MARTHA DALILA SEDANO-PARTIDA

Chemical and biological potential of *Hyptis* Jacq. (Lamiaceae)

Potencial químico e biológico de *Hyptis* Jacq. (Lamiaceae)

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Dedico con todo mi corazón este trabajo a

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mi pajarito, mi cuñado y al amor de mi vida,

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List of tables

List of figures

CHAPTER III Supplemental information

CHAPTER IV

CHAPTER V

Sedano-Partida, M. D. Chemical and biological potential of *Hyptis* Jacq. (Lamiaceae). [Thesis]. São Paulo: Institute of Bioscience, University of São Paulo; 2018

Abstract

Flavonoids and other phenolics are groups of natural bioactive compounds widely distributed in edible plants and are well documented to possess biological potential. *Hyptis* (Lamiaceae) is used in Brazilian folk medicine to treat various diseases. The aim of this study was to evaluate the antioxidant, anti-acetylcholinesterase, cytotoxic, antiviral and antibacterial potential of *Hyptis radicans* and *Hyptis multibracteata* by isolating and characterizing major constituents and their biological activities. *H. radicans* and *H. multibracteata* were dried, powdered and macerated in 70% ethanol which resulted in a crude ethanol extract (EE) for each species. EE were dissolved in 50% methanol and then was fractionated by partition with hexane and ethyl acetate; were obtained three phases: hexane phase (HP), ethyl acetate phase (EAP) and hydromethanol phase (HMP). EAP from *H. radicans* was the sample that presented the highest levels of total phenolic content, especially flavonoids, and was the sample with the high antioxidant activity with promising values of EC_{50}: DPPH (32.12 μg mL^{-1}), ABTS (5.04 μg mL^{-1}), Metal chelator assay (42.36 μg mL^{-1}), TBARS (40.46 μg mL^{-1}) and nonsite-Specific Hydroxyl Radical-Mediated 2-Deoxy-D-ribose Degradation *(*NS-Spe) with EC_{50} of 75.08 μg mL^{-1}. EE from *H. radicans* showed high antioxidant activity for FRAP and ORAC with EC50 of 6.01 and 2.68 μg mL^{-1}, respectively and had the highest amount of rosmarinic acid (17.64 mg *ρ*-CE g^{-1}). HMP from *H. radicans* showed high antioxidant activity in Site-Specific Hydroxyl Radical-Mediated 2-Deoxy-D-ribose Degradation (S-Spe) assay with EC50 of 0.32 μg mL^{-1} and had the highest content of chlorogenic acid derivatives. Regarding the results of cytotoxicity, HP from *H. multibracteata* induced the death of more than 80% of RAW 264.7 Cell Lines at 100 μg mL^{-1}. Nepetoidin B, isolated from *H. multibracteata* had the best EC_{50} (52.73 μg mL^{-1}) for anti-acetylcholinesterase activity. Antibacterial activity was evaluated *in vitro* against two Gram-negative bacteria, *Pseudomonas aeruginosa* and *Escherichia coli*, and a Gram-positive *Bacillus subtilis*. Phases from *H. multibracteata* were more effective on inhibiting *B. subtillis* with MIC_{50} of 23.6 μg mL^{-1} and 12.13 μg mL^{-1} for HP and EAP, respectively. HP was also activity against *P. aeruginosa* with MIC_{50} of 37.55 μg mL^{-1}. EE and HMP phase from *H. radicans* showed moderate anti-HIV-1 activity (MIC_{50} 159 μg mL^{-1}; MIC_{50} 180 μg mL^{-1}). Contents of total phenolic were not the main sample feature to define this activity, but there

was correlation between Rosmarinic acid contents and anti-HIV1 activity of *H. radicans*. Cirsimaritin and litospermic acid A were isolated for the first time, being the first time that they are described for the genus *Hyptis*. This study provides the first evidence of chemical and biological potential for these two Brazilian native species of *Hyptis*.

Keywords

Hyptis; flavonoids; nepetoidins; rosmarinic acid; lithospermic acid A; antioxidant; anti-HIV; cytotoxicity; antibacterial.

Sedano-Partida, M. D. Potencial químico e biologica de *Hyptis* Jacq. (Lamiaceae). [Thesis]. São Paulo: Instituto de Biociências, Universidade de São Paulo; 2018

Resumo

Flavonoides e outros compostos fenólicos são grupos de compostos bioativos naturais amplamente distribuídos em plantas e estão bem documentados por possuírem potencial biológico. *Hyptis* (Lamiaceae) é usado na medicina popular brasileira para tratar várias doenças. O objetivo deste estudo foi avaliar o potencial antioxidante, anti-acetilcolinesterase, citotóxico, antiviral e antibacteriano de *Hyptis radicans* e *Hyptis multibracteata*, isolar e identificar substâncias e correlacionar as atividades biológicas com a quantidade de compostos fenólicos e substâncias isoladas. *H. radicans* e *H. multibracteata* foram secas, pulverizadas e maceradas em etanol 70%, resultando em extrato etanólico bruto (EE). EE foi dissolvido em metanol 50% e depois foi fracionado por partição com hexano e acetato de etila, o que resultou em três fases: fase hexânica (HP), fase acetato de etila (EAP) e fase hidrometanólica (HMP). EAP de *H. radicans* foi a amostra que apresentou os maiores teores de conteúdo fenólico, principalmente flavonoides, e foi a amostra com a maior atividade antioxidante, com valores promissores de EC_{50}: DPPH (32,12 μg mL^{-1}), ABTS (5,04 μg mL^{-1}), Quelante de metais (42,36 μg mL^{-1}), TBARS (40,46 μg mL^{-1}) e Degradação da 2-deoxy-D-ribose de sitio não especifico mediada pelo radical hidroxil (NS-Spe) com EC_{50} de 75,08 μg mL^{-1}. EE de *H. radicans* apresentou a maior atividade antioxidante para FRAP e ORAC com EC_{50} de 6,01 e 2,68 μg mL^{-1}, respectivamente, e apresentou a maior quantidade de ácido rosmarínico (17,64 mg ρ-CE g-1). HMP de *H. radicans* apresentou a mais alta atividade antioxidante no ensaio de Degradação da 2-deoxy-D-ribose de sitio especifico mediada pelo radical hidroxil (S-Spe) com EC_{50} de 0,32 μg mL^{-1} e apresentou o maior teor de derivados de ácido clorogênico. Em relação aos resultados da citotoxicidade, HP de *H. multibracteata* induziu a morte de mais de 80% das células do tipo RAW 264.7 com uma concentração de 100 μg mL^{-1}. A Nepetoidina B isolada de *H. multibracteata* apresentou a melhor EC_{50} (52,73 μg mL^{-1}) para atividade anti-acetilcolinesterase. A atividade antibacteriana foi avaliada *in vitro* contra duas bactérias Gram-negativas, *Pseudomonas aeruginosa* e *Escherichia coli*, e uma bacteria Gram-positiva. *Bacillus subtilis*. Fases de *H. multibracteata* foram mais eficazes na inibição de *B. subtillis* com MIC50 23,6 μg mL^{-1} e 12,13 μg mL^{-1} para HP e EAP, respectivamente. HP também apresentou atividade contra *P. aeruginosa* com MIC_{50} de 37,55 μg mL^{-1}. EE e HMP de *H. radicans* mostraram moderada

atividade anti-HIV-1 (MIC_{50} 159 μg mL^{-1}; MIC50 180 μg mL^{-1}). Não há correlação entre o conteúdo total de fenólicos e esta atividade biológica, mas sim entre a quantidade de ácido rosmarínico das fases e a atividade anti-HIV1 de *H. radicans*. Foram isoladas pela primera vez a Cirsimaritina e o ácido litospermico A, sendo esta a primeira vez que se descrevem para o gênero *Hyptis*. Este estudo fornece a primeira evidência do potencial químico e biológico para estas duas espécies nativas de *Hyptis*.

Palavras chave:

Hyptis; Flavonoides; nepetoidinas; ácido rosmarínico; ácido litospermico A; antioxidante, anti-HIV; citotoxicidade; antibacterial.

Summary

CHAPTER I

Plant-derived drug discovery and special metabolism

Historical perspective

Throughout the history of the mankind, humans did depend on the nature in how to satisfy their basic needs. The first documented reports related to medicinal applications of plants dates back to 2,600 BCE and they report the existence of a sophisticated medical system in Mesopotamia, comprising about 1,000 plant-derived medicines. In these derivatives are included oils of *Cedrus* Trew species (cedar) and *Cupressus sempervirens L. (*cypress), *Glycyrrhiza glabra L.* (licorice), *Commiphora* Jacq. species (myrrh), and *Papaver somniferum* L. (poppy juice), all these which nowadays are still in use for the treatment of diseases who vary from coughs and colds to parasitic infections and inflammation. The Egyptian medicine dates from approximate 2,900 BCE, but the best-known record is the *Ebers Papyrus* dating from 1,500 BCE, which describes about 700 varieties of drugs, mostly originating of plants (Borchardt, 2002). The Chinese *Compendium of Materia Medica* has been extensively documented over the centuries (Huang, 1998), having the first report, known as the Wu Shi Er Bing Fang, dated from 1,100 BCE, and containing 52 prescriptions. Late, we have the, *Shennong Herbal* (~100 BCE), which contains 350 types of drugs, and the *Tang Herbal* written in 659 CE and containing 850 varieties of drugs. In the same way also exist documents where could be find the description of substances or plant-derived drugs in the Indian Ayurvedic system (dated from before 1,000 BCE). For example, the *Charaka Samhitas* and the *Sushruta Samhitas* report 341 and 516 drugs, respectively (Kapoor, 1990; Dev, 1999).

In Western world, the knowledge of the medicinal application of plants is mostly founded in two cultures: the Greek and Roman. In the first century the written documentation of the Greek physician Dioscorides was specifically important; for the Roman culture in the first century Pliny the Elder and Galen (2nd Century CE) were the two representatives in the area of medicinal plant descriptions (Sneader, 2005). Thanks to the Greek and Roman cultures, the Arabs, who already had medical experience, did preserve a great amount of knowledge during the Dark and Middle ages, in between the 5th and the 12th century and were improved with information received from traditional medicine from China and India.

Johannes Gutenberg assists in the revival of Greek and Roman knowledge in the 15th and 16th centuries with the invention of the printing press. As a result, several influential books on herbalism were compiled and widely distributed in Europe, for example *The Mainz Herbal* (1484) and The German Herbal (1485), both edited by Peter Schöffer, a Gutenberg's partner; the *Herbarium Vivae Eicones* by Otto Brunfels (1530), the *Kreütter Buch* by Hieronymus Bock in 1546 (written in German) and *De Historia Stirpium* by Leonhart Fuchs that was published in Latin (1542) and also in German (1543) (Sneader, 2005).

During all this time, the use of medicinal plants was applied on empirical grounds, since there was no mechanistic knowledge of its pharmacological activities or active constituents.

It was in the 18th century that the foundations for rational clinical investigation of medicinal herbs were laid by researcher Anton von Störck, who studied poisonous herbs such as *Aconitum* and *Colchicum*, and William Whitering who studied foxglove (*Digitalis* L.) for the treatment of edema (Sneader, 2005).

Later, at the beginning of the 19th century, the German apothecary assistant Friedrich Sertürner succeeded in isolating morphine from opium, an analgesic and sleep inducing agent, which was named after the Greek God, Morpheus. This was the first isolated constituent of a plant and Sertürner published the method of isolation and crystallization, as well as the crystal structure and pharmacological properties of morphine (which he studied in stray dogs and self-experiments) (Sertürner, 1817).

As expected, thanks to this research, other similar studies on medicinal plants were originated in the following decades of the 19th century, and many bioactive natural products were isolated and identified, mainly alkaloids (quinine, caffeine, nicotine, codeine, atropine, colchicine, cocaine, capsaicin) (Felter and Lloyd, 1898; Hosztafi, 1997; Sneader, 2005; Kruse, 2007; Zenk and Juenger, 2007; Corson and Crews, 2007; Kaiser, 2008). Between 1981 and 2010, 1,073 new chemical entities were approved, of which only 36% were purely synthetic and more than 50% were derived or inspired by nature (Newman and Cragg, 2012). A substantial number of these compounds have been discovered in higher plants (Kinghorn et al., 2011).

At the moment we can find very remarkable examples related to the importance that have acquired the natural products and their importance for modern pharmacotherapy, specifically in the area of anticancer agents. An example of this is paclitaxel and its derivatives from yew (*Taxus* L.) species, vincristine and vinblastine from Madagascar

periwinkle (*Catharanthus roseus* (L.) G. Don), and camptothecin and their analogs originally discovered in the Chinese tree *Camptotheca acuminate* Decne (Cragg and Newman, 2013; Kinghorn et al., 2011).

Another notable example is galanthamine, which is a cholinesterase inhibitor and has been approved for the treatment of Alzheimer's disease, discovered in *Galanthus nivalis* L. (Mashkovsky and Kruglikova-Lvova, 1951 *apud* Atanasov et al., 2015), and the important antimalarial and potential anti-cancer agent artemisinin originally derived from the traditional Chinese herb *Artemisia annua* L. (Klayman et al., 1984).

Caventou and Pelletier were the first to report, in 1820, the isolation of the anti-malaria drug quinine from the bark of *Cinchona L.* species (e. g., *C. officinalis L.*) (Buss and Waigh, 1995). Quinine occurs naturally in the bark of *Cinchona* trees in South America and had long been used by indigenous groups in the Amazon for the treatment of fever. It was first introduced into Europe in the early 1600s for the treatment of malaria. Quinine formed the basis for the synthesis of the commonly used antimalarial drugs being one of the oldest malaria remedies known. Chloroquine and mefloquine replaced quinine in the mid-20th century, but with the emergence of resistance to both these drugs in many tropical regions, another plant long used in the treatment of fevers in Traditional Chinese Medicine, *A. annua*, gained prominence *(*Wongsrichanalai et al., 2002). As described, for millennia, medicinal plants have been an invaluable resource for therapeutic agents. Nowadays many therapeutic agents are botanical drugs or directly derived therefrom (Kinghorn et al., 2011).

Natural products as drug candidates: significance and advantages against synthetic compounds

There is a wealth of available and well-documented ethnopharmacological information on the traditional uses of natural drugs, which is a great advantage because it provides evidences for therapeutically effective compounds in humans (Heinrich and Gibbons, 2001; Corson and Crews, 2007; Heinrich, 2010; Kinghorn et al., 2011). According to the information above, 122 compounds derived from plants used worldwide as therapeutic agents were analyzed and it was revealed that 80% have an identical or related use to indications for which these pure compounds were prescribed in ethnomedicine (Farnsworth et al., 1985; Fabricant and Farnsworth, 2001).

In addition, it has been shown that natural products used for the development of medicines are highly likely to be used traditionally. An example is the discovery of the anti-

cancer agent taxol, from *Taxus brevifolia* Nutt., which discovery was done with a random screening approach. Later on, it came to light that the plant has been used by western Indian cultures as a medicine (Heinrich, 2010).

Because natural drugs are made by or in living organisms, these products possess properties that are evolutionarily optimized to serve in different biological functions, as they are part of the body's metabolism, for example binding to specific target proteins or other biomolecules (Hunter, 2008; Appendino et al., 2010).

Natural compounds are highly diverse and often provide highly specific biological activities. This stems from the proposition that essentially all natural products have some ability to bind to the receptor. The natural molecules, however, differ substantially from the synthetic ones. The main structural differences between natural and combinatorial compounds originate mainly from properties introduced to make combinatorial synthesis more efficient. These include the number of chiral centers, the prevalence of aromatic rings, the introduction of complex ring systems, and the degree of saturation of the molecule, as well as the number and proportions of different heteroatoms.

The chiral separation method is challenging and expensive. Therefore, the creation of molecules with a low number of chiral centers is favorable. Synthetic compounds tend to have a much smaller number of chiral centers, and in addition a lower molecular weight, a higher number of freely rotatable bonds, higher chain lengths, a lower number of rings, less oxygen but more nitrogen, sulfur and halogen atoms, a lower number of acceptors and donors of Lipinski-type H-bonds and higher calculated octanol-water partition coefficients (cLogP values). Other differences are the complexity of ring systems and the degree of saturation (Stahura et al., 2000; Feher and Schmidt, 2003; Atasanov et al., 2015).

For example, because of the stereospecificity of most biological targets, it is likely that many non-stereospecific synthetic analogues, created, for example, by the introduction of aromatic rings, represent non-optimal compromises, especially in terms of selectivity and this occurs more frequently in the case of combinatorial synthesis compounds. The greater flexibility of combinatorial products is likely to have entropic consequences detrimental to the binding of these compounds. It may also affect negatively their ability to induce conformational changes in the receptor required for biological function. Also, the production process of synthetic analogs radically alters the number and ratios of different types of atoms, such as nitrogen, oxygen, sulfides and halogens. These distributions in turn

have a direct impact on the donor and acceptor patterns available to complement the receptor surface properties (Feher and Schmidt, 2003).

These factors, which are structural differences, specifically the significant number of chiral centers, low size and high flexibility, make the synthetic products weaker and less specific than natural products (Feher and Schmidt, 2003). Natural products have selective and specific biological actions due to the binding affinities to relevant proteins in their biological functions, and during biosynthesis a greater diversity and chemical complexity are developed than for their synthetic analogues (Clardy and Walsh, 2004; Koehn and Carter, 2005). They often have less advantageous absorption, distribution, metabolism, excretion and toxicity properties. In view of these facts, it is interesting to consider that the search for the replacement of natural compounds with synthetic ones is usually based on exactly these kinds of 'unfavorable' modifications.

The main focus of the pharmaceutical industry was for a time led to synthetic compound libraries and high throughput screening, with the aim of discovering new drug derivatives (Beutler, 2009; David et al., 2015). But, the results obtained did not meet expectations, and this is evident when the decreasing number of drugs that reach the market is observed (David et al., 2015). Because of this, the interest in products based on natural products has been revitalized for the discovery of new drugs, where broad interdisciplinary research approaches are required due to their high complexity, but at the same time high specificity as mentioned in previous paragraphs (Heinrich, 2010a).

Plants have been the basis for medical treatments through much of the human history. Nowadays, researchers are increasingly interested in medicinal plants as alternative medicine, due to their good pharmacological properties, fewer side effects, and low cost (Sayah et al., 2017).

Where do these medicinal substances originate in plants?

What is a special (or secondary) metabolite and which is their role in plants?

Land plants have colonized the vast majority of the Earth's surface due to rich levels of specialization and intricate relationships with other organisms. During this process land plants had (and still have) to face a number of challenges imposed by the terrestrial environment. These organisms are autotrophic stationary, dealing with biotic and abiotic stress factors such as the coexistence of herbivores and pathogens in their immediate environment, pollination and seed dispersal (specially angiosperms), and climate variations.

Therefore, and because of these challenges, land plants have developed special biochemical pathways that allow them to synthesize a series of chemicals, also called secondary metabolites or special metabolites, that are produced regularly in response to specific environmental stimuli, such as herbivore induced-damage, pathogens attack, enhanced concentration of air pollutants etc. (Reymond et al., 2000; Hermsmeier et al., 2001).

The function of these special metabolites is to increase the general plant ability plant to survive and overcome local challenges, allowing them to interact with their environment. They play no role in primary metabolic needs and may be unique to specific species or genera (Harborne, 2014). The energy invested in the synthesis of these special metabolites, which is usually much higher than that required to synthesize primary metabolites, is an indicator of the importance of these substances for the survival of plants (Gershenzon, 2017; Hong et al., 2016). Among the functions of the special metabolites are: protective roles as antioxidant, free radical-scavenging, UV light-absorbing, and defend the plant against microorganisms such as bacteria, fungi, and virus. They also manage inter-plant relationships, acting as allelopathic defenders of the plant's growing space against competitor plants. More complex roles include dictating or modifying the plant's relationship with more complex organisms (Harborne, 2014; Wink, 2003; Tahara, 2007).

One of the main roles of special metabolism is feeding deterrence. For that reason, many of these substances are bitter and/or toxic to potential herbivores, affecting the central and peripheral nervous system of the herbivore. In this regard, special metabolites often act as agonists or antagonists of neurotransmitter systems or form structural analogs of endogenous hormones (Wink, 2000; Miller and Heyland, 2010; Rattan, 2010). In addition to that defense mechanism, plants also have to foster a number of symbiotic relationships. One of the obvious roles in this series of mechanisms is the attraction of pollinators and other symbionts, using colors and scents or indirect defenses, by attracting natural enemies of its herbivorous attackers. In this way, it provides an attractive chemical environment for the predator or alternatively, it may be a direct response to tissue damage by the herbivore, resulting in the synthesis and release of a set of substances that are attractive to natural herbivore predators (Harborne, 2014; Wink, 2003; Tahara, 2007). There are more than 100,000 special metabolites already described in plants, ranging from simple alkaloids (structurally) to phytosterols and more complex polyphenolic molecules (Dillard and German, 2000).

Special metabolism and major groups of plant special metabolites

All living cells possess similar pathways for the synthesis of components such as sugars, amino acids, nitrogenous bases, carbohydrates, proteins, and nucleotides, being these are molecules essential for energy production and cell constitution and plant development. Plant special metabolites are derived from the products of primary metabolism but have a much more limited taxonomic distribution. They can be broadly classified according to their structure and biosynthetic pathways; however, it should be appreciated that many special metabolites are also derived by combining elements of all these biosynthetic pathways (Fig. 1.1).

These diversified compounds can be divided into three main categories: terpenes, nitrogenous compounds and phenolic compounds, based on their chemical structure. Amines, cyanogenic glycosides, glucosinolates, acetylenes and psoralens, are other minor groups that cannot be included in these three large groups (Fang et al., 2011 *apud* Russell and Duthie, 2011).

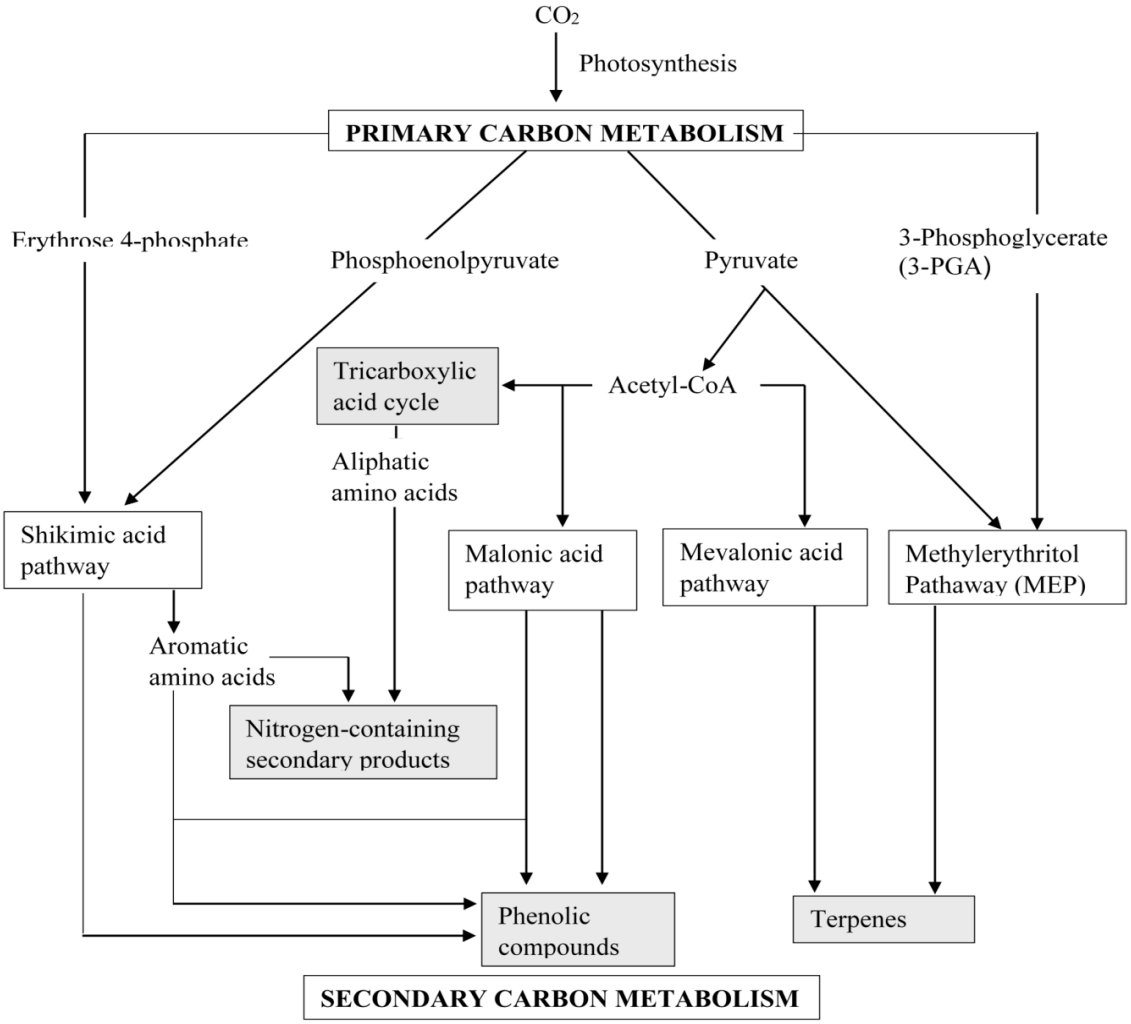

Figure 1.1. A simple schematic representation of the major secondary metabolites in plants (Ncube and Van Staden, 2015)

Terpenes

Terpenes represent the most abundant and structurally diverse group of plant special metabolites, in which more than 36,000 structures have been identified. They are a structurally diverse group of hydrocarbons derived from the five-carbon precursors: isopentyl diphosphate (IPP) or dimethylallyl diphosphate (DMPP), synthesized, in photosynthetic organisms, by mevalonate and methylerythritol phosphate pathways.

Terpenes are classified according to the degree of isoprene incorporation as follow: hemiterpenes (C_5), monoterpenes $(C_5)_2$, sesquiterpenes $(C_5)_3$, diterpenes $(C_5)_4$, sesterpenes $(C_5)_5$, triterpenes $(C_5)_6$, tetraterpenes (or carotenoids) $(C_5)_8$, and through to higher polymers such as rubber $(C_5)_{>100}$. Isoprene units are often joined in a head-to-tail and head-to-head linkage and a few terpene structures are formed by irregular head-to-middle linkage. After the basic terpene skeletons are formed, subsequent modifications occur which give rise to different structures such as steroids like cholesterol, ergosterol, sitosterol and stigmasterol, which are synthesized from a triterpene precursor. Among the modifications that the basic terpene skeleton receives are: reduction, isomerization, oxidation, conjugation and degradation (Grayson, 2000; Croteau et al., 2000; Maimone and Baran, 2007).

Pharmacological active molecules derived from terpenes include, for example, the herbal tranquilizer valtrate, the major component of valerian (*Valeriana officinalis* L.) and the anti-cancer drug taxol, extracted originally from the Pacific Yew (*Taxus brevifolia*) (Hayes et al., 2008).

Nitrogenous compounds: alkaloids

Alkaloids are a group of alkaline, low molecular weight and nitrogen containing compounds. They are the most widely distributed nitrogenous special metabolites and are found not only in plants, but also in microorganism, playing an important role in plant defense systems. Alkaloid containing plants were, for mankind, the original "*materia medica*" and many are still in use today as prescription drugs, such as vinblastine, quinine, atropine, and camptothecin. There are more than 12,000 alkaloids reported for 100 families of plants, being especially abundant in Fabaceae, Solanaceae, Menispermaceae, Papaveraceae, Ranunculaceae, Apocynaceae and Berberidaceae. Can be classified on the basis of the plants from which they were isolated, their chemical structures, and the biosynthetic origins. This last feature has an obvious advantage of reflecting the relationship between biosynthetic pathways and the chemical structures. Alkaloids could thus be further

classified into three groups according to their biosynthesis origin: true alkaloid, protoalkaloid, and pseudoalkaloid (Buchanan et al., 2000; Dewick, 2009).

The alkaloids, have contributed mainly providing neurotoxins, poisons and traditional psychedelics, among which are some that come from *Atropa belladonna* L. like, atropine, scopolamine, and hyosciamine, to this chemical group also belong the most consumed social drugs, nicotine, caffeine, methamphetamine (ephedrine), cocaine and opiates (Zenk and Juenger, 2007). This group also provides the cholinesterase inhibiting treatments routinely prescribed for the cholinergic deregulation of Alzheimer's disease, such as galantamine, huperzine, physostigmine, and rivastigmine (Mukherjee et al., 2007).

Phenolic compounds

Phenolic compounds are ubiquitously found across plant, with ~10,000 structures identified. Structurally, they share at least 1 aromatic hydrocarbon ring with 1 or more hydroxyl groups attached and are synthesized via the shikimate pathway alone or in combination with the acetate-*malonate pathway*. The simplest compound with this structural motif is the phenol molecule, which itself does not occur in plants. Phenolic compounds range from simple low-molecular mass, such as the simple phenylpropanoids, coumarins, and benzoic acid derivatives, to more complex structures such as flavanoids, stilbenes, and tannins. Flavonoids represent the largest, most diverse group, encompassing some 6,000 compounds, all of which share a common underlying structure of two 6-carbon rings, with a 3-carbon bridge, which usually forms a 3rd ring. Flavanoids can then be subdivided according to modifications of this basic skeleton into: chalcones, flavones, flavonols, flavanones, isoflavones, flavan-3-ols, and anthocyanins (Bowsher and Tobin, 2008; Yang et al., 2012).

