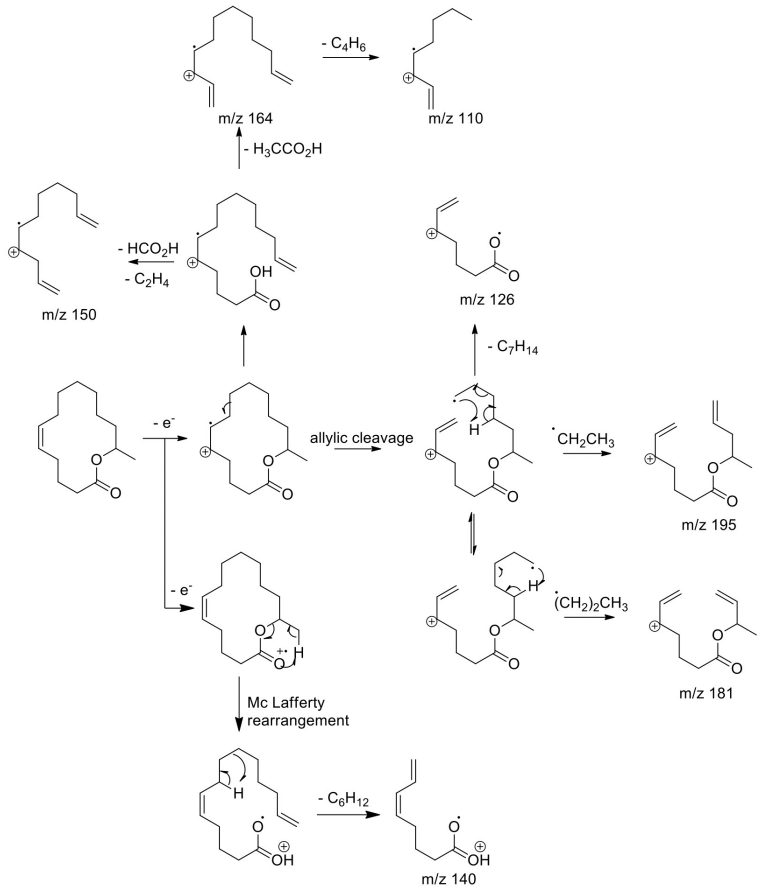

Figure 5. EIMS fragmentation of (5Z,13R)-tetradec-5-en-13-olide (**8**)

2.5.2 Identification of Erythronolide B

Erythronolide B (m/z 425.2523), a macrocyclic lactone from Streptomyces bacteria was subjected to tandem mass analysis. This compound showed a strong affinity with Na^+ adduct to form the pseudo-molecular ion. The base peak m/z 407.2392 was observed alongside m/z 327.1776, m/z 309.1678, m/z 291.1565, m/z 265.1765, m/z 253.1420, m/z 247.1668, m/z 235.1312, m/z 223.0943, m/z 207.1352, and m/z 179.1053; m/z 407.2392 differed from the molecular ion peak of 18 Da corresponding to the loss of H_2O. As depicted in figure 6, two proposals were made for this ion either the dehydration occurred at α and β-positions of the ketone or

it happened at the same locations for the ester function. The lactone opening was made via a McLafferty pathway manner and the resulted ion eliminated $C_6H_{10}O$ m/z 327.1776 which in turn lost successively two H_2O to afford respectively m/z 309.1678, and m/z 291.1565; a C-C sp3 bond cleavage was observed at near OH functions with a [1,3]-rearrangement of the hydrogen atom. Thus, this particular mechanism afforded m/z 265.1765 and m/z 253.1420 [22]. This latter ion lost H2O (18 Da) and CO (28 Da) yielding 235.1312 and m/z 207.1352.

As observed in the fragmentation pattern of erythronolide B, similar MS^2 behavior was also found for 6-deoxyerythronolide B. In figure 7, ion fragments from H_2O elimination, lactone opening ring through McLafferty pathway and cleavage of the C-C sp3 bond located in α to OH groups [22].

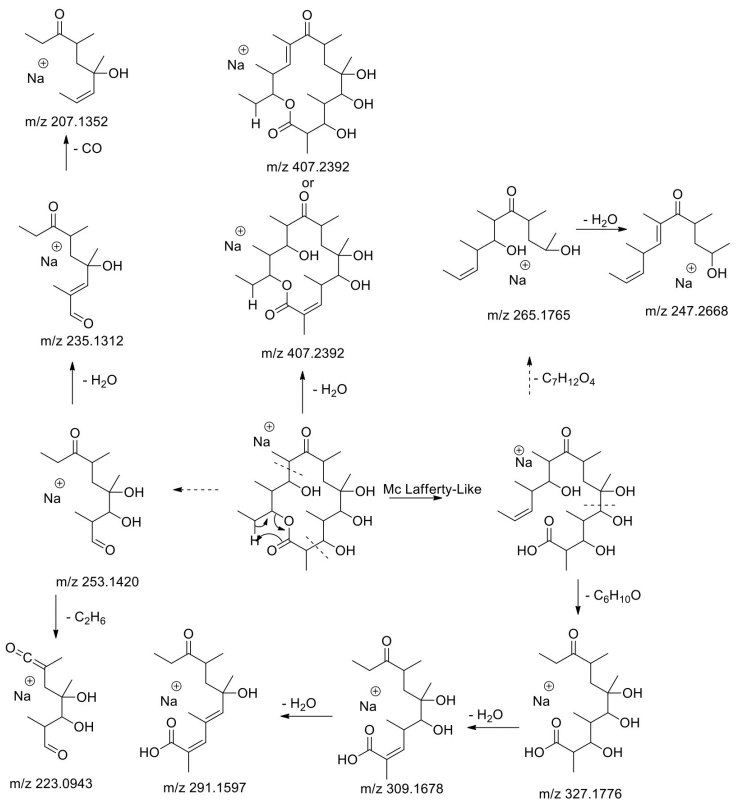

Figure 6. ESI-FTICR MS/MS fragmentation of Erythronolide B (**9**).

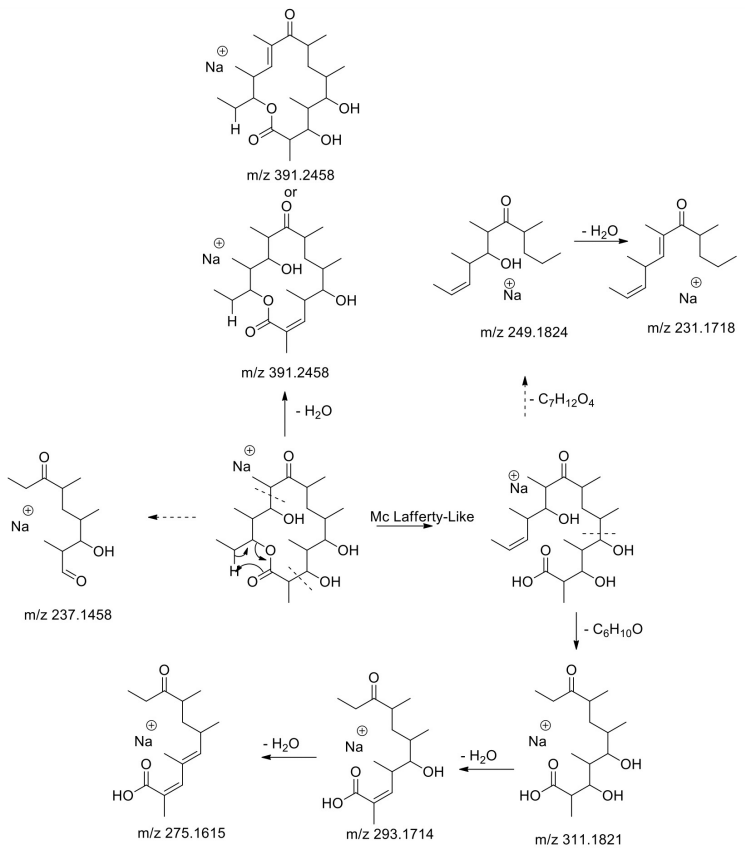

Figure 7. ESI-FTICR MS/MS fragmentation of 6-deoxyerythronolide B (**10**).

2.6 Identification of cyclopeptides by using MS2 analysis

Cyclopeptides consist in sequential and cyclic arrangement of amino acids attached to each other by peptide bonds or amide bond. Their identification is usually performed by using nuclear magnetic resonance, mass spectrometry, chemical transformation and enzymatic degradation. Depending on the number of amino acids involved in the cyclopeptide backbone, NMR data can result in many overlapped signals leading to a challenging task. For instance, cyclic peptide containing exclusively amino acids lacking aromatic rings and with hydrophobic side chain can lead a very difficult characterization work. However, mass spectrometry analysis provides some well-known information about the tandem mass fragmentation of cyclopeptides.

As discussed by Eckart in 1994, gas-phase protonation occurs in any amine of the amino acid in a particular cyclopeptide leading to the ring opening at different positions and affording different sequences of amino acids (Fig. 8). Thereafter, the elimination of a neutral amino acid species or neutral peptide moiety takes place to give different ions which enable the construction of the whole structure [23]. Since these peptide bonds do not have the same enthalpy, the applied energy of collision or the energy used to accelerate the inert atom will only trigger the cleavage of some of them.

In the particular case of arenariphilin B (**11**), all the fragmentation occurred from possibilities 3 and 4 [24]. Both ring openings provided crucial information on the sequence of amino acid in compound **11** (Fig. 9).

2.6.1 Identification of of clausenlanin B (12)

The case of clausenlanin B (**12**) only revealed one possibility of ring opening in the ESIMS analysis. The opening occurred on the peptide bond linking glycine and leucine. So, the elimination of leucine units allowed the confirmation of the sequence of amino acids in the lost portion not those remaining in the observed fragment m/z 270 [25]. Amino acids present in m/z 270 have 6 possible sequences therefore, NMR long-range correlations and/or the application of different collision energy can alternatively be helpful to determine the complete sequence.

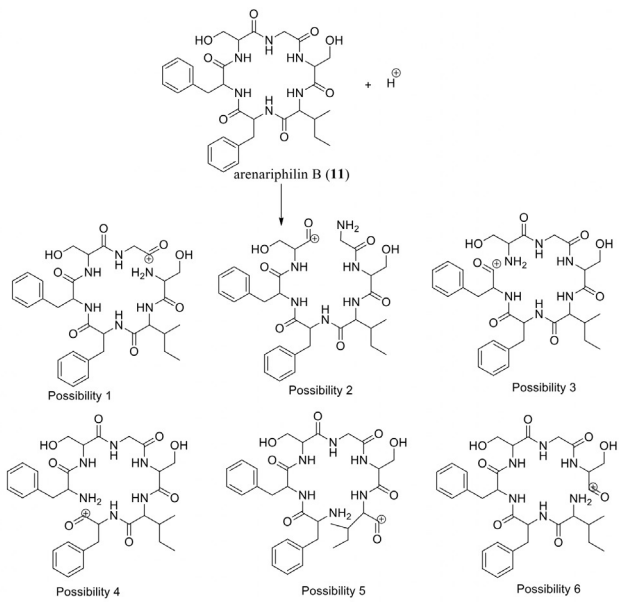

Figure 8. Possibilities of the ring opening in arenariphilin B (**11**) FAB-MS analysis.

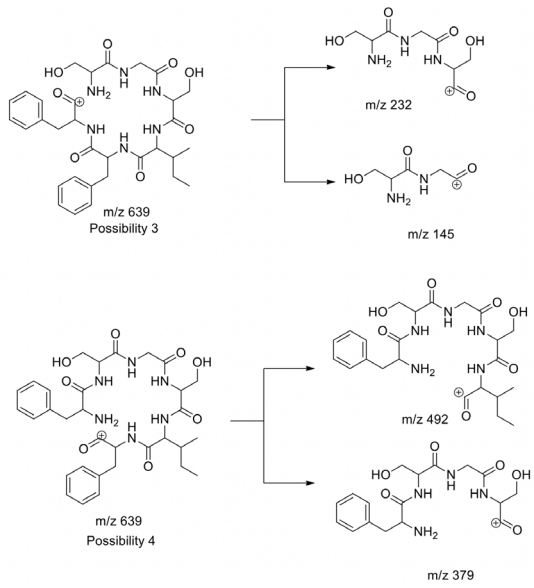

Figure 9. Fragmentation pattern of arenariphilin B (**11**) FAB-MS analysis.

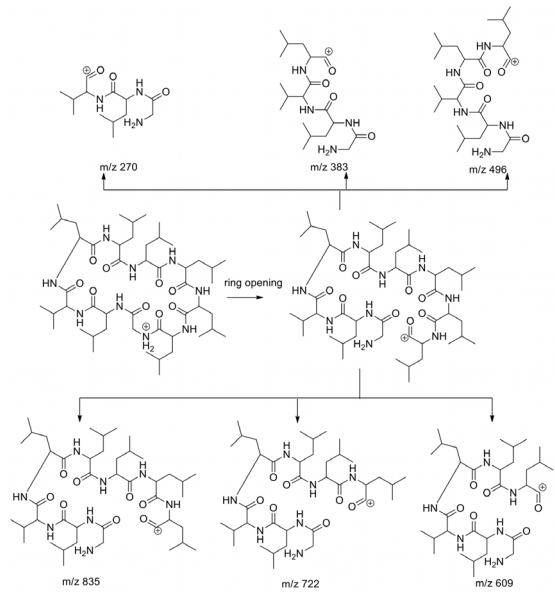

Figure 10. Fragmention pattern of clausenlanin B (**12**) ESI-MS analysis.

2.7 Identification of triterpenes by MS2 analysis

2.7.1 Cyclopropanic triterpenes: case of donellanic acid B (13)

Triterpenes cannot be easily identified only by MS analysis, its structure required more information from different techniques. So, 1D NMR data are generally helpful to define the triterpene skeleton and the 2D NMR maps enables to elucidate the structure. MS analysis will rather provide fragment ions that will confirm some functionalities and their position. Furthermore their fragmentation behavior can serve as a fingerprint to track similar and/or related components. Tandem mass analysis of a cyclopropane triterpene (donellanic acid B, **13**) using CID in an ESI-QTOF/MS equipment furnished the spectra depicted in figure 11. Diagnostics of fragment ions revealed that the double in ring C can react with H+ to form a secondary ion species on carbon atom C-12. This ion can underwent different allylic cleavage with a [1,3] position charge delocalization. The observed fragmentation pathway produced many daughter ions such as m/z 251.1623, m/z 263.1603, and m/z 237.1449 [26]. The ion m/z 251.1623 lost HCO_2H (46 Da) to afford m/z 205.1522 while m/z 237.1449 gave m/z 175.1405 after eliminating CO2 (44 Da) and H_2O (18 Da).

2.7.2 Tetracyclic triterpenes: case of pachymic acid (14)

Positive mode ionization ESI-MS analysis of pachymic acid (m/z 529) (**14**) gave the ion m/z 511 from the loss of H_2O (18 Da) which in turn provided m/z 451 after eliminating H_3CCO_2H (60 Da). The lighter fragment m/z 295 was formed from after the elimination of the side chain, H_2O and H_3CCO_2H based on the mechanism showed in figure 13 [27]. Negative mode ionization of the same compound did not afford many fragments. Only m/z 465 and m/z 449 were obtained after the precursor lost m/z 62 (CO_2 and H_2O) and m/z 78 (H_3CCO_2H and H2O), respectively [27].

2.7.3 Steroids: case of two androstane skeletons (15 and 16)

Steroids are biosynthetically related to triterpenes and both groups result from the cyclisation of squalene. Identification of steroids structures also relies on NMR data however every skeleton has a characteristic fragmentation pattern. So, 5α-androstan-17β-ol-3-one (**15**) and 17β-methyl-5α-androstan-17β-ol-3-one (**16**) were subjected to ESI-CID-MS2 from which both compounds shared similar features on their fragmentation pattern (Fig. 14). Compound **15** lost two H_2O (36 Da) from the ketone and OH groups to afford m/z 255 which in turn lost C_3H_4 (40 Da) to afford m/z 215. The ion m/z 199 was formed after m/z 215 eliminated CH4 (16 Da). The fragment ion m/z 255 also lost a C_4H_6 (52 Da) portion leading to the opening of ring A and affording m/z 201 which turn produced m/z 185 after losing CH_4 (14 Da). Similar behavior was observed in the tandem mass spectrum

of **16** in which identical neutral species were lost to afford fragment ions with 14 Da heavier than those obtained for compound **15** (Fig. 14) [28].

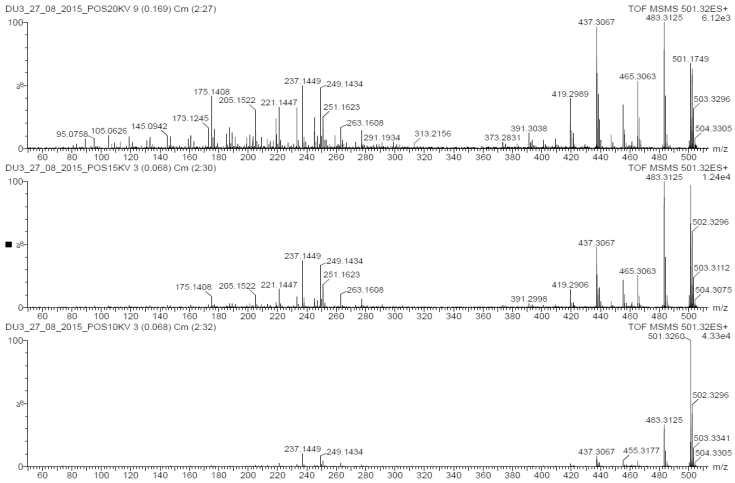

Figure 11. ESI- CID-QTOF/MS^2 spectra of donellalic acid B (**13**) with collision energy of 20 eV, 30 eV, and 40 eV.

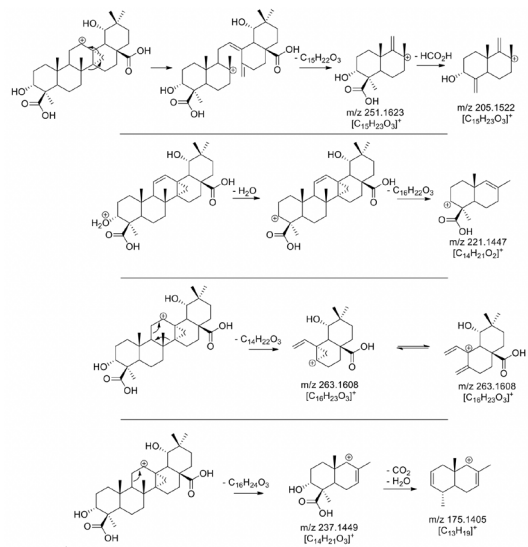

Figure 12. Proposal of the donellalic acid B (**13**) fragmentation pattern.

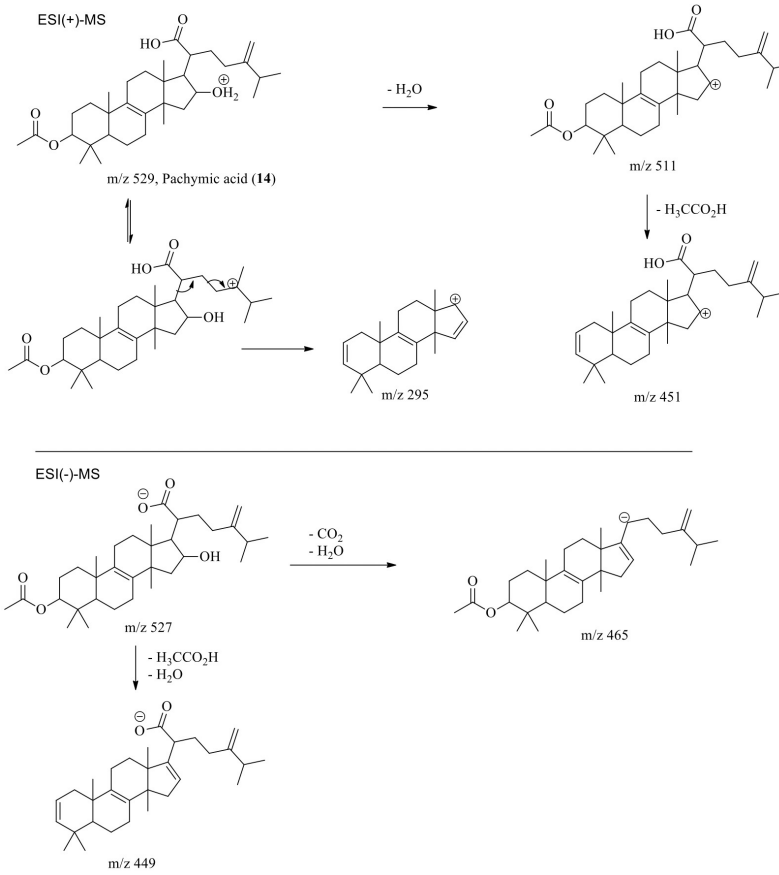

Figure 13. Proposal of the pachymic acid (**14**) fragmentation pattern.

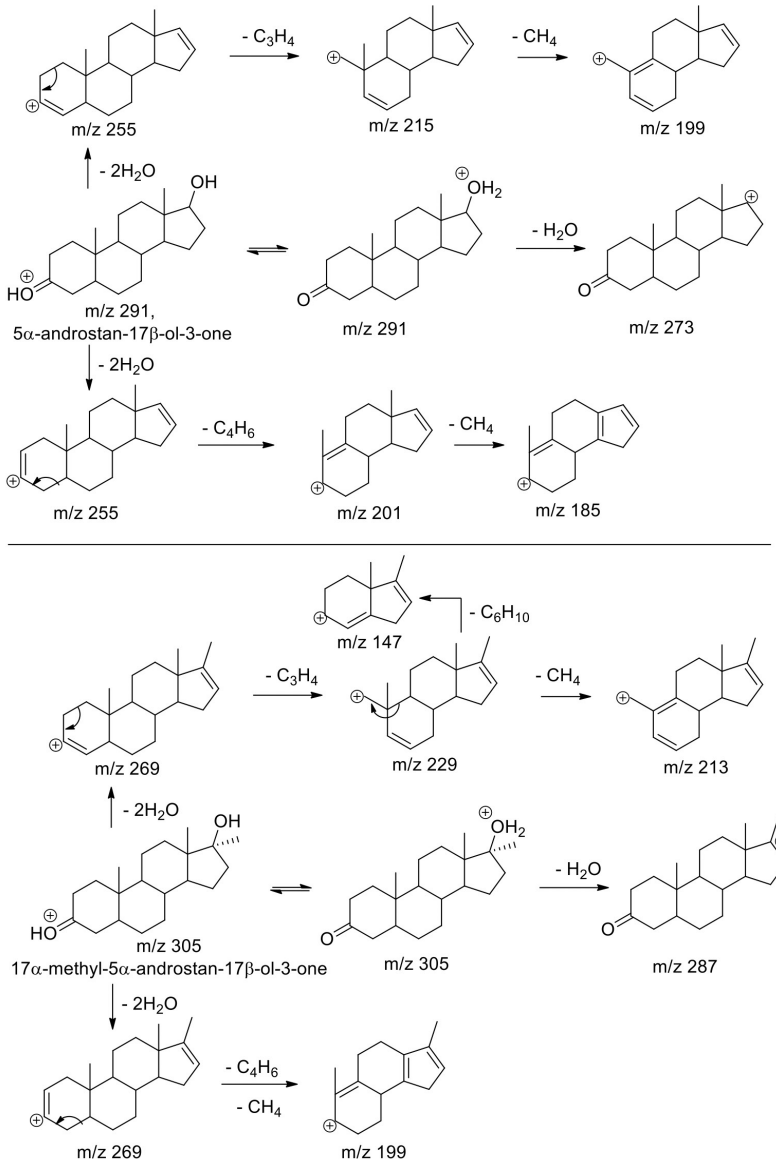

Figure 14. Proposal of two androstane steroids (**15-16**) fragmentation pattern.

2.7.4 Pentacyclic triterpenes: Case of hop-16-ene, lup-20(29)-ene, olean-12-en-3β-ol and urs-12-en-3β-ol (17-20)

Electronic impact analysis of pentacyclic triterpenes including hop-16-ene (**17**) and lup-20(29)-ene (**18**) gave features mostly similar in the fragmentation pattern. These two compounds have the same mass value and differ from each other by the position of the isopropyl moiety, the Me-28 group and the double bond [29]. However, some of the similar fragments seemed to be formed by a different mechanism as observed with the ion m/z 367. In hop-16-ene skeleton, the isopropane radical (43 Da) can be lost from m/z 410 by an allylic cleavage while in lup-20(29)-ene, the 5-membered ring needs to be opened somehow before it loses propene (42 Da) and a hydrogen radical (1 Da). This observation might support why the intensity of the fragment peak m/z 367 is greater in **17** fragmentations (21%) than in **18** (5%). The fragment ion m/z 218 formed by the opening of ring C, was exclusively present in the fragments list of compound **18** not in **17** fragmentation pattern. It seems like the radical could easily be stabilized by the π orbital of the isopropene olefinic bond in compound **18**, a feature that **17** do not contain (Fig. 15). Most importantly, in both compounds, m/z 191 was the base peak ion and the other fragments were attributed to portions containing either rings A and B or rings D and E [29].

Pentacyclic triterpenes containing exclusively 6-membered fused rings such as olean-12-en-3β-ol (**19**) and urs-12-en-3β-ol (**20**) (m/z 426) showed in their EI-MS spectra, m/z 218 as a base peak ion (Fig. 16). This MS behavior is completely different from that observed for triterpenes containing a 5-membered ring as ring E (lupane and hopane skeletons) [29]. Furthermore, our proposal suggests that m/z 218 was formed by an initial cleavage of the covalent bond C_8-C_{14} in lup-20(29)-ene skeleton (Fig. 15) followed by a homolytic rupture of C_9-C_{11} whereas in oleanane and ursane skeleton, it occurs from the allylic rearrangement due to the position C_{12}-C_{13} of the olefinic bond (Fig. 16). Compounds **19** and **20** on the other hand displayed similar mass behavior than 17 and 18 conducting to the formation of m/z 217 different from m/z 191 by 16 Da (compounds **17** and **18**), corresponding to an oxygen atom. The above mentioned data represent characteristic features that can facilitate the identification of each of this skeleton.

2.7.5 Triterpenic saponin: Case of hederagenin (21)

Tandem mass analyses of saponins usually provide information about the sequence of sugars sequenced in branching or linear pattern. Sometimes, the carbohydrate can be attached to the genin on different locations that can diagnostically be revealed by MS data. Nevertheless, this technique does not determine the exact position of the osidic bonds. This later exclusively requires

long-range NMR correlations techniques to be established. Metabolites composed of a carbohydrates sequence present characteristic elimination in their mass spectra. For instance, a hexose can be lost from the rest of the molecule 162 Da or 180 Da (Fig. 17) [30].

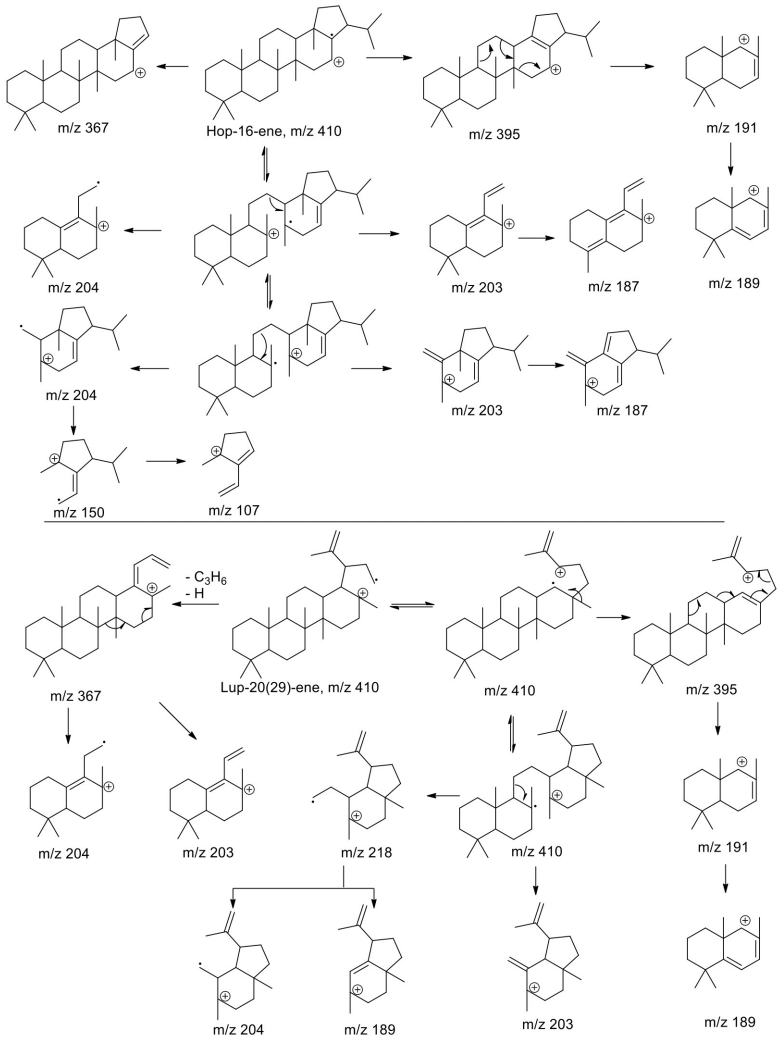

Figure 15. Proposal of the fragmentation pattern of two pentacyclic triterpenes containing a 5-membered ring (**17-18**).

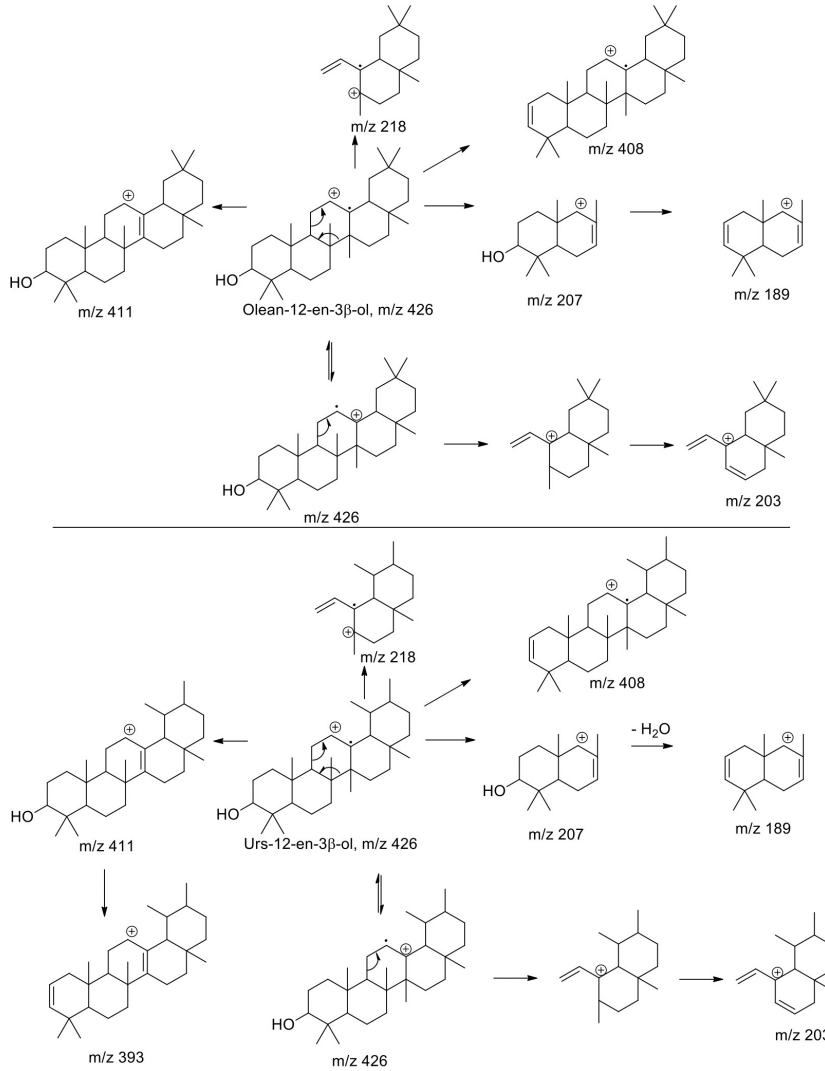

Figure 16. Proposal of the fragmentation pattern of two pentacyclic triterpenes containing 6-membered fused rings (**19-20**).

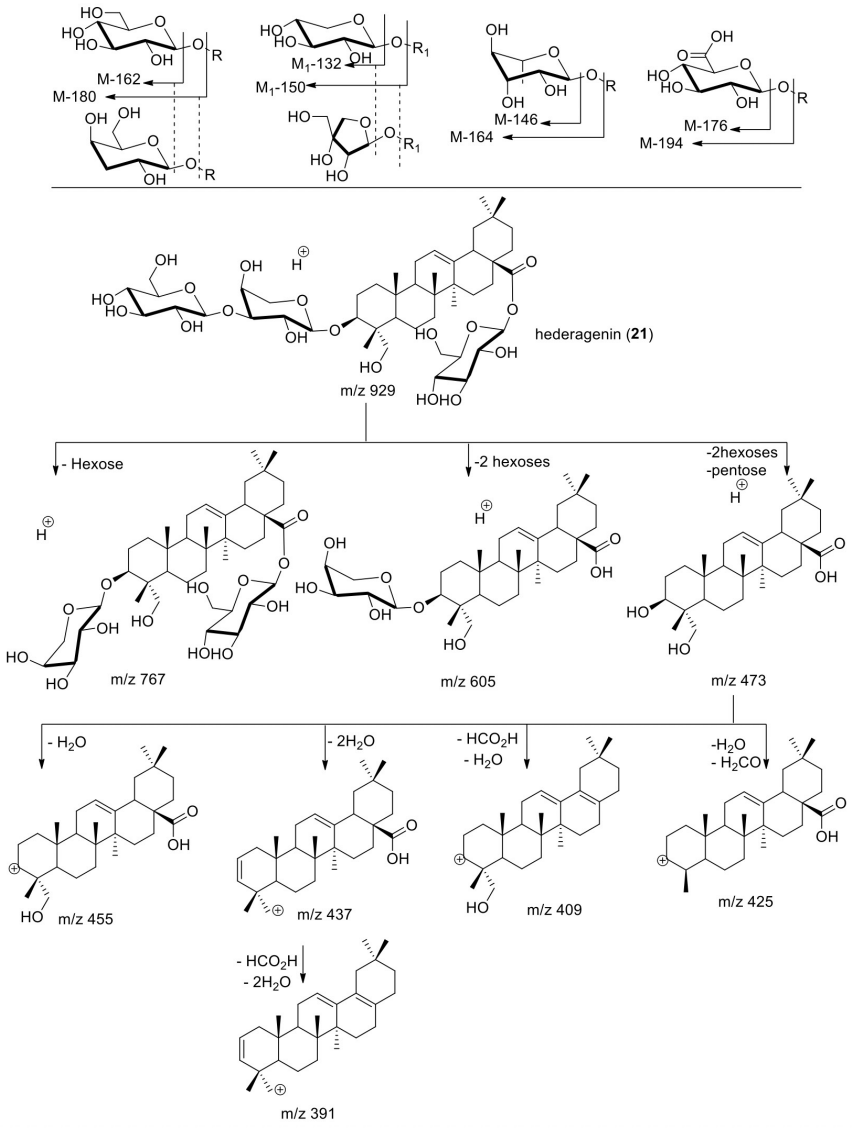

Figure 17. Proposal of the fragmentation pattern of the pentacyclic triterpene saponin, hederagenin (**21**).

Unfortunately, the mass data are not useful for the stereochemistry assignment of the sugar or cannot justify whether the sugar skeleton is a pyrane or a furan. The molecular weight reduces of 132 Da or 150 when the compound loses a pyrano- or furopentose. A loss of deoxyhexose moiety and uronic acid portion will decrease the mass value of the molecule by 146 Da and 164 Da in the first case, and 176 Da and 194 Da in the second [30]. This fact can easily be applied to the MS^2 fragmentation of hederagenin m/z 929 (**21**) which gave ions at m/z 797, m/z 605 and m/z 473 corresponding to the loss of one hexose (glucose, 162 Da), two hexoses (324 Da) and two hexoses and one pyranopentose (456 Da) respectively. Moreover, two interesting fragments revealing the type of functionalities were formed after the precursor lost a carbinol as formaldehyde (30 Da) and by undergoing a decarboxylation as formic acid (46 Da) [31]. Both neutral species lost justify the presence in the compound structure of a CH_2OH group and a CO_2H group. The identification of the entire aglycone will need high collision energy.

2.8 Identification of sesquiterpenes by MS analysis

2.8.1 Case of an eremophylan and eudesman sesquiterpenes (22 and 23)

Copound **22** just reveals that ionization by using EI can occur in any particular bond of the scaffold. As shown in figure 18, when the ionization is on the sesquiterpene double bond C_2-C_3, a radical methyl group can be eliminated to form the ion m/z 217 through an allylic cleavage. Moreover, if the α,β-exomethylene is the ionization spot, a ring B opening of the decalin can afford m/z 204 after losing C_2H_4 (28 Da). Ionization of the carbonyl group can either lead to a loss of a radical OH group (17 Da) followed by a decarboxylation (28 Da) to produce m/z 187. In the other hand, dehydration (18 Da) occurs to give the radical ion species m/z 214. Depending to the presence of some function groups, sesquiterpenes like other metabolites in EI can afford fragment ions through only a part of the well-established mechanisms [32].

Arsasin (**23**, m/z 266) is an eudesman sesquiterpene type bearing a γ-butyrolactone ring. The compound also contains an OH groups and a ketone group. So, two mechanisms namely the loss of H_2O and the RDA rearrangement typically observed in EI was obtained. Both afforded the radical ions m/z 248 and m/z 100. As shown in figure 18, the RDA ring opening took place after the enolization of the ketone function. In addition, a α-cleavage occurs on both δ C-C bonds of the CO function. The radical ion formed underwent a subsequent loss of OH radical and hydrocarbon portion to yield m/z 167 when the second lost C_2H_2O, H radical, and the lactone portion to produce m/z 151 (Fig. 8). Similar

mass value (m/z 151) was found when m/z 167 eliminated CH_4 (16 Da) and an accurate mass measurement can unequivocally enable a good assignment of the ion structure. Further ions with lighter mass values were obtained from m/z 167 which lost H_2O from the lactone ring opening and yielded m/z 149. This latter in turn rearranged the charge position affording a species with the positive charge on the CO group. Subsequently, this ion specie underwent separately a RDA ring opening to afford m/z 121 and lost CO to produce m/z 121 [33].

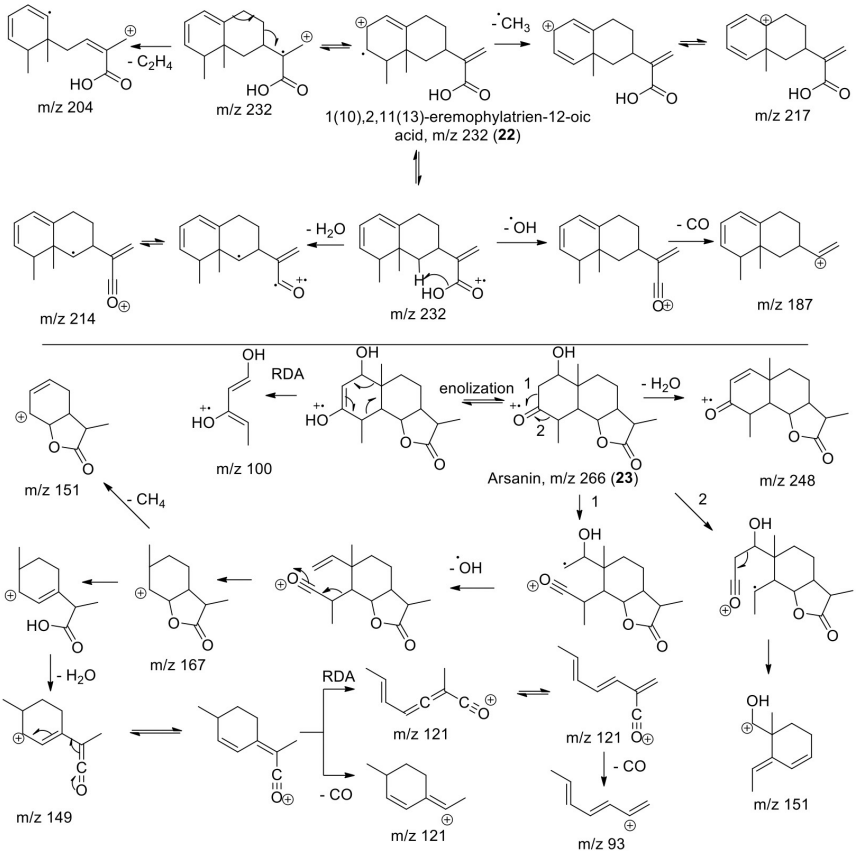

Figure 18. Proposal of the fragmentation pattern of 1(10),2,11(13)-eremophylatrien-12-oic acid (**22**) and arsanin (**23**).

2.9 Identification of diterpenes by MS analysis

2.9.1 Case of isopimarane diterpenes (24 and 25)

Compound **24** was detected as a protonated ion at m/z 307.2624 and the precursor formed was dissociated by CID-MS^2. Two daughter ions were formed by sequential loss of one and two H_2O to afford m/z 289.2524 and 271.2410 respectively (Fig. 19 and 20). Two postulates of the mechanism were enounced the first was the charge-remote elimination (R1) and the second was the charge-driven concerted reaction (R2) [34]. However, it is well-known that the precursor dissociation occurring in the collision chamber tends to produce stable ions. So, the calculation of their internal energy of each ion may allow the prediction of which ion is formed among those presented in figure 20 with the same mass value. The ion m/z 165.1157 was produced by m/z 289.2524 by the mechanisms outlined above however, the fragment resulting from the RDA rearrangement might seems the most stable. The application of both mechanisms also allowed to find m/z 219.1735 and to propose its structure as depicted in figure 20.

Compactol (**25**), a position isomer of **24** also provided ions at m/z 289.2339 and m/z 271.2411 resulting from the loss of one and two H_2O, respectively (Fig. 21 and 22). Figure 22 revealed that if OH-7 and OH-8 are eliminated through R1 and R2, respectively, the positive charge at C-8 can lead to m/z 187.1322 via R2. Moreover, the positive charge located the exocyclic olefin induced the loss of C_2H_4 (28 Da) and the ion formed lost in turn H_2O (18 Da) and C_3H_6 (42 Da) to provide m/z 219.1815 [34].

2.9.2 Case of neo-clerodane diterpenes (26)

Analysis of teucrin A (m/z 343, **26**) in a negative mode ionization using a triple quadrupole devise equipped with a CAD chamber afforded a fragment at m/z 325 after losing H2O (18 Da) (Fig. 23). Furthermore, a loss of the furan ring from the precursor followed by two decarboxylation reactions produced m/z 203. While a rearrangement of the spirobutyrolactone ring afforded m/z 219 from the parent ion, this latter also underwent a decarboxylation to yield m/z 299. The ion m/z 249 was formed after the parent lost the vinylfuran portion a decarboxylation of this daughter ion furnished m/z 205 [35].

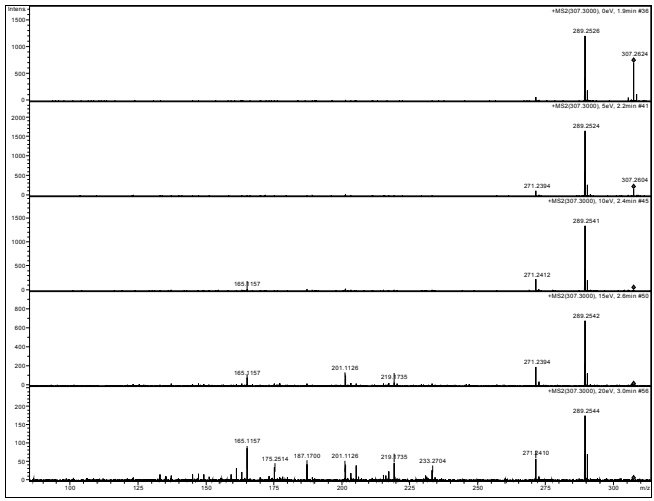

Figure 19. Mass spectra of isopimarane (**24**) [34].

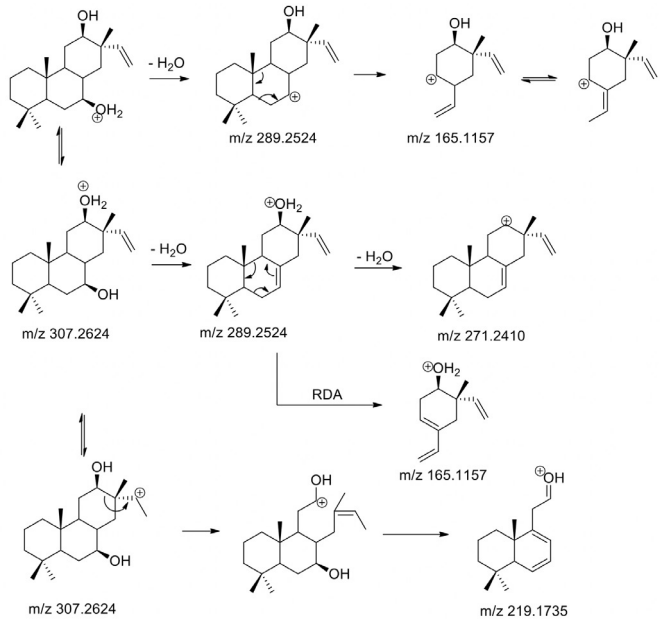

Figure 20. Proposal of the fragmentation pattern of isopimarane (**24**).

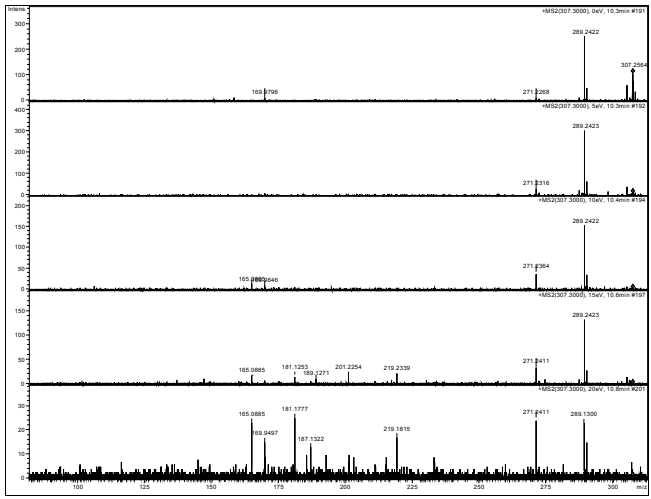

Figure 21. Mass spectra of compactol (**25**) [34].

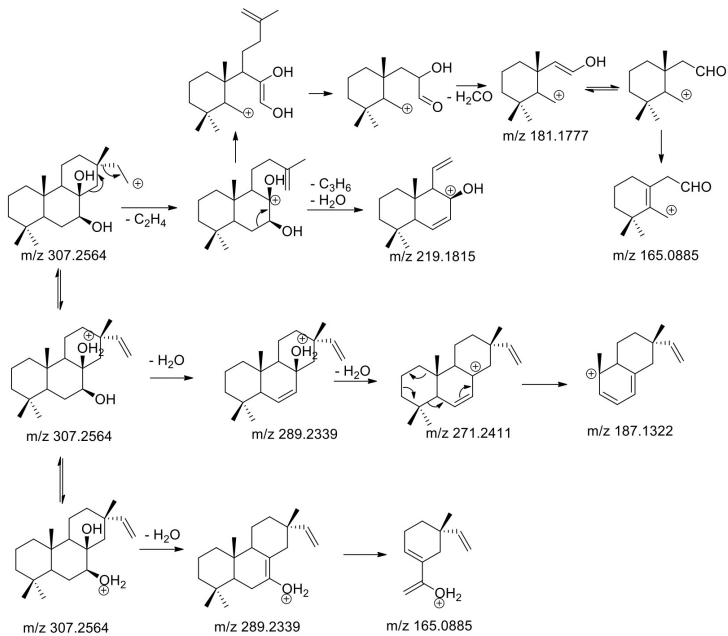

Figure 22. Proposal of the fragmentation pattern of compactol (**25**).

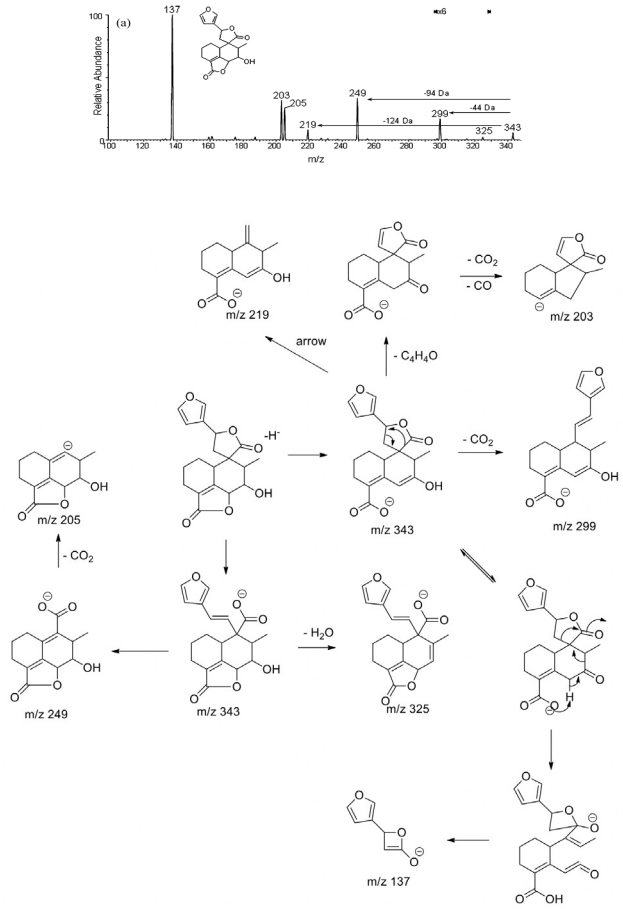

Figure 23. Mass spectrum of teucrin A (**26**) and a proposal of its fragmentation pattern [35].

2.10 Identification of polymethoxyflavones by MS analysis

Polymethoxyflavones (PMF, **27-30**) are natural flavonoids with two or more methoxyl on their basic benzo-γ-pyrone skeleton. These flavones are widespread in angiosperms families including Asteraceae, Lamiaceae and Rutaceae [36]. Additionally, they are well-known metabolites in *Citrus* and *Ageratum* genus [37, 38]. PMF stands out for their pharmacological properties, mainly anti-inflammatory and antiproliferative [39-42].

The structures of PMF usually differ in the number, type and position of substitution on the flavone skeleton, for example, several members are

bearing hydroxyl and dioxymethylene patterns. Furthermore, variations on benzo-γ-pyrone skeleton are also found, including polymethoxyflavanones and polymethoxychalcones. In this context, mass spectrometry is an important tool to support the identification of these phenylpropanoids derivatives.

The fragmentation pattern of PMF has been investigated using ESI-MS/MS behavior of standards belonging to this class of compounds [43-45]. In general, PMF MS/MS spectra in positive mode present the diagnostic fragmentation ions of m/z $[M+H-15]^+$, $[M+H-30]^+$, $[M+H-45]^+$, and $[M+H-60]^+$, which are associated with two fragmentation mechanisms namely methyl radical loss and α-elimination of formaldehyde. While $[M+H-15]^+$ corresponds to the loss of one methyl radical, $[M+H-30]^+$ could be related to the loss of a formaldehyde unit or two methyl radicals, with the formation of a dioxetane ring. The only way to differentiate these fragment ions is the use of an accurate measurement in mass spectrometry, 30.047 Da corresponds to two methyl radicals units, as opposed to 30.0106 Da which matches the formaldehyde unit. Considering this fact, to elucidate the other product ions, a careful analysis is mandatory, since $[M+H-45]^+$ could be either the loss of three methyl radicals or one methyl radical plus formaldehyde. The same fact need to be considered when occurs the ion m/z $[M+H-60]^+$, which might be related to two formaldehyde units loss or four methyl radicals (Fig. 24). This fragmentation pathway turn to be the predominant diagnostic daughter ions observed in PMF MS spectra.

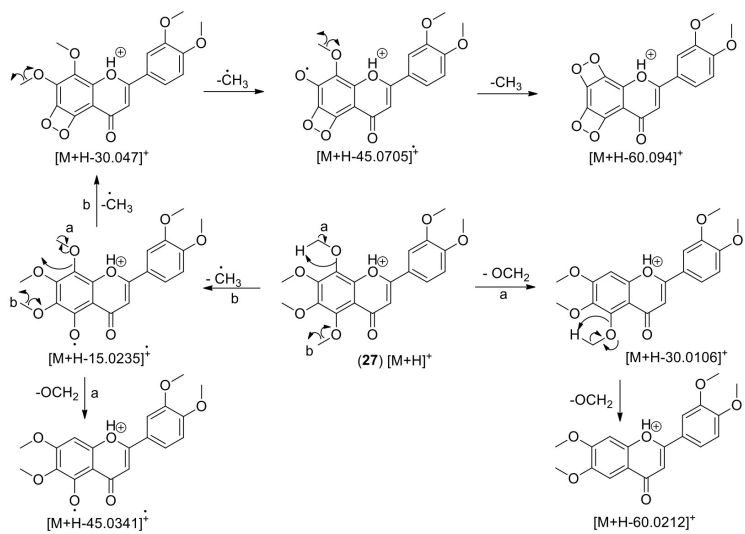

Figure 24. Illustration of the methyl radical fragmentation and α-elimination of formaldehyde in PMF (**27**).

Other eliminations can be also observed during the PMF dissociation, which include the loss of methane $[M+H-16]^+$, water $[M+H-18]^+$, carbon monoxide $[M+H-28]^+$, methoxyl radical $[M+H-31]^+$, water plus methyl radical $[M+H-33]^+$, carbon monoxide plus methyl radical $[M+H-43]^+$, carbon dioxide $[M+H-44]^+$, carbon monoxide plus water $[M+H-46]^+$, formaldehyde or two methyl radicals plus water $[M+H-48]^+$, and formaldehyde or two methyl radicals plus methoxyl radical $[M+H-61]^+$ (Fig. 25). This dissociation pathway is similar to those observed when using APCI-MS/MS [46]. Overall, the fragmentation behavior outlined above formed the MS^2 fingerprint of PMF.

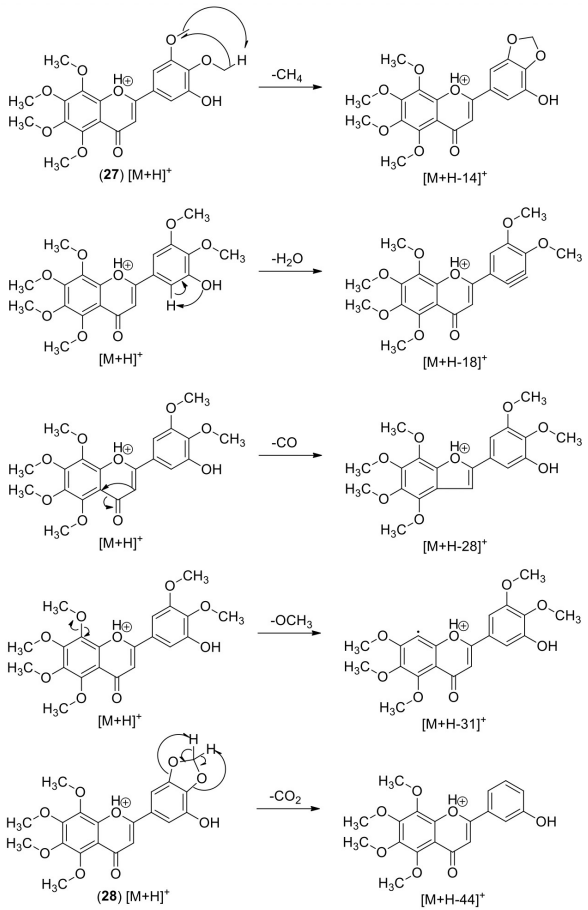

Figure 25. Illustration of common neutral species lost during the PMF fragmentation

Regarding the methane loss, this elimination occurs when there are two *ortho*-methoxyl groups attached to the aromatic rings and leads to the formation of a methylenedioxy derivative. Wang and Zhang [45] clearly described this MS^2 behavior of PMF bearing ortho MeO groups. Moreover, this fragmentation pattern also strongly indicated the presence of such substituents arrangement and therefore, is not definitely observed in mono methoxylated PMF. MS feature similar to PMF bearing O-dimethoxy groups has also been observed in MS/MS spectra of PMF with three or four neighboring methoxy groups [47]. However, the relative intensity of this ion is lower when compared with those with two *ortho*-methoxyl groups [47]. This information could be used to judge the distribution of the methoxyl groups on the benzo-γ-pyrone skeleton. The loss of carbon dioxide in PMF apparently via a radical pathway usually originates from a methylenedioxy group and this latter in turn can alternatively be formed from the rearrangement of an ortho-diMeO system after eliminating CH_4 [45]. Nevertheless, the function OCH_2O can also be from the original molecule and in a subsequently observation, CO_2 loss indicate the presence of methylene dioxide moiety in the PMF structure while CH_4 justifies the presence of ortho-diMeO groups. Retro Diels-Alder (RDA) is also usefull as a fragmentation mechanism occurring in PMF. This mechanism is often found in MS^2 spectrum of flavonoids and it is quite helpful to determine the methoxy group's distribution among the flavonoids rings A and B.

Considering other types of polymethoxylated flavonoids such as polymethoxylated flavanones, their MS/MS analysis indicate that the most frequent fragmentation pattern involves a) RDA cleavage from 1,3-position of C-ring and b) α-cleavage from 4,10-position followed by a cleavage from 1,2-position with 1-3hydrogene rearrangement of C-ring. Similarly, for polymethoxylated chalcones the main fragmentation pattern includes a) left and right α-cleavages (Fig. 26). Consequently, it is easy to distinguish polymethoxyflavones, from polymethoxyflavanones and from polymethoxychalcones. However, it is difficult to differentiate polymethoxyflavanones from polymethoxychalcones when both have the same *m/z* value. In this particular case, the difference in UV spectra between polymethoxyflavanone (λ_{max} 320 nm) and polymethoxychalcone (λ_{max} 330-370 nm) help to solve the problem [43, 48]. A bathochromic shift can be observed in chalcones due to a strong the mesomeric effect and the extension of the electrons delocalization. Ring C in flavanone contain a δ-bond which limited the extension.

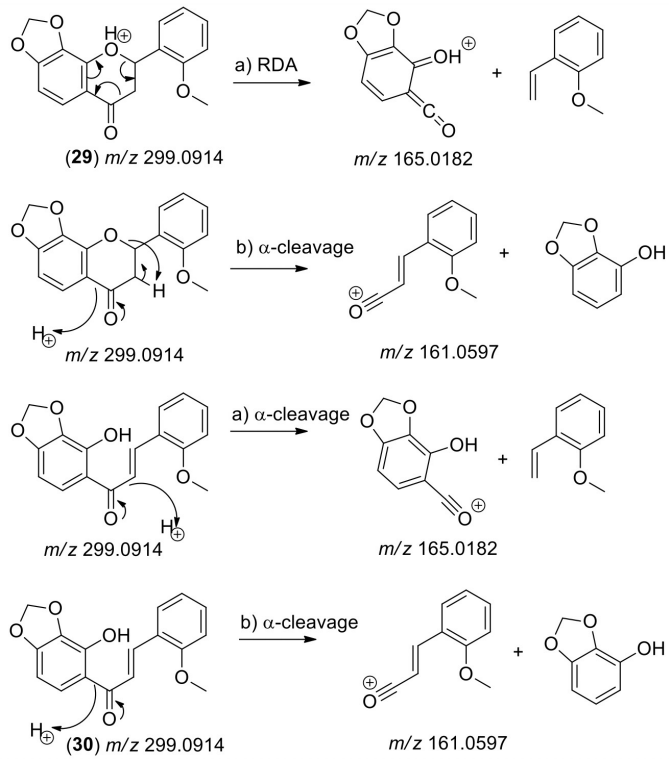

Figure 26. Illustration of fragmentation pattern of polymethoxyflavanones and polymethoxychalcones

2.11 Dereplication study in toxicology by using LC-ESI-MS²

MS spectrometry in it application is fast for the identification and requires low concentrated sample. So, LC-MS analysis of the DCM extract of the urine from a patient exposed to prednisolone (**30**) was performed on an UPLC-ESI-QTOFMS. The chromatogram (Fig. 27) revealed the presence of this drug (**30**) and a great quantity of its metabolite (**31**) (Table 1). LC-CID-MS/MS analysis (Fig. 28-31) was carried out in order to identify these two compounds and the fragmentation pattern led to the structure of prednisolone and prednisone. Moreover, caffeine and theobromine were detected in the fluid of the patient. Exploration of compound **31** fragmentation pattern corresponded to those reported in the literature [49]. It is worthy to mention that toxicology study by using mass spectrometry as tool requires many careful steps to be respect because of the presence of proteins in the human fluids. These proteins can make impossible the interpretation of the

result from the matrix. Therefore, separation, extraction, and detection are the steps that need to be well established before identification and/or quantification.

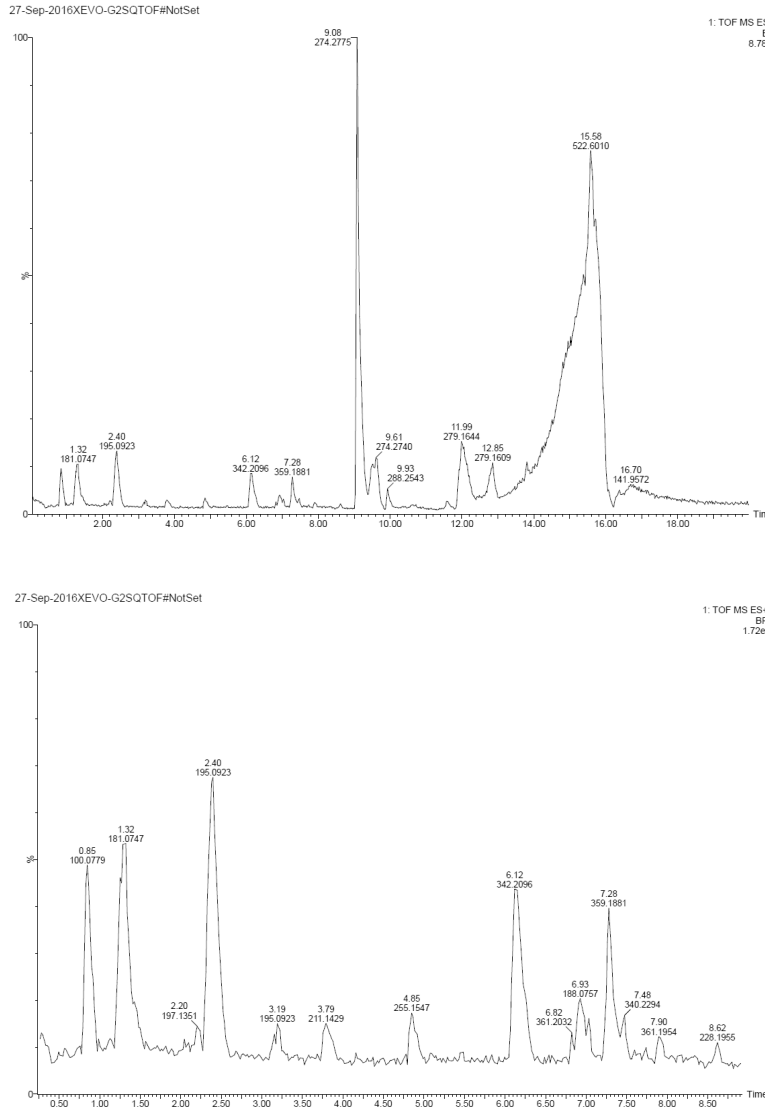

Figure 27. UPLC-ESI-MS of the methylene chloride extract of a patient urine exposed to prednisolone

Table 1. Dereplication of the DCM extract of a patient urine exposed to prednisolone

RT (min)	Mass detected (m/z)	Molecular formula	Proposal of the structure
1.32	181.0743	$C_7H_8N_4O_2$	Theobromine
2.40	195.0872	$C_8H_{10}N_4O_2$	Caffeine
6.12	342.2083	$C_{21}H_{27}NO_3$	Piperidine alkaloid type
6.82	361.2034	$C_{21}H_{28}O_5$	Prednisolone
7.28	359.1851	$C_{21}H_{26}O_5$	Prednisone

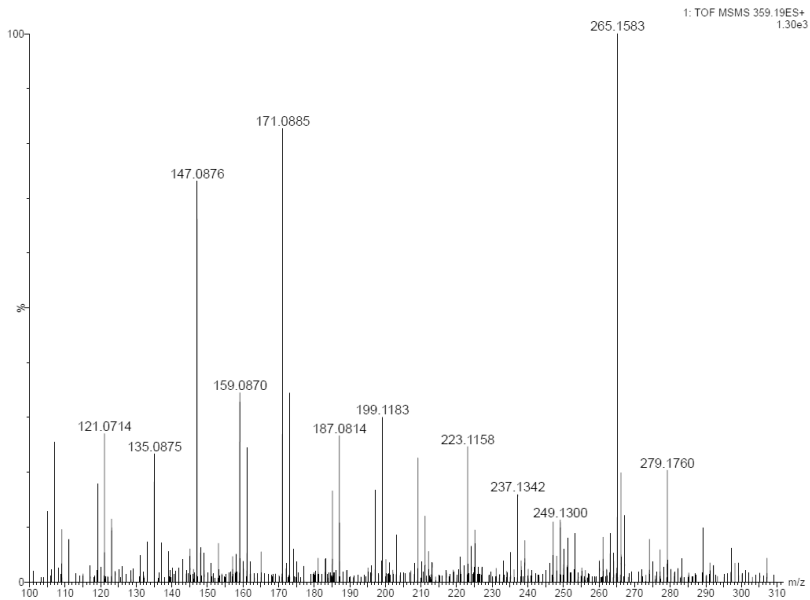

Figure 28. HRESI-CID-MS/MS spectrum of prednisolone (**31**)

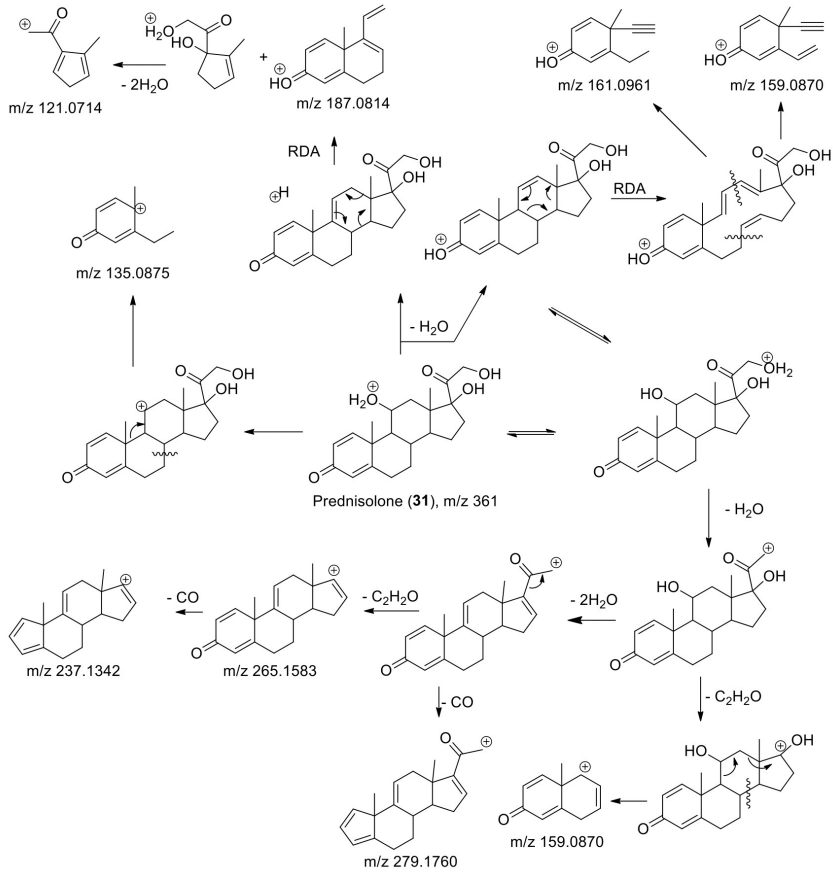

Figure 29. Proposal of the fragmentation pattern of prednisolone (**31**)

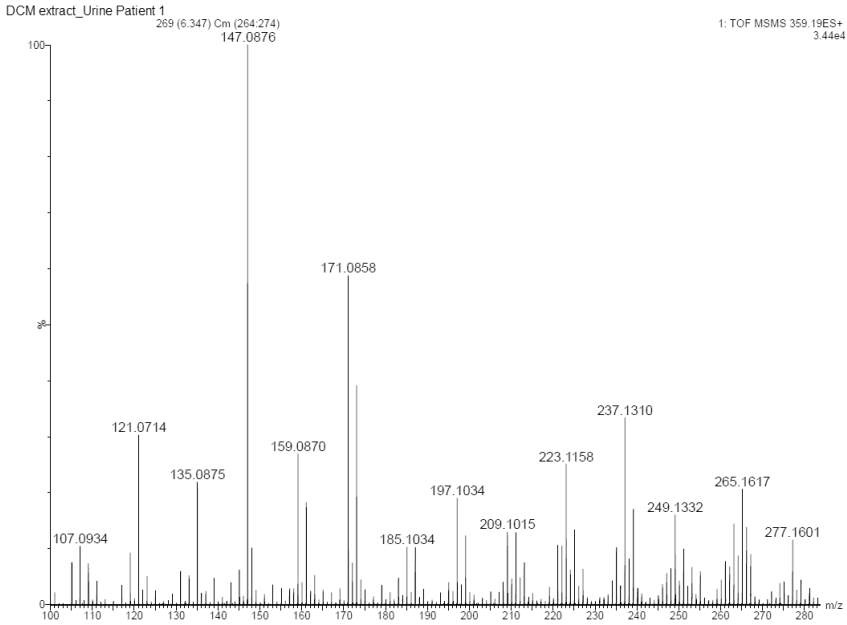

Figure 30. HRESI-CID-MS/MS spectrum of prednisone (**32**)

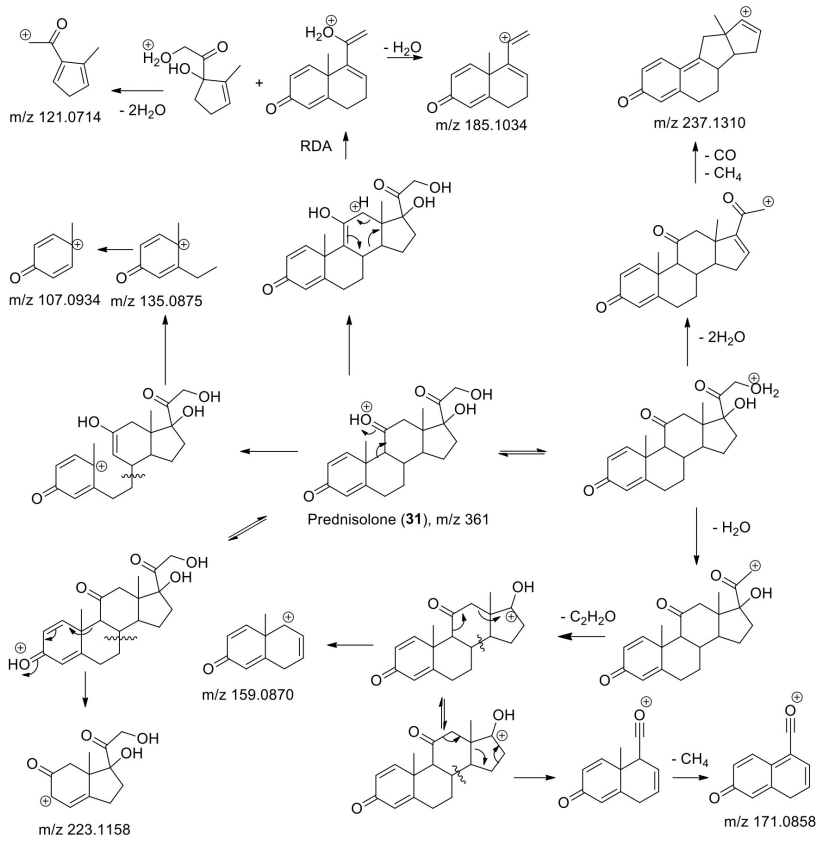

Figure 31. Proposal of the fragmentation pattern of prednisone (**32**)

Conclusions

The chapter has focused a particular emphasis on fragmentation mechanism of rare secondary metabolites. As outlined in the figures, some compounds such as cyclopeptides can easily be identified while others including carbohydrates require the use of further analytical techniques to reveal their full identity. However, mass spectrometry can be associated with chemotaxonomy information in order to propose a structure with high probability of certainty even though NMR analysis can provide equivocal evidences. Dereplication in mass spectrometry which is mostly based on a simultaneous separation and identification of components in a mixture can save time from the routine phytochemical study since it can be used as a powerful tool to track new metabolites. This technique has been

applied in many areas such as metabolomics, ecological chemistry relationship between living species, toxicology and so forth.

Conflicts of Interest

The authors declare no conflict of interest.

References and Notes

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Chapter 6

Coumarins: Synthetic Approaches and Pharmacological Importance

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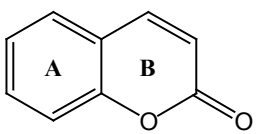
1. Concepts and Classification

Coumarins constitute one of the major classes of secondary metabolites 1 and as such play essential role in the survival of their producing organisms [2-3]. They are widely distributed in plants, but are also found in bacteria and fungi species. In these organisms, they play important functions in defense mechanism, such as protection against herbivorism and other stress events, as well as interaction with microorganisms. [4-6]

Chemically, they are lactones of *o*-hydroxide-cinnamic acid, therefore belonging to the class of phenolic compounds (compounds with at least one hydroxyl (-OH) attached to a benzene ring). Its minimum structure is formed by the fusion of two cyclic systems, one benzene and one α-pyrone (rings A and B respectively) (Figure 1) [7-9] to give 1,2-benzopyrones.

Figure 1. Minimal structure of coumarins: 1,2-benzopyrone ring.



According to their structural variations, coumarins can be classified as: [10,11]

i. *Single coumarins*: They are characterized by presenting exclusively the fundamental nucleus of coumarin (1-benzopyran-2-one ring) substituted in any/or both rings of the cyclic system with the presence of substituents (hydroxyl, alkyl, amine, among others) (Figure 2);

ii. *Furanocoumarins*: They are characterized by presenting a furan ring fused with a single coumarin. According to the spatial position adopted by the

furan ring, two major classes of isomers are generated: Psolaren (linear) and Angelicin (angular) derivatives (Figure 3);

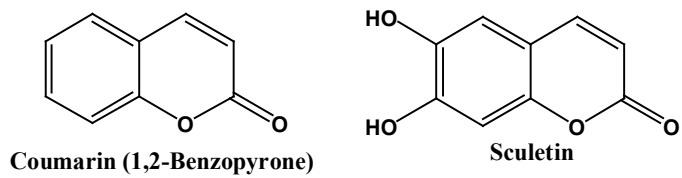


Figure 2. Examples of single coumarins.

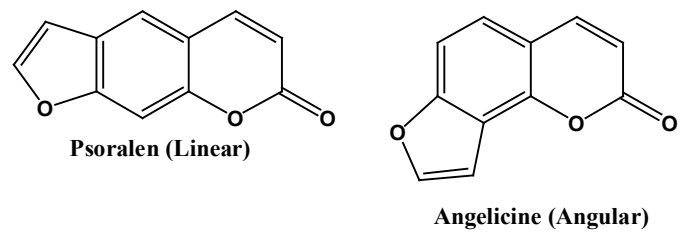


Figure 3. Examples of furanocoumarins.

iii. *Pyranocoumarins*: Characterized by presenting a pyran ring fused with a single coumarin. In the same way as observed with furanocoumarins, depending on the fusion position two most common isomers can be observed: linear (Xantiletin) and angular (Visnadin) (Figure 4);

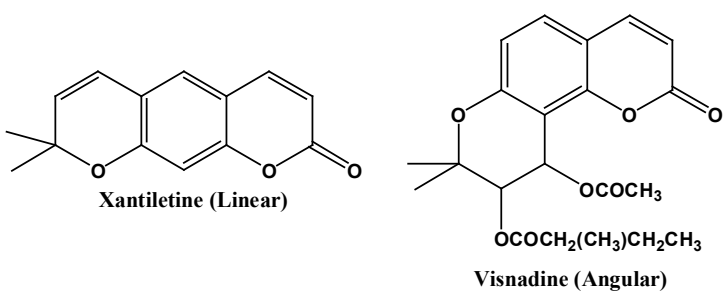


Figure 4. Examples of piranocoumarins.

iv. *Bis-coumarins and Tricoumarins*: Coumarins formed by the association of two or three subunits of simple coumarins (Figure 5);

One of the hallmarks of the coumarin compounds is their characteristic odor, which explain why these compounds were utilized, commonly, as food flavorings agents. However, this first industrial use was discontinued after the observation of considerable hepatic toxicity, which resulted in the interdiction of their uses as an additive for the *Food and Drug Administration* (FDA), [12] although their use continue to be common in the cleaning and cosmetics industry. [13]

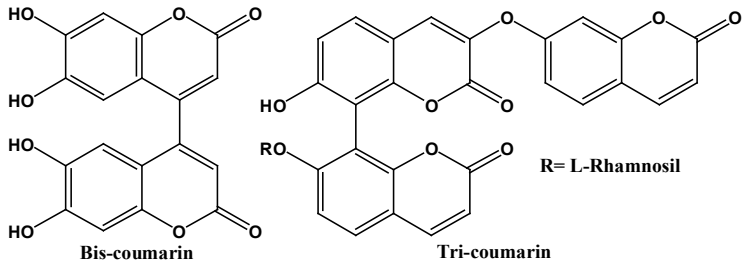


Figure 5. Examples of bis- and tri-coumarins.

v. Phenylcoumarins: Single coumarins substituted by a phenyl ring (Figure 6);

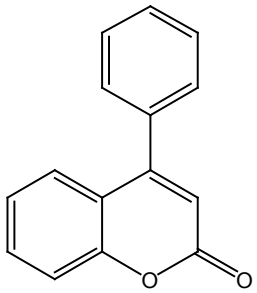


Figure 6. Example of phenylcoumarins.

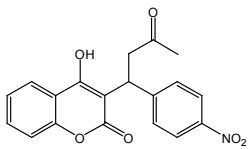
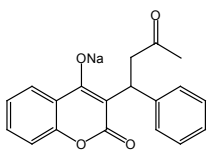
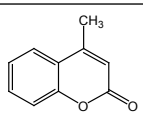
The first isolated constituent of this group (1,2-benzopyrone – Figure 1) was isolated for the first time, in 1822, from *Dupterix odorata*, [14] popularly known by the name of cumaru, name that gave rise to this class of secondary metabolites. [15]

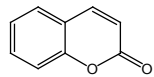
In plants, this group is represented for more than 1.500 different chemical structures present in more than 800 species, distributed in several parts of these plants, such as fruits, flowers, roots, leaves and stalk, [16,13] besides being able to be found also in microrganisms, as fungi and bacteria. [13]

Its great highlight in the literature, besides their alimentary and cosmetic properties, is given for its great medicine value. [17-21] Thus, reports of their biological and pharmacological activities are contained in scientific publications, where are described activities: anti-HIV, [22] hepatoprotective, [23] anti-inflammatory, [24] antiallergic, [19] antimicrobial, [25] antimitotic, [19] antitumor, [26] antioxidant, [27] cardiovascular, [9] antiplatelet, [28] antidepressant, [29] anticoagulant, [30] among others.

In table 1 are show some commercial drugs which have as main active principle a coumarin derivative.

Table 1. Some commercial drugs with a coumarin ring as main active principle.

Commercial Name	Chemical Structure	IUPAC Name	Indications:
Acenocoumarol®		4-hydroxy-3-[1-(4-nitrophenyl)-3-oxobutyl]-2*H*-cromen-2-one.	Treatment and prevention of thrombotic diseases; prevention of cerebral embolism, deep vein thrombosis, pulmonary embolism, thromboembolism in infarction and transient ischemic attack; treatment of deep vein thrombose and myocardial infarction
Warfarin®		4-hydroxy-3-(3-oxo-1-phenylbutyl)-2*H*-cromen-2-one sodium	Treatment of retinal vascular occlusion, pulmonary embolism, cardiomyopathy, atrial fibrillation and palpitation, cerebral embolism, transient cerebral ischemia, arterial embolism and thrombose.
Cantabiline®		4-methyl-2*H*-cromen-2-one	Sympathetic treatment of pain related to dyspeptic disorders.

Varicoss®		2H-1-benzopyran-2-one or 2H-cromen-2-one	Varicose syndromes, varicose veins, hemorrhoids, leg ulcers; phlebitis, thrombophlebitis, periphlebitis, arteritis, lymphangitis; prophylaxis of pre- and postoperative thrombosis and in pregnancy, prophylaxis and treatment of edema.

2. Biosynthesis

The distribution of coumarins is predominant in angiosperms. In the literature, the families most mentioned by the presence of members of this group are: Apiaceae, Rutaceae, Asteraceae, Fabaceae, Oleaceae, Moraceae and Thymeleaceae; possessing a considerable number of the citations also in Amaranthaceae, Cyperaceae, Dipsacaceae, Goodeniaceae, Guttiferae, Leguminosae, Pittosporaceae, Rosaceae, Samydaceae and Solanaceae. [15]

In plant cells the stock of coumarins occurs in the vacuoles in the form of glicosidic *o*-coumarinic acid. Biotic and abiotic stress events are responsible for their transformation through enzymatic hydrolysis and excessive lactonization, being released by the disruption of their cells, [31] therefore, oftentimes, their biosynthesis is tissue-specific and regulated with development, [32] for greater occurrence in areas most involved in the defense mechanism, [33] with important role in the plant physiology and biochemistry. [28]

Biogenetically, the production of coumarin compounds starts by the metabolism of the amino acid *L*-phenylalanine, which by enzymatic deamination of phenylalanine amonialiase (PAL) (Step **A** – Figure 7), making the interface between the primary and the secondary metabolisms in the production these phenolic derivatives. This deamination produces the *trans*-cinnamic acid, which is hydroxylated by cinnamate-4'-hydroxylase (Step **B** – Figure 7), resulting in one of the main precursors of this pathway, the *p*-hydroxy-cinnamic acid (*p*-coumarinic acid).

The *p*-coumarinic acid is hydroxylated in the C-2' position (*orto*-hydroxylation, Step **C** – Figure 7), followed by photocatalytic isomerization of the double bond (isomerization to *E* for *Z* – Step **D** - Figure 7). Spontaneous lactonization of the *Z* isomer (Step **E** – Figure 7) given a single coumarin, 7-hydroxy-coumarin (Umbelliferone) (Figure 7).

Others authors describe that biogenetic derivation of the coumarins occurs from the chiquimic acid route, however, a relevant number of observations indicates that their derivation have origin in a mixed route involving the chiquimic acid and acetate routes (Figure 8). [13,31,34]

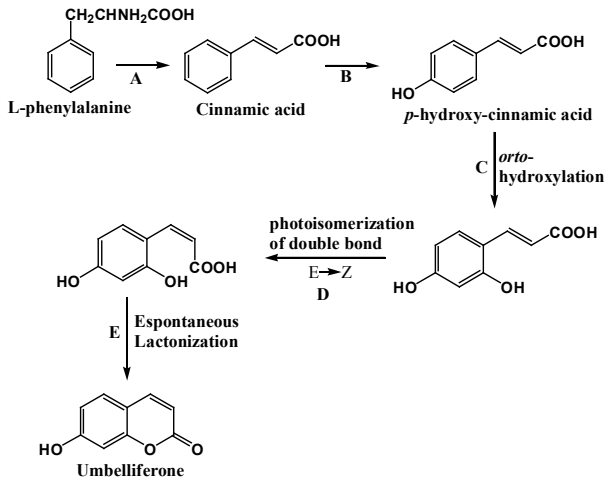


Figure 7. Biosynthetic pathway of coumarins, from *L*-phenylalanine precursor.

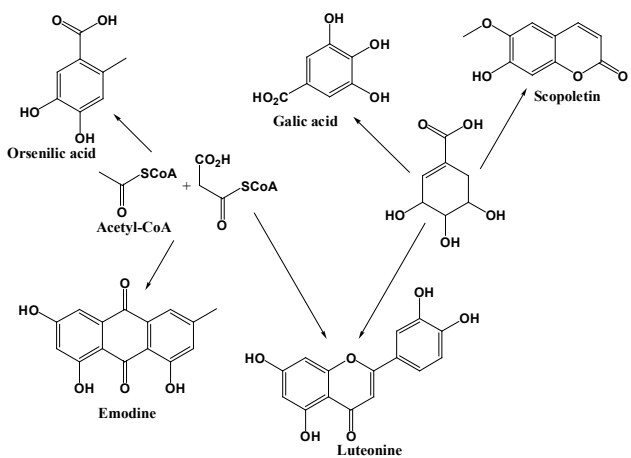


Figure 8. Representation of the mixed pathway of the chiquimic acid and acetate to obtain coumarins.

Following steps of derivation may exist, depending on plant genetic characteristics, which will reflected in the existence of specific routs in the biogenesis of coumarins, and production of their great structural diversity. [31,34,13]

Microrganisms also biosynthetize coumarin components, although there are evidences that their biosynthesis and derivations may occur by different biosynthetic routes, thus generating biochemically distinct analogues. [16] These natural compounds can be also susceptible to modifications in their original structures by chemical transformations and biotransformation. [10,35]

After all, the direct use of natural products, like coumarins, for industrial, pharmaceutic and food uses, in most cases, is not commercially feasible whereas the abundance of these chemical compounds in nature is low, making large-scale production difficult. This situation worsens due of the complex processes of purification involved, with many steps, which lead to losses, further reducing the final yield of the isolated compound. [36]

3. Synthetic Methodologies to Obtain Coumarins Derivatives

Instead to the difficulties found in the isolation of natural products, the synthetic procedures present themselves as an excellent alternative to obtaining natural products and their derivatives, like the coumarins. The synthetic methodologies present a wide range of possibilities (kinds of reactions and reactive conditions) that allow obtaining any chemical structure, for more complex it may seem to be, whether it is previously known or rationally planned.

The organic-synthetic procedures provide fast and efficient methods, low cost, and are shown as important alternatives for obtaining natural products and/or their derivatives, like the coumarins, [37,38] besides allowing the rational design of pharmacologically promising compounds. [39]

The organic synthesis of natural products can be classified in two approaches, the semi-synthesis and the total synthesis: [38]

i. Semi-synthesis: The main chemical structure (scaffold) of the natural products are used as starting material and small chemical modifications are generated in its chemical structure by pontual chemical transformations, affording various derivatives, which can be obtained by reactions of: addition, elimination, substitution, and conversion of substituents or rearrangements, among others (Figure 9);

ii. Total Synthesis: The main scaffold of the natural product is completelly obtained by synthetic procedures (Figure 10). In the definition of the synthetic route to be used, the choice of the starting reagents also allows the placement of several functional groups in different ring positions, generating a big number of derivatives.

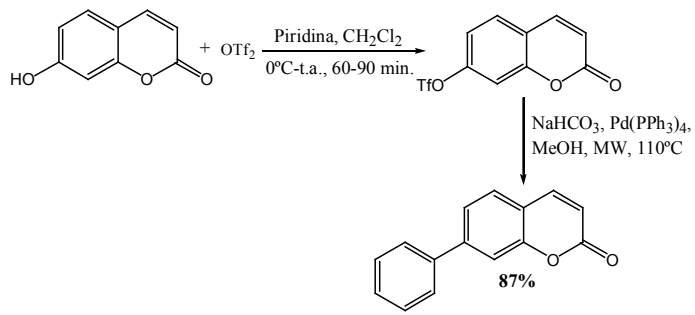


Figure 9. Example of derivatization of coumarins by semi-synthetic procedures.

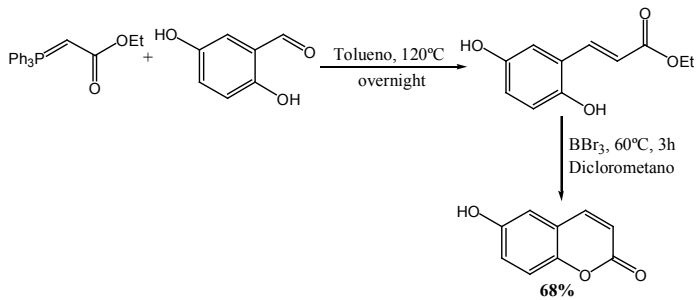


Figure 10. Example of total synthesis of 6-hydroxy-coumarin.

Coumarins are considered privileged structures in the design of novel bioactive compounds, [40,41] because synthetic modifications can be performed in practically all positions of their base core (1,2-benzopyrone), [28] generally often compounds with large biological or therapeutic applications. [15,42]

The interest in the development of synthetic routes to obtain coumarins and derivatives emerged from the 1800s, and actually the main synthetic routes utilized involving: Pechmann reaction, [43-48] Wittig reaction, [49,50] Knoevenagel Condensation, [51-53] and Perkin reactions. [54-56] Recently, methodologies making the use of transition metal-catalyzed cross-coupling reactions has also been highlighted in the formation of coumarin core. [57-60]

3.1 Pechmann Reaction

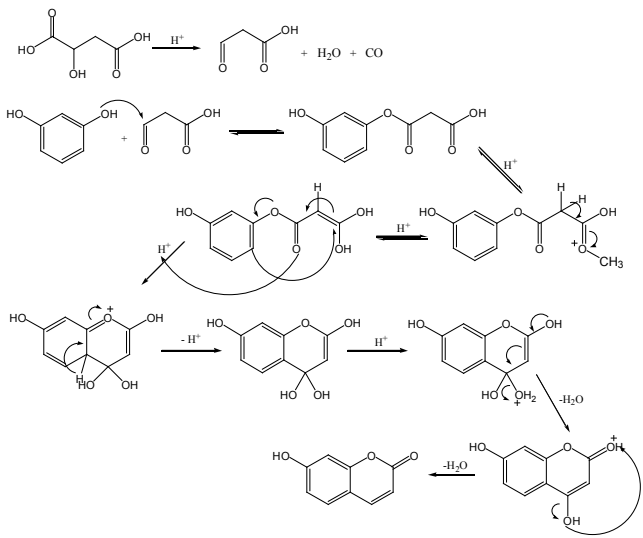
One of the main synthetic methodologies used to formation of coumarin rings is the Pechmann reaction, which utilize simple and inexpensive starting materials

and, generally, provide the desired coumarin in good yields. Their reactional conditions are characterized by the use of acidic medium, generally through the adition of sulfuric acid, but there are also methodologies which utilize aluminum chloride, phosphorus pentoxide, trifluoroacetic acid, among others. [61-64,47]

The greatest problems observed by utilization of the Pechmann reaction are: the need to use acids in large excess, long reaction time and high temperatures, that generally result in the formation of many undesirable sub-products. [47,65]

Synthetically, the coumarin ring, via Pechmann, is obtained through the condensation of malic acid, [43] or β-keto-esters [66] with phenolic compounds, in the presence of excess of a strong acid.

For this condensation to occur, initially the activation of malic acid is necessary. The activation occur through the conversion of malic acid to malonaldehyde acid, with lose of carbon monoxide (CO) and water (H_2O). After this preparation step, the condensation of the aldehyde moiety with the phenol generates an unstable intermediary, which, in acidic medium, will undergo cyclization and H_2O release, for formation of the final coumarin ring (Scheme 1). Depending on the standard substitution of the starting materials, the final coumarin product can present substitutions in their benzene, or heterocyclic (pyrone), or in both moieties. [62]



Scheme 1. Reaction mechanism of the Pechmann reaction to obtain de coumarins

The nature of this reaction depending of three factors: (a) the nature of the starting phenol; (b) the nature of the β-keto esters (or malic acid derivatives) and (c) the condenser agent. [62]

For the phenol, electron donor substituents favor the condensation, such as alkyl, hydroxyl, alkoxyl groups, and, in smallest scale, halogens; unlike electron withdrawing groups, such as nitro, sulfoxide, carboxylic acid, ester, ketone, nitrile and aldehyde, disadvantage. [67]

The standard of substitution of the coumarin ring can be also obtained by this methodology, depending of the starting materials used. For example, the reaction between 3-methylphenol and ethyl 4-chloroacetoacetate, in excess of sulfuric acid, provides the 4,7-disubstituted coumarin, in 90% yield (Figure 11) [68].

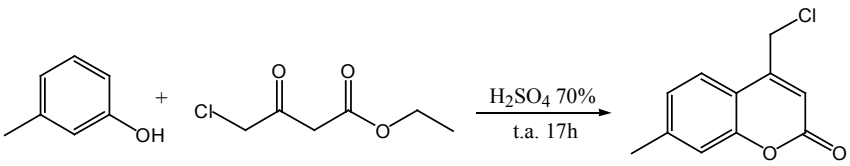


Figure 11. Synthesis of coumarin derivative by Pechmann reaction.

The utilization of others acids, like Lewis acids, is also possible. The use of aluminum chloride ($AlCl_3$) allow the condensation between a phenol-ketone derivative and ethyl acetoacetate, for formation of 5-hydroxy-6-acetyl-4-methylcoumarin in 41% yield (Figure 12). [69]

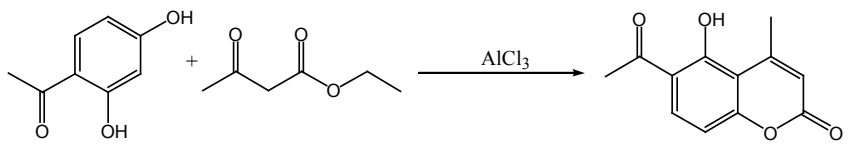


Figure 12. Utilization of Pechmann's reaction in the synthesis of coumarin derivatives.

3.2 Wittig Reaction

Wittig reaction is a second synthetic possibility for the obtainment of synthetic coumarin derivatives. It is characterized as a reaction that occurs between triphenylphosphonic salts and carbonyl compounds, with aromatic aldehydes, generating intermediates alkenes, that after cyclization step induced by Lewis acids, provide the coumarin derivatives. [70,71]

This procedure possess some advantages, when compared to the difficulties of Pechmann reaction, because use milder conditions [70] and alkaline medium. [71] The phosphorans reactants utilized for Wittig reaction own high nucleophilic character, by the formation of carbanions in their structures, which makes them highly reactive at low temperatures, for reaction with others carbonyl groups. [71]

The olefinic triphenylphosphorane possess, due to the existence of ressonance effect, ressonance contributors that transit between the olefinic and ylidium tautomers (Figure 13). Their more reactive form (carbanion), in a first step, reacts with carbonyl compounds, at the same times as the high affinity between the phosphorus and oxygen atoms. The possibility of expansion of the valency of the first, allows the formation of a P-O bond and construction of a four members ring, being a not very stable transition state and, therefore, easily undone to form a novel *trans*-olefinic product and release of triphenylphosphine oxyde. The final step is characterized for a simple cyclization induced by a Lewis acid (Scheme 2). [71]

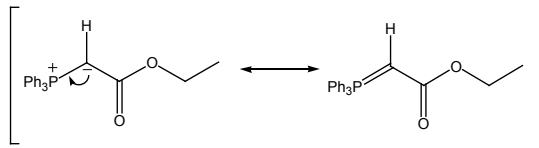
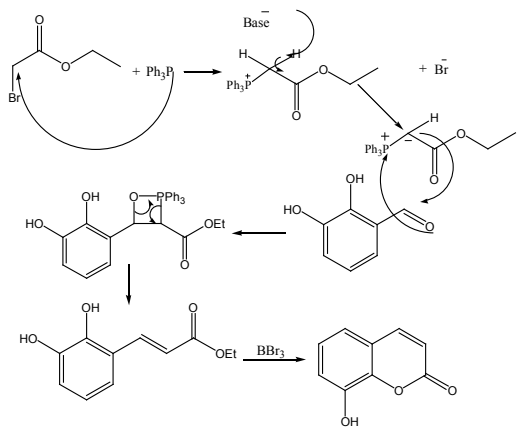


Figure 13. Ressonance contributors of the triphenylphosphorane, in the ylidium (first) and olefinic (second) tautomers.



Scheme 2. Mechanism of Wittig reaction.

The presence of electron withdrawing groups in the aromatic aldehyde, such as nitro and halogen are known for decrease the efficiency of this reaction, being the same, benefited by the presence of the electron donor groups, as alkyl, hydroxyl and methoxyl groups. [72]

The adaptation of this methodology allows the synthesis of natural coumarin derivative Aiapin in good yields, through the condensation between a phosphorus ylide and 4,5-methylenedioxy-2-hydroxybenzaldehyde, in the presence of *N*,*N*-diethylaniline (Figure 14). [73]

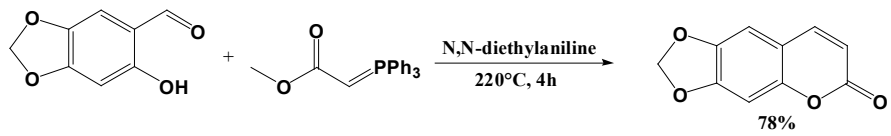


Figure 14. Wittig reaction for synthesis of coumarins.

Coumarin derivatives can also be obtained through the Wittig reaction, athwart of the reaction of methyl-chloroacetate with *o*-hydroxybenzaldehyde, in the presence of triphenylphosphine and sodium methoxide (NaOMe), in reaction medium with ionic liquid [bmim]BF_4, providing the 1,2-benzopyrone in 72% yield (Figure 15). [65]

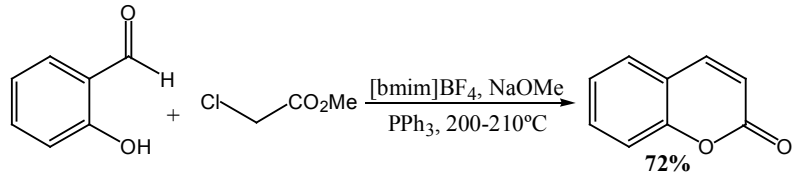


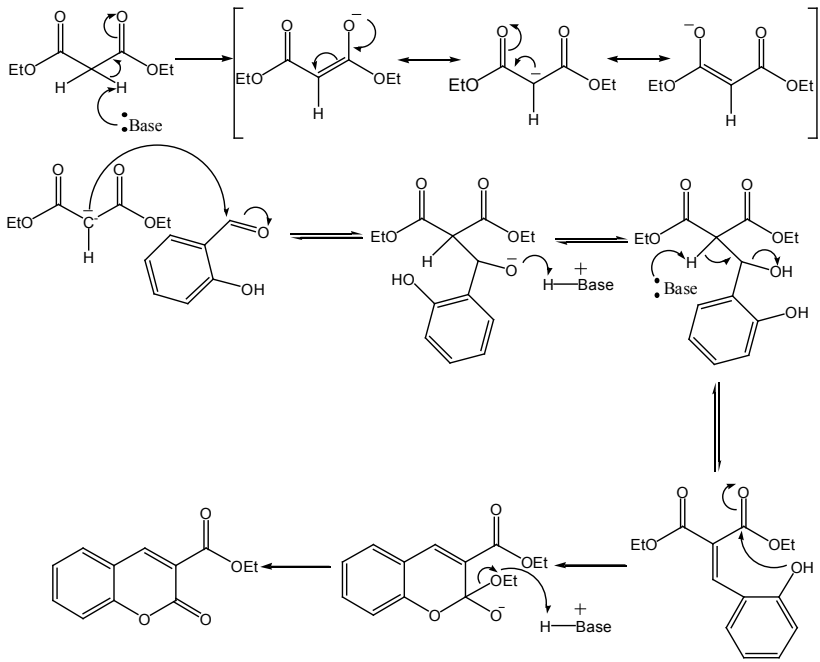
Figure 15. Total synthesis of coumarin ring, via Wittig reaction.

3.3 Knoevenagel condensation

The total synthesis of coumarins via Knoevenagel condensation is possible through the utilization of aldehydes or ketones (generally, hydroxybenzaldehydes for coumarins) and activated methylene compounds, in basic media, for formation of C-C bonds. [65,74,75]

The Knoevenagel reaction involving aldehydes and dicarbonyl compounds, this reaction occurs through an aldol condensation, generating α,β-unsaturated products, which after intra-molecular esterification, allow the desired coumarin. [5]

The Knoevenagel reaction mechanism start by the formation of a carbanion, by withdrawal, through the attack of the base, of an acidic hidrogen of the active methylene group. This carbanion possess high reactivity and reacts with the carbonyl carbon of one aldehyde, allowing the formation of a C-C bond. The presence of the basic media and release of water molecules allows the formation of an alkene intermediate that, after intramolecular cyclization step, provide the final coumarin ring, with substitution pattern depending of the starting products utilized. (Scheme 3).



Scheme 3. Reactional mechanism of the Knoevenagel reaction for coumarin synthesis.

The Knoevenagel reaction can be also utilized in the synhtesis of various coumarin derivatives. For example, from the reaction of *o*-hydroxylated aromatic ketones and monoethyl malonate, in alkaline media and in the utilization of dichloroethane (Figure 16). [76]

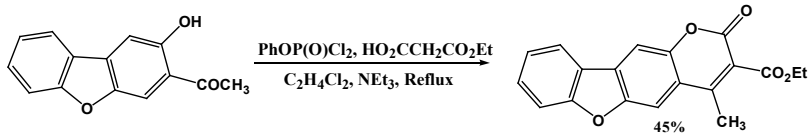


Figure 16. Utilization of Knoevenagel protocol for coumarin synthesis from aromatic ketones.

This methodology can also be used, in aqueous medium, from reaction between *o*-hydroxybenzaldehyde derivatives and active methylene compounds, in alkaline media with the use of phosphorous derivatives [75] (Figure 17).

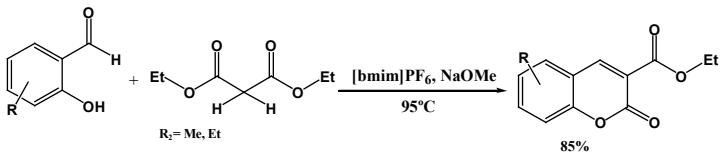
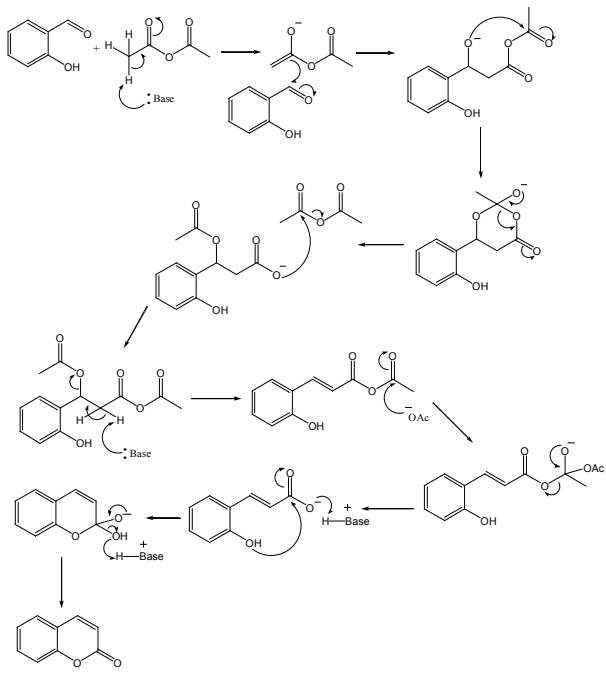


Figure 17. Formation of synthetic coumarins, via Knoevenagel condensation.

3.4 Perkin reaction

The Perkin reaction, described first in 1868 by W. H. Perkin, is characterized by condensation of salicylaldehydes derivatives with carboxylic acids or anhydrides, in alkaline medium, giving coumarins. [5]

The condensation of the aldehyde with the acid (or anhydride) happens due to an addition process, like as an aldol condensation, in the formation of an α,β-unsaturated product, [77] which suffers lactonization for formation of the coumarin ring [78]. For this condensation the use of one base is essential for the deprotonation of an acid hydrogen of anhydride (or acid) in the formation of a double bond and negative charge on your oxygen. This allows that the double bond to attack the electrophilic carbon of the aldehyde carbonyl, in the condensation process. The existence of negative charge in oxygen allow a second attack on carbonyl from anhydride, in the lactonization process. Electrons movement is responsible for open the ring and participate of the reaction of a new anhydride molecule, on which the intermediary formed when suffering with the deprotonation by the base, form a phenolic alkene that, after nucleophilic attack, electronic transfers and action of the conjugate acid of the base (protonated base), allows a new cyclization, generating the coumarin precursor. Their remaining hydroxyl still deprotonates more one conjugate acid molecule of the base, for finishing the lactonic ring and complete the formation of the coumarin (Scheme 4).



Scheme 4. Reactional mechanism of the Perkin reaction for total synthesis of coumarins.

With regard to the chemical reactivity, the substitution pattern of the salicylaldehyde derivatives directly affects the speed of the reaction and the yield of the final product. The presence of electron withdrawing groups contribute for maintaining a high polarity in the aldehyde group, and facilitate the condensation reaction with acid groups or anhydrides, providing generally products in a shorter time and with higher yields. [77]

Salicylaldehyde derivatives refluxed with acetic anhydride, in alkaline medium of triethylamine, via Perkin reaction, can be utilized in the synthesis of coumarin derivatives in good yields (70%) (Figure 18). [80]

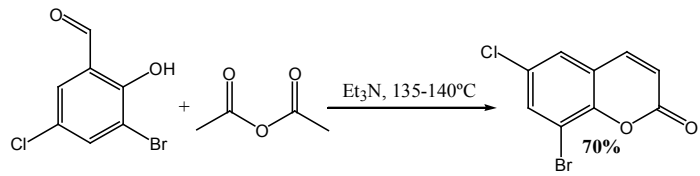


Figure 18. Perkin method for coumarin synthesis.

Methoxy- and hydroxy-coumarin derivatives can be obtained by Perkin reaction through of the reaction between *o*-hydroxybenzaldehydes and arylacetic acids with use of DCC, and DMSO as solvent at 110°C, for a obtaining, firstly, of 3-(methoxy-phenyl)-coumarins, which were utilized for acid hydrolysis and formation of their respective hydroxylated derivatives in 82-92% yields (Figure 19). [79]

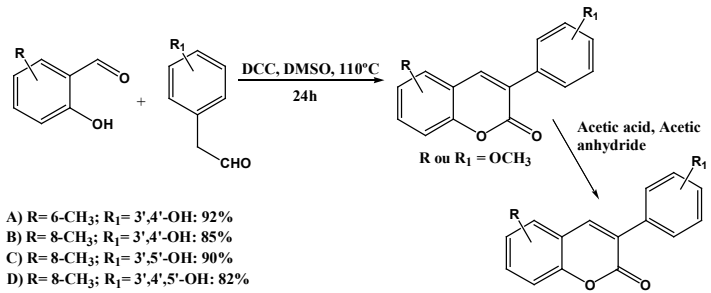


Figure 19. Perkin reaction for obtaining coumarins.

3.5 Transition Metal-Catalyzed Reactions

Among the new synthetic procedures developed as alternatives for total synthesis of coumarins and their derivatives, the transition metal-catalyzed reactions has gained highlights.

Palladium 0 sources (Pd(0)) are described as catalysts in the obtaining coumarins on reactions between *ortho*-halogenated phenols and alkynes, in the presence of carbon monoxide (CO). These reactions are characterized by the use of mild conditions and allow the insertion of several functional groups (Figure 20). [58]

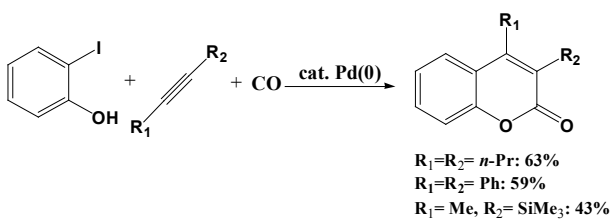


Figure 20. Obtaining coumarins by palladium(0)-catalyzed reactions.

Heck reaction is another alternative for coumarin synthesis using palladium II (Pd(II)) sources. In figure 21 is presented an example of this reaction, that was lead through of the use of palladium II as catalyst in alkaline medium for provide 4,6,8-trisubstituted coumarins in good to moderate yields. [59]

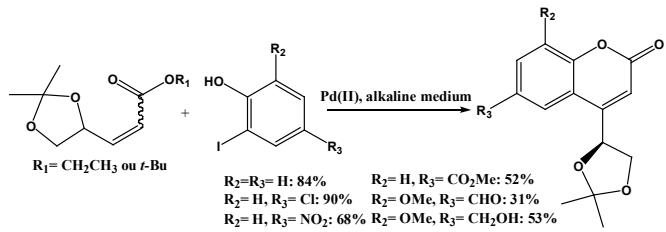


Figure 21. Coumarins obtained via Heck reaction.

3.6 Coumarin Derivatizations

The great diversity of synhetic methodologies that can be utilized to obtain the coumarin ring, allows that, during the synthetic planning a wide range of substituents that can be inserted at the different positions of their main ring, including nitrogen, carboxyl, hydroxyl, halogen groups, among others (Figure 22), enabling the formation of mono- or poly-substituted structures.

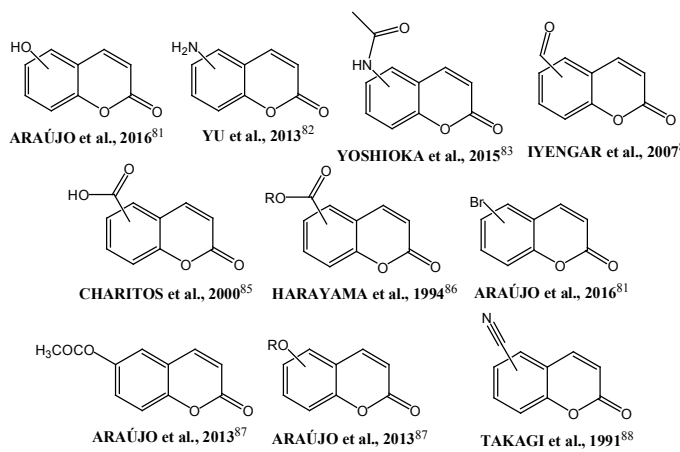


Figure 22. Diversity of substituents in coumarin rings.

Beyond the synthetic possibilities that allow the direct obtainment of polysubstituted coumarins, such as those previously presented, coumarins still allow that aditional substitutions are made in all positions of their main ring, including among others, alkylation, acetylation, nitration, carboxylation, condensation, organometallic-catalyzed coupling reactions, among many others.

Araújo et al. (2013) [87] carried out synthetic derivatization of hydroxycoumarins from alkylation, acetylation and nitration reactions. Conditions for alkylation involved the utilization of different alkyl halides, via substitution reactions, in alkaline medium and in moderate to excellent yields. Substitution reacions have also allowed acetylation procedures, in alkaline medium, and in the utilization of acetic anhydride, allowing derivations in excellent yields; and of nitration, from acid mixture (nitric acid/acetic acid) (Figure 23).

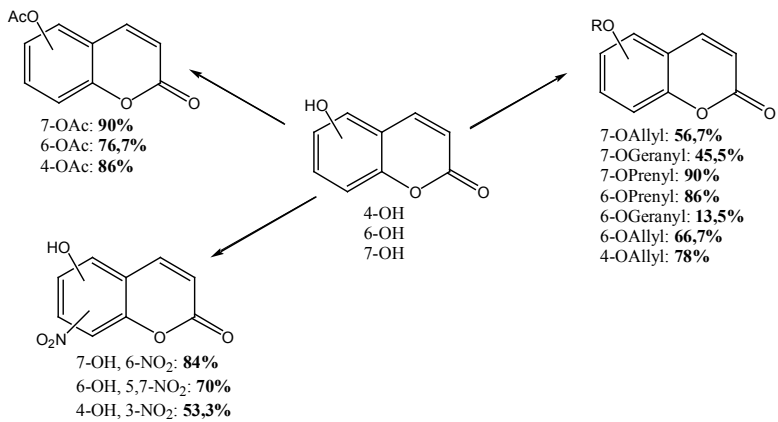


Figure 23. Alkylation, acetylation and nitration reactions in the derivation of coumarin rings.

The derivatization of coumarin derivatives, as well as with others classes of organic molecules, may also envolve the transition between different functional groups with different characteristics.

Coumarins aminated can be converted to nitrile coumarins, which, after acid hydrolysis, may provide carboxylated derivatives, as demonstrated in the Figure 24. [85]

Transition metal-catalyzed cross-coupling reactions have highlighted as new alternatives of synthetic derivation of coumarin rings and formation of a carbon-carbon and/or a carbon-nitrogen bonds [89].

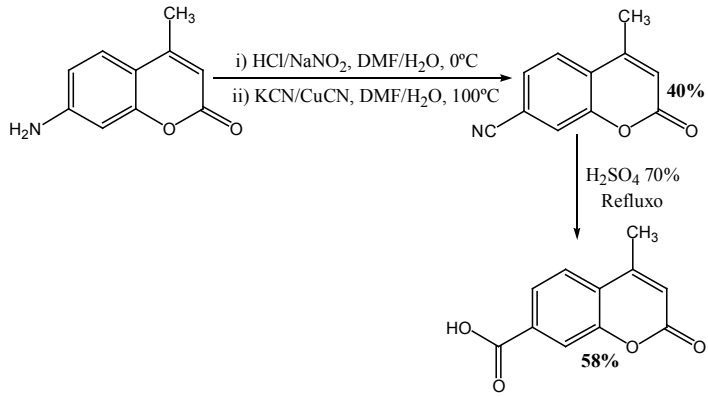


Figure 24. Insertion of carboxylic acid group in coumarin.

Utilization of Suzuki-Miyaura, Negishi and Sonogashira protocols, in the formation of carbon-carbon bonds, has allowed obtaining a variety of coumarin derivatives. The use of triflate-coumarins (or halogenated coumarins) as starting materials, in the utilization of appropriate conditions for each reactional procedure, involving the metal catalysis, providing various substituted coumarins (Figure 25). [81]

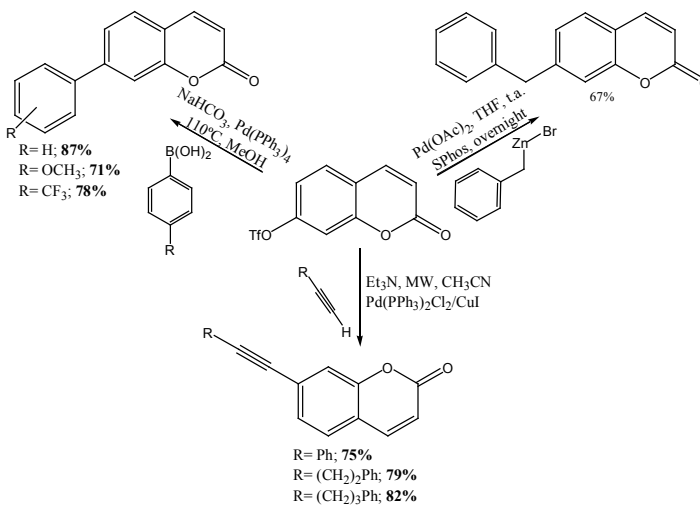


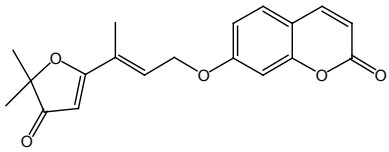
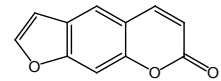
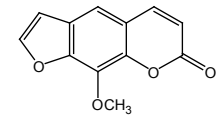
Figure 25. Examples of coumarin derivatizations via transition metal-catalyzed cross-coupling reactions.

4. Synthetic Procedures to Obtaining Natural Coumarins

As already mentioned above, the advantages in the use of the synthetic procedures, when compared to the difficulties in the isolation of natural products, has stimulated the development of synthetic routes for obtaining many natural products, such as coumarins.

Next, are presented one of the possible synthetic routes aiming to obtain three natual coumarins, randomly selected: the Geiparvarin, isolated of the *Geijera parviflora* Lindl [90]; the Psolaren, isolated of the *Psoralea corylifolia*; and the Xanthotoxin, isolated of *Fagara zanthoxyloides*, (Table 2).

Table 2. Chemical structures of the Geiparvarin, Psolaren and Xanthotoxin.

Geiparvarin	**Psoralen**	**Xanthotoxin**
		

Carotti et al. (2002) [91] described a synthetic methodology for obtaining the natural coumarin Geiparvarin, and some of their derivatives. The route start by the reaction between 2,2-dimethyl-ethyl-3-(2*H*)-furanone and 7-(2-oxoethoxy) coumarin, providing an aldol intermediary, which by Stork-Kraus dehydratation give the desired coumarin with a yield superior that 95% (Figure 26).

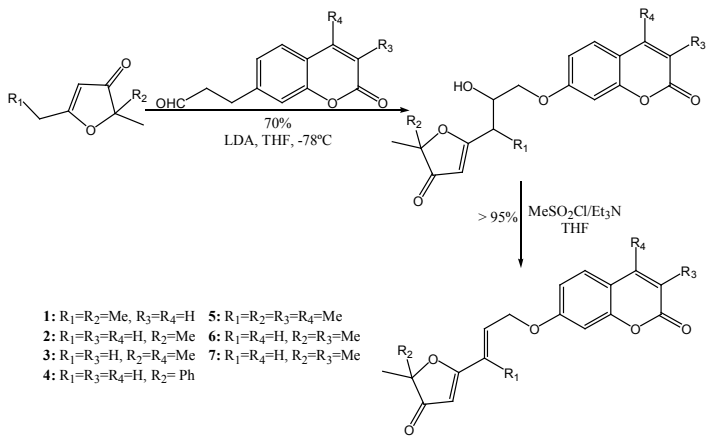
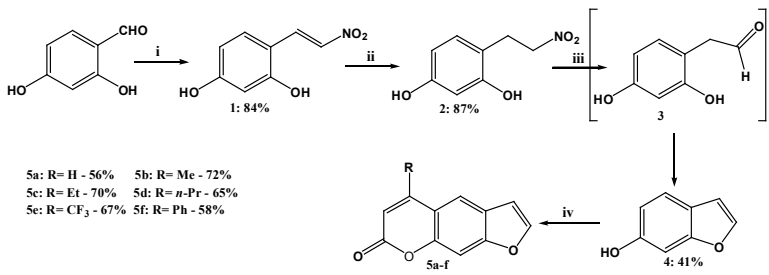


Figure 26. Total synthesis of Geiparvarin.

Psolaren and its derivatives (triciclyc coumarins) were synthesized through of a reactional methodology with more one step, starting by a mixture of 2,4-dihydroxybenzaldehyde and nitromethane stirred at 120°C in the presence of acetic acid (AcOH) and ammonium acetate (NH_4OAc), for provide the first intermediary 5-hydroxy-2-(2-nitroethenyl)phenol, which could be converted in the next intermediary by the treatment with sodium borohydride ($NaBH_4$) in *i*-PrOH-THF at room temperature. Nef reaction allows the conversion of the nitro to one aldehyde group, being, their corresponding intermediary, directly cyclized in the Nef reactional conditions. Reaction of this last intermediary with β-ketoesters derivatives and methanesulfonic acid (CH_3SO_3H) or ethylpropiolate and zinc chloride ($ZnCl_2$), providing the formation of the final furanocoumarins (Figura 27) [92-94].



Reagents and conditions: (**i**) CH_3NO_2, AcOH-NH_4OAc, 120°C, 45 min; (**ii**) $NaBH_4$, *i*-PrOH-THF (1:4), r.t., 40 min; (**iii**) Nef reaction; (**iv**) **5b-f**: *beta*-ketoesters, CH_3SO_3H; **5a**: ethylpropiolate, $ZnCl_2$.

Figura 27. Total synthesis of Psoralen and derivatives.

Xantothoxin (8-methoxypsoralen) have received great attention in the scientific literature, due their photoactive properties [95]. It is a marketed drug know by the names Oxsoralen, Deltasoralen, Uvadex and Meladinine. It is used in the treatment of psoriasis, vitiligo, and cutaneous lymphomas in association with exposition of the skin to UVA light. Herrou (2015) shown the synthesis of xantothoxin starting by an acetylation reaction on benzaldehyde derivative (compound I), for formation of the intermediary II, which is submitted to nitration reaction to obtaining III. This, proceed for formation of the intermediary IV, through a basic hydrolysis procedure, followed by Knoevenagel condensation (compound V) and intramolecular cyclization (compound VI), allowing the obtainment of 7-hydroxy-8-methoxy-coumarin, in 90%.

This coumarin derivative, so, is submitted to a new acetylation protocol, for formation of the intermediary VII, followed by Fries transposition reaction, allows the rearrangement in the formation of the compound VIII, and, finally, cyclization, allowing the formation of final xantothoxin (IX) with 80% yield (Figure 28) [96].

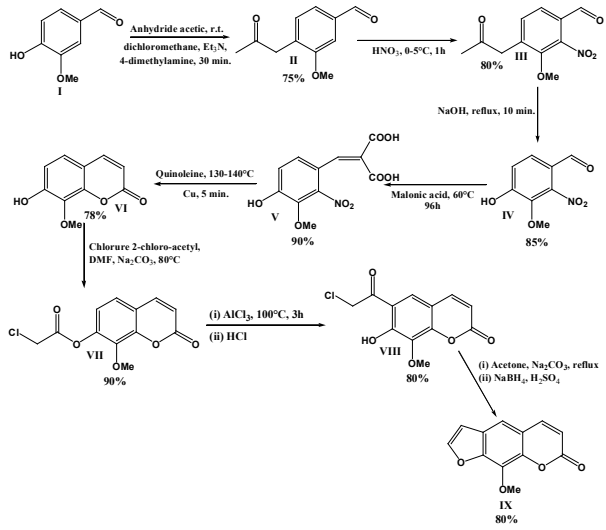


Figure 28. Total synthesis of Xantothoxin.

5. Conclusions

Coumarins are an important class of natural products of great importance, both for the plants and for the microorganism producers, them for their industrial uses as food additives, flavorings and pharmacologically bioactive compounds. Their isolation, from natural sources, often presents some difficulties, due to the impossibility of isolation of large amounts of products, as well as losses during the purification process, responsible for the high cost of many of these products.