Phenolic compounds and flavonoids in particular, are ubiquitous in plants and therefore represent an important component of a normal human diet. Epidemiological studies have suggested associations between consumption of phenolic-rich foods or beverages and various diseases, such as stroke, cardiovascular disease, and cancer (Steffen, 2006) and neurologic disorders such as dementia/Alzheimer's disease (Commenges et al., 2000; Vingtdeux et al., 2008).

Aims of the present study

In the world there are a great number of plant species, which produce a diversity of bioactive compounds with different chemical scaffolds. According to previous estimates, only 6% of existing plant species have been systematically pharmacologically investigated, and only about 15% were studied phytochemically (Fabricant and Farnsworth, 2001; Verpoorte, 1998 and 2000). Although today the percentage of species is better characterized by increased interest in this phytochemical area, it is still conceivable that there are a large number of plant compounds that are not well pharmacologically researched, especially if we consider the approximately 310,000 plant species described (IUCN, 2015). Unfortunately, a significant decline in global plant species is expected in the coming years as a result of climate change and anthropogenic factors that jeopardize these potential sources of new natural drugs, and therefore urgent measures are needed to access different species (Maclean and Wilson, 2011; Thomas et al., 2004).

Another point to highlight is the ethnobotanical knowledge about the traditional pharmacological use that is disappearing. With the increase of globalization, this information is in danger of being lost forever and it is being lost faster than the loss of the biodiversity (Appendino et al., 2010).

In the context of the discovery of drugs of plant origin, it is highly advantageous when the species under study come from regions of high biodiversity and endemism, as the chemical diversity of natural products can reflect the biodiversity of their organisms of origin and an example of megadiverse country is Brazil (Barbosa et al., 2012; Henrich and Beutler, 2013). These estimates gain importance when considering the broad potential of the active principles contained in nature and which have not yet been identified and evaluated in a medical context. This fact attracts the attention of the pharmaceutical industry that sees in plant diversity a feasible source for new medicines.

The present study aimed to access phytochemically to Brazilian native species: *Hyptis radicans* Jacq. and *Hyptis multibracteata* Poit. Both are species of occurrence in the Atlantic Rainforest of State of São Paulo and are easily found in the Paranapiacaba region. Furthermore, both are species that do not count on studies on their chemical composition, have not been evaluated for their biological activities and, therefore, are promising models for prospecting studies of natural bioactive substances.

The main objectives of the present study were:

- To isolate and identify substances present in both species; and
- To evaluate the antioxidant, antibacterial and anti-HIV potential of *H. multibracteata* and *H. radicans*;

This study is divided in 5 chapters and a final consideration as follow:

Chapter 1: Plant-derived drug discovery and special metabolism.

Chapter 2: *Hyptis* Jacq.: a general chemical profile review - *manuscript submited to Chemistry & Biodiversity.*

Chapter 3: Botanical aspects & chemical description of *H. radicans* and *H. multibracteata.*

Chapter 4: Antioxidant, anti-acetyl cholinesterase and cytotoxic potential of *Hyptis* spp – *part of the results published at Industrial Crops & Products 112 (2018) 705–715.*

Chapter 5: Anti-HIV-1 and antibacterial potential of *Hyptis radicans* (Pohl) Harley & J.F.B. Pastore and *Hyptis multibracteata* Benth. (Lamiaceae) – *manuscript submited to Journal of Herbal Medicine.*

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CHAPTER II

Hyptis Jacq.: a general chemical profile review

Lamiaceae is the sixth largest angiosperm family, presenting a cosmopolitan distribution with approximately 258 genera and 7,193 species widely distributed in the tropical regions, but absent in cold regions of high altitudes (Judd *et al*., 2002; Li et al., 2016). In Brazil, there are 46 genera and 525 native species (BFG, 2015; Harley et al., 2015). It is a family with global economic importance, since many species have been used either as medicine, as a condiment or, more rarely, as food basically in all continents and cultures. It has also been used as ornamental plant, being appreciated for their aroma or for their flowers (Fernández-Alonso and Rivera-Díaz, 2006).

Many Lamiaceae species produce volatile oils with medicinal properties and other species have landscape potential (Harley and Reynolds, 1992); therefore, some species are relatively well studied chemically. The basil (*Ocimum* L.), skullcaps (*Scutellaria* L.), horehound (*Marrubium* L.), mint, garden mint (*Mentha* L.), classic oregano and marjoram (*Origanum* L.), sage (*Salvia* L. and *Lepechinia* Willd.), mexican mint (*Plectranthus* L'Hér.), andean oregano (*Minthostachys* (Benth.) Spach), savory (*Satureja* L.), bushmints (*Hyptis* Jacq.*)*, thyme (*Thymus* L.), lemon balm (*Melissa* L.) and rosemary (*Rosmarinus* L.) can be cited as examples of the most common species of Lamiaceae. Furthermore, spices have been used for many years to enhance sensory attributes, such as the taste and aroma of foods, and to improve the overall quality of a product (Wang et al., 1996; Fernández-Alonso and Rivera-Díaz, 2006; Velasco and Williams, 2011).

Many Lamiaceae are included in the list of medicinal plants, once it has been reported substances isolated from plants of this family with antimicrobial, cytotoxic, antioxidant, anti-inflammatory, and insecticide activities (Falcão and Menezes, 2003). According to Richardson (1992), were found in Lamiaceae metabolites with cardiovascular activities, as labdane diterpenes in *Plectranthus forskohlii* Willd., antineoplastic agents, as sesquiterpenes isolated from *Salvia hypoleuca* Benth., hallucinogenic compounds from *Salvia divinorum* Epl. & Jativa, terpenoids and phenolic substances with allopathic effects isolated from *Leonurus sibiricus* L., and several flavonoids, which have been frequently used for chemotaxonomic purposes (Tomas-Barberan and Wollenweber, 1990).

Consumption of spices has been implicated in the prevention of cardiovascular diseases, carcinogenesis, inflammation, and atherosclerosis. Several studies showed that Lamiaceae herbs have a potent antioxidant and antibacterial activities, they are a source of phytochemical compounds which beneficial effect on health, or they play an active role in amelioration of diseases, mostly due to the content and type of phenolic compounds. Rosmarinic acid, eugenol, carvacrol, and thymol, which are the major components of volatile oils are phenolic compounds primarily responsible for the antibacterial properties reported for some Lamiaceae volatile oils (Shan et al., 2005; Hinneburg et al.,2006; Hossain et al., 2008; Tajkarimi et al., 2010).

Traditionally, aromatic herbs, such as rosemary (*Rosmarinus officinalis* L.), sage (*Salvia officinalis* L.), thyme (*Thymus vulgaris* L.) and lavender (*Lavandula angustifolia* Mill.), which are native to the Mediterranean region and cultivated world-wide, and balm (*Melissa officinalis* L.) and spearmint (*Mentha spicata* L.), common plants in Britain and other European countries, have been used in folk remedies for exhaustion, weakness, depression, memory enhancement, circulation improvement and strengthening fragile blood vessels. Researchers have found that these plants are a source of compounds possessing high antioxidant capacity (Zheng and Wang, 2001), anti-inflammatory (Al-Sereiti et al., 1999), anti-allergy (Ito et al., 1998) and anti-depression activitivies (Takeda et al., 2002).

In relation to chemical composition, Lamiaceae arouses great interest in the study of secondary metabolites, such as flavonoid, alkaloids, terpenes, coumarins, lignoids and volatile oils (Ferreira, 2009). Chlorogenic acid and 3,4-dihydroxyphenyl ethanoid glycosides are compounds frequently reported for Lamiaceae species (Basílio et al., 2007).

The family is divided in 12 subfamilies: Ajugoideae, Callicarpoideae, Cymarioideae, Lamioideae, Nepetoideae, Prostantheroideae, Peronematoideae, Premnoideae, Scutellarioideae, Tectonoideae, and Viticoideae (Li et al., 2016; Li and Olmstead, 2017). Nepetoideae is the largest subfamily, comprising almost 50% of the genera and species, comprising the majority of the volatile oil-rich genera of Lamiaceae, and, consequentely, it has most of the species with greater economic interest, such as mint, sage, rosemary, thyme and basil (Grayer et al., 2003; Zhong et al., 2010; Harley and Pastore 2012). Nepetoideae comprises 118 genera and ca. 3,400 species widely distributed in tropical and temperate areas. This subfamily presents as putative synapomorpies the presence of rosmarinic acid,

together with morphological features as hexacolpate and three-celled pollen, investing embryos, myxocarpy, and gynobasic style (Li et al., 2016).

Three tribes are recognized for Nepetoideae: Elsholtzie, Mentheae and Ocimeae, all well supported by molecular studies. Ocimeae is divided into seven subtribes according to Harley and colleagues (2004) and Zhong and colleagues (2010): Hanceolinae (C. Y. Wu) A. J. Paton & Harley, Isodoninae J. S. Zhong, J. Li & H. W. Li, Lavandulinae Endl., Ociminae (Dumort.) Schimidt, Plectranthinae Endl., Siphocranioninae J. S. Zhong, J. Li & H. W. Li and Hyptidinae Endl. Hyptidinae occurs predominantly in the New World (Pastore, 2010), while remaining subtribes are predominantly from the Old World, especially from Europe.

Hyptidinae is one of the most diverse clades of the New World mints, comprising approximately 400 species, extending from the southern United States to the Caribbean and south to Argentina, but the majority of its species occurring in the Neotropical region (Harley et al., 2004). Recently, a phylogenetic study based on the molecular data evidenced the need for major changes in the classification of Hyptidinae. Based on this new evidence, it was proposed the recognition of 12 new genera in the subtribe, totaling 19 genera. The choice in recognizing a substancial number of new genera was made as a way to avoid that *Hyptis* included more than 400 species, and, consequently, presenting a broad range of morphology, which would make difficult to understand its taxonomy. In addition, numerous genera are more easily characterized by a set of morphological features, sometimes by anatomical and chromossomal characters as well (Harley and Pastore, 2012). In this way, Hyptidinae in its current classification comprises *Asterohyptis* Epling, *Cantinoa* Harley & J.F.B. Pastore, *Condea* Adans., *Cyanocephalus* (Pohl ex Benth.) Harley & J.F.B. Pastore, *Eplingiella* Harley & J.F.B. Pastore, *Eriope* Humb. & Bonpl. ex Benth., *Eriopidion* Harley, *Gymneia* (Benth.) Harley & J.F.B. Pastore, *Hypenia* (Mart. ex Benth.) Harley, *Hyptidendron* Harley, *Hyptis* Jacq., *Leptohyptis* Harley & J.F.B. Pastore, *Martianthus* Harley & J.F.B. Pastore, *Marsypianthes* Mart. ex Benth., *Medusantha* Harley & J.F.B. Pastore, *Mesosphaerum* P. Browne, *Oocephalus* (Benth.) Harley & J.F.B. Pastore, *Physominthe* Harley & J.F.B. Pastore and *Rhaphiodon* Schauer.

Many taxa of Hyptidinae, especially those from *Hyptis*, present great variability of secondary metabolites, particularly volatile oils (Zhong et al., 2010; Oliveira et al., 2011). *Hyptis sensu stricto* comprises about 144 species been characterized by aromatic herbs or shrubs, with quadrangular stems and epipetalous stamens (Basílio et al., 2007; Harley and Pastore, 2012).

From the chemical point of view, *Hyptis* shows a wide variety of chemical constituents, including flavonoids (Abedini et al., 2013), monoterpenes (Fernández-Alonso et al., 2003; Abedini et al., 2013; Tafurt-Garcia et al., 2014), diterpenes (Urones et al., 1998; Alvarez et al., 2015), triterpenes (Lee et al., 1988; Kashiwada et al., 1998; Deng et al., 2009), brevipolides (Suárez-Ortiz et al., 2017), lactons (Almtorp et al., 1991), steroids (Costa et al., 2008; Tang et al., 2014). *Hyptis* species are commonly used in the treatment of gastrointestinal infections, skin infections, pain, headache, cramps, stomach discomfort, menstrual pain, treatment of flu/fever (Rocha et al., 2009B; Pedroso et al., 2017), for antiulcer and anti-inflamatory problems (Jesus et al., 2009), as anti-parasitic and insect repellent (Ortega et al., 2014), in the treatment of snakebites and scorpion stings (Costa et al., 2008). Despite its potential use as medication, heretofore few species were studied with proven medicinal action.

The phytochemical, biological and pharmacological interests in *Hyptis* taxa starded in 1952 when *Hyptis suaveolens* (L.) Poit was used as precursor in the treatment of various infections (Nayak and Guha, 1952). Other species reported on the literature are *Hyptis oblongifolia* Benth. and *Hyptis pectinata* (L.) Poit. (Pereda-Miranda and Delgado, 1990; Pereda-Miranda et al., 1993), *Hyptis spicigera* Lam. (Kini et al., 1993; Pereda-Miranda and Delgado, 1990), and *Hyptis ovalifolia* Pohl. (Souza et al., 2003).

Hyptis species are also known to be used in folk medicine for the treatment of various diseases, such as influenza and constipation (*Hyptis fruticosa* Salzm. ex Benth); respiratory diseases (*Hyptis macrostachys* Benth); stomach and intestinal disorders and bactericidal (*Hyptis martiusii* Benth.); colic and liver diseases (*Hyptis pectinata*); nasal and atrial disorders (*Hyptis umbrosa* Salzm. ex Benth.), and *Hyptis suaveolens* to combat fever (Judd et al., 2002, Agra et al., 2008; Coutinho et al., 2008).

Due to the new circumscription of the genus (Harley and Pastore 2012) some chemically important species, such as *Hyptis suaveolens*, *H. oblongifolia, H. pectinata, Hyptis spicigera, H. ovalifolia, H. macrostachys, H. fruticosa, H. martiusii, H. umbrosa* and more, were placed into other genera. Facing the recent evidences provided by the phylogenetic study performed in Hyptidinae, the major changes in the systematic of this subtribe, especially in *Hyptis*, and aiming to review the literature regarding the chemical composition and economic uses for *Hyptis*, the present study provides a bibliographical survey of all scientific articles published from 1906 up to March 2018 using databases SciFinder (879 papers), Web of Science (528 papers) and SciELO (96 papers). *Hyptis* and

Peltodon were used as keywords and a triage of articles were performed using genus delimitation *sensu* Harley & Pastore (2012). Peltodon were included as keyword once this genus is now a section according to Harley & Pastore (2012).

Table 2.1. Ethnopharmacological/pharmacological, economic uses, and chemical composition reported for *Hyptis sensu* Harley and Pastore (2012). SciFinder, Web of Science and SciELO databases were as a source of information (1906 to 2018).

Species	Chemical constituents	Uses/Biological Activities
Hyptis alata (Raf.) Shinners		**Insecticidal activity:** methanolic extract of roots was slightly attractant to ovipositing *Aedes aegypti* mosquitoes; Methanolic extract of stems caused 57% mortality of *Aedes aegypti* mosquitoes after 24 h of exposure, and 90% mortality after 7 days of exposure (Dees et al., 2014A; Dees et al., 2014B); hexane extract showed female mortality of *Aedes aegypti* after 24 hours of exposure (Cudd et al., 2014).
Hyptis atrorubens Poit.	**Volatile oils:** Hexanal, α-Pinene, β-Pinene, 1-Octen-3-one, 1-Octen-3-ol, 3-Octanol, Limonene, γ-Terpinene, Linalool, Pinocarveol, Myrtenal, α-Terpineol, Myrtenol, 1-Octenyl-3-propanoate, Isothymylmethylether, Thymol, Eugenol, α-Cubebene, β-Damascenone, α-Copaene, β-Copaene, β-Bourbonene, β-Elemene, α-Gurjunene, β-Caryophyllene, Caryophyllene oxide, *cis*-α-Bergamotene, Aromadendrene, *allo*-Aromadendrene, *epi*-β-Santalene, *trans*-β-Farnesene, α-Humulene, α-Humulene epoxide, β-Santalene, 9-*epi*-Caryophyllene, β-Ionone epoxide, β-Ionone, γ-Muurolene, 2-Tridecanone, Germacrene-D, α-Selinene, α-Muurolene, β-Bisabolene, γ-Cadinene, δ-Cadinene, α-Cadinol, δ-Cadinol, Calamenene, Lauric acid, Nerolidol, Palustrol, Spathulenol, Salvial-4(14)-en-1-one, Viridiflorol, Caryophylla-3(15),7(14)-dien-6-ol, Caryophylla-3,8(13)-dien-5-ol, α-Bisabolol, Hexahydrofarnesyl acetone, Neophytadiene; Pentadecanoic acid, Palmitic acid, Ethyl palmitate, Linoleic acid, Methyl linoleate, Ethyl linoleate, Stearic acid; Phytol, Farnesyl acetone, Isophytol, Phytyl acetate (Kerdudo et al., 2016).	**Antibacterial activity:** at 0.5% extracts were activie against: *Staphylococcus aureus* ATCC 6538 and *Bacillus cereus* ATCC 14579 gram (+) bacteria, and *Escherichia coli* ATCC 8739 gram (-) bacteria (Kerdudo et al., 2016); methanol extract of aerial parts active against *Staphylococcus epidermidis* and *Enterococcus faecalis* (Gram(+) bacteria), *Burkholderia cepacia* and *Stenotrophomonas maltophilia* (Gram(-) bacteria), at < 0.3 mg mL^{-1} (Abedini et al., 2012); hydromethanolic extracts active against: *Acinetobacter baumannii* 9010 (MIC 5 mg mL^{-1}), *Acinetobacter baumannii* 9011 (MIC 2.5 mg mL^{-1}), *Proteus mirabilis* 11060 (MIC2.5 mg mL^{-1}), *Proteus mirabilis* 11061 (MIC 5 mg mL^{-1}), *Providencia stuartii* 11038 (MIC 10 mg mL^{-1}), *Stenotrophomonas maltophilia* (MIC 0.3 mg mL^{-1}), *Pseudomonas aeruginosa* 8131 (MIC 2.5 mg mL^{-1}), *Pseudomonas aeruginosa* ATCC 27583 (MIC 1.2 mg mL^{-1}) – all Gram(-) bacteria; *Mycobacterium smegmatis* 5003 (MIC 0.6 mg mL^{-1}), *Staphylococcus aureus* 8146 (MIC 1.2 mg mL^{-1}), *Staphylococcus aureus* 8147 (MIC 1.2 mg mL^{-1}), *Staphylococcus epidermidis* 10282 (0.3 mg mL^{-1}), *Staphylococcus epidermidis* 5001 (MIC 0.3 mg mL^{-1}), *Corynebacterium* T25-17 (MIC 0.6 mg mL^{-1}), *Enterococcus* sp. 8152 (MIC 1.2 mg mL^{-1}), *Enterococcus* sp. 8153 (MIC 1.2 mg mL^{-1}), *Enterococcus faecalis* C159-6 (MIC 0.3 mg mL^{-1}), *Staphylococcus lugdunensis* T26A3 (MIC 0.6 mg mL^{-1}), *Staphylococcus warneri* T12A12 (MIC 0.6 mg mL^{-1}) - all Gram(+) bacteria (Abedini et al., 2013).

Table 2.1. continued.

Species	Chemical constituents	Uses/Biological Activities
Hyptis atrorubens Poit	**Phenolic Compounds**: Rosmarinic acid, Methyl rosmarinate, Quercetin-3-*O*-glucoside (isoquercetin), Quercetin-3-*O*-galactoside (hyperoside) (Abedini et al., 2013).	**Antifungal activity:** active agaisnt *Aspergillus niger* ATCC 16404 (fungi), *Candida albicans* ATCC 10231 (yeast) (Kerdudo et al., 2016); *Candida krusei* (MIC 0.3 mg mL^{-1}), *Candida glabrata* (MIC 10 mg mL^{-1}), *Candida kefyr* (MIC 5 mg mL^{-1}), *Candida albicans* (MIC 10 mg mL^{-1}), *Candida parapsilosis* (MIC 2.5 mg mL^{-1}); Active against the dermatophytes *Microsporum canis* (MIC 1.2 mg mL^{-1}), *Trichophyton rubrum* (MIC 0.6 mg mL^{-1}), *Trichophyton mentagrophytes* (MIC 0.6 mg mL^{-1}), *Trichophyton soudanense* (MIC 2.5 mg mL^{-1}), *Trichophyton tonsurans* (MIC 0.6 mg mL^{-1}) (Abedini et al., 2013). **Ethnopharmacological/pharmacological uses:** Traditional medicinal remedy in French West Indies (Abedini et al., 2012). **Trophic relationship:** is a floral resource for the specimens of *Apis mellifera*, a strong trophic relationship between these species was demonstrated in a secondary forest region, Alcantara, MA, Brazil (Gonçalves et al., 1996). **Parasitism**: Host for *Puccinia insititia* and *Puccinia medellinensis* (Baxter, 1961).
Hyptis brachiata Briq.	**Volatile oils**: α-Tuyene, α-Pinene, Sabinene, β-Pinene, 1-Octen-3-ol, 3-Octanol, α-Felandrene, p-Cimene, Limonene, β-Felandrene, 1,8-Cineol, trans-Verbenol, Terpinen-4-ol, α-Terpineol, Bornil acetate, β-Elemene, δ-Elemene, α-Cubebene, β-Cubebene, Eugenol, Isoledene, α-Copaene, β-Bourbonene, α-Gurjunene, E-Caryophyllene, β-Copaene, α-Guaiene, Aromadendrene, α-Humulene, allo-Aromadendrene, γ-Muurolene, Germacrene D, β-Selinene, epi-Cubebol, α-Selinene, α-Muurolene, Bicyclogermacrene, β-Bisabolene, Germacrene A, γ-Cadinene, δ-Cadinene, trans-Calamenene, trans-γ-Bisabolene, 10-epi-Cubebol, α-Cadinene, Germacrene B, Palustrol, Germacrene D-4-ol, Caryophyllene oxide, Globulol, Viridiflorol, Humulene	**Lectin activity:** Considerable lectin activity (66.6%) was detected from nutlets (Fernández-Alonso et al., 2003).

Table 2.1. continued.

Species	Chemical constituents	Uses/Biological Activities
Hyptis brachiata Briq.	epoxide II, 1,10-di-epi-Cubenol, τ-Cadinol, τ-Muurolol, δ-Cadinol, α-Cadinol (Tafurt-Garcia et al., 2014). Presence of mucilage; polysaccharide was found to be absent or undetectable (Fernández-Alonso et al., 2003).	
Hyptis brevipes Poit.	**Brevipolides**: A to F (Deng et al., 2009); A to J (Suárez-Ortiz et al., 2013); K, L, M, N, and O (Suárez-Ortiz et al., 2017). **Phenolic compounds:** 5-hydroxy-7,4'-dimethoxy-flavon-3-ol, 5-hydroxy-7-methoxy-2-(4`-methoxy-phenyl)-chromen-4-one; 5,3′-dihydroxy-3,7,4′-trimethoxyflavone (ayanin), 3,5,3′-trihydroxy-7,4′-dimethoxyflavone (ombuin), and 5,6,3′-trihydroxy-3,7,4′-trimethoxyflavone (Deng et al., 2009); Rosmarinic acid and 6-hydroxycaffeic acid (Pedersen, 2000). **Triterpenes and Steroids**: Ursolic acid, 2α-hydroxyursolic acid (maslinic acid); Daucosterol (Deng et al., 2009). **Volatile oils**: D-Limonene, 1-Octen-3-ol, α-Cubebene, Copaene, Linalool, (–)-β-Elemene, Caryophyllene, Benzene acetaldehyde, Humulene, *cis, cis, cis*-1,1,4, 8-Tetramethyl-4,7, 10-cycloundecatriene, Borneol, Germacrene D, Longifolene, (–)-β-Cadinene, α-Sesquiphellandrene, ç-Elemene, l-Calamenene, Thymol, α-Isosafrole, Caryophyllene oxide, Methyl eugenol, Humulene epoxide II, Cubenol, Viridiflorol, Spathulenol, 3-Allylguaiacol, Phytol, Palmitic acid (Xu et al., 2013). **Leaf volatile oils**: δ-Cadinene, (E) Ocimene, δ-Elemene, δ-Eudesmol, δ-Muurolene, 12-Oxabicyclo (9.1.0)dodec-4) dienes 1,5,8-tetramethyl, 1H-Cycloprople azulene, 1a, 2, 3,4,7-tetransethyl, [1aR(1aL, 4L, 4aL)], 1H-Cycloproly azulene, decahydro 1,1,7-trimethyl-4-methylene [1aR(1aL,	**Antibacterial activity:** antibacterial properties (Gupta et al., 1996; Zollo et al., 1998); using disk diffusion method, methylene chloride and methanol extracts (at 100 mg mL^{-1}) had no effect on *Bacillus subtilis* Gram(+), 25–50% more active than the control against *Staphylococcus* Gram(+), 25% or less active than the control against *Escherichia coli* and *Xanthomonas campestri*, both Gram(-) bacteria (Goun et al, 2003); Volatile oil active agianst *Staphylococcus aureus* (MIC 3.125 μg mL^{-1}), *Bacillus subtilis* (MIC 3.125 μg mL^{-1}) both Gram(+) bacteria, *Escherichia coli* (MIC 6.25 μg mL^{-1}), and *Pseudomonas aeruginosa* (MIC 3.125 μg mL^{-1}), Gram(-) bacteria (Xu et al., 2013). **Antifungal activity:** antifungical properties (Gupta et al., 1996; Zollo et al., 1998); using disk diffusion method, methylene chloride and methanol extract (at 100 mg mL^{-1}) had no effect on *Candida albicans*, 50% and more active than the control against *Pythium ultimu,* 25% or less active than the control against *Rhizoctonia solani, Sclerotium rolfsii,* and *Aspergillus fumigatus*, and equal active as the control against *Phytophthora parasítica* (Goun et al., 2003). Volatile oil active agianst *Fusarium graminearum* (MIC 3.125 μg mL^{-1}), *Botrytis cinerea* (MIC 6.25 μg mL^{-1}), *Exerohilum turcicum* (MIC 6.25 μg mL^{-1}), and *Lecannosticta acicola* (MIC 3.125 μg mL^{-1}) (Xu et al., 2013). **Antioxidant activity:** volatile oils exhibited notable antiradicalar activity, SC_{50} of 2.019± 0.25 μg mL^{-1} (DPPH assay) (Xu et al., 2013). **Citotoxicity:** DNA intercalation properties and high Brine shrimp lethality activity (Gupta et al., 1996)**;** volatile oil LC_{50} of 60.8±9.04 μg mL^{-1} (Xu et al., 2013); potential antitumor activities (Xu et al., 2013); Brevipolides (B and F) and 5,6-dihydro-α-pyrone derivative exhibited ED_{50} values of 6.1, 6.7, and 3.6 μM against MCF-7 cells; Brevipolides (A,

Table 2.1. continued.

Species	Chemical constituents	Uses/Biological Activities
Hyptis brevipes Poit.	1CB, 7L, 7aB, 7bL)], 1-Octen-3-ol, 2,3,4-Triflurobenzoie acid-4-tetrancyl ester, 2,6,10-Cycloundecatriene-1-one, 2,6,7,7-tetramethyl, 2-Pentadecanone, 6,10,14-trimethyl, 3-Carene, 5-Nonanol,2,8-Dimethyl, Aromadendrene oxide, β-Bisabolene, β-Elemene, β-Linalool, β-Methyl erotoric acid, β-Pinene, Camphone, Camphor, Carotol, Caryophyllene oxide, Cedrene, Copaene, Cubenol, Cycloisolongi folene, 8,9-dehydro, Eucalyptol, Farnesol isomer a, Flavan, Germacrene D, Germacrene D-4-ol, Hexyl valerate, Hotrienol, Isomerlegol, Isopropyl cyclohexene, Juniper camphor, α-Bergamotene, α-Bourbonene, α-Caryophyllene, α-Cubenene, Ledol, Limonene, α-Muurolene, Longipinocarveol, α-Pinene, α-Selinene, α-Thujene, Neryl acetate (Bhuiyan and Begum, 2010). **Inflorescence volatile oils**: δ-Gvrjunenep oxide (2), δ-Elemene, δ-Muurolene, [+] 1-Cyano-d-Camphidine, 1,5,5,8-Tetramethyl-12-Oxabicyclo [9.1.0] dodeca-3,7-diene, 1-Octen-3-ol, 3-Isopnpytricyc[4,3,1(2.5)] oxide-3-en-10-ol, Abieta-8(140,9(11).1.2-triene, Aexyl-2-methylbatyrath, Aromadendrene oxide, Bisabolene, Caryophyllene, Caryophyllene oxide, Copaene, Curcumene, Germacrene D, Kamran 18-al, 17-(acetylony)-, [4B], Lanceol-*cis*, α-Bnyphyllene, α- Bourbonene, α-Cadinol, α-Crbenene, Ledene alcohol, Linalool, Longipinocerleol *trans,* Neoclovene-(1), dihydro, Nerolidyl propionate, Nonanal, O-Cymene, Oleyl alcohol, Patchoulane, Phytol, Retinal, Solavetivone, Spathulenol, *Trans* -undec-4-enal, *Trans*-Nerolidol (Bhuiyan and Begum, 2010).	B and F) and 5,6,3′-trihydroxy-3,7,4′-trimethoxyflavone exhibited ED_{50} values of 5.8, 6.1, 7.5, and 3.6 μM against HT-29 cells, respectively; 5,6-dihydro-α-pyrone derivative is active in an enzyme-based ELISA NF-κB assay, with an ED_{50} value of 15.3 μM; Brevipolide C, 5,6-dihydro-α-pyrone derivative and 5,6,3′-trihydroxy-3,7,4′-trimethoxyflavone showed ED_{50} values of 8.5, 75, and 310 nM, respectively in a mitochondrial transmembrane potential assay (Deng et al., 2009); moderate DNA intercalating effect (19.7%) of methanol extract with an LC_{50} < 10 ppm. Inhibition (>20%) of crown gall tumors using the potato disc assay (Gupta et al., 1996); Brevipolides G-J exhibited moderate activity (ED_{50} 0.3–8.0 μg mL^{-1}) against a variety of tumor cell lines (Suárez-Ortiz et al., 2013); Brevipolides K to O active against cancer cells from colon (HCT-15), breast (MCF-7), cervix (HeLa), prostate (PC-3), nasopharyngeal (KB), and laryngeal epidermoid carcinoma (Hep-2) (Suárez-Ortiz et al., 2017). **Ethnopharmacological/pharmacological uses:** used for treatment of gastrointestinal infections, cramps, and pain, as well as skin infections (Correa, 1931); in folk medicine used for the treatment of asthma, malaria, and different types of cancer (Parker et al., 2007; Bhuiyan and Begum, 2010); used in the southern Sahara to treat asthma and malaria (Adjanohoun et al., 1986); growing widely in Bangladesh, may be utilized as a source for the isolation of germacrene D and caryophyllene oxide (Bhuiyan and Begum, 2010). **Insecticidal activity:** Methanol extract (Sakr and Roshdy, 2015) and dichloromethane extract (Sakr et al., 2013) as effective in the control program of the cotton leaf worm *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae) and could serve as potential natural insecticide for controlling *S. littoralis* larvae. **Ecosystem services**: effective translocating Zn and Pb when using for phytoremediation, can be potentially used to remove heavy metal from contaminated sites; most effective translocating the As element (Boechat et al., 2016); used as a pest-repelling plant for cereal conservation

Table 2.1. continued.