In this context, the synthetic approaches were developed aiming, and showing to be efficient in the total and/or partial synthesis of several natural products including coumarins and their derivatives, allowing to obtain them in large amounts and low costs, for their various industrial uses.

In this chapter were presented the main synthetic routes for obtaining coumarins and their derivatives, including the Knoevenagel Condensation, and the Pechmann, Wittig and Perkin reactions, and the use of transition metal-catalyzed for cross-coupling reactions, which allow the production of a wide range of polysubstituted coumarin derivatives in good yields.

Conflicts of Interest

"The authors declare no conflict of interest".

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Chapter 7

5-(*n*-alk(en)yl)-resorcinols as vertile naturally-occurring compounds: an updated overview on their biological and chemical aspects

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1. Introduction

5-(n-alk(en)yl)-resorcinols (AR) are naturally-occurring non-isoprenoid lipids belonging to the group of phenolics, very uncommon and often compared to fatty acids by their uncomplicated chemical structure where the carboxyl group is substituted by a 1,3-dihydroxybenzene ring [1]. Therefore, AR are usually abbreviated as fatty acids usage describing alkyl chain length and degree of unsaturation (e.g., C15:0, C15:1, C15:1(6Z), or C15:2 $\Delta^{6,8}$), whose names are often based on the resorcinol structure (e.g., 5-*n*-pentadecylresorcinol, 1) instead systematic IUPAC nomenclature (e.g., 5-*n*-pentadecylbenzene-1,3-diol) or trivial names (which sometimes cause confusion since 5-*n*-pentadecylresorcinol has as synonims adipostatin A, hydrobilobol, and cardol).

AR are considered amphipathic compounds since the aromatic ring provides them a hydrophilic capacity and the alkyl chain a hydrophobic one and, as a consequence, they have a strong affinity to cellular membranes modifying their structures and properties [2-4], since AR have the capacity to interact to them and to destabilize them or to make structural arrangements in their shape and features [5]. AR are considered lipids since they have an octanol/water partition coefficient (Log PO/W) between 7-10, which varies regarding the length of the alk(en)yl chain existing in the molecule [3].

AR were first identified in species of the Ginkgoaceae family [6]; and *Anacardium occidentale* was recognized as an significant source of alkylresorcinols even for chemical industry applications [1]. AR have also been found in various organisms such as bacteria, fungi, algae and even animals [1]. However, the most common source corresponds to plant organisms and, within these organisms, species belonging to the family Poaceae such as wheat, rye and triticale, register the highest concentrations of these compounds mainly in the fruit pericarp of each kind of plant, so Gramineae family has become a focus of interest for AR

[7]. These compounds have been the issue of several studies because different biological activities are attributed for AR such as antioxidant potential, antifungal capacity and growth inhibition of cancer cells, among others [1,8], and they are potential biomarkers in whole-grain human diets [1].

2. Chemical Features and Biosynthesis of 5-(*n*-alk(en)yl)-resorcinols

AR have a wide range of chemical structures but the moiety variation is dependent on the biological source. However, AR are essentially composed by an aromatic ring, two hydroxyl groups in *meta* position (1,3-substitution) and a saturated or unsaturated aliphatic chain (i.e., 5-*n*-alkyl, 5-alkenyl), sometimes with functional groups at alkyl chain (i.e., 5-oxoalkyl, 5-hydroxyalkylresorcinols), having 5 to 31 carbon atoms (Figure 1) [9]. Some AR have additional alkyl chains attached to the resorcinol moiety (ussually at C2 and/or C4); Other AR have two alk(en)yl chain-connected resorcinol rings (called 5,5'[alkanediyl]diresorcinols or bisalkylresorcinols) and sulfate-derivatized AR also occur particularly in fungi [1].

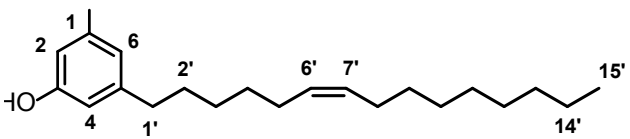


Figure 1. Basic structure of 5-(*n*-alk(en)yl)-resorcinols (AR) (e.g., 5-pentadecen-6(Z)-enylresorcinol, C15:1(6Z) or C15:1 Δ^6)

5-(*n*-alk(en)yl)-resorcinols are secondary metabolites that have been reported in different groups of living beings (animals, plants, bacteria, fungi, among others), as well as involved in numerous biochemical, structural and physiological processes [1]. However, research has mainly focused on their presence in plants and microorganisms, comprising attempts to elucidate the multiple processes and functions they perform. Since a limited number of organisms synthesize them with diverse purposes, there are different moments into the development and life cycles. AR are mainly synthesized in plant organisms with some unsaturation degrees whereas fungi and bacteria mostly synthesize saturated chains-containing AR [1].

AR are phenolic lipids and they are synthesized by biological organisms through the acetate pathway; hence their proximity to fatty acids and polyketides [1]. Biosynthesis process is mediated by the enzyme type-III Polyketide Synthase

(PKS III), involving C2 units derived from malonyl-SCoA or acetyl-CoA. Chain elongation process is provided by several additions of malonyl-SCoA molecules by means of claisen-type condensation. These condensations produce an intermediate compound that produces the cyclic compound 6-alkylresorcinolic acid by and aldol reaction. 6-alkylresorcinolic acid is then decarboxylated originating the final product, 5-*n*-alkylresorcinol. Conversion of these intermediates to AR is catalyzed by a group of enzymes called alkylresorcinol synthases (ARS), a type-III polyketide synthase, which are susceptible to receive various acetyl-CoA fatty acid starters. The synthesis of alkylresorcinols having side chains with more carbons, the route has some modifications, since the 6-alkylresorcinolic acid binds to an acetyl-CoA and produces 6-(2'-oxoalkyl)-resorcinolic acid, which in turn undergoes a reduction and subsequent dehydration forming chain-elongated alkylresorcinols [10, 11].

Biosynthesis of these compounds is mediated by gene regulation, which varies between taxa and species, mainly by the multiple regulators involved in the expression of genes responsible for the synthesis of these compounds. In the bacterium *Azotobacter vinelandii* (a gram negative nitrogen-fixing bacterium present in the soil), the molecular mechanism of AR synthesis has been already described, which. This bacterium uses these phenolic lipids as components of membranes to build cysts, which encapsulate primary cells of this organism on problematic and hard environmental conditions. This process guarantees its survival for long time without losing water and/or nutrients [12].

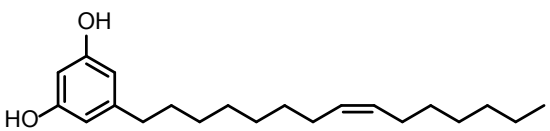
Synthesis of these compounds is therefore triggered by the transcription of the arsABCD operon. This gene cluster is expressed when the Kinase GacS sensor is turned on in response to any environmental stress and, as a consequence, it stimulates the GacA regulator, which encodes those genes responsible for the synthesis of membrane constituents of the cysts including AR [13-16]. On this signaling process by kinases, the genes rpoS (RNA polymerase sigmaS) are firstly activated, and they then encodes sigma factors to be attached to DNA fragments and therefore accelerate the transcription of particular genes needed to begin the formation of the cyst [16, 17]. rpoS regulates the expression of arpR, which is the regulator of the arsABCD operon [15, 18]. arsB and arsC are encoding genes for type-III polyketide synthase (PKS), but arsA and arsD encode for the type-I fatty acid synthase (FAS) [19]. Initially, arsA and arsD synthesize long chains of fatty acids, which are subsequently taken by arsB and arsC. arsB specifically incorporates chains of fatty acids esters and synthesizes AR by aldolic condensations. From the same starting chains through a similar process, arsC synthesizes tri and tetraketides pyrones by lactonization [20-22].

Mechanism has not yet been fully described for plants; however, genes responsible for AR biosynthesis in plants have been already described. These genes

have been found in *Oriza sativa* and *Sorghum bicolor*, calculated within a region of 30 kb on chromosome 5. These genes encode for PKS III enzymes responsible for the synthesis of phenolic lipids [23, 24]. There are several PKS III enzymes, which are classified by cyclization reactions to obtain aromatic rings. Among them, stilbene synthase (STS) uses aldol-type condensations, whereas chalcone synthase (CHS) uses claisen-type condensations [23]. In *Sorghum bicolor*, two genes have been described to codify for two enzymes that synthesize AR. They have been named ARS1 and ARS2. These enzymes mediate the biosynthesis pathway of sorgoleone, which has an AR as its precursor [24]; In contrast, in *Oriza sativa* were found particular genes encoding for enzymes to synthesize ARs, but were named ARSS1 and ARSS2 [25]. These genes have been reported to encode PKS III and comprise a gene family under investigation, since by homology analysis (BLAST) has been demonstrated to have a high similarity percentage. In addition, when performing alignments with sequences from other species, highest homology between sequences was found to those belonging to the Poaceae family [23-25].

3. Sources of 5-(*n*-alk(en)yl)-resorcinols

5-(*n*-alk(en)yl)-resorcinols have been reported in several plants, fungi and bacteria, and only in one animal species. AR were initially extracted from Ginkgoaceae plants, specifically *Ginkgo biloba*. They were mainly found in fruit pulp comprising amounts of 400 mg per kg plant material [6]. The first of these isolated metabolites was 5-*n*-pentadecen-8(Z)-enyl-resorcinol, named bilobol **1**. The structure of AR is almost dependent on the biological source. Thus, majority of AR found in Poaceae plants are saturated carbon chain compounds; other plants produce saturated and/or functionlized carbon chain analogues; AR sulfates are produced by fungi and dialkylresorcinols by bacteria and mosses.



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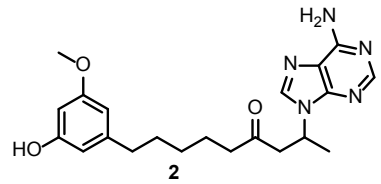
AR are interesting molecules because of structural and biological reasons. Structural differences are mainly due to the alkyl chain length having odd number of carbons - from 5 (1,3-dihydroxy-5-pentylbenzene) to 29 (1,3-dihydroxy-5-nonacosylbenzene) - as well as instaurated chains (1-4 *Z*-configurated double

bonds), oxygenated chains (functional groups at 1 and/or 2 position), and other derivatives by substitutions on hydroxyl groups of resorcinol moiety [26].

Studies on these compounds were very limited during years 2000-2007, but a rise was occurred from 2008 to present. The last review of this topic [1] described most reports are related to higher plants (11 families), as well as 3 bacterial families, 2 algae families, 2 mosses species, 2 fungal families and 1 animal family. This last one is quite particular interesting because it makes reference to a marine sponge (Haliclona genus), so there is a possibly assumption related to its biosynthesis through the same pathway to that of other species. From this review to date, AR have been reported in other several species listed in Table 1. There is something to be highlighted since more detailed studies have been carried out allowing to establish more accurately the presence of compounds not previously reported and also those novel structures such as adeninealkylresorcinol **2**, a compound isolated from the fungal endophyte *Lasiodiplodia sp*., having in its structure a nitrogenous base [27]. The survey is complemented by the report of AR in pseudocereals like *Chenopodium quinoa* [28], and several species from the family Myrsinaceae (see Table 1) like *Oncostemon bojerianum* with the isolation of *bis*-5-alkylresorcinols.



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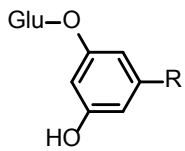
AR have also been reported in some plants belonging to the Fabaceae family such as *Pisum sativum* L, *Ononis pubescens* and *Ononis natrix* [8, 29] and in woody plants such as *Grevillea robusta*, better known as silky oak, from which some AR glucosides **3-8** were isolated [30].



3. R = $CH_2)_2CH{=}CH\ (CH_2)OH$
4. R = $CH_2(CH_2)_3CH_2OH$
5. R = $CH_2(CH_2)_3COOH$
6. R = $CH_2(CH_2)_2CH_2OH$
7. R= $CH_2(CH_2)_4COOH$
8. $CH_2)_4HCOOH$
$_2$

However, the largest reported source of AR is plant organisms specifically related to the Poaceae family (commonly known as grasses), which is integrated

by a large number of species of economic importance worldwide. However, AR have been found mainly in wheat, rye and triticale and in low amounts in products derived from maize, oats and rice [52] especially in bran and intermediate layers of the fruit (hyaline layer, testa and intermediate pericarp), comprising amounts ca. 16000 μg AR per g dry basis. AR are also found in the outer pericarp but in lesser amounts (75-99 μg AR per g dry basis) [7].

Table 1. Additional reports over 2000 year of AR sources in different species

Family	Species	Ref.
	Apatococcus constipatus	[31]
	Merulius incarnatus S	[32]
	Azotobacter sp	[33, 34]
	Pseudomonas sp.	[33]
	Aspergillus sp.	[35]
	Lasiodiplodia sp.	[27]
Tamaricaceae	*Tamarix canariensis*	[36]
Myrsinaceae	*Cybianthus magnus*	[37]
Myrsinaceae	*Oncostemon bojerianum*	[38]
	Ardisia elliptica	[39]
	Embelia schimperi	[40]
	Labisia pumila	[41]
(Proteaceae)	*Grevillea robusta*	[42, 43]
	Mangifera indica L.)	[44-46]
Iridaceae	*Iris sp*	[47]
Asteraceae	*Cichorium spinosum*	[48]
Liliaceae	*Urginea indica L*	[49]
Anacardiaceae	*Lithraea molleoides*	[50]
Fabaceae	*Ononis natrix L*	[51]
	Pisum sativum L	[8]
Amaranthaceae	*Chenopodium quinoa*	[28]

Unrecognized AR have also been detected in some flours, which are the food base in some countries, such as industrially milled wheat flour, which registers 491 μg AR/g as average value, whereas in manually ground flours they have 710 μg AR/g as average value. Taking this into account, from the AR content, it is

possible to determine the bran amount to be contained in different cereal products [53]. This difference between flours is due to the fact that industrial sieves have a small mesh size to avoid pericarp parts of these grains can pass [52]. On these sense, whole rye flour has AR average amounts of 972 μg AR/g whereas refined flours have 90 μg AR/g, rye bran recorded 2758 μg AR/g [54] and triticale bran 0.62-0.86 g AR/100 g plant material [55].

4. Extraction and analyses of 5-(*n*-alk(en)yl)-resorcinols

4.1. Extraction

Since the octanol/water partition coefficient (log P_{OW}) for AR is 7-10 range, they have a very low solubility in water. In addition to their amphipathic nature, they can be extracted using solvents of intermediate polarity such as ethyl acetate, methanol or acetone [56]. Thus, methanol can difficultly extract AR with chains greater than 23 carbons and ethyl acetate has a good extraction efficiency, but not as much as that of acetone [57]. This solvent is recurrently used for these extractions because it is more selective, generates less environmental impact and health compared to other solvents [56, 58].

Extraction by maceration or conventional extraction consists in leaving the biological material in contact to the solvent during some time (1-2 days). In some studies, acetone or ethyl acetate are chosen as conventional solvents at 1:40 ratio for periods between 24 and 48 hours under constant stirring and at temperatures below 24 °C to avoid compound decomposition, followed by evaporation of extraction solvents under reduced pressure [59-61]. This method, although effective and cheap, is not exhaustive, so most of the times not all of the compounds are extracted due to the above-mentioned polarity variation. On this context, it is necessary to consider that the utilization of the AR-rich extract is limited only for analytical purposes depending on the solvent used for maceration due to FDA and EPA regulations. Therefore, other technically-improved methodologies have been used like accelerated solvent extraction (ASE). In the AR case, ethyl acetate, water and propanol have been employed by a semi-automatic technique using a Dionex ASE 200 equipment, at high temperatures (100 °C) and high pressure (> 1000 psi), resulting in rapid (5 min) and more efficient extractions [62]. Another strategy allowing the rapid AR extraction using smaller solvent amounts is the use of ultrasound-assisted protocols, so taking in advantage the cavitation phenomenon, the AR release from biological material is accelerated by tissue rupture and the solubility is also increased by generation of point temperatures up to 2000 ° C [44].

Soxhlet apparatus-mediated extractions have as advantage the use of small amount of solvent required for exhaustive extraction due to the cyclic process, being very useful for analytical purposes. Various solvents such as acetone, ethyl acetate, chloroform, cyclohexane, diethyl ether, and hexane have been employed. Cyclohexane has been found to be a more selective solvent for AR extraction by Soxhlet, allowing the separation of a wide AR variety as well [56, 63].

Supercritical fluid extraction (SFE) is one of the most innovative and emerging methods. A supercritical fluid is a matter state at determined pressure and temperature conditions, where the substance behaves like a gas (it covers the entire space where it is), but it has the density of a liquid (0.1-1 g/mL) and, therefore, it can act as solvent for extractions. The substance often used for SFE is carbon dioxide (CO_2) which acts as a low polarity solvent, similar to hexane. In this method, the plant material is extracted at 304.2 K and 7.38 MPa using CO_2 to behave as supercritical fluid [64]. There is a further consideration to SFE since this method is beneficial to the environment because it does not generate hazardous waste [65] and it has been found that the AR extraction by supercritical fluids with CO_2 provides therefore cleaner extracts [66]. However, when extracted by pure CO_2, not all AR can be obtained, so co-solvent use is usually necessary, such as methanol or ethanol [9]. As an advantage of SFE, step-by-step extractions can be then conducted using variations of temperature (40 ° C - 70 ° C) as a fundamental parameter to increase extraction efficiency, pressure (40-50 MPa) and co-solvent (0.06% -10%) depending on the matrix to be extracted [55, 67, 68]. On comparing the composition of extracts obtained from barley and wheat by SCF with those obtained by maceration using ethyl acetate, there are no significant differences in composition and yield, so in a large-scale process for AR extraction, the recommended methodology to be used is SFE (commercially-available SFE extractors had been already developed), minimizing the use of solvents and environmental impact [69].

4.2 Qualitative and quantitative analyses of 5-(*n*-alk(en)yl)-resorcinols

A qualitative method for detecting AR is thin layer chromatography on silica gel plates (normal phase) using binary systems $CHCl_3$-acetone (95:5-85:15) and $CHCl_3$-methanol (85:15) [59] and alumina using mobile phases composed by methanol-water (90:10-85:15). In both cases, AR are revealed with phosphomolybdic acid, vanillin or Fast Blue B [1]. Solvent system can vary regarding the type of extracted AR, however, it must be a intermediate polarity solvent system due to their lipophilic nature [3]. For quantitative analysis, different techniques have been developed over the years based on spectrophotometry (see Table 2). However, these colorimetric methods are generalized (i.e., focused on total content determinations) and they do not accordingly provide structural information (e.g., related to the differentiation of the carbon chain or double bonds) [70].

High performance liquid chromatography (HPLC) is also a methodology used for the AR analysis, allowing detection of those chromatographic signals corresponding to AR in a clearer way to that of TLC [57]. For the analysis of these compounds by HPLC is preferably performed in reverse phase, since by its amphipathic nature, this method ensures compounds can be quickly separated and recognized at higher resolution [78]. When a C18 reverse phase column is available, it is necessary to use binary phases to ensure separation of these compounds. It has been found that using MeOH:water mixtures (7:3 ratio) and MeOH:IPA (8:2 ratio) in gradient elution gives suitable separations and no AR retention within column might occur. Gradient methods have also been reported using methanol and water as mobile phases [79], allowing detection by UV-Vis of a wide AR variety and their identification can be performed when LC system is coupled to tandem mass spectrometry [57].

Table 2. Spectrofotometric methods for AR quantification

Methods	Observations/considerations	Ref
Fluorimetric method. Reaction between AR with $CHCl_3$ and potassium hydroxide (KOH).	Daily Calibration Curve	[71]
Diazonium salt of sulfanilic acid	Low reagent stability, fresh preparation and rapid color loss (*ca*. 15 minutes).	[72]
Diazonium salt of *p*-nitroaniline	Sensitivity of this method was not comparable with previous ones	[1]
Fast Blue B (FBB®)	Highly specific for AR and excellent sensitivity (1-10 μg AR).	[73, 74]
FBB-$ZnCl_2$	Increased sensitivity of the test in comparison to [73] (0.1 μg AR) and higher stability of the products of reaction (3 h).	[75]
Fast Blue RR ½$ZnCl_2$ (FBRR®).	Reduction of incubation (to 20 minutes) and a microscale method in comparison to [75].	[76]
Sulfanilic acid (*in-situ* diazotized)	Low-cost, reproducible, highly sensitive, stability problems solved [72]	[77]

On the other hand, the analysis by gas chromatography (GC) is conditioned to previous derivatization since the AR can not be directly analyzed by this technique unless longer analysis times and higher temperatures are employed [57]. AR derivatization can be carried out using bis-(trimethylsilyl)-trifluoroacetamide + 1% trimethylchlorosilane derivatizing reagent at 75 °C for 30-60 min [55, 80] or trimethylchlorosilane (TMCS) [81] affording the corresponding trimethylsilyl ethers (TMS) (see Figure 1). Their TMS derivatives detection by GC-MS can be achieved making a filter taking into account as peak base the fragment derived from the McLafferty arrangement (i.e., *m/z* 268) and from the molecular ion is realized the peak annotation and/or putative identification of AR within sample [28, 57, 82, 83]. For the structural determination of AR, the information obtained by mass spectrometry and ^{1}H and ^{13}C nuclear magnetic resonance is conventionally used. In addition, oxidation reactions are also used to determine the position of the double bond in the side chain [1, 84].

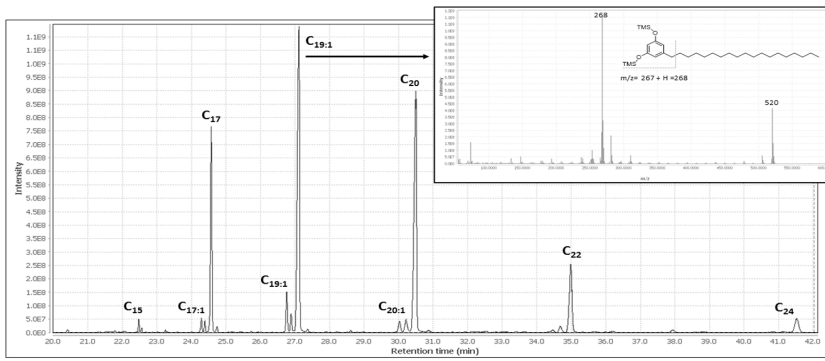


Figure 2. GC/MS chromatogram of an AR-rich mixture obtained from *Triticum secale* seeds derivatized by TMCS.

5. Biological properties and activities of 5-(*n*-alk(en)yl)resorcinols

AR have different biological properties by their chemical structure and amphipathic feature (see Table 3). These compounds mainly have a strong affinity for lipid bilayers and biological membranes [85]. Alkyl group have a great interaction to the bilayer-forming lipids (through apolar-type interactions), causing membranes to be permeabilized by the arrangement occured by this

interaction [1, 80]. This fact was observed in erythrocytes since the permeability increases in membrane water channels because of the interaction to 5-(*n*-alk(en) yl)-resorcinols [2, 86]. However, several studies have shown that coating the liposomes improves the stability and stiffness of the liposomal layer in adequate amounts, but when coated with long chain AR, it loses size and, at high amounts, membrane permeates, losing its functions [3]. Liposomes have advantages because they are vesicles composed by double phospholipid membrane and they are useful for transporting different substrates through the cell or tissues, such as sphingomyelin and cholesterol as most stable substrates. However, their performance can be increased by mixing them to resorcinolic lipids to improve their properties and to be thus able to transport solutes at greater efficiency [87]. Its amphipathic and cell membrane penetration capacity varies regarding the length of the AR side chain [5]. In addition, AR produced by the *Azotobacter* genus interact to bacterial phospholipids by structurally bilayers modification and it has been evidenced that they suppress mitochondrial respiration in the presence of NAD-dependent substrates [34, 88].

Its antioxidant potential can be attributed to the two hydroxyl groups since they can donate their hydrogens. In this way, AR can reduce free radicals existing in the environment, which are generated by naturally-occurring oxidative reactions into a biological systems [80, 89]. *In vitro* tests show that AR nature have an antioxidant potential by inhibiting copper-mediated oxidation of human LDL [90], but this antioxidant potential is influenced by the alkyl chain length [1]. Antioxidant potential of AR-rich extracts from wheat can change depending on genotype and environment where the wheat crop is established [65, 91]. However, other studies call into a question this potential regarding radical scavenging in comparison to other compounds such as α-tocopherol [92].

Table 3. Biological activity reported for AR isolated from different sources.

Activity		Source	Ref
Antioxidant	DPPH	5-*n*-pentadecilresorcinol	[82]
	FRAP – DPPH Inhibition of LDL	5-*n*-alkylresorcinols ($C_{15:0}$, $C_{17:0}$, $C_{19:0}$, $C_{21:0}$, and $C_{23:0}$)	[90]
	DPPH - CL	Rye bran alkylresorcinols ($C_{15:0}$–$C_{25:0}$)	[89]

Antifungal	*Aspergillus niger and Penicillium crysogenum.*	*Hordeum vulgare*	[1]
	Fusarium graminearum, F. culmorum, F. avenaceum and F. poae	Wheat seeds	[93]
	Rihizoctonia solani and Rhizoctonia ceralis	Rye grain	[94]
Cytotoxic	A2780 ovarian cancer cell line	*Oncostemon bojerianum*	[38]
	Breast carcinoma (MCF-7), lung carcinoma (NCI-H460), and central nervous system carcinoma (SF-268) cell lines.	*Grevillea robusta leaves*	[42]
	Breast (MCF-7), colon (HCT-116) and prostate (PC-3)	*Labisia pumila Leaves*	[41]
	colon cancer cell lines (HCT-116 and HT-29)	Wheat bran	[95]
Nematicide	*Caenorhabditis elegans* and the infective larvae of *Trichostrongylus colubriformis*	*Lithraea molleoides.*	[50]
PAF	PAF antagonism	Ardisia elliptica leaves	[39]
Antimutagenic		Salvado de centeno	[96]
Effects on DNA	Rompimiento del DNA	*Hakea trifurcata*	[97]
	cell line HT29 colon cancer	5-n-Alkylresorcinols (C15:0, C17:0, C19:0, C21:0, and C23:0)	[98]
Bactericide	*Escherichia coli*	Rice seedlings	[99]
	Staphylococcus aureus	*Merulius incarnatus*	[32]
Antiparasite	*Leishmania sp*	*Merulius incarnatus*	[32]

AR are also believed to be agents with potential for inhibition of cell growth and fungal spores germination [1], which is useful on protecting seeds since they are only found to be mainly accumulated in bran or hyaline layer [100]. As antimutagenic agents, several studies demonstrated the *in vitro* mutagenicity to be reduced when lymphocytes are exposed to direct mutagens such as daunorubicin and methyl methanesulphonate [96]. Concerning AR as antifungal agents, some studies have been conducted against *Fusarium culmorum*, exhibiting an inhibitory effect against mycelial growth at 400 mg/mL. This effect was also reported

against *Rihizoctonia solani* and *Rhizoctonia ceralis* at 100 mg/mL [8]. Recently, a study explored the activity of AR-depurated fractions of grain flour of barley against *Fusarium oxysporum*, exhibiting ED_{50} values between 3.3-24.0 μg/mL [106]. Unsaturated and lower-chain AR have been found to be 2-4 times more active than those saturated and higher chain-containing AR.

On the other hand, several studies had demonstrated AR analogues can interact to several enzymes and these interactions are especially interesting [1]. In Table 4 is exposed the different studied enzymes interacting to AR.

Table 4. Different AR-interacting enzymes.

Enzymes	Observations	Ref
Ca^{2+}-calmodulin ATPase	Cereal-derived AR stimulate this enzyme. Weak inhibition was detected at higher concentrations.	[107]
Acetylcholinesterase	Cereal-derived AR inhibit this enzyme at 1890 mM. Effect depends on the alkyl chain length.	[107]
Prostaglandin H_2-synthetase	Short alkyl chain AR (C1-C3) are potent inhibitors of this enzyme.	[108]
Glycerol-3-phosphate dehydrogenase	Long alkyl chain AR present in whole grains and effectively inhibit this enzyme and prevent the accumulation of triacylglycerol. C21:0 is the strongest inhibitor	[109, 110]
Lipoxygenase-1	Cashew nut shell oil compounds were found not to be an inhibitor but cereal-derived AR showed inhibiting activity. Most efficient inhibitor was C23:0.	[111, 112]
Neuraminidase	C15:0 disulfate is an inhibitor from *Streptomyces*.	[113]

AR are consumed by humans at a rate of 40 mg per cereal serving and, due to its characteristics, it has been mentioned that by interaction to proteins and biological membranes, AR can alter their structure and function, affecting some biological processes [101], but recent studies have shown AR intake is unrelated to any disease and they are considered safe for human consumption [46, 100, 102, 103]. On the other hand, it has been found the consumption of AR-rich foods is related to the decrease of blood glucose levels [104] and favors the excretion of cholesterol in mice [105]. AR are absorbed and at least partially metabolized, and due to their high contents in grass products they are currently under further investigations as biomarkers of whole grain intake.

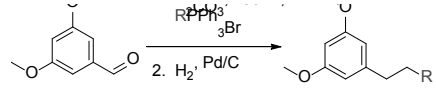
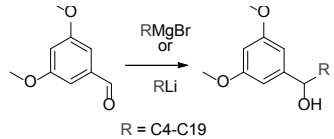
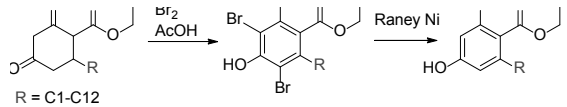
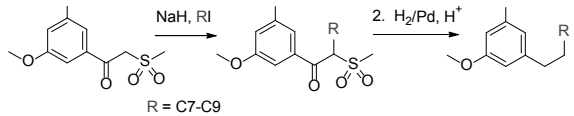
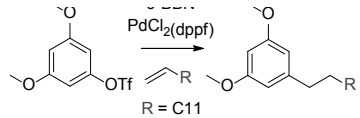
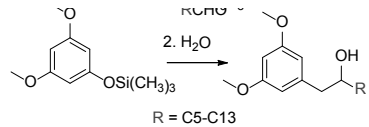
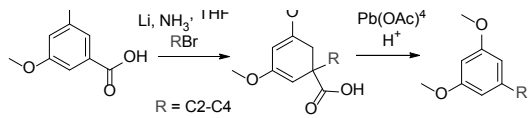
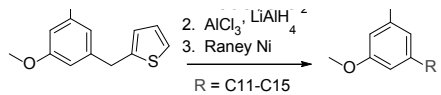
6. Synthesis of 5-(*n*-alk(en)yl)resorcinols

Some synthetic approaches/strategies for AR synthesis have been published since an efficient preparation method is continuously needed for further analytical, metabolic, biosynthetic, bioactivity or pharmacology studies. The main concern for the AR synthesis (for short or long-chain AR) is the formation of C–C bond between the aromatic ring and the alkyl chain and but also depending on the desired chain length [114]. Hence, Grignard or alkyllithium reactions have been commonly exploited to prepare AR analogues starting from 3,5-dimethoxybenzaldehyde, but those reported multistep syntheses involve techniques that are time-consuming, require an inert atmosphere and give highly variable overall yields. On this context, several reactions have been previously reported for AR syntheses and they are listed in Table 5 as a survey of the reported strategies.

7. Conclusions

AR are the group of natural phenolic lipids more extensively studied due to their chemical and biological aspects as well as the linkage among biology, biochemistry, and chemistry, making these metabolites versatile compounds to be exploited in different fields and purposes. Such properties and possible functions have located AR to be the main subject of several researchers worldwide, to the aim of describing and/or establishing their functionality and use at different levels. Hence, the information about the biosynthesis on plants, fungi and bacteria has involved important advances during the last years but it still requires further studies for creating a detailed picture of such molecular events. Meanwhile, more AR sources had been described and discovered, but Poaceae and Anacrdiaceae families still remain the most important ones. Moreover, in addition to the use of these compounds as starting materials in the semisynthesis of several bioactive compounds, several strategies have been developed to suitable synthesis based on the C-C bond between alkyl chain and aromatic ring. These syntheses are continuously required for further studies on analytical, metabolic, biosynthetic, bioactivity or pharmacology aims. Thus, interdisciplinary investigations are then required for depicting the versatility of these natural compounds from their contents of novel/known sources to practical applications.

Table 5. Reported multistep AR syntheses using different strategies.

Strategy (overall yield range)	Key Step for the Multistep Synthesis	Ref
Wittig reaction (65-81%)		[114]
Grignard and alkyllithium reactions (18-59%)		[115-117]
Cyclohexane aromatization (61-66%)		[118]
β-keto Sulfone-mediated condensation (45-60%)		[119]
Suzuki coupling (>90%)		[97]
Lithium-mediated condensation of TMS derivatives (40-50%)		[120]
Reductive alkylation/ oxidative decarboxylation		[121]
Substitution on thiophene derivatives (26-35%)		[122]

List of abbreviations

AR	5-(*n*-alk(en)yl)-resorcinols
BBN	Borabicyclononane
GC/MS	Gas Chromatography Mass Spectrometry
HPLC	High performance Liquid Chromatography
LC/MS	Liquid Chromatography coupled to Mass Spectrometry
NMR	Nuclear Magnetic Resonance
PKS	Polyketide synthase
SFE	Supercritical Fluids Extraction
THF	Tetrahydrofurane
TMCS	trimethylchlorosilane
TMS	trimethylsilane

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Conflicts of Interest

The authors declare that they have no conflict of interest. This article does not contain any studies with human or animal subjects.

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Chapter 8

Essential oil vapors as alternative strategy for new antimicrobial agents

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1. Introduction

Humans have been using aromatic plants since ancient times for their preservative, flavoring and medicinal properties. Their use is recorded by the ancient Egyptians, Chinese and Ayurveda therapy for treating diseases, in rituals and religious ceremonies. Aromatic oils and resins, such as cedar and myrrh, were used in the Egyptian mummification process. The Chinese traditional medicine describes the use of several aromatic substances that are still used by eastern medical therapy today. The traditional Indian medicine Ayurveda also has a history of incorporating aromatic oils and resins into their preparations, listing more than 700 substances like cinnamon, ginger, myrrh and sandalwood. Hippocrates, known as the 'father of medicine', strongly believed in the medicinal benefits of fumigation with aromatic oils, even using this practice to combat the bubonic plague in Athens [1,2].

During the Black Death (1347-1351) in Europe, there was a growth in usage of aromatic products because the plague was believed to be caught by breathing foul air (miasmatic theory of disease). People used to carry small flower bouquets and breathed through nosegays filled with aromatic herbs, which were a distinctive part of the Plague Doctors costume. Another tools used in defense against Black Death were to wash the death bodies with water and enveloping

them in incense smoke, also important people walked behind torch bearers, known as plague torches that burned fragrant herbs [3].

The antimicrobial properties of aromatic plants are very often attributed to essential oils (EOs), which are a complex mixture of volatile lipophilic compounds, *i.e.* like oils, with heterogeneous chemical structures. The International Organization for Standardization (ISO) in their Vocabulary of Natural Materials (ISO/D1S9235.2) [4] defines an essential oil as a product made by distillation with either water or steam or by mechanical processing of citrus rinds or by dry distillation of natural materials.

These metabolites are commonly found in the leaves, twigs, wood pulp or bark tissue of higher plants, but also widely found in bryophytes, such as the liverworts. In principle, all plants can produce these volatile compounds, however quite often they are only found in trace amounts. Among the worldwide known plant species (estimated at 220,000–450,000 [5]) only a small fraction, about 2000 plant species distributed in about 60 families, produce these volatile oils [6]. Moreover, from these plants only a few have been investigated for the presence of antimicrobial compounds [7,8].

The so called "aromatic plants" or "essential oil plants" are those plant species that can deliver an essential oil of commercial interest. These species are mostly found in Lauraceae, Myrtaceae, Rutaceae, Asteraceae, Rosaceae, Pinaceae, Apiaceae, Myristicaceae, Lamiaceae and Santalaceae. Essential oils are relatively rare in monocotyledons, they can be found in Poaceae (especially in the genus *Cymbopogon* and *Vetiveria*), in Zingiberaceae, for example in *Alpinia* and *Curcuma* species, and in Acoraceae, typically in *Acourus calamus* L. [9].

In nature, essential oils play an important role in the plant physiology and ecology, presenting several protective effects [10,11]. Concerning their medicinal effects, the complex chemical composition of a volatile oil turns it difficult to relate their pharmacological activity with the isolated substances. Generally, the action attributed to an isolated compound may not be accurate due to possible interactions that may occur between the different oil components. Based on the historical use of essential oils, the antimicrobial action of these compounds is one of the most verified activity, it has been assayed against a variety of pathogenic bacteria, including certain antibiotic resistant strains. Some volatile oils are also active against lower fungi responsible for mycoses and yeasts [2,12].

Infectious diseases are the major cause of morbidity and mortality killing more people worldwide than any other single death cause. The emergence and widespread of antimicrobial resistant and cross-resistant of microorganisms, proposes that efficient antimicrobial products are required to completely inhibit and kill bacteria. Antimicrobial resistance is amplified when bacteria form biofilms. It is a natural tendency of microorganisms to attach to biotic or abiotic surfaces,

to multiply and to embed themselves in a slimy matrix, resulting in biofilms. Biofilms are the leading example of physiological adaptation and are one of the most important sources of bacterial resistance to antimicrobials. It is now recognized that most bacterial-associated infections, including endocarditis, dental caries, middle ear infections, osteomyelitis, medical device-related infections and chronic lung infections in cystic fibrosis patients are problematic because of biofilms. Bacteria in biofilms demonstrate intrinsic resistance to antimicrobial stress more effectively than the planktonic counterparts. Antimicrobial concentrations necessary to inhibit bacterial biofilms can be up to 10-1000 times higher than those needed to inhibit the same bacteria grown planktonically [13]. In parallel with the increasing problem of resistance, there is a conspicuous lack of development of new antibacterial products. In fact, traditional methods of antibiotic discovery have failed to keep pace with the evolution of bacterial resistance to antimicrobial therapy.

There is growing evidence that essential oils in vapor phase are effective antimicrobial systems. Essential oils in vapor phase have the advantage that they can treat large areas without requiring direct contact with surfaces. This can make them suitable for use as disinfectant of rooms and as air decontaminants [14]. Furthermore, these vapors also represent an interesting alternative due to emerging resistance of microorganisms against synthetic agents, including methicillin resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas fluorescens* [15,16]. There are already several studies confirming that vapor phases of essential oils are more effective as antimicrobials than their liquid phases [16-19].

One of the earliest reports of antimicrobial activity was at the end of the XIX century that only was continued in 1960 [20], but up to date there is no standard screening assay accepted. Different methods have been used by different investigators, but none has good characteristics for high throughput screening of large sample quantities. In this chapter, we discuss the current knowledge concerning the methods employed to assess the antimicrobial activity of essential oils in the vapor phase and their advantages, also we present the most significant achievements in this field to date.

2. Methods used to assess vapor phase antimicrobial activity of essential oils

The evaluation of the vapor phase antimicrobial activity consists of exposing the microorganisms to an atmosphere rich in essential oil vapors and verifying their growth and, when possible, determining the values for the minimum inhibitory concentration (MIC). The exposure to the oil vapors is made using basically two methods: vapor diffusion and the adapted disc diffusion method. As there is no official or standard method, these two methods are used indiscriminately and, depending on the objective, screening or the MIC determination, there are still some variations possible for each of them.

The first assays to evaluate the essential oil vapors on microbes were in liquid medium by allowing the direct contact through sealed glass tubes [21]. Later, in the decades 1950-1960, it was developed the adapted disc diffusion method, consisting of a paper disc impregnated with the oil that was placed on the lid of the Petri dish which is then inverted and incubated. The results were determined by measuring the inhibition zones [20]. The main feature of this method is the direct contact between the inoculated medium and the oil vapors, sometimes it is also called inverted plate method, vapor-agar contact method or micro atmosphere method [22]. Although several adaptations appeared in the last decades, like the vapor dispersion, open plates, inoculum type, etc. [23-27], they are all based on the same principle (**Figure 1**).

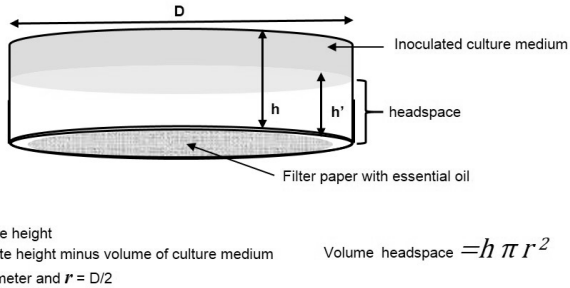

Figure 1. Schematic representation of the inverted plate method model

In this method, the vapor dispersion can be obtained through an impregnated filter paper or small flasks containing the liquid oil. The paper disc is preferred to the flask because of the volatilization speed [25,27]. Paper size is also important for the atmosphere homogeneity, but the evaporation process occurs gradually and the vapor composition may vary because the oil components have different vapor pressures [28]. Using paper discs that cover the whole dish lid allows the distribution of the essential oil on the total agar surface. However, the oil needs to be solubilized in ethyl acetate to ensure a homogeneous impregnation of the disc. The oil solution is applied always with the same volume, but with different concentrations, which allows the MIC determination. In such cases, one should wait 1 min before closing the dishes to avoid the effect of the solvent on the microorganism growth [29]. Besides the MIC, by aerating the dishes, where there was 100% inhibition, to eliminate the oil vapors and re-incubation, it is possible to determine if the action was only of growth inhibition (bacteriostatic/fungistatic) or lethal (bactericidal/fungicide) with no need of re-inoculation [23]. In most cases, MIC results are obtained by visual observation of growth absence

or formation of inhibition zones, as result of the inoculum density of 10^4 e 10^5 colony forming units (CFU) per dish, which will result in uniform microbial film upon the whole medium surface. However, if the microbial density is reduced up to 3 X 10^2 CFU, the number of colonies can be easily counted after the incubation, giving a better measurement of the oil vapor potency [23].

Recently, some adaptations were proposed to optimize the screening time of this methodology. The first modification used a four sectioned Petri dish, allowing to test one essential oil against different microorganisms at the time. The authors claimed the variation provided a more uniform headspace and reduced material and labor costs [30,31]. In this variation, only 6 Petri dishes are needed to test one essential oil in 6 concentrations against four microorganisms while 24 dishes are needed for the same experiment with the classic method.

Another modification to increase the assay performance developed a sealed experimental apparatus for simultaneous evaluation of vapor phase antimicrobial activities of essential oils at different concentrations without creating gradients. The microbial growth was observe using a differential medium in which there was a color change due to the metabolism of glucose-fermenting microorganisms. The inverted plate principle was the same, but this new container consisted of two sections, an upper chamber or lid with seven wells, each containing a solid medium inoculated with the targeted microorganism, and a lower chamber also with seven wells were the essential oils vapor were generated from an impregnated with the oil solution in the desired concentration. These two sections fit hermetically together forming a 1 mL headspace chamber The MIC is considered the lowest concentration of oil that did not cause a change in the medium color, and the lethal concentration can be obtained by sampling those with color change, re-inoculation in fresh medium and incubation [32]. The main advantages are the low amounts of essential oil needed for the assay as well as those from culture medium, and this apparatus allows to test up to 5 concentrations of an essential oil at the same time. However, as the microbial growth occurs in an air-tight environment the growth of aerobic bacteria can be affected.

The variation called vapor-agar contact method was developed to mimic a practical approach for controlling fungi in storage facilities for food products. In this method, the volatilization is carried out in an air-tight vessel of larger dimensions and the open inoculated Petri dishes placed into this vessel. The volatiles are dispersed either by an impregnated paper disc or a small vial placed at the side of the dishes [26]. Depending on the headspace dimensions, the saturation time will vary, requiring a good homogenization of the atmosphere for obtaining reproducible results (Figure 2).

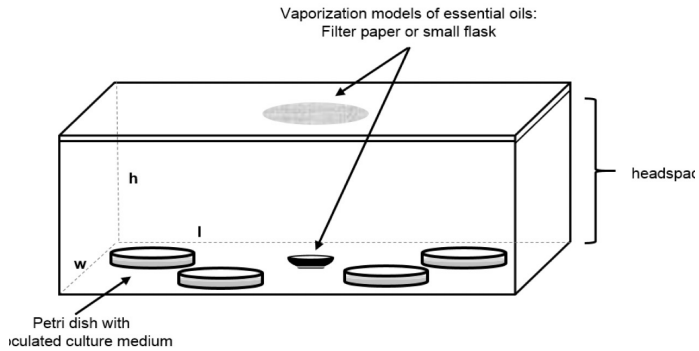

Figure 2. Schematic representation of the vapor diffusion model

The vapor diffusion method is like this last variation. It uses a closed vessel, with volumes ranging from 1 to 9 liters, filled with the vapors but the Petri dishes are placed closed in its interior. The mass or volume of the essential oil is known, which allows to calculate the essential oil concentrations, expressed in oil mass or volume per unit of the container volume. The microbial inoculum can be made by adding a cut of the culture medium (plug), with desired dimensions from a previously cultured plate with the test microorganism, in the center of the dish to be assayed. Alternatively, the inoculum can be made by pour plate or spread plate methods with a microbial density of 10^3 to 10^6 CFU per plate. Afterwards, the plates are closed and inserted into the container containing the essential oil. However, the dish arrangement, side by side, stacked or overlapping, will influence the gas exchange between the container atmosphere and that of the dish headspace [16,29,33,34]. Gas exchange and atmosphere homogeneity can be promoted by introducing a magnetic bar acting as a propeller, causing an internal atmosphere currents in the vessel which increases the homogenization rate of the gas phases (vessel and dish headspace). The oil vapor concentrations and composition can be sampled with air tight syringe from a sealed hole (septum) in the vessel top [28]. However, even with such care, there is no way to assess whether the dish internal headspace is the same as the vessel atmosphere, or even if the equalization moment between the gas phases occurs. The homogenization of the internal atmosphere of the dish is an important factor to be considered, since the incubation process take from 18 to 24 h for bacteria and 48 to 72 h for fungi.

The vapor dispersion method is another factor to be accounted for the test end results. If the essential oil vapors are generated by a small vial containing the essential oil inside the test vessel, the evaporation is slow, taking longer to saturate the internal atmosphere with the oil, but when they are generated by an

oil impregnated filter paper, the evaporation is faster due to the larger contact surface. For filter papers with diameters larger than 8 cm, it is recommended to dilute the volatile oils in ethyl acetate to ensure a homogeneous distribution over the whole surface. These precautions with volatilization are important because the slow rate of evaporation results in MIC values much higher than those obtained with rapid evaporation [29].

The diffusion vapor methodology is very suitable for screening studies because it allows testing various microorganisms at the same time, defining their different susceptibilities to a determined essential oil. However, with the use of larger vessels with more incubating dishes, the results may not be reproducible for each Petri dish.

As essential oils are a complex mixture of compounds with different physicochemical properties, it is important to analyze the actual composition of the headspace during the experiment. In the vapor diffusion method, this is easily by placing a sampling septum in the vapor container [28], but in the disc diffusion this task is more complicated. In order to analyze the vapor composition in a Petri dish headspace, a special dish was elaborated with a side septum, allowing the introduction of a SPME fiber to sample the atmosphere [27]. However, it is important to keep in mind that composition of the fresh liquid oils might be different from that obtained in the vapor phase. In one study with *Mentha piperita* L. the liquid essential oil was composed by 27.0% monoterpene hydrocarbons and 63.3% oxygenated monoterpenes, while the vapor phase analysis the respective compositions for both classes were 62.8 and 29.3% [25]. These findings might be accounted for the higher volatile of the monoterpene hydrocarbons. Besides the proportion differences, chemical conversions are also expected. There is a proposition that some monoterpenes can be converted in other compounds by polymerization, dehydrogenation and/or oxidation, such as limonene into *p*-cymene [35]. Analyzing the vapor phase composition of *Hesperozygis myrtoides* (A.St.-Hil. ex Benth.) Epling essential oil, it contained menthone, a compound not previously found in the liquid oil. As the concentration of limonene was lower in the vapor phase than in the liquid oil, menthone could be originated in the vapor phase as the product of an oxidative process from limonene [23].

Both methods, vapor diffusion and adapted disc diffusion, meet the objective of assessing the most effective oils against Gram positive and negative bacteria, fungi and yeasts. However, in all of them the atmosphere composition directly in contact with the microorganism may vary, as discussed above. Therefore, the evaluation of the evaporation dynamics, considering the concentration of the components and the modifications caused by oxidative processes, should be an integral part when assaying the vapor phase antimicrobial activity.

3. Antimicrobial activity of essential oil vapors

The essential oils activity combined with their volatility predisposes them for application in closed environments such as healthcare units with occupied environments [36,37], heritage libraries and archives [38], beehives [30], home and work places [39] and pathogen control in foodstuffs [40]. Essential oils in vapor phase have the advantage that they can treat large areas without requiring direct contact with surfaces. In all studies found, dealing with essential oil vapors, the aim was to screen the oils for eliminating or growth control of the microorganisms. Up to now, the essential oils of 146 plant species have already been evaluated for their vapor phase antimicrobial activity against different Gram-negative and Gram-positive bacteria, molds and yeasts. which had their essential oils evaluated by this methodology. However, it is difficult to compare the literature data because the results depend on several methodological factors besides the composition variability within the same plant species.

Despite the wide number of plant species producing essential oils, generally, the targeted species for antimicrobial evaluation in the vapor phase are those obtained from commercial spices and other natural flavorings, such as *Syzygium aromaticum* (L.) Merr. & L.M.Perry, *Thymus vulgaris* Willk., *Allium sativum* L., *Cinnamomum zeylanicum* Blume [30,41] or yet from consecrated antimicrobial species already used medicinally, such as Eucalyptus globulus and *Melaleuca alternifolia* Cheel [29,30]. The vapor phase studies using these essential oils have several advantages, once they are easily obtained from companies, in sufficient volumes for screening test and scale-up purposes, besides they have already been evaluated for their antimicrobial activity in the liquid phase model [42].

Among the studied species, 18 have been evaluated using the two vapor phase methodologies available (vapor and disc diffusion) (**Table 1**). Another difficulty found to compare the results was that there was no consensus on the units used to express the MIC values, the published data contained MIC's reported in μL/L, mg/L, μL/cm3 and % v/v. The results presented in Table 1 were normalized to mg/L, when possible, as a form of comparing the different results obtained for the essential oils using the different methodologies. It was not possible to convert the MIC's values when presented in μL/mL, since the authors did not show the density data for each oil tested.

Additionally, there was a considerable number or plant species (127) that were not included in this analysis because other potency units are still accepted, such as direct inhibition zone measurements, percentage of growth inhibition, days to reduce the spore germination to zero and even subjective results like weak, strong and efficient.

The microorganisms that entered this compilation (**Table 1**) were the Gram-positive *Staphylococcus aureus* and *Listeria monocytogenes*, the Gram-negative *Pseudomonas aeruginosa*, *Salmonella enteritidis* and *Escherichia coli*, and the molds *Aspergillus brasiliensis* and *A. flavus* and the yeast *Candida albicans*.

The most active vapor against S. aureus was found for the *H. myrtoides* oil, with MIC value of 0.392 mg/L [23]. This oil is composed mainly for two oxygenated monoterpenes pulegone (30-48%) and iso-menthone (15-32%), which can be the active compounds, since they have shown strong antimicrobial activity in liquid phase studies [43]. Unlike most of the other studies, this is a wild native species, demonstrating the importance of testing different sources, not only the commercially available essential oils.

C. zeylanicum and *T. vulgaris* also presented expressive activity against *S. aureus* strains, if compared to the other oils, with MIC values of 6.25 mg/L [29]. Both oils contained phenolics as major compounds, cinnamaldehyde (63%) and thymol (25%), respectively. As these compounds are usually recognized as the most active antibacterial [43], it would be expected that these oils were more active than that of *H. myrtoides*. However, there are no information about the vapor phase antimicrobial activity for pulegone or iso-menthone.

Among the plant species selected in the Table 1, *Armoracia rusticana* G.Gaertn., B.Mey. & Scherb. presented the highest activity against all the other bacteria tested, thus for the strains of *E. coli*, *L. monocytogenes*, *P. aeruginosa* and *S. enteritidis* the MIC values were 8.3 μL/L [44]. The antimicrobial activity of the isothiocyanates, pungent flavors present as the major compounds of this essential oil, has also been reported the agar diffusion method [45] and like the other essential oil components, its mechanism of action is related to alterations in the cell membranes [46].

Concerning the fungi, *H. myrtoides* essential oil was also one of the most active against *C. albicans*, presenting MIC values of 0.833 mg/L [23] while the molds, *A. flavus* and *A. brasiliensis*, were most sensitive to the *C. zeylanicum* oil, with MIC's values of 13.1 μL/L [44] and 5.625 mg/L [47], respectively.

There are reports, using the same vapor phase methodology, for the same essential oils from different origins presenting distinct chemical composition (**Table 1**). The vapors from *T. vulgaris* oil from three chemotypes carvacrol, thymol and geraniol were evaluated against *E. coli*. The two first chemotypes presented the same MIC value (12.5 mg/L) while the geraniol rich chemotype was much less active with MIC > 1600 mg/L. Testing the same oils against *S. aureus* the thymol chemotype was the most active (MIC = 6.25 mg/L) followed by the carvacrol (MIC-= 12.5 mg/L) and again the geraniol reich oil was the less active (50 mg/L) [29]. Different chemotypes of *T. vulgaris* from USA (linalool, citral and geraniol) were assayed against *S. enteritidis* and *A. brasiliensis*, using the disc diffusion technique, showing no activity [30].

Table 1. Overview of selected studies testing the antimicrobial activity of essential oils by using vapor phase conditions

Plant species	Oil origin	Major compounds	Microorganism	Activity (MIC)		Reference
				Vapor difusion	Disc difusion	
Allium sativum L.	Czech Republic	ND	*E. coli* (ATCC 25922)	ND	530 µL/L[B]	[30,44]
			L. monocytogenes (ATCC 7644)	ND	8.3 µL/L[B]	
	Czech Republic	ND	*P. aeruginosa* (ATCC 27853)	ND	530 µL/L[B]	
	USA	Diallyl disulfide (43%), diallyl trisulfide (27%)	*P. aeruginosa* (ATCC 27853)	ND	250 µL/L	
	Czech Republic	ND	*S. enteritidis* (ATCC13076)	ND	260 µL/L[B]	
	USA	Diallyl disulfide (43%), diallyl trisulfide (27%)	*S. enteritidis* (ATCC 13076)	ND	NA	
	Czech Republic	ND	*S. aureus* (ATCC 25923)	ND	8.3 µL/L[B]	
	USA	Diallyl disulfide (43%), diallyl trisulfide (27%)	*S. aureus* (ATCC 25923)	ND	250 µL/L	
	USA	Allyl isothiocyanate (63%), β-phenylethyl isothiocianate (23%)	*A. brasiliensis*[A]	ND	31.25 µL/L	
Armoracia rusticana Ç. Gaertn., B. Mey & Scherb.	Czech Republic	ND	*E. coli* (ATCC 25922)	ND	8.3 µL/L[B]	[30,44],
			L. monocytogenes (ATCC 7644)	ND	8.3 µL/L[B]	
	Czech Republic	ND	*P. aeruginosa* (ATCC 27853)	ND	8.3 µL/L[B]	
	USA	Allyl isothiocyanate (63%), β-phenylethyl isothiocianate (23%)	*P. aeruginosa* (ATCC 27853)	ND	31.25 µL/L	

Table 1. Overview of selected studies testing the antimicrobial activity of essential oils by using vapor phase conditions

Plant species	Oil origin	Major compounds	Microorganism	Activity (MIC)		Reference
				Vapor difusion	Disc difusion	
Armoracia rusticana Ç. Gaertn., B. Mey & Scherb. (continued)	Czech Republic	ND	*S. enteritidis* (ATCC13076)	ND	8.3 µL/L[B]	[30, 44]
	USA	Allyl isothiocyanate (63%), β-phenylethyl isothiocianate (23%)	*S. enteritidis* (ATCC 13076)	ND	31.25 µL/L	
	Czech Republic	ND	*S. aureus* (ATCC 25923)	ND	8.3 µL/L[B]	
	USA	Allyl isothiocyanate (63%), β-phenylethyl isothiocianate (23%)	*S. aureus* (ATCC 25923)	ND	31.25 µL/L	
Cinnamomum zeylanicum Blume	Poland	ND	*A. brasiliensis*[A] (ATCC 16404)	ND	5.625 mg/L	[27, 29, 47]
	Spain	ND	*A. flavus* (CECT 2687)	ND	13.1 µL/L	
			C. albicans (ATCC 64550)	ND	13.1 µL/L	
	Germany	Cinnamaldehyde (63%)	*E. coli* NIHJ	12.5 mg/LC	ND	
	Germany	Cinnamaldehyde (63%)	*E. coli* NIHJ	>100 mg/LC	ND	
	Spain	ND	*E. coli* (ATCC 29252)	ND	17.5 µL/L	
	Germany	Cinnamaldeyde (63%)	*S. aureus* 209P	6.25 mg/LC	ND	
	Germany	Cinnamaldeyde (63%)	*S. aureus* 209P	>100 mg/LC	ND	
	Spain	ND	*St. aureus* (ATCC 29213)	ND	34.9 µL/L	

Table 1. Overview of selected studies testing the antimicrobial activity of essential oils by using vapor phase conditions

Plant species	Oil origin	Major compounds	Microorganism	Activity (MIC)		Reference
				Vapor difusion	Disc difusion	
Coriandrum sativum L.	France	Linalool (73%)	*S. aureus* 209P	50 mg/L[c]	ND	[29,30]
	USA	Linalool (66%)	*S. aureus* (ATCC 25923)	ND	500 µL/L	
Cymbopogon citratus Stapf	France	Geranial (37%), neral (32%)	*S. aureus* 209P	12.5 mg/L[c]	ND	
	France	Geranial (37%), neral (32%)	*S. aureus* 209P	>100 mg/L[c]	ND	
	USA	Citronellal (38%), geraniol (23%), citronellol (13%)	*S. aureus* (ATCC 25923)	ND	250 µL/L	[29,30]
Hesperozygis myrtoides (St. Hill. Ex Benth.) Epling	Brazil	Pulegone (30-48%), iso-menthone (15-32%)	*A. brasiliensis* (ATCC 16404)	ND	378 mg/L	[23]
			C. albicans (ATCC 10231)	ND	0.833 mg/L	
			E. coli (ATCC 8739)	ND	>3000 mg/L	
			P. aeruginosa (ATCC 9027)	ND	2600 mg/L	
			S. aureus (ATCC 6538)	ND	0.392 mg/L	
Juniperus communis L.	USA	α-pinene (38%), myrcene (12%), sabinene (11%)	*S. aureus* (ATCC 25923)	ND	NA	[30]
	USA	α-pinene (39%), sabinene (15%)	*S. aureus* (ATCC 25923)	ND	500 µL/L	
Lavandula angustifolia Mill.	France	Linalyl scetate (36%), linalool (30%)	*E. coli* NIHJ	>800 mg/L[c]	ND	[29,44]
	France	Linalyl scetate (36%), linalool (30%)	*E. coli* NIHJ	>800 mg/L[c]	ND	
	NS	Czeth Republic	*E. coli* (ATCC 25922)	ND	NA	

Table 1. Overview of selected studies testing the antimicrobial activity of essential oils by using vapor phase conditions

Plant species	Oil origin	Major compounds	Microorganism	Activity (MIC)		Reference
				Vapor difusion	Disc difusion	
Lavandula angustifolia Mill. (continued)	France	Linalool (30%), linalyl scetate (36%)	*S. aureus* 209P	100 mg/L[c]	ND	[29,30,44]
	France	Linalool (30%), linalyl scetate (36%)	*S. aureus* 209P	>800 mg/L[c]	ND	
	Czech Republic	ND	*S. aureus* (ATCC 25923)	ND	NA	
	USA	Linalyl acetate (45%), linalool (36%)	*S. aureus* (ATCC 25923)	ND	NA	
Lavandula latifolia Medik.	France	Linalool (46%), 1,8-cineole (23%)	*S. aureus* 209P	50 mg/L[c]	ND	[29,30]
	USA	Linalyl acetate (25%), linalool (23%)	*S. aureus* (ATCC 25923)	ND	NA	
Melaleuca alternifolia Cheel	Australia	Terpinen-4-ol (42%), γ-terpinene (17%)	*S. aureus* 209P	50 mg/L[c]	ND	[29,30]
	Australia	Terpinen-4-ol (42%), γ-terpinene (17%)	*S. aureus* 209P	800 mg/L[c]	ND	
	USA	Terpinen-4-ol (44%), γ-terpinene (19%)	*S. aureus* (ATCC 25923)	ND	NA	
Mentha x piperita L.	France	Menthol (63%), p-menthone (19%)	*E. coli* NIHJ	> 1600 mg/L[c]	ND	[29,44]
	Czech Republic	ND	*E. coli* (ATCC 25922)	ND	NA	
	France	Menthol (63%), p-menthone (19%)	*S. aureus* 209P	25 mg/L[c]	ND	
	Czech Republic	ND	*S. aureus* (ATCC 25923)	ND	NA	

Table 1. Overview of selected studies testing the antimicrobial activity of essential oils by using vapor phase conditions

Plant species	Oil origin	Major compounds	Microorganism	Activity (MIC)		Reference
				Vapor difusion	Disc difusion	
Origanum marjorana L.	Czech Republic	ND	*S. aureus* (ATCC 25923)	ND	530 μL/L[B]	[30,44]
	USA	Terpinen-4-ol (24%), E-sabinene hydrate (16%), γ-terpinene (13%)	*S. aureus* (ATCC 25923)	ND	NA	
	USA	Terpinen-4-ol (26%), linalool (16%), γ-terpinene (12%)	*S. aureus* (ATCC 25923)	ND	NA	
Origanum vulgare L.	Spain	ND	*A. flavus* (CECT 2687)	ND	17.5 μL/L	[45,48]
			C. albicans (ATCC 64550)	ND	17.5 μL/L	
	Spain	ND	*E. coli* (ATCC 29252)	ND	13.1 μL/L	
	Czech Republic	ND	*E. coli* (ATCC 25922)	ND	66 μL/L[B]	
	Spain	ND	*L. monocytogenes* (ATCC 7644)	ND	26.2 μL/L	
Origanum vulgare L. (continued)	Czech Republic	ND	*L. monocytogenes* (ATCC 7644)	ND	66 μL/L[B]	[30,44,48]
	Spain	ND	*P. aeruginosa* (ATCC 27)	ND	175 μL/L	
	Czech Republic	ND	*P. aeruginosa* (ATCC 27853)	ND	NA	
	USA	Carvacrol (92%)	*P. aeruginosa* (ATCC 27853)	ND	NA	
	Czech Republic	ND	*S. enteritidis* (ATCC13076)	ND	NA	

Table 1. Overview of selected studies testing the antimicrobial activity of essential oils by using vapor phase conditions

Plant species	Oil origin	Major compounds	Microorganism	Activity (MIC)		Reference
				Vapor difusion	Disc difusion	
	USA	Carvacrol (92%)	*S. enteritidis* (ATCC 13076)	ND	62.5 µL/L	[30,44,48]
	Spain	ND	*S. aureus* (ATCC 29213)	ND	13.1 µL/L	
	Czech Republic	ND	*S. aureus* (ATCC 25923)	ND	17 µL/L[B]	
	USA	Carvacrol (92%)	*S. aureus* (ATCC 25923)	ND	62.5 µL/L	
Rosmarinus officinalis L.	France	α-pinene (24%), 1,8-cineole (23%)	*E. coli* NIHJ	> 1600 mg/L[C]	ND	[27,30,48]
	Spain	ND	*E. coli* (ATCC 29252)	ND	NA	
	France	α-pinene (24%), 1,8-cineole (23%)	*S. aureus* 209P	100 mg/L[C]	ND	
	Spain	ND	*S. aureus* (ATCC 29213)	ND	NA	
	USA	1,8-cineole (44%), α-pinene (13%), camphor (11%)	*S. aureus* (ATCC 25923)	ND	NA	
	USA	α-pinene (23%), 1,8-cineole (22%)	*S. aureus* (ATCC 25923)	ND	NA	
Salvia officinalis L.	USA	β-thujone (15%), 1,8-cineole (15%), α-pinene (12%)	*A. brasiliensis*[A] (ATCC 6275)	ND	NA	[18,30]
	Italy	α-thujone[E] (29%), camphor (22%)	*A. brasiliensis*[A]	ND	125000-250000 µL/L[D]	
	USA	β-thujone (15%), 1,8-cineole (15%), α-pinene (12%)	*P. aeruginosa* (ATCC 27853)	ND	NA	

Table 1. Overview of selected studies testing the antimicrobial activity of essential oils by using vapor phase conditions

Plant species	Oil origin	Major compounds	Microorganism	Activity (MIC)		Reference
				Vapor difusion	Disc difusion	
Salvia officinalis L. (continued)	Czech Republic	ND	*P. aeruginosa* (ATCC 27853)	ND	NA	[30,44]
	USA	β-thujone (15%), 1,8-cineole (15%), α-pinene (12%)	*S. aureus* (ATCC 25923)	ND	NA	
	Czech Republic	ND	*S. aureus* (ATCC 25923)	ND	NA	
Syzygium aromaticum L.	Italy	Eugenol (77%)	*A. brasiliensis*[A]	ND	62500-125000 µL/L[D]	[18,27,30, 49]
	USA	Eugenol (88%)	*A. brasiliensis*[A]	ND	500 µL/L	
	Italy	Eugenol (77%)	*A. flavus*	ND	62500-125000 µL/L[D]	
	Spain	ND	*A. flavus* (CECT 2687)	ND	17.5 µL/L	
			A. flavus (CECT 2687)	ND	17.5 µL/L	
	Spain	ND	*C. albicans* (ATCC 64550)	ND	13.1 µL/L	
	Korea	ND	*C. albicans* KCTC 7965	2500000 µL/L[D]	ND	
	Spain	ND	*S. aureus* (ATCC 29213)	ND	26 µL/L	
	USA	Eugenol (88%)	*S. aureus* (ATCC 25923)	ND	250 µL/L	
Thymus serpyllum L.	Czech Republic	ND	*S. enteritidis* (ATCC13076)	ND	NA	[44]

Table 1. Overview of selected studies testing the antimicrobial activity of essential oils by using vapor phase conditions

Plant species	Oil origin	Major compounds	Microorganism	Activity (MIC)		Reference
				Vapor difusion	Disc difusion	
Thymus serpyllum L. (continued)	USA	Carvacrol (15%), p-cymene (14%), thymol (12%)	*S. enteritidis* (ATCC 13076)	ND	250 µL/L	[30, 44]
	Czech Republic	ND	*S. aureus* (ATCC 25923)	ND	33 µL/L[B]	
	USA	Carvacrol (15%), p-cymene (14%), thymol (12%)	*S. aureus* (ATCC 25923)	ND	250 µL/L	
Thymus vulgaris L.	USA	Thymol (42%), p-cymene (29%)	*A. brasiliensis*[A] (ATCC6275)	ND	250 µL/L	[18,29,30, 44,48]
	USA	Linalool (58%), thymol (15%)	*A. brasiliensis*[A] (ATCC6275)	ND	250 µL/L	
	USA	Citral (45%), chrysanthenyl acetate (31%)	*A. brasiliensis*[A] (ATCC6275)	ND	250 µL/L	
	USA	Geraniol (63%)	*A. brasiliensis*[A] (ATCC6275)	ND	125 µL/L	
	Italy (Red)	Thymol (26%), p-cymene (16%)	*A. brasiliensis*[A]	ND	7800 µL/L[D]	
	Spain	ND	*C. albicans* (ATCC 64550)	ND	26.2 µL/L	
	Spain	ND	*E. coli* (ATCC 29252)	ND	52.4 µL/L	
	Czech Republic	ND	*E. coli* (ATCC 25922)	ND	33 µL/L[B]	
	Japan (Wild)	Carvacrol (80%)	*E. coli* NIHJ	12.5 mg/L[C]	ND	
	Japan (Wild)	Carvacrol (80%)	*E. coli* NIHJ	>100 mg/L[C]	ND	

Table 1. Overview of selected studies testing the antimicrobial activity of essential oils by using vapor phase conditions

Plant species	Oil origin	Major compounds	Microorganism	Activity (MIC)		Reference
				Vapor difusion	Disc difusion	
Thymus vulgaris L. (continued)	Germany (Red)	Limonene (25%), thymol (25%)	*E. coli* NIHJ	12.5 mg/L[C]	ND	[29, 30, 44, 48]
	France (geraniol)	Geranyl acetate (55%), geraniol (32%)	*E. coli* NIHJ	> 1600 mg/L[C]	ND	
	Spain	ND	*L. monocytogenes* (ATCC 7644)	ND	175 µL/L	
	Czech Republic	ND	*L. monocytogenes* (ATCC 7644)	ND	260 µL/L[B]	
	Czech Republic	ND	*S. enteritidis* (ATCC13076)	ND	33 µL/L[B]	
	USA	Thymol (42%), p-cymene (29%)	*S. enteritidis* (ATCC 13076)	ND	NA	
	USA	Linalool (58%), thymol (15%)	*S. enteritidis* (ATCC 13076)	ND	125 µL/L	
	USA	Citral (45%), chrysanthenyl acetate (31%)	*S. enteritidis* (ATCC 13076)	ND	NA	
	USA	Geraniol (63%)	*S. enteritidis* (ATCC 13076)	ND	NA	
	Spain	ND	*S. aureus* (ATCC 29213)	ND	87.3 µL/L	
	Czech Republic	ND	*S. aureus* (ATCC 25923)	ND	17 µL/L[B]	
	USA	Thymol (42%), p-cymene (29%)	*S. aureus* (ATCC 25923)	ND	NA	
	USA	Linalool (58%), thymol (15%)	*S. aureus* (ATCC 25923)	ND	125 µL/L	

Table 1. Overview of selected studies testing the antimicrobial activity of essential oils by using vapor phase conditions

Plant species	Oil origin	Major compounds	Microorganism	Activity (MIC)		Reference
				Vapor difusion	Disc difusion	
Thymus vulgaris L. (continued)	USA	Citral (45%), chrysanthenyl acetate (31%)	*S. aureus* (ATCC 25923)	ND	250 µL/L	[29, 30]
	USA	Geraniol (63%)	*S. aureus* (ATCC 25923)	ND	125 µL/L	
	Japan (Wild)	Carvacrol (80%)	*S. aureus* 209P	12.5 mg/L[C]	ND	
	Japan (Wild)	Carvacrol (80%)	*S. aureus* 209P	>100 mg/L[C]	ND	
	Germany (Red)	Limonene (25%), thymol (25%)	*S. aureus* 209P	6.25 mg/L[C]	ND	
	France (geraniol)	Geranyl acetate (55%), geraniol (32%)	*S. aureus* 209P	50 mg/L[C]	ND	

Legend: [A]: originally *Aspergillus niger*;
[B]: originally cm^3;
[C]: originally MID (minimum inhibition dose);
[D]: originally %, v/v;
[E]: originally cis-thujone;
ND: not determined;
NA: not active.

Besides the differences in activity that are caused directly from the distinct chemical composition, the method used for determining the activity (vapor or disc diffusion) may also influence the results as can be seen in Table 1. Nowadays, the disc diffusion method is the most used, but within this technique there are also several variations, such as paper disc size and form, different sizes of the vials used for oil dispersion, essential oil dilution and volume added. In two independent studies, the vapor of *T. vulgaris* essential oils, with similar composition, were evaluated against the same *L. monocytogenes* strain, using similar methods varying only the paper disc diameter, 6 mm [44] and 10 mm [48]. The MIC obtained with the smaller disc (260 μL/L) was higher than that obtained for the bigger one (175 mg/mL). Changing the dispersion method from paper discs to oil vessels, the MIC obtained are also different. The antifungal activity for clove (*S. aromaticum*) essential oils against *A. brasiliensis* was much lower when it was determined using a glass slide for dispersing the vapors (MIC between 62500 – 125000 μL/L) when compared to paper disc (MIC =- 500 μL/L) [18,30]. Similar findings were obtained for the *T. vulgaris* essential oils against the same fungus MIC of 7800 μL/L with the glass slide [18] and MIC's varying between 125 -250 μL/L, depending on the oil composition [30]. These results indicate that the in the disc diffusion method the vapor dispersion mode is very important for the results obtained and that to allow comparisons a standard procedure should be employed.

The use of the vapor diffusion method seems to be restricted to few research groups aiming to propose practical applications for essential oil vapors against airborne life-threatening pathogens [29], dermatophytic fungi [49] or descontaminate heritage papers and textiles [38,47]. As previously discussed, this method presents difficulties to standardize the vapor atmosphere leading to different MIC values, which can be emphasized by the volume and format of the boxes used in the tests [30]. However, no different assays presenting MIC values were found in the literature that allowed a direct comparison of these variables. On the other hand, there were results that allowed the comparison between the vapor diffusion experiments with those employing the disc diffusion. *C. zeylanicum* oil vapors showed activity against *E. coli* and *S. aureus*, but the highest activities were reported for the vapor diffusion method for the two bacteria, with respective MIC's of 12.5 μL/L and 6.25 mg/L [29,48]. As discussed in the previous methodology, the antimicrobial activity is dependent upon the evaporation rate of the essential oil. In a systematic study using *C. zeylanicum*, *Cymbopogon citratus* Stapf, *Lavandula angustifolia* Mill., *M. alternifolia* and *T. vulgaris* oils against *E. coli* and *S. aureus*, the paper discs allowed a rapid evaporation of the oils, comparing with the vessel dispersion, affording lower MIC values [29]. Although several studies have discussed the vapor oil activity, only a few have evaluated the evaporation dynamics of the

individual constituents. The headspace evaluation of the main constituents from *C. zeylanicum*, *T. vulgaris*, *M. alternifolia* and *Citrus aurantiifolia* (Christm.) Swingleoils showed that both monoterpenes (limonene, citral and terpine-4-ol) and the phenolic compound (thymol) reach the maximal vapor levels around 1 hour [29]. Experiments to determinate the complete vapor composition were also performed. In an analysis of *L. angustifolia* oil vapors, the oxygenated monoterpenes linalool, eucalyptol and linalyl acetate were the most abundant, differently from the liquid oil that presented camphor as the most abundant one [30].

The oxidation from some oil components was also observed in the vapor phase, in some experiments there was the formation of alcohols, acids and furan [29]. As the oxygenated products are considered more potent as antimicrobials [43] than their hydrocarbons counterparts, this phenomenon, summed with the volatility previously discussed, could explain in part why the oil vapors are more able to exert an antimicrobial effect than when in the liquid phase. Furthermore, it is assumed that the free oils components would easily link to the bacteria or fungi lipophilic cell wall, due to direct contact, compared to those in liquid phase that are surrounded by a solvent envelope [29]. However, more studies are necessary to establish the real mechanism of action for the vapor components and their synergistic interactions.

Vapor phase antimicrobial active of pure compounds

Not only the whole essential oils have been tested for their antimicrobial activity in vapor phase, but also some isolated pure compounds as can be seen in Table 2. The presented data contain the results found in the literature for the pure compounds where the authors expressed the Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) or Minimal Fungicidal Concentration (MFC). The concentrations were normalized to mg/L for comparison purposes. Grouping the compounds by classes, oxygenated monoterpenes and phenylpropanoids were compounds with the highest activity. In Figure 2. the structures of the most active compounds of each group is represented.

Among the oxygenated monoterpenes, citral and thymol were the most active compounds. These two substances were tested by vapor diffusion method against 2 Gram negative (*Haemophilus influenza*, *E. coli*) and 3 Gram positive bacteria (*Streptococcus pyogenes*, *S. pneumonia* and *S. aureus*). Citral presented MIC values ranging from 3.13 to 12.5 mg/L, depending on the strain, whereas thymol inhibited the growth of tested bacteria at the concentration of 3.13 mg/L [29]. Thymol, despite being an oxygenated monoterpene, is also a phenolic compound, what can explain its higher potency. The most accepted mechanism of action for the essential oils' antimicrobial effect is thanks to their hydrophobicity, which

by disturbing lipid layer from the bacterial cell membrane causes cell leakage and disruption leading to the microorganism death. Phenolics, additionally, can disrupt the proton motive force (PMF), electron flow and active transport in the pathogen cells, being considered the strongest antimicrobial compounds [43]. Unfortunately, carvacrol, that differs from thymol only by the hydroxyl position, was not tested in the same study. However, its vapor phase antimicrobial activity was evaluated in another experiment by adapted disc diffusion assay against *Salmonella choleraesuis*, *L. monocytogenes*, *A. flavus* and *C. albicans* presenting MIC values between 10.65 – 21.30 mg/L [48].

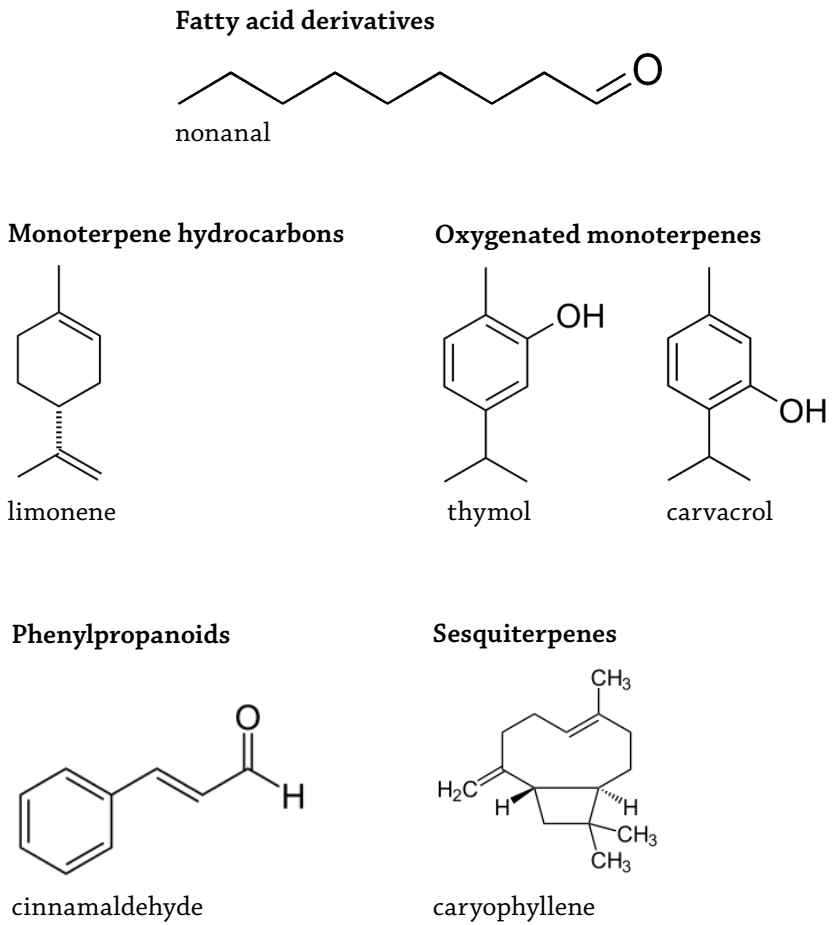

Figure 2. Structures of the most active compounds of each class.

Phenylpropanoids' vapors also showed good antimicrobial effects, considering its phenolic characteristics as mentioned before. Cinnamaldehyde, was the most potent compound of the group, demonstrating inhibitory activity against *H. influenza*, *E. coli*, *S. pyogenes*, *S. pneumonia* and *S. aureus* in concentrations between 1.56 – 6.25 mg/L [29], and MIC's of 4.62 – 22.89 mg/L against *S. choleraesuis*, *L. monocytogenes*, *A. flavus* and *C. albicans* [48]. Although it possesses a phenyl group, it does not always disintegrate the microorganisms' cell membrane [50], but rather inhibits amino acid decarboxylases activities by binding to the proteins through the carbonyl group [51]. Eugenol, recognized as an antimicrobial agent, surprisingly did not give good results when tested in vapor phase. It was only able to inhibit the growth of *S. choleraesuis* in 20% and in 52% for *A. flavus* [48], as no actual MIC or MBC values were presented, these results were not listed in Table 2. In another experiment, eugenol MFC was estimated against three Candida species isolated from infected patients by adapted disc diffusion method [52], however, after unit's normalization the MIC values varied between 2650 and 5300 mg/L, whereas the value obtained by the microdilution technique for liquid phase was few times lower giving only 625 mg/L [53].

Monoterpene hydrocarbons were not very active in the vapor phase, since their MIC values were higher than 200 mg/L for D-limomene and more than 400 mg/L for α-pinene for *H. influenza*, *E. coli*, *S. pyogenes*, *S. pneumonia* and *S. aureus*. On the other hand, in the same experiment, fatty acid derivatives, such as nonanal and octanal, showed an antibacterial activity higher than that observed for the monoterpene hydrocarbons with MIC values between 12.5 – 50 mg/L [29]. Another monoterpene hydrocarbon, p-cymene, did not show any growth inhibition of *S. choleraesuis*, *L. monocytogenes*, *A. flavus* or *C. albicans* in the vapor phase [48]. Despites its lipophilic properties, it is not effective when used alone but it demonstrated certain synergism when combined with other compounds. In a liquid phase study with *Bacillus cereus*, *p*-cymene seemed to be incorporated in the cell membrane lipid layer, which probably facilitated the carvacrol transport through the membrane [54]. The compounds belonging to these last two classes may not be very active by themselves but they can improve the effect of other molecules that act directly on cell's metabolism.

Sesquiterpene hydrocarbons are a group that was not presented in the table due to lack of published MIC for the vapor phase. One of the most known representative of this class is β-caryophyllene. It did not inhibit the growth of any tested bacteria in vapor phase by adapted disc diffusion method [48]. On the contrary, the same compound, when evaluated in liquid phase by disc diffusion method, demonstrated high antimicrobial effect; MIC values obtained against *S. aureus*, *E. coli* and *A. brasiliensis* after normalization were less than 2mg/L [55].

It must be mentioned that depending on the method used, different MIC values were obtained for the same compound. For example, using adapted disc diffusion method, thymol vapors presented less inhibitory activity against E. coli (MIC = 200 mg/L, [56]) than in the vapor diffusion method (MIC = 3.13 mg/L [29]). Similarly, menthol activity was also lower when adapted disc diffusion was used against *E. coli*. The MIC value was 2500mg/L [56], when the MIC obtained with the vapor diffusion assay was > 50 mg/L. Since the strains used in two works were different, it is also possible, that the results might be influenced by different susceptibility from the test bacteria. Therefore, it is not possible to affirm if these two methods will always give different results and if there is an exact correlation among them, but for sure that the method used has an impact on the obtained results.

Another difficult task is also to estimate whether the essential oils are more or less potent than their isolated compounds. As an example, when thyme oil was evaluated for its antimicrobial activity by vapor diffusion method [48], MIC values against *L. monocytogenes* and *A. flavus* were 175 μL/L, and the values for thymol, the main thyme oil component, were only 21.8 and 43.6 μL/L, respectively (units given by the author). In this case, thymol could be accounted as the responsible for the antimicrobial and antifungal effects of the essential oil. On the other hand, in the same study, carvacrol and cinnamaldehyde expressed very similar results to their original essential oils from oregano and cinnamon. It means that, in some cases essential oils can be more effective due to cumulated synergistic action of different ingredients providing wide spectrum of action. Finally, it should be also considered, that the amount of each substance may vary for oils of different origin within the same species.

To conclude, the antimicrobial activity of essential oils' pure compounds varied depending on many factors including method of evaluation and bacteria used as the main ones. Apart from the fact, that most of the authors believe that Gram positive bacteria are slightly more susceptible to essential oils' compounds than Gram negative bacteria [43], we did not observe such tendency. Anyhow, some bacteria were found to be more sensitive than others. For instance, *H. influenza* was more susceptible to pure essential oils' compounds from the group of the Gram-negative bacteria, and *S. aureus* was the least sensitive of the Grampositive bacteria.

Table 2. Antimicrobial activity of essential oils' compounds in vapor phase expressed in Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration or Minimal Fungicidal Concentration (MBC/MFC) normalized to mg/L.

Group	Isolated Compound	Microorganism	MIC [mg/L]	MBC/MFC [mg/L]	Method	Ref
Fatty acid derivatives	nonanal	*Haemophilus influenza* (ATCC 33391)	12.5*	ND	Vapor diffusion	[29]
		Streptococcus pyogenes (ATCC 12344)	12.5*	ND		
		Streptococcus pneumoniae (IP-692)	12.5*	ND		
		Streptococcus pneumonia (PRC-53)	12.5*	ND		
		Staphylococcus aureus (209P)	25*	ND		
		Escherichia coli (NIHJ)	> 50*	ND		
	octanal	*Haemophilus influenza* (ATCC 33391)	12.5*	ND	Vapor diffusion	[29]
		Streptococcus pyogenes (ATCC 12344)	25*	ND		
		Streptococcus pneumoniae (IP-692)	25*	ND		
		Streptococcus pneumoniae (PRC-53)	25*	ND		
		Staphylococcus aureus (209P)	50*	ND		
		Escherichia coli (NIHJ)	25*	ND		

Group	Isolated Compound	Microorganism	MIC [mg/L]	MBC/MFC [mg/L]	Method	Ref
Monoterpene hydrocarbons	D-limonene	*Haemophilus influenza* (ATCC 33391)	200*	ND	Vapor diffusion	[29]
		Streptococcus pyogenes (ATCC 12344)	400*	ND		
		Streptococcus pneumoniae (IP-692)	200*	ND		
		Streptococcus pneumonia (PRC-53)	400*	ND		
		Staphylococcus aureus (209P)	800*	ND		
		Escherichia coli (NIHJ)	800*	ND		
	α-pinene	*Candida albicans* (n = 26)	ND	4290 - 8580**	Adapted disc diffusion	[52]
		Candida glabrata (n = 10)	ND	4290 - 8580**		
		Candida tropicalis(n = 10)	ND	8580**		
		Haemophilus influenza (ATCC 33391)	> 800*	ND	Vapor diffusion	[29]
		Streptococcus pyogenes (ATCC 12344)	800*	ND		
		Streptococcus pneumonia (IP-692)	400*	ND		
		Streptococcus pneumonia (PRC-53)	400*	ND		
		Staphylococcus aureus (209P)	800*	ND		
		Escherichia coli (NIHJ)	> 800*	ND		

Group	Isolated Compound	Microorganism	MIC [mg/L]	MBC/MFC [mg/L]	Method	Ref
Oxygenated monoterpenes	camphor	*Haemophilus influenza* (ATCC 33391)	50*	ND	Vapor diffusion	[29]
		Streptococcus pyogenes (ATCC 12344)	100*	ND		
		Streptococcus pneumonia (IP-692)	100*	ND		
		Streptococcus pneumoniae (PRC-53)	100*	ND		
		Staphylococcus aureus (209P)	400*	ND		
		Escherichia coli (NIHJ)	>400*	ND		
	carvacrol	*Salmonella choleraesuis* (CECT 4000)	10.65***	ND	Adapted disc diffusion	[48]
		Listeria monocytogenes (ATCC 7644)	21.30***	ND		
		Aspergillus flavus (CECT2687)	21.30***	ND		
		Candida albicans (ATCC 64550)	10.65***	ND		
		Candida albicans (n = 26)	ND	1220 - 9770**	Adapted disc diffusion	[52]
		Candida glabrata (n = 10)	ND	2440 - 9770**		
		Candida tropicalis (n = 10)	ND	2440 - 9770**		
		Aggregatibacter actinomycetemcomitans (ATCC 33384)	200	200	Adapted disc diffusion	[56]
		Streptococcus mutans (ATCC 25175)	400	600		
		Methicillin-resistant *Staphylococcus aureus* (MRSA, ATCC 33591)	400	600		
		Escherichia coli (ATCC 10798)	400	400		

Group	Isolated Compound	Microorganism	MIC [mg/L]	MBC/MFC [mg/L]	Method	Ref
	1,8-cineole	*Haemophilus influenza* (ATCC 33391)	25*	ND		
		Streptococcus pyogenes (ATCC 12344)	200*	ND	Vapor diffusion	[29]
		Streptococcus pneumonia (IP-692)	200*	ND		
		Streptococcus pneumoniae (PRC-53)	200*	ND		
		Staphylococcus aureus (209P)	> 200*	ND	Vapor diffusion	[29]
		Escherichia coli (NIHJ)	> 200*	ND		
	citral	*Haemophilus influenza* (ATCC 33391)	3.13*	ND		
		Streptococcus pyogenes (ATCC 12344)	3.13*	ND		
		Streptococcus pneumonia (IP-692)	6.25*	ND	Vapor diffusion	[29]
		Streptococcus pneumoniae (PRC-53)	6.25*	ND		
		Staphylococcus aureus (209P)	12.5*	ND		
		Escherichia coli (NIHJ)	> 12.5*	ND		
	geraniol	*Haemophilus influenza* (ATCC 33391)	6.25*	ND		
		Streptococcus pyogenes (ATCC 12344)	12.5*	ND		
		Streptococcus pneumonia (IP-692)	6.25*	ND	Vapor diffusion	[29]
		Streptococcus pneumonia (PRC-53)	6.25*	ND		
		Staphylococcus aureus (209P)	> 25*	ND		
		Escherichia coli (NIHJ)	> 25*	ND		

Group	Isolated Compound	Microorganism	MIC [mg/L]	MBC/MFC [mg/L]	Method	Ref
	hinokitiol	*Aggregatibacter actinomycetemcomitans* (ATCC 33384)	40	40	Adapted disc diffusion	[56]
		Streptococcus mutans (ATCC 25175)	40	100		
		Methicillin-resistant *Staphylococcus aureus* (MRSA, ATCC 33591)	60	60		
		Escherichia coli (ATCC 10798)	40	100		
	linalool	*Candida albicans* (n = 26)	ND	4290**	Adapted disc diffusion	[52]
		Candida glabrata (n = 10)	ND	4290**		
		Candida tropicalis (n = 10)	ND	8580**		
		Haemophilus influenza (ATCC 33391)	12.5*	ND	Vapor diffusion	[29]
		Streptococcus pyogenes (ATCC 12344)	25*	ND		
		Streptococcus pneumoniae (IP-692)	25*	ND		
		Streptococcus pneumoniae (PRC-53)	25*	ND	Vapor diffusion	[29]
		Staphylococcus aureus (209P)	50*	ND		
		Escherichia coli (NIHJ)	50*	ND		
	linalyl acetate	*Candida albicans* (n = 26)	ND	8950 - > 8950**	Adapted disc diffusion	[52]
		Candida glabrata (n = 10)	ND	8950 - > 8950 **		
		Candida tropicalis (n = 10)	ND	8950 - > 8950**		

Group	Isolated Compound	Microorganism	MIC [mg/L]	MBC/MFC [mg/L]	Method	Ref
	menthol	*Aggregatibacter actinomycetemcomitans* (ATCC 33384)	500	1000	Adapted disc diffusion	[56]
		Streptococcus mutans (ATCC 25175)	1000	1000		
		Methicillin-resistant *Staphylococcus aureus* (MRSA, ATCC 33591)	1000	1000		
		Staphylococcus aureus (209P)	25*	ND		
		Escherichia coli (ATCC 10798)	2500	2500	Vapor diffusion	[29]
		Escherichia coli (NIHJ)	> 50*	ND		
		Haemophilus influenza (ATCC 33391)	6.25*	ND		
		Streptococcus pyogenes (ATCC 12344)	25*	ND		
		Streptococcus pneumonia (IP-692)	25*	ND		
		Streptococcus pneumonia (PRC-53)	25*	ND		
	menthone	*Haemophilus influenzae* (ATCC 33391)	50*	ND	Vapor diffusion	[29]
		Streptococcus pyogenes (ATCC 12344)	200*	ND		
		Streptococcus pneumonia (IP-692)	200*	ND		
		Streptococcus pneumonia (PRC-53)	200*	ND		
		Staphylococcus aureus (209P)	200*	ND		
		Escherichia coli (NIHJ)	> 400*	ND		

Group	Isolated Compound	Microorganism	MIC [mg/L]	MBC/MFC [mg/L]	Method	Ref
	perillaldehyde	*Haemophilus influenzae* (ATCC 33391)	12.5*	ND	Vapor diffusion	[29]
		Streptococcus pyogenes (ATCC 12344)	12.5*	ND		
		Streptococcus pneumoniae (IP-692)	12.5*	ND		
		Streptococcus pneumoniae (PRC-53)	12.5*	ND		
		Staphylococcus aureus (209P)	50*	ND		
		Escherichia coli (NIHJ)	> 50*	ND		
	terpinen-4-ol	*Haemophilus influenzae* (ATCC 33391)	12.5*	ND	Vapor diffusion	[29]
		Streptococcus pyogenes (ATCC 12344)	25*	ND		
		Streptococcus pneumonia (IP-692)	25*	ND		
		Streptococcus pneumoniae (PRC-53)	25*	ND		
		Staphylococcus aureus (209P)	25*	ND		
		Escherichia coli (NIHJ)	25*	ND		
	thymol	*Salmonella choleraesuis* (CECT 4000)	10.46***	ND	Adapted disc diffusion	[48]
		Listeria monocytogenes (ATCC 7644)	20.93***	ND		
		Aspergillus flavus (CECT 2687)	41.86***	ND		
		Candida albicans (ATCC 64550)	10.46***	ND		

Group	Isolated Compound	Microorganism	MIC [mg/L]	MBC/MFC [mg/L]	Method	Ref
	thymol (continued)	*Candida albicans* (n = 26)	ND	1200-2400**	Adapted disc diffusion	[52]
		Candida glabrata (n = 10)	ND	2400**		
		Candida tropicalis (n = 10)	ND	2400 - 4800**		
		Aggregatibacter actinomycetemcomitans (ATCC 33384)	100	200	Adapted disc diffusion	[56]
		Streptococcus mutans (ATCC 25175)	200	400		
		Staphylococcus aureus (209P)	3.13*	ND	Vapor diffusion	[29]
		Methicillin-resistant *Staphylococcus aureus* (MRSA, ATCC 33591)	200	200	Adapted disc diffusion	[56]
		Escherichia coli (ATCC 10798)	200	400		
		Escherichia coli (NIHJ)	> 3.13*	ND	Vapor diffusion	[29]
		Haemophilus influenza (ATCC 33391)	3.13*	ND		
		Streptococcus pyogenes (ATCC 12344)	3.13*	ND		
		Streptococcus pneumoniae (IP-692)	3.13*	ND		
		Streptococcus pneumoniae (PRC-53)	3.13*	ND		

Group	Isolated Compound	Microorganism	MIC [mg/L]	MBC/MFC [mg/L]	Method	Ref
Phenylpropanoids	cinnamaldehyde	*Salmonella choleraesuis* (CECT 4000)	4.62***	ND	Adapted disc diffusion	[48]
		Listeria monocytogenes (ATCC 7644)	22.89***	ND		
		Aspergillus flavus (CECT 2687)	22.89***	ND		
		Candida albicans (ATCC 64550)	4.62***	ND		
		Haemophilus influenza (ATCC 33391)	1.56*	ND	Vapor diffusion	[29]
		Streptococcus pyogenes (ATCC 12344)	3.13*	ND		
		Streptococcus pneumoniae (IP-692)	3.13*	ND		
		Streptococcus pneumonia (PRC-53)	3.13*	ND		
		Staphylococcus aureus (209P)	6.25*	ND		
		Escherichia coli (NIHJ)	6.25**	ND		
	eugenol	*Candida albicans* (n = 26)	ND	5300**	Adapted disc diffusion	[52]
		Candida glabrata (n = 10)	ND	2650**		
		Candida tropicalis (n = 10)	ND	5300**		

* In the reference, the MIC was termed minimal inhibition dose (MID)

** Values were normalized to mg/L from the units given by the author (% - v/v)

*** Values were normalized to mg/L from the units given by the author (μL/L)

Conclusions

There is growing evidence that essential oil vapors are effective as an antimicrobial system and that they present some advantages over the use in liquid phase such as increase in activity, relatively low concentrations, ability to treat large areas without requiring direct contact with surfaces. This can make them suitable for using as disinfectant of rooms, air decontaminants, and food preservatives. However, there is not a general method available for assessing the antimicrobial activity in the vapor phase, and consistency concerning effectiveness parameters to decide which essential oil vapor will be effective against which type of microorganism, and consequently their spectrum of activity. Regulatory agencies should pay more attention to this subject, as it was already done for evaluation of putative surface disinfectants that has a generally accepted method to evaluate new germicidal candidates [24]. Moreover, more research is needed towards understanding their mode of action, and future experiments must include the headspace composition analysis due to the chemical transformation that occur in the vapor phase. Although most essential oil have been used by humans for centuries, vapors probable toxicological effects should also be studied because some terpenes can react with oxidants such as ozone, hydroxyl and nitrate radicals form high molecular weight oxidation products such as aldehydes, ketones and organic acids.

Due to its environmentally friendly characteristics, the antibacterial activity of essential oil vapors might be an interesting option in hospital environments due to their ability in preventing biofilm formation or removing it, since the microbial biofilms are an important route for cross-contamination [14], or to be used as inhalation therapy against bacterial respiratory tract pathogens [29]. Additionally, the use of essential oil vapors can be a new strategy for developing therapies for infections avoiding bacterial resistance.

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Conflicts of Interest

The authors declare no conflict of interest.

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Chapter 9

Advances in studies of anxiolytic and antidepressant activity with essential oils and their chemical components by inhalation

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Introduction

Essential Oils

Essential oils (EO), also known as volatile oils or ethereal oils, are composed by complex mixtures of volatile and lipophilic substances obtained from vegetable raw materials. In general, they have a pleasant odor and are found in liquid form.

The number of components of the essential oils can range from 20 to 200, and for being complex mixtures, they have different concentrations among them. Those composed from 20 to 95% of the mixture are called major constituents, from 1 to 20% are referred to as secondary constituents, and below 1% are called trace components. So far, about 3,000 distinct chemicals were found in essential oils, some examples are organic acids, simple and terpenic alcohols, ketones, aldehydes, phenols, terpene hydrocarbons, peroxides, ethers, esters, lactones, oxides, and coumarins. In the majority of the essential oils, however, the structures of the phenylpropanoids and terpenoids stand out due to their greater quantity [1].

Several essential oils with pharmacological activity are described in the literature, among them, some have a notorious capacity to modulate the central nervous system (CNS), promoting anxiolytic and antidepressant effects. It is

the case of *Asarum heterotropoides*, a native plant in the Asian continent, whose main constituents are methyl-eugenol (22.58%), pentadecane (6.78%) and 2,3,5-trimethoxytoluene (5.54%). Pharmacological tests revealed that this essential oil has a broad spectrum of action, as well as antinociceptive, anti-inflammatory, antiallergic, antioxidant, anti-anxiety and antidepressant effects [2].

Pharmacological trials using anxiety models demonstrated that the essential oils of *Citrus aurantium*, *Cymbopogon citratus*, *Lavendula augustifolia* and *Lippia alba* presented anxiolytic drug profile. In addition, the essential oil of *Citrus sinensis* presented an acute effect in the clinical phase in healthy volunteers. It has also been reported that some of the aforementioned essential oils do not have action mechanisms based on GABAergic pathway, which may be a breakthrough in the discovery of a new class of effective anxiolytics in clinical practice and with fewer side effects [3].

These studies involving essential oils demonstrate its capacity to contribute with the scientific progress in the pharmacology field. Natural products, in general, present a great biodiversity yet to be understood. Many of the chemical constituents of essential oils work synergistically to promote its effect, so it is necessary to study these complex mixtures to elucidate their true potential.

Due to its CNS action, essential oils are an alternative for the treatment of depressive and anxiety disorders. Mood disorders, which are those, in general, related to depression or euphoria, and anxiety disorders, which implicate in an abnormal regulation of fear, it consists of the most severe mental disorders. In both cases, the main symptoms, involves an emotional component, which reflects on physiological, behavioral and cognitive changes [5].

Depression

According to the symptoms, the previous history of the disorder, the pattern of family transmission and the response to treatment, depression can be classified into two categories: unipolar depression, which is diagnosed in people who present only depressive episodes, and bipolar disorder, whose diagnosis occurs in people who have both depressive and mania episodes [4,7]. In this chapter we will emphasize the unipolar depressive disorder.

According to the Diagnostic and Statistical Manual of Mental Disorders (DSM), unipolar, episodic, primary depression, which is at least two weeks long, is classified as major depression. This type of depression may have an early onset, promoting a number of changes in the patient's life quality, and the way more severe forms involve psychological symptoms, including delusions and hallucinations, the most serious outcome of which is suicide. According to

the report published in 2014 by the World Health Organization (WHO), each 40 seconds a person commits suicide in the world. Brazil appears as the eighth country with the highest number of cases. What makes these data more alarming is that more than 90% of suicides are associated with mental disorders, being depression the major cause. The WHO still reported that about 322 million people across the globe suffer from depression, and the estimate is that by 2020 it will be a more disabling disease in the world, demonstrating that this disorder deserves increased attention [5,7,9].

Some aspects may favor the probability of the individual developing depressive episodes, among them are temperamental factors, whose negative affectivity is the most relevant; environmental factors, especially adverse experiences during childhood, mainly stressful events; genetic and physiological, in which first-degree relatives of depressed individuals are more likely to develop the disease; and course modifiers, in which basically any non-mood disorder may increase the individual's risk of developing depression [5].

However, the major cause of depression is related to a deficiency in monoaminergic synaptic transmission. The monoamines, serotonin, noradrenaline, and dopamine are synthesized in small nuclei of neurons present in the brainstem from a precursor amino acid, and can be stored in synaptic vesicles or metabolized by the enzyme monoamine oxidase (MAO). These nuclei project themselves to the whole brain, as also emits projections to the spinal cord. This connectivity allows the monoaminergic neurons to establish coordinated responses and to influence several functions, such as alertness, motivation, attention, among other cognitive and emotional states [4,8].

Once released into the synaptic cleft, these neurotransmitters are able to bind to their respective receptors and promote a cascade of intracellular reactions or suffer reuptake by transporters present in the presynaptic membrane [4,8].

The main drugs used to treat depression have the monoaminergic neural systems as target. Its main purposes are to inhibit MAO, preventing monoamines degradation, and to inhibit the monoamine transporters, promoting their greater availability in the synaptic cleft [8].

Anxiety

Anxiety disorders encompass disorders that share characteristics of both fear and excessive anxiety, as well as their related emotional disturbances. Fear can be defined as a psychological, behavioral and cognitive response that occurs through a threat stimulus, resulting from this stimulus a transient adaptive response. In parallel, anxiety is a long-term response to danger stimuli, and

these stimuli may be signaled by well-defined circumstances of the danger, or by vague indications that there may be consequences that harm the individual. In this context, the absence of a sense of security can cause the signs of anxiety to persist. When anxiety extends even in the absence of real risk, or through an exacerbated response to a possible threat, it can generate distress and make the individual incapacitated [4,5].

According to the report published in 2017 by the WHO, Brazil has the highest prevalence of anxiety disorders in the world. About 9.3% of Brazilians have some anxiety disorder. The growth of this disorder is registered mainly in countries with low income, because with the population growth more people arrive at the ages in which the anxiety is frequent. Approximately 264 million people worldwide suffer from anxiety disorders. Of this total for the year 2015, there was a substantial increase of 14.9% over the year 2005. This shows that mood disorders are being widely misinterpreted, and many affected individuals are being stigmatized and not given proper attention by health systems. Epidemiological research also shows that these disorders have had a huge economic impact, in which their early onset influences both the learning ability of the young individual and the working ability of adult individuals [9].

Anxiety disorders are distinguishable from each other through the types of objects or situations that cause fear, anxiety, avoidance behavior or associated cognitive ideation. Currently, they are categorized into panic disorder, posttraumatic stress disorder, generalized anxiety disorder, social anxiety disorder, simple phobias and obsessive-compulsive disorder.

As with depression, first-degree relatives of individuals with anxiety disorders are more likely to have this disorder than the rest of the population. Studies have shown that the progress of some of the anxiety disorders is defined primarily by genetic inheritance [4,5].

Treatment

Anxiety disorders are commonly treated through pharmacological therapy and psychological treatment methods.[10] Pharmacotherapy is the first line of treatment for this condition, but many patients continue to be symptomatic and are commonly submitted to a combination of drugs.[11] The great range of side effects and high cost of this treatment it is a problem not only for patients but also for public health.

Aromatherapy

Complementary therapies and alternative medicine are interventions that have been noticed as effective as conventional pharmacotherapy, an increasing

number of patients with anxiety disorders are being treated by this alternative therapy with or without conventional treatment [12].

Due to the constant need of identify new treatments for anxiety disorders in recent years. Aromatherapy has reemerged in the alternative medicine field. Many reports have been made about its effectiveness in reducing stress and improving mood [13]. Furthermore, a large number of studies have shown promising results for the relief of depression, anxiety and other stress-related illnesses. Aromatherapy is the part of phytotherapy based on the therapeutic power of aromatic plants that uses their essential oils, by inhalation, application or baths, helping to relief health problems and improve quality of life in general [14].

Aromatherapy has been applied extensively throughout the world for mental disorders treatment [15,16]. Ancient civilizations such as Egypt, China and India have used aromatherapy as an alternative therapy for at least 6,000 years [17]. Today, many researchers believe that the inhalation of aromas may represent a possible alternative to the traditional pharmacological treatment of anxiety, and in fact, it is already being used in several conditions, not only on anxiety, but in other disorders as depression and chronic pain [14].

The administration of substances by inhalation can exert psychopharmacological action quickly, once the essential oils molecules, when inhaled, are processed by the vomeronasal organ, directly reaching the limbic system and hypothalamus via olfactory bulb. These signals stimulate the release of neurotransmitters such as serotonin (5-HT) and endorphin. 5-HT, which is derived from the tryptophan, is stored in vesicles in the presynaptic neuron and once stimulated by the nerve impulse, will be released into the synaptic cleft. Then, a 5-HT will act on its metabotropic receptor to regulate gene expression. Stimulation by essential oils can inhibit 5-HT uptake via serotonin transporters as well as the feedback control of the 5-HT release via 5-HT_{1B} regulatory auto-receptor in the presynaptic neuron. This contributes to the relief of mood disorders like stress, anxiety and depression [16,17].

On the other hand, gaseous or volatile drugs, when administered by inhalation, can also have alveolar absorption from the respiratory tract, with consequent systemic effects. The volatile molecules pathway, via the respiratory tract, allows psychoactive drugs to enter the bloodstream quickly, via the pulmonary route. An extra inspiration may produce a marked increase in the concentration of the drug in the arterial blood, which is carried directly to the brain [18]. Many effects from aromatic plants and their essential oils have been reported, and central activity is intensely documented, with evidences of sedative, anxiolytic and antidepressant effects [19].

2. Essential Oils, isolated constituents and its anxiolytic or antidepressant actions

A search conducted on electronic databases PubMed, SciELO and Periódicos CAPES, was delimited using the following criteria: Articles published in the last 5 years (2012-2017); studies with animals and humans, regardless of gender and route of administration: inhalation. The descriptors were: *"anxiety", "antidepressant", "aromatherapy"*, "*inhalation*" and *"essential oil"*.

Table 1 represents plants that have essential oils in its composition and Table 2 represents isolated constituents of essential oils. In table 1 columns describe plant species, time of exposure to essential oil, the animal species studied, tests used to assess the anxiolytic and/or antidepressant activity, the observed effect (if anxiolytic and/or antidepressant), the possible mechanism of action of essential oil, the effective doses tested, the possible alteration in the motor activity and main constituents of the essential oil. Table 2 described the main information of the principal constituent of each essential oil studied as: time of exposure of the animal/individual to the constituent by inhalation, the tests used to assess the anxiolytic and/or antidepressant activity, the effect observed (if anxiolytic and/or antidepressant), possible mechanism of action, effective dose tested and effect of the constituent on locomotion. The doses described represent values that have effectively altered the parameters tested; some papers cited in the tables reported additional doses, however they were not included in the table, if no effect was reported. Articles that did not present behavioral tests or possible mechanism of action were not considered. Only tests related to anxiolytic and/or antidepressant activities were describe in the tables.

The following plants containing essential oils described in Table 1 are: *Asarum heterotropoides, Cananga odorata, Chamaecyparis obtusa, Chamaemelum nobile, Citrus junus, Citrus limon, Citrus sinensis, Coriandrum sativum, Lavandula angustifolia, Lavandula hybrida, Litsea glaucescens, Matricaria recutita Ocimum gratissimum, Ocimum sanctum L. and Ocimum basilicum L., Piper guineense,* and *Pimpinella peregrina, Salvia sclarea L., Salvia miltiorrhiza, Santalum album, Thujopsis dolabrata, Vetiveria zizanioides*. The main constituents of the essential oils described in Table 2 are: Benzyl benzoate, linalool and bezylic acid, R-(-)-linalool, 3,5-Dimethoxytoluene, (+) – limonene and α-pinene.

Table 1. Principal data of the anxiolytic and / or antidepressant activity of essential oils obtained from vegetable species studied

Aromatic plant	Route of administration	Animal species	Model used	Observed effect	Mechanism of action	Effective tested doses	Effect on motor activity	Major Constituents	Reference
Asarum heterotropoides	Acute inhalation	Mice (male)	Forced swimming test / Tail suspension test	Antidepressant	↑ Expression of 5-HT neurons in the raphe nucleus and ↑ of CRF expression in the paraventricular nucleus	1 e 2g	Not evaluated	Methyl Eugenol	[2]
Anthriscus nemorosa	Inhalation (21 consecutive days)	Rats (male)	Y-maze and radial arm-maze test/ Forced swimming test	Anxiolytic / Antidepressant	---	1% e 3%	Not evaluated	---	[20]
Cananga odorata	Acute and 7 days inhalation	Mice (male and female)	Open field test / elevated plus maze test/ light and dark box test	Anxiolytic after chronic exposure, only in males	↓ of dopamine in striatum and ↑serotonin in hippocampus	Oils diluted in 10 ml of emulsion with 1% tween 80 water solution	No change	Benzyl Benzoate (20.25%), Linalool (11%), Benzyl Salicylate (9.53%), Benzyl Alcohol (9.1%), Benzyl Acetate (7.48%), Geraniol (6.79%) and Methyl Benzoate (6.08%)	[21]

Aromatic plant	Route of administration	Animal species	Model used	Observed effect	Mechanism of action	Effective tested doses	Effect on motor activity	Major Constituents	Reference
Chamaecyparis obtusa	Inhalation (7 consecutive days)	Rats	Elevated plus maze test	Anxiolytic	Prevents increased expression of *CCL-2* and *IL-6* in hippocampus	16 µl/ per cage (inhalation 1 h/day) and 27 µl/ per cage (inhalation 2h/day)	Not evaluated	A-Pinene, B-Pinene, B-Phellandrene, Terpinyl Acetate, δ-3-Carene, Limonene, P-Cimene, Camphene, A-Terpinolene and Others	[22]
Chamaemelum nobile, Matricaria recutita L	Inalação (14 continuous days)	Rats (male)	Open field test/Forced swim test	Antidepressant	↑ Expression of parvalbumin mRNA in hippocampus	400 µL	No change	A-Pinene	[23]
Citrus junus	Acute inhalation	Mice (male)	Open field test/ Elevated plus maze test/ Light/dark box test	Anxiolytic	---	3,4 e 6,7 mg/L	Increased activity with essential oil and no change with (+) -limonene	(+) -Limonene	[24]
Citrus junus	Acute inhalation (in two moments)	Woman	HRV power spectral analysis - Profile of Mood States (POMS)	Anxiolytic	- ↓ Sympathetic activity and ↑ parasympathetic activity	10 µl de yuzu essential oil	Not evaluated	Limonene (78.02 %), γ-Terpinene (9.32 %)	[25]
Citrus limon (L.)	Acute inhalation	Mice (male)	Elevated plus maze test / Open field test	Anxiolytic	---	200 e 400 µL	Not evaluated	A-Pineno, B-Pineno, Mirceno, *Trans*-Geraniol, γ-Terpineno and (-)-D-Limoneno,	[26]

Aromatic plant	Route of administration	Animal species	Model used	Observed effect	Mechanism of action	Effective tested doses	Effect on motor activity	Major Constituents	Reference
Coriandrum sativum var. microcarpum	Acute inhalation (60 min)	Rats (male)	Forced swimming test/ Elevated plus maze test	Anxiolytic / Antidepressant	---	1% e 3%	Increased	Linalool (69.36%), γ-Terpinene (7,73%)	[27]
Lavandula angustifolia and *Lavandula hybrida.*	Inhalation (60 min for 7 continuous days)	Rats (male)	Y-maze, elevated plus-maze, forced swimming, radial arm-maze	Anxiolytic / Antidepressant	---	200 µL	Not evaluated	*L. Angustifolia Linalool* (28.0%), Linalyl Acetate (17%,) Terpinen-4-Ol (3.3%), Lavandulyl Acetate (8.3%) *L. Hybrid Linalool* (21.5%), Linalyl Acetate (22.5%), Terpinen-4-Ol (16.7%), Lavandulyl Acetate (8.4%)	[28]
Lavandula angustifolia, Salvia sclarea L., Santalum album, and *Citrus sinensis*	Inhalation (45 min for 10 continuous days)	Rats (female)	Elevated plus-maze	Anxiolytic	Metabolic alterations and gabaergic	---	Not evaluated	Linalool and Linalyl Acetate	[29]
Lavendula augustifolia	Inhalation	Sheep (female)	Test agitation score, crosses over the central lines of the isolation box, vocalizations.	Anxiolytic in calm sheep with no effect on nervous sheep	---	10%	Reduced in calm sheep and increased in nervous sheep	---	[30]

Aromatic plant	Route of administration	Animal species	Model used	Observed effect	Mechanism of action	Effective tested doses	Effect on motor activity	Major Constituents	Reference
Lavandula angustifolia	Inhalation	Mice (male)	Elevated Plus-Maze Test and Marble-burying test	Anxiolytic	Serotonergic but not through $GABA_A$/ benzodiazepine receptors	5%	No change	Linalool (46.5%) and Linalyl Acetate (53.5%)	[31]
Lavandula angustifolia	Inhalation	Mice (male)	Marble-burying test	Anxiolytic in anomic animals	---	5%	No change	Linalool (46.5%) Aad Linalyl Acetate (53.5%)	[32]
Ocimum gratissimum L.	Acute inhalation	Mice (male)	Open field / Light/dark transition / Tail suspension	Anxiolytic / Antidepressant	---	4×10^{-4} mg (anxiolytic) / 4×10^{-6} mg (antide-pressant)	No change	Timol (68%)	[33]
Ocimum sanctum L. and *Ocimum basilicum L.*	Inhalation (21 consecutive days)	Rats (male)	Forced swimming test/ Elevated plus maze test	Anxiolytic / Antidepressant	---	OS: anxiolytic and anti-depressant: 1% e 3% OB: Anxiolytic 3% Anti-depressant 1% e 3%	No change	OB: Camphor, β -Elemene , Bornyl-Acetate, Estragole (15.57%); Eugenol (2,64%); 1,8-Cineole (3,29%) OS: Camphor, β-Elemene, Bornyl-Acetate, Estragole (7,59%); Eugenol (1,39%); 1,8-Cineole (3,9%)	[34]

Aromatic plant	Route of administration	Animal species	Model used	Observed effect	Mechanism of action	Effective tested doses	Effect on motor activity	Major Constituents	Reference
Pimpinella peregrina	Inhalation (21 consecutive days)	Rats (male)	Y-Maze Test / Radial Arm-Maze Test / Forced Swimming Test	Anxiolytic / Antidepressant	Anxiolytic effect through the GABAergic pathway ($GABA_A$ receptor). Antidepressant effect by increasing 5-HT and norepinephrine.	---	200 µL of P. *peregrina* 1% and 3% for 15 min	Trans-Pinocarveol (35.1 %), Pregeijerene (15. 1 %), A-Cubebene (12.4 %), (+)-Epi-Bicycloses-quiphellandrene (7.5 %), A-Terpineol (6.7 %)	[35]
Piper guineense	Acute inhalation	Mice (male)	Open field / Light/dark transition	Anxiolytic	---	Anxiolytic 4.0×10^{-6} mg per cage	Decreased	Linalool (41.8%); 3,5-Dime-thoxytoluene (10.9%)	[36]
Salvia species (Clary sage oil)	Acute inhalation	Meno-pausal women	Beck Depression Inventory-I e II (KBDI-I e KBDI-II) e Korean version of Self-rating Depression Scale	Antidepressant	Decreased cortisol levels and increased 5-HT levels	Inhalation of 0.1 ml of oil per 5 minutes	Not evaluated	Linalyl Acetate (63.99%); Linalool (20.99%)	[37]
Santalum album L.	Acute inhalation	Mice (male)	Elevated Plus-Maze Test	Anxiolytic	Not evaluated	4 µL/air	Not evaluated	Z)-A-Santalol (51.1%); (Z)-B-Santalol (28.5%)	[24]
Thujopsis dolabrata	Inhalation	Rats (male)	Elevated Plus-Maze Test	Inhibition of stress-induced anxiety	Not evaluated	50 µL	Not evaluated	---	[38]
Vetiveria zizanioides (L.)	Acute inhalation	Rats (male)	Elevated Plus-Maze Test	Anxiolytic	Increased expression of c-fos in the central amygdaloid nucleus	2.5% for 7 minutes	Not evaluated	---	[39]

Table 2. Essential data of the anxiolytic and / or antidepressant activity of isolated chemical constituents of essential oils

Oil essential	Route of administration	Animal species	Model used	Observed effect	Mechanism of action	Effective tested doses	Effect on motor activity	Reference
Benzyl benzoate, linalool and benzylic acid	Inhalation (acute and chronic)	Mice (male)	Open field test / Elevated plus maze test/ Light and dark box test	Anxiolytic in males only	↓ of dopamine in the striatum and ↑ serotonin in the hippocampus	Benzyl benzoate 2%, Linalool 0.55%, benzylic acid 0.45%	Benzyl benzoate and benzylic acid: no effect. Linalool decreased	[21]
R-(–)-Linalool	Acute inhalation	Mice (male)	Open field test	Sedative	Unvalued	4.0×10^{-5} and 4.0×10^{-3} mg/ per cage	Decreased	[36]
3,5-Dimethoxytoluene	Acute inhalation	Mice (male)	Open field test	Sedative	Unvalued	4.0×10^{-5} and 4.0×10^{-2} mg/ per cage	Decreased	[36]
(+)-limonene	Inhalation	Mice (male)	Elevated plus maze test	Anxiolytic	Not gabaergic	0.5% e 1.0%	Not evaluated	[40]
α-pinene	Chronic inhalation	Rats (male)	Open field test / Forced swimming test	Antidepressant	↑Expression of parvalbumin mRNA in the hippocampus	400 µL	No change	[23]

3. Discussion on essential oils, their chemical constituents and their use in depression and anxiety

Anxiety is an extremely prevalent disorder among people and this state may be related to external or internal stimuli, which results in a set of behavioral, physiological, hormonal and autonomic reactions [41]. Stress would be the main triggering factor for anxiety states, which varies, depending on the nature, duration, intensity and age of perception of the stimulus. Symptoms vary, since muscular tension, sleep disturbances, fatigue, concentrating difficulty, irritability, restlessness and excessive worry for an extended time.

Depression is a disease that affects one in ten people at least once in a lifetime and with enough intensity to need drug treatment [42]. There is an intersection between depression and anxiety. Although they are different disorders, anxiety is the main cause of depression [43].

Symptoms of depression include anxiety, anhedonia, fatigue, sleep and cognitive disorders, suicidal thoughts, among others. In addition to the symptoms, there is an increased vulnerability to cardiovascular diseases, gastrointestinal disorders, type 2 diabetes and dementia. The triggering factors of depression are diverse and depend on the individual's age. Severe stressful events, hormonal changes, nervous degeneration among several other factors, contribute to its appearance [2, 22].

The neural circuitry triggering anxiety activation involves the following structures: the central nucleus of the amygdala (CeA), the paraventricular nucleus of the hypothalamus (PVN), the dorsomedial hypothalamic nucleus (DMH), the ventromedial hypothalamic nucleus (VMH), the lateral hypothalamic area (LH), the striated and caudate-putamen (CP), the nucleus accumbens (AC) and the acumbens shell (AS) [44]. Some studies corroborate the role of the amygdala in anxiety. Saiyudthong and collaborators [39] evaluated the expression of the *c-fos* gene, a marker of neuronal activity in the central amygdaloid nucleus, by subjecting mice to 2.5% vetiver essential oil inhalation prior to the elevated plus maze test. Vetiver oil in mice increased the expression of *c-fos* after the elevated plus maze test similarly to the standard, diazepam, indicating an anxiolytic activity. It is interesting to mention that only the central amygdaloid nucleus showed an increase in *c-fos* expression, and no other part of the amygdala. In this study, other parts of the brain associated with anxiety were not investigated.

The major regions affected in depression are: right and left amygdala, pregenual anterior cingulate cortex, raphe nucleus, hippocampus, and prefrontal cortex [45].

There is a wide variety of drugs used to treat anxiety, being benzodiazepines, often the first choice. Although they are relatively safe, continued use may lead to tolerance and loss of efficacy. The adverse effects associated with its use, include muscle relaxation, mild amnesia, dose-dependent sedation, dependence,

abstinence symptoms related to drug withdrawal [46]. These effects prevent some day-to-day activities from being carried out safely, such as driving vehicles. Benzodiazepines act through $GABA_A$ receptors, leading to CNS depression [35].

Other classes of drugs used to treat depression are serotonin reuptake inhibitors (SSRIs), serotonin and noradrenaline reuptake inhibitors (SNRIs), serotonin and noradrenaline reuptake inhibitors (SNRIs) and monoamine oxidase inhibitors (MAOIs).

Several studies have reported, consistently, reduced levels of dopamine and its metabolites in depressed patients. A previous study has shown that antidepressant drugs improve dopaminergic function through increasing D2 receptor expression and the release of dopamine in the synaptic cleft [47]. Two serotonergic receptors stand out in studies involving the pathophysiology of depression: the 5-HT1A receptor and the 5-HT2A / 2C receptor. Many studies have suggested that the activation of 5-HT2 receptors may be related to the regulation of mood disorders. These receptors distributed through cerebral structures important in the pathophysiology of depression, such as the prefrontal cortex and the hippocampus [48]. The inclusion of the 5-HT1A receptor antagonists and 5-HT2C also found support in the literature as effective in the treatment of depression [49]. The monoaminergic hypothesis postulates that depressed patients have a deficit in serotonin levels, however, this effect is usually related to other factors such as the corticotropin-releasing factor.

The inhalation of *Asarum heterotropoides* essential oil was evaluated using behavioral forced swimming and the tail suspension tests. Immunohistochemical analysis revealed that the oil of *A. heterotropoides* 1% increased serotonin levels in the raphe nucleus and reduced levels of tyrosine hydroxylase in the locus ceruleus and also corticotropin releasing factor (CRF) in the paraventricular nucleus, which would explain the reduction in depressive behavior of the animals [2].