Species	Chemical constituents	Uses/Biological Activities
Hyptis brevipes Poit.		(Adjanohoun et al., 1986; Seyoum et al., 2002; Parker et al., 2007; Bhuiyan and Begum, 2010). **Physiological studies:** Effects of temperature and photoperiod on flowering: the number of inflorescences formed as well as the floral index vary according to day-length x temperature x inductive cycle number. Long days are inhibitory to flowering, either suppressing it completely (when symmetrically intercalated among 24 inductive cycles) or preventing the floral index from increasing (Zaidan et al., 1991). **Parasitism***:* Host for *Puccinia medellinensis* (Mayor.) (Baxter 1961). **Patent in food industry and cosmetics:** extract from diferent species including leaves and fruit of *H. brevipes* useful as a food preservative without damaging to food quality, inhibiting *Bacillus subtilis* and *Escherichia coli* (Patent No. JP 2000136141; Oshima et al., 2000); Patent No. JP 11106311 (Mitani et al., 2000) for a cosmetic lotion and other manufacturing, including other plant extracts as hyaluronidase inhibitors **Xanthine oxidase (XO) inhibitory activity:** acetone extracts of aerial parts (at 10 μg mL^{-1}) had 13% of XO inibhitory activity; ethanolic extract (at 10 μg mL^{-1}) had 28% of XO inibhitory activity; acetone extracts of root (at 10 μg mL^{-1}) had 3% of XO inibhitory activity; ethanolic extracts of root showed IC_{50} of 6.2 μg mL^{-1} (González et al., 1995).
Hyptis campestris (Pohl) Harley & J.F.B. Pastore	**Phenolic compounds**: Rosmarinic acid (Santos et al., 2018).	**Antioxidant activity:** antiradicalar activity of ethanolic extract (DPPH, EC_{50} 78.95 μg mL^{-1}; ABTS, EC_{50} 21.93 μg mL^{-1}), metal reduction (FRAP, EC_{50} 15.69 μg), and peroxidation (ORAC, EC_{50} 2.03 μg mL^{-1}) (Santos et al., 2018).

Table 2.1. continued.

Species	Chemical constituents	Uses/Biological Activities
Hyptis capitata Jacq.	**Phenolic compounds**: 5-hydroxy-4',7-dimethoxyflavone (apigenin-4',7-dimethyl ether), 2,3-di(3',4'-methylenedioxybenzyl)-2-buten-4-olide (Lignan); Rosmarinic acid (Almtorp et al., 1991). **Lactons**: 10-*epi*-olguine and 4-deacetoxy-10-*epi*-olguine (Jupudi et al., 2002); 10-*epi*-olguine (isomer), 5,6-dihydro-a-pyrone (Almtorp et al., 1991). **Triterpenes**: hyptatic acid A (2α,3β,24-trihydroxyolean-12-en-28-oic acid or 24-hydroxymaslinic acid) and B (2α,3β,19α,24-tetrahydroxyurs-12-en-28-oic acid or 24-hydroxytormentic acid) (Yamagishi et al., 1988; García-Granados et al., 2007), 2-α-hydroxyursolic acid, tormentic acid and maslinic acid (Yamagishi et al., 1988); oleanolic and ursolic acids, (Almtorp et al., 1991; Kashiwada et al., 1998; Lee et al., 1988), Pomolic acid (Kashiwada et al., 1998; Lee et al., 1988).	**Anti-HIV activity**: whole plant demonstrated significant anti-HIV activity (EC_{50}< 20 μg mL-1). Oleanolic acid and Pomolic acid were identified as the anti-HIV agents (Kashiwada et al., 1998). **Citotoxicity:** methanolic extract showed significant cytotoxicity in the lymphocytic leukemia cells P-388 and L-1210 as well as the human lung carcinoma cell A-549; It also demonstrated marginal cytotoxicity in the KB and the human colon (HCT-8) and mammary (MCF-7) tumor cells (Lee et al., 1988); Hyptatic acid-A (ED_{50} 4.2 μg ml^{-1}) and 2 α - hydroxyursolic acid (ED_{50} 2.7 μg ml^{-1}) demonstrated significant *in vitro* cytotoxicity in human colon HCT-8 tumour cells (Yamagishi et al., 1988); Patent No. CN 104945461 for anti-tumor application of hyptatic acid A (Liu and Yang et al., 2015). **Ethnopharmacological/pharmacological uses:** used by traditional healers from Antioquia and Chocó for snakebites; ethanolic extracts of leaves, branches and stem had *in vitro* neutralizing ability against the haemorrhagic effect of *Bothrops atrox* venom (24 ± 3^{e} % of neutralization) (Otero et al., 2000); used as a medicinal plant by the Cayapa indians (Peru) to cure fungal diseases (Kvist and Holm-Nielsen, 1987); used as a medicinal plant in Taiwan against colds, fever, and asthma (Lee et al., 1988). **Ecosystem services***:* Cu hyperaccumulator (up to 800 ug g^{-1} dry weight Cu in hairyroots) (Nedelkoska and Doran, 2000); Extract as a part of a selective weed killer called 2,4-D in sugar-cane fields, coffee plantations, pastures, swamps, and plantations of minor crops (Van Overbeek and Velez, 1946). **Parasitism***:* Host for *Dicaeoma Hyptidis* (Lagerh.) and *Puccinia medellinensis* (Mayor) (Baxter 1961). **Patent**: patent of method to extract asprellic acid from *H. capitata*, Patent No. 102887935 (Su, 2013); used in cosmetic composition for lightening the skin, eliminating freckles and improving and homogenizing complexion, Patent No. WO 9843608 (Greff, 1998).

Table 2.1. continued.

Species	Chemical constituents	Uses/Biological Activities
Hyptis comaroides (Briq.) Harley & J.F.B. Pastore	**Phenolic compounds**: Rosmarinic acid (Santos et al., 2018). **Terpenes**: Abietane diterpenes: 7-α-acetoxyroyleanone, 7-α-hydroxyroyleanone (horminone), royleanone, 7-ketoroyleanone, 7-α-ethoxyroyleanone, iguestol, deoxyneocryptotanshinone, 12-hydroxy-11-metoxyabieta-8,11,13-trien-7-one, inuroyleanol, sugiol, cryptojaponol, and orthosiphonol (Fronza, 2011; Fronza et al., 2011).	**Anti-HIV activity:** ethanolic extract showed $EC_{50}$of 33.9 μg mL^{-1} on the inihibition of reverse transcriptase assay (Sedano-Partida et al., 2015). **Antioxidant activity:** antiradicalar activity of ethanolic extract (DPPH, EC_{50} 29.66 μg mL^{-1}; ABTS, EC_{50} 8.84 μg mL^{-1}), metal reduction (FRAP, EC_{50} 5.88 μg mL^{-1}), and peroxidation (ORAC, EC_{50} 0.96 μg mL^{-1}) (Santos et al., 2018). **Citotoxicity:** in a human pancreatic cancer cell line (MIA PaCa-2). 7-α-acetoxyroyleanone, the major compound, was one of the most active with an IC_{50} value of 4.7 μM from 12 isolated diterpenes (Fronza, 2011; Fronza et al., 2012).
Hyptis conferta Pohl ex Benth.	**Inflorescence volatile oils:** Alcanfor, Bornil acetate, β-Bourbonene, E-Caryophyllene, α-Humulene, γ-Muurolene, Germacrene D, *epi*-Cubebol, γ-Cadinene, δ-Cadinene, *trans*-Calamenene, Spathulenol, Sesquiterpene (M+. 220, BP 119; BP 105; M+. NR, BP 96), Caryophyllene oxide, Humulene epoxide II, 1,10-di-*epi*-Cubenol, τ-Cadinol, Caryophyl-4(12),8(13)-dien5-ol, δ-Cadinol, *cis*-Calamenen-10-ol, Hexahydrofarnesyl acetone (Tafurt-Garcia et al., 2014). *H. Conferta* var. *conferta:* Sabinene, Limonene, Terpinolene, β-Bourbonene, β-Elemene, β-Caryophyllene, α-Humulene, Germacrene D, bicyclogermacrene, *trans*-β-Guaiene, γ-Cadinene, δ-Cadinene, Germacrene B, Spathulenol, β-copaen-4α-ol, 1,10-di-*epi*-cubenol, T-Cadinol, α-Cadinol, α-Bisabolol (Ferreira et al., 2005). *H. conferta* var. *agustata* (Briq.): 2-(*E*)-Hexenol, 2-Hexanol, α-Pinene, Camphene, Sabinene, β-Pinene, Myrcene, α-Phellandrene, Limonene, (*Z*)-β-Ocimene, Phenyl acetaldehyde, (*E*)-β-ocimene, Acetophenone, Terpinolene, Linalool, Nonanal, α-Terpineol, α-Copaene,	**Antioxidant activity:** total phenolic content of 0.107 ± 0.009 g GA g $extract^{-1}$; antiradicalar activity (DPPH, EC_{50} $4.50x10^{-7}$±$4x10^{-8}$ Kg extract μmol DPPH); Total Antioxidant Activity of 721 ± 27 mmol trolox Kg $extract^{-1}$ (Tafurt-García et al., 2015). **Parasitism**: Host for *Dicaeoma hyptidis* (Lagerh.) (Baxter, 1961).

Table 2.1. continued.

Species	Chemical constituents	Uses/Biological Activities
Hyptis conferta Pohl ex Benth.	β-Bourbonene, β-Elemene, β-Caryophyllene, Geranyl acetone, α-Humulene, Germacrene D, Bicyclogermacrene, α-Muurolene, *cis*-Calamenene, δ-Cadinene, Spathulenol, Caryophyllene oxide, Humulene epoxide II, α-Muurolol (Ferreira et al., 2005).	
Hyptis crenata Pohl ex Benth.	**Volatile oils: α-Pinene,** β-Pinene**, Camphene, Myrcene, α-Terpinene, *p*-Cymene, 1,8-Cineole, γ-Terpinene, Camphor, Terpineol-4, β-Caryophyllene, Aromadendrene, Ledene, Vridiflorol, Caryophyllene oxide (Diniz et al., 2013);** Camphor, Bornyl acetate, Thymol, α-Terpineol, α-Phellandrene, γ-Terpinene, α-Terpinene, *p*-Cymene, Myrcene, Limonene, 1,8-Cineole, α-Eudesmol, γ-Eudesmol, α-Cadinol, Aromadendrene, Globulol, Carvacrol, Borneol, Viridiflorol, 4-Terpineol, Ledol, Thymol methyl ether, Spathulenol, α-Humulene, *trans*-Linalool oxide, Viridiflorene, 9-*epi*-*trans*-Caryophyllene, 14-Hydroxy-9-*epi*-β-caryophyllene (Violante et al, 2012A), Bis-*seco*-abietane diterpenoid: hyptisolide A (Yun et al., 2014) **Volatile oils (leaves and stems):** α-Thujene, α-Pinene, Camphene, Sabinene, β-Pinene, Myrcene, α-Phellandrene, α-Terpinene, *p*-Cymene, Limonene, 1,8- α-Thujene, α-Pinene, β-Pinene, Camphene, Camphene hydrate, Sabinene, *cis*-Sabinene hydrate, Myrcene, α-Phellandrene, α-Terpinene, γ-Terpinene, *p*-Cymene, Limonene, 1,8-Cineole, Terpinolene, Camphor, Borneol, Isoborneol, 4-Terpineol, α-Terpineol, α-longipinene, β–Caryophyllene, Caryophyllene oxide, aromadendrene, *allo*-Aromadendrene, β–Chamigrene, α-Himachalene, β–Himachalene, β–Himalachene oxide, β–Dehydro-*ar*-himachalene, Himachalol, Pentyl propanoate, Decane, Isobutyl 3—methylbutanoate, 3-methylbutyl butanoate,	**Antibacterial activity:** volatile oil as bactericidal against *Staphylococcus aureus* and *Enterococcus faecalis,* both are Gram(+) bacteria (Violante et al., 2012A); ethanolic, hexane, dichloromethane, ethyl acetate and hydromethanolic extract showed MIC>1000 μg mL^{-1} against *Escherichia coli* ATCC25922, *Klebsiella pneumonia* ATCC700603, and *Pseudomonas aeruginosa* ATCC27853, Gram(-) bacteria; against *Enterococcus faecalis* ATCC29218: ethanolic extract MIC 500 μg mL^{-1}, dichloromethane MIC 62,5 μg mL^{-1}, ethyl acetate MIC 31,3 μg mL^{-1}, hydrometathanolic and hexane extracts MIC>1000 μg mL^{-1}, against *Staphylococcus aureus* ATCC 25923: ethanolic extract MIC 250 μg mL^{-1}, hexane MIC 125 μg mL^{-1}, dichloromethane MIC 62,5 μg mL^{-1}, ethyl acetate MIC 125 μg mL^{-1}, and hydrometathanolic extract MIC>1000 μg mL^{-1}, both are Gram(+) bacteria (Violante et al., 2012B). **Antifungal activity**: ethanolic, hexane, dichloromethane, ethyl acetate and hydrimetanolic extract showed MIC>1000 μg mL^{-1} against *Candida albicans* ATCC90028, *Candida glabrata* ATCC9030, and *Candida tropicalis* ATCC760; against *Candida krusei* ATCC 6258: ethanolic extract MIC 125 μg mL^{-1}, hexane and hydrometathanolic extracts MIC>1000 μg mL^{-1}, dichloromethane and ethyl acetate extracts MIC 250 μg mL^{-1}; against *Candida parapsilosis* ATCC22019: ethanolic, hexane, ethyl acetate, and hydrometathanolic extracts MIC>1000 μg mL^{-1}, dichloromethane MIC 500 μg mL^{-1}; against *Cryptococcus neoformans* ATCC32045: ethanolic and hexane extracts MIC 125 μg mL^{-1}, dichloromethane, ethyl acetate and hydrometathanolic extracts MIC>1000 μg mL^{-1} Violante et al., 2012B); ethanolic extract showed some systemic effect against the cucumber (*Cucumis sativus* L.) powdery mildew (*Sphaerotheca fuliqinea*) (Stadnik et al, 2003).

Table 2.1. continued.

Species	Chemical constituents	Uses/Biological Activities
Hyptis crenata Pohl ex Benth.	3-methylbutyl 2-methylpropanoate, (*Z*)-β–Ocimene, (*E*)-β–Ocimene, Octanol, Linalool, Isopentyl 2-methylbutanoate, Isopentyl isovalerate, 1,3,8-*p*-Menthatriene, *endo*-Fenchol, *trans*-2-Pinanol, *trans*-Pinocarveol, *cis*-Verbenol, Pinocarvone, Pinocampheol, Naphthalene, *p*-Cymen-8-ol, Butyl hexanoate, Myrtenol, Dodecane, Decanal, *cis*-3-Hexanyl 2-methylbutanoate, Bornyl acetate, Safrole, Tridecane, α-Cubenene, α-Ylangene, α-Copaene, Hexyl hexanoate, β–Elemene, Cyperene, Tetradecane, α-Gurjunene, β–Gurjunene, , α-Humulene, γ-Muurolene, Germacrene D, β–Selinene, Valencene, α-Selinene, Bicyclogermacrene, β–Himachalene, Germacrene A, δ-3-Carene, Terpinen-4-ol, (*E,E*)-α-Farnesene, β–Calacorene, α-Calacorene, Spathulenol, Humulene epoxide II, 1-*epi*-Cubenol, *epi*- α-Muurolol, α-Muurolol, α-Cadinol, 14-Hydroxy-9-*epi*-β–caryophyllene (Scramin et al., 2000; Zoghbi et al., 2002; Rebelo et al., 2009).	**Antinociceptive effect**: decoction extract showed antinociceptive effects in two mouse models of pain at 15 mg kg^{-1} b.w (Rocha et al., 2009A); decoction extract tested in 8 C57/BL6 mice and showed no mortality (Rocha et al., 2009B). **Antioxidant activity**: antiradicalar activity of methanol extract (DPPH, EC_{50} 16.7 μg mL^{-1}); total phenolics 373.0 mg GAE g^{-1} and Trolox equivalent of 226.8 mg TE g^{-1} (Rebelo et al, 2009). **Anti-tick properties**: volatile oil at 2.5% inhibited significantly tn vivo oviposition of engorged females of the catle tick *Rhipicephalus* (Boophilus) *microplus* using the adult immersion test, with an effectiveness of 94.4% (Violante et al., 2012A). **Citotoxity:** extract and fractions non-toxic to *Artemia salina*, LD_{50}>1000 mg mL^{-1} (Violante et al., 2012B); using brine shrimp lethality assay volatile oil and methanol extract showed LC_{50} of 6.7 and 13.0 μg mL^{-1} (Rebelo et al., 2009). **Ethnopharmacological/pharmacological uses**: traditional use for mild pain headache, stomach, discomfort, menstrual pain, treatment of flu/fever (Rocha et al., 2009B); one of the most cited species used for antiulcer and anti-inflammatory problems in Mato Grosso (Brazil) (Jesus et al., 2009). **Gastroprotective action**: volatile oil from aerial parts reduced the rat gastric lesions and increased mucus production and gatrointestinl emptying (Diniz et al., 2013). **Hepatoprotective effect**: volatile oil normalized serum alkaline phosphatase, alanine aminotransferase, bilirubin levels, inhibited morphological changes, inhibited the elevation in epatic lipid peroxidation, inhibited the reduction of glutathione peroxidase activity induced by sepsis (Lima et al., 2018). **Patent:** extract is part of a naturally hydroplastic mask for removal or progressive reduction of hair growth, Patent No. BR 2008000859 (Duarte, 2009); extract is part of a serine protease inhibitors for treatment of skin diseases and rough skin, Patent No. JP 2001240551

Table 2.1. continued.

Species	Chemical constituents	Uses/Biological Activities
Hyptis crenata Pohl ex Benth.		(Kobayashi et al., 2001A); extract is part of a gelatinase inhibitors to prevent the degradation of elastins, lamins, and dermal basal membrane, Patent No. JP 2001172157 (Kobayashi et al., 2001B). **Weed infestation:** it is considered a harmful plant present in irrigated rice production areas of the cooperatives: Cooperatives Mista Rural Vale do Javaés and Cooperativa Agroindustrial Rio Formoso, in Formoso do Araguaia-TO, Brazil (Erasmo et al., 2004).
Hyptis dilatata Benth. in DC.	**Diterpenes:** 7-ethoxyrosmanol and carnosol Epimethylrosmanol, epiethylrosmanol, rosmanol, methylrosmanol, esquirolin B (Urones et al., 1998; Alvarez et al., 2015). **Volatile oils (aerial parts):** α-Pinene, β-Pinene, Camphene, Sabinene, Myrcene, α-Felandrene, β-Felandrene, *p*-Cimene, o-Cimene, Limonene, 1,8-Cineol, δ-3-Carene, *cis*-β-Ocimene, α-Terpinene, γ-Terpinene, *trans*-4-Tuyanol, *m*-Cimeneno, Fenchona, *p*-Menta-2,4(8)-dieno, α-terpinolene, *cis*-4-Tuyanol, Alcanphor, Canfene hydrate, Borneol, Terpinen-4-ol, *p*-Cimen-8-ol, α-Terpineol, Mirtenol, Carveol acetate, Bornyl acetate, Myrtenyl acetate, Eugenol, Isoledene, α-Copaene, Isobornyl propanoate, Longifolene, α-Gurjunene, *cis*-Caryophyllene, β-Ylangene, α-Guaiene, Aromadendrene, Espirolepechinene, α-Panasinsene, α-Humulene, *allo*-Aromadendrene, γ-Gurjunene, β-Selinene, δ-Selinene, Valencene, Viridiflorene, Eremofilene, α-Muurolene, γ-Cadinene, δ-Cadinene, *trans*-Calamenene, β-Calacorene, Palustrol, Caryophyllene oxide, Globulol, Viridiflorol, Caryophyl4(12),8(13)-dien5-ol, isopropylidenenorcarane, *allo*-Ocimene, 4-carene, isopregol, camphor, α-terpineol, dihydrocarveol, 3-caren-10-al, davanone, ledene, δ-	**Antibacterial activity**: volatile oil showed Quorum sensing inhibitory activity (Alvarez et al., 2015). **Antioxidant activity:** antiradicalar activity (EC_{50} $4.60x10^{-7}$ Kg μmol^{-1} of DPPH); total antioxidant activity of 903 ± 64 mmol of trolox Kg $extract^{-1}$; total phenolic content of 0.071 ± 0.002 g GA g $extract^{-1}$ (Tafurt-García et al., 2015). **Cytotoxic activity:** Inducing of 50% growth inhibition (GI_{50}), total growth inhibition (TGI), and 50% cytotoxicity (LC_{50}) after the 24-h of incubation period: PC-3 (prostate carcinome): GI_{50} 31, TGI 36 and LC_{50} 1500 μg mL^{-1}, MCF7 (breast carcinome): GI_{50} 45, TGI 707 and LC_{50} 900 μg mL^{-1}, HT-29 (colon cancinome): GI_{50}:22, TGI 131 and LC_{50} 1500 μg mL^{-1}, MDA-MB-231 (breast carcinome): GI_{50} 7, TGI 37 and LC_{50}:98 μg mL^{-1}, 4T1 (mouse mammary carcinome): GI_{50} 25, TGI 37 and LC_{50} 1500 μg mL^{-1}, RAW-267 (mouse leukemic monocyte macrophage cell line): GI_{50} 40, TGI 83 and LC_{50} 1500 μg mL^{-1} (Taylor et al., 2013). **Ethnopharmacological/pharmacological uses**: in Panama leaves are used as topical anti-parasitic in animals, in folk medicine and as an insect repellent (Ortega et al., 2014). **Endophytic interaction**: *Pestalotiopsis manguiferae* was isolated from *Hyptis dilatata* (Ortega et al., 2014).

Table 2.1. continued.

Species	Chemical constituents	Uses/Biological Activities
Hyptis dilatata Benth. in DC.	Cadinene, palustrol, Iedol, (Tafurt-Garcia et al., 2014; Alvarez et al., 2015).	
Hyptis divaricata Pohl ex Benth.	Resinous exudates did not show flavonoid profiles (Park et al., 2002).	**Propolis botanical Origin**: brown propolis (also called propolis type 6) collected near Salvador, Bahia State, has resins from *Hyptis divaricata*. Bees were observed visiting mainly their buds and unexpanded leaves (Park et al., 2002). Propolis ethanol extract showed inhibitory effects on TPA-Induced Inflammation in Mice (TPA: 12-O-tetradecanoylphorbol-13-acatate as a promoter) (Yasukawa et al., 2012).
Hyptis goyavensis A.St.-Hil. ex Benth.	**Volatile oils (aerial parts)**: α-Pinene, β-pinene, Camphene, Δ-Carene, *p*-Cymene, 1,8-Cineole, Camphor, Borneol, Myrcene, α-Phellandrene, Estragol, Calarene, β-Caryophyllene, Fenchol, α-Terpinene, γ-Terpinene, α-Terpinolene, Linalol, 4-Terpineol, α-Terpineol (Luz et al., 1984).	
Hyptis lacustris A.St.-Hil. ex Benth.	**Phenolic compounds**: Rosmarinic acid (Santos et al., 2018).	**Antioxidant activity**: antiradicalar activity of ethanolic extract (DPPH, EC_{50} 24.79 μg mL^{-1}; ABTS, EC_{50} 9.03 μg mL^{-1}), metal reduction (FRAP, EC_{50} 5.35 μg mL^{-1}), and peroxidation (ORAC, EC_{50} 1.49 μg mL^{-1}) (Santos et al., 2018). **Anti-Leishmania:** ethanol extract of leaves tested against axenic amastigotes of *Leishmania amazonensis* (strain MHOM/BR/76/LTB-012) - IC_{50} 10±7.2 μg mL^{-1} (Valadeau et al., 2009). **Ethnopharmacological/pharmacological uses**: leaves and exuding sap used by the yanesha community for the treatment of cutaneous leishmaniosis (*Mareñets) (Valadeau et al., 2009)*.

Table 2.1. continued.

Species	Chemical constituents	Uses/Biological Activities
Hyptis lanceolata Poir.	Ethanolic extract of the leaves, **Alkaloid:** Imidodicarbonimidic diamide, N, N-dimethyl**; Fatty acids:** Tetradecanoic acid, 9-Octadecenoic acid, 12-hydroxy, hexadecanoic acid methyl Ester, hexadecanoic acid ethyl Ester, 9,12,15- Octadecatrienoic acid, ethyl ester; **Alcohol:** 2-hexadecen-1-ol, 3, 7, 11, 15-tetramethyl, **Hydrocarbon:** 3-eicosyne**.** **Volatile oils (aerial parts):** α-Thujene, α-Pinene, β-Pinene, β-Selinene, α-Selinene, α-Phelandrene, α-Terpinene, β-Phelandrene, *p*-Cymene, Limonene, *trans*-β-Ocimene, *cis*-β-Ocimene, γ-Terpinene, α-Terpinolene, Linalool, Terpieneol-4, Thymol, Carvacrol α-Copaene, β-Bourbonene, β-Elemene, β-Caryophyllene, Germacrene D, Amorphene, α-Humulene, β-Selinene, *allo*-Aromadendrene, γ-Muurolene, Bicyclogermacrene, α-Farnesene, γ-Cadinene, δ-Cadinene, Spathulenol, T-Muurolol, T-Cadinol, α-Cadinol, *cis*-Sabinene hydrate, Linalol, Camphor, Terpinen-4-ol, α-Terpineol, Thymol, Octen-3-ol, Myrtenal, β-Elemene, δ-Elemene, α-Copaene, β-Bourbonene, β-Caryophyllene, β-Copaene, α-Guaiene, α-Humulene, γ-Muurolene, Germacrene B, Germacrene D, *trans*-Calamenene, *cis*-Nerolidol, , α-Thujene, Myrcene, α-Cubebene, *cis*-β-Farnesene, Guaiene, Caryophyllene epoxide, Humulene epoxide, T-Muurolol, α-Cadinol, T-Cadinol, Torreyol (Zollo et al., 1998; Tchoumbougnang et al., 2005, Koba et al., 2007). **Phenolic compounds**: Nepetoidins A and B (Grayer et al., 2003); Rosmarinic acid and 6-hydroxycaffeic acid (Pedersen, 2000).	**Antifungal activity:** volatile oil active against fungi in the microatmosphere assay after 7 days of incubation: *Microsporum gypseum* (MIQ 15 µL), *Aureobasidium pullulans* (MIQ 15 µL), *Candida albicans* (MIQ>15 µL), *Aspergillus flavus* (MIQ>15 µL), *Trichoderma viride* (MIQ>15 µL); after 7 days of incubation agianst *Microsporum gypseum* (MIC 5000 ppm), *Aureobasidium pullulans* (MIC 10.000 ppm), *Aspergillus flavus* (MIC 10.000 ppm), *Trichophyton rubrum* (MIC 625 ppm), *Candida albicans* (MIC 10.000 ppm), *Cryptococcus neoformans* (10.000) (Zollo et al., 1998). **Ethnopharmacological/pharmacological uses:** leaves are used for the treatment of cutaneous and subcutaneous parasitic infections such as eczema, ringworm, rashes, athlete foot; used as painkillers in herbal medicine in Nigeria; leaves are used by the Ijo people of South Eastern Nigeria as a headache cure; roots are used in the treatment and or management of pulmonary troubles (Burkill, 1994 *apud* Igwe, 2014).

Table 2.1. continued.