Similarly, Chioca and collaborators [31] used in their study the inhalation of the essential oil of lavender (*Lavandula angustifolia*) in mice and verified an anxiolytic-type effect in the elevated plus-maze and marble-burying tests of this essential oil at 5% concentration when compared to the control group. The mechanism of action suggested is the participation of the 5-HT1A serotonergic receptor, since they found that the 5-HT1A serotonergic receptor antagonist (WAY100635) blocked the effect of the essential oil and serotonergic receptor agonist (8-OH-DPAT). On the other hand, pre-treatment with the $GABA_A$ receptor antagonist did not alter the behavior of the animals treated with the essential oil in the marble-burying test. In addition, lavender oil did not modify the [3H] flunitrazepam binding of the $GABA_A$ receptor at the benzodiazepine-binding site. Regarding the locomotor activity of the animals, this was not altered when compared to the control group.

These anxiolytic-type effect findings without alteration of the locomotor activity are corroborated by previous studies [50, 19, 51 and 52] and the authors suggest that the major constituents linalol (46.5%) and linalyl acetate (53.5%) are the main responsible for this anxiolytic effect via the 5-HT1A receptor.

The *Cananga odorat*a plant has an essential oil called ylang-ylang, recognized for its activity on cognitive functions and studied by Zhang and colleagues [21]. In this study, the researchers tested the anxiolytic effect of essential oil and three isolated compounds (benzyl benzoate, linalool e benzyl alcohol) in male and female mice. Males presented anxiolytic effects after acute or chronic inhalation of essential oil and an isolated constituent (Benzyl benzoate), but females did not. In this study, the authors measured levels of serotonin in the hippocampus and dopamine in the striatum of male and female mice. Zhang and collaborators [21] concluded that benzyl benzoate could enhance serotonin synthesis and the activation of serotonergic neurons could suppress dopaminergic neurons. It is important to mention that women are apparently more likely to be affected by emotional nature of disorders and stress-related [53]. Several studies seek to relate hormonal variations of females with mood swings probably associated with 17β-estradiol levels however the results are inconclusive [54, 55, 21].

Some essential oils have also been shown to be capable of increasing plasma serotonin levels in animals and humans, as clary sage oil. This oil, in addition, increased serotonin levels, and reduced cortisol and TSH levels in women [37]. In this study, the authors used 5-HT, cortisol and TSH as biomarkers in menopausal women with no serious diseases. Depressed patients have high levels of cortisol and TSH, consequence of a hypothalamic-limbic activation, with an increase in CRH and consequent increase of cortisol. TSH levels of depressed people are lower because of deficient secretion of TRH. If thyroid hormones are deficient, the humor is blunt. This group was chosen because menopause women may have memory problems, depression, headaches, anxiety and hot flushes. The aim was to demonstrate that the essential oil was able to elevate the biomarkers and alleviate the symptoms of the menopause.

There is an important factor in the control of anxiety: the autonomic nervous system. This system regulates all physiological parameters in the human body and is important to maintain homeostasis. Stress is a factor related to anxiety and stressful stimuli can activate diverse pathways such as endocrine and neuroendocrine [56]. (It is well described that the activity of the HPA axis (Hypothalamus-pituitary-adrenal) is increased in depressed patients. The release of both CRH and vasopressin promotes the release of ACTH by the pituitary that leads, consequently, the release of cortisol. Cortisol has potent immunosuppressive actions. In association with this axis, there is also the

activation of the sympathetic-adrenal axis, raising levels of epinephrine and norepinephrine [56]. The elevation of these catecholamines affects, among other factors, heart rate and heart rate variability (HRV). HRV is a general parameter used as a marker of sympathetic and parasympathetic activity of the myocardium. Some studies have used the heart rate variability (HRV) as a parameter to establish the anxiolytic activity of oils such as yuzo (*Citrus junus*). This study, conducted by Matsumoto and colleagues [25], with young women (between 20 and 30 years) at two different moments of the menstrual cycle (follicular and late luteal phases) tested the inhaling influence of yuzu oil for 10 minutes, compared to water inhalation (control). The results of the experiments confirmed the effect of essential oil in reducing the stress of the test group by increasing the activity of the parasympathetic system and reducing the effect of the sympathetic system. The activity of yuzo oil could be important in reducing the symptoms of premenstrual syndrome and premenstrual dysphoric disorder.

The mechanism of action of essential oils and their constituents can involve cell mechanism involving different pathways of monoamines and their receptors, in addition to the HPA axis.

Several evidences have shown that mitochondrial dysfunction and parvalbumin could be relevant factors to the pathogenesis of depression, among other neuropsychiatric disorders [58, 57]. The study of Kong [23] found evidences that support these previous studies by identifying three proteins in the hippocampus and four proteins in the prefrontal cortex after inhalation treatment with a component of the essential oil of the roman chamomile, the α-pinene.

Studies that are more recent have shown that depression may be a result of oxidative phosphorylation in cells present in the hippocampus and frontal cortex, structures related to depression [58]. Cytochrome C oxidase 6C-2 subunit and cytochrome C oxidase 7A2, inhibitor of ATPase in the hippocampus and cytochrome C oxidase 6C-2 subunit, subunit of ATP synthase and the acyl carrier protein and subunit 6 of the cytochrome b-c1 complex found in the prefrontal cortex were expressed in greater numbers (up-regulation) when rats were treated for 14 days with α-pinene. The behavioral results of the open field and forced swim tests were superior when compared to control animals, showing an antidepressant effect. This result suggests that there is a relationship between the variation of the expression of proteins involved in oxidative phosphorylation and depression. This same study, Kong et al. [23] verified that the protein parvalbumin and their mRNA would be present in greater amounts when stimulated by the α-pinene in GABAergic interneurons present in the hippocampus, compared to control animals. This finding finds support in studies that found parvalbumin is lower in depressed patients [59]. Treatment with antidepressants could reverse

this situation [60]. Parvalbumin is a slow calcium binding protein present in GABAergic neurons that helps to control calcium levels during neuronal firing [61].

There are several substances derived of plants containing essential oils that can modulate the activity of $GABA_A$ receptors. Recently, Aydin and collaborators [35] published an article reporting the activity of the *Pimpinela peregrina*, demonstrating through the inhalation of the oil, an activity similar to anxiolytic and antidepressant drugs. The isolated constituents that could have pharmacological activity probably are: trans-pinocarveol, pregeirejerene and α-cubene. Anxiolytic activity was demonstrated by the elevated plus maze test and using diazepam as a positive control that acts on GABAergic type A receptors. Antidepressant activity was demonstrated in the forced swim test using tramadol, a positive control drug capable of inhibiting the reuptake of serotonin and noradrenaline.

The (+) - limonene is an isolated constituent isolated and presented anxiolytic effect, but not via the $GABA_A$ receptor pathway at the benzodiazepine binding site. At concentrations of 0.5 and 1.0%, (+) - limonene increased the number of entries and the permanence time of the animals in the open arms of the elevated plus maze test, effect not observed in the concentration of 2.5%. However, flumazenil - an antagonist of the of the $GABA_A$ benzodiazepine receptor was not able to block the anxiolytic effect observed in the animals treated with (+)-limonene 1%, thus suggesting that this GABAergic binding site is not involved in this behavior. The authors worked with the hypothesis what the negative result found in the highest dose tested (2.5%), perhaps can be attributed to a specific effect of day and associating that, the animal variability [40]. The Souto-Maior study [62] carried out with linalool oxide found similar results, that is, the highest concentration used did not present the best behavioral result.

Advances in studies of anxiolytic and antidepressant activity with essential oils and their chemical components by inhalation can be found in studies of pro-inflammatory cytokines and their relationship to the HPA axis. The hypotheses cited above are valid, however, there is a confluence of factors where inflammatory cytokines may alter the transmission of monoamines, glutamate, among other excitatory neurotransmitters, increase the resistance of CRH receptors to influence neurogenesis of hippocampal cells in adults, influencing the response of antidepressants [45]. In depressed patients, occurs a hypoactivity of glucocorticoid receptors, which could at least in part explain the poor effect of glucocorticoids in depression [63]. Usually, glucocorticoids inhibit the production of inflammatory cytokines as part of the negative feedback of the HPA axis. This inhibition, however, appears to be dysfunctional during both chronic and acute depressive episodes [64]. The increase of cytokines *IL-1, IL-2, IL-4, IL-6, TNF*-α and *INF*-γ, promote

the release of CRH leading to an overstimulation of the HPA axis, resulting in an increase of cortisol [64]. During depression states, both cortisol and pro-inflammatory cytokines are increased, which suggests a failure in the regulation of HPA axis feedback mechanisms [63]. High levels of circulating corticosteroids in plasma appear to be unable to inhibit the production of pro-inflammatory cytokines in depressed patients, suggesting a hypoactivity of the glucocorticoid receptors in the cells of the immune system [65]. Antidepressants appear to be able to increase the sensitivity of glucocorticoid receptors [66]. The study carried out by Park e collaborators [22] with the plant *Chamaecyparis obtusa*, to verify the anxiolytic activity using the elevated plus maze test. Found that the essential oil was able to reduce the expression of 5 genes including *IL-6* and *CCL-2* proteins in the hippocampus of rats undergoing maternal separation stress compared to control animals. The animals not treated with the essential oil (control) and that underwent the separation stress, experienced an increase in the expression of *IL-6* and *CCL-2*. Elevated levels of cytokines (*IL-1β, IL-6*) and chemokines (*CCL-2* and *CCL-3*) were found in the plasma of depressed animals submitted to the same model of maternal separation [67]. *IL-6* is a cytokine widely studied and there is a correlation between their levels and the severity of depression [68]. A reduction of its levels usually occurs in the presence of antidepressants [69]. Similarly, increased levels of *CCL-2* can be found in depressed patients and their levels are reduced when the patient are treated with antidepressants [70,71].

The search for the anxiolytic and / or antidepressant therapeutic potential of aromatic plants, essential oils and its constituents isolated through the inhalation route, in animal models and in humans, has been intensified in recent years, as we have seen in this chapter. A more recent approach is the metabolomics research, which has established itself as a successful analytical tool, can capture the subtle metabolic changes, either in tissue samples or bio-fluids (plasma, urine and cerebrospinal fluid) resulting from exposure to essential oils and their constituents isolated and provide the basis for locating the pathways of the behavioral effect of these molecules.

Recently in 2012, Wu and colleagues [29] have published an important work, which demonstrates the advances in research in aromatherapy: they conducted a behavioral animal study and metabolomics analysis through rigorous analytical methods, which captured their real impact on animal biology, with gas chromatography time-of-flight mass spectrometry (GC-TOFMS). This study detected that the inhalation of a mixture of essential oils obtained from four different aromatic plants (*Lavandula angustifolia, Salvia sclarea L., Santalum álbum e Citrus sinensis*) for 10 consecutive days, induced endogenous metabolic changes in both urine and brain tissue of animals; as well as behavioral alterations in

the anxiolytic-type elevated plus maze test. Among the metabolic changes in the brain tissue, they showed increased levels of carbohydrates and low levels of neurotransmitters (tryptophan, serine, glycine, aspartate, tyrosine, cysteine, phenylalanine, hypotaurine, histidine and asparagine), amino acids and fatty acids. Other studies have shown that carbohydrates have anxiolytic effects [72] and significantly higher levels of carbohydrates in this study, can be due to the therapeutic effects of essential oils on anxiety. The metabolism of carbohydrates promotes the oxidation of fatty acids [73] and, fatty acids can also be synthesized from carbohydrates. A decrease in fatty acids in brain tissue may be due to increased levels of carbohydrates after inhalation of essential oils. It is believed that the decrease in brain histamine may result in higher levels of anxiety [74]. Phenylalanine can be biosynthesized in phenylethylamine (PEA) by enzymatic decarboxylation and, high levels of PEA are observed in anxious individuals [75]. Increased histamine and decreased phenylalanine in the brain after inhalation of aromas further support the therapeutic effects of essential oils on anxiety. It is insightful to point out that because of the blood-brain barrier, brain metabolism is independent of peripheral circulation and, as a result, the metabolic alterations induced by the inhalation of aromas were different between urine and brain tissue. There have been observed in urine: elevation of aspartate, carbohydrates (sucrose, maltose, fructose and glucose), nucleosides and organic acids like lactate and pyruvate. Excessive loss of metabolites in the brain can be observed through elevated levels of metabolites in the urine. After inhalation of essential oils, a large number of neurotransmitters in the brain decreased, although only aspartate has increased significantly in the urine of the animals.

Interestingly, the constituents of these various essential oils were not found in brain tissues and urine samples from rats, presumably because these organic compounds are volatile and have low concentration, making it difficult detection in bio-fluids and brain tissue. This metabolomics approach can capture light metabolic changes resulting of exposure to essential oils and provide the basis for locating the pathways affected in the anxiety/depression behavior helping and facilitating the mechanistic understanding of the effect of aromatic plants, essential oils and their isolated constituents.

4. Conclusions

In conclusion, the existing data show that several essential oils and its isolated constituents present potential for their use in the treatment of anxiety and depression disorders. This statement finds support in the articles that demonstrated the possible mechanisms of action, using different animal models. Some anxiolytic mechanisms of action of current medications are well established,

such as increasing levels of serotonin in the hippocampus and reduction of dopamine in the striatum, as well as increasing noradrenaline or activating $GABA_A$ receptors or even increasing parasympathetic activity and reduction of sympathetic activity. Inhalation of essential oils and reduction of cortisol levels, increasing levels of serotonin and noradrenaline are also well reported effects in articles on antidepressant activity. However, there is evidence of anxiolytic effects by the reduction of pro-inflammatory cytokines such as *IL-6* and *CCL-2* and the increase of parvalbumin in depression demonstrating that there are other pathways involved in the pathophysiology of anxiety and depression. Some articles report that the altered metabolism of carbohydrate, lipid proteins (amino acids) can affect the production of neurotransmitters and promote anxiety disorders. Therefore, further studies are needed to group existing data and to draw a broad perspective on the treatment of anxiety and depression. The fact that there are essential oils that act through diverse mechanisms of action through the inhalation confers to this option of treatment a new horizon to be explored.

List of abbreviations

5-HT - 5-hydroxytryptamine
$5\text{-}HT_{1A, 2A, 2C}$ - Subtypes receptor -hydroxytryptamine
AC - Nucleus Accumbens
ACTH - Adrenocorticotropic hormone
AS - Acumbens Shell
c-fos - Proto-oncogene
CCL-2 - Chemokine C-C motif ligand 2
CCL-3 - Chemokine C-C motif ligand 3
CeA - Central nucleus of the amygdala
CNS - Central Nervous System
CP - Caudate-Putamen
CRF - Corticotropin releasing factor
DMH - Dorsomedial Hpothalamic Nucleus
DSM - Diagnostic and Statistical Manual of Mental Disorders
EO - Essential oils
GABA - γ-aminobutyric acid
GC-TOFMS - Gas chromatography time-of-flight mass spectrometry
HRV - Heart rate variability
IL-1 - Interleucina 1
IL-2 - Interleucina 2
IL-4 - Interleucina 4
IL-6 - Interleucina 6

INF-γ, - Interferon gamma
LH - Lateral Hypothalamic Area
MAO - Enzyme Monoamine Oxidase
mRNA - Messenger RNA
PEA - Phenylethylamine
PVN - Paraventricular Nucleus Of The Hypothalamus
TNF-α - Tumor necrosis factor- alpha
TSH - Thyroid-stimulating hormone
VMH - Ventromedial Hypothalamic Nucleus
WHO - World Health Organization

Conflicts of Interest

"The authors declare no conflict of interest".

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Chapter 10

Therapeutic potential of polyphenols on the cardiovascular system: focus on northeastern Brazilian grape byproducts and red wines

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1. Introduction

Cardiovascular diseases (CVDs), such as hypertension, coronary heart diseases and stroke, are the major cause of death worldwide. An estimated 17,7 million people died from CVDs in 2015, which represent 31% of all global deaths [1]. On the other hand, most CVDs can be prevented by addressing behavioral risk factors such as tobacco use, obesity and physical inactivity. In addition, healthy nutrition and pharmacological tricks also can be added in order to promote cardiovascular protection [2-4].

In fact, even in the presence of high fat consumption, the French population present low rate of mortality for CVDs, being known by "French paradox", which was explained, at least in part, by the regular consumption of red wine by the French people [5].

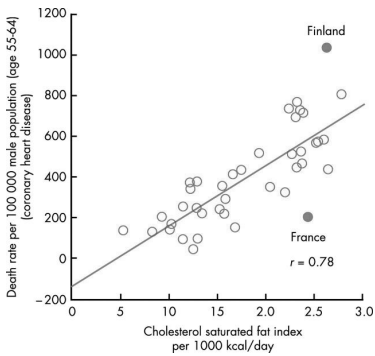

Figure [6].

The São Francisco Valley region on the Brazilian northeastern is a second largest producer of refined grapes and red wine in the country [7, 8]. Inserted in semi-arid tropical climate, at 8–9S (latitude) and around 40W (longitude), this region has specific features such as, hot climate, abundant water irrigation or drought, and light/UV intensity, which severely affect phenolic metabolism and increased, grape phenolic content [9, 10]. Chemical contents undergo variations which depend on some kind of factors, such as environmental, and technique used in vinification process [11].

Red wine or grape pomace, a winemaking byproduct composed of pressed skins, seeds, and stems have been assigned to high polyphenolic compound content. It has become an important economical alternative to wine industry [12-16]. High levels of polyphenolic compounds remain in the skin matrix of grape pomace after enological fermentation, and the use of adequate methods to breakdown the cell wall in grape pomace tissues is indispensable for effective phenolic content extraction [17].

Wine phenolic compounds include flavonoids and nonflavonoids. The most significant subclasses of flavonoids in red wine are anthocyanins (such as delphinidin-, cyanidin-, and malvidin-3O-glucosides), flavonols (myricetin, quercetin, and their corresponding glucosides), 2-dihydroflavonol derivatives (dihydroquercetin-3-O-rhamnoside and dihydromyricetin-3O-rhamnoside), and flavanols (cathechin, epicatechin, oligomeric, and polymeric procyanidin, also called condensed tannins). Red wine also contains several classes of nonflavonoid compounds, such as phenolic acids (protocatechuic acid, vanillic acid, gallic acid), hydroxycinnamic acids (such as p-coumaricacid,caftaric acid and caffeic acid) and stilbenes (cis- and trans-resveratrol being their major constituents and their corresponding glucosides acids, cis- and trans-piceid).

Today, more than 8000 polyphenols are defined [14]. They are synthesized by plants as secondary metabolites by two different biosynthetic pathways: the shikimate and the polyacetate pathways, which explain a high structural diversity. In addition, this structural diversity due to these two pathways is enhanced by the possibility of a simultaneous participation of both pathways to the development of flavonoids [15].

Several pathways probably contribute to the beneficial action of polyphenolic compounds on the cardiovascular system. Experimental evidences shown association with the platelet aggregation inhibition, decrease in low-density lipoprotein oxidation, reduction of endothelin synthesis, inhibition of smooth muscle cell proliferation, inhibition of expression of prothrombotic and proatherosclerotic molecules such as monocyte-chemoattractant protein-1, and increase in endothelial-type nitric oxide (NO)-synthase expression and activity

[16, 19-22]. However, the mechanisms underlying the cardioprotection effect elicited by red wine consumption is not fully understood.

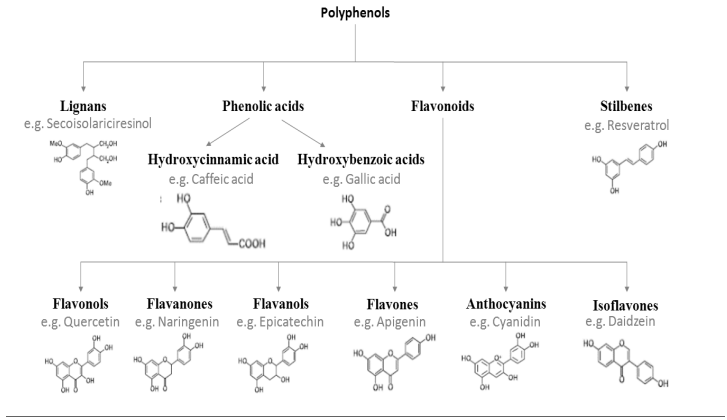

Figure adapted [18].

Despite the existence of several studies with wines from different parts of the world, the potential use of fermented and non-fermented grapes byproducts of winemaking (pomace) and red wine grape varieties of northeastern Brazilian as sources for novel therapeutics to prevent cardiovascular disease has received scant attention. In this chapter, we will compare the quantitative and the qualitative chemical composition of phenolic compounds in grapes byproducts and red wines from São Francisco Valley. In addition, we will summarized of the biological activity of phenolic extracts prepared from several red wine grape varieties and their fermented byproduct of winemaking (pomace) on the cardiovascular system, especially on the molecular mechanisms which involve the vascular endothelium.

2. Methods

2.1 Standards and Reagents

2.1.1 Grape/Red Wine GASH and RSCS

Phenylephrine hydrochloride (Sigma, cod. P6126), Acetylcholine chloride (Sigma, cod. A6625), Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME,

Sigma, cod. N5751), charybdotoxin (Sigma, cod. C7802), indomethacin (Sigma, cod. I7378), apamin (Sigma, cod. A1289), Dihydroethidium (Sigma, cod. D7008), 2,2-Diphenyl-1-picrylhydrazyl (Sigma, cod. D9132), Folin-Ciocalteu's (Sigma, cod. 47641), ascorbic acid,1H-[1,2,4] oxadiazolo[4,3-a]-quinoxalin-1-one (ODQ), atropine (Sigma, MO, United States), gallic acid, (Sigma-Aldrich®), 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI - Invitrogen Molecular ProbesTM cod. D1306),Dako Fluorescence Mounting Medium (cod. S3023), diaminofluoresceindiacetate (DAF-2DA, Calbiochem, Foster City, CA, USA), quercetin, myricetin, kaempferol from Cayman Chemicals (Ann Arbor, MI, USA), Na2CO3, NaCl, KCl, KH2PO4, NaHCO3, C6H12O6, CaCl2, MgSO4, ethylenediaminetetraacetic Acid (EDTA), ethanol, methanol, from Vetec®.

2.2 Obtainment and Preparation of extracts

2.2.1 Grape

The grape Vitisvinifera (L.) var. Petit Verdot from the São Francisco Valley, and used in the process of wine production was used in our studies. The samples were collected in two stages of the wine production: one type was supplied after separation of the must in the first step of fermentation (fermented grape), and the other type was unfermented grape sampled before the winemaking process. Both samples originated from the São Francisco river valley region in Brazil, and were provided by EMBRAPA as collected from the 2011 crop of a local vineyard. The skins from unfermented (UGS) and fermented (FGS) grape pomace, were manually separated from the remainder of the plant material and crushed. The material was submitted to the freeze-drying process, and then ground. The particle size was homogenized at lower than 0.71 mm mesh. The homogenized powder was submitted to an extraction procedure by ultrasound with a mixture of ethanol-water (50:50) used as the solvent; the liquid-to-solid ratio was 5g of dry weight per 100 milliliter of solvent [23-26]. The resultant extract was dried under vacuum pressure by rotary evaporator and then lyophilized for 24 hours under the vacuum pressure of 0.024 mbar (Free Zone 6 LABCONCO®) with prior freezing at -80°C. The dried samples were stored frozen at -22ºC prior the use in the experiments [27].

2.2.2 Red Wine GASH and RSCS

Preparation of the alcohol-free lyophilized red wine. The red wine from Cabernet Sauvignon (vintage 2006) was made from red grape varieties (Vitis vinifera L.) grown in the semiarid climate with high sunlight exposure in the São Francisco river valley and provided by the Winery Santa Maria (Lagoa Grande, Pernambuco, Brazil) and red wine GASH (Shiraz, 2005). To obtain an

alcohol-free lyophilized extract of RSCS and GASH, the wine ethanol was first evaporated under low pressure at 55 °C to obtain approximately 50% of the original volume. Then, the residual liquid was lyophilized using a LABCONCO Freezone1 lyophilizer (Kansas City, MO, USA), and kept at –20 °C until use [28].

2.3 Determination of the phenols content

2.3.1 Grape

The total phenolic content of the different lyophilized samples was determined using the Folin-Ciocalteu's (F-C) method, with gallic acid as a standard, in methanol medium. This method was based on the reaction of phenolic compounds with a colorimetric reagent detected spectroscopically at a wavelength of 750 nm [29, 30].Briefly, an aliquot of 100 μL of each sample, standard, or 95% (v/v) methanol blank, was added in duplicate to 2 mL microtubes; adding 200 μL 10% (v/v) of F–C reagent and vortexing thoroughly. In the next step, adding 800 μL 700 mM Na2CO3 to each tube and incubating the assay tubes at room temperature for 2 h; transfering 200 μL of sample, standard or blank from the assay tube to a clear 96-well microplate and reading the absorbance of each well at 750 nm. Solutions of gallic acid (ranging from 0.5 to 1 mg/mL) were analyzed in a similar manner to construct a calibration curve. Each sample was analyzed in triplicate and the total phenolic content was expressed as micrograms of gallic acid equivalent/milligram of dry weight (μg GAE/mg DW). Data are expressed as mean ± SEM [31].

2.3.2 Red wine GASH and RSCS

The total phenolics were determined by the Folin–Ciocalteau method, and the results were expressed in galic acid equivalent per liter of wine. The concentration of trans-resveratrol, cis-resveratrol, and quercetin in GASH and the concentration of quercetin, kaempferol and myricetin in RSCS both determined by previously validated methods [20].Briefly, the method used high performance liquid chromatography with a photodiode array detector with a C8 column of 15-cm 34.6-mm internal diameter and particles of 5.0-mm diameter and a C8 pre-column of 1.0-cm 34.0-mm internal diameter.

Samples were injected through a 100-mL loop. The mobile phase used was a mixture of MeCN: aqueous 0.1% formic acid (25:75, vol/vol) delivered at a flow rate of 2.0 mL/min. The detection of resveratrol (both cis and trans isomers) was at 285 nm, whereas quercetin was quantified at 370 nm and the detection of quercetin, kaempferol and myricetin was quantified at 370 nm. A photodiode array detector was used. The samples were analyzed in triplicate, and the concentration of phenolic was expressed as g/mL of red wine.

2.4 Chromatographic Determination of Phenolic Compounds

2.4.1 Grape

Reversed-phase high performance liquid chromatography (HPLC) method was used to analyze the phenolic compounds present in the samples; using the separation module (LC-20 AT, Shimadzu Corporation, Japan) equipped with a C18 column (Vydac, 218 TP, 250 4.6 mm, 5 μm particle size, Sigma–Aldrich, St. Louis, MO, USA). The samples were eluted with a gradient system consisting of solvent A (2% acetic acid, v/v), and solvent B (acetonitrile: methanol, 2:1, v/v), used as the mobile phase, at a flow rate of 1 mL/min. The samples (20 μL) were directly injected after filtration through a 0.45 μm membrane filter. The gradient system started from 90% A at 0 min, to 80% A at 10 min, 70% A at 15 min, 60% A at 25 min, 50% A at 30–40 min, 75% A at 42 min, and 90% A at 44 min. A photodiode array detector (Rheodyne, USA) was used, and the peaks of the phenolic compounds were monitored at 280 nm [32]. The Gower similarity coefficient was used to determine relations between the chemical compositions of FGS and UGS, in PAST 3.11 software.

2.5 Radical-Scavenger Activity

2.5.1 Grape

The modified DPPH● method performed by Sánchez-Moreno and colleagues was used [30]. The standard curve was performed (3.94, 7.89, 11.83, 15.77, 19.71, 23.66, and 27.60 μg/mL of DPPH● in methanol) for the DPPH● (μg/mL) reaction medium concentration, determined by linear regression: A490nm = 0.01116*[DPPH●] t – 0.01031, r2 = 0.99. Aliquots of the sample extract or the positive control (ascorbic acid) were prepared at different concentrations and then added to the DPPH 19.71 μg/mL solution (diluted in methanol). The absorbance at 490 nm was measured until the 30 min steady state to better understand the antioxidant behavior, and to design the protocol for the optimal range [33,34]. The remaining DPPH● (% DPPHrem) percentage was calculated as the ratio between DPPH concentration at each reaction time and DPPH concentration at the initial time (t = 0), using the following equation: % DPPHrem [DPPH●]/([DPPH●]t=0)*100. The percentage of remaining steady state DPPH● against the sample concentration was graphically plotted to obtain the amount of antioxidant sample necessary to decrease the initial DPPH● concentration by 50% (EC50). The antioxidant reducing power (ARP), an important antioxidant parameter, was calculated as an inverse of the EC50 value, such that the larger ARP value was related to more efficient antioxidant activity [30, 35].

2.6 Experimental animals and preparations

2.6.1 Grape/Red Wine GASH and RSCS

Twelve week-old male Wistar rats (Rattus norvegicus) weighing around 250 – 300 g were used in all experimental protocols. The animals were housed in groups of four, and given four days to acclimate to the housing facility. The environmental experimental conditions were a room temperature of21ºC ± 1ºC, humidity of 60% ± 10%, lighting of 325 lux, and a 12:12 light/dark cycle with lights on at 06:00 and off at 18:00, and the environmental enrichment included bedding. The animals were housed in 410X340X160 mm cages (Beira-Mar-BHG, Brazil) and given access to food and water ad libitum. During housing, the animals' health status was monitored twice daily. No adverse events were observed. The study conformed to the International Guide of Care and the Use of Laboratory Animals, and all experimental protocols were submitted to and approved by the Federal University of Paraíba Ethics Committee on Animal Use (CEUA/UFPB n° 1505/13 and 0310/08).

2.7Vascular reactivity studies

2.7.1 Grape

2.7.1.1 Small Mesenteric Arteries

Before each experiment, male Wistar rats (wt, 250 – 300 g) were anesthetized with a pentobarbital sodium (100 mg/kg, i.p.) injection (dissolved in 0.9% sterile saline), and were euthanized by cervical dislocation without suffering. The third order branches of the superior mesenteric arteries were identified, dissected, and ring segments (1.5 – 2.0 mm in length), were mounted in the Mulvany apparatus, composed of a small vessel chamber, and a myograph for isometric tension measurements (DMT® myograph 610M, Aarhus N, Denmark). In certain experiments, the endothelium was removed by rubbing the intima with a single hair [36]. The rings were suspended in organ baths containing Krebs-bicarbonate solution (mM: NaCl 119, KCl 4.7, KH2PO4 1.17, MgSO4 1.18, CaCl2 2.5, NaHCO3 25, EDTA 0.027 and D-glucose 5.5; pH 7.4 and 37 °C) and aerated with a mixture of 95% O2 and 5% CO2. After an initial equilibration period of 60 min, the integrity of the endothelium was pre-assessed by contracting the tissues with phenylephrine (PE, 1 - 10 μM) and then adding acetylcholine (1 – 10 μM). Tissues in which the acetylcholine reversed the phenylephrine-induced tone by more than 90% were designated as endothelium-intact rings and tissues in which acetylcholine caused less than 10% relaxation were designated as endothelium-denuded rings. The

vasodilator response induced by the lyophilized grape skins extract, FGS or UGS (10 – 3000 μg/mL), was evaluated in endothelium-intact or endothelium-denuded mesenteric artery rings pre-contracted with PE 10 μM. In the endothelium-intact rings, L-NAME (100 μM), charybdotoxin (50 nM), or apamin (50 nM) was added to the organ bath at least 20 min before addition of PE (10 μM). These concentrations were respectively chosen for having been shown to inhibit nitric oxide (NO) and endothelium-derived relaxing factor (EDHF) responses in mesenteric rings [37].

2.7.2 Red Wine GASH and RSCS

2.7.2.1 Superior Mesenteric Artery

Vascular Reactivity Studies in Isolated Rat Superior Mesenteric Artery Rings Rats were euthanized by stunning and bleeding. The superior mesenteric artery was removed and cleaned from connective tissue and fat. Whenever appropriated, the endothelium was removed by gently rubbing the intimal surface of the vessels. Rings (1–2 mm) were obtained and placed in physiological Tyrode's solution [Tyrode's solution composition was (in mmol/L): 158.3 NaCl; 4.0 KCl; 2.0 CaCl2; 1.05 MgCl2;0.42 NaH2PO4; 10.0 NaHCO3; 5.6 glucose], maintained to 37C and gassed with carbogenic mixture (95% O2 and 5% CO2) and maintained at pH 7.4. All preparations were stabilized under a resting tension of 0.75g for 1 hour. The solution was replaced every 15 minutes to prevent the accumulation of metabolites [4]. The force of contraction was isometrically recorded by a force transducer (Miobath-4, WPI, Sarasota, FL) coupled to an amplifier recorder (Miobath-4, WPI) and to a computer equipped with an analog-to-digital converter board. The presence of functional endothelium was assessed by the ability of acetylcholine (10 mM) to induce 85% relaxation of vessels pre-contracted with Phe (10 mM). Less than 10% of relaxation to acetylcholine was taken as evidence that the vessel segments were functionally denuded of endothelium [23].

2.7.2.2 Protocols with GASH

The preparations were exposed to L-NAME (100 mM), an NO synthase (NOS) inhibitor [24], ODQ (10 mM), a soluble guanylatecyclase (sGC) inhibitor [25], and atropine (1 nM), a competitive antagonist for the muscarinic acetylcholine receptor [26]. These inhibitors were added 30 minutes before the application of Phe. In the tonic phase of the second contraction, GASH (Log 25 to 3mg/mL) was cumulatively added to preparations until a maximum response for the addition was observed as indicated by a plateau response (ca. 3 minutes). Inhibition was calculated by comparing the response elicited by GASH before and after the inhibitors or antagonists were added to the preparation mixture.

2.7.2.3 Protocols with RSCS

In some experiments, rings were exposed to either the NO synthase inhibitor L-NAME (100 M) [38], ODQ (10 M), a soluble guanylylcyclase (sGC) inhibitor [39], charybdotoxin (CTX, 0.2 M) + apamin (0.2 M), two inhibitors of calcium-dependent K+ channels involved in endothelium-dependent hyperpolarization (EDH) mediated relaxation [40], or tempol (100 M), a SOD mimetic [41]. Rings were exposed to an inhibitor for 30 min before the addition of phenylephrine. Once a stable contraction was reached, a concentration relaxation curve to RSCS was constructed.

2.8 Measurement of arterial pressure and heart rate

2.8.1 Red Wine RSCS

For blood pressure and heart rate determinations, protocols used were similar to those previously described [42]. Briefly, following ketamine and xylazine (75 and 10 mg/kg, i.p., respectively) administration, polyethylene catheters were inserted into the lower abdominal aorta and the inferior vena cava of rats through the left femoral artery and vein, respectively. Both catheters were filled with a heparinized saline solution, tunnelled subcutaneously, exteriorized and sutured at the dorsal surface of the neck. Twenty-four hours after the surgical procedure, experiments were performed in conscious rats. Changes in blood pressure and heart rate were recorded using a pressure transducer coupled to an acquisition system (PowerLab, AD Instruments, Australia) connected to a computer installed with LabChart 5.0 software (AD Instruments). In one series of experiments, once cardiovascular parameters had stabilized, increasing doses of RSCS (10, 30, 90 mg/kg) were randomly administered i.v. Successive injections were performed at 15 min intervals in order to allow changes in arterial pressure to develop. Thereafter, 30 min after baseline recovery, L-NAME (20 mg/kg, i.v., a NO synthase inhibitor) was administered for 30 min before the administration of increasing doses of RSCS. In a second series of experiments, after an adaptation period, 12 rats were treated orally with L-NAME (40 mg/kg/day) dissolved in the drinking water. Twelve days after the beginning of the L-NAME treatment, when rats were hypertensive, 6 rats were treated with L-NAME plus vehicle (saline), and 6 with L-NAME plus RSCS (100 mg/kg/day) from day 12 until day 21. Mean arterial blood pressure and heart rate were determined at day 21.

2.9 Determination of Vascular Oxidative Stress

2.9.1 Grape

The redox-sensitive fluorescent dye dihydroethidium (DHE) was used to evaluate in situ ROS formation. On the day of vascular reactivity studies, mesenteric artery rings (3 to 4 mm in length) were embedded in OCT compound and frozen in a nitrogen bath for cryostat sections. DHE (2.5 μM) was then applied onto the unfixed 14 μm mesenteric artery cryosections for 30 min at 37ºC in a light-protected humidified chamber to determine in situ formation of ROS [43]. To determine the nature of ROS reductions, the rings were incubated with FGS or UGS for 15 min at 37ºC before adding DHE. Sections were then washed three times and DAPI (Molecular ProbesTM) was added for 5 min following by further washing (twice) before being mounted in Fluorescence Mounting Medium (DAKO©), and coverslipped. Images were obtained with a Fluorescence Eclipse Ti-U Nikon® microscope. Quantification of the staining levels was performed using NIS-element® software. It is important to note that this experimental protocol was performed only because we had removed and frozen, mesenteric artery parts at –80ºC from the rats whose third branch arteries had been used to evaluate vascular reactivity. Thus, no additional animals were used, reducing the number of animals used in the study.

2.10 Cultured rabbit aortic endothelial cells (RAEC)

Cultured rabbit aortic endothelial cells line (The cell line established from primary culture of RAEC were gifts from Dr. Helena B. Nader at UNIFESP, São Paulo, Brazil). RAEC were grown in 24-well plates using F12 medium (F12 Coon's modification) supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin) as previously described [44].

2.10.1 Red Wine RSCS and GASH

2.10.1.1 Determination of nitric oxide formation by endothelial cells

DAF-2 DA, a fluorescent indicator that enables the direct detection of NO under physiological conditions by flow cytometry [42, 45] was used in a Cultured rabbit aortic endothelial cells. To determine the formation of NO in RAEC, cells were exposed to RSCS (100 and 300 g/mL) and GASH (100 and 300 mg/mL) for 5 min and 10 M DAF-2 DA for 30 min at 37 °C. RAEC and GASH were also stimulated with ACh (10 M) for 15 min. To verify that the fluorescent signal obtained after the addition of DAF-2 DA was dependent on the presence of NO, RAEC was pre-incubated with L-NAME (100 M) for 30 min before the addition of DAF-2 DA and RSCS (100 and 300 g/mL) and GASH (100 and 300 mg/mL). Cells were then washed twice with phosphate buffered saline

solution containing bovine serum albumin and analysed (10,000 cells per sample) by flow cytometry using the FACSCalibur equipment (Becton Dickinson, San Jose, CA, USA). Data were analysed by FACSCalibur Set-up – Cell Quest Pro Software. Changes in NO formation were expressed as percent changes of fluorescence.

2.10.1.2 Determination of superoxide anions formation by endothelial cells

The fluorescent probe dihydroethidium (DHE) was used to evaluate the intracellular formation of superoxide anions using a fluorescent reader. Cultured rabbit aortic endothelial cells (5 × 105 /well) were treated with RSCS (300 g/mL) or 1 M angiotensin II (positive control), and then they were loaded with dihydroethidium (DHE, 5 M) in Tyrode solution for 30 min in the dark at room temperature. After the incubation period, fluorescence was monitored using a spectrofluorometer (Tecan, Salzburg, Austria) with an excitation at 396 nm and an emission at 590 nm. Results are expressed as percentage of relative fluorescence units.

2.11 Cultured porcine coronary artery endothelial cell

Porcine coronary artery endothelial cells were isolated and cultured as previously described [46].Briefly, endothelial cells were isolated from freshly dissected porcine coronary arteries by collagenase treatment (type I, Worthington, 1 mg/mL for 12 min at 37 °C), and cultured in flasks containing MCDB 131 medium (Invitrogen, Saint Aubin, France) supplemented with 15% fetal bovine serum, fungizone (250 g/mL), penicillin (100 U/mL), streptomycin (100 U/mL), and L-glutamine (2 mM) (all from Cambrex, Saint-Beauzire, France). All experiments were performed with confluent cultures of cells used at first passage. Cells were incubated with serum-free culture medium (with 0.1% fetal bovine serum) for 5 h prior to treatment.

2.11.1 Red Wine RSCS

2.11.1.1 Western blot analysis

Thereafter, cells were incubated for 30 min with either RSCS (100 and 300 g/mL) or tempol (10 M) + RSCS (300 g/mL). Cells were then washed twice with phosphate-buffered saline solution containing bovine serum albumin and analysed by Western blotting [47]. Western blot analyses were performed in cultured porcine endothelial cells washed twice with phosphate-buffered saline solution and then lysed in extraction buffer. Total proteins (20 g) were separated on 10% SDS–polyacrylamide gels at 80 V for 2 h. Separated proteins were transferred electrophoretically onto polyvinylidinedifluoride membranes (Amersham, Les Ulis, France) at 100 V for 120 min. Membranes were blocked with blocking buffer containing 3% bovine serum albumin, Tris buffered saline solution (Euromedex,

Souffelweyersheim, France) and 0.1% Tween 20 (TBS-T) for 1 h. To detect the phosphorylated proteins, membranes were incubated with the respective primary antibody (p-Akt Ser473 and p-eNOS Ser1177 (1:1000), β-tubulin, Cell Signaling Technology (Danvers, MA, USA), 1:10,000 dilution) overnight at 4 °C. After washing, membranes were incubated with the secondary antibody (peroxidase-labelled anti-rabbit or anti-mouse IgG; Cell Signaling Technology, 1:5000 or 1:20,000 dilution, respectively) at room temperature for 60 min. Pre-stained markers (Euromedex) were used for molecular mass determinations. Immunoreactive bands were detected by enhanced chemiluminescence (Amersham, Les Ulis, France).

2.12 Statistical Analysis

The minimum number of animals was chosen to allow adequate statistical analysis. Values are expressed as mean ± S.E.M. Statistical significance was determined (when appropriate) by using the student's T test with GraphPad Prism software, version 6.0 (GraphPad Software Inc., La Jolla, CA, USA). The student's T test was chosen in accordance with the number of animals per group, ranging from 4 to 7. All phytochemical measurements were carried out in triplicate. Relaxation responses are expressed as a percentage of the phenylephrine contraction effect (at 10 μM). Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1 Phenolic compounds as natural antioxidants in grape and red wine byproducts.

In the present investigation, the polyphenols were extracted from fermented and non-fermented grapes byproducts of winemaking (pomace) and red wine grape varieties of northeastern Brazilian by chromatographic methods, consisted of total phenolic, flavonoids, stilbenes and tannins contents.

Initially, the concentration of grape or wine phenolics was estimated by analyzing for total phenol by the Folin-Ciocalteu procedure and results are expressed in micrograms per milliliter or molar equivalents of gallic acid. A calibration curve was prepared using concentrations of gallic acid ranging from 0 to 500 mg. Considering the total phenolic composition obtained by Folin-Ciocalteu's (F-C) method, as summarized in the Table 1, we found that FGS (185.53 ± 14.73 μg/mg DW) presented about seven times higher total phenolics than that found in UGS (25.29 ± 0.30 μg/mg of DW).

Lucena et al. [31] evaluated the antioxidant activity and total phenolic contents from eight wines from the São Francisco Valley. They have demonstrated that these red wines possess twice as much total phenolic contents as data from literature, with

a total phenolic content ranging from 3,2 to 5,9 GAE/L, 5,9 GAE/L corresponding to Tannat and Syrah wines (Table 1). The most concentrated fraction of polyphenols was with Petite Sirah red wine with 677,8 ± 2,6 mg GAE/g [31]. Interestingly, a high sunlight-exposed grape can increase quantitative composition of polyphenols in wine up to ten times [48]. Luciano et al. have also determined the concentration of total phenolic compounds. In the Shiraz 2005 from the São Francisco Valley, it was 2971 ± 214 mg GAE/L[31]. On the other hand, Ribeiro et al. [20] has enabled to discover that Cabernet-Sauvignon studied contains 4,2 mg GAE/L of total phenolic contents.

Lucena et al. and Soares de Andrade et al. studies have highlighted, by different methods, that there are less anthocyanin in the red wine from São Francisco Valley than in red wines already studied such as in southern Brazilian and Chile red wine [31, 49]. In fact, Lucena et al. have determined an anthocyanin content ranging from 5,2 ± 0,02 mg AM/L for Merlot to 20,7 ± 0,09 for Ruby Cabernet wine, using the pH-differential method [31] (Table1). In addition, Soares de Andrade *et al.* have performed their researches with HPLC-UV-Vis [49]. This observation may be explained by the frequent harvests that inhibits the possibility of the grape to synthesize anthocyanins. The most abundant anthocyanin in studied wines from the São Francisco Valley was Malvidin (approximately 30 mg/L), according to the results of Soares de Andrade *et al.* [49].

Table 1. Total phenolic content and total monomeric anthocyanin content the different studied red wines produced in the São Francisco Valley

RED WINES	Total phenolic content (gGAE/L)	Total monomeric anthocyanin content (mgAM/L)	Ref.
Petite Sirah 2005	5,2 ± 0,2	9,2 ± 0,02	[31]
Tannat 2002	5,9 ± 0,5	9,7 ± 0,08	
Syrah 2005	5,9 ± 0,2	13,9 ± 0,05	
Cabernet Sauvignon 2005	5,4 ± 0,2	11,2 ± 0,01	
Cabernet Sauvignon 2006	3,2 ± 0,2	10,8 ± 0,02	
Ruby Cabernet 2006	5,4 ± 0,3	20,7 ± 0,09	
Shiraz 2003	5,0 ± 0,1	14,3 ± 0,04	
Merlot 2003	4,7 ± 0,2	5,2 ± 0,02	
Cabernet Sauvignon	$4{,}2.10^{-3}$		[20]
Shiraz 2005	2,971 ± 0,214		[4]

Values are expressed as mean ± SEM

In addition to the total phenolic content and the total monomeric anthocyanin content, some phenolic compounds in particular have been searched such as cis-, trans-resveratrol, quercetin as well as myricetin and kaempferol, by HPLC. It has been observed that there are more cis-resveratrol and quercetin in the red wine from São Francisco Valley than in other wines. Moreover, cis-resveratrol is up to 5 times higher than that of trans-resveratrol [31]. The levels of cis-resveratrol varied from 0,72 to 5,49 mg/L, while the levels of trans-resveratrol were lower, varying from 0,04 to 1,26 mg/L. Finally, concentration of quercetin in the wines samples varied from 3,2 to 5,9 mg/L [31]. The Shiraz 2005 studied by Luciano et al., the quantity of cis-resveratrol, trans-resveratrol and quercetin were, respectively, 8,32 ± 0,0013, 0,34 ± 0,0017 and 2,16 ± 0,0115 μg/mL [4]. It has been found quercetin, myricetin and kaempferol in the red wine sample of Cabernet Sauvignon studied by Ribeiro et al. whose levels were, respectively, 6,37 ± 0,29, 3,25 ± 0,13 and 0,07 ± 0,03 mg/mL [20] (Table 2).

Table 2. Concentration of *cis-*, *trans-*resveratrol, quercetin, myricetin and kaempferol in the studied red wine samples

RED WINES	Quantitative composition of polyphenols (mg/mL)					Ref.
	Cis-resveratrol	Trans-resveratrol	Quercetin	Myricetin	Kaempferol	
Petite Sirah 2005	2,02 ± 0,01	0,51 ± 0,04	1,80 ± 0,07			(48)
Tannat 2002	5,49 ± 0,46	1,26 ± 0,10	5,56 ± 0,43			
Syrah 2005	2,85 ± 0,24	0,19 ± 0,04	6,05 ± 0,39			
Cabernet Sauvignon 2005	0,72 ± 0,70	0,04 ± 0,01	0,23 ± 0,01			
Cabernet Sauvignon 2006	2,27 ± 0,09	0,55 ± 0,01	3,18 ± 0,06			
Ruby Cabernet 2006	1,41 ± 0,01	0,69 ± 0,02	1,10 ± 0,01			
Shiraz 2003	5,40 ± 0,01	0,21 ± 0,02	1,55 ± 0,02			
Merlot 2003	1,15 ± 0,02	0,16 ± 0,01	1,03 ± 0,04			
Cabernet Sauvignon			6,37 ± 0,29	3,25 ± 0,13	0,07 ± 0,03	(50)
Shiraz 2005	8,32 ± 0,0013*	0,34 ± 0,0017*	2,16 ± 0, 0115*			(52)

Values are expressed as mean ± SEM

In addition to the research on the qualitative and quantitative phenolic composition of grapes byproducts and red wine from northeast of Brazil, recently, much attention has focused on the protective effects of naturally occurring antioxidants in biological systems. Many papers have reported the correlation between antioxidant

activity and total phenolic, flavonoids, anthocyanins and tannins content, and the dependence of these properties on varieties, vintage and wineries [50]. Our studies associated the polyphenolic composition with the antioxidant activity measurement by DPPH assay expressed in terms of vitamin C concentrations.

The antioxidant activity (ARP) presented by fermented and non-fermented grapes byproducts of winemaking (pomace) samples, was evaluated, and we found that EC50 values were 0.50 ± 0.05, 1.10 ± 0.14 and 1.91 ± 0.42, for Ascorbic Acid (AA), FGS, and UGS. This relationship between samples confirms a greater ARP of FGS, with 0.91, almost twice that of UGS 0.52, and half that of the Ascorbic Acid potential scavenger 2.00 (Table 3).

Table 3. Antioxidant reducing power and EC50 values obtained in DPPH assay from fermented (FGS) and non-fermented (UGS) grapes byproducts

SAMPLES	DPPH	
	EC50 values (μg of ext. / μg of DPPH)	Antioxidant activity (ARP)
AA	0.50 ± 0.05	2.00
FGS	1.10 ± 0.14 *	0.91
UGS-	1.91 ± 0.42 **;#	0.52

Mean values ± SEM of triplicate are shown. (AA), Ascorbic Acid; (FGS), Fermented Grape Skin; (UGS), Unfermented Grape Skin; * significant when compared to AA, $P < 0.05$; ** significant when compared to AA, $P < 0.01$; # significant when compared to FGS and AA

The relationship between the structure of flavonoids and their antioxidant potential has been intensively studied; Lucena *et al.* have highlighted the antioxidant activity of the polyphenols contained therein. They have discovered that antioxidant activity of polyphenols founded in the São Francisco Valley red wines is positively correlated with the polyphenolic composition, namely the flavonols, by DPPH and ABTS radical-scavenging assay [31].

3.2 Polyphenols founded the São Francisco Valley grape byproducts and their cardiovascular effects

3.2.1 Vascular protective antioxidant effects from grapes byproducts

In vitro, radical scavenger activity induced by fermented (FGS) and non-fermented (UGS) grapes byproducts was confirmed in small mesenteric artery

rings (n=4). Reactive oxygen species (ROS) fluorescence intensity to DHE dye in treated vessels (FGS and UGS) was significantly reduced in comparison to artery rings in basal conditions (Figure 1) [8]. Our previous results suggest a protective effect of grapes byproducts from northeastern Brazilian in the pathways that regulated oxidative stress on the cardiovascular system.

Vascular protection induced by grape byproducts

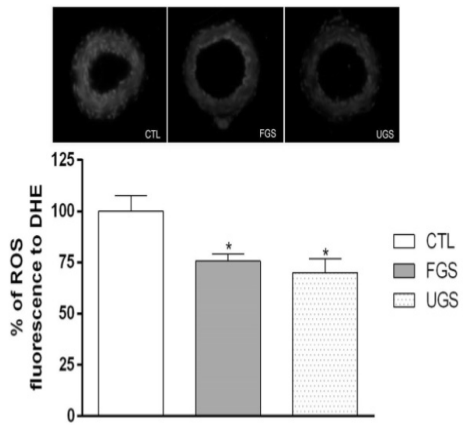

Figure 1: ROS measurement in intact mesenteric arteries. Reduced effects of superoxide fluorescence dye to DHE in normotensive small mesenteric artery sections exposed to vehicle (CTL), fermented (FGS) or unfermented (UGS) grape skin extracts. Data are expressed as mean ± SEM of 4 experiments. *significant when compared to controls (CTL), $P < 0.05$

3.2.2 Unraveling the vascular effects induced by grapes byproducts of winemaking and red wine grape varieties of northeastern Brazilian

Thus, to know if this effect is due to the São Francisco grapes byproducts or red wine (SFRW), our vascular studies have performed experiments in isolated rat superior mesenteric arteries, independently of neurohumoral influence. In rat mesenteric artery rings with an intact endothelium, São Francisco grapes byproducts (table 4) or red wine (GASH or RSCS; table 5) induced potent concentration-dependent relaxations of rings contracted with phenylephrine. In rings without endothelium, the vasorelaxant effect elicited by grapes byproducts or red wine (SFRW) was significantly attenuated (table 4 and 5).

The endothelial-dependent response was assessed in the presence of L-NAME, to prevent endothelial NO-formation, and in presence of charybdotoxin plus apamin, to inhibit EDHF-mediated responses. In these conditions, the

vasorelaxation induced by FGS was significantly attenuated (maximum relaxation = 97.1% ± 12.1%; EC50 = 495.2 μg/ mL ± 93.8 μg/mL, n = 4, Fig. 2) suggesting a strong participation of both, NO and EDHF, in the response induced by FGS [8].

Table 4. Vasorelaxant effect induced by fermented (FGS) and unfermented (UGS) grape skin extracts

CONDITION	FGS	UGS
Intact endothelium (E+)	EC_{50}= 80.50 ± 14.9	EC_{50}= 768.0 ± 99.4*
Denuded endothelium (E-)	EC_{50}= 545.0 ± 146.8*	- -
L-NAME+Caribdotoxin+Apamin	EC_{50}= 495.2 ± 93.8*	- -

Values are expressed as mean ± SEM. *significant when compared to intact endothelium from FGS, P < 0.05

In order to further investigate the mechanisms underlying the effects of northeastern brazilian red wine, the vasorelaxation was evaluated in the presence of either a NOS inhibitor (L-NAME) or a selective inhibitor of soluble guanylylcyclase (ODQ). Both of these treatments significantly reduced the red wine-induced endothelium-dependent relaxation in mesenteric artery rings (table 5). In contrast, the combination of charybdotoxin plus apamin, inhibitors of endothelium-dependent hyperpolarization mediated relaxation, did not significantly affect the relaxation induced by RSCS (maximal relaxation was 78.3 ± 5.8%, table 5).

Table 5. Vasorelaxant effect induced by red wine grape varieties of northeastern Brazilian

CONDITION	GASH	RSCS
Intact endothelium (E+)	E_{max}= 87.2 ± 6.5 %	E_{max}= 87.2 ± 3.2 %
Denuded endothelium (E-)	E_{max}= 28.4 ± 4.9 % *	E_{max}= 32.0 ± 2.0 % *
L-NAME	E_{max}= 23.4 ± 5.1 %*	E_{max}= 22.6 ± 3.7 %*
Caribdotoxin + Apamin	- -	E_{max}= 78.3 ± 5.8 %
ODQ	E_{max}= 11.8 ± 2.7 %*	E_{max}= 37.0 ± 4.6 %*

Values are expressed as mean ± SEM. *significant when compared to intact endothelium

3.2.3 Red wine of northeastern Brazilian (SFRW) induces NO production in endothelial cells

The quantification of the bioavailability of NO was performed using flow cytometry analysis based on fluorescent intensity levels of DAF2-DA, which increase in proportion to the amount of NO released by the endothelial cells in basal or stimulated conditions [42]. In cultured endothelial cells, GASH (100 mg/mL: 58 ± 1 and 300 mg/mL: 82 ± 7.9; % of fluorescence, n = 5) or RSCS control: 10.88 ± 2.34%, RSCS100 μg/mL: 79.90 ± 0.98% and 300 μg/mL: 295.20 ± 1.79% of fluorescence, n = 4) increased the cell-associated DAF fluorescence level compared with that in untreated control cells (Fig. 2).

As shown in Fig. 3, in the presence of L-NAME (100 μM) the GASH-response (100 μg/mL: 29.5± 0.5 and 300 μg/mL: 30.2± 12.1; % of fluorescence) or RSCS-induced increase in the DAF fluorescence level (L-NAME+ RSCS 100 μg/mL: 40.59 ± 0.54% and L-NAME + RSCS 300 μg/mL: 98.80 ± 9.86% of fluorescence) was significantly attenuated indicating the involvement of NO (figure 2).

Red wine from Northeastern Brazilian induces eNOS-derived NO formation

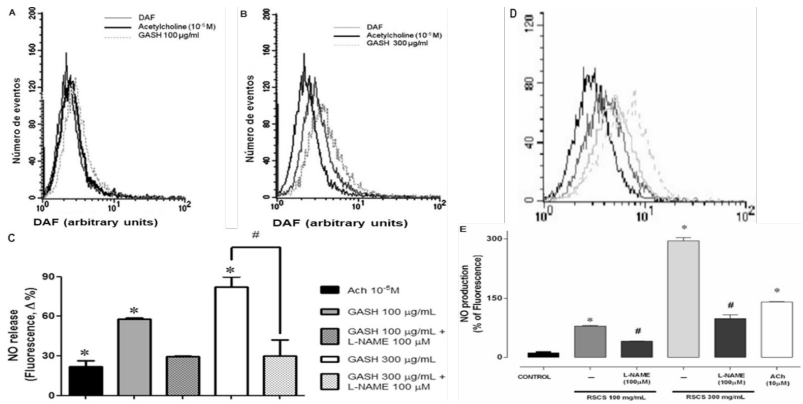

Figure 2. Determination of NO formation using diaminofluoresceindiacetate (DAF-2DA) in cultured rabbit aorta endothelial cells. (A-B) Representative flow cytometry findings are shown. (C) Bar graph showing the effects of either GASH (100 and 300 μg/mL) 10 min before and 30 min after treatment of cells with L-NAME (100 μM), or ACh (10 μM, used as a positive control; n = 4). (D) Representative flow cytometry findings of NO production on rabbit aortic endothelial cells, representative of 4 separate experiments (in duplicate). (E) Bar graph showing the effects of either GASH (100 and 300 μg/mL) 10 min before and 30 min after treatment of cells with L-NAME (100 μM), or ACh (10 μM, used as a positive control; n = 4).* P <0.05 versus control; #p <0.05 versus red wine.

3.2.4 Red wine of northeastern Brazilian (SFRW) induces redox-sensitive mechanism in endothelial cells

The formation of superoxide anions in endothelial cells was assessed using the redox-sensitive probe dihydroethidium (DHE). Treatment of endothelial cells with GASH or RSCS significantly increased the DHE fluorescence signal compared with that observed in control cells (Fig. 3). In addition, an increased DHE fluorescence signal was also observed in response to angiotensin II. Furthermore, in isolated rat mesenteric artery rings, the endothelium-dependent relaxation induced by GASH or RSCS was significantly reduced in the presence of the n-acetylcysteine, a radical scavenger (10μM) or tempol (100 μM), a SOD mimetic, respectively (figure 3).

Red wine from Northeastern Brazilian induces redox-sensitive mechanisms

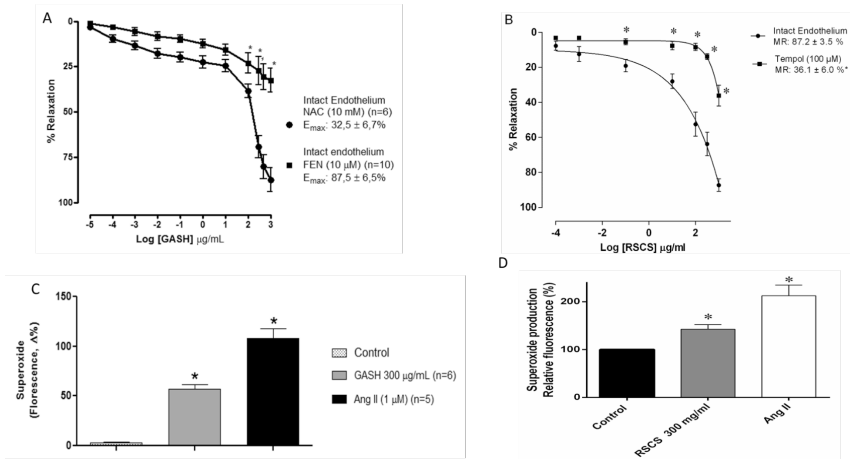

Figure 3. Red wine GASH or RSCS increases the formation of superoxide anions in endothelial cells. (A) Superoxide anion formation induced by GASH (C) or RSCS (300 μg/mL) (D) and Ang II (1 μM) in rabbit aorta cultured endothelial cells (n = 4, experiments performed in triplicate). (B) Concentration–response curves showing the relaxant effect induced by GASH or RSCS in the absence or presence of 10 μM NAC (A) or 100 μM tempol (B). MR means the maximum response. Results are expressed as mean ± S.E.M *P < 0.05.

3.2.5 RSCS stimulates the phosphorylation of Akt and eNOS in cultured endothelial cells

Experiments were performed to characterize the signaling pathway leading to eNOS activation and subsequent NO formation in response to RSCS. Exposure of cells

to RSCS (100 and300 μg/mL) for 30 min markedly increased the phosphorylation level of Akt at Ser 473 and eNOS at Ser1177 (Fig. 4). RSCS-induced phosphorylation of Akt and eNOS was significantly reduced by a 15-min pre-treatment period of cells with the SOD mimetic, tempol (100 μM, Fig. 4B and C).

RSCS increases Akt and eNOS phosphorylation

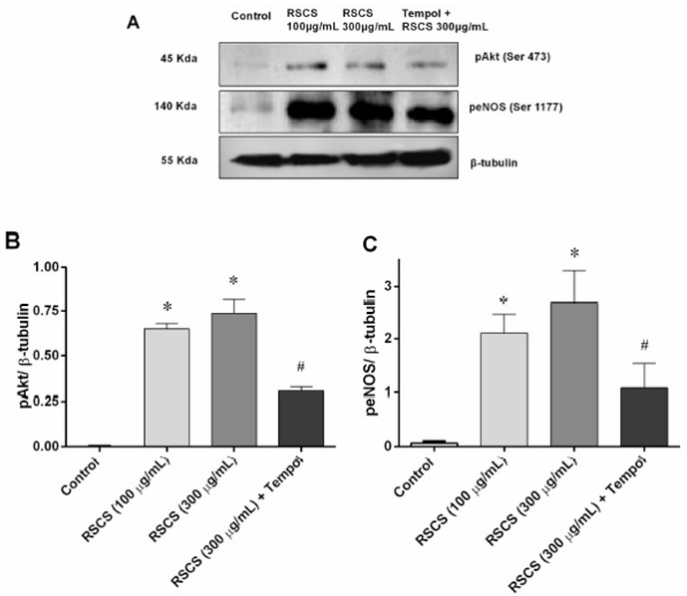

Figure 4. RSCS causes a concentration-dependent phosphorylation of Akt (Ser 473) and eNOS (Ser 1177). Endothelial cells were incubated either with solvent, RSCS for 30 min in the presence or absence of tempol (100 μM, 10 min pre-treatment). The levels of p-Akt and p-eNOS were determined by Western blot analysis. (A) Representative immunoblots, and corresponding cumulative data of (B) p-Akt and (C) p-eNOS. n = 4 different experiments. *P < 0.05 versus control; #P < 0.05versus RSCS 300 μg/mL.

3.2.6 RSCS decreases blood pressure in normotensive rats

Systemic hemodynamic changes induced by RSCS were studied in normotensive un-anaesthetized rats. In normotensive conscious rats, baseline values of mean arterial pressure and heart rate were 110 ± 1 mmHg and 378 ± 5 bpm, respectively. Acute administration of RSCS (10, 30 and 90 mg/kg, i.v.,randomly) induced hypotension associated with tachycardia (–25.4 ± 1.5;

–35.8 ± 4.5; –57.6 ± 6.0 mmHg, n = 6, and 62.1 ± 11.2;70.3 ± 10.2; 102.8 ± 9.1 bpm, n = 6, respectively; Fig. 5).

The hypotensive and tachycardia responses were significantly attenuated after acute inhibition of the NO synthase with L-NAME (20 mg/kg) (–6.0 ± 0.8; –10.2 ± 0.8; –21.7 ± 2.0 mmHg, and12.0 ± 4.0; 13.0 ± 4.0; 32.0 ± 11.0 bpm, respectively; Fig. 5).

RSCS decrease blood pressure by NO-dependent mechanism

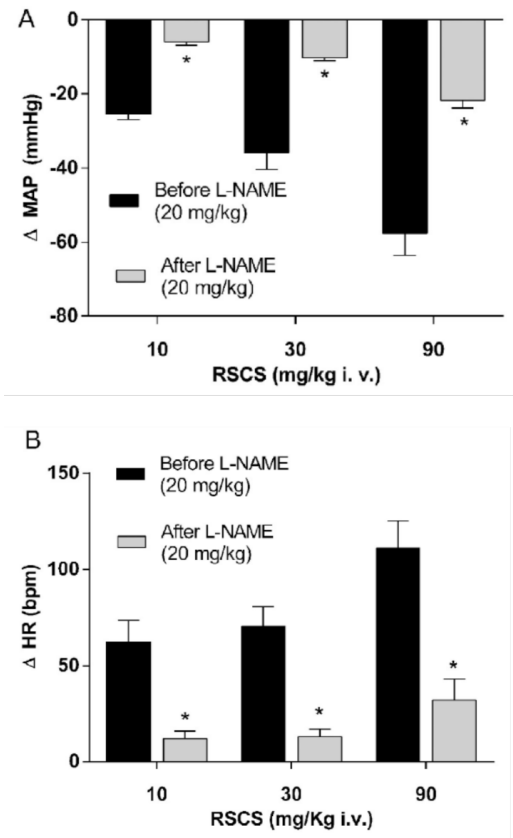

Figure 5. RSCS induces hypotension and tachycardia mediated by NO in normotensive conscious rats. Changes in mean arterial pressure (MAP, A) and heart rate (HR, B) following the acute administration of increasing doses of RSCS (mg/kg, i.v.) in conscious normotensive rats either before or after administration of the NO synthase inhibitor L-NAME (20 mg/kg, n = 6). Values are expressed as mean ± S.E.M. *P < 0.05.

3.2.7 The antihypertensive effects induced by Red wine of northeastern Brazilian

Daily administration of L-NAME (40 mg/kg/day) induced a progressive increase in systolic blood pressure of L-NAME group from121 ± 6.53 mmHg and reached 172.5± 6.26 mmHg at the end of treatment. Interestingly, oral intake of RSCS (100 mg/kg/day) significantly reduced the mean arterial pressure in chronically L-NAME-treated hypertensive rats (values were 172.5 ± 6.3 and143.7 ± 4.7 mmHg), while the heart rate was not affected (421.6 ± 11.0 and 399.5 ± 9.0 bpm; Table 6).

Table 6. RSCS induced antihypertensive effects in L-NAME-induced hypertensive rats.

	Control rats	L-NAME Hypertensive rats	L-NAME Hypertensive rats treated with RSCS 100 mg/kg/day	n
MAP (mmHg)	121 ± 6.53	172.5 ± 6.26	143.75 ± 4.73 *	6
HR (bpm)	395 ± 8.90	399.5 ± 27.37	421. 6 ± 16	6

Values are expressed as mean ± S.E.M. *P < 0.05.

Additionally, oral intake of GASH (100 mg/kg/day) during 7 days significantly reduced the mean arterial pressure in chronically L-NAME-treated hypertensive rats, while the heart rate was not affected (Figure 6). In SHR rats the oral intake of GASH during 7 or 21 days significantly decreased high blood pressure (Figure 6), but did not induced any alteration in the heart rate (Figure 6).

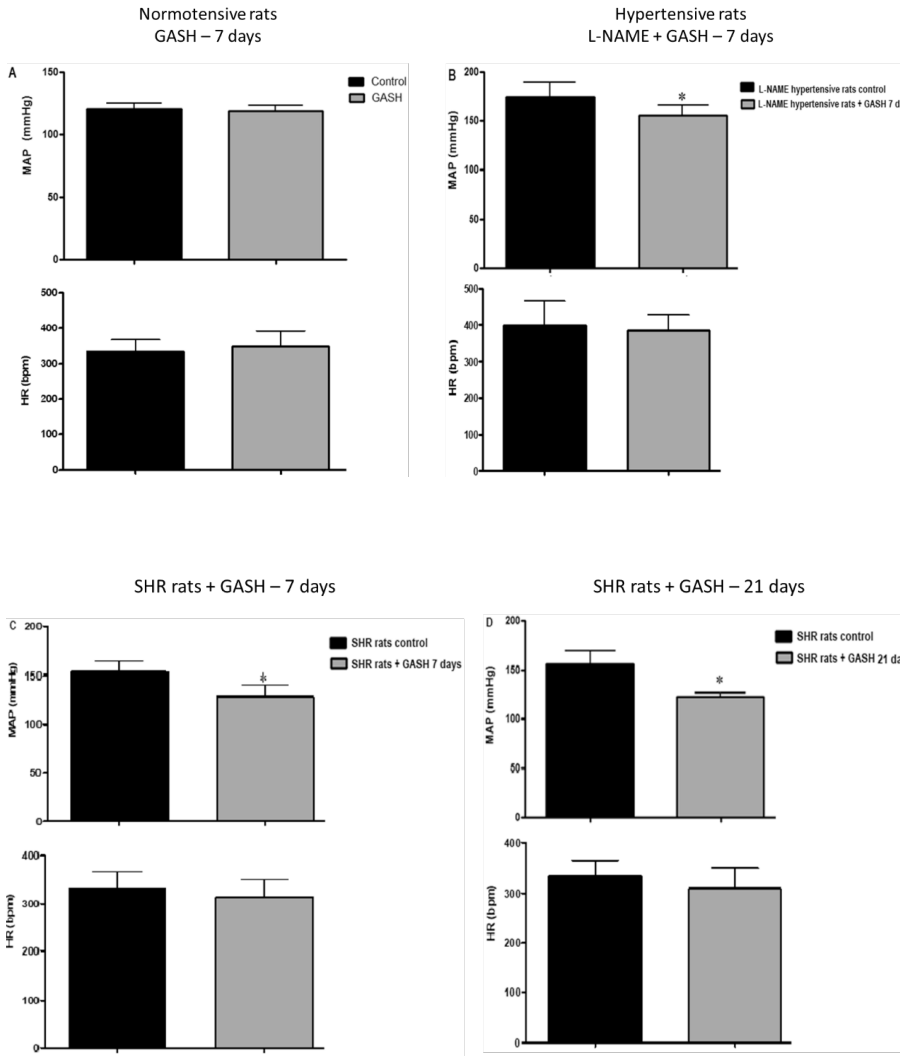

Figure 6. (A) Bars representative of the hypotensive effect of chronic treatment (7 days) with GASH (100 mg / kg / day) in normotensive rats. (B) Bars representative of the hypotensive effect of chronic treatment (7 days) with GASH (100 mg / kg / day) in L-NAME hypertensive animals. (C) Representative bar graphs of the hypotensive action induced by chronic treatment (7 days) with GASH (100 mg / kg / day) in SHRs. (D) Bars graphs of the hypotensive effect evoked by chronic treatment (21 days) with GASH (100 mg / kg / day) in SHR animals L-NAME. *p<0,05 vs. Control.

4 Discussion

The São Francisco River Valley, located in northeastern Brazil, has very different terrain and climate characteristics compared to other traditional wine-making region, which is characterized by the temperate climate. The major findings, in summary, were as follows: (1) The fermented (FGS) and non-fermented grapes (UGS) byproducts of winemaking (pomace) and red wine grape varieties of northeastern Brazilian, in recent studies, have shown high levels of total phenolics being particularly rich in flavonoids and non-flavonoids compounds and (2) exhibits antioxidant activities. (3) In mesenteric artery, grape- and red wine-derived byproducts induces endothelium-dependent vasorelaxion, and (4) the molecular mechanisms induced by grape- and red wine-derived byproducts involves increases in NO activation pathway in microvessels and endothelium cell culture in a dose-dependent manner.

4.1 Polyphenols agents from grape and red wine byproducts of northeastern Brazilian.

Northeast of Brazil disposes of particular climatic conditions, that differs from the rest of the country. This region present a semi-arid climate, with a high sunlight exposure almost all year. Despite particular climatic and environmental characteristics, this area, situated at 8-9S (latitude) and around 40W (longitude), is the second major producer of wine of the country [51].It can even be said that this region has a unique viticulture style, in comparison to others viniculture in the world. High temperatures and water stress have an influence on the synthesis of phenolic compounds, such polyphenols.

It has been demonstrated that limited irrigation can increase this phenolic compounds such tannins and anthocyanin content in grapes [12]. Furthermore, because of the high humidity, risk of fungal or bacterial infection for the plant are increased, which can cause an enhanced production of polyphenols [52]. Effects of sunlight-exposure have been researched. Price *et al*. [13] have observed that total phenolic content were higher in samples of wines made from exposed in comparison to shaded wines. Moreover, vinification techniques add some activity but are not necessary to obtain active polyphenols. Fermentation seems to enhance active content to the already existing active phenolic compounds in grapes [53].

Formerly known as “vegetable tannins”, polyphenols constitute one of the most distributed groups of natural products a structural class of mainly organic compounds, including a large enough number of di- or/and trihydroxyphenyl units, their solubility depends mainly on the molecular size, the hydroxyl groups, and the length of hydrocarbons [14]. It may range from simple phenols to compounds

highly polymerized but also molecules with one phenol ring, such as phenolic acids and phenolic alcohols.

Grape pomace contains polyphenols having useful bioactive components. The major polyphenolic compounds found in grapes and wine can, in general, be classified into three main groups: (1) tannins and pro-anthocyanidins, (2) simple flavonoids (catechins, flavonols, and anthocyanins), and (3) phenolic acids (mainly benzoic and hydroxycinnamic acids). In grape pomace, are anthocyanins, catechins, flavan-3-ols, flavonols, and polyphenolic acids hydroxybenzoic, hydroxycinnamic acids, alcohols, and stilbenes.

Reported studies are mainly focused in the content of phytochemicals of grape-wine byproducts extracts are related on quantitative data as total phenolic contents. The Folin-Ciocalteu's method is widely-used to assess total polyphenolic contents, although different polyphenolic compounds show different responses in the Folin-Ciocalteu's assay [54].In this method, Fermented (FSG) and non-fermented grape skins (UGS) from São Francisco Valley has great potential as a source of total polyphenolic compounds [8]. Similarly, few studies have worked on polyphenols from Northeastern of Brazil, Lucena *et al.* [31] have demonstrated that red wines from São Francisco valley possess twice as much total phenolic contents as data from literature, with a total phenolic content corresponding to Tannat and Syrah wines. Furthermore, we demonstrated published higher contains of total phenolic contents present in the Shiraz (GASH) or Cabernet-Sauvignon (RSCS) red wine from the São Francisco Valley published by Luciano *et al.* [4] and Ribeiro *et al.* [20], respectively. Besides the relative composition of the grapes-wine- byproducts of winemaking, it is of utmost importance to determine their individual chemical characteristics to recognize possible bioactive properties.

Thus, the complexity of the extracts makes it necessary to apply highly sensitive and selective analytical methods to isolate different groups of phenolics, such as anthocyanins, procyanidins, flavanols, isoflavones, flavonols, phenolic acids, flavanones, and stilbenes. The polyphenol content of the extraction of fermented and non-fermented grapes byproducts of winemaking (pomace) varieties of northeastern Brazilian, thus, potentially constitutes a very abundant and relatively inexpensive source of a wide range of polyphenols including monomeric and oligomeric pro-anthocyanidins, flavan-3-ols and a diversity of anthocyanin glycosides. Our previous results published by Albuquerque *et al.* [8] showed the content were highly variable depending on grape variety and type of extract (whole fruit vs fermented pomace), bioactive compounds such ferrulic acid, sinapic acid, hesperetin and chrysin, which were present only in the fermented samples (FGS). Interestingly, catechin, 2,5-dihydroxybenzoic, and syringic acid are present in higher amounts in FGS compared to UGS (FGS

presented twice the catechin and three times the amount of 2,5-dihidroxibenzoic and syringic acid than UGS).

To begin with studies with red wine demonstrate that quantification of quercetin, cis-resveratrol, and trans-resveratrol levels in GASH is consistent with the findings in the literature suggesting that the levels of cis-resveratrol are considerably higher than those of trans-resveratrol in some Brazilian wines [31, 55]. Among stilbene monomers, trans-resveratrol has been the most widely studied grapevine phytoalexin for its role on human health, although a low concentration of this compound was found in wine [56, 57].Therefore, trans-resveratrol has been suggested as one of the components in red wine that may be beneficial to human health. On the other hand, cis-resveratrol is typically found at lower concentrations and is often less biologically active than trans-forms.[57, 58].

Additionally, analysis of the RSCS has indicated a high level of flavonoids and, in particular, quercetin, myricetin and kaempferol. Furthermore, the most abundant anthocyanin in studied wines from the São Francisco Valley was malvidin. These observations support previous results that revealed the malvidin and other derivates, have also been identified and characterized in wines, however different studies have highlighted that there are less anthocyanin in the red wine from São Francisco Valley than in southern Brazilian red wines and Chile red wine [31, 49, 59].

4.2 Beneficial properties of Northeastern Brazilian Red Wine Products: highlight on antioxidant activities

Phenolic compounds can be found in grape varieties and wines, where they are known to have a key role in growth, pigmentation, protection against solar radiation, resistance against pathogens or environmental stresses in plant [54]. Grapes byproducts of winemaking (pomace) and red wine grape varieties of northeastern Brazilian demonstrated a valuable source of polyphenols, including flavonoids and can be used as antioxidants, which might be utilized as functional food components. In particular, these chemical structures are related to the antioxidant characteristics as reducing agents (i.e., by donating hydrogen-quenching free radicals).

In this way, the high content of phenolic compounds provides a basis for the antioxidant activity reported in the DPPH analysis, which like others studies, with red wine, establishes a positive correlation between antioxidant activity and the total polyphenolic content of the samples [54]. The kinetic behavior of the antioxidant activity for the fermented and non-fermented grapes byproducts of winemaking (pomace) of northeastern Brazilian, previous studies demonstrated that FGS was more relevant than for UGS. Similar studies have shown the positive

effect of the fermentation process; which yields higher antiradical activity than for unfermented samples [60]. Thus, the potent antioxidant activity found in FGS was probably due to the fermentation process, which probably induced chemical changes in the phenolic composition of the extract; as a consequence of pomace cell structure degradation and polyphenol releases [60, 61].

Indeed, grape- red wine- byproducts should be considered a rich potential source of natural phytochemicals that are particularly suitable for cardiovascular use. For this reason, our observations for FGS provide evidence that its potential effects are related to the presence of a greater number of polyphenolic compounds and radical-scavenger activity. Thus, we investigated in vitro the radical oxygen species and superoxide products production in small mesenteric artery rings it was found marked tissue antioxidant effect. These effects on ROS reduction in vascular smooth muscle cells are relevant to several disorders such as hypertension, which is related to an increase in the production of the superoxide anion and hydrogen peroxide, and decreased bioavailability of antioxidants [62]. As a matter of fact, therapies targeted at decreasing ROS generation in vascular smooth muscle cells may be useful for minimizing vascular injury, thereby preventing or reducing end-organ hypertensive damage [63, 64].

Beyond, these effects, several studies have discovered phenolic compounds potential action on health [16, 65]. In fact, most of polyphenols found in grape- or red wine byproducts are known exhibit antioxidant activities. Analysis of the GASH or RSCS red wine extract has indicated a concentration of total phenolics is significantly higher than the average reported in the literature for red wines. In particular, quercetin, cis- and trans- resveratrol, myricetin and kaempferol, that have been shown previously to have antioxidant and cardiovascular properties[66].

Polyphenols derivatives of benzoic acid and stilbene such as cis-resveratrol, which are found in large amounts in São Francisco Valley region red wines; and flavonoids, such as quercetin and catechins are closely associated with both antioxidant capacity and beneficial biological effect including cardioprotective actions [62, 67]. Consequently, grape- wine- byproducts is considered a valuable source of phytochemicals that may be recovered as functional compounds for drug development with pharmacological proprieties.

4.3 Cardioprotective actions of grape and red wine byproducts of northeastern Brazilian: Beyond antioxidant activities

Recent studies indicate that regular polyphenol-rich beverages (red wine and tea) and foods (chocolate, fruit, and vegetables) intake is associated with a protective effect on the cardiovascular system in humans and animals [68]. Furthermore, previous studies have shown additional protective effects of

cabernet wines such as reduced levels of lipid peroxidation, of vascular endothelial dysfunction, and of oxidative stress in acute coronary syndrome in both preclinical and clinical studies [65, 69, 70].

In addition, it has been known for many years that some flavonoids such as quercetin produce vasorelaxation and inhibit platelet aggregation [43].In addition, the polyphenol quercetin decreased blood pressure in several models of hypertension in rats including the spontaneously hypertensive rat, the L-NAME hypertensive rat, and the DOCA-salt hypertensive rat [71]. The antihypertensive effect was also associated with an improvement of morphological and functional changes in the heart, blood vessels and kidney [72].

The present study has demonstrated cardiovascular studies with grape byproducts (FGS and UGS), lyophilized red wine of the São Francisco valley region, GASH and RSCS. Indeed, our recent studies have shown that fermented (FGS) and non-fermented grapes (UGS) byproducts of winemaking (pomace) and red wine grape varieties of northeastern Brazilian, reduced the risk of cardiovascular disease, by produces vasorelaxation effects in mesenteric arteries, at least in part, for the presence of a high level of total polyphenols and flavonoids founded.

The experimental protocols performed in mesenteric arteries from rats to evaluate the effects of fermented (FGS) and non-fermented grapes (UGS) byproducts of winemaking (pomace) and red wine grape varieties of northeastern Brazilian on vascular reactivity. Indeed, vascular reactivity studies indicated that fermented (FGS) and non-fermented grapes (UGS) byproducts of winemaking (pomace) and red wine grape varieties of northeastern Brazilian is able to cause concentration-dependent relaxations in rat superior mesenteric artery rings contracted with phenylephrine.

Since the vascular endothelium is well known to have a pivotal role in the regulation of vascular tone, experiments were performed to determine its contribution to the vasorelaxant activity of the fermented (FGS) and non-fermented grapes (UGS) byproducts of winemaking (pomace) and red wine grape varieties of northeastern Brazilian. Although these products caused pronounced relaxations in rings with endothelium, indicating the key role of the endothelium. Numerous studies have reported the ability of several sources of polyphenols such as tea catechins, Aronia melanocarpa and grape juices and other red wines to cause endothelial dependent relaxation in aorta, pulmonary, coronary and mesenteric arteries [4, 43, 73]. In addition, Brazilian red wine presented vasorelaxant effect in isolated mesenteric vascular [74].

The results previous obtained so far show consistently that the polyphenols in grape- red wine byproducts have the capacity to improve endothelial control of vascular tone. Interestingly, in grape byproducts studies was demonstrated

that vasorelaxation induced by FGS was about 10 times more potent than that induced by UGS. Considering this strong difference between FGS and UGS we found that under this controlled condition, the FGS effects was significantly attenuated after removal of the functional endothelium. The endothelium-dependent vasodilatation induced by FGS in the mesenteric artery rings of rats demonstrated was in accordance with other our previous findings showing that red wine grape varieties of northeastern Brazilian (GASH and RSCS) also induce an endothelium-dependent vasorelaxant effect.

In blood vessels, endothelial cells play a critical role in maintaining local homeostasis, via the release of various autacoids on vascular and circulating cells. Among these factors, the NO, which is synthesized by endothelial NOS after activation of the enzyme by calcium–calmodulin or phosporylation/desphosporylation, has attracted much attention because of its multiple vasoprotective properties [75]. In addition to NO, EDH plays often an important role in the vasorelaxation mediated by polyphenols [76].In order to verify whether the endothelium-dependent relaxation to the fermented (FGS) byproducts of winemaking (pomace) in mesenteric rings pre-incubated with selective inhibitors of the NO and EDHF pathways, FGS induced a similar response to that presented for preparations in the absence of endothelium. These results strongly suggest that the mechanism by which these effects occur seems to be dependent on endothelium-derivative relaxant factors such as NO and EDHF. The data demonstrates the importance of these two pathways in the endothelial response induced by FGS. Indeed, the improvement of vascular tone by grape polyphenols, induced by the formation of NO and stimulation of EDHF pathways is well established [75].

It is well known that NO-induced relaxation occurs predominantly through the activation of sGC in vascular smooth muscle leading to accumulation of cGMP [76] and subsequent cGMP-dependent protein kinase (PKG) activation [77]. Our studies have demonstrated the involvement of the NO/soluble guanylylcyclase pathway induced by red wine grape varieties of northeastern Brazilian (GASH and RSCS). In presence of the NO synthase inhibitor, L-NAME or the soluble guanylylcyclase inhibitor, ODQ the GASH and RSCS effects was attenuated, indicating the involvement of the NO/soluble guanylylcyclase pathway. These findings corroborate with previous observations and also those of others indicating that red wine polyphenols are potent endothelium-dependent vasodilators in the rat mesenteric artery [78] and the porcine coronary artery [79], and that these effects involve endothelial NO synthase.

However, in the RSCS study, charybdotoxin plus apamin, inhibitors of endothelium-dependent hyperpolarization-mediated responses, affected only

minimally relaxations to RSCS, indicating that the EDH component is only a minor component of the vasorelaxant effect to the northeastern Brazilian red wine.

Nitric oxide is generated by the endothelial NO synthase and it is a major regulator of vascular tone and blood pressure. Furthermore, after its synthesis and release, NO diffuses to adjacent smooth muscle cells and causes them to relax [80, 81]. Therefore, many studies have been performed to directly measure vascular relaxation elicited by natural products and found that the vasorelaxant effect is related to stimulation of NO release from vascular tissues [42, 82, 83]. The half-life of NO in the body is very short. Thus, the direct measurement of NOS activity and NO production in biological systems of activated endothelial cells has become increasingly important, and several methods for assaying NO in biological systems have been reported [84].

To extend our findings from functional experiments, we performed biochemical assay using a very sensitive technique for NO analysis, where we measured NO levels as an index of NO production before and after GASH or RSCS red wines administration. Quantification of the bioavailability of NO can be performed by using flow cytometry analysis based on fluorescent intensity levels of DAF2-DA, which increase in proportion to the amount of NO released by endothelial cells under baseline or stimulated conditions. The combination of DAF2 with NO, in the presence of oxygen, produces a highly fluorescent compound detected in the cytoplasm [85]. The reaction is specific since it is prevented by the NO synthase inhibitor, L-NAME. To extend the present findings from functional experiments, we have determined NO levels as an index of NO formation before and after GASH or RSCS treatment.

The GASH or RSCS was able to significantly increase NO levels in rabbit endothelial cells, suggesting that the increase of NO levels induced by GASH or RSCS is endothelium-dependent. Comparing the vasorelaxant effects and increased formation of NO induced by ACh and GASH or RSCS, it appears that although ACh induced the formation of a lower amount of NO compared to GASH or RSCS, its relaxant effect was higher than that induced by the red wines. Such a difference might be explained by the fact that endothelium-dependent ACh-induced relaxations involves, besides NO, the activation of endothelial small (SKCa) and intermediate conductance Ca2+ -activated K+ channels (IKCa) inducing hyperpolarization of the endothelium. Thus, it produces the subsequent relaxation of the vascular smooth muscle [72, 86] whereas the vasorelaxant effect of GASH or RSCS seems to involve predominantly the NO component.

Of note, pretreatment with L-NAME decreased NO production corroborating with the results in mesenteric artery rings. Our results are in accordance with

the reports from the literature showing that the treatment of human umbilical vein endothelial cells with red wine also increased the NO production [87].

Chronic treatment with phenolic compounds in red wine has been shown to reduce blood pressure and vascular dysfunction by several mechanisms. Indeed, the redox-sensitive Akt pathway has been shown to mediate the stimulatory effect of polyphenols and other natural products on the endothelial formation of NO by eNOS [16-88]. In order to characterize the signaling pathway involved in NO formation and eNOS activation, further experiments were performed in endothelial cells.

The beneficial effect of polyphenols on hypertension and its chronic cardiovascular consequences may be best explained by their ability to restore an optimal endothelium-derived NO formation, which, besides decreasing vascular tone, prevents the induction of endothelial senescence and vascular smooth muscle proliferation and migration, thereby contributing to maintain an optimal endothelial and vascular function.

Natural products and polyphenol rich sources induce NO-mediated relaxations in different types of arteries, and appear to involve redox-sensitive events [42, 68, 88]. Several studies have shown that some polyphenolic compounds such as epigallocatechin-3-gallate produce superoxide anions and hydrogen peroxide following auto-oxidation [70, 89]. Procyanidins condensed tanins oligomers can protect endothelial cells from membrane lipid oxidation and cytotoxicity by scavenging peroxynitrite [70]. The role of endothelial protective effects of polyphenols has been associated with the intracellular formation superoxide anions and the subsequent activation of the redox-sensitive PI3-kinase/Akt pathway leading to eNOS activation and the subsequent formation of NO [41, 70, 78]. However, the specific mechanism involved in the endothelial pro-oxidant effect of polyphenols is still unknown.

Our previous findings indicated that the GASH or RSCS-induced relaxations in mesenteric artery rings were strongly reduced by antioxidant inhibitors, such N-acetylcysteine, a radical scavenger or tempol, a superoxide dismutase (SOD) mimetic. These findings strongly suggest a key role for intracellular superoxide anions in the GASH or RSCS-induced endothelial-mediated relaxation. The increase of ROS was confirmed by experiments using cultured endothelial cells and the oxidative fluorescent probe DHE. GASH or RSCS significantly increased the formation of superoxide anions in endothelial cells. As a matter of fact, besides their direct antioxidant properties in vascular smooth muscle cells reported in several models of cardiovascular diseases, other activities of polyphenols may be related to the increased redox-sensitive mechanisms in the endothelial layer [90].

A vascular source of ROS activated by red wines has been suggested to involve the activation of NADPH oxidase [91]. In addition, an important

mechanism frequently overlooked in considering the biological effects of polyphenols is their ability to interact with receptors capable of initiating cell signaling [92]. Polyphenols are able to regulate the activity of cell surface growth factor receptors, especially receptor tyrosine kinases, including the EGF receptor, the VEGF receptor, the IGF receptor and the insulin receptor [73]. The activation of such receptors can increase the formation of ROS and the subsequent activation of redox-sensitive events. Indeed, such a mechanism has been suggested to contribute to the protective effect of polyphenols against endothelial dysfunction in an experimental model of hypertension [92].

To understanding the role of redox-sensitive effects in endothelial cells, our studies demonstrated the mechanisms of endothelial protection induced by RSCS northeastern Brazilian red wine. Endothelial NO synthase is mostly expressed in endothelial cells and can be activated in response to red wine and several polyphenol-rich sources, in part, through the Akt-dependent phosphorylation of eNOS at Ser1177 [47]. A similar activator mechanism was observed in the present study since RSCS increased the phosphorylation of eNOS at Ser1177 and of the Akt at Ser473. Furthermore, the stimulatory effect of RSCS on Akt at Ser473 and eNOS at Ser1177 was significantly reduced by pretreatment of endothelial cells with the SOD mimetic tempol indicating the involvement of a redox-sensitive event. The endothelial protective effect of grape- red wine byproducts has been attributable, at least in part, to their high level of polyphenols. Previous studies have shown that, besides red wine, other natural products containing high levels of polyphenols, such as grape-derived products and tea catechins, and also omega-3 fatty acids are able to increase the intracellular formation of superoxide anions leading to the subsequent activation of the redox-sensitive PI3-kinase/Akt pathway and, hence, eNOS derived NO formation and vasorelaxation [47, 70].

Bearing in mind that a reduction of peripheral vascular resistance can lead to a decreased arterial blood pressure, we have hypothesized that GASH or RSCS may possibly act by reducing vascular tone and exhibits antihypertensive effects. The present findings indicate that the acute intravenous administration of GASH or RSCS caused pronounced hypotension in normotensive rats, RSCS was significantly attenuated after acute NO synthase blockade, indicating the involvement of NO.

Thereafter, experiments were performed to determine whether oral administration of GASH or RSCS are also able to decrease an established high level of blood pressure induced by the chronic administration of a NO synthase inhibitor to rats. GASH or RSCS was evaluated at a dose of 100 mg/kg, which corresponds to about two glasses of wine (approximately 210 ml) for an adult daily. Regular intake of such a moderate volume of wine has been associated with cardiovascular

protective effects [73]. The present findings indicate that such a low dose of the GASH or RSCS treatment was able to significantly reduce the L-NAME-induced high level of blood pressure without affecting heart rate. Previous studies by Soares de Moura *et al.* [49] have also indicated that oral administration of a lyophilized southern Brazilian red wine induced an antihypertensive effect in L-NAME-treated rats. In addition, in SHR rats GASH produced similar antihypertensive effects after oral intake in duration of 7 or 21 days.

The present study has evaluated the level of flavonoids and other phenolic compounds present in the grape pomace byproducts and lyophilized red wine of the São Francisco valley region GASH and RSCS. Indeed, recent studies have shown that regular consumption of polyphenol-rich fruits and vegetables reduced the risk of cardiovascular disease, and flavonoids were the most important polyphenol category in most type of red wines [93]. Our analysis of the grape byproduct and red wine grape varieties of northeastern Brazilian extract has indicated antioxidant actions and vasodilator properties have been corroborate with other studies [94]. Thus, it is likely that products from northeastern Brazilian, including flavonoids founded, are responsible, at least in part, to induce molecular mechanisms in endothelial cells to protect cardiovascular system.

Conclusion

In conclusion, using different sophisticated *in vivo* and *in vitro* approaches, the present review demonstrate that grape byproducts and alcohol-free lyophilized red wine from the Brazilian São Francisco valley presented a high level of phenolic compounds, especially flavonoids. Taken together, the results obtained so far suggest that components described in this work support the proposed use of grape or wine byproducts to drug discovery.

The data described here demonstrated that grape byproducts and alcohol-free lyophilized red wine might be of great interest for its antiradical/antioxidant effects. In addition, red wine grape varieties of northeastern Brazilian induces hypotension in rats most likely by reducing peripheral vascular resistance subsequent to the induction of endothelium-dependent vasorelaxant effect, provided cardiovascular protection induced by their molecular mechanisms, including the activation of nitric oxide pathways via an increased formation of NO and a redox-sensitive event.

Finally, taken together, components and biological activities described in this work support the proposed use of grape byproducts, obtained from the wine production process, as a potential source of polyphenolic compounds used in the food industry (nutraceutical, and/or pharmaceutical industries). This review highlights the therapeutic potential of polyphenols from northeastern brazilian red wine products on the cardiovascular system.

List of abbreviations

AA: ascorbic acid; ARP: antioxidant reducing power; DAPI: 4′,6-Diamidino-2-Phenylindole, dihydrochloride; DHE: dihydroethidium; DPPH: 2,2-Diphenyl-1-Picrylhydrazyl; EDHF: endothelium-derived hyperpolarizing factor; EDTA: ethylenediaminetetraacetic acid; F-C: Folin-Ciocalteu's; FGS: fermented grape pomace; HPLC: reversed-phase high performance liquid chromatography; LNAME: Nω-Nitro-L-Arginine methyl ester hydrochloride; NO: nitric oxide; PE: phenylephrine; ROS: reactive oxygen species; UGS: unfermented grape pomace

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Ethics approval and consent to participate

This study conforms to the International Guide for Care and the Use of Laboratory Animals and in accordance with the local Ethics Committee on Animal Use of the Federal University of Paraíba (CEUA/UFPB n° 1505/13; 0310/08).

Conflicts of Interest

The authors report no conflict of interests, and are responsible for the both the content and development of this paper.

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Chapter 11

Biological and Immunological roles of Alkaloids

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1. Introduction

Alkaloids are natural products able to regulate biological systems by interacting with micro- and macromolecules when inside the body [1]. These molecules are secondary metabolites produced by a variety of organisms, such as plants, marine animals, bacteria and fungi and they are widely distributed in nature with different structures, biosynthesis routes and many pharmacological activities [2–4].

Alkaloids are well defined as crystalline substances that bound with acids to form salts and they are derived from the amino acid biosynthesis or transamination processes [5]. However, alkaloids form a group of substances that are, actually, difficult to define because they present a variety of structures that have no clear boundary among alkaloids and natural complex amines [6].

In 1983, Peletier defined alkaloid as "an organic substance of natural origin, cyclic, containing a nitrogen in the negative oxidation state and whose distribution is limited among the living organisms [7]. This definition of alkaloid remains up to today.

There are many different alkaloid classification systems due to the variety of their chemical structure, biochemistry, botanical origin and pharmacological activity. A didactic classification is based on their amino acids and two categories are considered: non-heterocyclic or atypical alkaloids called 'proto-alkaloids' or biological amines; and heterocyclic or typical alkaloids subdivided according to their ring structure. Heterocyclic or typical alkaloids are derived from plant sources, have a basic character, one or more nitrogen atoms, usually in a heterocyclic ring and generally demonstrating a physiological action on man and/or other animals. An example of a typical alkaloid (aromatic alkaloid) are the tetrahydroisoquinoline

alkaloids commonly found in numerous structurally diverse natural products that exhibit a wide range of biological and pharmacological activities[6,8,9].

Despite of their ability to induce pharmacological activities only in the 19th century, alkaloids have been isolated and defined their therapeutic activities. These studies allowed the introduction of several types of alkaloids as drugs to treat numerous diseases.

2. Classification of alkaloids

The main purpose of chemical compound classification is for the scientific study and further therapeutic usage. Alkaloids are classified according to (1) biological and ecological nature, (2) technological innovation and chemical relationship, (3) structure and (4) biosynthetic pathways (Table 1).

Table 1. Alkaloid classification

(1) Biological and ecological nature	
1. Neutral or weakly basic molecules	
2. Animal derivatives	
3. Marine alkaloids	
4. Moss alkaloids	
5. Derived from fungi and bacteria	
6. Non-natural alkaloids (analogues or structurally modified from natural alkaloids	
(2) Chemical nature and technological innovation	
Classification	Description
1. Natural alkaloids	Molecules naturally synthesized by living organisms
2. Biomimic and bionic alkaloids	Natural alkaloids artificially copied in the laboratory.
3. Synthetic alkaloids	Fully planned in synthetic laboratory
(3) Basic chemical nucleus of alkaloids	
1. Acridones	2. Phenylethylamines
3. Aromatics	4. Piperidines
5. Carbolines	6. Purines
7. Ephedras	8. Pyrolidines
9. Ergots	10. Pyrrolizidines
11. Imidazoles	12. Pyrroloindoles
13. Indoles	14. Pyrydines
15. Bisindoles	16. Sesquiterpenes
17. Indolizidines	18. Simple tetrahydroisoquinolines

19. Manzamines	20. Steroids
21. Oxindoles	22. Tropanes
23. Quinoles	24. Terpenoids
25. Quinozolines	26. Diterpenes
27. Quinolizidines	28. Triterpenes
29. Phenylisoquinolines	
(4) Biosynthetic origin, molecular precursors and chemical structure	
Classification	Derived
True alkaloids	Amino acids
Protoalkaloids	Amino acids
Pseudoalkaloids	Non amino acid derivative

[10–13]

2.1 True Alkaloids

True alkaloids are derived from amino acids and share a heterocyclic ring with nitrogen. These compounds are highly reactive and, even at low doses, present biological activity. True alkaloids form water-soluble salts; moreover, most of them are well-defined crystalline substances that unite with acids to form salts. These molecules occur in plants (1) in the free state, (2) as salts, and (3) as N-oxides.

These alkaloids occur in a limited number of species and families which decarboxylated amino acids are condensed with a non-nitrogenous structural moiety. The primary precursors of true alkaloids are amino acids such as L-ornithine, L-lysine, L-phenylalanine/L-tyrosine, L-tryptophan, L-histidine, and anthranilic acid or nicotinic acid (Table 2) [5,11,14].

Table 2. Amino acids precursors and chemical groups of alkaloids formed

Precursor	**Chemical groups of Alkaloids**	**Examples**
L-ornithine	Pyrroline	cuscohygrine and hygrine
	Tropane	atropine, cocaine, hyoscyamine and scopolamine/hyoscine
	Pyrrolizidine	acetyl-lycopamine, acetyl-intermedine, europina, homospermidine and ilamine

L-lysine	Piperidine	anaferine, lobelanine, lobeline, N-methylpelletierine, pelletierine, piperidine, piperine, pseudopelletierine and sedamine
	Quinolizidine	cytisine, lupanine, sparteine
	Indolizine	castanospermine and swansonine
L-tyrosine	Phenylethylamino	adrenaline, anhalamine, dopamine, noradrenaline and tyramine
	Simple tetrahydroisoquinoline	codeine, morphine, norcoclaurine, papaverine, tetrandrine, thebaine and tubocurarine
	Phenethylisoquinoline	autumnaline, crinine, floramultine, galanthamine, galanthine, haemanthamine, lycorine, lycorenine, maritidine, oxomaritidine and vittatine
L-tryptophan	Indole	arundacine, arundamine, psilocin, serotonin, tryptamine, zolmitriptan, elaeagnine, harmine, ajmalicine, catharanthine, secologanin and tabersonine
	Quinoline	chloroquinine, cinchonidine, quinine and quinidine
	Pyrroloindole	chimonantheine, chimonantheine, corynantheine, corynantheidine, dihydrocorynantheine and corynanthine
	Ergot	ergobine, ergotamine, ergocomine, ergocornine, ergocristine, ergosine and ergostine
L-histidine	Imidazole	histamine, pilocarpine and pilosine
	Manzamine	xestomanzamine A and B
L-arginine	Marine	saxitoxin, tetrodotoxin, 1-acetyl-β-carboline, 7-bromo-1-ethyl-β-carboline, eudistomidin B, G, H, I, J and K, marinacarboline A – D, seragadine A, veriabine A and B

Anthranilic acid	Quinazoline	Peganine
	Quinoline	acetylfolidine, acutine, bucharine, dictamnine, dubunidine, γ-fagarine, flindersine, foliosidine, glycoperine, haplophyllidine, haplopine, helietidine, kokusaginine, maculosine, prefamine, perforine, polifidine and skimmianine
	Acridone	acrsonycine and rutacridone
Nicotinic acid	Pyridine	anabasine, cassinine, celapanin, evoline, evonoline, evorine, maymyrsine and nicotine

[10,15–30]

2.2 Protoalkaloids

Protoalkaloids are compounds, in which the amino acid derivate nitrogen atom does not belong to a heterocyclic bond of the chemical structure of the molecule. These compounds are derivatives from L-tyrosine and L-tryptophan (Table 3) [7]. In addition protoalkaloids are molecules with a perfect closed ring and structurally simple. However, they consist a minority of all alkaloids and can be as natural, biomimic, bionic, and synthetic alkaloids

Table 3. Precursor Compound and Chemical Group of Alkaloids of Protoalkaloids

Precursor Compound	Chemical Group of Alkaloids	Examples
L-tyrosine	Phenylethylamino alkaloids	Hordenine and mescaline
L-tryptophan	Terpenoid indole alkaloids	Yohimbine

[31].

2.3 Pseudoalkaloids

Pseudoalkaloids are compounds that the basic carbon skeletons are not derived from amino acids. They can also result from the amination and transamination reactions of the different pathways connected with precursors or postcursors of amino acids (Table 4). Pseudoalkaloids can be as natural, biomimic, bionic, and synthetic alkaloids. [5,14].

Table 4. Precursor Compound and Chemical Group of Alkaloids of Pseudoalkaloids

Precursor Compound	Chemical Group of Alkaloids	Examples
Acetate	Piperidine alkaloids	Coniine, coniceine and pinidine
	Sesquiterpene alkaloids	Cassinine, celapanin, evonine, evonoline, evorine, maymyrsine, regelidine and wilforine
Pyruvic acid	Ephedra alkaloids	Ephedrine and norephedrine
Ferulic acid	Aromatic alkaloids	Capsaicin
Geraniol	Terpenoid alkaloids	Aconitine, actinidine, atisine and gentianine
Saponins	Steroid alkaloids	Cholestane, conessine, cyclopamine and jervine
Adenine/guanine	Purine alkaloids	Caffeine, theacrine, theobromine and theophylline

[32]

3. Alkaloids and their biological activities

As a therapeutic molecule, alkaloids have been described to present biological effects such as antitumor, anticholinergic, diuretic, sympathomimetic, antiviral, and antihypertensive. In addition the alkaloids can act on the CNS (central nervous system) and others systems as hypnoanalgesic, antidepressant, myorelaxant, antimicrobial, antiemetic and anti-inflammatory [7](Figure 1).

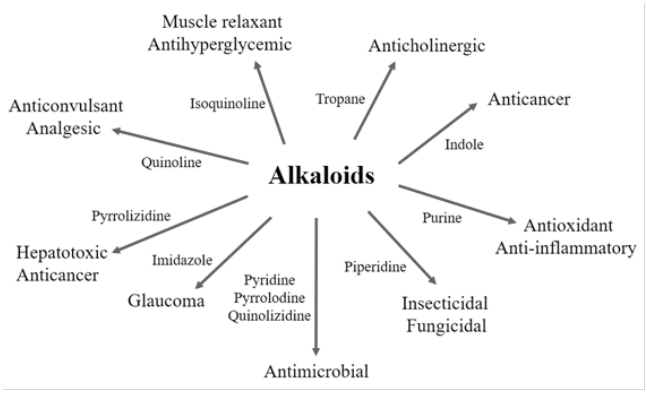


Figure 1. Alkaloid and its biological effects.

However, there are several reports indicating the toxic effects of alkaloids in humans thus, it is necessary to study alkaloids in different experimental models to understand the exact mechanism of action of these molecules in order to have the real knowledge of their effect.

Therefore, we are going to describe below some classes of alkaloids including tropane, indole, purine, piperidine, imidazole, pyrrolizidine, pyrrolidine, quinolizidine and isoquinoline and its pharmacological purposes.

3.2 Tropane

Tropane alkaloids have the 8-azabicyclo octane nucleus and are derived from the amino acid ornithine [2,33]. Examples of tropane alkaloids: scopolamine, hyoscyamine, cocaine and atropine that have several legitimate medicinal uses. This group of alkaloids is well-known for anticholinergic activities [34].

3.3 Indole

Indole alkaloids have in their chemical structure, serotonin (hydroxytryptamine or 5-HT). More than two thousand compounds are classified into this alkaloid class, among them, the most studied; due to their pharmacological effects are vincamine, vincristine, vinblastine, strychnine, ajmalicine and ajmaline. Vinblastine and vincristine, so-called "Spindle Poison", are often used as anticancer drugs [2].

3.4 Purine

Purine alkaloids, also so-called "Xanthenes", are derived from purine (adenine and guanine). Caffeine, theobromine, theophylline and aminophylline are the most important members of this alkaloid class. They have many beneficial properties, such as antioxidant and anti-inflammatory features, in addition they offer protect from diabetes, hyperlipidemia and obesity [35,36].

3.5 Piperidine

Piperidine alkaloids possess a saturated heterocyclic ring and are well-known for their toxicity. However, they also have biological activities such as, bactericidal, antihistaminic, anticancer, herbicidal, insecticidal, fungicidal, central nervous system stimulant and depressant effects. The well-known members of piperidine alkaloids are coniine, lobeline and cynapine [37].

3.6 Pyridine

Pyridine alkaloids are similar to piperidine alkaloids, except by their heterocyclic nitrogen containing unsaturated nucleus. Important members of

pyridine alkaloids are anabasin, nicotine, anatabin, anatabine and epibatidine. They have been found to exhibit strong antimicrobial properties [38].

3.7 Imidazole

Imidazole alkaloids are derived from amino acid L-histidine containing imidazole ring. Pilocarpine is an important member of this group alkaloid. The product is valuable in ophthalmic practices and eye disorders treatment, such as glaucoma [39].

3.8 Pyrrolizine

Pyrrolizidine alkaloids have two five-membered rings (necine base), which share a nitrogen in position 4. Senecionine, heliotrine and clivorine are the common examples of pyrrolizidine alkaloids. They have hepatotoxic effects, however their glycosidase inhibitory activity makes them an important compound for treatment of cancer and diabetes [40].

Pyrrolidine alkaloid class contain five-membered, N-containing rings that are derived from amino acids ornithine (or arginine in some cases) and lysine, with addition of acetate/malonate units. Putrescine, hygrine and cuscohygrine are some of important pyrrolidine alkaloids examples. These alkaloids present antimicrobial properties against a wide range of microorganisms [41].

3.9 Quinolizidine

Quinolizidine alkaloids consist of two six-membered fused rings that share nitrogen and show simple and complex structural variations. In addition to lupinine and lupanine alkaloids, cytisine and sparteine are the two most widely distributed quinolizidine alkaloids. They exhibit antimicrobial properties against a wide range of microorganisms [42].

3.10 Aromatic alkaloids

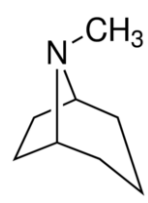
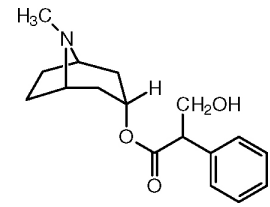
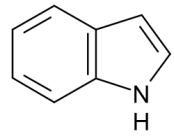
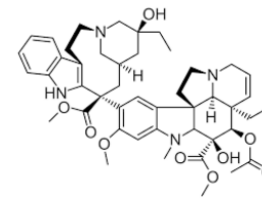
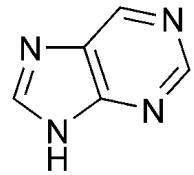
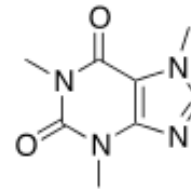
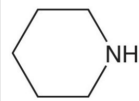
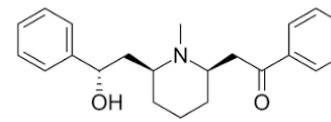
Aromatic alkaloids, so-called “Benzopyridines”, are formed by the fusion of a benzene ring with a pyridine ring, presenting quinoline and isoquinoline nuclei. Examples of quinolines alkaloids are quinine, camptothecin, echinopsine, homocamptothecin, chinidin, cinchonidin, folipdine and dihydroquinine. This class of compounds presents important biological activities, such as: antimalarial, anti-bacterial, antifungal, anthelmintic, cardiot onic, anticonvulsant, anti-inflammatory and analgesic activities [43].

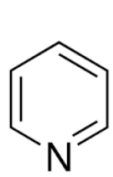
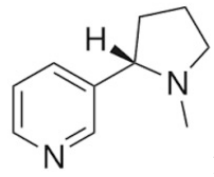
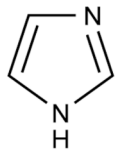
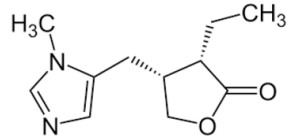
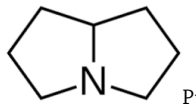
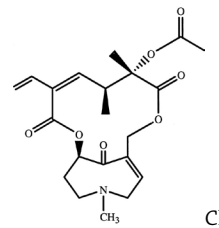
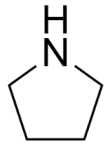
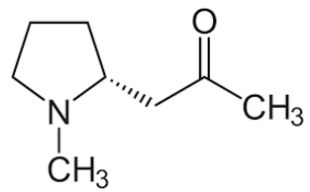
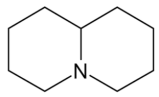
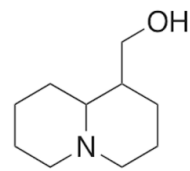
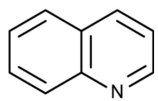
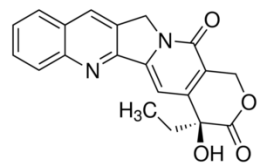
Isoquinoline alkaloids are structural isomers of quinoline alkaloids. These alkaloids are further subdivided into simple-isoquinolines, benzylisoquinolines, morphine alkaloids, phthalide isoquinolines, protoberberines and ipecac alkaloids,

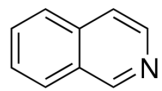
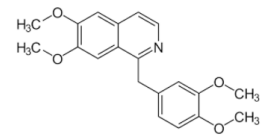
based on an addition of groups. Narcotines, protopines, morphine, codeine and thebaine belong to this alkaloid group. They have analgesic effect as well as some can be narcotic drug (morphine), cough suppressant (codeine) and muscle relaxant [44]. It also have antitumor properties associated with papaverine and noscapine, and antimicrobial activity linked to sanguinarine [44]. Additionally, this alkaloid class are known to exhibit biological activities, such as antihyperglycemic, antitumor and antibacterial activities [45].

The general structures and an example of each classes of alkaloids are shown in table 5.

Table 5. Classes of alkaloids and their chemical structures

Type of Alkaloids	Structures
Tropane Alkaloid	 Tropane  Atropine
Indole Alkaloid	 Indole  Vimblastine
Purine Alkaloid	 Purine  Caffeine
Piperidine Alkaloid	 Piperidine  Lobeline

Pyridine Alkaloid	 Pyridine  Nicotine
Imidazole Alkaloid	 Imidazole  Pilocarpine
Pyrrolizidine Alkaloid	 Pyrrolizidine  Clivorine
Pyrrolidine Alkaloid	 Pyrrolidine  Hygrine
Quinolizidine Alkaloid	 Quinolizidine  Lupinine
Quinoline Alkaloid	 Quinoline  Camptothecin

Isoquinoline Alkaloid	 Isoquinoline  Papaverine

4. The immune system and alkaloids

4.1 Immune system and the maintenance of health

The immune system of the vertebrates is composed by primary lymphoid organs (bone marrow and thymus) and secondary organs (lymph nodes, spleen and mucosa associated lymphoid tissues), high endothelium venules, leukocytes, tissue resident cells and several molecules such as proteins of the complement system and cytokines secreted by an array of blood, epithelium and mucosal cells. Its functionality is didactically divided on natural immunity (innate immune response) and adaptive immunity (adaptive immune response). The immunity function is basically to maintenance of homeostasis by defending the body against pathogenic- or non-self- agents and autoreactive reactions [46–48]blood vessels deliver lymphoid tissue inducer cells that initiate and sustain the development of lymphoid tissues. In adults, the blood vessels are structurally distinct from those in other organs due to the requirement for high levels of lymphocyte recruitment under non-inflammatory conditions. In lymph nodes (LNs.

The innate immune response is the first barrier for pathogens such as bacteria, fungi, virus, parasites and pollutants. Innate response is also known as natural or non-specific immune responses because of its limited capability of pathogen discrimination. These mechanisms are represented by defense barriers classified as physical (i.e. skin, mucosa, hair, nails, urine flow), chemical (i.e. enzymes, gastric acid, antibiotic peptides and alfa/beta interferons) and biologic (i.e. microbiota) which inhibits the pathogenic microorganism grows preventing serious infections of gastrointestinal, urinary, respiratory tracts and others [49].

As part of innate immune cells, phagocytes mainly represented by tissue resident macrophages and dendritic cells and blood leukocytes as neutrophils and monocytes eliminate pathogens by recognizing them via membrane cell proteins named Toll like receptor (TLR) which are described as the main pathogen associated pattern recognition (PAMP) receptors. TLR recognize different microorganism PAMPs such as sugars, lipids, proteins and nucleic acid which allowing activation of phagocytes and secreting of reactive oxygen intermediate (ROI) species, nitric oxide (NO) and lizossomal enzymes responsible for intracellular pathogen

killing. However, sometimes innate immune response is not enough to eliminate pathogens properly which leads the immune system activating a second and more specialized defense mechanism strategy called the adaptive immune response [49]

The adaptive immune response is composed by T and B cells that present specific receptors to recognize specific aggressive agents. These activated cells produce molecules such as cytokines and immunoglobulins (Ig) that together protect the organism against the invader [50].

According to the pathogens different T cell population may be activated such as TCD4+cells/T helper (Th) cells or TCD8+cells that are cytokine-secreting cells or citolytic lymphocytes respectively. Th cells might differentiate in Th cell subtypes mainly described as Th1, Th2, Treg or Th17 cells that are able to secret an array of cytokines able to regulate the whole immune response since the innate immune response to adaptive immune response. Firstly, Th1 and Th17 cytokines (IFN-γ and IL-17 family) improve the cell recruitment and phagocyte response in terms of enhancing phagocytosis rate as well as increasing ROIs (reactive oxygen intermediate) and NO (nitrogen oxide) generation allowing a faster and stronger pathogen clearance by the innate immune response [51].

In addition, Th1 response is the main anti-tumor defense mechanism in humans. IFN-γ is well described as a pivotal stimulus to catalytic T cell activation and tumor cell apoptosis induction [52]. Secondly, Th2 cells derived cytokines (IL-4, IL-5, IL-13) are necessary to induce B cell action and parasite-specific IgE production besides eosinophil cell recruitment to eliminate parasites commonly located at gut mucosa where they grow and cause nutrient depletion, bowel disease symptoms such as abdominal pain and diarrhea [53]. Equally important to maintenance of homeostasis, Treg cells are required to balance Th responses by secreting regulatory (IL-10) and immunossupressive (TGF-β) cytokines [54]. IL-10 is largely described as anti-inflammatory molecule produced by different cell sources including Treg cells. It has been well documented that IL-10 cytokine physiological levels are pointed as a preventive factor to avoid chronic inflammatory diseases such as asthma, rheumatoid arthritis and multiple sclerosis. Also, TGF-β secreted by Treg regulates pathological immune reactions which could be causing tissue damaging as those known as autoimmune diseases [55,56]memory and effector functions, and their role in CAR-T therapy--a cellular adoptive immunotherapy with T cells expressing chimeric antigen receptor. The CAR-T cells recognize tumor antigens and induce cytotoxic activities against tumor cells. Recently, differences in T cell functions and the role of memory and effector T cells were shown to be important in CAR-T cell immunotherapy. The CD4\u207a subsets (Th1, Th2, Th9, Th17, Th22, Treg, and Tfh. Both innate and

adaptive immune responses induce inflammatory process that underlies a wide variety of physiological and pathological mechanisms.

The classic targets of inflammation, infection and tissue injury, trigger the recruitment of leukocytes and plasma proteins to the affected tissue site and induce innate and/or adaptive immune responses. These immune responses rely mainly on tissue-resident macrophages and are intermediate between the basal homeostatic state and a classic inflammatory response [57]. The concept of inflammation and its terms such as acute and chronic have come down to us from such remote antiquity that they carry with them, inevitably, imprecise usage [58].

The terms acute and chronic represent two distinct aspects of the inflammatory reaction that relies on biologic and histologic events. In summary, local injuries of all sorts induce an immediate, acute immune response, which is acts primarily on the microcirculation, with two main effects: a) exudation of fluid, and b) exudation of white blood cells, primarily polymorphonuclear leukocytes (PMNs). This is nonspecific response. Therefore, a typical focus of chronic inflammation, as caused by, for example, tuberculosis, mononuclear cells including mainly lymphocytes and plasma cells show up with a highly specific response. Thus, local tissue injury can elicit two types of response: one immediate and nonspecific and the other delayed and highly specific. Of note, cells and their secreted cytokines regulate immune responses but also exogenous stimuli might regulate immune response as well as the anti-inflammatory drugs [59]. Accordingly these perspectives, new molecules have been tested for their potential effect in regulation inflammatory reaction and several natural products classes have been described for this purpose such as alkaloids [60].

Therefore, in this chapter we aim to describe some alkaloids that have their action on different branches of the immune system by using *in vivo* and *in vitro* inflammatory methodologies. We are focusing mainly on bisbenzylisoquinoline, tetraisoquinoline, morphynamic alkaloids.

4.2 Bisbenzylisoquinoline alkaloids

Bisbenzylisoquinoline (BBI) alkaloids are defined by two monomeric benzylisoquinoline (BI) units, which are linked by ether bridges. In addition to the ether linkage, methylenoxy bridging or direct carbon-carbon bounding is also found between the two BI units. They present a variety of structural standards with differences on their structures, such as the number of aromatic oxygen substituents, the number and nature of ether bridges (e.g. diphenyl ether or benzylphenyl ether), and the sites on the two BI units at which the ether or the carbon carbon bond originates [61].

Groups of BBI alkaloids are classified based on their structure differences. An individual member of alkaloids, in each group, differs in: a) the nature of the oxygenated substituents (OH, OMe, OCH2O); b) the nature of substitution of the two nitrogen atoms (NH, XMe, N-Me2, NO); c) the degree of unsaturation of the hetero rings and d) the stereochemistry of the two asymmetric centers [61]. In addition, BBI alkaloids vary in nature and in relative proportion in different parts of the plant, for instance: leaves of Menispermum canadense Linn, contain no alkaloid; whereas, other parts of the plant, stem, root, and rhizome contain a huge amount of alkaloids [61].

Bisbenzylisoquinoline (BBI) alkaloids have been used as medicine in a various pathologies, such as hepatic injury and immunological diseases [62]. In addition, BBI alkaloids are major components of some antirheumatic remedies; they also possess anti-inflammatory [63–65], immunomodulatory [63,66] and antimalarial activities [67–69].

Besides, they present anti-inflammatory activities based on their ability to prevent the synthesis or action of some pro-inflammatory cytokines [70], cytotoxic toward some cancer cell lines and human tumor [51] and protection against lethal toxicity induced by lipopolysaccharide from bacillus of *Calmette-Guerin* [71].

Thereby, this group of alkaloids has been widely studied due to their potential pharmacological effects. Another important aspect of this group of alkaloids is that, approximately, two hundred compounds belong to different families of plants such as Ranunculaceae, Menispermaceae, Berberidaceae, Annonaceae, Lauraceae and Monimiaceae [61,72,73].

Menispermaceae family is well known for producing different types of alkaloids [74]. Several studies with different species of this family showed the presence of bisbenzylisoquinoline alkaloids (warifteine and methylwarifteine); morphinandienoni alkaloid (milonine); aporfinic alkaloid (laurifoline) [75]; bisbenzyltretaisoquinoline alkaloid (roraimine) and oxaporfinic alkaloid (liriodenine) [76].

Cissampelos sympodialis Eichl. (Menispermaceae), a species found in Northeast and Southeast Brazil, has been used in folk medicine for to treat rheumatism, colds, asthma, malaria, fever, pain, edema, urethritis, cystitis, ulcers and menstrual disorders [77]. Phytochemical analyses of the roots of this plant revealed the presence of several alkaloids mainly warifteine and its methylated form - methylwarifteine [62] and in the leaf a high amount of milonine (morphinandienoni alkaloid). Studies showed that the leaf hydroalcoholic extract of *C. sympodialis* presented an immunodulatory effect on spleen cells from allergic and non-allergic mice by enhancing the production of IL-10 and IFN-γ as well as the enhancing of IL-10 levels in mice macrophage cultures [78,79].These data scientifically demonstrated, for the first time, that the plant has immunomodulatory effect.

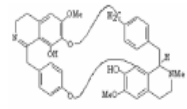
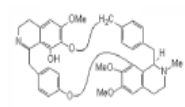
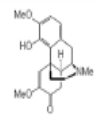
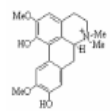
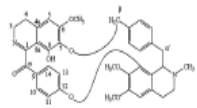
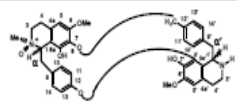
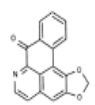
Structure	Compound name	References
	Warifteine	Barbosa et al., 1997
	Methilwarifteine	Barbosa et al., 1997
	Milonine	Barbosa et al., 1997
	Laurifoline	Alencar, 1994
	Roraimine	De Lira et al., 2002
	Simpodialine β-*N*-oxide	Alencar, 1994
	Liriodenine	De Lira et al., 2001

Figure 2. The chemical structures of Alkaloids of *Cissampelos sympodialis* Eichl [80].