Species	Chemical constituents	Uses/Biological Activities
Hyptis lantanifolia Poit.		**Anti-HIV activity:** water extract of leaves showed IC_{50} 7 μg mL^{-1} using the reverse transcriptase assay (Matsuse et al., 1998). **Xanthine oxidase (XO) inhibitory activity:** acetone extract of aerial parts, 10 μg mL^{-1} had 29% of XO inibhitory activity; ethanolic extract, IC_{50} 2.1 μg mL^{-1}; acetone extracts of roots, IC_{50} 3.8 μg mL^{-1}; and ethanolic extract of root (IC_{50} 2.9 μg mL^{-1}) (González et al., 1995). **Parasitism**: host to the fungus *Colletotrichum guaranicola* in guaraná crops in four municipalities of Amazonas (Brazil) (Miléo et al., 2007); host to *Puccinia insititia* (Baxter 1961). that may represent a strong potential of plant pathogen inoculums (Miléo et al., 2007).
Hyptis lappulacea Mart. ex Benth.	**Phenolic compounds**: Rosmarinic acid (Santos et al., 2018).	**Anti-HIV activity:** crude ethanol extract showed EC_{50} of 865.0 μg mL^{-1} on the inihibition of reverse transcriptase assay (Sedano-Partida et al., 2015) **Antioxidant activity:** antiradicalar activity of ethanolic extract (DPPH, EC_{50} 12.33 μg mL^{-1}; ABTS, EC_{50} 6.13 μg mL^{-1}), metal reduction (FRAP, EC_{50} 8.83 μg mL^{-1}), and peroxidation (ORAC, EC_{50} 0.56 μg mL^{-1}) (Santos et al., 2018).
Hyptis marrubioides Epling	**Volatile oils:** β-Bourbonene, Caryophylla-4(14),8(15)-dien-5β-ol, Caryophylla-4(14),8(15)-dien-5α-ol, Caryophyllene oxide, α-Caryophyllene, β-Caryophyllene, Caryophyllenol, α-Copaene, β-Copaen-4α-ol, δ-Cadinene, γ-Cadinene, Cedrol, Cadalene, α-Eudesmol, β-Eudesmol, Eudesma-4(15),7-dien-1β-ol, Eudesm-4(14)-en-11-ol, Eudesma-4(15),7-dien-1β-ol, Kaur-16-ene, Methyl 3β-hydroxy-urs-12-en-28-oate, Heptadec-1-ene, Methyl hexadecanoate, Methyl 9,12,15-octadecatrienoate, Methyl heptadecanoate, α-Thyjone, β-Thujone, Linalool, Gremacerne D, Linalol, *cis*-Thujone, *trans*-Thujone, *iso*-3-Thujanol, *cis*-Verbenol, *cis*-3-Pinocanphone, Terpinen-4-ol, α-Terpineol, 1-Terpineol, α-Humulene,	**Ecosystem services:** volatile oil investigated for pest control in agriculture, such as the prevention and control of Asian soybean rust (Silva et al., 2012B) and treatment of *Colletotrichum truncatum*-infected soybean seeds (Silva et al., 2012A); volatile oil is efficient in controlling soybean rust, by preventive treatment, mainly at higher concentrations (Da Silva et al., 2012). **Endophytic interaction**: it was evaluated the response to inoculation with endophytical bactria and fungus. τ-cadinol and caryophyllene oxide were produced only in plnats inoculated with endophytical bacteria. Methyl hexadecanoate, methyl heptadecanoate, methyl 9,12,15-octadecatrienoate, and methyl 3β-hydroxy-urs-12-en-28-oate were overexpressed only in plants treated with endophytic fungi (Vitorino et al., 2013).

Table 2.1. continued.

Species	Chemical constituents	Uses/Biological Activities
Hyptis marrubioides Epling	γ-Himachalene, γ-Muurolene, *trans*-Muurola-4(14),5-diene, Italicene epoxide, Ocidentalol, , Salvial-4(14)-en-1-ona, Muurol-4,10(14)-dien-1β-ol, α-Muurolol, *cis*-Calamenen-10-ol, 14-Hydroxi-(*Z*)-caryophyllene, Germacra-4(15),5,10(14)-trien-1α-ol, *iso*-3-Thujanol, α-Pinocarveol, β-Verbenol, β-3-Pinocanphona, Methyl myrtenate, Dendrolasine, eugenol, *epi*-longipinanol, *trans*-Sesquisabinene hydrate, Guaiol, Selin-11-en-4-α-ol, 14-Hydroxy-9-epi-β-caryophyllene (Sales et al., 2007; Botrel et al., 2009; McNeil et al., 2011; Botrel et al., 2010A; Botrel et al., 2010B; Vitorino et al, 2013). *When cultivated in greenhouse***:** *trans*-Thujone, *iso*-3-Tujanol, *trans*-Sabinol, 3-Thujanol, *cis*-3-Pinocanphona, α-Terpineol, α-Copaene, β-Caryophyllene, α-Humulene, γ-Muurolene, *trans*-Muurola-4(14),5-diene, γ-Cadinene, δ-Cadinene, *cis*-Muurol-5-en-4α-ol, Ocidentalol, *cis*-Nerolidol, Caryophyllnee oxide, Rosifoliol, Muurola-4,10(14)-dien-1β-ol, Caryophylla-4(14),8(15)-dien-5α-ol, Caryophylla-4(14),8(15)-dien-5β-ol, α-Muurolol, α-Cadinol, *cis*-Calamenen-10-ol, Bulnesol, Kusinol, Germacra-4(15),5,10(14)-trien-1α-ol, Eudesma-4(15),7-dien-1β-ol (Botrel et al., 2010B)**.**	**Ethnopharmacological/pharmacological uses:** gastrointestinal infections, skin infections, pain, and cramps (Pedroso et al., 2017). **Fungitoxic activity:** scanning electron microscopy revealed that the oil and decoction extract exhibited fungitoxic activity on *Oidium eucalypti* (powdery mildew) (Silva et al., 2014A); decotion extract were efficient in reducing the severity of powdery mildew in eucalyptus ministumps; volatile oil (0.05 – 2%) inhibits the *in vitro* spore germination of *Phakopsora pachyrhizin* (Silva et al., 2013B); volatile oil reduces the disease severity by 33 to 41%, whereas the commercial fungicide (pyraclostrobin + epoxyconazole) reduced the severity by 61% (Silva et al., 2014B). **Physiologycal studies**: the use of organic fertilizer application improved soil fertility, increased dry biomass and resulted in higher voltile oil yield (Sales et al., 2009B); volatile oil content was not influenced when exposed to different levels (20, 60 and 100%) of natural light irradiance. The oil chemical composition did not show changes with the different levels of irradiance, except for iso-3-Thujanol and δ-Cadinene, which presented higher concentrations in plants grown at 100 and 60% irradiance (Sales et al., 2009A); red light induces plant growth and increases leaf number and dry weight of seedlings cultivated *in vitro*, whereas blue and white lights promote rutin accumulation (Pedroso et al., 2017); nodal culture method is effective for micropropagation of *H. marrubioides.* Plants grown in greenhouse, wild and *in vitro* do not affect the chemical composition of the volatile oil (Botrel et al., 2013); light did not affect germination of *H. marrubioides* seeds, but a temperature of 20°C retarded the germination process, although the percentage of germinating seeds was not affected; seeds that were harvested at the more mature stage (dark brown color) could be stored for up to 18 months (Sales et al., 2011); *Hyptis marrubioides* was used for the evaluation of a reliable methodology for assessing the in vitro photosynthetic competence (Costa et al., 2014).

Table 2.1. continued.

Species	Chemical constituents	Uses/Biological Activities
Hyptis meridionalis Harley & J.F.B. Pastore	**Phenolic compounds**: Rosmarinic acid (Santos et al., 2018).	**Antioxidant activity:** antiradicalar activity of ethanolic extract (DPPH, EC_{50} 30.47 μg mL^{-1}; ABTS, EC_{50} 8.94 μg mL^{-1}), metal reduction (FRAP, EC_{50} 7.53 μg mL^{-1}), and peroxidation (ORAC, EC_{50} 0.84 μg mL^{-1}) (Santos et al., 2018).
Hyptis monticola Mart. ex Benth.	**Volatile oils:** α-Pinene; Camphene; Sabinene; β-Pinene; Myrcene; Limonene; *cis*-β-Ocimene; *trans*-β-Ocimene; γ-Terpinene; Linalool; 2-*cis*-Hexenylpropanoate; Terpinen-4-ol; α–Terpineol; *trans*-Methyl cinnamate; δ-Elemene; α-Cubenene; α-Copaene; β-Elemene; *trans*-Caryophyllene; *cis*-Thujopsene; α-Humulene; *allo*-Aromandendrene; *trans*-Cadina-1(6),4-diene; Germacrene D; Bicyclogermacrene; α-Muurolene; γ-Cadinene; δ-Cadinene; Hedycaryol; Germacrene B; Nerolidol; Spathulenol; Caryophyllene oxide; Guaiol; 10-*epi*-γ-Eudesmol; *epi*-α–Cadinol; β-Eudesmol; α-Eudesmol (Perera et al., 2017).	
Hyptis multibracteata Benth.	**Phenolic compounds**: Rosmarinic acid (Santos et al., 2018).	**Anti-HIV activity:** crude ethanol extract showed EC_{50} of 1096.0 μg mL^{-1} on the inihibition of reverse transcriptase (Sedano-Partida et al., 2015). **Antioxidant activity** antiradicalar activity of ethanolic extract (DPPH, EC_{50} 72.19 μg mL^{-1}; ABTS, EC_{50} 23.17 μg mL^{-1}), metal reduction (FRAP, EC_{50} 17.60 μg mL^{-1}), and peroxidation (ORAC, EC_{50} 30.09 μg mL^{-1}) (Santos et al., 2018).
Hyptis obtusiflora Presl ex Benth.		**Ethnopharmacological/pharmacologhical uses:** Leaves and exuding sap used by the yanesha community for the treatment of cutaneous leishmaniasis (*Mareñets) (Valadeau et al., 2009).* **Xanthine oxidase (XO) inhibitory activity:** acetone extract of aerial parts (IC_{50}: 5.3 μg mL^{-1}), ethanolic extract (IC_{50}: 1.4 μg mL^{-1}), also tested for acetone extract of root (IC_{50}: 24.3 μg mL^{-1}) and for ethanolic extract of root (IC_{50}: 1.1 μg mL^{-1}) (González et al., 1995).

Table 2.1. continued.

Species	Chemical constituents	Uses/Biological Activities
Hyptis passerina Mart. ex Benth.	**Inflorescence volatile oils:** β-Pinene, Limonene, Linalool, Methyl salycilate, Bornyl acetate, *Trans*-Pinocarvyl acetate, α-Cedrene, β-Damascenone, δ-Elemene, β-Caryophyllene, α-Gurjene, Aromadendrene, Geranylacetone, α-Humulene, β-Acoradiene, α-Curcumene, Bicyclogermacrene, γ-Acoradiene, δ-Cadinene, Nerolidol, Spathulenol, Caryophyllene oxide, β-*epi*-Acorenol, Acorenol isomer, Farnesylacetone, Abietatriene (Zellner et al., 2009). **Leaves volatile oils:** β-Pinene, Limonene, Linalool, β-Damascenone, δ-Elemene, β-Caryophyllene, α-Gurjene, Aromadendrene, Geranylacetone, β-Acoradiene, α-Curcumene, Bicyclogermacrene, γ-Acoradiene, δ-Cadinene, Spathulenol, Caryophyllene oxide, Isospathulenol, β-*epi*-Acorenol, Acorenol isomer, Farnesylacetone, Abietatriene (Zellner et al., 2009).	**Antimicrobial activity:** at concentration levels of 500.0 μg and 250.0 μg, leaf-derived oil inhibited the growth of *Staphylococcus aureus* ATCC 2592, Gram (+) and *Pseudomonas aeruginosa* ATCC 15422, Gram (-). Also tested against *Staphylococcus epidermidis* ATCC 1499, Gram (+); flower-derived oil at at 500.0 μg was more active against *Pseudomonas aeruginosa* ATCC 15422, Gram (-) (Zellner et al., 2009). **Antifungal activity:** volatile oil (leaf and flower) active against *Candida albicans* ATCC 10231 (Zellner et al., 2009). **Odour-activity:** volatile oil could be applied as fixative in perfume formulations due to the high concentration of sesquiterpenes and diterpenes (Zellner et al., 2009).
Hyptis radicans (Pohl) Harley & J.F.B. Pastore	**Phenolic compounds**: Rosmarinic acid (Santos et al., 2018; Pedersen, 2000). 6-Hydroxyrosmarinic acid (Pedersen, 2000). **Triterpenes and Steroids:** β-Amirin, α-Amirin, Ursolic acid, 2a,3b,19a-tri-hydroxi-urs-12-en-28-oic acid, 3β-Methyl hydroxi-28-ursolate, Sitosterol-3-*O*-β-D-glycopiranoside, Stigmasterol-3-*O*-β-D-glycopiranoside, Stigmasterol, β-Sitosterol (Costa et al., 2008).	**Anti-HIV activity:** ethanol extract showed EC_{50} of 158.7 μg mL^{-1} on the inihibition of reverse transcriptase (Sedano-Partida et al., 2015). **Antioxidant activity:** antiradicalar activity of ethanolic extract (DPPH, EC_{50} 37.61 μg mL^{-1}; ABTS, EC_{50} 6.01 μg mL^{-1}), metal reduction (FRAP, EC_{50} 6.01 μg mL^{-1}), and peroxidation (ORAC, EC_{50} 2.68 μg mL^{-1}) (Santos et al., 2018). Leaves from *H. radicans* were tested on DPPH with an IC_{50} of 4.46 ± 1.32 μg mL^{-1}, reducing power, IC_{50} of 23.23 ± 0.07 μg mL^{-1} and β-carotene/linoleic acid inhibition % at 40 μg mL^{-1} was 50.33 ± 14.30 (Scio et al., 2012). **Antiedematogenic activity**: after 2 h, the ethanol extract of flowers showed blocking action, reducing 23.39% of the edematogenic activity of the venom of *Bothrops atrox*, in relation to the control animals (Costa et al., 2008).

Table 2.1. continued.

Species	Chemical constituents	Uses/Biological Activities
Hyptis radicans (Pohl) Harley & J.F.B. Pastore		**Ethnopharmacological/pharmacologhical uses:** is used in the treatment of snakebites and scorpion stings in the Amazon, where most of the snakebite incidents involve *Bothrops atrox* (Costa et al., 2008). Expectorant, pertussis, cough, asthma, sneezing, carminative, dermatites, scorpion and snake bites, antispasmodic, syphilitic, parasitosis, diuretic (Scio et al., 2012).
Hyptis ramosa Pohl ex Benth.	**Phenolic compounds**: Nepetoidins A and B (Grayer et al., 2003).	
Hyptis recurvata Poit.	**Leaves volatile oils:** α-Pinene, β-Pinene, Myrcene, 1,8-cineole, *trans*-β-Ocimene, *cis*-β-Ocimene, Linalool, δ-Terpineol, Terpinen-4-ol, α-Terpineol, *p*-Eugenol, β-Caryophyllene, α-Humulene, β-Selinene, α-Selinene, α-Panasinsene (Leclercq et al., 2000).	
Hyptis rhomboidea M Martens & Galeotti	**Triterpenes and Steroids**: Ursolic acid, Oleanolic acid, Ilexgenin A, β-Amyrin, Cholest-5-ene-3beta, 4beta-diol (Tang et al., 2014). **Phenolic compounds**: Vanillactic acid, Ethyl caffeate, Methyl rosmarinate, Kaempferol 3-*O*-alpha-L-rhamnopyranosyl-(1-->6)-β-D-glucopyranoside, Kaempferol 3-*O*-alpha-L-rhamnopyranosyl-(1-->6)-β-D-glucopyranoside, Kaempferol 3-*O*-β-D-glucopyranoside (astragalin) (Tang et al., 2014); Nepetoidin A and B, Apigenin, Kaempferol, Anisofolin-A, Quercetin, Hyprhombins A to E, Hyprhombin B methyl ester Epihyprhombin B (Tsai and Lee, 2014). **Volatile oils**: 1,8-Cineole, 1-Octen-3-ol, Thujopsene-I3, α-Cubebene, Ylangene, Copaene, Linalool, Isocaryophyllene, β-Elemene, Aristolene, Benzene acetaldehyde, Humulene, *cis,cis,cis*-1,1,4,8-Tetramethyl-4,7,10-cycloundecatriene, 4,11-Selinadiene, Cedrene,	**Allelopathic activity:** aqueous extracts (0.05, 0.1 g/mL) significantly inhibited the seed germination and seedling growth of *Brassica campestris* L., *Raphanus sativus* L., and *Oryza sativa* L., lower concentrations had a positive effect on the seed germination and seedling growth of *Lactuca sativa* L. (Wang et al., 2015). **Antibacterial activity:** volatile oil active against *Staphylococcus aureus* (MIC 12.5 μg mL^{-1}), *Bacillus subtilis* (MIC 12.5 μg mL^{-1}), Gram (+) bacteria; *Escherichia coli* (MIC 6.25 μg mL^{-1}), *Pseudomonas aeruginosa* (MIC 6.25 μg mL^{-1}), Gram (-) bacteria (Xu et al., 2013). **Antifungal activity**: volatile oil active against *Fusarium graminearum* (MIC 25 μg mL^{-1}), *Botrytis cinerea* (MIC 12.5 μg mL^{-1}), *Exerohilum turcicum* (MIC 25 μg mL^{-1}), *Lecannosticta acicola* (MIC 25 μg mL^{-1}) (Xu et al., 2013); ethyl caffeate and methyl rosmarinate isolated from the *H. rhomboidea* inhibit *Sclerotinia sclerotiorum* (MIC 16.2 mg L^{-1}), *Sclerotinia minor* (MIC 16.2 mg L^{-1}), and *Exserohilum turcicum* (MIC, 8.1 mg L^{-1}) (Tang et al., 2014).

Table 2.1. continued.

Species	Chemical constituents	Uses/Biological Activities
Hyptis rhomboidea M Martens & Galeotti	Borneol, Germacrene D, α-Gurjunene, α-Muurolene, γ-Muurolene, β-Cadinene, γ-Elemene, l-Calamenene, Thymol, Butylated hydroxytoluene, Caryophyllene oxide, Methyl eugenol, Globulol, *cis*-Asarone, β-Eudesmol, Phytol, Palmitic acid (Xu et al., 2013).	**Antioxidant activity:** ethyl acetate phase and ethanolic extract have a great antioxidant activity when tested the ability of scavenging DPPH and ABTS, and against FRAP assay (Li et al., 2014); volatile oil active on DPPH assay (SC_{50} 2.591± 0.15 μg mL^{-1}) (Xu et al., 2013). **Citotoxicity:** Ethanol extracts can improve a tumor-burdened mice inflammation factors, improve the survival quality of H_{22} tumor mice, and enhance immunity and antitumor activity: inhibitory effect on the digestive tumor cells. In solid tumor model, the inhibitory rate is up to 68.84%. In ascites tumor model, could slow down weight gain in mice and prolong the survival time; in immunodeficiency model, could improve the serum TNF-α and, IL-2 levels, increase SOD activity, and reduce MDA content (Cui et al., 2017); Volatile oil LC_{50} 65.9±6.55 μg mL^{-1} of Brine shrimp lethality activity (Xu et al., 2013). **Patent**: is a part of the Chinese medicinal pesticide for garden plants and flowers, Patent No.CN 106857715 (Lin, H. 2017); An herbicide including tribenuron-Me, azone, *H. rhomboidea* extract, sodium lignosulfonate, sunflower leaf extract, glyphosate isopropylamine salt, and water, Patent No. CN 105494457 (Zhang, 2016); Germicidal composition containing thiophanate-methyl and *H. rhomboidea* extract, Patent No. CN 105165912 (Shen, 2015). **Xanthine oxidase (XO) inhibitory activity:** ethanolic extract active against xanthine oxidase; Hyprhombin C and epihyprhombin B isolated from *H. rhomboidea* more active than allopurinol, with IC_{50} values of 0.6 and 2.0 μM, respectively (IC_{50}: 0.6 and 2 μM, respectivelly) (Tsai and Lee, 2014).
Hyptis saxatilis A.St.-Hil. ex Benth.		**Anti-Fungic activity:** ethanolic extract investigated against the dermatophytes: *Microsporum canis*, *Microsporum gypseum*, *Trichophyton rubrum*, *Trichophyton mentagrophytes* (the study did not show the results) (Souza et al., 2002).
Hyptis shaferi Britton		**Parasitism:** Host to *Dicaeoma hyptidis* (Lagerh.) (Baxter, 1961).

Table 2.1. continued.

Species	Chemical constituents	Uses/Biological Activities
Hyptis sinuata Pohl ex Benth.	Polysaccharide was found to be absent or undetectable (Fernández-Alonso et al., 2003). Contents of Chlorophyll a (0.39 g m^{-2}) b (0.26 g m^{-2}), and a+B (0.66 g m^{-2}) (Castrillo et al., 2001).	**Lectin activity**: Considerable lectin activity (65.6 %) (Fernández-Alonso et al., 2003). **Physiologycal studies**: populations from different shaded environments presented reduced contents of sugar and no diferences on the proteins contentes (Castrillo et al., 2005).
Hyptis velutina Pohl ex Benth.	**Leaves volatile oils:** 1-octen-3-ol, β-caryophyllene, α-Humulene, γ-Muurolene, Bicyclogermacrene, β-copaen-4-α-ol (Batista et al., 2003).	
Hyptis villosa Pohl ex Benth.	**Volatile oils**: α-Thujene, Sabinene, Limonene, *cis*-Piperitol acetate, α-Copaene, α-Bourbonene, β-Bourbonene, β-Elemene, *cis*-Caryophyllene, β-Ylangene, β-Gurjunene, α-Humulene, *allo*-Aromadendrene, γ-Muurolene, Bicyclogermacrene, *cis*-β-guaiene, γ-Cadinene, 7-*epi*-α-Selinene, δ-Cadinene, Kessane, *trans*-Nerolidol, Germacrene B, *cis*-nerolidol, Spathulenol, Globulol, Viridiflorol, Rosifoliol, Humulene epoxide II, 1,10-di-epi-Cubenol, 1-epi-Cubenol, epi-α-Cadinol, α-Cadinol, Khusinol, 5-neo-Cedranol, Eudesm-7(11)-en-4-ol acetate (Silva et al., 2013A).	

MIC: minimal inhibitory concentration; **SC_{50}**: 50% reduction of total free radical; **EC_{50}**: half maximal effective concentration; **LC_{50}**: Lethal Concentration 50%; **IC_{50}**: inhibitory concentration 50%; **ED_{50}**: median effective dose; **MIC50**: minimal inhibitory concentration 50%; **MIQ**: minimal inhibitory quantity (µL).

Results and discussion

Many of the articles found corresponded to chemical description and biological activities of species that are no longer placed into *Hyptis*. Species names recognized by Epling (1949) were reviewed and only names accepted *sensu* Harley and Pastore (2012) were used. As presently circumscribed, *Hyptis* comprises 144 species (Suplemental 2.1) but only 31 of them have some type of record in the literature for pharmaceutical, agricultural, chemical description, etc. This number of species corresponds to 20.9% of the total, but within this percentage some of them have only one use or biological activity described as: *H. alata* (insecticidal activity), *H. brachiata* (lectin activity), *H. campestris* and *H. meridionalis* (antioxidant activity), *H. saxatilis* (antifungic activity). *Hyptis divaricata* was reported as propolis botanical origin, and *H. shaferi* as host for parasites (Table 2.1).

For 25 species were found reports regarding their ethnopharmacological and/or pharmacological and/or chemical composition. As biological activities most cited in the literature for *Hyptis* species, antioxidant activity was reported for 9.45% of species (14 spp.), antibacterial activity was reported for only 4.05% of species (6 spp.), and antifungical activity could be found reported for 5.40% (8 spp) of *Hyptis* species. *Hyptis atrorubens, H. brevipes* and *H. crenata* are the most studied species regarding these three biological activities. The anti-HIV activity was tested for six species, *Hyptis capitata*, *H. comaroides*, *H. lantanifolia*, *H. lappulacea*, *H. multibracteata* and *H. radicans* (4.05% of total). Less accessed was the cytotoxicity of extracts obtained from *Hyptis* species, evaluated only for six species: *Hyptis brevipes*, *H. capitata*, *H comaroides H. crenata*, *H. dilatata* and *H. rhomboidea,* corresponding to 4.2% of total number of species. In regard to the ethnopharmacological/pharmacological uses, were found studies reporting 11 species used in traditional medicine: *Hyptis atrorubens*, *H. brevipes*, *H. capitata*, *H. crenata*, *H. dilatata*, *H. lacustris*, *H. lanceolata*, *H. marrubioides*, *H. multibracteata*, *H. obtusiflora* and *H. radicans,* corresponding to 7.43% of total *Hyptis* species and showing the importance of this genus for some tradictional communities. Furthermore, ethnopharmacological studies are shortcuts in the search for new bioactive substances, being one important tool in bioprospecting programs (Atanasov et al., 2015).

Other biological activities were tested only for one species, such as anti-tick properties (*H. crenata*), gastroprotective action (*H. crenata),* bioestimulant activity (*H. marrubioides),*

antiedematogenic activity (*H. radicans*), allelopathic activity (*H. rhomboidea*), antinociceptive effect (*H. crenata*), and anti-Leishmanicide (*H. lacustris*). Regarding the insecticidal activity, only *Hyptis alata* and *H. brevipes* (1.35%) were studied.

In relation to the chemical constituents, volatile oil was described for 11.49% of total *Hyptis* species: *H. atrorubens*, *H. brachiata*, *H. brevipes*, *H. conferta*, *H. crenata*, *H. dilatata*, *H. goyavensis*, *H. lanceolata*, *H. marrubioides*, *H. multibracteata*. *H. monticola*, *H. passerina*, *H. radicans*, *H. recurvata*, *H. rhomboidea*, *H. velutina* and *H. villosa*, which means that little more than tenth part has been studied in terms of the chemical potential of the volatile oil.

The most repeated compounds in the chemical composition of *Hyptis* were natural bicyclic sesquiterpenes, like Caryophyllene. In nature, it mainly occurs as *trans*-caryophyllene ((*cis*)-β-caryophyllene) mixed with small amounts of its isomers, (*trans*)-β-caryophyllene, and α-humulene (also known as α-caryophyllene), as well as its derivative Caryophyllene oxide (Fidyt et al., 2016). Derivatives of this sesquiterpene appeared in 15 species of *Hyptis*.

Cadinenes are also bicyclic sesquiterpenes and have been further divided into four classes of compounds based on the nature of the ring fusion and the orientation of the isopropyl group at C7. The four classes are the cadinanes, the muurolanes, the bulgaranes and the amorphanes (Borg-Karlson et al., 1981). Substances of that class were described as volatile oil constituents of 12 *Hyptis* species.

Pinenes have two active constitutional isomers: α- and β-pinene and are bicyclic monoterpenes. Both structural isomers have enantiomers known in nature as (–)-α-pinene (more common in European pines), (+)-α-pinene (more common in North America), (–)-β-pinene and (+)-β-pinene (Silva et al., 2012C). Pinenes were reported for 11 species of *Hyptis*.

Monoterpenes as terpineol derivatives appear as four isomers, α-, β-, γ-, and terpinen-4-ol. β- and γ-terpineol differ only by the location of the double bond. Terpineol is usually a mixture of these isomers with α-terpineol as the major constituent (Yuasa and Yuasa, 2006; Baptistella et al., 2009). Derivatives of these monoterpenes were found in nine species of *Hyptis*.

Regarding the non-volatile compounds, they were reported for only 6.08% of species: *Hyptis atrorubens*, *H. brevipes*, *H. capitata*, *H. lacustris, H. lanceolata*, *H. multibracteata*, *H. radicans*, *H. ramosa,* and *H. rhomboidea.* For some species, the description of one susbtance is found, such as rosmarinic acid, as in the case of *H. campestris*, *H. lappulacea*, *H. meridionalis*

and *H. comaroides*, and nepetoidins A and B for *H. ramosa*. These three substances are also comomly found in Nepetoideae taxa.

Conclusions

Although a considerable number of papers was found in databases: 879 in SciFinder, 528 in Web of Science and 96 in SciELO, only 20% of *Hyptis sensu* Harley and Pastore (2012) have been studied. The majority of species was studied regarding their volatile oil composition; remaning poorly explored the polar constituents. Furthermore, based on the articles published, it was possible to notice that these species are characterized by the presence of substances with promising pharmacological potential, mainly antimicrobial, antifungal, cytotoxic, anti-inflammatory, and anti-HIV, pointing to a great relevance of *Hyptis* to bioprospecting studies.

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Supplementary information 2.1 - *Hyptis* species sensu Harley and Pastore (2012) presenting no reports in literature regarding their chemical composition and biological activities.

Hyptis actinocephala Griseb., *Hyptis adamantium* A.St.-Hil. ex Benth, *Hyptis alpestris* A. St.-Hil. ex Benth., *Hyptis alutacea* Pohl ex Benth, *Hyptis amaurocaulos* Briq., *Hyptis ammotropha* Wright ex Griseb., *Hyptis angulosa* Schott ex Benth., *Hyptis angustifolia* Pohl ex Benth., *Hyptis arenaria* Benth. in DC., *Hyptis argentea* Epling & Mathias, *Hyptis armillata* Epling, *Hyptis asteroides* A.St.-Hil. ex Benth., *Hyptis australis* Epling, *Hyptis bahiensis* Harley, *Hyptis balansae* Briq., *Hyptis brachypoda* Epling, *Hyptis caduca* Epling, *Hyptis caespitosa* A.St.-Hil. ex Benth., *Hyptis colligata* Epling & Jativa, *Hyptis colubrimontis* Epling & Játiva, *Hyptis complicata* A.St.-Hil. ex Benth., *Hyptis corymbosa* Benth. in DC., *Hyptis crassipes* Epling, *Hyptis cruciformis* Epling, *Hyptis dictyodea* Pohl ex Benth., *Hyptis ditassoides* Mart. ex Benth., *Hyptis dumetorum* Morong, *Hyptis eriocauloides* Rich., *Hyptis fallax* Harley, *Hyptis ferruginosa* Pohl ex Benth., *Hyptis florida* Benth., *Hyptis frondosa* S. Moore, *Hyptis fulva* Epling, *Hyptis gardneri* Briq., *Hyptis guanchezii* Harley, *Hyptis hamatidens* Epling & Jativa, *Hyptis hassleri* Briq., *Hyptis havanensis* Britton ex Epling, *Hyptis heterophylla* Benth. in DC., *Hyptis hilarii* Benth., *Hyptis hirsuta* Kunth, *Hyptis hispida* Benth. in DC., *Hyptis homalophylla* Pohl ex Benth., *Hyptis huberi* Harley, *Hyptis humilis* Benth. in DC., *Hyptis hygrobia* Briq., *Hyptis imbricata* Pohl ex Benth., *Hyptis imbricatiformis* Harley, *Hyptis inodora* Schrank, *Hyptis intermedia* Epling, *Hyptis involucrata* Benth., *Hyptis kramerioides* Harley & J.F.B.Pastore ,*Hyptis laciniata* Benth., *Hyptis lagenaria* A.St.-Hil. ex Benth., *Hyptis lanuginosa* Glaz. ex Epling, *Hyptis lappacea* Benth., *Hyptis lavandulacea* Pohl ex Benth., *Hyptis leptoclada* Benth. in DC., *Hyptis linarioides* Pohl ex Benth., *Hyptis longifolia* Pohl ex Benth., *Hyptis lorentziana* O.Hoffm., *Hyptis loseneriana* Pilg., *Hyptis lucida* Pohl ex Benth., *Hyptis lutescens* Pohl ex Benth., *Hyptis luticola* Epling, *Hyptis macrocephala* M.Martens & Galeotti, *Hyptis mariannarum* Briq., *Hyptis maya* Harley, *Hyptis microphylla* Pohl ex Benth., *Hyptis microsphaera* Epling, *Hyptis minutifolia* Griseb., *Hyptis mollis* Pohl ex Benth., *Hyptis muelleri* Briq., *Hyptis nigrescens* Pohl ex Benth., *Hyptis nudicaulis*

Benth., *Hyptis obtecta* Benth. in DC., *Hyptis odorata* Benth., *Hyptis orbiculata* Pohl ex Benth., *Hyptis origanoides* Pohl ex Benth., *Hyptis ovata* Pohl ex Benth., *Hyptis pachyarthra* Briq., *Hyptis pachycephala* Epling, *Hyptis pachyphylla* Epling, *Hyptis paludosa* A.St.-Hil. ex Benth., *Hyptis parkeri* Benth., *Hyptis paupercula* Epling, *Hyptis penaeoides* Taub., *Hyptis personata* Epling, *Hyptis petiolaris* Pohl ex Benth., *Hyptis proteoides* A.St.-Hil. ex Benth., *Hyptis pseudosinuata* Epling, *Hyptis pulchella* Briq., *Hyptis pulegioides* Pohl ex Benth., *Hyptis pusilla* (Pohl) Harley & J.F.B.Pastore, *Hyptis pycnocephala* Benth. in DC., *Hyptis pyriformis* Epling & Játiva, *Hyptis remota* Pohl ex Benth., *Hyptis rhypidiophylla* Briq., *Hyptis riparia* Harley, *Hyptis rondonii* Epling, *Hyptis rotundifolia* Benth., *Hyptis rubiginosa* Benth., *Hyptis salicina* J.A.Schmidt, *Hyptis savannarum* Briq., *Hyptis sericea* Benth., *Hyptis subviolacea* Briq., *Hyptis tetracephala* Bordignon, *Hyptis tetragona* Pohl ex Benth., *Hyptis tricephala* A.St.-Hil. ex Benth., *Hyptis tumidicalyx* Epling & Játiva, *Hyptis turnerifolia* Mart. ex Benth., *Hyptis uliginosa* A.St.-Hil. ex Benth., *Hyptis uncinata* Benth., *Hyptis vilis* Kunth & Bouche, *Hyptis vimínea* Epling, *Hyptis xanthiocephala* Mart. ex Benth.

CHAPTER III

Botanical aspects & chemical description of *Hyptis radicans* and *Hyptis multibracteata*

General taxonomic and morphological aspects of Hyptis Jacq.

Lamiaceae includes about 258 genera and 7,193 species with cosmopolitan distribution (Harley, 2012). In Brazil, 34 genera and 498 species are found; many of them are endemic to the northeastern semi-arid region, with emphasis on *Hyptis* Jacq. and *Rhaphiodon* Schauer (Harley, 2012). This family comprises herbaceous to shrubby plants, mainly characterized by stem and tetradular branches, when young; heavily zygomorphic flowers, bilabiate, and ovary stiletto ginobasic; and by the presence of rosmarinic acid (Pedersen, 2000; Harley et al., 2004).

This family has great economic importance mainly for the presence of volatile oils and for the diverse species cultivated as ornamental. Among the most important cultivated species are mint and peppermint and related species (*Mentha* spp.), basil (*Ocimum* spp.), oregano (*Origanum* spp.), rosemary (*Rosmarinus officinalis* L.), salvias (*Salvia* spp.) and lavenders (*Lavandula* spp.), all used as seasoning or in the manufacture of perfumes. Most of the species economic importante, especially due to the presence of volatile oils, are included in the subfamily Nepetoideae (Pastore, 2010).

Lamiaceae is divided into 12 subfamilies, being Nepetoideae the largest one. It comprises almost 50% of the genera and species, and the majority of the volatile oil-rich genera of Lamiaceae, and thus most of the species with greater economic interes, (Grayer et al., 2003; Zhong et al., 2010; Harley and Pastore 2012). According to Li and colleagues (2016), Nepetoideae comprises 118 genera and ca. 3,400 species widely distributed in tropical and temperate areas. Among synapomorpies for this subfamily, the presence of rosmarinic acid, together with morphological features as hexacolpate and three-celled pollen, investing embryos, myxocarpy, and gynobasic style, are accepted. The most representative genera in number of species are: *Salvia* L. (800 species) followed by *Clerodendrum* L. (400,) and *Hyptis* Jacq. (144) (Pastore 2010; Harley et al., 2004; Harley and Pastore, 2012).

Based on molecular data, Harley and Pastore (2012) proposed the monophyly of *Hyptis* after a new circumscription, with about 144 species included in 10 sections distributed in tropical and subtropical zones from North America to the Caribbean and southward to Argentina and Peru, often occurring in humid savannas. A few species can be found in the Old-World tropics, mainly as weeds.

Silva-Luz and colleagues (2012) described the morphology of *Hyptis* as follows: shrubs, sub-shrubs or perennial or annual herbs, strongly aromatic or odoriferous; leaves decussed or not, petiolate or sessile, discolores or colorings, various patterns of venation, usually leathery or characte to membranous, usually dressed; inflorescence usually hemispherical, sometimes spherical, persistent bractheolar chapters; flower campanula or tubular calyx, subactinomorph, 5-lobed, the same or sub-lobed, elliptical, narrowly elliptic, lanceolate, linear or triangular, subulate or flat lobes, with or without conspicuous appendix expanded and complanated; and fruit with 4 nules, usually flattened, glabrous or tomentosa, lenticellate, smooth or verrucous.

Hyptis is known to be used in folk medicine for the treatment of various diseases. For example, *Hyptis atrorubens* Poit., used as traditional medicinal remedy in French West Indies (Abedini et al., 2012); *Hyptis brevipes* Poit., used in folk medicine for the treatment of asthma, malaria, and different types of cancer (Adjanohoun et al., 1986; Parker et al., 2007; Bhuiyan and Begum, 2010); *Hyptis capitata* Jacq. used by traditional healers from Antioquia and Chocó for snakebites (Otero et al., 2000); *Hyptis crenata* Pohl ex Benth. traditional use for mild pain headache, stomach, discomfort, menstrual pain, treatment of flu/fever (Rocha et al., 2009B), it is also one of the most cited species used for antiulcer and anti-inflamatory problems in Mato Grosso (Brazil) (Jesus et al., 2009); and *Hyptis lacustris* A.St.-Hil. ex Benth. used by the Yanesha community for the treatment of cutaneous leishmaniosis (*Mareñets) (Valadeau et al., 2009)*.

The present study accessed *Hyptis radicans* (Pohl) Harley & J.F.B. Pastore and *Hyptis multibracteata* Benth. in order to analyze their chemical compotion and biological activities. Both species are native to Brazilian biomes and has no data in literature regarding the chemical composition.

Hyptis radicans has *Peltodon radicans* Pohl as basyonym and *Clinopodium repens* Vell., *Peltodon radicans* var. *tenuicaulis* Tolm., and *Peltodon repens* (Vell.) Kuntze as heterotypic. It is an herb with an earthling substrate, is native but is not endemic to Brazil. In relation to its geographical distribution, there are confirmed occurrences in the Midwest (Mato Grosso do

Sul), Southeastern (Minas Gerais, Rio de Janeiro, and São Paulo) and South (Paraná and Santa Catarina) regions of Brazil (figure 3.1). The phytogeographical domains in which it has been found are Brazilian Central Savannah (Cerrado) and Atlantic Forest, being very commom to areas with the following type of vegetation: Antropogenic Area, Altitude Field, Clean Field, Rock Formation, Ombrophilous Forest (= Rainforest), Mixed Ombrophilous Forest (Flora do Brasil 2020). *H. radicans* was traditionally treated as *Peltodon radicans*, however *Peltodon* emerged among *Hyptis* species in a recent molecular phylogenetic analyzes and for this reason was reduced to a section within *Hyptis* by Harley and Pastore (2012). The species is easily recognized by the chalice lobes with conspicuous expanded and convoluted appendages, very conspicuous oval involucral bractulae, and strongly attenuated base leaves (Silva-Luz et al., 2012).

Hyptis multibracteata has *Hyptis marginata* J. A. Schmidt as relevant synonym, *Mesosphaerum marginatum* (J. A. Schmidt) Kuntze as heterotypic, and *Mesosphaerum multibracteatum* (Benth.) Kuntze as homotypic. It is an herb with an earthling substrate, is native and endemic of Brazil. In relation to its geographical distribution, there are confirmed occurrences in Northeast (Bahia), Southeast (Espírito Santo, Minas Gerais, Rio de Janeiro, São Paulo) and South (Santa Catarina) regions of Brazil (figure 3.1). Atlantic forest is the phytogeographical domain in which it has been found. It mainly appears where there is the following type of vegetation: Ombrophilous Forest (= Rain Forest) and Restinga (Flora do Brasil 2020).

In relation to chemical constituents, Lamiaceae arouses great interest in the study of secondary metabolites, such as flavonoid, alkaloids, terpenes, coumarins, lignoids and volatile oils (Ferreira, 2009). Chlorogenic acid and 3,4-dihydroxyphenyl ethanoid glycosides are compounds frequently reported for Lamiaceae species (Basílio et al., 2006). A review of data already published regarding the chemical composition and human uses of *Hyptis* species (*sensu* Harley and Pastore, 2012) was discussed in Chapter II (Table 2.1).

Figure 3.1. (A) Geographic distribution of *Hyptis radicans* (Pohl) Harley & J.F.B. Pastore. This species is accepted, and is native to Brazil South, Brazil Southeast and Brazil West-Central. **(B)** Geographical distribution of *Hyptis multibracteata* Benth. This species is accepted, and is native to Brazil South, Brazil Northeast and Brazil Southeast. (Images font: powo.science.kew.org by Royal Botanic Gardens & Plants of the World Online).

Realizing the chemical importance of *Hyptis*, the therapeutic uses of many species in contries from Latin America, and the absence of phytochemical and pharmacological studies of *H. radicans* and *H. multibracteata*, two native Brazilian species, were important factors for our selection of species for chemical profiling, aiming to contribute to the description of the Brazilian chemical diversity, as well as providing data to chemotaxanomic studies. The present study aims to determine the phytochemical profile of extracts derived from *Hyptis radicans* (Pohl) Harley & J.F.B. Pastore and *Hyptis multibracteata* Benth.

Material and methods

Plant Material

Individuals from the same population of *Hyptis radicans* and *Hyptis multibracteata* (Figure 3.2) were collected in the morning during the dry season in July near to the Biological Reserve of Alto da Serra de Paranapiacaba (Atlantic forest) in the municipality of Santo André, São Paulo, Brazil (23° 46' 25" S e 23° 40' 18" W; authorization number CNPQ 010894/2014-4). Aerial parts were stored for identification and voucher specimens (Silva-Luz 295 and Silva-Luz 294) were deposited at SPF Herbarium (University of São Paulo, Brazil).

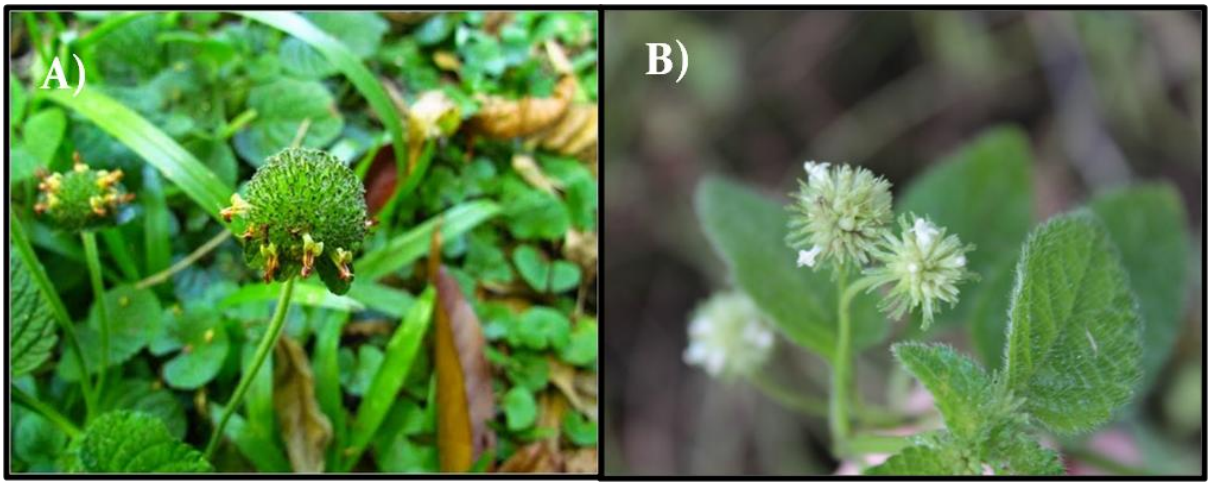

Figure 3.2. *Hyptis* species used in this study. A) *H. radicans and* B) *H. multibracteata* (Photos: Furlan CM).

Extraction and Fractionation (plant material and sample preparation)

H. radicans and *H. multibracteata* were dried at 40°C, powdered and macerated in 70% ethanol for 7 days at room temperature in the dark. Crude ethanol extract (**EE**) were concentrated under reduced pressure below 50°C using a rotary evaporator and freeze-dried. **EE** (2 g each) was dissolved in 100 mL of 50% methanol and then was fractionated by partition with the same volume of hexane and ethyl acetate for four times, originating three phases: hexane (**HP**), ethyl acetate (**EAP**), and hydromethanol phase (**HMP**) (Figure 3.3). All phases were concentrated under reduced pressure below 50°C by a rotary evaporator and freeze-dried.

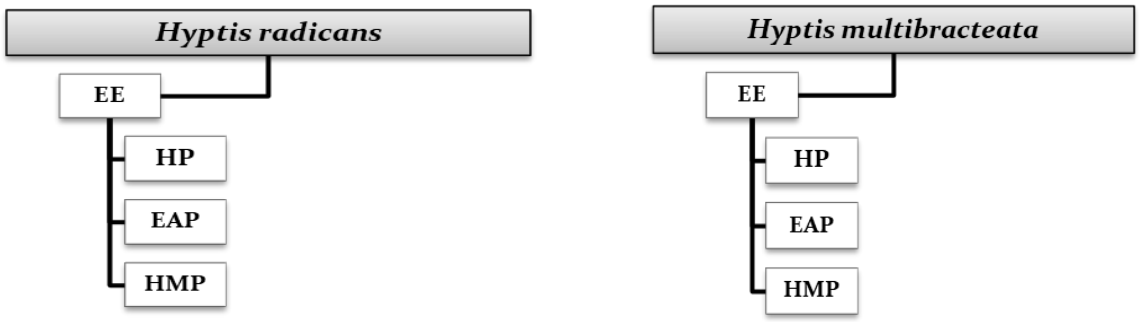

Figure 3.3. Flowcharts of crude extract and phases of *H. radicans* and *H. multibracteata*: EE: crude ethanol extract; HP: hexane phase; EAP: ethyl acetate phase; HMP: hydromethanol phase.

Chemical analyzes

All freeze-dried extracts and phases (**EE**, **HP**, **EAP** and **HMP**) for both species were dissolved in DMSO (2 mg mL^{-1}) and analyzed by HPLC-DAD (LC1260 - Agilent Technologies) using a Zorbax C18 (150 X 4.6 mm, 3.5 µm) at 40°C. Solvent gradient used was 0.1% acetic acid (AcOH) and acetonitrile (CH_3CN) starting with 10% CH_3CN (0-6 min), increasing from 10% to 15% (6-7 min), isocratic for 15 min in 15% (7-22 min), increasing from 15% to 50% (22-32 min), ranging from 50% to 100% (32-42 min), isocratic for 8 min in 100% (42-50 min) (Santos et al., 2016). Solvent flow was 1.0 mL min^{-1}; injection volume 3 µL, and detection at 352 nm and 280 nm. For the quantification of the constituents, areas in the chromatogram (HPLC-DAD) were compared to calibration curves obtained using authentic samples of *p*-coumaric acid (0.75 a 150 µg mL^{-1}) to quantify cinnamic acid derivatives, and luteolin (0.75 a 150 µg mL^{-1}) to quantify flavonoids. Results are expressed as mg g^{-1} of dry extract equivalent to the standard used. Authentic samples of cinnamic acid derivatives, flavones and flavonols *O* and *C*-glycosides were used for the preliminary identification of the constituents by their UV-Vis absorption spectra and retention times.

All freeze-dried extracts and phases dissolved in DMSO (2 mg mL^{-1}) were also analyzed by GC-EIMS. It was used a capillary column HP-5MS (5% phenyl, 95% polydimethylsiloxane - 30 m x 0.25 mm x 0.25 µm film thickness) with helium as mobile phase (1.0 mL min^{-1}). The column temperature program was 150°C, raising 5°C min^{-1}, until 325°C. The EIMS analysis employed an ionization voltage of 70 eV and an ion source temperature of 230°C. The GC-EIMS

identification was performed by computer matching of 850 with NIST mass spectral search program version 2.of and literature.

Isolation of bioactive compounds and identification by Nuclear Magnetic Resonance (NMR)

Part of the crude extract (1.5 g) was fractionated by exclusion Column Chromatography using sephadex LH-20 (GE Healthcare) as adsorbent and methanol as mobile phase, generating 6 fractions (**HRF1**, **HRF2**, **HRF3**, **HRF4**, **HRF5**, **HRF6**) for *H. radicans* and 4 fractions (**HMF1**, **HMF2**, **HMF3**, **HMF4**) for *H. multibracteata,* using a UV detector for fraction colletion.

Fractions **HRF2**, **HRF3**, **HRF4** and **HRF5** from *H. radicans* were subjected to semi-preparative HPLC (1200 Agilent Technologies) coupled to a DAD and using a Zorbax Eclipse XDB-C18 column (250 mm x 9.4 mm x 5.0 µm) at 40°C. Solvents used were 0.1% acetic acid (AcOH) and acetonitrile (CH_3CN), starting with 10% of CH_3CN (0 min), increasing to 25% (0–6 min); raising to 40 % (7-10 min), raising to 45% (10-15 min), raising to 50% (15-18 min), to 60% (18-20 min), raising to 70% (20-24 min) and increasing to 100 % (24-28 min) and 5 min of post-run. Solvent flow rate was 4.1 mL min^{-1} (0–28 min); injection volume of 400 µL, and detection at 352 and 280 nm.

HMF2 and **HMF3** from *H. multibracteata* were subjected to semi-preparative HPLC (1200 Agilent Technologies) coupled to a DAD and using a Zorbax Eclipse XDB-C18 column (250 mm x 9.4 mm x 5.0 µm) at 40°C. Solvents used were 0.1% acetic acid (AcOH) and acetonitrile (CH_3CN), starting with 10% of CH_3CN (0 min), increasing to 25% (0–8 min); raising to 40% (8-15 min), raising to 80% (15-20 min) and increasing to 100% (20-26 min) with 5 min of post-run. Solvent flow rate was 4.1 mL min^{-1} (0–26 min); injection volume of 400 µL, and detection at 352 and 280 nm.

All fractions were analyzed by analytical HPLC-DAD under the same methodology described above to check purity. Isolated substances were analyzed by ^{1}H NMR in a Bruker Avance III at 300 MHz and ^{13}C NMR was analyzed at 75 MHz in deuterated methanol (CD_3OD) or deuterated chloroform ($CDCl_3$). Two-dimensionals analyses were also performed. All data were processed using MestReNova software 9.0.1-13254 version (Mestrelab Research S. L.). The identification of compounds was performed with collaboration of Prof. Dr. Marcelo José Pena Ferreira (Department of botany, University of São Paulo).

Results and discussions

Extraction yield

On this study, initially the target components were phenolic compounds, since volatile oils constituents and other terpenes were already described for Lamiaceae species. The strategy was to extract a broad spectrum of phenolic classes. The initial crude extracts were prepared using 70% aqueous ethanol (solvent penetration ability is strong), since this solvent has proven in practice to be suitable to extract a broad range of bioactive molecules. Using 70% aqueous alcohol therefore, may be a simple and effective way of extracting polar and non-polar components in one extraction, thus recovering major, if not all, components for bioactivity screening. According to Liu (2008) this simple method does not favor the extraction of any components, but rather broadband components.

Yields of crude hydroethanol extract (**EE**) of *H. radicans* and *H. multibracteata* were 14.37% and 3.93%, respectively. *H. radicans* hydromethanol phase (**HMP**) showed the highest yield (52%), while *H. multibracteata* hexane phase (**HP**) yielding 49% (Table 3.1). Results point out to a less polar composition of *H. multibracteata* due to the low yield of the crude ethanol extract, and the highest yield of its hexane phase (Table 3.1).

Compared to other species belonging to the same genus, *H. radicans* and *H. multibracteata* presented an **EE** yield higher than that verified by Rebelo and colleagues (2009) for *Hyptis crenata* Pohl and Benth. (0.9%), but a lower yield when compared with *H. lacustris* (17.39 %) (Sala-Carvalho, 2017).

Table 3.1. Yield (%) of crude ethanol extract (**EE**) and its phases from *H. radicans* and *H. multibracteata:* hexane phase (**HP**), ethyl acetate phase (**EAP**), and hydromethanol phase (**HMP**).

Sample	*H. radicans*	*H. multibracteata*
EE	14.4	3.9
EAP	14.0	10.0
HP	27.0	49.0
HMP	52.0	40.0

Isolation and identification of non-volatiles constituents

First step to isolation: HPLC-DAD to see chemical profiles

After yield calculations, extract and its phases were submitted to qualitative and quantitative analysis by HPLC-DAD. Figure 3.4 shows the chromatograms obtained at 280 nm due to the greater variety of substances detected in this wavelength.

Main phenolic classes detected by HPLC-DAD in ethanol extract and phases of both *Hyptis* species analyzed are presented in Table 3.2. Phenolic classes were established using their UV-VIS absorption spectra (240–600 nm). Phenolic classes observed for both *Hyptis* spp were: phenolic acids, flavonoids, chlorogenic acid derivatives, cinnamic acid derivatives, nepetoidins, and rosmarinic acid. These last compounds, nepetoidins and rosmarinic acid, are caffeic acid esters, as chlorogenic derivatives, but they are characteristic constituents in the subfamily Nepetoideae and weres quantified separately. These phenolic classes were established according to their UV–Vis spectral features (Markham, 1982).

Comparing the total phenolic content of the ethanol extracts (Table 3.2), were observed higher phenolic content in *H. radicans* (53.03 mg *ρ*-CLE g^{-1} - milligrams equivalents of *p*-cumaric acid/luteolin per gram of dry mass) than *H. multibracteata* (33.66 mg *ρ*-CLE g^{-1}). Flavonoid content ranged from 1.74 to 33.29 mg LE g^{-1} (milligrams equivalents of luteolin per gram of dry mass) for *H. radicans*, and 3.10 to 11.87 mg LE g^{-1} for *H. multibracteata.* **EAP** in *H. radicans* showed the highest contents of flavonoids 33.29 mg LE g^{-1} and for *H. multibracteata,* **EE** showed the highest value, 11.87 mg LE g^{-1}.

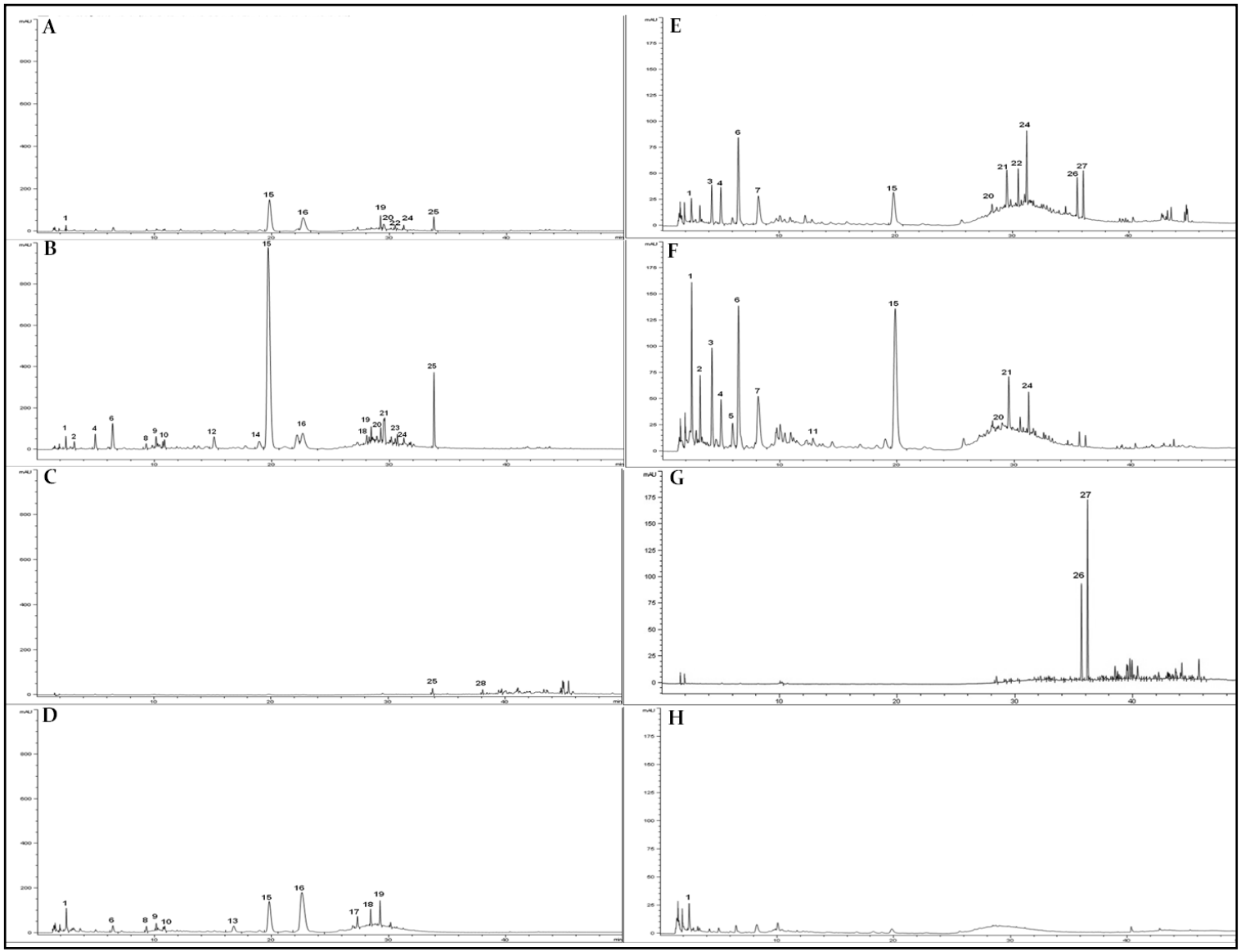

Figure 3.4. Chromatograms obtained by HPLC-DAD (280 nm) of extract and phases of: *H. radicans* - **A.** Ethanol extract, **B.** Ethyl acetate phase, **C**. Hexane phase, **D**. Hydromethanol phase; and *H. multibracteata*, **E**. Ethanol extract, **F**. Ethyl acetate phase, **G.** Hexane phase, **H.** Hydromethanol phase. Numbers above each peak correspond to identifications shown in **table 3.3. All c**hromatograms are on the same scale for comparison purposes.

Chlorogenic acid derivatives ranged from 21.97 to 41.18 mg ρ-CE g^{-1} (table 3.2) for *H. radicans,* while *H. multibracteata* **EE** and **EAP** showed values near to 9 mg ρ-CE g^{-1}. On the other hand, cinnamic acid derivatives were detected only in **EAP** and **HMP** from *H. radicans* (2.02 and 1.75 mg ρ-CE g^{-1}, respectively). Table 3.2 is important for discussions of chapters IV and V.

As expected, nepetoidins were detected in both species (**EE** and **EAP**), since they are considered as chemotaxonomic markers of Nepetoideae by Grayer and colleagues (2003). *H. multibracteata* showed higher amount of nepetoidins in **EE** (6.95 mg ρ-CE g^{-1}). Regarding the content of rosmarinic acid, **EE** from *H. radicans* showed the highest amount of this compound (17.64 mg ρ-CE g^{-1}) and **EE** from *H. mutibracteata* showed the smallest amount of rosmarinic acid (3.89 mg ρ-CE g^{-1}).