4.2.1 Warifteine

Warifteine (C36H36N2O6) (figure 3), a bisbenzylisoquinoline alkaloid, is the predominant componentof root bark of *C. sympodialis* [62,81]. Several studies have demonstrated promising pharmacological effects of this alkaloid on biological systems including immunomodulation by decreasing the production of immunoglobulin, antidepressant effect and inhibition of eosinophil recruitment to the lungs in experimental model of asthma [77,82].

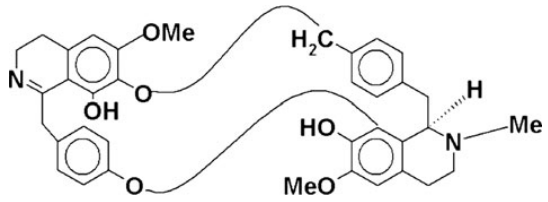


Figure 3. The chemical structure of warifteine.

Warifteine is an amorphous yellow crystal compound, chemically named (R)-2,8,13,13a,14,15,16,25-Octahydro- 18,30- dimethoxy-14 -methyl -4,6:9,12:21,24 -trietheno-3H pyrido (3',2':14,15) (1,11) dioxacycloeicosino (2,3,4- ij) isoquinoline-5,19-diol with molecular weight of 592.68084 g/mol. In addition, this molecule is insoluble in polar solvents but in acidic conditions becomes a water-soluble salt, allowing the in vivo and in vitro analysis without addition of other toxic solvents. Warifteine becomes an important compound marker for the extract standardization of *Cissampelos sympodialis* as well as a candidate for a phytomedicine [83].

Pharmacological effects of warifteine demonstrated that the molecule was able to relax smooth muscle independently of endothelium, i.e., it did not only control the muscle tone in vessels but also relaxed the bronchioles muscles [81]. Warifteine then becomes an important tool in attempting to prevent or reverse the respiratory distress occurring during asthmatic attacks [84]. To evaluate the alkaloid activity on mast cell degranulation, IgE anti-DNP-BSA sensitized mast cells were treated with warifteine and, after DNP-BSA challenges it was observed that the molecule inhibited the mast cell degranulation by measuring histamine release. Histamine is one of the molecule that contributes to the muscle contradiction in several medical conditions including allergic reaction. To corroborate with this data, warifteine treatment inhibited mice scratching behavior mediated by 48/80 compound as well as hyperalgesia reaction [82]. Therefore, warifteine modulates, mast cells, vessels and nerves.

Several experimental models have been employed for analyzing B cell *response in vitro*. Anti-IgM and T-independent type 2 antigens (TI-2) have been used as tools for studying B cell signaling [85–87]. In this context, it was observed that warifteine inhibited both B cell proliferation and immunoglobulin secretion induced by TLR ligands (LPS, Pam3Cys and CpG oligodeoxynucleotide) or anti-IgM. These effects were not due to a toxicity since warifteine neither induced alteration in propidium iodide labeling of fresh spleen B cells nor modified XTT metabolization by the B cell line A20. As mechanism of actions, warifteine treatment attenuated intracellular calcium levels, the phosphorylation of mitogen-activated protein kinase (MAPK) ERK and the intracellular levels of transcription

factor NFκB and increased the cAMP level. *In vivo* study, warifteine treatment inhibited the production of anti-TNP-ficoll in BALB/c mice immunized with TI-2 antigen (TNP-ficoll)[88]. Taken together, the data showed that the alkaloid of *Cissampelos sympodialis* is responsible for the B cell modulatory effect.

In addition warifteine inhibits the eosinophil activity. One of the feature of asthma is a pulmonary chronic inflammation with degeneration of bronchial epithelium in an eosinophil-dependent mechanism. Eosinophils release cationic proteins, chemotactic agents (eotaxin) and eicosanoids (cis-LT) [89]. Treatment with warifteine inhibited eosinophil migration into the pleural and bronchoalveolar cavities of OVA sensitized BALB/c mice [77]. Warifteine, also, decreased cis-leukotriene and eotaxin secretion suggesting the alkaloid was controlling the inflammatory process by regulating eosinophils. Thus, this information provides strong support of the modulatory effect of warifteine on allergic and inflammatory reactions.

Cissampelos simpodialis also naturally produces the methylate form of warifteine named methylwarifteine. [90] threatening OVA-sensitized animals with methylwarifteine by inhalation rout observed diminishing of the number of LTCD3+ and eosinophils into the bronchial space suggesting that the methylation process improved the molecule activity. It was also shown significant and reproducible inhibitory activity of methylwarifteine to human nasopharynx carcinoma cells [91].

4.2.2 Curine

Curine is the major constituent of the root bark of *Chondrodendron platyphyllum* (Menispermaceae), which is a medicinal plant found in Northeast Brazil. The folk medicine uses the plant to treat malaria, fever, pain, swelling, urethritis, cystitis and ulcers [92]. Three alkaloids have been identified from this plant: curine, isocurine, and 12-O-metilcurine, and curine is the major constituent [93]. The curine and isocurine presented a vasodilatory effect in the rat artery as well as anti-inflammatory activity in a LPS-induced pleurisy model [93]. The molecular structure of curine is presented on figure 4 [92].

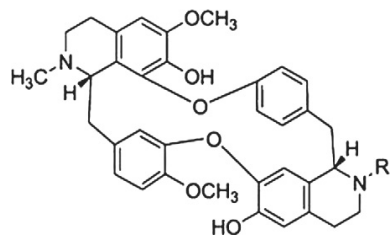


Figure 4. The chemical structure of curine

Curine is an alkaloid with some biological properties. Recently, Medeiros et al. (2011) demonstrated that curine decreased intracellular Ca 2+ transients in A7r5 cells, possibly through a direct blockade of L-type Ca 2+ channels. In addition [93], showed that curine have a vasodilator effect associated with the inhibition of calcium channels.

The first study in pulmonary inflammatory allergic model, conducted by Ribeiro-Filho and colleagues (2013), demonstrated that oral administration of curine significantly inhibited pulmonary allergic eosinophilic inflammation and eosinophil lipid body formation. Curine treatment also reduced eotaxin and IL-13 production triggered by allergen and inhibited the calcium-induced tracheal contractile response, suggesting that the mechanism by which curine exerts its biological effects in pulmonary inflammation is through the inhibition of a calcium-dependent response. A toxicological evaluation showed that curine, orally administered, did not alter the biochemical, hematological, behavioral and physical parameters postulating that curine presented low toxicity.

Besides the potent anti-allergic activity of curine this alkaloid was also studied in experimental models of acute inflammation. Leite and colleagues (2014) analyzed the effect of curine in inflammatory nociception by using the acetic acid writhing experimental model. For these experiments, they used indomethacin and morphine as standard pharmacological controls. The authors observed that curine and indomethacin presented similar but partially inhibitory effects, whereas the writhing response was completely inhibited by morphine, suggesting that curine presents an analgesic effect by anti-inflammatory mechanisms independently of neurogenic mechanisms. To prove this hypothesis, formalin test was used. This test characterizes the nociception by neurogenic or inflammatory mechanisms. In the first phase (neurogenic phase), curine or indomethacin presented no effect in the licking behavior. However, the second phase (inflammatory phase) was significantly inhibited by curine, indomethacin, as well as morphine, confirming that the analgesic effect of curine is dependent of anti-inflammatory mechanisms. Taken all the data together, the authors demonstrated, for the first time, that curine presents anti-inflammatory and analgesic property.

In addition Leite and colleagues (2014) also demonstrate the anti edematogenic (another cardinal signal of inflammatory process) effect of curine. Animals pretreated with curine showed significant inhibition of the paw edema formation caused by carrageenan as well as decreased of vascular permeability induced by acetic acid suggesting that its anti-edematogenic property was associated with the inhibition of vascular permeability.

4.3 Tetrahydroquinoline alkaloid

Tetrahydroisoquinoline (THI) alkaloids present pharmacological actions on inflammation (TSOYI et al., 2008). Studies have shown that THI analogs are effectively inhibitors of iNOS expression on many lipopolysaccharide (LPS) activated cells [94–96]YS 49, on NO production and iNOS protein expression were investigated in cultured rat aortic vascular smooth muscle cells (RAVSMC. These molecules present one asymmetric center in their structure and exist as enantiomers, which encourage the development of new drugs instead of using racemic mixtures[97].

4.3.1 MHTP

MHTP (2-methoxy-4-(7- methoxy-1,2,3,4-tetrahydroisoquinolin-1-yl) phenol) (Figure 5) is an unprecedented tetrahydroquinoline alkaloid synthesized by Pictet– Spengler reaction (Figure 6). This condensation method is considered the most important method for alkaloid synthesis and to produce new compound prospections with therapeutic properties [98,99].

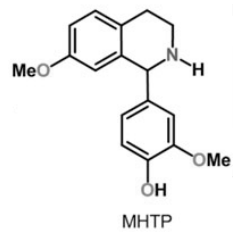


Figure 5. The chemical structure of MHTP [2-methoxy-4-(7- methoxy-1,2,3,4-tetrahydroisoquinolin-1-yl) phenol][98].

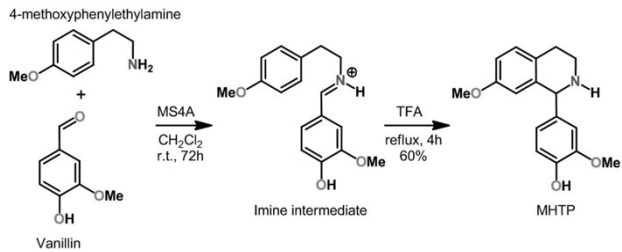


Figure 6. MHTP synthesis 2-methoxy-4-(7- methoxy-1,2,3,4-tetrahydroisoquinolin-1-yl) phenol (MHTP) was synthesized by the Pictet–Spengler cyclization, using p-(OH)- phenylethylamine and vanillin.

MHTP is chemically similar to cryptostilins I, II and III isolated from *Cryptostylis fulva* (Orchidaceae) (figure 7), which have been described to have biological properties. Numerous cryptostilin analogues present antagonist effects for the dopamine D1 receptor [100,101] and for substance P, which is involved in the transmission of pain and neurogenic inflammation.

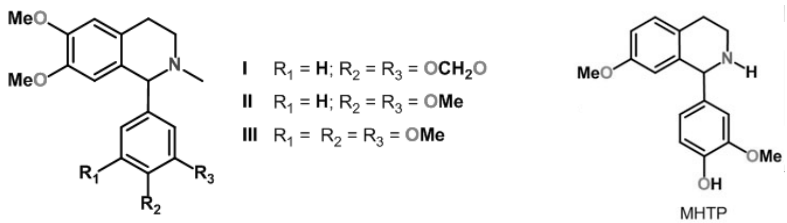


Figure 7. Chemical structure of Cryptostilins I, II, III Source: MUNCHHOF; MEYERS (1995)

MHTP is also chemically similar to the synthetic isoquinoline alkaloids CKD712 [(S) -1- (α-naphthylmethyl) -6,7-dihydroxy-1,2,3,4- tetrahydroisoquinoline] and THI52 (1-naphthylethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline) (figure 8) that have been described to present anti-inflammatory activity [33,96].

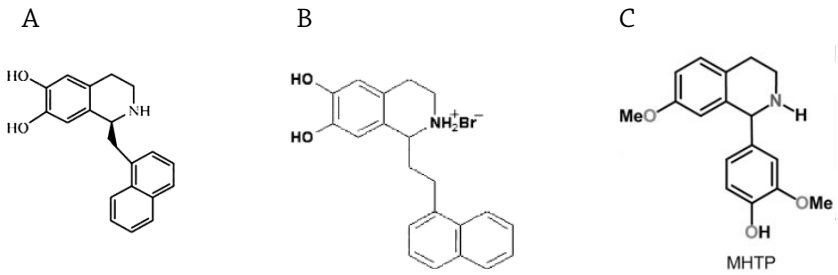


Figure 8. The chemical structure of alkaloid CKD712 (A); THI52 (B); MHTP (C) [96,97].

In our laboratory we showed [102] the anti-edematogenic property of MHTP by using the carrageenan-induced paw edema model. The oral treatment with MHTP at dose of 2.5 mg/kg inhibited significantly the paw edema formation in Swiss mice, which was analyzed at 1 h, 2 h, 3 h, 4 h, 5 h and 24h after carrageenan-inducted edema. In addition, MHTP paw edema inhibition was dependent of the PGE2 modulation on the inflamed site demonstrated one of its mechanism of action. In addition, Oliveira and colleagues (2015)

also evaluated the MHTP effect in inflammatory cell migration and protein extravasation on carrageenan-induced peritonitis model. Animals treated with MHTP presented decreasing on polymorphonuclear leukocyte migration to the inflamed site and protein extraversion on the carrageenan-inflamed peritoneum. Another important observation, of this study was that in LPS-induced acute lung injure (ALI) mice pre-treated with MHTP presented significantly diminishing of polymorphonuclear leukocyte migration into the inflamed lung corroborating with the anti-inflammatory property of the alkaloid.

Taken all data together it was summarize that MHTP, a synthetic alkaloid, presents anti-edematogenic effect by inhibiting PGE2 activity, anti-inflammatory effect by inhibiting leukocyte migration as well as by reducing the protein extraversion to the inflamed tissue implicating that MHTP presents potential to became a drug to be included on the arsenal of anti-inflammatory medicines.

4.3.2 CKD712

CKD712 is a synthetic tetrahydroisoquinoline alkaloid of (S)-1-(α-naphthylmethyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline and it is a (s)-form of the YS-49 enantiomer, a derivative of higenamine. This molecule presents great potential as anti-inflammatory, anti-septic or anti-cancer. Recent studies have shown that CKD712 can be used for the treatment of disorders such as sepsis [103]. Besides, CKD712 inhibited iNOS on activated macrophages through the negative regulation of Janus kinase2 (JAK2)/signal transducer and the inhibition of the activator of transcription 1 (STAT-1) phosphorylation and heme oxygenase-1 (HO-1) induction (Tsoyi et al., 2008), showing that CKD712 acts on multiple targets on the inflammatory process [103].

Furthermore, studies with CKD712 demonstrated that the molecule presented protective effect against LPS-mediated platelet aggregation by attenuating the pro-inflammatory response of LPS-induced endothelial cells. In addition, CKD712 inhibited the expression of vascular adhesion molecule-1 (VCAM-1) by a negative regulation of phosphoinositide 3-kinase (PI3K)/ protein kinase B (AKT) pathway [104].

4.3.3 THI52

THI52 (1 – naphthylethyl – 6, 7 – dihydroxy – 1, 2, 3, 4 -tetrahydroisoquinoline) (figure 9) is a synthetic isoquinoline alkaloid. In a study developed by Kang et al (2003), the authors showed the inhibitory effect of THI 52 on TNF-α mRNA and iNOS mRNA expressions and iNOS protein production and NO production *in vitro* and *in vivo* experimental models. These effects were related with NF-kB modulation.

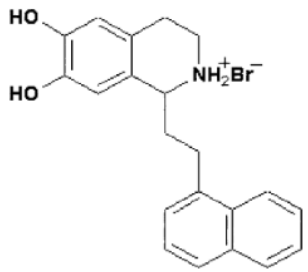


Figure 9. The chemical structure of THI52 [96]

4.4 Morphynandienone alkaloids

4.4.1 Morphine

The primary source of morphine (C17H19NO3) is from poppy straw of the opium poppy (*Papaver somniferum*) and is the main representative alkaloid in opium and the prototype opiate analgesic and narcotic drugs and it is the first alkaloid used as therapeutic drug for pain releases. Morphine has its widespread effects in the central nervous system and on smooth muscle. Currently, it is estimated that about 10,000 alkaloids are known as drugs [6] and morphine was the first active alkaloid isolated from a plant between 1803 and 1805 by Friedrich Sertürner. In addition, morphine was more widely used after the invention of the hypodermic syringe in 1853–1855. Friedrich Sertürner originally named the substance morphium due to the Greek God of Dreams, Morpheus because its sleepiness property.

Pharmacological studies demonstrated that morphine induces a relaxed and drowsy state, however presented several side effects such as depression of the respiratory, circulatory, and gastrointestinal systems. The opioid agonists cause varying degrees of hypotension and bradycardia by central-nervous-system actions [105].

4.4.2 Milonine

Milonine, a novel 8,14-dihydromorphinandienone alkaloid (figure 10) was isolated from the leaves of *Cissampelos sympodialis* EICH (Menispermaceae). The molecule presents a morphinanic skeleton, a structural variant previously unknown in this genus [81]. Preliminary studies showed that milonine has spasmolytic effects in a similar way of warifteine [81] and promotes endothelium-dependent smooth muscle relaxation.

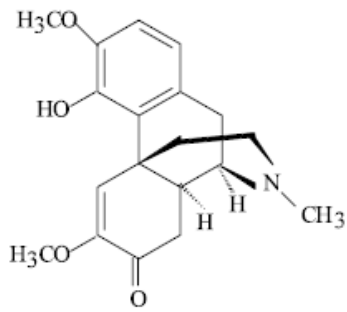


Figure 10. The chemical structure of milonine [80].

More recently, it was demonstrated that the milonine inhibited the mast cell degranulation and scratching behavior on experimental models of allergy. Milonine also decreased the 48/80 compound-edema formation independently of histamine release. In anaphylactic shock reaction, milonine increased the time of animal survival with decreasing of mast cell degranulation. Taken these data together, milonine is an alkaloid with anti-allergic properties by decreasing mast cell degranulation rather than acting on histamine effect [106].

Silva and colleagues (2017) demonstrated that milonine reduced the mice paw edema formation induced by LPS, PGE2 (prostaglandin 2) or BK (bradicinine) independently of 5-HT (hydroxitripatmine); decreased the acetic acid induced-peritoneum exudate fluid maintaining the tissue morphology as well as inhibited the polymorphonuclear cell migration to the inflamed peritoneum. These effects were related with decreasing of TNF-α and IL-1β levels in the peritoneum. In addition, in nociceptive experimental model, milonine decreased the frequency of abdominal writhing induced by acetic acid but did not increased the time elapsed between the contact of the animal with the hot plate and the paw licking reaction. Milonine drastically reduced the nociceptive behavior of paw licking induced by formalin after 10 min of observation. Therefore, the authors hypothesizes that milonine presents anti-edematogenic and anti-inflammatory activities by inhibiting mediators that are responsible for the initiation of the inflammatory and analgesic processes [107].

4.5 Isoquinoline

Isoquinoline alkaloids and their related analogues present many important effects on inflammatory processes e.g. inhibited LPS-induced activation of NF-κB, TNF-α and NO production. Therefore, these classes of alkaloids have been used as anti-inflammatory agents [72,94,108–110].

4.5.1 Berberine

Rhizoma coptidis is a highly valued traditional Chinese medicine, which has been widely applied in complementary and alternative medicines in China, Korea, India, Japan, and other Asian countries, especially for the treatment of dysentery, cancer, diabetes mellitus, and eczema [111–113]berberine, have drawn extensive attention toward their antineoplastic effects in the recent years. The antineoplastic effects are related to the Chinese Medicine (CM. In China, berberine hydrochloride has been approved as anti- dysentery however, in recent years, berberine has been described to present anticancer activities in various types of cancer [114,115] with synergistic anticancer effects in combination with chemotherapeutic agents [116] or radiotherapy [117]. These studies suggested that berberine might have potential as adjuvant effect for anticancer drugs.

In addition, berberine presented anti-hyperglycaemic, anti-hyperlipidaemic effects reducing weight gain in Type 2 Diabetes patients [118]. Another important aspect of this alkaloid is the inhibitory effect on PGE2 level and on cyclooxygenase2 (COX2) expression [119]. Therefore, the predominant clinical uses of berberine include bacterial diarrhea, intestinal parasite infections, and ocular trachoma infections [120]. Side effects of berberine as a medicine can result from high dosages and include gastrointestinal discomfort, dyspnea, lowered blood pressure, and cardiac damage [120].

4.5.2 Tetrandine

Tetrandrine is a bisbenzylisoquinoline alkaloid originally extracted from the root of *Stephania tetrandra*, a Chinese herbs. Tetrandrine (C38H42N2O6) (figure 11) has been used in China to treat silicosis, autoimmune disorders, pulmonary inflammatory diseases, cardiovascular diseases and hypertension. In addition, some studies showed that tetrandrine has pharmacological potential in cancer therapy by inhibiting the cell proliferation by inducting tumor cell apoptosis [121].

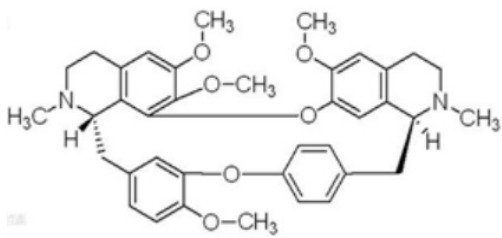


Figure 11. The chemical structure of Tetrandrine [121].

Immunosuppressive, anti-proliferative and free radical scavenging effects have been reported to tetrandrine. This alkaloid acts by non-selectively inhibition of voltage-gated Ca2+ channels activity, G1 blockade of the G1 phase of the cell cycle and apoptosis in various cell types [122]a bis-benzyl-isoquinoline alkaloid, on voltage-gated Ca2+ currents (ICa. Sakurai and colleagues (2015) reported that tetrandrine inhibited Ebola virus infection both *in vitro* and *in vivo* by inhibiting endosomal calcium channels. The alkaloid can also inhibit lipid peroxidation by blocking ROS production to protect several types of cells from oxidative stress [123,124]but there is no currently approved therapy. Cells take up Ebola virus by macropinocytosis, followed by trafficking through endosomal vesicles. However, few factors controlling endosomal virus movement are known. Here we find that Ebola virus entry into host cells requires the endosomal calcium channels called two-pore channels (TPCs. However the alkaloid exhibited anti-tumor effect by trigging ROS accumulation [121]. Moreover, tetrandrine has anti-inflammatory and anti-fibrogenic actions useful in the treatment of lung silicosis, liver cirrhosis, and rheumatoid arthritis [125].

4.5.3 Vincristine

Vincristine (C46H56N4O10) (figure 12) is an antitumor alkaloid isolated from Catharanthus roseus also known as Vinca rosea (Merck, 11th ed.). The antitumor activity of the molecule is due primarily to inhibition of mitosis at metaphase through its interaction with tubulin. Also interferes with the cyclic adenosine monophosphate (cAMP) and glutathione metabolism, calmodulin-dependent Ca2+-transport ATPase activity, cellular respiration and nucleic acid and lipid biosynthesis, inhibited calcium-calmodulin regulated cAMP phosphodiesterase, DNA repair and the RNA synthesis mechanisms, blocking the DNA-dependent RNA polymerase [126].

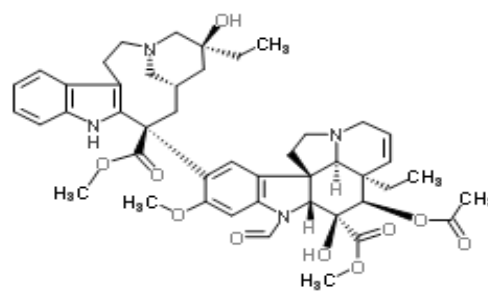


Figure 12. The chemical structure of Vincristine [126]

Therefore, vincristine is a vinca alkaloid antineoplastic agent used to treat several types of cancers including breast cancer, Hodgkin's disease, Kaposi's sarcoma, and testicular cancer. The vinca alkaloids are structurally similar compounds comprised of 2 multi-ringed units, vindoline and catharanthine. The vinca alkaloids have become clinically useful since the discovery of their antitumour properties in 1959. Vincristine binds to the microtubular proteins of the mitotic spindle, leading to crystallization of the microtubule and mitotic arrest or cell death. The vinca alkaloids are considered to be cell cycle phase-specific. Vincristine and other anti-cancer drugs are well reported to exert direct and indirect effects on sensory nerves to induce peripheral neuropathy characterized by progressive motor, sensory, and autonomic involvement [127].

4.5.4 Vinblastine

Robert Noble and Charles Thomas Beer were the first scientists to isolate vinblastine and characterized as a chemotherapeutic agent [126]. Vinblastine (C46H58N4O9) (figure 13) is a natural alkaloid isolated from the plant *Vinca rosea* and is a dimeric indole alkaloid formed by coupling of vindoline and catharanthine catalyzed by horseradish peroxidase [128]. It acts through the disruption of mitotic spindle assembly and arrest of tumor cells in the M phase of the cell cycle by inhibiting the formation of micotubule. Vinblastine, similar to vincristine, also elevate the cyclic adenosine monophosphate (cAMP), and glutathione metabolism, calmodulin-dependent Ca2+ -transport ATPase activity, cellular respiration, and nucleic acid and lipid biosynthesis. As vinca alkaloid, vinblastine, in tumor cells, inhibits the DNA repair and the RNA synthesis mechanisms, blocking the DNA-dependent RNA polymerase [126].

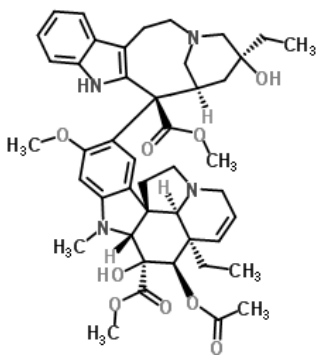


Figure 13. The chemical structure of Vinblastine [126].

Vinblastine had also some favorable effects in the treatment of certain forms of neoplastic diseases in man, particularly in the treatment of Hodgkin's disease [126].

5. Conclusion

The immune system is responsible for the homeostasis of the vertebrate organism by defending it from non-self-organisms or non-self-substances. Several biological alterations evoke this system to be renew the tissue, however the activated immune system can also provoke pathological conditions such as auto-inflammation or autoimmunity.

Even though inflammation is responsible to eradicate the pathogen it can also provoke tissue injures, therefore the pharmaceutical industries have been developing drugs to treat such conditions.

Alkaloids are natural molecules with several biological activities acting on biological targets that regulate biological functions such as expression and/or production of inflammatory and neurotransmitter mediators that regulate the homeostatic conditions. As describe here there are a lot of kind of alkaloids that act on inflammatory and allergic diseases as well as cancer state. In addition, these molecules can ameliorate several human conditions e.g dysenteries, seasickness, and fever.

Therefore, taken all information presented in this review it is clear to understand the importance of alkaloids used as pharmacological drugs to treat human disorders.

Acknowledgments

All the authors contributed to writing, analysis, language revision and design of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Chapter 12

Profile of the Graduate Program in Natural and Synthetic Bioactive Products of the UFPB: Balance of the Quadrennium 2013-2016, Challenges and Perspectives

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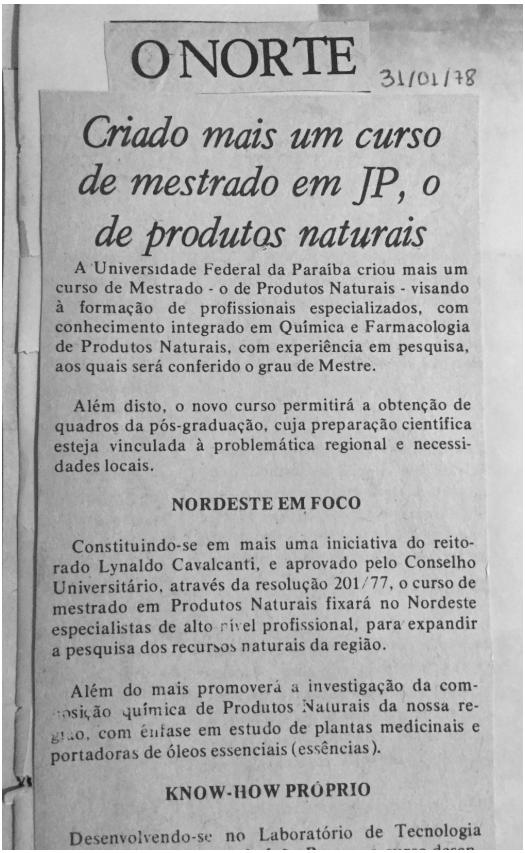
1. History and Contextualization of the Program

The Graduate Program in Natural and Synthetic Bioactive Products (PgPNSB) at the Health Sciences Center (CCS) of the Federal University of Paraiba (UFPB) was started in 1977 as a Master's Program in Natural Products (Pharmacology and Chemistry), springing enthusiastically out of the entrepreneurial mind of its creator, Prof. Delby Fernandes de Medeiros, the then-Director of the Laboratory of Pharmaceutical Technology (LTF), with the support of Prof. Linaldo Cavalcanti, the Rector of UFPB (Figure 1).

Since its inception, it has continued to be a very innovative program; it was the first graduate program in natural products in Brazil to propose a multidisciplinary integration between the areas of pharmacology and chemistry, highlighting the importance of interdisciplinary studies focused on the research and development of natural products. Furthermore, to address the lack of qualified human resources in Brazil, particularly in the Northeast region, its permanent faculty comprised primarily foreign researchers from different countries, such as India, Germany, England, Italy, Poland, and France.

Throughout its history, the PgPNSB has held large events (1984 to 1990) on a national level: 3 editions of the Symposium on Natural Products (SIMPRONAT), an event for its own graduate program, and the 11th edition of the Symposium of Brazilian Medicinal Plants (SPMB), which was a joint edition with the III SIMPRONAT, in 1990. These events were attended by renowned Brazilian and foreign researchers specialized in the area of natural products and medicinal plants, who provided PgPNSB students and professors with a great opportunity for national and international scientific exchange, enabling formal collaborations with foreign institutions to train highly qualified human resources at the doctoral

level, primarily in Scotland and France, who later joined the faculty of the Graduate Program in Natural Products.



O NORTE 31/01/78

Criado mais um curso de mestrado em JP, o de produtos naturais

A Universidade Federal da Paraíba criou mais um curso de Mestrado - o de Produtos Naturais - visando à formação de profissionais especializados, com conhecimento integrado em Química e Farmacologia de Produtos Naturais, com experiência em pesquisa, aos quais será conferido o grau de Mestre.

Além disto, o novo curso permitirá a obtenção de quadros da pós-graduação, cuja preparação científica esteja vinculada à problemática regional e necessidades locais.

NORDESTE EM FOCO

Constituindo-se em mais uma iniciativa do reitorado Lynaldo Cavalcanti, e aprovado pelo Conselho Universitário, através da resolução 201/77, o curso de mestrado em Produtos Naturais fixará no Nordeste especialistas de alto nível profissional, para expandir a pesquisa dos recursos naturais da região.

Além do mais promoverá a investigação da composição química de Produtos Naturais da nossa região, com ênfase em estudo de plantas medicinais e portadoras de óleos essenciais (essências).

KNOW-HOW PRÓPRIO

Desenvolvendo-se no Laboratório de Tecnologia

Figure 1. Article from the newspaper O NORTE about the creation of the Master's Program in Natural Products in 1977. The first cohort of students entered the program in 1978.

The restructuring of the PgPNSB was approved by Resolution No. 90/97 of the UFPB Higher Council for Teaching, Research and Extension (CONSEPE), dated October 14, 1997. Thereafter, it began to operate at the masters and doctoral levels, as well as along broader fields, not only with natural products from medicinal plants but also with marine organisms and studies with bioactive synthetic products. It was eventually renamed the Graduate Program in Natural and Synthetic Bioactive Products.

After the creation of the doctoral program, the PgPNSB welcomed its first cohort of doctoral students in March 1998 and, in the second half of that same year, received its first accreditation from the Brazilian Federal Agency for the Support and Evaluation of Graduate Education (CAPES), with a grade of 4. From 2001 to 2003, it received a grade of 5; and from 2004 to 2006, it received a grade of 6, denoting excellence. However, for the following triennium (2007-2009), the Technical Administrative Council of the CAPES, unfortunately, gave the PgPNSB a grade of 5, causing great concern and sadness among its members, professors, and students. In the 2010-2012 triennial evaluation, after much effort by all, the program once again received a grade of 6, denoting excellence, making it the only program in the North/Northeast and Central-West of Brazil to receive this designation in the pharmaceutical area.

It should be noted that 2010 was a decisive year with regard to the accreditation of faculty and the addition of new professors to the PgPNSB's permanent staff. Some of these changes were made by the program's administration and board, resulting in the reaccreditation of faculty, which reduced the number of professors comprising the group of collaborators, according to the percentage suggested by the Health Committee. This task was also carried out in 2013, with the end result being a total of 27 accredited professors: 21 permanent faculty and 6 collaborators. In 2014, 1 additional professor was accredited to strengthen the chemical and biological control research field, bringing it to a total of 22 permanent faculty. In 2015, this number remained the same. This variation is quite significant compared with the 2007-2009 triennium, which had a total of 36 professors, of whom 16 were collaborators (approximately 60%).

Returning to the cycle of scientific events, in 2010, the PgPNSB once again hosted the SPMB, now in its 21st edition, 20 years after the 11th edition. This event brought together approximately 2000 participants and 106 speakers from Brazil and abroad, involving plenary lectures, mini-courses, lectures, oral presentations, and 1,300 panel presentations. The PgPNSB organized the 4th edition of the SIMPRONAT in October 2012, returning to the tradition of fostering scientific exchange in their areas of expertise between professors and students from the PgPNSB and renowned researchers from other Higher Education Institutions (HEIs) and abroad. These meetings have not only strengthened the program's objectives, but they have also created important opportunities for discussing research and its importance in the national context with representatives from funding agencies.

After 38 years, the PgPNSB, in addition to fostering qualified research on Brazil's natural products, is also responsible for training highly qualified human resources, corresponding to 323 masters and 197 doctors, a total of 520

professionals in the pharmaceutical area, who primarily work in teaching and research in Brazil and abroad.

The PgPNSB is an established program formed by a permanent faculty with 22 qualified researchers with excellent scientific productivity in the program's 2 areas of concentration. Ten of these researchers work in the area of pharmaceutical chemistry, and twelve of them work in pharmacology.

Over the last 38 years (1977-2015), there has been a clear evolution in the academic activities of the PgPNSB's faculty—both in quality and quantity—since its first cohort of master's students in 1978. The PgPNSB can currently be characterized as an established program, not only because of its regional and national scientific importance and the financial support it has received from funding agencies since it was founded, but primarily for its training of qualified human resources, who spread the knowledge acquired in the program through the creation and consolidation of new graduate programs in Northeastern Brazil, such as Graduate Programs in Health Sciences (Universidade Federal de Alagoas [Federal University of Alagoas] – UFAL; UFM), Pharmaceutical Sciences (Universidade Estadual da Paraíba [State University of Paraíba]; UEPB; UFAL), Natural Resources of Semi-Arid Regions (Universidade Federal do Vale do São Francisco [Federal University of Vale do São Francisco]; UNIVASF), and Development and Technological Innovation in Medicines (PGDITM) (UFPB; Federal University of Rio Grande do Norte; UFRN; Universidade Federal Rural de Pernambuco [Federal Rural University of Pernambuco]; UFRPE), among others.

The PgPNSB has been a very strong element of the integration and consolidation between the North/Northeast region and the rest of the country, as it has made a strong contribution to training professors from HEIs in these regions, as can be seen by its participation as a cooperating institution in the CAPES Institutional Qualification Program/Institutional Program for Teacher and Technical Training (PQI/PICDT) at 7 HEIs, 6 located in the Northeast (Universidade Federal do Maranhão [Federal University of Maranhão]; UFMA; UFAL; Universidade Federal da Bahia [Federal University of Bahia]; UFBA; Universidade Federal do Ceará [Federal University of Ceará]; UFC; Universidade Regional do Cariri [Regional University of Cariri]; URCA; UEPB) and 1 in the North (Federal University of Roraima; UFRR) of Brazil. Approximately 25 of the professors participating in the PQI/PICDT and the PgPNSB received their degrees from the program.

Another indicator of the program's evolution and improvement in quality is the increase in the number of professors in the permanent faculty who have received Research Productivity (PQ) grants from the National Council for Scientific and Technological Development (CNPq), which has been growing each year

since 2005 when there were only 9. There are currently 17 professors: with 7 level 1 researchers (2 level 1A, 1 level 1B, 1 level 1C, and 3 level 1D), 10 level 2 researchers, and 1 researcher with a Tutorial Education Program (PET) CAPES grant. Thus, 80% of the permanent faculty currently comprises researchers with PQ grants from the CNPq.

Although new programs in the pharmaceutical area have emerged in the Northeast region, a majority thanks to the academic impact of the PgPNSB, there has been a demonstrable increase in the program's attractiveness to candidates in recent years, primarily due to the following factors: (a) the search for a program with a higher grade, offering higher qualifications; (b) the option to choose only 1 area of concentration, pharmacology or pharmaceutical chemistry, and 1 supervisor, which saves time and enables a greater concentration of activity on dissertation/thesis work; (c) a reduction in the number of credits for the 2 areas: 22 for the master's degree and 35 for the doctorate; (d) the presence of a significant number of students who received grants from the Institutional Scientific Initiation Scholarship Program (PIBIC), as well as Scientific Initiation (IC) grants from the CNPq (40-50%); upon entering the program, they already have some theoretical knowledge and basic experimental scientific training, which is necessary for the development of their graduate research; and (d) a team of highly qualified multidisciplinary professors, many of whom have international experience, who provide a wider range of options when choosing research lines. This set of factors has also contributed to a reduction in the students' average time-to-degree, with a mean of approximately 22.94 months for the master's degree and 49.08 months for the doctorate.

With regard to the student body, they come from different states around Brazil, particularly the Northeast, and from abroad. It is important to note that the human resources trained by the PgPNSB, even those in the final phase of their doctorate, are already in the labor market, working primarily in teaching and research.

Although scientific production has developed greatly since the program's creation, it strengthened quantitatively and qualitatively in the 2010-2012 triennium, with an increased number of publications in high-impact scientific journals, scoring at the highest Qualis CAPES levels (Qualis is the Brazilian official system for classifying scientific production). This production is basically concentrated in levels A1 to B2, with a predominance in B1 and B2, and fewer publications in the lower levels, B3 to B5. These results reflect the joint efforts undertaken by the program's administration, the permanent faculty of the PgPNSB, and all involved in the PgPNSB to increase the number of publications in journals with a higher impact factor, classified as level A according to Qualis

CAPES, to attain a level necessary for the program to maintain its grade of 6, a score it has already achieved. In the 2007-2009 triennium, the permanent faculty published 196 articles in indexed journals, distributed in Qualis A2, B1, B2, B3, B4 and consolidated mainly in levels B3 and B4. This evolution is quite noticeable when compared to data from the 2010-2012 triennium, in which more than 266 (107 in 2012) articles were published in the Qualis CAPES Journals portal: 12 A1 articles, 13 A2 articles, 79 B1 articles, 87 B2 articles, 5 B3 articles, 11 B4 articles and 10 B5 articles. These figures show the qualitative evolution of the program's scientific production, particularly with regard to publications by the permanent faculty, which, for the first time, were published in Qualis A1 journals, as well as a considerable increase in patent applications. The data from 2013 is similar to that from 2012, demonstrating the consistent number of publications aiming at the highest levels. In addition, it should be noted that approximately 183 (66 in 2012) of the articles involve the participation of PgPNSB students and/or IC fellows, which corresponds to approximately 75% of the scientific production involving student participation, demonstrating the ability of the program and its research groups to perform experimental research, as well as publish their results in high-impact journals. It should be noted that during the first, second and third years of the 2013-2016 quadrennium, these numbers have been maintained. These data demonstrate the evolution of Qualis A1 scientific production and the higher concentration of Qualis A1 to B3 articles.

Another relevant indicator, which is very important for the PgPNSB, was the technical production recorded for the 2010-2013 triennium through product development: 18 patent applications were filed, some involving student participation. Of these, 16 are linked to the production of pharmaceuticals and similar areas of focus, including 1 product that is being developed in collaboration with a pharmaceutical company, and 2 are linked to the development of computer programs for biological research (microbiological and DNA).

With regard to obtaining funds, the program has received approval for several projects submitted to different funding agencies, including the following: Funding Authority for Studies and Projects (FINEP), CNPq and CAPES. In the 2010-2012 triennium, the program was able to obtain a total of R$ 5,155,548.00, which concluded in 2012. Currently, the PgPNSB has conducted several projects that have been approved by funding agencies, such as CNPq, CAPES and FINEP, as well as others in collaboration with other institutions and individual projects. Among the group's major projects, several were initiated in 2008 and continued until 2012-2013: 1) Ministry of Science and Technology (MCT)/ FINEP/Infrastructure Fund (CT-INFRA) – Alternative Energy Sources Incentive Program (PROINFRA), amount: R$ 1,353,275.00; 2) MCT/CNPq/National Fund for

Scientific and Technological Development (FNDCT)/Research Foundation (FAP) Call for Proposals No. 71/2010 - National Institute for Science and Technology (INCT) in a Tropical Marine Environment, amount: R$ 2,820,000.00; 3) CAPES Institutional Research Equipment Call for Proposals, amount: R$ 185,000.00; 4) MCT/National Institute for Semi-Arid Regions (INSA)/CNPq Call for Proposals 35/2010 (Process: 562730/2010-9) - Term: 2010-2012, amount: R$ 250,000.00; 5) Support Program for Centers of Excellence (PRONEX)/CNPq/Paraíba State Research Foundation (FAPESQ-PB) Call for Proposals No. 04/2008 - Term: 2009-2012, amount: R$ 1,020,000.00; 6) MCT/CNPq/Ministry of Education (MEC)/CAPES/Agribusiness Fund (CT-AGRO)/Water Resources Fund (CT-HIDRO)/FAPS/Brazilian Agricultural Research Corporation (EMBRAPA) No. 22/2010, amount: R$ 150,000.00; 7) CAPES Call for Proposals 17/2009 National Program for the Support and Development of Botany (PNADB) - Term: 2010-2013, amount: R$ 111,193.75; 8) MCT/FINEP/CT-INFRA - PROINFRA 02/2010, amount: R$ 1,294,040.00; 9) Call for Proposals No. 25/2011 – Research Equipment (PRÓ-EQUIPAMENTOS)/MEC/CAPES, amount: R$ 299,531.16; 10) Ministry of Science, Technology and Innovations (MCTI)/FINEP/CT-INFRA - PROINFRA 01/2011, amount: R$ 1,246,810.00; and 11) Call for Proposals No. 024/2012 – PRÓ-EQUIPAMENTOS/MEC/CAPES, amount: R$ 211,447.00.

The PgPNSB also has the support of different individual projects, faculty projects, or institutional projects financed by national funding agencies, obtained through projects submitted to calls for proposals from the CNPq, state FAPs, and the Banco do Nordeste do Brasil (BNB), totaling approximately R$ 1,600,291.00 in the 2010-2012 triennium. Other network projects received a total of R$ 4,633,400.00, and projects for product development received approximately R$ 2,864,148.32, which joined equipment projects (R$ 5,101,296.91) and Science without Borders (R$ 417,000.00). These resources have enabled improvements to the program's infrastructure to acquire large and small equipment, as well as to pay for laboratory consumables. The total resources allocated to the PgPNSB in the 2010-2013 triennium was approximately R$ 14,666,136.00. However, the resources from PROINFRA 2009, 2010 and 2011 have not yet been made available to the PgPNSB. Regarding routine services, the main funding source for maintaining the laboratories, vivarium, classrooms, and auditorium, which is necessary for complete operation of the program's academic activities, is the CAPES Graduate Support Program (PROAP-CAPES), which has enabled not only the renovation and maintenance of the auditorium and classrooms, with audiovisual resources and air-conditioning, and supported the vivarium and the pharmaceutical chemistry and pharmacology laboratories, but also made it possible for approximately 120 external examiners from other HEIs to participate

in thesis and dissertation defenses and seminars. Additionally, it has allowed faculty and students to participate in national and international events held around the country, with the support of travel costs or per diem.

Although it is a relatively new program compared with those in other regions and even in other countries, the tradition and qualifications of the PgPNSB can be demonstrated by several factors: 1) it has a stable faculty; 2) 80% of the faculty has PQ grants from the CNPq; and 3) it has been integrated internationally since its creation, as the Master's Program in Natural Products could only have been created with the participation of foreign professors from other countries such as Germany, India, Sweden, and England, strengthening and increasing the qualifications of the program.

Currently, keeping with its tradition, the PgPNSB has several projects involving bilateral, individual, formal, and informal collaborations with countries from South America (Argentina and Colombia) and Europe (Greece, Scotland, United Kingdom, France and Switzerland), as well as the United States, Australia, and Pakistan, some of which are financed by international funding agencies, such as the National Science Foundation, with the participation of researchers from foreign institutions, who have supported the program not only by teaching subjects and giving lectures and seminars at the PgPNSB but also by receiving our professors on sandwich fellowships. These exchanges create the opportunity for collaboration among professors, and have also increased the quality of their publications; there are already a significant number of joint publications in high-impact journals, strengthening the program and contributing to the higher qualification of human resources at the international level. In the current phase, the PgPNSB faculty and administration are committed to maintaining the program's indices and grade of 6. Some of these actions are summarized below:

- Increase its international presence: a. high value placed on student foreign language proficiency; b. sandwich internships, c. postdoctoral studies abroad; d. encourage the presence of foreign students; e. support the participation of visiting professors from foreign institutions; f. increase the number of formal bilateral agreements with foreign institutions; and g. encourage the reception of fellows from The World Academy of Sciences (TWAS);
- Accredit new qualified professors with international experience, or from Brazil's Programs of Excellence, to support the permanent faculty;
- Continue to obtain funds to develop the infrastructure by acquiring equipment and maintaining laboratories and classrooms to include new techniques available for the PgPNSB research lines;
- Review the accreditation rules;

- Review Regulation No. 32/2015, which regulates our program;
- Increase the number of doctoral fellowships;
- Strengthen the program's visibility on the university website, as well as its Facebook and Twitter accounts, by preparing brochures and organizing systematic scientific meetings, as well as participating in/organizing the Higher Studies Program/CAPES;
- Improve the doctoral student flow to achieve a shorter time-to-degree;
- Prioritize the development of level A to B1 scientific production; and
- Encourage the filing of patents for developed products, strengthening technological innovation.

In addition to the items related to scientific production, the PgPNSB will encourage actions related to social inclusion, strengthening the items listed below:

- Transfer of technology to the National Pharmaceutical Industry;
- Support drug analysis requirements from the regulatory sector (compounding pharmacies);
- Deaf Inclusion through Science, with the development of an experimental approach and didactic material for teaching the sciences to deaf students, promoting social inclusion;
- Train employees working in regulatory agencies in the pharmaceutical sector (the State Health Surveillance Agency - AGEVISA and the National Health Surveillance Agency - ANVISA) and employees at UFPB and other HEIs;
- Professor participation in ANVISA technical committees (Technical Commission for Herbal Medicine - CATEF, the Brazilian Pharmacopoeia); and
- Academic impact through the representative participation of graduates at new or newly opened HEIs (UNIVASF, Universidade Federal de Campina Grande [Federal University of Campina Grande] - UFCG), UFCG-Cuité, UFRN) and regulatory agencies in the pharmaceutical sector (ANVISA and AGEVISA-PB), among others.

In a broader analysis of the PgPNSB, examination of the proposals involving ongoing projects, faculty, scientific production, actions related to social inclusion and the program's international presence, and particularly its importance to the training of Brazil's human resources, especially for the North and Northeast region, its importance for the region, and therefore the country, becomes evident. This importance is visible not only through its pioneering actions and tradition, but primarily through its academic impact, with an innovative and critical proposal for training qualified human resources to meet the demand of the Northeast region. Thus, while the PgPNSB contributes to the creation of scientific knowledge in Brazil, it also contributes to the establishment of human resources

and the region's economic and sociocultural development. Those involved with the PgPNSB are confident of achieving the proposed objectives, and to this end, they are all reassessing priorities and joining efforts to consolidate what has been achieved historically, as well as setting new goals to collectively maintain the space and importance earned by the PgPNSB in the community.

2. General and Specific Objectives

The PgPNSB was initiated in 1997, 20 years after the creation of the Master's Program in Natural Products (Pharmacology and Chemistry). At that time, it was the first graduate program in Brazil to propose a multidisciplinary integration between pharmacology and chemistry, highlighting the importance of interdisciplinarity in studies focused on research and development in the natural products area. With this restructuring, a doctoral degree was added to the graduate program, and it also began to focus on a broader research area, not only on natural products but also on the study of synthetic products with biological activity.

Currently, the program carries out its activities to meet the following objectives:

1. Train specialized human resources for teaching and research, with integrated knowledge in the areas of pharmacology and pharmaceutical chemistry, at the master's and doctoral level;
2. Contribute to technical and scientific research, linked to regional problems and local demands, to improve the sociocultural level of the North, Northeast, and Central-West regions and, consequently, Brazil;
3. Promote the establishment of technically and scientifically qualified specialized human resources in the North, Northeast, and Central-West regions of Brazil to meet the country's regional development needs;
4. Expand research on natural resources (plant and marine), especially in the Northeast region, to allow them to be used and to develop our own technology, minimizing dependence on foreign expertise; and
5. Perform research aimed at the development and technological innovation of natural and synthetic bioactive products, enabling the transfer of technology to the national productive sector, with a special focus on solving regional problems.

With the restructuring of the Master's Program in Natural Products (Pharmacology and Chemistry), the original objective was maintained, and the PgPNSB remained in the broad pharmaceutical area, covering 2 areas of concentration, each with 3 research lines: pharmacology - a) biological characterization of natural and synthetic bioactive products, b) research on

biologically active natural products to obtain new sources of raw materials, and c) toxicological evaluation of natural and synthetic bioactive products; and pharmaceutical chemistry - a) research and development of natural products to obtain biologically active substances, b) physicochemical and biological quality control, and c) partial or total synthesis of biologically active substances. These research lines are comprehensive considering the interdisciplinarity of the area and its interface with biological sciences, health sciences, technological sciences, and innovation. As a result, the PgPNSB faculty comprises pharmacists, chemists, doctors, botanists, veterinarians, and biologists with doctoral degrees, and approximately 80% have already completed postdoctoral studies in Brazil and/or abroad. The program's permanent faculty comprises professors from UFPB, who are working in different centers and departments at that institution. While physically operating in the UFPB LTF, the PgPNSB has, since its creation, been academically linked to the UFPB CCS, although it has no direct link to any department. The physical structure of the PgPNSB has classrooms, facilities for professors, and different laboratories, which encourage collaborative studies among researchers and strengthen research lines. In contrast, the diversity of subjects and because the professors, although located in different departments, have spaces and laboratories at the LTF, encourage exchange, collaboration, and integrated development of their research.

Currently, the program's faculty includes professors from different regions of the country, most of whom have obtained their doctoral or postdoctoral degrees abroad or at other HEIs in Brazil, who belong to the following Departments and Centers: Department of Pharmaceutical Sciences (DCF-CCS), Department of Physiology and Pathology (DFP-CCS), Molecular Biology – Center for Exact and Nature Sciences (CCEN), Biotechnology Department – Center for Biotechnology (DB-CBiotec), Department of Cellular and Molecular Biology (DBCM-CBiotec), Department of Statistics (DECCEN), Foreign and Modern Languages - Center for Human Sciences, Literatures and Arts (DLEM-CCHLA), and the Department of Education Methodology at the Education Center (CE-CCEN).

As the only graduate program in the pharmaceutical area with a grade of 6 in the North and Northeast regions, as well as the Center-West of Brazil, the PgPNSB has a great responsibility of training professors and technicians with multidisciplinary knowledge of the pharmaceutical area, including chemistry, quality control, pharmacology, and the toxicology of natural (plant and marine) and synthetic products, as well as botanical studies. Furthermore, the PgPNSB has a key role in attracting young people from the region, primarily recent graduates in health and related areas, and encouraging them to improve their knowledge and scientific qualifications. Another important factor is that the

PgPNSB develops its research activities to encourage the utilization and rational exploitation of natural resources in all regions of Brazil, although primarily in the Northeast Region, contributing to the technical and scientific development of the region and, consequently, the country and thus reducing regional asymmetries in Brazilian research and graduate programs.

3. Profile of Graduates

Graduates of the PgPNSB have the training and knowledge to work in the pharmaceutical area, especially in the sectors of pharmaceutical chemistry and the pharmacology of natural products. Over the course of the program, the student studies 6 required subjects in 2 areas of concentration (pharmaceutical chemistry and pharmacology), which provides the program's graduates with theoretical and practical knowledge in both pharmaceutical chemistry and pharmacology. Graduates of the PgPNSB are professionals who are qualified to work in natural products research with resourcefulness and have an ability to carry out projects, obtain funds, and advise at the scientific initiation, master's, and doctoral levels, as well as write and publish their research data in high-impact journals. Graduates of the PgPNSB are also fully qualified for teaching; they are qualified professionals with sufficient resourcefulness and education, skills acquired over the course of the program through seminars and study groups, among others, as corroborated by the number of graduates currently working in different sectors, both research and teaching in the public or private sectors, in practically every state in Brazil.

4. Curriculum Structure

Our curriculum structure has been developed to meet the program's characteristics, providing a solid theoretical and practical training in the foundations demanded by the areas of concentration (pharmaceutical chemistry and pharmacology). All the subjects have been reviewed and have an appropriate and up-to-date bibliography. The program maintains a harmonious relationship between teaching and research activities. The research lines are consistent with the program's objectives. We maintain exchanges with centers of excellence at home and abroad (France, Greece, England, Germany, the United States, and Australia). The program maintains an appropriate supervisor load, with an average of 5 supervisees/supervisors. The supervising professors all hold doctoral degrees and have experience in research and supervision; most of them are researchers with respected experience in their research area.

The PgPNSB has a minimum duration of 12 months and a maximum duration of 24 months to complete the master's and a minimum duration of 24 months and a maximum of 48 months to complete the doctorate.

To complete the required credits, the student must take the following:

- For the master's degree, 12 (twelve) credits in required subjects and at least 10 credits in elective subjects; and
- For the doctorate, 8 credits in required subjects and at least 27 credits in elective subjects.

Each credit corresponds to 15 theoretical class hours or 30 practical class hours.

The academic activities related to the Teaching Internship are developed according to the following criteria:

- For the master's students: 1 credit over 1 academic period;
- For the doctoral students: 2 credits over 2 academic periods (1 credit per period), consecutive or not.

The internship is required for master's and doctoral students who are CAPES fellows and is elective for other students in the graduate program.

Doctoral candidates can use their master's credits up to a limit of 22 credits, regardless of the year they completed their master's degree.

To obtain the master's degree, the student will be required to take an English language proficiency exam up to 1 year after enrolling in the program, and for the doctorate, a second exam will be required in another foreign language in addition to English proficiency; the student may choose among Spanish, French, or German.

If a doctoral student has graduated from a master's program with a proficiency exam that was not conducted in English, the proficiency exam in the second language must be in English.

In the case of foreign students whose language in their country of origin is 1 of those mentioned above, a proficiency exam will be required in Portuguese.

To obtain the doctoral degree, the student must be approved in a Qualification Examination, which must be completed at least 6 months before the date of the public thesis defense.

The academic year will consist of 2 periods, the beginning and end of which will be determined by the academic calendar by the program administration. The current curricular structure maintains the integrated and multidisciplinary training demanded by the natural products area.

5. Innovative Training Experiences

Since its creation, the PgPNSB has demonstrated itself to be a program focused on innovative training. It was the first program to train professionals with integrated knowledge in the pharmacology and chemistry of natural products. Years later, the areas were broken up, yet the program continued to innovate using

state-of-the-art technology for research, thus training qualified human resources with extensive knowledge of the mentioned areas. With regard to teaching, lectures are innovative, with internet available in the classroom and real-time consultations and discussions of current topics in the natural products area. With this vision and almost 40 years of experience, the PgPNSB can be considered an innovative program, training qualified human resources in the pharmacology and pharmaceutical chemistry of natural products in the Northeast region.

6. Laboratory Infrastructure

6.1. Laboratories

Analytical Center

Coordinator: Prof. Josean Fechine Tavares, PhD

Built with resources from CT-INFRA 2005, the UFPB Analytical Center opened in December 2006, with an area of 330 m2, which has allowed a leap in the program's quality. In early 2007, the Analytical Center began operations; it is responsible for analyzing materials and chemical substances, which contributes to the development of the program's research and supports the training of specialized human resources. In addition to providing services to other universities and regional agencies, the Analytical Center offers its services to companies that have an academic link with UFPB, making it possible for other institutions to utilize state-of-the-art technology to analyze their products. The Analytical Center has the following equipment, most of which were acquired with CT-INFRA funds:

Equipment: 1 Varian 500 MHz nuclear magnetic resonance spectrometer coupled to HPLC, which was the second machine of this type in Brazil, 1 Varian 200 MHz nuclear magnetic resonance spectrometer, 1 Varian HPLC, 1 Raman infrared device for analyzing the 12000 to 400 cm-1 region, 2 PerkinElmer UV/Vis devices, 1 semi-industrial lyophilizer, 1 spray dry machine, 1 nitrogen plant to supply all the liquid nitrogen for the 2 nuclear magnetic resonance (NMR) devices (to reduce costs and provide the liquid nitrogen required for the 200 and 500 MHz NMR devices and for other research groups at UFPB, UFCG, and the Universidade Estadual de Campina Grande (State University of Campina Grande; UECG)), 1 Bruker high-resolution LC/MS/MS mass spectrometer, 2 Shimadzu high-pressure liquid chromatographs, and 1 polarimeter. Additionally, 1 supercritical fluid extractor and 1 solid phase extractor are currently being acquired, all with CT-INFRA funds. In 2012, the Analytical Center was authorized to acquire another, 600 MHz, resonance machine. These funds have not yet been released.

Immunology Laboratory (Research Institute for Drugs and Medicine – IpeFarM)

Coordinator: Prof. Márcia Regina Piuvezam, PhD

Equipment: 1 enzyme-linked immunosorbent assay (ELISA) reader, 1 Nikon optical microscope, 1 analytical scale, 1 inverted microscope, 1 pH meter, 1 microwave, 1 water bath, 1 refrigerated centrifuge, 1 laminar flow hood, 1 CO2 oven, 1 Millipore Milli-Q, 1 refrigerator, 1 freezer, 3 desktop computers, and 1 printer.

Psychopharmacology Laboratory (IpeFarM)

Coordinator: Prof. Reinaldo Nóbrega de Almeida, PhD

Equipment: 1 tail flick, 1 hot plate, 1 electroshock, 1 active avoidance, 1 microscope, 1 plantar test, 1 animal activity meter, comprising 1 central module and 4 activity boxes, 1 hole board, 1 rota rod, 1 skinner device, 1 water bath, 1 refrigerator, 3 desktop computers, and 1 printer.

Toxicology Laboratory (IpeFarM)

Coordinator: Prof. Marianna Vieira Sobral, PhD

Equipment: 1 Olympus BX51-BF-III biological microscope with UIS infinity-corrected objective lens, with a digital camera attached, 1 hematological device, 1 sonicator, 1 Taimin optical microscope, 1 centrifuge, 1 freezer, 1 refrigerator, 1 reverse osmosis, 1 pH meter, 1 analytical scale, 4 desktop computers, 2 printers, 1 water ultra-purification system, 1 fluorescence system with an HBO V-50 mercury lamp, 1 vertical autoclave, and 1 water bath

Prof. George Thomas Pharmacology Laboratory (IpeFarM)

Coordinator: Prof. Bagnólia Araújo da Silva, PhD

Equipment: 3 chemographs, 2 physiographs, 1 plethysmograph, 7 isometric transducers, 3 thermostatic pumps, 1 tmb-4m amplifier, 1 horizontal freezer, 1 electric stimulator and 1 peristaltic pump, 1 hotplate with magnetic stirrer, 1 water bath, 1 refrigerator, 1 heating mantle, 1 scanner, 1 pH meter, 1 analytical scale, 2 desktop computers, and 2 printers.

Cardiovascular Laboratory (IpeFarM)

Coordinator: Prof. Isac Almeida de Medeiros, PhD

Equipment: 1 isolated organ bath, 1 carbogen tank, 1 4-channel bath system, 1 CVMs data acquisition system, 1 analytical scale, 1 infusion pump, 1 2-channel bath system for isolated atrium, 2 refrigerators, 1 physiograph, 1 heating mantle, 1 TBM4M signal amplifier, 1 pH meter, 1 isolated organ bath, 1 carbogen tank, 1 4-channel bath system, 1 CVM data acquisition system, 1 TBM4M signal amplifier, 5 computers, 2 printers, and 1 scanner.

Laboratory for the Neural Control of Circulation and Hypertension (LCNCHA)

Coordinator: Prof. Valdir de Andrade Braga, PhD

Equipment: 2 digital stereotactic equipment, 1 cryostat microtome, ice machine, real-time PCR, gel photo-documentation system, portable ultrasound, luminometer, blood pressure recording system, ventilation recording system, tank system for organ baths, telemetry system for recording blood pressure and heart rate, nerve recording system, stereomicroscope, inverted fluorescence microscope, artificial ventilation machine, inhalation anesthetic system, multifunction printer, peristaltic pump, and 4 computers.