In relation to the total phenolic content detected by HPLC-DAD, **EAP** from *H. radicans* was the phase showing the highest amounts of these compounds (63.82 ρ-CE g^{-1}). However, **HMP** from *H. multibracteata* showed 163.64 times less amount of total phenolic compounds (0.39 ρ-CE g^{-1}) when compared to *H. radicans*.

Table 3.2. Content of phenolic compounds (mg g^{-1}) of crude ethanol extract (**EE**) and phases of *H. radicans* and *H. multibracteata* analyzed by HPLC-DAD detected in 280 nm: hexane phase (**HP**), ethyl acetate phase (**EAP**), hydromethanol phase (**HMP**).

Phenolic classes	***H. radicans***				***H. multibracteata***			
	EE	**EAP**	**HP**	**HMP**	**EE**	**EAP**	**HP**	**HMP**
Phenolic acids[a]	0.50	0.81	-	1.61	0.69	2.21	-	0.39
Flavonoids[b]	9.74	33.29	1.74	8.99	11.87	10.73	3.10	-
Chlorogenic acid derivatives[a]	21.97	25.55	-	41.18	9.15	9.25	-	-
Cinnamic acid derivatives[a]	-	2.02	-	1.75	-	-	-	-
Nepetoidin A and B	3.18	2.15	-	-	6.95	1.45	-	-
Rosmarinic acid	17.64	-	-	8.27	3.89	8.08	-	-
NI					1.11		0.64	
Total phenolic content	53.03	63.82	1.74	61.80	33.66	34.70	3.75	0.39

-: Not detected; [a] milligrams equivalents of ρ-coumaric acid per gram of dry mass (mg ρ-CE g^{-1}); [b] milligrams equivalents of luteolin per gram of dry mass (mg LE g^{-1}); NI: Not identified.

Methods used to extract phenolic compounds can also interfere in their quantification since some other reducing compounds from non-phenolic origin can also be extracted. However, several studies have demonstrated that extracts obtained by maceration with polar solvents followed by filtration, similar to the methodology used in the present study, are recommended for extraction of phenolic substances (Iloki-Assanga et al., 2015).

Santos and colleagues (2018) studied the phenolic profiles of ethanolic extracts from seven *Hyptis* spp and used a very similar classification of constituents. The results for phenolic acids in the ethanol extract showed *H. radicans* collected in Santo André, São Paulo during the rain season (humid season) does not present this class of substance. However, **EE** from the same species collected during the dry season in the same area presented 0.50 mg ρ-CE g^{-1} of phenolic acids. Authors sampled populations of seven species occurring in the Atlantic rainforest and Cerrado areas and content of phenolic compounds varied between biomes, *i.e.*, for the same species, populations from the Atlantic forest showed higher content of phenolic compounds than populations from Cerrado.Analyzing the content of flavonoids, *H. multibracteata* showest lower content of this phenolic class (11.87 mg *L*E g^{-1}) when comparing with *H. campestris* (114.43 mg *L*E g^{-1}), *H. comaroides* (72.47 mg *L*E g^{-1}), *H. lappulacea* (28.89 mg *L*E g^{-1}), and *H. meridionalis* (76.91 mg *L*E g^{-1}), but a higher content when comparing to *H. radicans* (9.74 mg *L*E g^{-1}) and *H. lacustris* (3.67 mg *L*E g^{-1}) (Santos et al., 2018).

Regarding rosmarinic acid, this phenolic compound is commonly found in Boraginaceae species and in Nepetoideae (Lamiaceae) (Petersen and Simmonds, 2003). *H. radicans* is among the species with the highest contents of this compound (17.64 mg ρ-CE g^{-1}), following *H. comaroides* (19.68 mg ρ-CE g^{-1}), the species with the highest rosmarinic content among the seven studied by Santos and colleagues (2018).

Second step to isolation: exclusion column chromatography using Sephadex®

Crude ethanol extract from both species were fractionated by exclusion Column Chromatography (CC, Sephadex®) generated 6 fractions (**HRF1**, **HRF2**, **HRF3**, **HRF4**, **HRF5**, **HRF6**) for *H. radicans* and 4 fractions (**HMF1**, **HMF2**, **HMF3**, **HMF4**) for *H. multibracteata,* using a UV detector. The following phases were selected for the next step, semi-preparative HPLC: **HRF2** (522.8 mg), **HRF3** (200 mg), **HRF4** (212.4 mg), and **HRF5** (64.1 mg) from *H. radicans*; and **HMF2** (412 mg) and **HMF3** (248.5 mg) from *H. multibracteata*.

Figures 3.5 and 3.6 show flowcharts of sub-fractions obtained using semi-preparative HPLC. The 4 fractions from *H. radicans* obtained by CC yielded 70 sub-fractions (Figure 3.5). For *Hyptis multibracteata*, from the 2 fractions obtained by CC, were obtained 34 sub-fractions (Figure 3.6). All sub-fractions were analyzed by HPLC-DAD to verify purity and select constituents successfully isolated to send for NMR ^{1}H analysis.

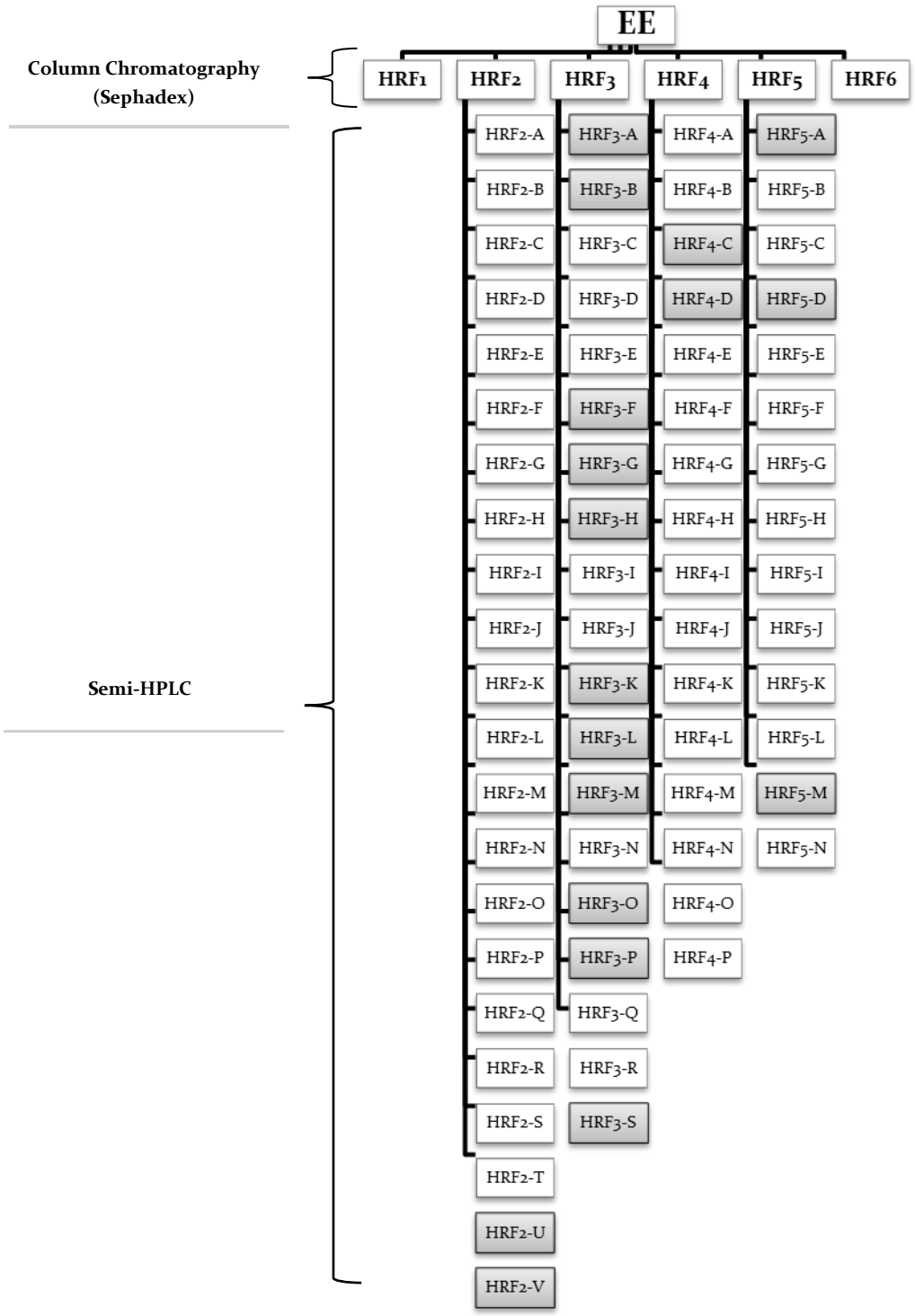

Figure 3.5. Flowchart of crude ethanol extract from *H. radicans*, its fractions and sub-fractions from Column Chromatography (Sephadex) and semi-preparative HPLC. Grey boxes represent sub-fractions with isolated substances according to data from HPLC-DAD.

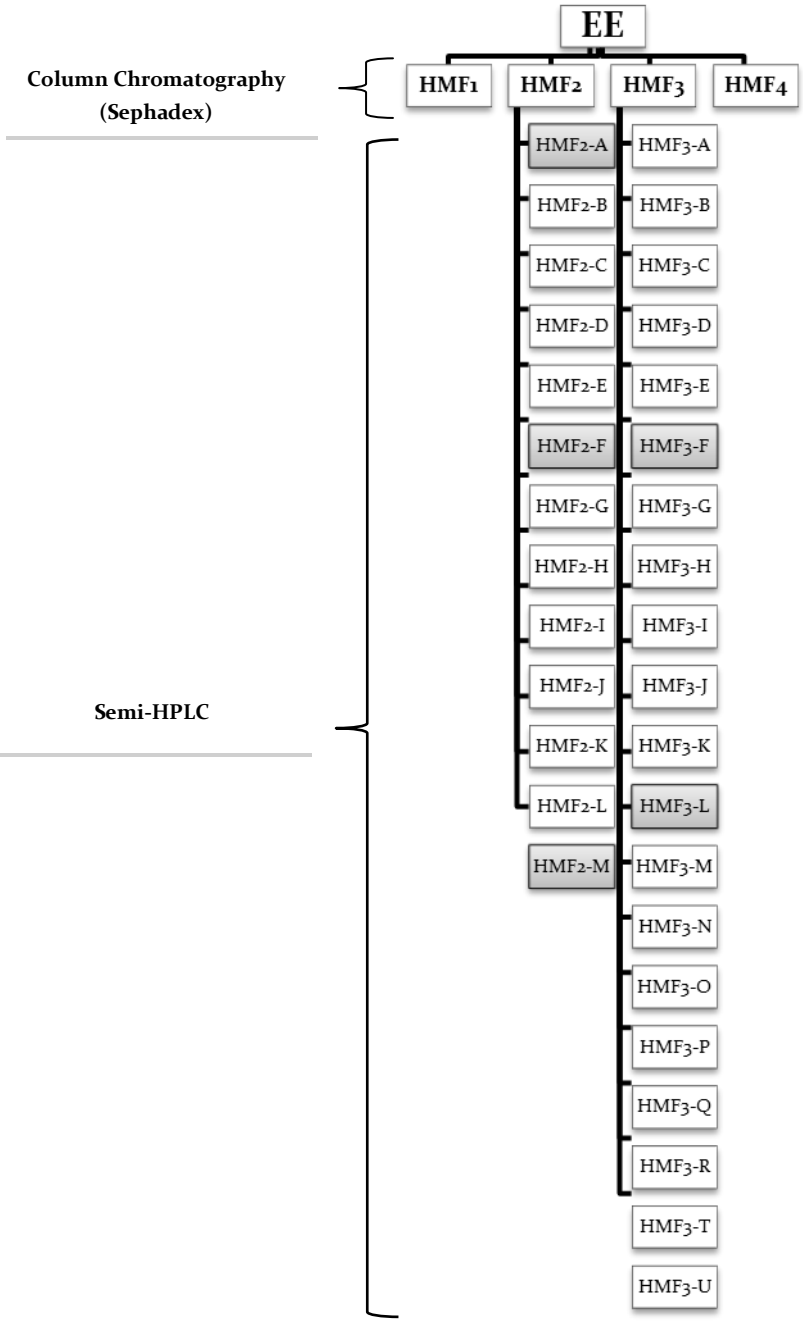

Figure 3.6. Flowchart of crude extract from *H. multibracteata*, its fractions and sub-fractions from Column Chromatography (Sephadex) and semi-preparative HPLC. Grey boxes represent sub-fractions with isolated substances according to data from HPLC-DAD.

After isolation…third step is identification

Gallic acid (**1**), Chlorogenic acid (**3**), Caffeic acid (**6**), *p*-Coumaric acid (**9**), Rutin (**11**), Rosmarinic acid (**15**) and Apigenin (**23**) were identified comparing the retention time and their UV/Vis spectra to authentic substances injected in the same analytical method in HPLC-DAD.

Rosmarinic acid (**15**) was isolated and yielded 9.4 mg. Sala-Carvalho (2017) isolated and analyzed rosmarinic acid from *Hyptis lacustris* using the same HPLC conditions and equipment used in the present work. Therefore, this substance was compared to the commercial rosmarinic acid (SigmaAldrich) and also confirmed using H^1 NMR by Sala-Carvalho (2017).

Constituents **2**, **7**, **8**, **10**, **12**, **13**, **21**, **27,** and **28** (Table 3.3; Figure 3.7) were proposed as flavonoids due to their UV/Visible absorption spectra features. Methanol spectra of flavones and flavonols exhibit two major absorption peaks in the region 240-400 nm. These two peaks are commonly referred to as Band I, usually 300-350 nm for flavones and 350-380 for flavonols, and Band II (usually 240-280 nm). Band I is considered to be associated with absorption of the B-ring cinnamoyl system, and Band II are associated with the absorption of the A-ring benzoyl system (Mabry et al., 1970). Substances **8**, **10**, **12,** and **28** were classified as flavones, all apigenin derivatives. Compounds **2**, **7**, **13**, and **27** were proposed as flavanones, due to an intense Band II absorption and a shoulder, or low intensity of Band I, near to 300 nm of their spectra (Mabry et al., 1970).

Compounds **4**, **5**, **14,** and **24** showed maxima absorption at 325 nm and a shoulder at 298 nm, spectra very similar to UV-Vis spectrum exhibited by chlorogenic acid (**3**), a caffeic acid derivative, characteristic of this class of phenolic compounds (Mussi de Mira et al., 2008).

Compounds **16** and **17** are also caffeic acid derivatives, formed by the condensation of two caffeic acid and one 3,4-dihydroxystyrene moieties (Tsai and Lee, 2014). Compound **16** was identified by ^1H and ^{13}C NMR as Lithospermic acid A. Compound **17** showed a similar UV-Vis spectrum of compound **16** suggesting they are similar substances, belonging to the same class (Table 3.3, Figure 3.7).

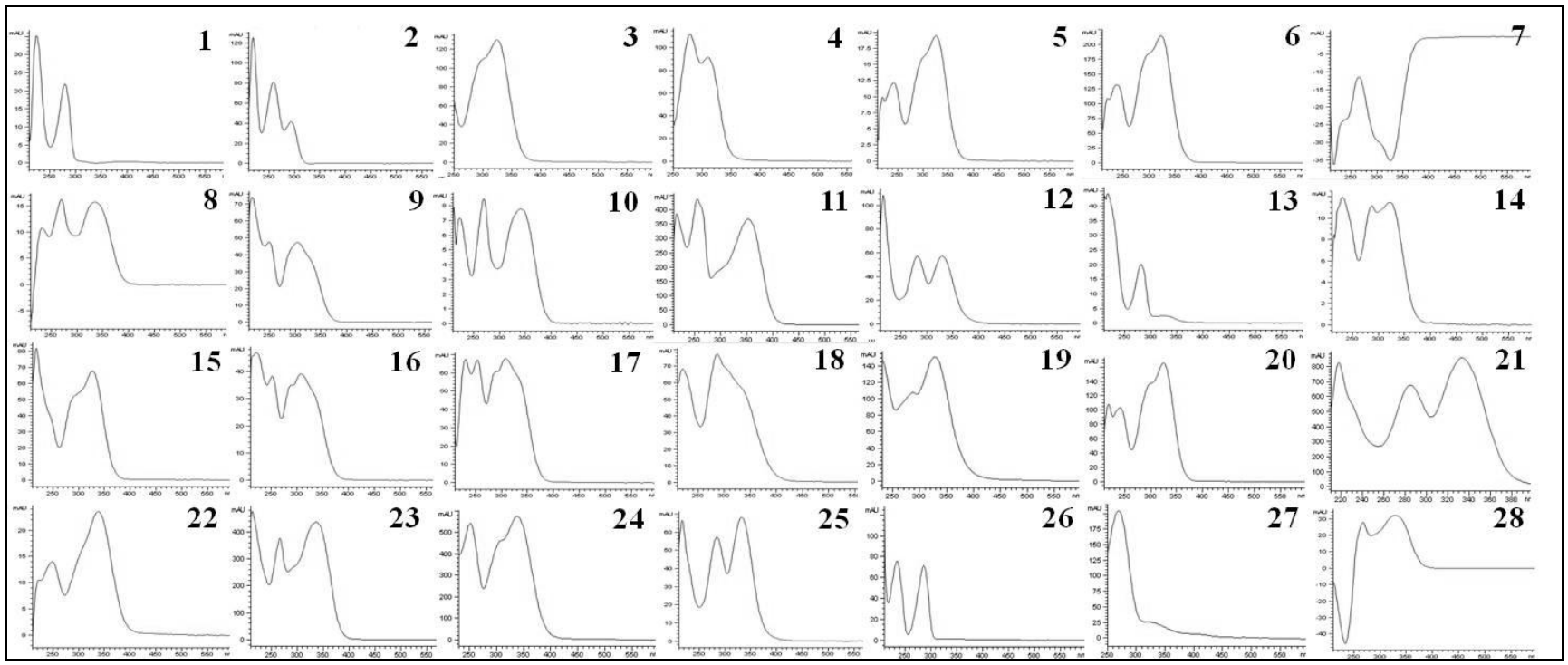

Figure 3.7. UV/Vis absorption spectra of the major constituents detected by HPLC-DAD (280 nm) in the crude ethanol extract and phases of *H. radicans* and *H. multibracteata*. Numbers correspond to the identification shown in **Table 3.3.**

Phenolic acids and flavonoids are frequently described in Lamiaceae species. Flavonoids and phenolic acids were reported as major constituents of *Rosmarinus officinalis*. Caffeic acid and rosmarinic acid were described for *Lavandula angustifolia* Miller (lavender), *Stachys officinalis* (L.) Trev. (Bruconic) showed caffeic acid, rosmarinic and chlorogenic acid, and *Hypenia salzmanii* (Benth.) Harley contains some phenolic derivatives as *p*-methoxycinnamic acid (Messana et al., 1990), some glycosylated phenylpropanoids, chlorogenic acid, protocatechinic acid, hydroquinone, and *thymohydroquinone* (Pedersen, 2000).

Among phenolic compounds, rosmarinic acid and cinnamic and chlorogenic acid derivatives are always described as major phenolic constituents in *Hyptis* (Falcão and Menezes, 2003). Table 2.1 (Chapter II) also pointed out to cinnamic and chlorogenic acid derivatives as major constituents of *Hyptis* species, like rosmarinic acid in *H. campestris*, *H. lappulacea*, *H. meridionalis* and *H. comaroides* (Santos et al., 2018), and nepetoidins A and B in *H. ramose* (Grayer et al., 2003). The present study corroborates the major presence of rosmarinic acid and chlorogenic acid derivatives in *H. radicans* and *H. multibracteata*.

Major constituents found in *H. radicans* and *H. multibracteata* are listed in table 3.3, together with their retention time, description of the UV/Vis spectra, as well as their identification.

Using semi-preparative HPLC was possible to isolate a large number of compounds from both species. However, quantities of each isolate compound limited the number of samples sending to NMR analyzes. It was possible to group sub-fractions composed by the same compound, increasing it mass, using HPLC-DAD.

Substances having sufficient mass (>4 mg) and without an authentic compound to compare to, were subjected to NMR ^{1}H analysis. Constituents **16**, **18**, **19**, **20**, **21**, **24**, **25**, and **26** were analyzed by NMR ^{1}H, but it was possible to identify only 4 of them due to the low-quality spectra and/or the presence of impurities.

Table 3.3. Major constituents of crude ethanol extract (**EE**) and phases (**EAP**, ethyl acetate; **HP**, hexane; and **HMP**, hydromethanol) of *H. radicans* and *H. multibracteata* analyzed by HPLC-DAD (280 nm) and expressed as mg g^{-1}. RT: retention time.

N	RT (min)	EE		EAP		HP		HMP		UV/ Vis (nm)	Suggestion
		Hrad	*Hmul*	*Hrad*	*Hmul*	*Hrad*	*Hmul*	*Hrad*	*Hmul*		
1	2.451	0.50 ^{P}	0.69 ^{P}	0.81 ^{P}	2.21 ^{P}			1.61 ^{P}	0.39 ^{P}	280	Gallic acid
2	3.164			1.36 ^{L}	2.12 ^{L}					260, 292	Flavanone
3	4.198		1.47 ^{P}		2.03 ^{P}					290 (sh), 330	Chlorogenic acid
4	4.947		1.53 ^{P}	1,65 ^{P}	1.06 ^{P}					280, 310	Chlorogenic acid Der.
5	5.947				0,77 ^{P}					298(sh), 325	Chlorogenic acid Der.
6	6.428		4.66 ^{P}	3.52 ^{P}	4.16 ^{P}			0.88 ^{P}		298 (sh), 320	Caffeic acid
7	8.205		6.63 ^{L}		6.64 ^{L}					262, 302 (sh)	Flavanone
8	9.287			1.17 ^{L}				1.10 ^{L}		270, 338	Apigenin Der.
9	10.129			0.97 ^{P}				0.61 ^{P}		310, 335 (sh)	*p*-coumaric acid
10	10.841			1.83 ^{L}				0.96 ^{L}		270, 345	Apigenin Der.
11	13.301				0.38 ^{L}					260, 270 (sh), 290 (sh), 360	Rutin
12	15.063			4.85 ^{L}						280, 330	Apigenin Der.
13	16.754							3.32 ^{L}		280, 330 (sh)	Flavanone
14	18.903			2.05 ^{P}						290, 320	Chlorogenic acid Der.
15	19.783	17.64 ^{P}	3.89 ^{P}		8.08 ^{P}			8.27 ^{P}		290 (sh), 325	Rosmarinic acid
16	22.642	20.59 ^{L}		14.37 ^{L}				37.86 ^{L}		252, 290 (sh), 310, 340 (sh)	Lithospermic acid A
17	27.324							2.44 ^{L}		255, 288 (sh), 310, 340 (sh)	Chlorogenic acid Der.
18	28.437			1.06 ^{P}				1.14 ^{P}		288, 340 (sh)	Cinnamic acid Der.
19	29.242	3.99 ^{L}		2.50 ^{L}				3.62 ^{L}		270 (sh), 290 (sh), 330	Flavonoid
20	29.338	1.39 ^{P}	1.48 ^{P}	3.96 ^{P}	1.24 ^{P}					295 (sh), 325	Ethyl caffeate
21	29.590		2.32 ^{L}	6.48 ^{L}	1.60 ^{L}					290, 334	Apigenin Der.

Tabela 3.3. continued.

N	RT (min)	EE		EAP		HP	HMP	UV/ Vis (nm)	Suggestion
22	30.480	1.41 ^{L}	2.24 ^{L}					240(sh), 250, 340	Nepetoidin A
23	30.658	0.69 ^{L}		1.74 ^{L}				270, 290 (sh), 340	Apigenin
24	31.217	1.76 ^{L}	4.71 ^{L}	2.15 ^{L}	1.45 ^{L}			252, 305 (sh), 340	Nepetoidin B
25	33.778	5.06 ^{L}		13.37 ^{L}		0.99 ^{L}		285, 332	Cirsimaritin
26	35.549		1.11 $^{\rho}$				0.64 $^{\rho}$	235, 290	NI
27	36.072		2.92 ^{L}				3.10 ^{L}	275, 330 (sh)	Flavanone
28	38.065					0.75 ^{L}		270, 330	Apigenin Der.

^{P}: Values expressed in milligrams of substances equivalent to *ρ*-cumaric acid per gram of extract (mg *p*CE g^{-1}); ^{L} : Values expressed in milligrams of substances equivalent to Luteolin per gram of extract (mg LE g^{-1}); **(sh)**: Shoulder.

NMR results

Substance (**16**) **3HRF5-D** corresponds to **Lithospermic acid A** (table 3.3) and presented the following characteristics in the methodologies used for its identification: mass: 71 mg, UV λ_{max} (methanol): 252, 290 (sh), 310, 340 (sh) nm and the ^{1}H, ^{13}C NMR and two-dimensional spectra (HMBC and HSQC) are showed in table 3.4 (Supplementary information, figures: 3.8.1-3.8.8). The ^{13} C NMR showed 27 signals, of which 20 signals were found between δ 115-150 and were related to aromatic ring carbons and double bond carbons suggesting the presence of 3 aromatic rings in the structure. Additionally, a signal at δ 166.91 referring to an ester and two signals at δ 172.85 and δ 174.52 referring to two carboxylic acids. A signal at δ 36.70 referring to a methylene carbon, a signal in δ 56.89 referring to a methane group bonded at an aromatic ring and two more signals at δ 74.07 and δ 87.94 referring to oxygenated methine carbons linked to a carboxylic group (C8"and C8'). ^{1}H NMR showed 10 signals referring to aromatic ring hydrogens of which two signals at δ 6.82 (d, *J* = 8.4 Hz) and δ 7.22 (d, *J* = 8.4 Hz) referring to hydrogens in *ortho* position in the aromatic ring. Two signals at δ 7.86 (d, *J* = 15.9 Hz) and δ 6.35 (d, *J* = 15.9 Hz), indicating two hydrogens in the double bond in *trans* configuration constituting a caffeic acid fragment in the structure with an additional substitution at C-2. Three signals at δ 6.63 (dd, *J* = 8.0, 2.0 Hz), δ 6.70 (d, *J* = 8.0 Hz) and δ 6.84 (d, J = 2.0 Hz) referring to an aromatic ring with two hydroxyl group in C3' and C4' positions. Three additional signals at δ 6.75 (dd, *J* = 8.2, 2.0 Hz), δ 6.78 (d, *J* = 8.2 Hz) and δ 6.82 (d, *J* = 2.0 Hz) were attributed to another aromatic ring with two hydroxyl groups in C3" and C4" positions. Two signals at δ 3.0 (dd, *J* = 14.2, 8.4 Hz) and 3.08 (dd, *J* = 14.2, 4.1 Hz) referring to two hydrogens of a methylene group at C7' and a signal at δ 5.14 (dd, *J* = 8.4, 4.1 Hz) referent to a hydrogen linked to an oxygenated carbon at C8'. A signal at δ 5.92 (d, *J* = 4.8 Hz) referring to an additional hydrogen bonded at an oxygenated carbon in C7". A signal at δ 4.38 (d, *J* = 4.8 Hz) referring to a hydrogen from a methine group bonded to aromatic ring at C8". The analysis of ^{1}H and ^{13}C NMR spectra indicates the presence of a rosmarinic acid derivative and a unit of caffeic acid from which its position in the structure was defined through two-dimensional analyzes, such as HMBC and HSQC spectra. HMBC Spectrum showed two important correlations to define the position of the third unit: where H7" and H8" correlated with C3 and C2 of the caffeic acid unit of rosmarinic acid indicating the formation of a dihydrofuran ring. Through the analysis of the

spectra, the presence of **lithospermic acid A** was suggested and confirmed in comparison with data from the literature (Table 3.4).

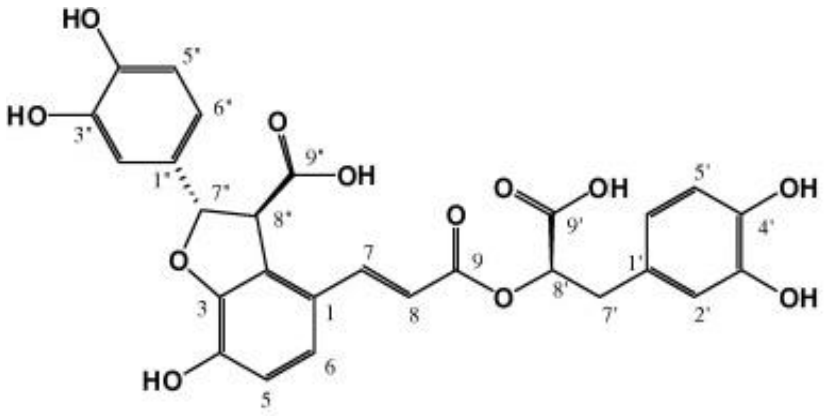

Figure 3.8. Structure of **Lithospermic acid A.**

Table 3.4. ^{1}H, ^{13}C-NMR and HMBC data of compound **Lithospermic acid A (16)** in CD_3OD compared to data from the literature in CD_3OD.

^{1}H position	**δ_H/ppm [mult., *J* in Hz]***	**δ_C/ppm****	**HMBC**	**δ_H/ppm[mult., *J* in Hz] (Thuong et al, 2009)$^{\Lambda}$**	**δ_H/ppm [mult., *J* in Hz] (Thuong et al, 2009)$^{\Lambda\Lambda}$**
1		123.29			
2		126.84			
3		147.44			
4		143.67			
5	6.82 (d, 8.4)	116.43	C_3; C_1	6.82 (d, 8.4)	6.82 (d, 8.4)
6	7.22 (d, 8.4)	120.17	C_2; C_4	7.20 (d, 8.4)	7.20 (d, 8.4)
7	7.86 (d, 15.9)	142.54	C_1; C_2; C_8; C_9	7.84 (d, 16.2)	7.84 (d, 16.2)
8	6.35 (d, 15.9)	115.02	C_9; C_1	6.32 (d, 16.2)	6.32 (d, 16.2)
9	-	166.91			
1'	-	128.31			
2'	6.84 (d, 2.0)	112.16	C_7'	6.8 (br s)	6.8 (br s)
3'	-	143.74			
4'	-	144.68			
5'	6.70 (d, 8.0)	115.02	C_1'; C_4'	6.68 (d, 8.1)	6.68 (d, 8.1)
6'	6.33 (dd, 8.0, 2.0)	116.95	C_7'; C_3'	6.61 (dd, 8.1, 1.8)	6.61 (dd, 8.1, 1.8)

Table 3.4. continued.

^{1}H position	δ_H/ppm [mult., *J* in Hz]*	δ_C/ppm**	HMBC	δ_H /ppm[mult., *J* in Hz] (Thuong et al, 2009)$^{\Delta}$	δ_H/ppm [mult., *J* in Hz] (Thuong et al, 2009)$^{\Delta\Delta}$
7'	3.0 (dd, 14.2, 8.4) 3.08 (dd, 14.2, 4.1)	36.70	C_8'; C_1'; C_9'; C_6'	3.05 (dd, 14.1, 8.4) 2.99 (dd, 14.1, 3.6)	3.05 (dd, 14.1, 8.4) 2.99 (dd, 14.1, 3.6)
8'	5.14 (dd, 8.4, 4.1)	74.07	C_7'; C_9'; C_1'	5.12 (dd, 8.0, 3.6)	5.12 (dd, 8.0, 3.6)
9'	-	172.85			
1''	-	132.6			
2''	6.82 (d, 2.0)	116.72	C_8''; C_4''	6.80 (s)	6.80 (s)
3''	-	145.18			
4''	-	145.27			
5''	6.78 (d, 8.2)	114.91	C_1''	6.77 (d, 8.1)	6.77 (d, 8.1)
6''	6.75 (dd, 8.2, 2.0)	120.39	C_8''; C_5''	6.72 (d, 8.1)	6.72 (d, 8.1)
7''	5.92 (d, 4.8)	56.89	C_2; C_3; C_1; C_1''; C_2''; C_9''	5.90 (d, 4.8)	5.90 (d, 4.8)
'8''	4.38 (d, 4.8)	87.94	C_2; C_1''; C_3; C_9''	4.36 (d, 4.5)	4.36 (d, 4.5)
9''	-	174.52			

Mult.: Multiplicity; **br s**: broad singlet; *: 500 MHz ^{1}H; **: 125 MHz 13C; $^{\Delta}$: 300 MHz; $^{\Delta\Delta}$: 75 MHz

Substance (**20) 2HRF2-V** corresponds to **ethyl caffeate** (Table 3.3) and presented the following characteristics in the methodologies used for its identification: mass: 38.3 mg, UV λ_{max} (methanol): 255, 348 nm and the ^{1}H NMR spectrum (Supplementary information, fig. 3.9.1) showed two signs at δ 6.7 and δ 7.55 both with coupling constant at *J*= 15.9 Hz suggesting the presence of α- and β- olefinic hydrogens with *trans* configuration, respectively. Signals at δ 6.96 (dd, *J*=8.3 and 2.2 Hz), δ 6.79 (d, *J*=8.2 Hz) and δ 7.05 (d, *J*=2.1 Hz) are characteristics of a 1,3,4-trisubstituted aromatic ring. Methylene group of the ethyl ester presents itself as a quartet at δ 4.23 (q, *J*=7.1 Hz) and the terminal methyl group as a triplet at δ 1.33 (t, *J*=7.1 Hz) (Supplementary information fig. 3.9.1). The above signs suggest the presence of the phenolic compound ethyl caffeate. Table 3.5 compares the experimental data with those found in the literature.

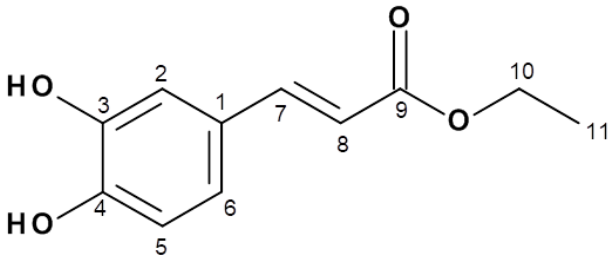

Figure 3.9. Structure of **ethyl caffeate.**

Table 3.5. ^{1}H NMR data for **Ethyl caffeate** (**20**) in CD_3OD compared to data from the literature in DMSO-d6.

^{1}H position	**δ_H/ppm [mult.; *J* in Hz]**	**δ_H/ppm [mult. *J* in Hz] (Shi et al, 2008)**
2	7.05 (d, 2.1)	7.03 (d, 2.0)
5	6.96 (dd, 8.3; 2.2)	6.98 (dd, 8.0; 2.0)
6	6.79 (d, 8.2)	6.75 (d, 8.0)
7	7.55 (d, 15.9)	7.46 (d, 16.0)
8	6.27 (d, 15.9)	6.24 (d, 16.0)
10	4.23 (q, 7.1)	4.14 (q, 7.2)
11	1.33 (t, 7.1)	1.22 (t, 7.2)

CD_3OD: deuterated methanol; **ppm:** parts per million

Substance (**24**) **2HMF2-M** corresponds to **Nepetoidin B** (table 3.3) and presented the following characteristics in the methodologies used for its identification: mass: 6.4 mg, UV λ_{max} (methanol): 252, 305 (sh), 340 nm and ^{1}H NMR: δ_H: The signals at 6.49 (d; *J*= 15.8 Hz) and δ 7.75 (d; *J*= 15.8 Hz) are indicating the α and β olefinic hydrogens with *trans* configuration corresponding to 7 and 8 positions, respectively. The signs at δ 7.15 (d; *J*= 2.1 Hz); δ 6.84 (d; *J*= 8.2 Hz) and δ 7.07 dd (J= 8.2 and 2.1 Hz) are characteristics of a 1,3,4-trisubstituted aromatic ring with two hydroxyls in *ortho*-position in A ring. These data suggest the presence of a caffeoyl moiety in the structure. The signals at δ 7.26 d (J= 7.2 Hz) and δ 5.65 d (J= 7.2 Hz) are

indicating the α and β olefinic hydrogens, respectively with the *cis* configuration corresponding to 7' and 8' positions. Additional signals at δ 7.32 d (J= 2.0 Hz), δ 6.77 d (J= 8.2 Hz) and δ 6.93 dd (J= 8.2; 2.0 Hz) are characteristics of a second 1,3,4-trisubstituted aromatic ring (B-ring) with two hydroxyl groups in *ortho*-positions. (Table 3.6, ^{1}H NMR, supplemental figure 3.10.1)

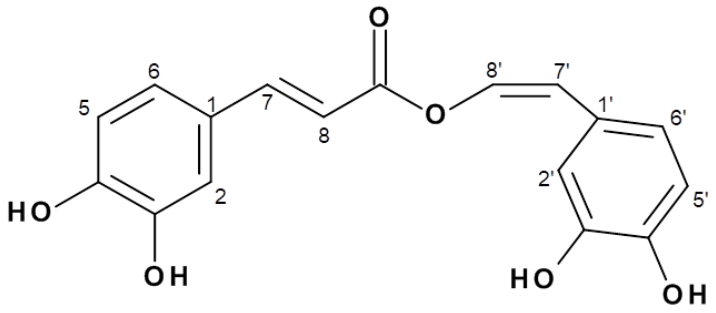

Figure 3.10. Structure of **Nepetoidin B.**

Table 3.6. ^{1}H NMR data for **Nepetoidin B** (**24**) in CD_3OD compared to data from the literature in DMSO-d6.

^{1}H position	**δ_H/ppm [mult.; *J* in Hz]**	**δ_H/ppm [mult.; *J* in Hz] (Nakanishi et al, 1990)**
7	6.49 (d, 15.8)	6.49 (d, 16.0)
8	7.75 (d, 15.8)	7.69 (d, 16.0)
2	7.15 (d, 2.1)	7.18 (d, 2.0)
5	6.84 (d, 8.2)	6.83 (d, 8.0)
6	7.07 (dd, 8.2; 2.1)	7.11 (dd, 8.0; 2.0)
7'	7.26 (d, 7.2)	7.20 (d, 7.5)
8'	5.65 (d, 7.2)	5.67 (d, 7.5)
2'	7.32 (d, 2.0)	7.24 (d, 2.0)
5'	6.77 (d, 8.2)	6.75 (d, 8.3)
6'	6.93 (dd, 8.2; 2.0)	6.91 (dd, 8.3; 2.0)

CD_3OD: deuterated methanol; **ppm:** parts per million

Substance (**25**) **2HRF2-X** corresponds to **Cirsimaritin** (table 3.3) and presented the following characteristics in the methodologies used for its identification: mass: 11 mg UV λ_{max} (methanol) 285, 332 nm and the resonance signals observed in the ^{1}H NMR spectrum (Supplemental figure. 3.11.1) indicated the presence of a singlet at δ 6.62, characteristic of an olefinic hydrogen, linked to C-3 of a flavone, and two doublets, with integration for two hydrogens each, at δ 7.86 (d, *J*= 9.0 Hz) and δ 7.00 (2H, d, *J*= 9.0 Hz) relating to a second-order coupling system of the *para*-substituted ring (ring B) of a flavonoid. In addition, the spectrum revealed a singlet at δ 6.58, integrating to a hydrogen, indicating that ring A of this compound was pentasubstituted. Two other singlets at δ 3.97 and δ 3.87, integrating to three hydrogens each, indicated the presence of two methoxyl groups attached to ring A (Table 3.7 and figure 3.11).

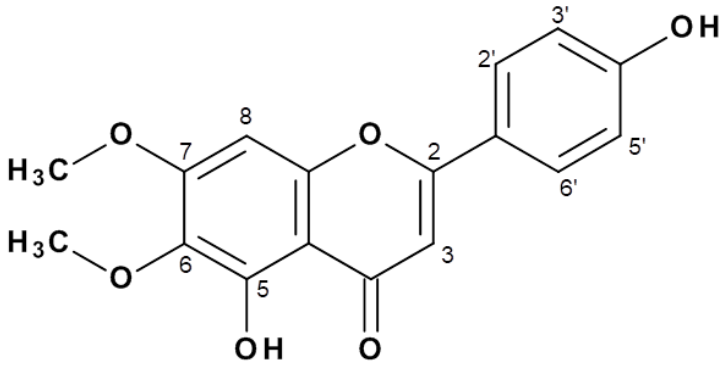

Figure 3.11. Structure of **Cirsimaritin.**

Table 3.7. ^{1}H NMR data for **Cirsimaritin** (25) in **$CDCl_3$**compared to data from the literature in **$CDCl_3$**.

^{1}H position	**δ_H/ppm [mult.; *J* in Hz]**	**δ_H/ppm [mult.; *J* in Hz] (Zuccolotto, 2017)**
3	6.62 (s)	6.59 (s)
8	6.58 (s)	6.55 (s)
2'6'	7.86 (d, 9.0)	7.79 (d, 8.8)
3'5'	7.00 (d, 9.0)	6.97 (d, 8.8)
OCH_3-C_6	3.97 (s)	3.97 (s)
OCH_3-C_7	3.87 (s)	3.93 (s)

CD_3OD: deuterated chloroform; **ppm:** parts per million

The most common phenolic acids in plant tissues are hydroxycinnamic acid derivatives. This broad class includes caffeic acids, chlorogenic acids, *o*-, *m*- and *p*-coumaric acids, ferulic acids, and sinapic acids (Castelluccio et al., 1995; Prasad et al., 2011). The combination of caffeic acid and quinic acid results in a broad class of caffeic acid esters named chlorogenic acids. As well as, rosmarinic acid is an ester of caffeic acid, but having 3,4-dihydroxyphenyl lactic acid in its structure (Petersen, 2013). Chlorogenic acid, or *trans*-5-*O*-caffeoyl-D-quinate, and caffeic acid, are common constituents of coffee and some fruits, which are the most important sources of these compounds among plants, but they are also frequently reported as constituents of Lamiaceae species, as *H radicans* and *H. multibracteata* (Basílio et al., 2006; Garambone and Rosa 2008).

Chlorogenic (**3**) and caffeic (**6**) acids, are being described for the first time in this study for species of the genus *Hyptis* according to the classification of Harley and Pastore (2012) (Table 2.1, Chapter II). The ethyl ester of caffeic acid called ethyl caffeate (**20**) was described for the first time for the genus in the ethanolic extract from *Hyptis rhomboidea* by Tsai and Lee (2014) and as major constituent of leaf infusion of *Mesosphaerum suaveolens* by Bezerra and colleagues (2017). Caffeic acid is one of the most widely distributed hydroxycinnamates and can be found in diverse forms such as esters and amides (Fu et al, 2010; Thomford et al., 2018).

Rosmarinic acid (**15**) is a major constituent of Lamiaceae, especially of Nepetoideae. It cannot be considered a taxonomic marker for this subfamily due to its occurrence in other 39 families as Boraginaceae and in some monocotyledons families (Kim et al., 2015). For *Hyptis* species *sensu* Harley and Pastore (2012), rosmarinic acid was reported for *H. capitata* (Almtorp et al., 1991), *H. brevipes* and *H. lanceolata* (Pedersen, 2000), and *H. atrorubens* (Abedini et al., 2013). More recently Santos and colleagues (2018) reported the presence of rosmarinic acid in crude ethanol extract of *H. campestris*, *H. comaroides*, *H. lacustris*, *H. lappulacea*, *H. meridionalis*, *H. radicans,* and *H. multibracteata*.

The best-known rosmarinic acid derivative is lithospermic acid, a conjugate of rosmarinic acid and an extra caffeic acid (Petersen and Simmonds, 2003). Lithospermic acid A (**16**) is a complex ester of caffeic acid characterized by the presence of a dihydrobenzofuran nucleus (Fig 3.8) (Watzke et al., 2006). It is the principal polyphenolic acid constituent of roots of *Lithospermum ruderale* Dougl. ex Lehm. and is also found in roots of *Lithospermum*

erythrorhizon both Boraginaceae (Kelley et al., 1975; Thuong et al., 2009). Lithospermic acid is one of the six major phenolic acids reported in the roots of *Salvia miltiorrhiza (*Danshen), Lamiaceae (Liu et al., 2006; Chan and Ho, 2015). It was also found in the aqueous ethanolic extract of the aerial parts of *Origanum vulgare* L. (Lin et al., 2003). This is the first time that this compound is described for *Hyptis* species and in this study was found only as *H. radicans* constituent (Tab. 3.3).

In addition, Grayer and colleagues (2003) identified other caffeic acid esters known as nepetoidin A and nepetoidin B in extracts of *H. ramose* and *H. lanceolata.* Nepetoidins were also reported by Tsai and Lee (2014) as constituents of *H. rhomboidea*. Grayer and colleagues (2003) analyzed 166 species from 78 Lamiaceae genera and correlated families and observed the presence of nepetoidins in 110 of 116 Nepetoideae species. Falcão and colleagues (2013) also observed the presence of these compounds in the vast majority of the species belonging to Nepetoideae and their absence in other subfamilies. The authors proposed these two nepetoidins as chemotaxonomic markers for Nepetoideae.

The present results corroborate rosmarinic acid (**15**), chlorogenic acids, and nepetoidins as common constituents of Nepotoideae. Furthermore, the results corroborate the presence of these constituents also in *Hyptis* species.

Another prominent phenolic acid is gallic acid**,** or 3,4,5-trihydroxybenzoic acid, consisting of a trihydroxylated phenolic structure. It is an intermediate of secondary plant metabolism in higher plants, which is found in a wide variety of vegetables, fruits, tea, coffee and wine (Grundhöfer et al., 2001; Nayeem et al., 2016). It was described in crude aqueous extract of *Mesosphaerum suaveolens (Lamiaceae)* (Bezerra et al., 2017; Thomford et al., 2018), therefore, this is the first time it is being described in *Hyptis sensu* Harley and Pastore (2012), found in *H. radicans* and *H. multibracteata*.

Three flavonoids were identified, being two flavones, Apigenin **(23)** and Cirsimatitin **(25)**, and the flavonol Rutine (**11**). Apigenin **(23)** is one of the most common flavones found in plant species and has gained particular interest in recent years as a beneficial agent and health promoting, due to its low intrinsic toxicity (Lin et al., 2008; Odonbayar et al., 2017). Cirsimatitin **(25)** is a flavone with a 5-hydroxy-6,7-dimethoxy-A-ring substitution and is considered typical of Lamiaceae. Both flavones were described and obtained from *Mesosphaerum pectinata* and *Plectranthus ecklonii,* while cirsimaritin was also described in *Condea undulata* (Gaspar-

Marques, 2008). According to the new classification of Harley and Pastore (2012), this would be the first time that cirsimaritin is being described for *Hyptis*; it was found in *H. radicans* as the second most abundant compound of the **EAP** (13.37 mg LE g^{-1}). Rutine **(11),** or 3′,4′,5,7-tetrahydroxy-flavone-3-rutinoside, is a flavonol diglycoside already described by Pedroso and colleagues (2017) in *Hyptis marrubioides* and by Okhale and colleagues (2018) in *Mesosphaerum pectinata*. However, major flavonoid constituents in *H. radicans* and *H. multibracteata* are flavones, especially apigenin derivatives.

Quantification and identification of nonpolar substances

The hexane phase of *H. radicans* and *H. multibracteata* were submitted to a qualitative analysis by GC-MS. Chromatograms obtained are shown in Figure 3.12 and mass spectra fragmentation is displayed in Table 3.8. Crude ethanol extract, ethyl acetate and hydrometanol phases, although presenting several polar compounds, also showed apolar substances. A relative percentage of these compounds in each extract and phase is presented in Table 3.7; compounds presenting <1% of abundance were not integrated and reported.

Constituents were analyzed by GC-MS with electrospray ionization (ESI) operated in negative mode using NIST Library 2.0f (2008) version. NIST Library suggestion was considered when the "mainlib" (only a single spectrum for each compound) and the "replib" (replicate spectra of compounds in the mainlib that are often encountered in various GC/MS analyses, like solvents, pesticide, food additives, toxins, etc.) got multiple hits for a single compound; and when match factor values were >850, indicating high probability of a correct hit (NIST, 2017).

Matchs given by the NIST library were also verified using databases of mass spectra such as: SciFinder, PubChem, NIST Chemistry WebBook (SRD 69), The Lipid Web, MassBank of North America (MoNA), GNPS Spectral Libraries. In some cases, when identification by the NIST library was not possible, tools for predicting spectra such as MoNA, interpretation of mass spectrometric fragmentation patterns and literature mass spectra were used and compared to each other.

Compound identification based on mass spectra has limited accuracy because of the number of isomeric forms and variability of chemical classes present in complex samples, for example volatile oils and crude extracts make the separation of the compounds by conventional gas chromatography (GC-MS) often unattainable. In addition, identification based on

similarities of mass spectra obtained experimentally with a library cannot be performed without doubt when the obtained spectra are extremely similar between the isomers present in a sample (Mühlen, 2009) and that is the reason why in the present study only suggestions are presented for the identifications obtained by GC-MS (Table 3.8).

As an important family of aromatic species (Lamiaceae), main compounds investigated were constituents of volatile oils. The main chemical constituents reported for Lamiaceae are eugenol, nerol, caryophyllene, terpinene, α-selinene, α and β-pinene, camphor and carvacrol, cineole, linalool, and fatty acids (Gayathiri et al., 2016).

In the present work we describe apolar constituents for crude ethanol extract and phases of *H. radicans* and *H. multibracteata.* All substances were identified using the NIST Library 2.of (2008) and by comparing the mass spectrum with those of the literature *(*Supplementary information).

Hexahydrofarnesyl acetone **(7)**, is a terpene ketone in which an (*E, E*)-farnesyl group is bonded to one of the α-methyls of acetone (Chemical Entities of Biological Interest, also known as ChEBI, 2012). It was described in *Hyptis atrorubens* (Kerdudo et al., 2016), *Hyptis conferta* (Tafurt-Garcia et al., 2014), *Hyptis brevipes Poit.* (Bhuiyan and Begum, 2010), and *Cantinoa spicigera* (Bogninou-Agbidinoukoun et al., 2012; Harley and Pastore, 2012).

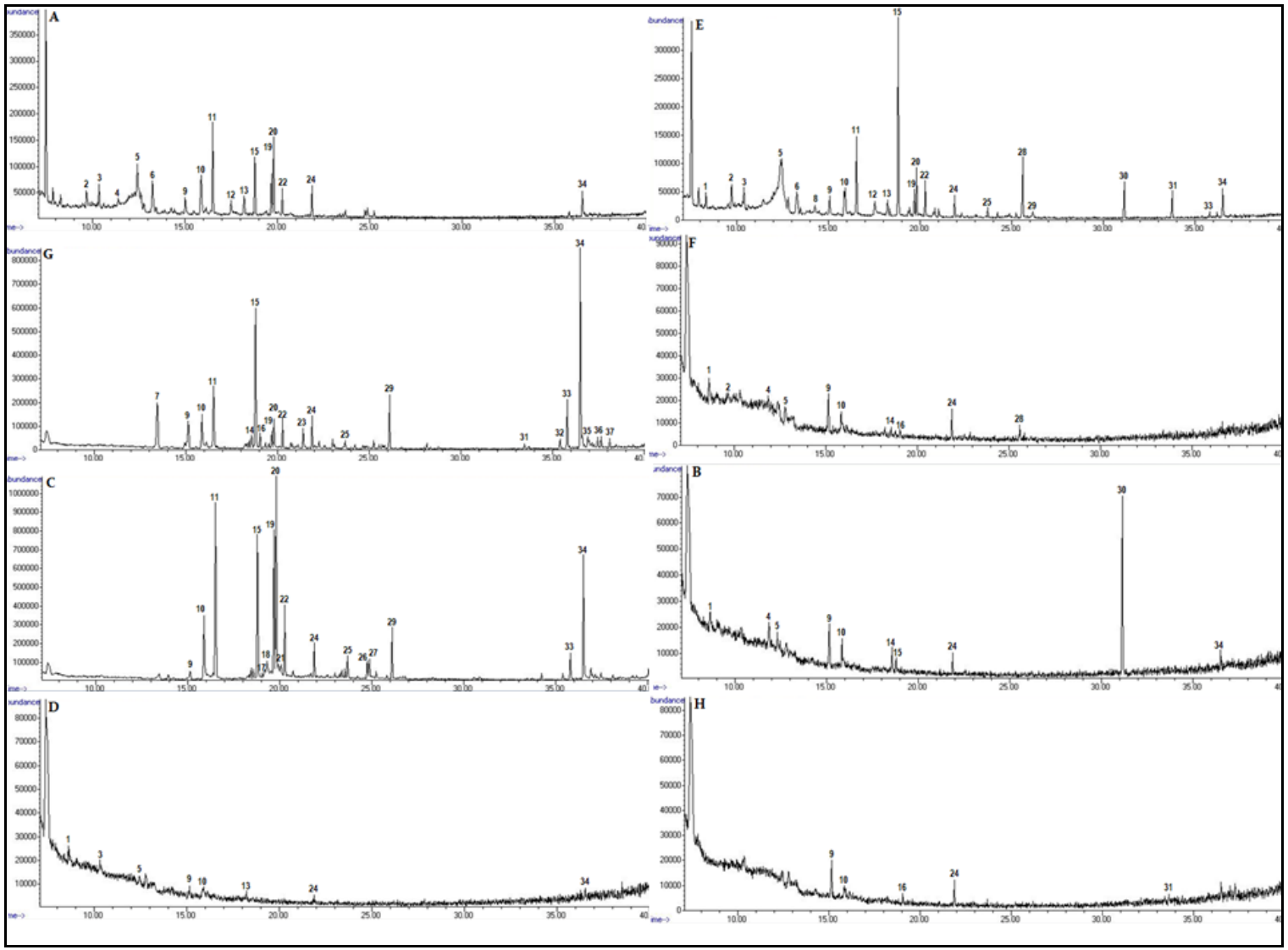

Figure 3.12. CG/MS Chromatograms of extract and phases of: *H. radicans* - **A**. Ethanol extract, **B**. ethyl acetate phase, **C**. hexane phase, **D**. Hydromethanol phase; and *H. multibracteata*, **E**. Ethanol extract, **F**. ethyl acetate phase, **G**. hexane phase, **H.** hydromethanol phase). Numbers above each peak correspond to the possible identifications shown in **table 3.8.**

Table 3.8. Relative abundance (%) and mass spectra fragmentation of constituents from crude ethanol extract (EE) and phases (EAP, ethyl acetate; HP, hexane; and HMP, hydromethanol) of *H. radicans* and *H. multibracteata* analyzed by GC-MS. RT: retention time.

N	RT	EE		EAP		HP		HMP		MM	MS: *m/z* (intensity, %)	Compound
	(min)	*Hr*	*Hm*	*Hr*	*Hm*	*Hr*	*Hm*	*Hr*	*Hm*			suggestion
1	8.639		1.44	5.67	8.82			5.44			63 (100); 78 (58); 60 (48); 61 (38); 65 (24); 64 (22); 79 (20); 62 (20); 80 (15); 57 (127)	NI
2	9.696	2.34	3.28		11.67						61 (100); 63 (95); 79 (89); 125 (75); 64 (58); 78 (50); 93 (26); 80 (25); 65 (21); 139 (13)	NI
3	10.367	2.68	2.21					1.09			79 (100); 61 (79); 63 (58); 125 (52); 64 (36); 78 (29); 93 (18); 80 (17); 139 (15); 65 (14)	NI
4	11.868	2.63		7.75	6.46						63 (100); 65 (46); 93 (29); 64 (25); 111 (24); 139 (21); 61 (20); 78 (20); 109 (19); 94 (14)	NI
5	12.330	7.22	6.02	4.41	5.51			4.31			124 (100); 63 (79); 65 (38); 80 (25); 94 (23); 78 (19); 61 (15); 64 (12); 139 (12); 79 (11)	NI
6	13.269	5.01	4.67								61 (100); 63 (15); 93 (92); 78 (70); 79 (52); 64 (49); 62 (37); 65 (26); 94 (25); 80 (23)	NI
7	13.462						8.73			266	58 (100); 71 (57); 57 (47); 55 (44); 59 (37); 69 (32); 85 (28); 95 (23); 109 (22); 56 (19)	Hexahydrofarnesyl acetone
8	14.284		1.82								63 (100); 57 (98); 64 (53); 76 (47); 93 (47); 212 (44); 79 (41); 69 (33); 78 (32); 80 (30)	NI
9	15.157	1.85	1.99	16.51	20.58	1.68	5.87	3.98	52.07	270	74 (100); 87 (66); 55 (30); 57 (15); 143 (14); 69 (13); 75 (11); 227 (76); 83 (65); 71 (63)	n-Hexadecanoic acid, methyl ester
10	15.903	8.95	2.58	4.30	4.93	7.30	5.99	4.20	7.11	256	60 (100); 55 (87); 57 (87); 71 (49); 69 (46); 129 (39); 83 (34); 61 (30); 85 (27); 97 (22)	n-Hexadecanoic acid

Table 3.8. Continued.

N	RT (min)	EE		EAP		HP		HMP		MM	MS: *m/z* (intensity, %)	Compound suggestion
		Hr	*Hm*	*Hr*	*Hm*	*Hr*	*Hm*	*Hr*	*Hm*			
11	16.524	9.59	6.90			15.71	7.96			284	88 (100); 101 (61); 55 (32); 57 (22); 70 (21); 69 (18); 89 (15); 61 (12); 157 (12); 60 (12)	Hexadecanoic acid, ethyl ester
12	17.506	2.47	2.03								93 (100); 61 (70); 79 (57); 63 (49); 78 (34); 64 (32); 111 (27); 171 (26); 80 (26); 65 (21)	NI
13	18.227	2.53	2.31					1.87			93 (100); 79 (31); 63 (28); 111 (24); 171 (19); 64 (16); 61 (14); 78 (17); 95 (94); 65 (91)	NI
14	18.579			6.34	4.99		2.39				79 (100); 63 (88); 67 (83); 80 (66); 95 (56); 55 (54); 93 (46); 65 (34); 64 (32); 94 (30)	NI
15	18.806	5.40	14.47	3.74		9.84	12.75			296	71 (100); 57 (34); 55 (32); 69 (26); 81 (24); 68 (21); 123 (19); 56 (17); 83 (15); 95 (15)	Phytol derivative
16	19.066				4.66		1.78		15.27	298	74 (100); 87 (79); 55 (40); 57 (29); 69 (22); 143 (16); 71 (12); 83 (12); 129 (94); 101 (77)	Methyl stearate
17	19.200					1.00				280	67 (100); 55 (78); 81 (67); 95 (52); 69 (48); 68 (46); 54 (42); 79 (37); 82 (36); 65 (31)	9,12-Octadecadienoic acid (*Z,Z*)
18	19.309					3.36					67 (100); 79 (93); 55 (84); 81 (55); 95 (53); 80 (50); 93 (38); 69 (37); 68 (34); 82 (30)	NI
19	19.695	4.35	2.39			10.82	2.09			308	67 (100); 81 (82); 55 (76); 95 (58); 79 (45); 68 (39); 82 (39); 54 (36); 69 (36); 109 (26)	9,12-Octadecadienoic acid (*Z, Z*)-ethyl ester
20	19.821		8.18			14.87	3.87			306	79 (100); 67 (69); 55 (57); 95 (51); 93 (47); 81 (45); 80 (38); 91 (28); 108 (28); 69 (26)	9,12,15-Octadecatrienoic acid, (*Z,Z,Z*)-
21	19.921					1.83					55 (100); 67 (58); 69 (56); 83 (38); 57 (37); 81 (37); 97 (35); 95 (34); 79 (32); 84 (27)	NI

Table 3.8. Continued.