Laboratory for the Genetics of Microorganisms (Department of Molecular Biology – DBM/CCEN)

Coordinator: Prof. José Pinto Siqueira Junior, PhD

Equipment: 2 electrophoresis devices, 4 tube stirrers (vortex), 2 culture chambers, 1 freezer, 4 refrigerators, 1 digital scale, 1 analytical scale, 1 pH meter, 4 water baths, 1 ultrasound, 2 magnetic stirrers, 1 13000 rpm centrifuge, 1 6000 rpm mini-centrifuge, 1 clinical centrifuge, 1 computer, 2 sterilization chambers, 2 autoclaves, 1 microwave oven, 2 laminar flow hoods, 1 benchtop distiller, 1 wall distiller, 2 thermal cyclers, 2 UVA sources, 2 UVB sources, 1 UVC source, 1 radiometer with 3 sensors (UV A, B and C), and 1 UVA dosimeter.

Multi-User Pharmacology Laboratory (IpeFarM)

Coordinator: Prof. Demétrius Antonio Machado de Araújo, PhD

Equipment: 1 magnetic stirrer, 2 inverted microscopes, 1 binocular microscope, 2 refrigerators, 1 clinical centrifuge, 3 desktop microcomputers, patch-clamp test device, refrigerated benchtop centrifuge, refrigerated plate centrifuge, 1 microplate reader, ultra-freezer, 2 flow hoods, 1 commercial freezer, 1 analytical scale, 1 photo-documentation system, 1 autoclave, 1 isolated organ bath, 1 real-time thermal cycler, electrophoresis tanks and power supplies, 1 osmometer, 1 pH meter, 1 glass capillary puller, and 1 CO2 incubator.

Developmental Biology Laboratory (DBM/CCEN)

Coordinator: Prof. Luis Fernando Marques, PhD

Equipment: 2 culture chambers (1 with photoperiod), 2 magnetic stirrers, 1 distiller, 4 phase contrast and dark field binocular optical microscopes, 1 fluorescence microscope with an image capture system, 1 binocular microscope, 4 drying ovens, 2 refrigerators, 1 freezer (-20o C), 1 clinical centrifuge, 1 microtome, 2 water baths, 1 analytical scale, 1 pH meter, 1 microwave for sterilization, and 1 water filtration sterilization system with UV radiation.

GIT Pharmacology Laboratory (IpeFarM)

Coordinator: Prof. Leônia Maria Batista, PhD

Equipment: 1 refrigerator, 1 freezer, 1 magnifying glass, 1 stereoscopic microscope, 1 pH meter, 1 sonicator, 1 computer, 1 vortex, 1 plethysmometer, 1 pH meter, 1 oven, and 1 scale.

Phytochemical Laboratories – 3 Laboratories Equipped with Fume Hoods (CBIOTEC)

Coordinator: Prof. José Maria Barbosa Filho, PhD, Prof. Maria de Fátima Vanderlei de Souza, PhD, Prof. Josean Fechine Tavares, PhD

Equipment: 9 rotary evaporators, 3 analytical scales, all laboratories are provided with externally generated vacuum and cold water, 3 drying ovens, 1 distiller, dozens of stirrers/heaters, 3 freezers and 3 refrigerators, 2 HPLC devices, 1 melting point apparatus, 3 computers, 1 medium-pressure chromatograph and 1 chromatotron, 1 ultraviolet camera, ultrasound devices, 3 scales, 1 DVM-156/60 vacuum pump, 1 LS 3000 C benchtop lyophilizer, 1 PFD III 220V digital melting point apparatus, 1 TE-394/3-MP drying and sterilizing chamber with air circulation and renewal, 1 manual chromatographic plate preparation device, 1 analytical scale, and 1 vacuum pump with compressed air.

Organic Synthesis Laboratory (IpeFarM)

Coordinator: Prof. José Maria Barbosa Filho, PhD

Equipment: 2 fume hoods, 2 rotary evaporators, 1 analytical scale, 1 freezer, 1 refrigerator, 1 vacuum pump, 1 high vacuum, 5 stirrers/heaters, 1 drying oven, a system for the refrigeration and drying of organic solvents, and 1 computer.

Prof. Thomas George Vivarium (IpeFarM)

Coordinator: Prof. Marianna Viera Sobral, PhD

For research activities involving animal experimentation, PgPNSB supervisors and students can use the Prof. Thomas George Vivarium on UFPB Campus I. The vivarium, recently built with R$ 950,000.00 from MS/ANVISA, has an area of 650 m2, operating within international standards, and serves as a reference for the entire Northeast region. The vivarium's head technician is a veterinarian, and there are also 3 vivarium technicians involved in mice, rat, guinea pig, and rabbit breeding and husbandry. Its facilities are equipped with an air filtration system that has ambient temperature control, culture and sterilization chambers, a refrigerated centrifuge, a refrigerator, a freezer and laminar flow hood, and a sanitary barrier for specific pathogen free animal breeding. The hygienic and sanitary control of the animals and facilities is carried out every 3 months.

Pharmaceutical and Molecular Analysis Laboratory – LAFAM (IpeFarM)

Coordinator: Prof. Eduardo Jesus de Oliveira, PhD

Equipment: High-performance liquid chromatograph, disintegration device, UV/Vis spectrophotometer, friability tester, durometer, conductivity meter, potentiometer, refractometer, electronic analytical scales, water purifier and deionizer system, laminar flow hood, pH meter, medium-pressure chromatography system, and a liquid chromatograph-mass spectrometer.

Taxonomy and Pharmacobotany Laboratory – TAXFAR (IpeFarM)

Coordinator: Prof. Maria de Fátima Agra, PhD

Physical area: Equipped with support benches for mounting plant tissue slides, a greenhouse for drying pressed material, and a fume hood.

Equipment: 1 Olympus optical microscope with a photographic camera for photomicrographs, 2 Leica optical microscopes with photographic cameras and image capture computer and software, 1 stereoscopic microscope with drawing attachment, 2 Leica stereoscopic microscopes with a digital system for image capture and measurement, 1 microwave oven, 1 refrigerator, 1 microtome, 1 water bath, 1 Apple laptop, 2 desktop computers, 1 paraffin dispenser, and a botanical reference collection with approximately 7,500 samples of plants used medicinally in Northeastern Brazil

7. IT Resources

The graduate program is completely computerized, and the administrative offices, auditorium, classrooms, laboratories, and student workspaces all have access to the internet, with support from the software necessary for the work they carry out in their research. Professors and students have computers in their respective areas, as well as access to the UFPB network, where they can connect their laptops to different research portals, such as CAPES Journals portal, TROPICOS-MOBOT (an online botanical database maintained by the Missouri Botanical Garden), and Google, among others.

These connected laboratories and classrooms, most equipped with wireless internet, facilitate direct online searches within these spaces, as well as other places, such as the administrative offices and laboratories, supporting not only research but also seminars and classrooms.

The scientific community—both faculty and students—have online access, not only from the university but also from their homes, to the CAPES Journals portal, as well as other databases like Scifinder (Chemical Abstracts) and more specific ones in the natural products area, such as Natural Products Alert (NAPRALERT), MOBOT, and the International Plant Names Index (IPNI), among others.

The fact that the CAPES Journals portal and Chemical Abstracts can be freely accessed by all public institutions has allowed programs in the less privileged regions of Brazil (North/Northeast/Central-West) to achieve gains in quality, productivity, and competitiveness.

8. Library

Central Library

With regard to the FUPB Library System, the bibliographic collection of the Central Library has approximately 360,135 physical books, 8,956 distinct titles, and 628 journal subscriptions distributed across the libraries of the 3 UFPB campuses. Among the journals subscribed by the library in 2013, there are some journals that are not available through the CAPES Journals portal, such as Planta Medica, Phytotherapy Research, British Journal of Pharmacology, Phytochemical Analysis, and Pharmaceutical Biology, among others. Eight volumes of the Dictionary of Natural Products were also purchased at a price of R$ 10,500.00. The graduate program also has its own small library, with specific books in the areas of botany, pharmacology, and chemistry.

CCS Library

With R$ 325,432.00 from the Treasury/CCS/UFPB, the Health Sciences Center Library was built with approximately 600 m2 of physical area on 2 floors. The lower floor has a Xerox machine to serve the academic body, lockers, a loan desk, a help desk for the users, a study area, an area for the library's collection, and a room for the library's administrative activities and technical operations. On the upper floor, there is another study area and also an area storing the library's collection. This floor also has an auditorium with capacity for 50 people, which is used for classes and training courses for employees, professors, and students. The CCS library also has a wheelchair platform, which helps in moving from one floor to the other, thus allowing access throughout the library to people with special needs. The library was built to meet the demands of the undergraduate and graduate programs in the health area.

9. Other Information

In addition to the laboratories, libraries, and IT resources, the PgPNSB infrastructure also has the following items listed below, which not only complement but are fundamental to the research work developed by PgPNSB professors and students in their dissertations and theses.

Automobile

The program has an automobile for sampling expeditions to collect natural products (plant and marine): 1 2010 Ford Ranger pickup, double cab, 4x4. Purchased with PRONEX-2008 funds.

PgPNSB Classrooms and Auditorium

The PgPNSB has the following:

- 1 auditorium with air-conditioning, audiovisual resources (digital projector, DVD player and sound system), and wireless access. The auditorium is the largest space, with a capacity for 100 (one hundred) people, where dissertation and thesis defenses are held, as well as seminars and lectures. In addition, when necessary, the program can use the CCS auditorium.
- 3 classrooms and 1 auditorium, all with air-conditioning and audiovisual resources (digital projector, overhead projector).

Prof. Lauro Pires Xavier Herbarium (CCEN)

Curator: Prof. Regina Maria de Vasconcellos Barbosa, PhD

The Herbarium has a biological collection of approximately 30,000 plant specimens documenting the local flora of the Brazilian Northeast, in particular Paraíba, occupying an area of 65 m^2, and also a fruit collection in an area of 30m^2, a microscopy room with an area of 30 m2, and a space for preparing botanical material with an area of 30 m^2.

Technical Support Workshops (CCEN)

A glass workshop with 60 m2 for making and repairing glassware for the laboratory in general with a glass lathe, blowtorches, and a technician.

Electronics Workshop with 30 m^2 specialized for repairing and maintaining high-precision analytical equipment, with an oscilloscope, power supplies, generators, multimeters, and an electronics technician.

A room for distilling and recovering solvents.

A room for preparing, drying and milling plant material: 2 forced-air ovens and 1 mid-level technician.

10. Integration with Undergraduates

10.1. Indicators of Integration with Undergraduates

The benefits generated by the integration of graduates and undergraduates in the PgPNSB have been evident since the program began; it has had a revitalizing impact on undergraduates, as observed through the growth in demand for IC. The current number of IC fellows is quite significant, with students coming from the

Pharmacy, Biology, Biotechnology, Medicine, and Chemistry programs, among others, all linked to research being developed at the PgPNSB. There are 55 CNPq-PIBIC fellows, 5 CNPq-Balcão fellows, 51 interns from the UFPB Volunteer Initiation Program (PIVIC, similar to the PIBIC, but without remuneration), and 14 Tutoria-PET fellows, totaling 124 undergraduate students (fellows and volunteers) actively participating in projects linked to graduate dissertations or theses. Approximately 80% of these students are applying to the PgPNSB, bringing with them not only the experience acquired during IC but also the possibility of developing their future experimental studies by continuing the project they began during IC, thereby reducing the time-to-degree as they already have preliminary training.

All professors in the program actively participate in undergraduate teaching and support the UFPB PIBIC and PIVIC programs. This contact enables the selection of students with academic potential who are engaged in different research groups where they participate in group seminars, attend dissertation and thesis defenses, and receive a range of experimental and theoretical training, as well as take advantage of scientific exchanges with professors from the PgPNSB and other HEIs, in visits and conferences at the PgPNSB.

Another element of integration with undergraduates was the participation of PgPNSB students as evaluators for oral and panel presentations at the UFPB IC conference.

Finally, the data show that students with experience in the IC program who enrolled in the master's program had better performance in the graduate program than students with no previous experience. The students with excellent performance in the graduate program and in the required subjects and those that come from the PIBIC and PIVIC programs are encouraged to enroll directly in the doctorate.

10.2. Teaching Internship

Graduates and Undergraduates.

The UFPB CONSEPE created the teaching internship in July 1999 following a recommendation by CAPES, making it a requirement for all Social Demand (DS) fellows. The program's committee also extended this internship as an elective to other students (CNPq fellows or not), and it has been occurring regularly since the second semester of 1999.

There has been a regular demand for the teaching internship, and it has presented important results for undergraduates. Following the recommendation of the Evaluation Committee, the program once again offered the pedagogical subject "Methodology of Higher Education" and Methodology of Scientific Research, which led students to develop a greater interest in teaching and research.

11. Exchanges

11.1. National Exchanges

- The program's faculty have maintained an intense collaboration with researchers from different HEIs and national research institutes when developing projects. These collaborations have been carried out with different institutions: Universidade Federal de São Paulo (Federal University of São Paulo; UNIFESP), University of Campinas (UNICAMP), Universidade Federal do Rio de Janeiro (Federal University of Rio de Janeiro; UFRJ), Universidade Federal de Minas Gerais (Federal University of Minas Gerais – UFMG), Universidade Estadual do Norte Fluminense (State University of Northern Rio de Janeiro – UENF), UFBA, UFAL, Universidade Federal de Pernambuco (Federal University of Pernambuco; UFPE), UFRN, UFC, Federal University of Sergipe (UFS), the Oswaldo Cruz Foundation in Bahia and Rio de Janeiro (Fiocruz-BA/RJ), and the Institute of Pharmaceutical Technology (Farmanguinhos/RJ.

• JOSE MARIA BARBOSA FILHO: Fiocruz-BA: Lain Carlos Pontes de Carvalho, Ricardo Ribeiro dos Santos and Milena Botelho Soares. UFBA: Camila Alexandrina Figueiredo, Neuza Maria Alcantara-Neves, Cristiane Flora Villarreal and Ramon dos Santos El-Bacha. Universidade Estadual Feira de Santana (Feira de Santana State University; UEFS): Ana Maria Giulietti. UFAL: Magna Suzana Alexandre Moreira, João Xavier de Araujo Júnior, Carlos Alberto de Simone. UFRJ: Ligia Maria Torres Pecanha, Raimundo Braz-Filho. Universidade Federal Rural do Rio de Janeiro (Federal Rural University of Rio de Janeiro; UFRRJ): Raimundo Braz-Filho. Universidade Severino Sombra (Severino Sombra University; USS): Marise Maleck de Oliveira Cabral. UFS: Lucindo José Quintans-Júnior. UFRPE: Tania Maria Sarmento da Silva, Celso Amorim Camara. UFPE: Ivan da Rocha Pitta, Maria do Carmo Alves Lima, Eliete Cavalcanti Silva, Cynthia Dias Rayol, Haroudo Satiro Xavier, Teresinha Goncalves da Silva. UFC: Francisca Clea Florenço de Souza, Silvânia Maria M. Vasconcelos and Glauce Socorro de Barros Viana. UFMG: Steyner de Franca Cortes, Virginia Soares Lemos and Jader dos Santos Cruz. University of São Paulo (USP): Dominique Corinne Hermine Fischer. UEPB: Raissa Mayer Ramalho Catão, Rossana Miranda Pessoa Antunes, Thulio Antunes de Arruda.

• Maria de Fatima Agra: Fiocruz-BA: Ricardo Ribeiro dos Santos and Milena Botelho P. Soares. UEFS: Ana Maria Giulietti and Alessandro Rapini. Rio de Janeiro Botanical Garden (JBRJ): Rafaella Forzza, Ariane Luna Peixoto. UFS: Lucindo Jose Quintans-Júnior, Ana Prata. UFRPE: Tania Maria Sarmento da Silva, Celso Amorim Camara. UFPE: Marccus Alves. UFC: Francisca Clea

Florenco de Souza and Iracema Loiola. UFMG: Joao Renato Sthemann. USP: Lucia Lohmann. Federal University of Rio Grande do Sul (UFRGS): Lilian Auler Mentz. Universidade Federal de Vicosa (Federal University of Vicosa; UFV): Marilia Ventrella. UFRN: Jomar Jardim. Universidade Estadual de Santa Cruz (State University of Santa Cruz; UESC): Emerson Rocha de Lucena. Universidade Estadual de Pernambuco (State University of Pernambuco; UPE): George Sydney Baracho. UEPB: Iranildo Alves and Kiriaki Nurit Silva.

- In addition to 2 PQI/CAPES projects with UFS and UFMA, there have been 2 exchanges as a cooperating institution, 1 with UEPB and 1 with UFRR, which demonstrates our program's importance to the North and Northeast regions, as a result of the level of work and quality of human resources training.
- The Botanical Sector collaborated with the following institutions: UFRN (Biology Department), UFRDS (Biology Department), UFPE (Plant Biology Graduate Program), UPE (Biological Sciences Department), UESC, Itabuna-Ilheus, Bahia, UEFS Herbarium (Herbario UEFS – HUEFS), JBRB (Taxonomy of Fanerogamas), the Herbarium at the National Institute of Amazonian Research (INPA), and the Herbarium at the Emilio Goeldi Museum in Minas Gerais, National University of Colombia, Science Institute

11.2. International Exchanges

The program's teaching faculty has maintained close bilateral collaborations with researchers from different foreign HEIs. Below are some collaborations that are currently under development.

Eduardo de Jesus Oliveira

- David Watson from the University of Strathclyde, Scotland, in studies involving the metabolism of quercetin and kaempferol in rat hepatocytes and the identification of flavonoids in biological fluids.
- University of Toledo, USA, PIANI cooperation program, to receive undergraduates as interns upon completion of their degree.
- Dr. Jean Luc Wolfender – University of Geneva, University of Lausanne, Switzerland. He visited our Program and gave a workshop on Introduction to Plant Metabolomics at the PgPNSB's invitation.

Emidio Vasconcelos Leitao da Cunha – Dr. Alexander I. Gray – Strathclyde Institute of Pharmacy, Scotland – He taught a 2-credit course at the PgPNSB's invitation: Applications of bi-dimensional NMR to chemistry of natural products.

Isac Almeida de Medeiros

- Prof. Jean Sassard and Christian Barres of the Departement de Physiologie et Pharmacolgie Clinique of Universite Claude Bernard (Claude Bernard

University), Lyon, France, in joint research involving the study of the effect of plant extract on the blood pressure of normotensive and unanesthetized rats.

- Prof. Valerie Schini-Kerth, of the Departement Pharmacologie et Physico-Chimie des Interactions Cellulaires et Moleculaires, of Faculte de Pharmacie, Louis Pasteur University, Strasbourg, France, in joint research on the transduction pathway involved in the effects induced by red wine byproducts from the Rio São Francisco Valley region.

  Jose Maria Barbosa Filho
- Dr. Jennifer M. Duringer of the Department of Biomedical Sciences, College of Veterinary Medicine, Oregon State University, USA, on the biological analysis and evaluation of natural products. This partnership began to generate its first results with a study published in 2008 (Chemistry and Biodiversity) and another in 2009 (Pharmaceutical Biology).

  Marcelo Sobral da Silva
- Magalys Casals Hung, Universidad de Oriente (University of Oriente; UDO), Phytochemical investigation of Cuban vegetal species for pharmaceutical application.

  Participation of professors and students in postdoctoral studies.

  Maria de Fatima Agra
- Formal agreement with the Department of Biology, University of Utah, Salt Lake City, USA, in cooperation with Dr. Lynn Bohs, a specialist in the molecular biology of Solanaceae, and doctoral student Stephen Stern, whose thesis is on the Brazilian Solanum subg. Leptostemonum, as part of a collaboration with the Planetary Biodiversity Inventory (PBI). Photos and videos of Dr. Lynn Bohs collecting the Solanaceae family at Proa. Agra can be found on the Natural History Museum website. This partnership resulted in the publication of an article.
- Dr. Sandra Knapp, specialist in Solanum sect. Geminata, Department of Botany, The Natural History Museum, Cromwell Road, London, United Kingdom, to make the Solanum sect. Erythrotrichum species available online, as part of the Solanaceae Source Project (http://www.nhm.ac.uk/research-curation/projects/solanaceaesource/taxonomy/description-detail.jsp?spnumber=2158).
- Dr. Clara Inez Orozco, Natural Science Institute, Universidad Nacional de Colombia, to develop the project Phylogenetic Analysis of Solanum sect. Crinitum and to study Solanum species from the Brazilian and Colombian Amazon. Fieldwork was carried out in Munchique, Colombia, located in the municipality of Popayan. This partnership has begun to generate its first results, and a student/professor study will be published at the

invitation of Caldasia in the commemorative issue for the anniversary of the Universidad Nacional de Colombia.

- Maria de Fatima Agra participated in 2008-2015 as the Brazilian representative in the international project "PBI Solanum: A Worldwide Treatment," which received external funding from the National Science Foundation (NSF) based in the United States. Solanaceae experts from around the world participated in this mega-project.
- Dr. Gloria Estela Barboza, Natural Science Institute, Multidisciplinary Institute of Plant Biology (IMBIV-CONICET) and School of Chemical Sciences, Universidad Nacional de Cordoba. CC 495, CP 5000. Cordoba, Argentina. Project: Diversity of Capsicum (Solanaceae) in Northeastern Brazil. This partnership resulted in the publication of an article in the journal Systematic Botany: The Capsicum (Solanaceae) species from the Brazilian caatinga: C. parvifolium, and 2 new species, their morphological and karyological features.
- Maria de Fatima Agra, with Dr. Wayt Thomas, New York Botanical Garden, for the project Atlantic Coastal Forest Plant Diversity in Northeastern Brazil, funded by the NSF, based in the United States, with the aim of conducting botanical research in the Atlantic Forest in Northeastern Brazil, thereby promoting its conservation.
  Maria de Fatima Vanderlei de Souza
- University of Athens/Prof. Vassilius Roussis and the PgPNSB - 2009/2015, doctoral sandwich fellowship granted to Anna Claudia de Andrade Tomaz. This partnership has begun to generate its first results, and a study is being prepared for submission for publication.
  Valdir Andrade Braga
  (a) Prof. Eugene Nalivaiko – Newcastle University, Australia. CAPES/ Foreign Visiting Professor (PVE) Project 088/2012 (term: 2012-2015).
  (b) Prof. Eric Lazartigues – Louisiana State University, United States of America. CNPq Fellowship for a doctoral sandwich fellowship granted to student Thyago Moreira de Queiroz (2013-2015).
  (c) Prof. Barkat Ali Khan – Gomal University – Pakistan. Joint article published (doi:10.4314/tjpr.v11i6.12).

12. Indicators of Solidarity, Academic Impact, Leadership, and Visibility

12.1. Solidarity

To reach this indicator, the PgPNSB has developed cooperation agreements and partnerships with level 3 and 4 programs and with research groups that do

not yet have graduate programs. The PgPNSB program has been a very strong element of integration and consolidation between the North-Northeast region and the rest of the country, as it has been making a strong contribution to training professors from HEIs in these regions. The program's professors participate in various projects in collaboration with different research groups around Brazil, financed by state and federal funding agencies. Many of these collaborations originate from the research tradition and pioneering spirit of our program, which is 39 years old, as well as the academic impact of the program's graduates, who have formed nascent groups throughout the region. In addition, different professors are collaborating on projects with professors from other UFPB departments, such as the departments of Chemistry, Pharmacy, Physiology and Pathology, Systematics and Ecology, Dentistry, and Molecular Biology. It should be noted that all the physical infrastructure and equipment built and acquired over the course of these 39 years is available to graduates, who are creating their own groups and beginning to establish their own research lines. This is an important factor because with the expansion of HEIs and the creation of federal institutes, our program's graduates have been absorbed throughout the region. To consolidate this growth, it is necessary for these new doctoral graduates to become established on these expansion campuses. In addition to the above, the faculty of the PgPNSB have acted on the following items.

1. Cooperation or association agreements with HEIs to promote the creation and/or consolidation of graduate programs.

- National Program for Academic Cooperation (PROCAD) 2014-2018 – Multidrug Resistance in Tumor Cells - Collaborative study to investigate phenotype and sensitivity to chemotherapeutic agents with different cell targets. PROCAD project, with interdisciplinary CAPES funding, involving 3 federal universities: UFRJ, UFRG and UFPB.
- In 2016, the PgPNSB/UFPB/UFCG interinstitutional master's degree (MINTER) was established with the Center for the Sustainable Development of Semi-Arid Regions (CDSA). This center is located in the city of Sumé, in Paraíba's Cariri region, 300 km from João Pessoa. The MINTER was designed for the association of the sponsoring (PgPNSB) and receiving institutions (CDSA/UFCG). It is immensely important for expanding knowledge of the plant species in Paraíba's Cariri region, as local cadres are being formed for experimental research on native medicinal plants, which have often been ignored by scientists. More important is the training of master's graduates who will integrate knowledge in the field through agro-ecophysiological plant studies with experimental studies in analytical laboratories to determine the bioactive chemical composition of plant

species. This knowledge is necessary and will provide a foundation for the sustainable development of the region while ensuring the ecological preservation of native forests. The results of the MINTER project were published on the CAPES Sucupira Platform on December 16, 2015, and it was highly rated by the Evaluation Committee. The MINTER Project Selection Call for Proposals was published on April 1, 2016, with a schedule of activities for completing the process that lasted until June 30, 2016. During the selection process, the applications of 21 candidates were accepted. Following the written exam, 11 candidates were approved for the 10 slots available. Only 1 candidate from the CDSA/UFCG, the receiving institution, applied, but they did not attend the written exam. At the end of the process, 10 candidates were admitted. They enrolled in the program and began classes in early August, with all 10 students participating in the required subjects. At the end of the first course, 2 students requested to leave due to force majeure, and the remaining 8 students continue to participate in the project's activities. The classes were offered at the receiving institution, which arranged with the administration to take good care of the students. By the end of December, 18 of the 22 credits required by the PgPNSB had already been offered; 4 of the credits, for Special Topics in Pharmaceutical Chemistry, were offered by 2 students at the receiving institution, CDSA/UFCG. The students are preparing projects with the integration of professors from both the sponsoring and receiving institution to provide more effective training with the creation of knowledge in the field and in laboratories.

2. Participation in joint projects with non-consolidated research groups.

- In 2016, the PgPNSB submitted a proposal to CT-INFRA, and it was approved. The project for this open call was developed in collaboration with the following graduate programs: PGDITM (level 4), Biotechnology (level 3), Biological Sciences (level 4), Food Technology (level 4), a program in the Physical Education Association (level 4), and the Nutrition program (level 4). With this project's approval, the partnerships with these programs were strengthened, and we contributed to their strengthening.
- In 2016, the results of the 2014 INCT project were released, and the proposal coordinated by Prof. Marcelo Sobral da Silva, PhD, was approved. This will represent a very strong collaborative link between different research groups throughout the North and Northeast regions and will help consolidate the pharmaceutical indicators for these regions.

3. Participation in subjects, seminars, and workshops in programs with a grade of 3 or 4.

- Some PgPNSB professors are accredited for the Master's in Biotechnology (level 3) and the PhD in Drug Development and Innovation (level 4). Thus, they regularly teach courses in these programs.

4. Programs in a broad association of HEIs.

Recently, the PgPNSB has also had a great partnership with the Northeast Network of Biotechnology (RENORBIO) institutional program and the new PGDITM network program (UFPB, UFRN, UFC and UFRPE). These collaborations are possible not only because of the multidisciplinarity of the PgPNSB, which enables interaction between different departments/programs, but also because of the physical proximity between these units on the UFPB Campus.

Over the course of the quadrennium, the PgPNSB achieved its objective for solidarity: it established partnerships with other lower-level programs, it created cooperation agreements with other HEIs through PROCAD, and members of the PgPNSB approved an INCT project involving more than 160 researchers. We also approved and are developing a MINTER with UFCG.

12.2. Academic Impact

The PgPNSB has contributed significantly to the strengthening of nascent groups at HEIs in the region. For example, undergraduate programs in the pharmaceutical area have recently been accredited at several HEIs, such as UNIVASF, UEPB, UFAL, and UFS, with a large presence of graduates from our graduate program. Another example is UEPB, located in the city of Campina Grande 130 km from João Pessoa, the faculty of which is basically composed of professors who graduated from our graduate program.

Altogether, there are approximately 200 master's graduates and 100 doctoral graduates from our program who are carrying out their activities at the different HEIs in the region, both public and private. Currently, there is at least 1 professor at each of the federal or state universities or private institutions in the Northeast region, particularly those that were founded more recently, such as UNIVASF, UFBA-Advanced Campus of Vitoria da Conquista, and UFPE-Advanced Campus of Serra Talhada, and in almost all the federal universities in the North region. Among the private HEIs that have been absorbing our graduates, the following are particularly noteworthy: University of Fortaleza (UNIFOR), Centro Universitário do Maranhão (University Center of Maranhão; UNICEUMA), Rural School of Pernambuco (FAAPE), Integrated Schools of Vitória de Santo Antão (FINTVISA), Universidade Potiguar (Potiguar University; UNP-RN), Universidade Tiradentes (Tiradentes University; UNIT-SE), Centro Universitário Nilton Lins (Nilton Lins University Center; UNINILTON-AM), School of Medical Sciences (FCM) in João Pessoa, Nova Esperança Schools of Nursing and Medicine (FACENE/

FAMENE, João Pessoa-PB), Maurício de Nassau School (FMN) in João Pessoa, and Santa Maria School (FSM) in Cajazeira. In 2014, with the establishment of IPeFarM at UFPB, several actions were undertaken to expand this impact. The first project, which is already underway, is the creation of medicinal plant cultivation centers in the Caatinga biome. This project will begin with the city of Sumé-PB working to form and strengthen a research group at UFCG. It should be noted that the faculty participate as collaborators in different research projects submitted by program graduates to calls for proposals from CAPES, CNPq, and research foundations. This collaboration enables an underlying support for infrastructure and training master's and doctoral students in specific techniques that contribute to the feasibility of the proposals.

12.3. Leadership

The programs have demonstrated their leadership in the area with the following actions.

1. Attracting students from different regions of the country and other countries.

- Since its creation, the PgPNSB has attracted students from different states and regions, achieving its goal of training human resources and reducing regional asymmetries. Below is a list of regularly enrolled students from different states and regions. In 2015, we received Lazaro Goncalves Cuinica from Moçambique through the CAPES-PEC project. We received the doctoral student Ania Ochoa Pacheco from the Universidad de Oriente in Santiago de Cuba for a 1-year sandwich internship under the supervision of Prof. Marcelo Sobral da Silva, PhD. We received the student Chonny Alexander Herrera Acevedo. Chemotaxonomic studies and virtual screening of lactonized sesquiterpenes isolated from the Asteraceae family with potential leishmanicidal and trypanocidal activity. Enrollment date: 2016. Dissertation (Master's Degree in Natural and Synthetic Bioactive Products); UFPB, CNPq. Open call: CNPq no. 05/2015 - 2015 Application Process for the Exchange Program for Graduate Students (PEC-PG)

2. Receiving researchers for postdoctoral studies.

- Program: National Postdoctoral Program/CAPES (PNPD/CAPES). Program/Project: PNPD 2013/0376 - 24001015015P5 - PNPD - UFPB/João Pessoa/Natural and Synthetic Bioactive Products. Modality: Postdoctoral Internship. Document 17637970310. Name: Fatima de Lourdes Assunção Araujo de Azevedo. Enrollment date according to the program's archives (05/2013)

- Junior Postdoctoral Fellow – project approved by the CNPq - Elisana Afonso Moura. During this internship, she developed a project for an analytic and bioanalytic characterization of riparin I under the supervision of Prof. Marcelo Sobral da Silva, PhD.

  3. Professors participating on committees related to the area for CNPq, CAPES, FINEP, MS, or international funding agencies.
- Eduardo de Jesus Oliveira - Member of the Technical and Multidisciplinary Commission to Develop and Update the National List of Medicinal Plants and Herbal Medicines at the Ministry of Health (MS), and the CATEF, MS/ANVISA. Ad-hoc scientific advisor to the São Paulo Research Foundation (FAPESP).
- Emidio Vasconcelos Leitão da Cunha - Member of the Editorial Board of the Revista Brasileira de Farmacognosia and the Editorial Board of Phytochemical Analysis. Member of the Technical Committee to Support Policies for Medicinal and Phytotherapeutic Plants in the Brazilian Pharmacopoeia, MS/ANVISA.
- Isac Almeida de Medeiros - Member of the National Board of the Forum of Pro-Rectors for Graduate Studies and Research (FOPROP). Consultant for FINEP.
- Jose Maria Barbosa Filho - Member of the RENORBIO Scientific Committee. Member of the Advisory Board of the Brazilian Society of Pharmaceutical Sciences.
- Marcelo Sobral da Silva - Member of the Advisory Committee for the pharmaceutical area at the CNPq. Consultant for FINEP.

  4. National or international awards received by professors involved in research and supervisory activities and by students. Rodrigo de Oliveira Formiga (master's student) - Anti-motility Pathways Involved In The Antidiarrheal Mechanisms of Action of *Maytenus erythroxylon* Reissek (Celastraceae) Ethanol Extract (Poster) - Honorable Mention at the 48th Brazilian Congress of Pharmacology and Experimental Therapeutics and the 21st Latin American Congress of Pharmacology (SBFTE) - Foz do Iguaçú. Klinger Antonio da Franca Rodrigues (PhD, 2016 student). Study: 2-Amino-Thiophenes Derivatives and Their Uses for the Treatment of Leishmaniasis. Event: 2nd Prize for Technological Innovation. Professor: Delby Fernandes de Medeiros. Year: 2016.

  5. Professors participating on the boards of national and international scientific associations.
- Jose Maria Barbosa Filho - Member of the RENORBIO Scientific Committee. Member of the Advisory Board for the Brazilian Society of Pharmaceutical Sciences;

- Margareth de Fátima F. Melo Diniz - Member of the International Society for the Promotion of Health Technology Assessment (HTA)

6. Professors participating in positions relevant to national health, education or science and technology policies.

- Isac Almeida de Medeiros - Member of the National Board of FOPREP.
- Margareth de Fátima F. Melo Diniz - Member of the Board of Directors of the Brazilian Hospital Services Company (EBSERH), representing the National Association of Directors of Federal Higher Education Institutions (ANDIFES).

Prof. Valdir de Andrade Braga: North-Northeast representative to the Brazilian Society of Physiology (SBFis) (2016-2018).

The development criteria have been met. Practically all graduates are involved in public service, carrying out teaching and research activities. Leadership has also been clearly demonstrated. The faculty attracted funds, students came from abroad for their doctorate, there were postdoctoral researchers, and professors are participating on scientific committees.

12.4. Visibility

On our new website (www.ufpb.br/pos/pgpnsb), there is information about our graduate program, such as the following: name and contact information for the permanent faculty, with a link to the Lattes Platform, their research group, and the professor's website. There is also information about all research lines, subjects offered, registration procedures, calls for proposals, ordinances, resolutions, and general reports, among others. Since March 2006, students have been required to make their successfully defended theses and dissertations available for download at the website of the UFPB Central Library via a link from the program's website. The homepage has been restructured and is updated weekly in 3 languages (Portuguese, English, and Spanish). In addition to the homepage, the PgPNSB has a presence on Twitter to disseminate information and news. It also has a presence on Facebook with significant engagement numbers.

12.5. Following the Graduates

The PgPNSB has contributed significantly to the strengthening of nascent groups at HEIs in the region. For example, undergraduate programs in the pharmaceutical area have recently been accredited at several HEIs, such as UNIVASF, UEPB, UFAL, and UFS, with a large presence of graduates from our graduate program. Another example is UEPB, located in the city of Campina Grande 130 km from João Pessoa, the faculty of which is basically composed of professors who graduated from our graduate program.

Altogether, there are approximately 200 master's graduates and 100 doctoral graduates from our program carrying out their activities at different HEIs in the region, both public and private. Currently, there is at least 1 professor at each of the federal or state universities or private institutions in the Northeast region, particularly those that were founded more recently, such as UNIVASF, UFBA-Advanced Campus of Vitoria da Conquista, UFPE-Advanced Campus of Serra Talhada, and in almost all the federal universities in the North region. Among the private HEIs that have been absorbing our graduates, the following are particularly noteworthy: UNIFOR, UNICEUMA, FAAPE, FINTVISA, UNP-RN, UNIT-SE, UNINILTON-AM, FCM, and FACENE/FAMENE.

Through a survey conducted on the Lattes Platform, it was possible to determine the activities of the PgPNSB graduates over the last 10 years.

2005

Liana Clébia Soares Lima de Morais – Professor - UFPB
Márcio Roberto Viana dos Santos – Professor - UFS
Evaleide Diniz de Oliveira – Professor - UFS
Carla Maria Lins de Vasconcelos – Professor - UFS
Eurica Adélia Nogueira Ribeiro – Professor - UFAL
Arquimedes Fernandes Monteiro de Melo - Professor at the Centro Universitário do Vale do Ipojuca (Vale do Ipojuca University Center; FAVIP)
José Farias da Mata – could not be located

2006

Cláudio Roberto Bezerra dos Santos - Professor - UFPB
Katy Lísias Gondim Dias - Professor - UFPB
Rita de Cássia Meneses de Oliveira - Professor - Universidade Federal do Piauí (Federal University of Piauí; UFPI)
Ana Cláudia Dantas de Medeiros - Professor - UEPB
Danielly Albuquerque da Costa - Professor - UFCG
Louisianny Guerra da Rocha - Professor- UFRN
Fábio Santos de Souza - Professor - UFPB
Francisca Maria Barros Souza - Professor - UFC
Ana Paula Barreto Gomes - Professor - UFRN
Rinalda Araújo Guerra de Oliveira - Professor - UFPB
Hilzeth de Luna Freire Pessoa - Professor - UFPB
Rilva Lopes de Sousa - Professor - UFPB
Denise Fernandes Coutinho - Professor - UFMA
Maria da Conceição Rodrigues Gonçalves - Professor - UFPB
Jackson Roberto Guedes da Silva Almeida - Professor - UNIVASF

Stanley Juan Chavez Gutierrez- Professor - UFPI

2007

Luciano Augusto de Araújo Ribeiro - Professor - UNIVASF
Maria das Graças Valverde Mariani Passos - Professor - UFBA
Saulo Rios Mariz - Professor - UFCG
Fladmir de Sousa Claudino - Professor - UFPB
Raíssa Mayer Ramalho Catão - Professor - UEPB
Rossana Miranda Pessoa Antunes - Professor - UEPB
José Alixandre de Sousa Luis - Professor - UFCG
Maurus Marques de Almeida Holanda - Professor - UFPB
Josean Fechine Tavares - Professor - UFPB
Marianna Vieira Sobral - Professor - UFPB
Aristides Medeiros Leite - Professor - UFPB
Carlúcia Ithamar Fernandes Franco - Professor - UEPB
Laudelina Rodrigues Magalhães - Prison officer - PE
Flávia Maria Mendonça do Amaral - Professor - UFMA
Horacinna Maria de Medeiros Cavalcante - Professor - FMN
Hosana Bandeira Santos - Collaborating researcher - UFPB
Júlia Beatriz Pereira de Souza - Professor - UFCG

2008

Fabiana de Andrade Cavalcante - Professor - UFPB
Maísa Freire Cartaxo Pires de Sá - Professor - UFPB
Julianeli Tolentino de Lima - Professor - UNIVASF
Darizy Flávia Silva - Professor - UFBA
Alessandra Teixeira Ramos - Professor - UEPB
Aldeídia Pereira de Oliveira - Professor - UFPI
Angela de Siqueira Figueirêdo - Professor - UFPB
Maria de Fátima Duques de Amorim - Professor - UFPB
Josimar dos Santos Medeiros - Professor - UEPB
Karla Veruska Marques Cavalcante - Professor - UFPB
Vanda Lúcia dos Santos - Professor - UEPB
Albanita de Jesus Rodrigues da Silva - Professor - UFRR
Xirley Pereira Nunes - Professor - UNIVASF
Marco Antonio Ventura Romero - Professor - UECE
Harley da Silva Alves - Professor - UEPB
Henrique Douglas Melo Coutinho - Professor - URCA
Alessandra Azevedo Nascimento - Professor - UNIFAP
Fernando Antonio de Medeiros - Professor - UNIFAP

2009

Daniele Idalino Janebro - Professor - UFPB
Fernando de Sousa Oliveira - Professor - UFCG
Irinaldo Diniz Basílio Júnior - Professor - UFAL
Karina Carla de Paula Medeiros - Professor - UFRN
Joelmir Lucena Veiga da Silva - Professor - UNINOVE
Robson Cavalcante Veras - Professor - UFCG
Susy Mary Souto de Oliveira - Professor - FCM/PB
Adalberto Coelho da Costa - Professor - UFPB
Marcos Antonio Alves de Medeiros - Professor - FACENE/PB
Alessandra Sousa Braz - Professor - UFPB
Alessandra Camillo da Silveira Castello Branco - Professor - Santo Agostinho School (FSA/PI)
Isabella Bezerra Wanderley de Queiroga - Ophthalmologist
Ana Carolina Pessoa Moreira - Professor - Independent School of the Northeast (FAINOR/BA)
José Guedes de Sena Filho - Pharmacist - EMBRAPA/SE

2010

Maria do Socorro de Sousa Cartagenes - Professor - UFMA
Rosimeire Ferreira dos Santos - Professor - UFPI
Nadábia Almeida Borges de Souza - Professor - UFPB
Marilene Lopes da Rocha - Professor - UEFS/BA
Leandra Eugênia Gomes de Oliveira - Professor - UESB/BA
Raline Mendonça dos Anjos - Professor - FCM/PB
Roberto Jefferson Bezerra do Nascimento - Professor - UNIVASF
Lupicínio Farias Torres - Professor - UFPB
Ricardo Dias de Castro - Professor - UFPB
Jader Freire Sobral Filho - Professor - UFPB

2011

Caliandra Maria Bezerra Luna Lima - Professor - UFPB
Kiriaki Nurit Silva - Professor - UFCG
Fabricia Costa Montenegro - could not be located
Islânia Gisélia Albuquerque Araújo - Professor - UFPB
Jana Luiza Toscano Mendes de Oliveira - Professor - UFPB
Wylly Araújo de Oliveira - Professor - UFCG
Roosevelt Albuquerque Gomes - Professor - FSM/PB
Egberto Santos Carmo - Professor - UFCG

Alethéia Lacerda da Silveira - Full Researcher at Cristalia Produtos Quimicos Farmaceuticos Ltda

Flávia Negromonte Souto Maior - Professor - UFCG

Patrícia Pinheiro Rafael de Sousa - Professor - UFPB

Walter Mendes de Oliveira Júnior - Professor - UFPB

Gabriela Lemos de Azevedo Maia - Professor - UNIVASF

João Euclides Fernandes Braga - Professor - UFPB

Aurylene Carlos de Oliveira - Pharmacist at the Emergency and Trauma Hospital

Camila Carolina de Menezes Patrício Santos - Professor - UFCG

Igara Oliveira Lima - Technician - UFPB

Vanine Gomes Mota - Technician - UFPB

Heloina de Sousa Falcão - Works in Chinese Medicine in London

Adriana Maria Fernandes de Oliveira - Professor - UFPB

Sócrates Golzio dos Santos - Technician - UFPB

Alexsandro Fernandes Marinho - Technician - UFPB

Juan Carlos Ramos Gonçalves - Professor - FACENE/PB

Marine Raquel Diniz da Rosa - Professor - UFPB

2012

Aldeide de Oliveira Batista Rocha - Professor - UFPB

Anna Claudia de Andrade Tomaz - Pharmacist at the Professor Alberto Antunes University Hospital

Antonio Claudio da Silva Lins - could not be located

Ciberio Landim Macedo - Full Professor at the Centro Universitário de João Pessoa (João Pessoa University Center /PB - UNIPÊ)

Clelia de Alencar Xavier Mota - Professor - FACENE/FAMENE/PB

Danielle Serafim Pinto - Professor - FACENE/PB

Ethiene Castellucci Estevam - Coordinator of the Biomedicine Program at the Santa Emilia de Rodat School (FASER)

Fillipe De Oliveira Pereira - Professor - UFPB

Flavia Cristina Fernandes Pimenta - Professor - UFPB

Ionaldo Jose Lima Diniz Basilio - Professor - UFPB

Maria Angélica Satyro Gomes - Professor - UFCG

Maria do Socorro de França e Silva - Professor - UFPB

Melissa Negro Luciano - Professor - UFSC

Monica Souza de Miranda Henriques - Professor - UFPB

Narlize Silva Lira - Visiting Professor at the National Business Education Service (SENAC/PB)

Neyres Zínia Taveira de Jesus - Professor - UFMG

Rogerio Alexandre Nunes dos Santos - Professor - Universidade de Cuiabá (University of Cuiabá; UNIC)

Severino Antonio de Lima Neto - Technician at the Paraíba Regional Electoral Court (TRE/PB)

Steno Lacerda de Oliveira - Professor - FCM/PB

Thais Josy Castro Freire de Assis - Professor - UFCG

Thais Porto Ribeiro - Visiting researcher - UFPB

Vinicius Nogueira Trajano - Professor - FECENE

Vivianne Marcelino de Medeiros - Coordinator of the FSM

2013

Ana Carolina de Carvalho Correia - Monitor for Chemistry at the Professor Theonilo Gama School

Ana Karina Holanda Leite Maia - Professor - FEMENE

Analúcia Guedes Silveira Cabral - Professor - FMN

Antonilêni Freire Duarte Medeiros - Professor - UNIPÊ

Camila Silva de Figueiredo - Professor - Instituto de Educação Superior da Paraíba (Paraíba Higher Education Institute; IESP)

Carolina Uchoa Guerra Barbosa de Lima - Professor - FMN

Charlane Kelly Souto Pereira - Professor - João Pessoa Anglo-American School (FAAJP) and São Vicente de Paula Nursing School (FESVIP)

Daysianne Pereira de Lira Uchoa - Professor - Patos Integrated Schools (FIP)

Denise Aline Casimiro Bezerra - Professor - IFPB

Fabiana Lima Silva - Professor - Universidade Paulista (Paulista University; UNIP)

Fabio de Souza Monteiro - Researcher - UFMA

Fabio Henrique Tenorio de Souza - Professor - FCM/PB

Fabíola Fialho Furtado Gouvêa - Professor - UFCG

Giciane Carvalho Vieira - Professor - UFPB

Hermann Ferreira Costa - Professor - FSM

Kelly Samara de Lira Mota - Professor - FECENE

Marcelo Cavalcante Duarte - Professor - UFAL

Marianne Guedes Fernandes - Deceased

Mirian Graciela da Silva Stiebbe Salvadori - Professor - UFPB

Rubens Batista Benedito - Pharmacist at the University Hospital (HU)

Thaisa Leite Rolim Wanderley - Professor - FMN

Tiago Bezerra de Sá De Sousa Nogueira - Professor - FIP/PB

Vivyanne dos Santos Falcão Silva - Professor - UFCG

Vicente Carlos de Oliveira Costa - Technician - UFPB
Wemerson Neves Matias - Professor - FSM

2014

Aline de Freitas Brito - Professor - UFPI
Andrea Maria Rolim da Paz - Professor - UNIPÊ
Antônia Rosângela Soares Penha - Biologist at the Onofre Lopes University Hospital (HUOL)
Bruna Priscilla Vasconcelos Dantas - Junior Postdoctoral Fellow
Heraldo Arcela de Carvalho Rocha - Professor - UFPB
João Carlos Lima Rodrigues Pita - Pharmacist at the HU
Michelline Viviane Marques das Neves - Professor - President Humberto Castelo Branco School
Mônica Lorena Dias Meirelles da Cunha - Professor - UFPB
Tangbadioa Herve Coulidiati - Professor - Université de Koudougou, Nigeria
Thyago Moreira de Queiroz - Junior Postdoctoral Fellow
Zilmara Vieira Pedrosa - UFPB Technician

2015

Abrahão Alves de Oliveira Filho - Professor - UFCG
Aline Lira Xavier – Pharmacist at the Pernambuco State Health Department (SES/PB)
Ana Silvia Suassuna Carneiro Lucio - Professor - São Francisco da Paraíba School (FASP) and FCM/PB
Augusto Lopes Souto - Professor - University of Brasilia (UnB)
Camila de Albuquerque Montenegro - Professor - FSM
Fabiola Lelis de Carvalho - Substitute Professor - UFPB
Gedson Rodrigues de Morais Lima - Pharmacist at the Januário Cicco Maternity School (MEJC)
Ingrid Christie Alexandrino Ribeiro de Melo - Military Pharmacist
Isis Fernandes Gomes - Military Pharmacist
Jacqueline Iris Vasconcelos Costa - could not be located
Janiere Pereira de Sousa - Técnico - UFPB
Jocelmo Cássio de Araujo Leite - Professor - UNIPÊ
Maria Clerya Alvino Leite - Professor - IFPB
Maria do Carmo de Alustau Fernandes - Professor - UFCG
Matheus Morais de Oliveira Monteiro - Technician - UFPB
Monica Moura De Almeida – could not be located
Niara Moura Porto - could not be located
Paula Ferreira dos Santos - could not be located

Rafael de Almeida Travassos - Professor - UFPB

Raquel Bezerra de Sa de Sousa Nogueira - Professor - FIP

Severino Gonçalves de Brito Filho – Pharmacist at the Ingá Municipal Health Office/PB

Viviane Araújo da Silva - Biologist at the Ana Bezerra University Hospital (HUAB)

2016

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13. Social Inclusion and Interfaces with Basic Education

13.1. Social Inclusion

The PgPNSB has a fundamental social role in the Northeast region. The PgPNSB can currently be characterized as an established program, not only because of its regional and national scientific importance and the financial support it has received from funding agencies since it was founded but also for its training of qualified human resource personnel who spread the knowledge acquired in

the program through the creation and consolidation of new graduate programs in Northeastern Brazil, such as Graduate Programs in Health Sciences (UFAL, UFM), Pharmaceutical Sciences (UEPB, UFAL), Natural Resources of Semi-Arid Regions (UNIVASF), and PGDITM (UFPB, UFRN, UFRPE).

The PgPNSB has been a very strong element of integration and development between the North/Northeast region and the rest of the country, as it has made a strong contribution to training professors from HEIs in these regions. This contribution can be evidenced through its participation as a cooperating institution in the CAPES PQI/PICDT, at 7 HEIs, 6 in the Northeast (UFMA, UFAL, UFBA, UFC, URCA, and UEPB) and 1 in the North (UFRR) of Brazil. Approximately 25 of the professors participating in the PQI/PICDT and the PgPNSB received their degrees from the program during the last triennium. Graduates from the PgPNSB are highly qualified, and their inclusion in research centers and public and private universities has contributed to the creation of strategies that will help society solve problems. Several social projects are being developed by researchers in the program, such as the following: Deaf Inclusion through Science: Developing an Experimental Approach to Teaching the Deaf. Continuing and supplementing the development of an experimental approach and didactic material for science education aimed at deaf students, promoting social inclusion. This project seeks to offer young deaf students the chance to integrate themselves into the advances of science and technology in a critical way, learning scientific concepts with those who actually carry out scientific activities and developing scientific method and thought, rather than simply receiving information. All previous editions of the programs for the deaf have been successful. Thus far, 17 programs have focused on this particular audience, involving the participation of more than 320 students and 80 professors.

In addition to promoting social inclusion through science, professors are expected to use this new approach to teaching at their schools to stimulate creativity. After all, this approach can serve as a source of inspiration for developing innovative teaching methodologies intended to encourage deaf students to enroll at federal universities. Finally, the development of didactic material is intended to disseminate science to deaf individuals. Including information about the program on our website and Facebook, as well as making didactic material available, leads deaf youth to become more interested in participating in the next editions and encourages them to attend university.

It is thus possible to include the deaf in the national science and technology system, creating new opportunities for employment, learning and teaching, while simultaneously stimulating their creativity in the field of science. This approach will allow for more inclusion of deaf youth in the current technological society.

Prof. Leônia Maria Batista, PhD, is developing a social inclusion project, as described below.

Elderly Health: Experiences in the Health/Illness Process Among the Residents of Vila Vicentina Júlia Freire

Coordinator: Prof. Leônia Maria Batista, PhD

Collaborator: Prof. Climério Avelino de Figueredo, PhD

Extension Students: UFPB students Ana Luiza Bezerra de Macêdo, Camyla Caroliny Neves de Andrade, Catarina Alves de Lima Serafim, Dafne Dayse Bezerra Macedo, Gabriel Rodrigues da Silva, Isabelle de Farias Oliveira, Jessielly Tuanne Mesquita da Silva Suamy Rabelo Rocha da Costa, Thassya Matias Ribeiro, Thaynara Amaral Leite, Wênia Brito Barreto do Nascimento, Wedna dos Santos, Miguel Moura.

This project was carried out at the Vila Vicentina Júlia Freire Long-Term Care Facility, located in Bairro da Torre in the municipality of João Pessoa-PB, as a way of experiencing the health/illness process of the elderly, a bottleneck in the curriculum of pharmaceutical training. This practical scenario was chosen because of its proximity to UFPB, thereby facilitating the movements of human and material resources to carry out the activities.

The first phase of the project lasted 6 months, from July to December 2016. It was approved by the Ongoing Extension Flow (FLUEX) and developed by members of the PET-UFPB Pharmacy, with an additional academic volunteer from the UFPB Pharmacy program, under the coordination of Prof. Leônia Maria Batista and the direct collaboration of Prof. Climério Avelino de Figueredo.

Objectives

General:

- Understand the health/illness process of the elderly people living at Vila Vicentina Júlia Freire and develop health care and promotion actions.

Specific:

- Understand the profile of the elderly residents of Vila Vicentina Júlia Freire;
- Conduct weekly follow-up of the elderly;
- Identify the illness process of the residents;
- Monitor the evolution of these residents' records;
- Have conversations about therapeutic practices with caregivers and the nursing staff; and
- Contribute to the organization of the institution's pharmacy.

The following reports were presented at ENEX based on the project:

1. Experience Report: Compreendendo a História de Vida de Idosos Institucionalizados [Understanding the Life Story of Institutionalized

Elderly Persons]. Ribeiro, T.M.; Serafim, C.A.L.; Moura, W.S.N.; Leite, T.A.; Batista, L.M.

2. Processo de Organização da Farmácia da Instituição Vila Vicentina Júlia Freire: Um Relato de Experiência [The Process of Organizing the Pharmacy at the Vila Vicentina Júlia Freire Facility: An Experience Report]. Silva, G.R.; Macêdo, A.L.B.; Andrade, C.C.N.; Macedo, D.D.B.; Batista, L.M.

13.2. Interfaces with Basic Education

The PgPNSB has sought to develop projects that allow basic education students to become more familiar with research. Professors from the program give lectures at primary and secondary public schools, discussing the importance of studying medicinal plants. One of the projects under development is the formation of an integrated unit for teaching exact and natural sciences, involving the Liceu Paraibano and research and graduate programs at UFPB. The main objective of this project is to promote greater interaction between research and graduate programs at UFPB and teaching activities for exact and natural sciences at secondary education institutions, aiming to make students aware of potential future careers and direct more and better students to the technological areas covered by the oil & gas, biofuel, and petrochemical industries. The project involves the participation of the Chemistry, Physics, Biology, Mathematics, and Geosciences departments and the Lyceu Paraibano Secondary School. The planned activities include lectures, mini-courses, science olympiad, and the preparation of kits that will be made available at the school. There are also plans to renovate a block of laboratories in the Department of Chemistry to provide an exclusive space for the project's activities. The program administration has encouraged actions like this to narrow the gap between the PgPNSB and basic education and to increase the interests of students in research.

14. Internationalization

This item has been demonstrated since the PgPNSB was first created. At that time, to ensure that the program succeeded, many foreign researchers came to Brazil from different countries, such as France, Germany, India, and Poland. Currently, the administration, professors, and students have been working to strengthen this item, and some of these efforts are highlighted below.

1. Enabling Thyago Moreira de Queiroz's doctoral sandwich internship at Louisiana State University/USA. External Supervisor: Eric Lazartigues. Term: 08/01/2013 to 07/31/2014.

2. We received the student Heather G. McGovern. Suny Oswego's Global Laboratory Network at Science labs around the world. 2013. Other

supervisory activities. (Biology) - State University of New York, SUNY Oswego Global Laboratory Program.
3. We received the student Htet Oo K. San. Suny Oswego's Global Laboratory network at science labs around the world. 2013. Other supervisory activities. (Biochemistry) - State University of New York, Suny Oswego Global Laboratory Program.
4. We received the student Chiamaka Agbasionwe. Suny Oswego's Global Laboratory network at science labs around the world. 2013. Other supervisory activities - State University of New York, Suny Oswego Global Laboratory Program.
5. Prof. Valdir de Andrade Braga, PhD, permanent member of the PgPNSB, gave the lecture "Brain reactive oxygen species: are they linked to neurogenic hypertension?" at the State University of New York, during a visit from October 1 to 6, 2013.
6. Prof. Valdir de Andrade Braga, PhD. Visited the Sanford-Burnham Medical Research Institute, Orlando, FL, USA. 06/30/2013 to 07/06/2013. Established a collaboration with Prof. Julio Ayala at the institute's Signaling and Disease Metabolic Program.
7. Presentation of a study at an international conference: Bondarenko, Evgeny (Australia); Guimarães, Drielle Dantas (IC); Averell, Lee (Australia); Braga, Valdir de Andrade; Hodgson, Deborah M. (Australia); Nalivaiko, Eugene (Australia). Central neuronal pathways mediating respiratory activation in response to alerting and stressful stimuli. XXIII Annual meeting of the Australasian Society for Psychophysiology (ASP 2013), Wollongong NSW–Australia, November 20 to 22, 2013. (doi: 10.3389/conf.fnhum.2013.213.00009).
8. Approved research project: Collaboration Between Brazil and Australia for Understanding Respiration and Emotion: How and Where are They Linked? Funding agency: CAPES Call for Proposals/Open Call: Special Visiting Reseacher/CNPq (Line 1 – Fellowship Open Call 61/2011) Term: 2012-2015 Researchers: Eugene Nalivaiko (Australia), Maria de Fátima Agra, Márcia Regina Piuvezam, Valdir de Andrade Braga (Coordinator).
9. Brazil-Cuba international cooperation project. Prof. Emidio Vasconcelos Leitão da Cunha taught a short course at the Universidad de Oriente in Santiago de Cuba in February 2013. For this same project, coordinated by Prof. Marcelo Sobral da Silva, PhD, we received PhD student Ania Ochoa Pacheco for a doctoral sandwich from 2012-2013.

10. Enabling a doctoral sandwich internship beginning in 2014. Caroline Duarte Siqueira, Doctoral Sandwich Program Abroad (PDSE). Supervisor abroad: Isido Gonzalez Collado at the Universidad de Cadiz in Spain.

15. Other Information (Additional Data)

The PgPNSB has made every effort to input the 2014 data into the Sucupira Platform, which was not an easy task due to the constant maintenance and changes to the system. We had to redo a particular task more than once to clear what was pending. The program numbers for the training of qualified human resources in the region are encouraging, with a total of 16 dissertations and 10 theses defended. Of the scientific production, 65% is linked to students. A large number of articles has been published, with the highest concentration in level A2-B3 journals. Efforts are being made to increase internationalization. In 2014, the student Caroline Duarte Siqueira embarked on a 12-month doctoral sandwich internship at the Universidad de Cadiz in Spain under the external supervision of Prof. Isido Gonzales Collado, PhD. Thus, the objectives and goals proposed for the program are being achieved, solidifying the PgPNSB as a level 6 program in the pharmaceutical area. In the 'proposal' item, there are character errors that we were unable to correct.

16. Conclusions

Based on the presented data, we consider the objectives of the CCS/UFPB Graduate Program in Natural and Synthetic Bioactive Products to have been successfully achieved. It is impossible to fathom its invaluable contribution to the training of human resources in the pharmaceutical area and, thus, the health area. Over 39 years of existence, the program was able to train 350 master's graduates and 400 doctoral graduates. These results have only been achieved because everyone involved with the program has applied, in practice, the power of cooperation and teamwork.

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Prof. Dr. Marcio Santos da Silva

Holds a degree from the Federal University of Pelotas - UFPel and a master's degree from the same university in the area of Green Chemistry. Has a PhD from the University of São Paulo - USP in the area of Organic Synthesis. Worked at Central Analytical-IQ/USP in the NMR area. He is Adjunct Professor II at the Center for Natural and Human Sciences-CCNH at the Federal University of ABC-UFABC. Has experience in the following subjects: Green Chemistry, Organocalocalcogenic Compounds, Organometallic Reagents and Nuclear Magnetic Resonance. He is currently coordinator of the Multiuser Center - CEM - Santo André campus.

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Prof. Dr. Marcus Tullius Scotti

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on Industrial Administration at University of São Paulo. After that, he started post-graduation in organic chemistry at the University of São Paulo in 2003, and finished his Master in 2005 and PhD in 2008. In January of 2009 he moved to João Pessoa and started to work as Professor of Organic Chemistry at Federal University of Paraíba, Brazil. At beginning of 2014 finished Pos-doc in cheminformatics at Universidade Nova de Lisboa, Portugal. Prof. Marcus research interests are in the area of chemistry of the natural products, acting on the following subjects: Cheminformatics, QSAR, Virtual Screening, natural products database, molecular descriptors and chemotaxonomy using several statistic tools and machine learning algorithms.

Profª. Drª. Margareth de Fátima Formiga Melo Diniz

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The book is intended for the scientific community that works with natural products, both those that investigate specific groups of natural compounds, and those who wish to further explore their potential as new medicinal products, addressing chemical, biological and pharmacological aspects.

Launched in celebration of the 40th anniversary of the Postgraduate Course on Natural and Synthetic Bioactive Products, from the Federal University of Paraíba (PgPNSB - UFPB), this book brings up-to-date texts on the pharmacochemistry and pharmacology of natural products through nationally and internationally renowned researchers.

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