N	RT (min)	EE		EAP		HP		HMP		MM	MS: *m*/*z* (intensity, %)	Compound suggestion
		Hr	*Hm*	*Hr*	*Hm*	*Hr*	*Hm*	*Hr*	*Hm*			
22	20.291	2.69	3.12			4.73	2.63			312	88 (100); 101 (62); 55 (36); 57 (29); 70 (21); 69 (20); 89 (16); 157 (14); 83 (13); 71 (12)	Octadecanoic acid, ethyl ester
23	21.415	8.06					2.15				71 (100); 57 (95); 55 (76); 83 (71); 69 (69); 95 (52); 81 (41); 97 (36); 111 (31); 109 (29)	Eicosanoic acid
24	21.884	2.96	2.04	4.62	9.39	2.36	2.81	0.76	22.47	377	61 (100); 57 (96); 55 (74); 71 (62); 239 (44); 69 (39); 85 (35); 83 (32); 97 (27); 95 (23)	Hexadecanoic acid, 4-nitrophenyl ester
25	23.696		1.01			1.53	1.04			340	88 (100); 101 (64); 55 (42); 57 (36); 69 (24); 89 (19); 70 (18); 71 (18); 83 (15); 97 (14)	Eicosanoic acid, ethyl ester
26	24.762					1.05					61 (100); 67 (97); 55 (68); 81 (64); 95 (56); 79 (44); 69 (28); 109 (25); 54 (22); 80 (20)	NI
27	24.887					1.44					61 (100); 79 (82); 67 (64); 55 (55); 95 (39); 93 (38); 81 (34); 80 (30); 69 (29); 107 (21)	NI
28	25.609		6.76		12.66					368	191 (100); 175 (75); 163 (65); 178 (59);57 (33); 135 (31); 91 (25); 368 (22); 119 (20); 141 (18)	Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-ethyl-
29	26.112		2.09			2.93	4.28			390	149 (100); 57 (35); 167 (25); 71 (21); 70 (19); 55 (16); 150 (11); 83 (76); 113 (76); 56 (72)	1,2-Benzenedicarboxylic acid, diisooctyl ester
30	31.145		3.06	24.57							57 (100); 71 (71); 85 (43); 55 (25); 56 (14); 69 (13); 97 (13); 83 (13); 99 (11); 113 (81)	Sulfurous acid, butyl dodecyl ester
31	33.763		2.50				1.08		3.07		57 (100); 71 (58); 207 (49); 73 (43); 85 (41); 55 (28); 69 (19); 83 (17); 281 (17); 56 (163)	NI

Table 3.8. Continued.

N	RT (min)	EE		EAP		HP		HMP		MM	MS: *m*/*z* (intensity, %)	Compound suggestion
		Hr	*Hm*	*Hr*	*Hm*	*Hr*	*Hm*	*Hr*	*Hm*			
32	35.407						1,66			400	55 (100); 95 (73); 79 (67); 145 (65); 71 (64); 69 (63); 57 (60); 93 (57); 105 (52); 107 (49)	24-α-Methyl-5-cholesten-3β-ol
33	35.801		1.47			2.18	4.64			412	55 (100); 83 (66); 69 (64); 81 (58); 79 (43); 67 (41); 93 (39); 97 (38); 95 (37); 159 (36)	Stigmasta-5,22-dien-3beta-ol
34	36.514	3.54	4.47	6.45		9.36	20.46	2.88		414	55 (100); 57 (86); 107 (75); 81 (72); 105 (72); 95 (66); 79 (63); 91 (62); 93 (60); 145 (59)	24-ethylcholest-5-en-3 beta-ol
35	36.925						1.31			426	218 (100); 203 (66); 55 (50); 95 (47); 105 (45); 69 (45); 57 (32); 81(31); 107 (31); 109 (28)	olean-12-en-3β-ol
36	37.471						1.28			426	218 (100); 55 (51); 95 (38); 203 (32); 189 (30); 69 (27); 67 (28) ;93 (28); 105 (26); 107 (26)	urs-12-en-3β-ol
37	37.647						1.12			410	174 (100); 55 (34); 81 (28); 187 (28); 159 (27); 410 (26); 161 (25); 79 (25); 93 (20); 175 (19)	3-Estigmasta-3,5-dien-7-ona
Fatty acids		38.45	28.21	25.43	39.56	60.0	36.19	8.94	96.92			
Terpenes		5.40	16.29	3.74	-	9.84	11.32	-	-			
Steroids		3.54	5.94	6.45	-	11.54	27.88	2.88	-			
Others		-	11.91	24.57	12.66	2.93	4.28	-	-			
NI		24.88	24.46	24.17	37.45	2.49	16.22	12.71	3.07			
Total		72.27	86.81	84.36	89.67	86.80	95.89	24.53	99.99			

MM: molecular mass of the compound; ***m*/*z***: mass/charge ratio of the fragments; -: No presence; NI: not identified.

Lipid components, especially fatty acids, are present in the most diverse forms of life, playing important roles in the structure of cell membranes and metabolic processes and are important energy source for plant tissues. In plant photosynthetic cells, the synthesis of fatty acids occurs in the stroma of chloroplasts (Heldt and Piechulla, 2004; Perini et al., 2010). Palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), and linolenic acids (18:3) are common fatty acids found in plant (Moire et al., 2004; Lim et al., 2017). Palmitic acid or n-Hexadecanoic acid (**10**) is the first fatty acid produced during lipogenesis (fatty acid synthesis), is the final product of the fatty acid synthetase, which can be elongated to stearic acid and other long-chain acids (www.drugbank.ca). Also, it is the most abundant saturated fatty acid in nature (www.lipidhome.co.uk). n-Hexadecanoic acid was described as constituent of *Hyptis atrorubens* Poit. (Kerkudo et al., 2016), *Hyptis brevipes,* and *Hyptis rhomboidea* (Xu et al., 2013). Another saturated fatty acid is Arachidic acid also called n-eicosanoic acid **(23)** and is commonly found in peanut oil. It can be formed by the hydrogenation of arachidonic acid. It was described in *Thymus mastichina* L. (Lamiaceae) (Barros et al., 2011). These two compounds belong to the class of organic compounds known as long-chain saturated fatty acids. These are fatty acids with an aliphatic tail that contains between 13 and 21 carbon atoms (www.hmdb.ca, A).

Fatty esters (esters of acids and alcohols of long chains) form one of the main classes in the composition of epicuticular waxes and are compounds containing a fatty acid that is esterified with a methyl group (www.hmdb.ca, B). They have the general structure RC(=O)OR', where R=fatty aliphatic tail or organyl group and R'=methyl group (de Siqueira et al., 2003). This class is composed mainly of derivatives of palmitic acid like Ethyl palmitate or hexadecanoic acid ethyl ester (**11**), this substance was already described in *Hyptis lanceolata* Poir. (Igwe, 2014) and methyl palmitate or n-Hexadecanoic acid methyl Ester (**9**), described in *Hyptis lanceolata* Poit*, Condea verticillate*, and *Cantinoa spicigera* (Onayade et al 1990; Kini et al., 1993; Igwe, 2014; Harley and Pastore, 2012). Methyl stearate or n-Octadecanoic acid, methyl ester (**16**) and ethyl stearate or octadecanoic acid, ethyl ester (**22**), are also members of the class of compounds known as fatty acid esters. Methyl stearate is a fat, oily, and waxy tasting compound found in cloves, which makes methyl stearate a potential biomarker for the consumption of this food product (www.hmdb.ca, B) and was described *Cantinoa spicigera* (Onayade et al., 1990). Ethyl stearate is a mild and waxy tasting compound found in coriander and sweet marjoram, which makes ethyl octadecanoate a potential biomarker for the consumption of these food products. It was described in *Phlomis*

bourgaei Boiss (Başer et al., 2008) and *Stachys swainsonii* subsp. *scyronica* (Boiss.) (Skaltsa et al., 2001), both Lamiaceae, and in species of other families such as *Raphanus raphanistrum* (Brassicaceae) (Marrelli et al., 2014) and *Sarcopoterium spinosum* (L.) Spach (Rosaceae) (Loizzo et al., 2013). Arachidic acid ethyl ester or eicosanoic acid ethyl ester **(25)**, was described by Ghalib and colleagues (2012) in *Cinnamomum iners* Reinw. ex Blume (Lauraceae).

Within the belonging class of organic compounds known as lineolic acids are derivatives of linoleic acid. Linoleic acid or 9,12-Octadecadienoic acid (*Z, Z*) (**19**), is a doubly unsaturated fatty acid, also known as an omega-6 fatty acid, 18 carbons long, with two CC double bonds at the 9- and 12-positions. Is an essential fatty acid in human nutrition, used in the biosynthesis of prostaglandins (via arachidonic acid) and cell membranes, and it is not synthesized by humans (www.hmdb.ca, C). Was described in *Hyptis atrorubens* (Kerdudo et al., 2016), *Mesosphaerum suaveolens,* and *Cantinoa spicigera* (Xu et al., 2013; Ladan et al., 2010). Another derivative is the Linoleic acid ethyl ester or 9,12-Octadecadienoic acid (*Z, Z*)-ethyl ester **(19)**, already described in *Hyptis atrorubens* (Kerdudo et al., 2016), *Heracleum sphondylium* L. (Apiaceae) (Fierascu et al., 2016), as well as, *Flourensia microphylla* (A. Gray) SF. Blake and *Flourensia retinophylla* B. L. Rob. (Asteraceae) (de Rodríguez et al, 2017). Linolenic acid methyl ester or 9,12,15-Octadecatrienoic acid, (*Z, Z, Z*) **(20)**, is a natural methylated form of α-linolenic acid (www.caymanchem.com) identified in some species of Lamiaceae as *Prunella vulgaris* L. (Golembiovska et al., 2014), *Micromeria dalmatica* Benth. (Radulović and Blagojević, 2012), and *Mentha aquatica* L. (Marrelli et al., 2014). The levels of the unsaturated fatty acids linoleic and linolenic acids are particularly important in the mechanism of plant defense, especially against pathogens (Walley et al., 2013).

Triterpenes and steroids are formed by the union of two molecules of farnesyl pyrophosphate (C15) joined tail-tail to form squalene (Dewick, 2009). Phytosteroids are modified triterpenoids containing the cyclopentaneperhydrophenanthrene tetracyclic system (rings A, B, C and D of structure 1) and are derived from cycloartanol but without the methyl group at C4 and C14. Steroids are found as free alcohol (3β-OH), esterified to fatty acids or as glycosides. They are constituents of membranes in plants, algae and fungi, influencing their permeability (Dewick, 2009). The basic sterol from which other sterol structures are defined is 5α-cholestan-3β-ol. The most commonly found phytosterols are 4-demethyl steroids such as β-sitosterol (the most abundant), stigmasterol, campesterol, and brassicasterol, according to Law (2000). The hexane phase of *H. multibracteata* showed the

first three steroids mentioned: Campesterol or 24-α-Methyl-5-cholesten-3β-ol **(32)**, Stigmasterol or Stigmasta-5,22-dien-3beta-ol **(33),** and β-sitosterol or 24-ethylcholest-5-en-3 beta-ol **(34)**. Campesterol was described in Lamiaceae in *Mesosphaerum suaveolens* (Misra et al., 1981) and *Leonotis nepetifolia* L. (Gayathiri et al., 2016), while Stigmasterol was described in *Hyptis capitata* Jacq. (Almtorp et al., 1991), *Gymneia platanifolia* (Araújo et al., 2005; Harley and Pastore, 2012), *Condea fasciculata* (Falcão et al., 2003; Harley and Pastore, 2012), and *Hyptis mutabilis* (Rich.) Briq. becomes *Cantinoa mutabilis* (Rich.) Harley & J.F.B.Pastore (Melo, 2003; Harley and Pastore, 2012). β-sitosterol was previous described in *Gymneia platanifolia* Mart. ex Benth (Araújo, et al., 2005), *Hyptis rhomboidea* M Martens & Galeotti (Lin et al., 1993), *Mesosphaerum suaveolens* (Rao and Nigam, 1972), and *Condea fasciculata* Benth (Falcão et al., 2003).

H. multibracteata also contains the triterpenes α-Amyrin (**36**) and β-amyrin (**35**). β-amyrin (olean-12-en-3β-ol) was described in *Hyptis rhomboidea* (Tang et al., 2014), *Condea verticillata* (Biggs et al., 2008; Harley and Pastore, 2012), and *Mesosphaerum suaveolens* (Misra et al., 1983), while α-amyrin (urs-12-en-3β-ol) was described in *Condea verticillata* (Biggs et al., 2008; Harley and Pastore, 2012) and *Mesosphaerum suaveolens* (Misra et al., 1983).

Regarding apolar substances, fatty acids and terpenes, especially phytol, were the most abundant compounds observed in *H. radicans* and *H. multibracteata*. For this last species, triterpenes were also observed.

Reports in literature differs from this study, just for citing two examples of this is the study of the volatile oils of *Hyptis lanceolata* that contained oxygen-containing monoterpenes (5.6 %) and oxygen-containing sesquiterpenes (6.1 %) but consisted mainly of sesquiterpenes hydrocarbons (69.7 %) and monoterpenes hydrocarbons (18.4 %) (Koba et al., 2007); and *Hyptis passerine* Mart. analyzed by GC-MS showed sesquiterpenes as major compounds. Previous studies on the constituents of *Hyptis* species have revealed sesquiterpenoids as major constituents (Table 2.1, Chapter II).

Conclusions

The present results corroborate rosmarinic acid, chlorogenic acids, and nepetoidins as common constituents of Nepetoideae. Furthermore, the results corroborate the presence of these constituents also in *Hyptis* species. Lithospermic acid A and cirsimaritin were described for the first time in this study for *Hyptis*, both found in *H. radicans*. Fatty acids and triterpenes are the most abundant kind of apolar substances in *H. radicans* and *H. multibracteata*. This differs from what is most reported in the literature; first because the majority of reports focused on volatile oils and in this study, we analyzed also the apolar constituents of aerial parts extracts.

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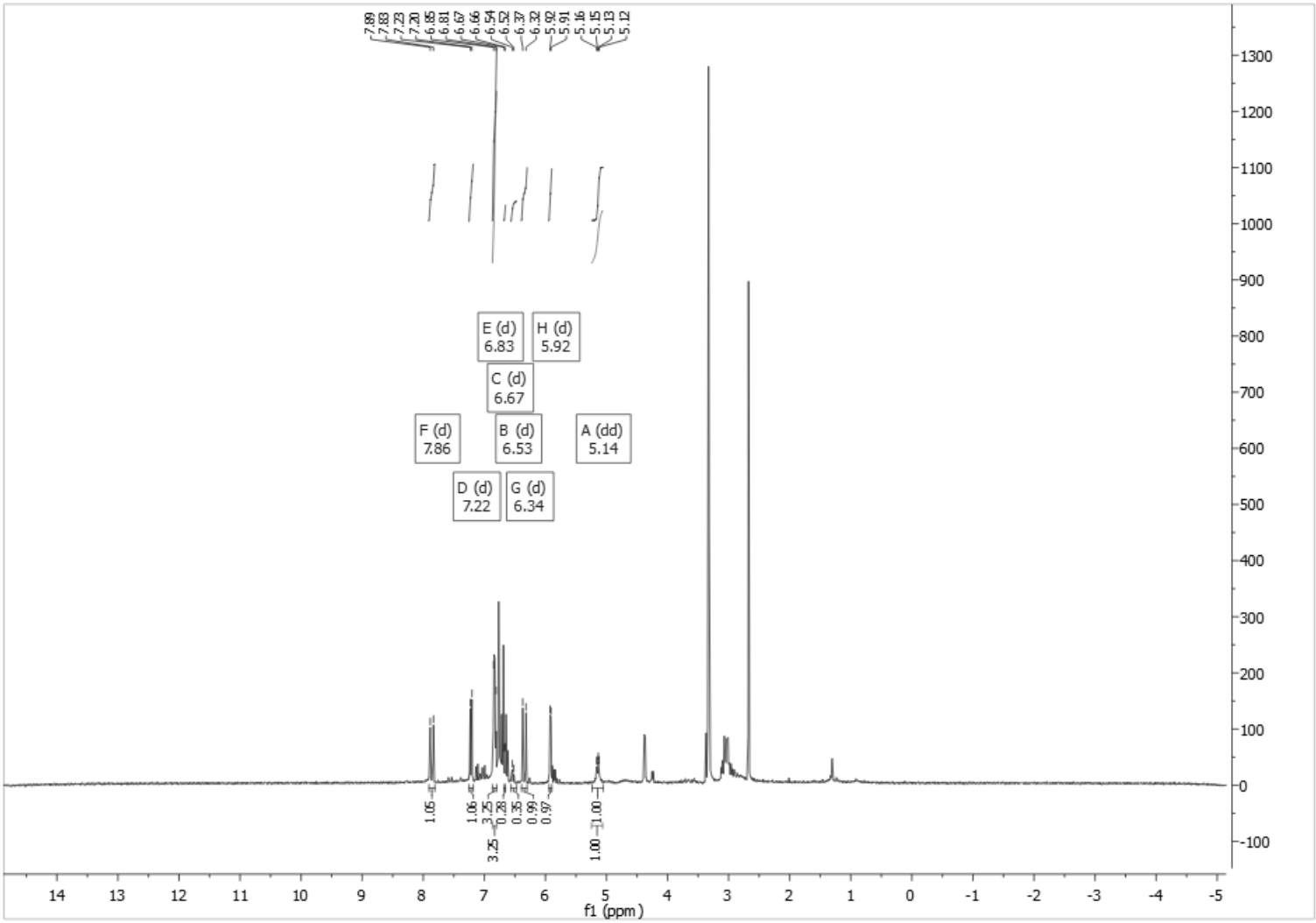
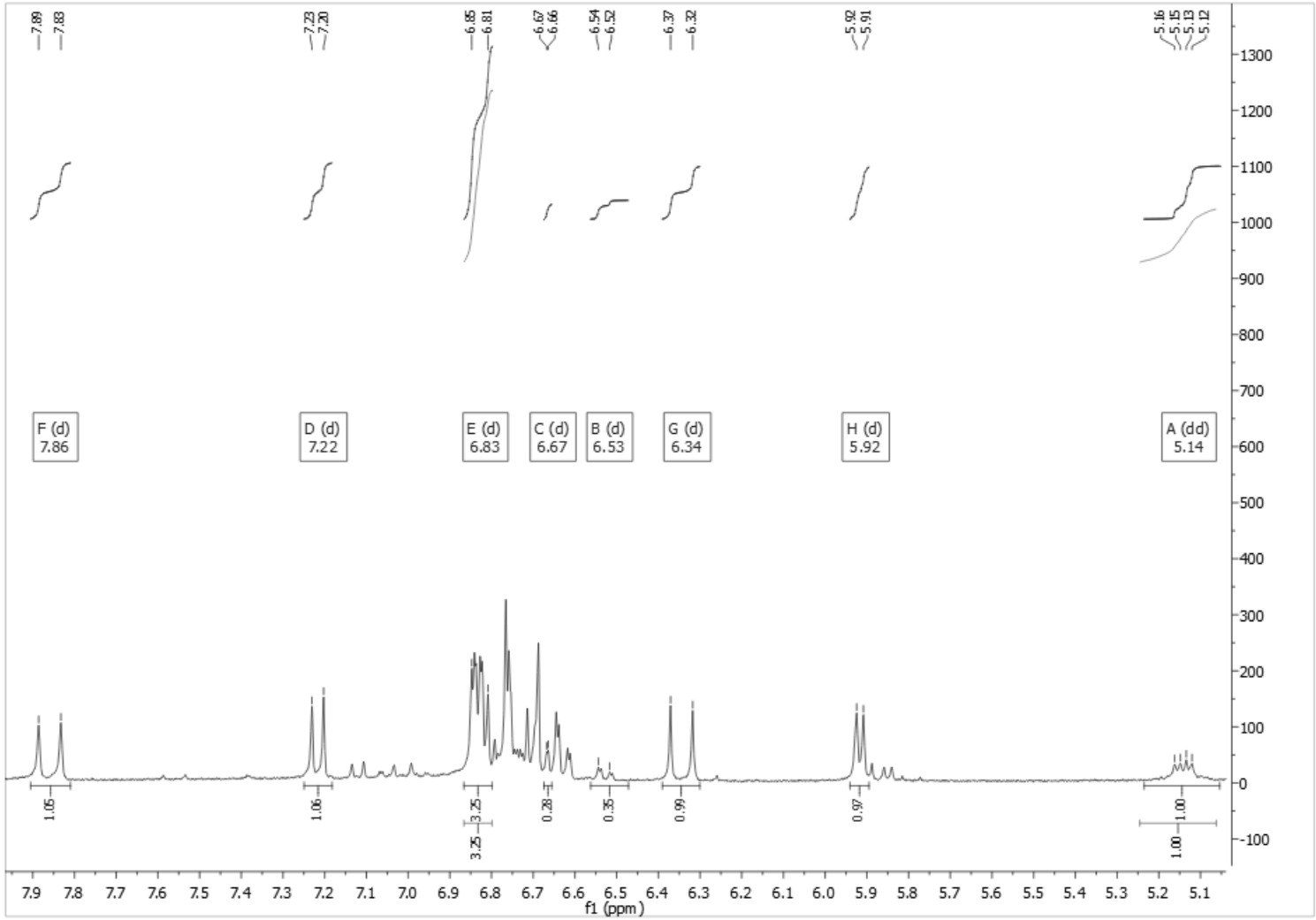
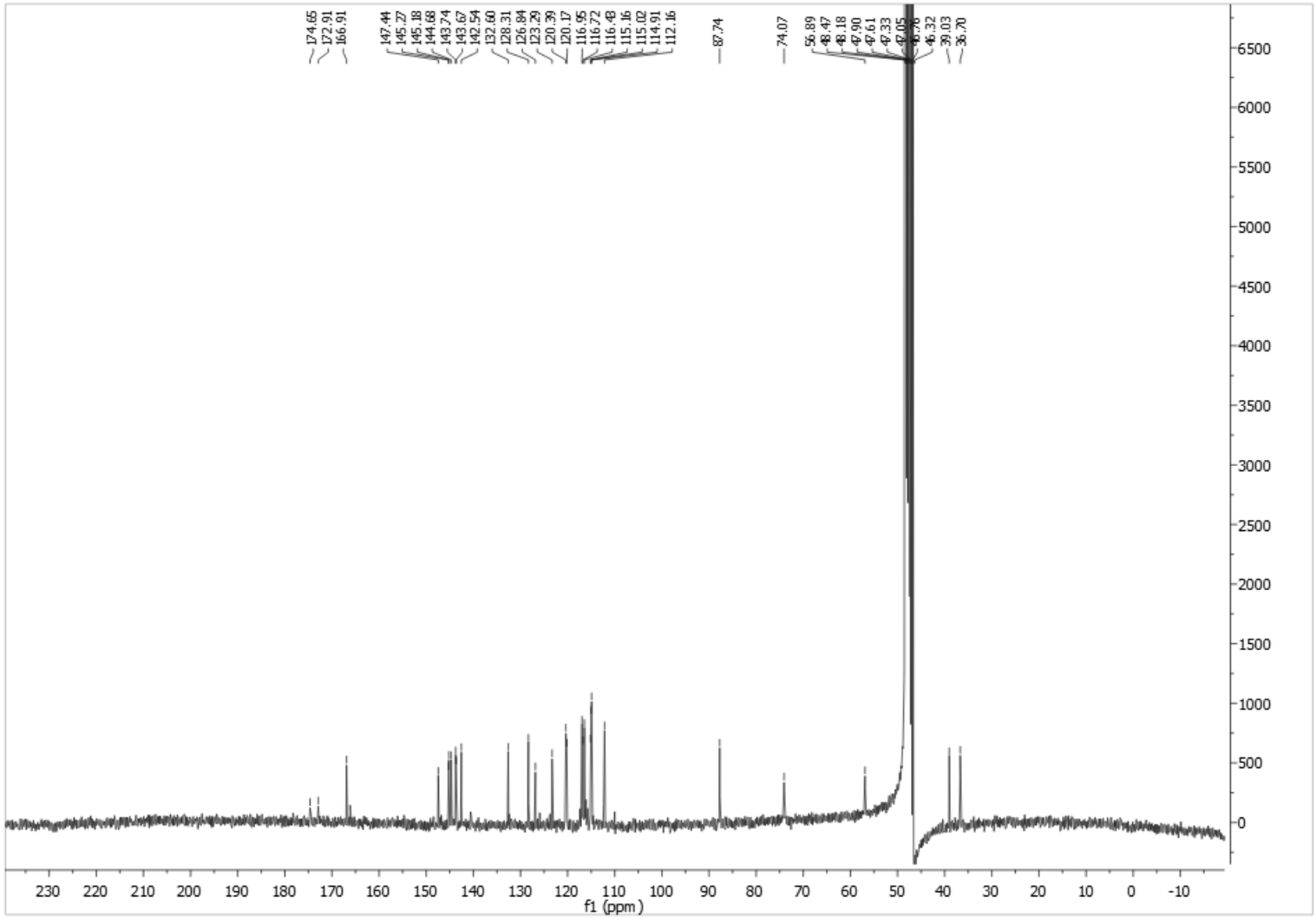
Supplementary information



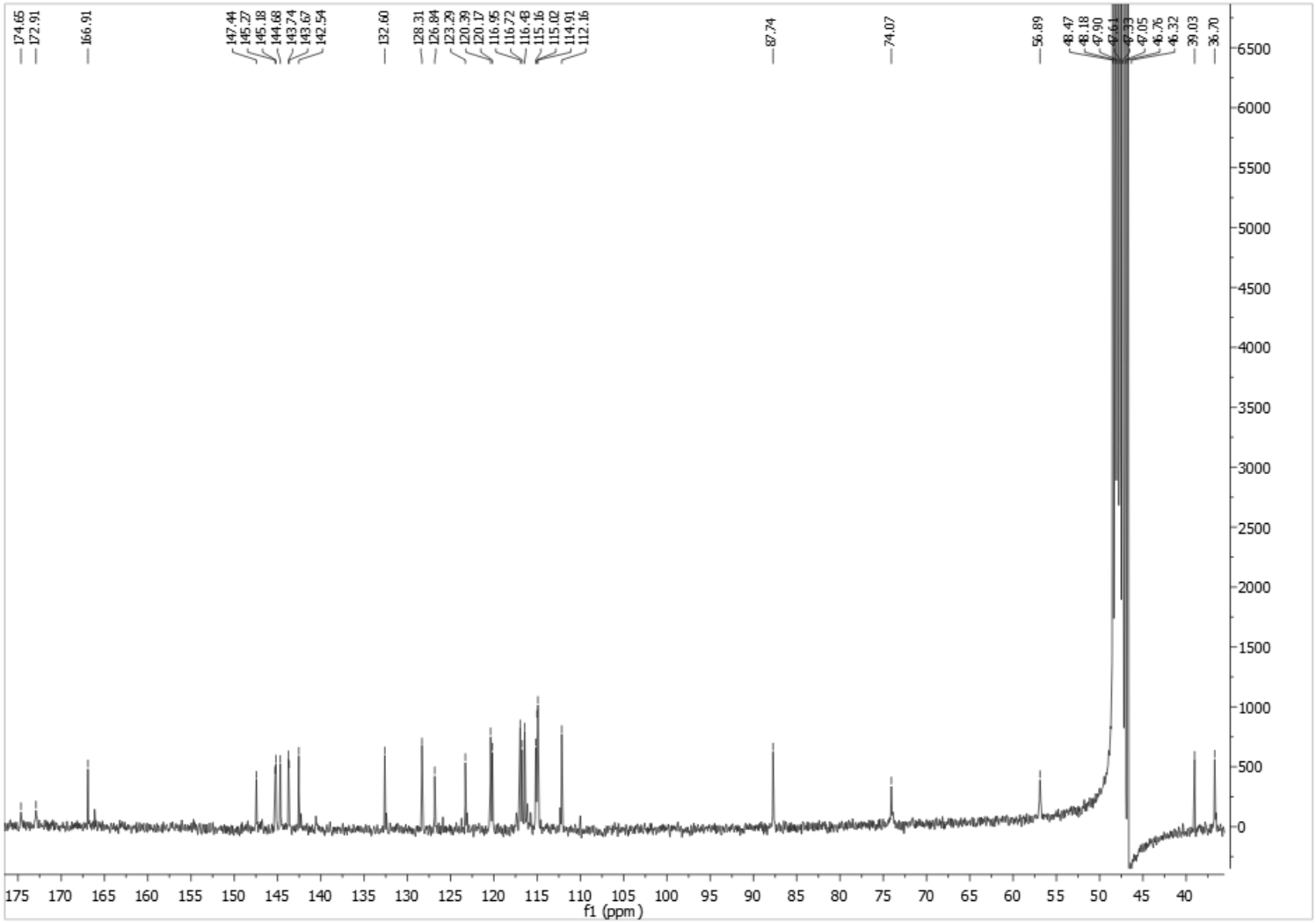
Supplemental figure 3.8.1. ^{1}H NMR spectra (CD_3OD) of **Lithospermic acid A** (**16**).



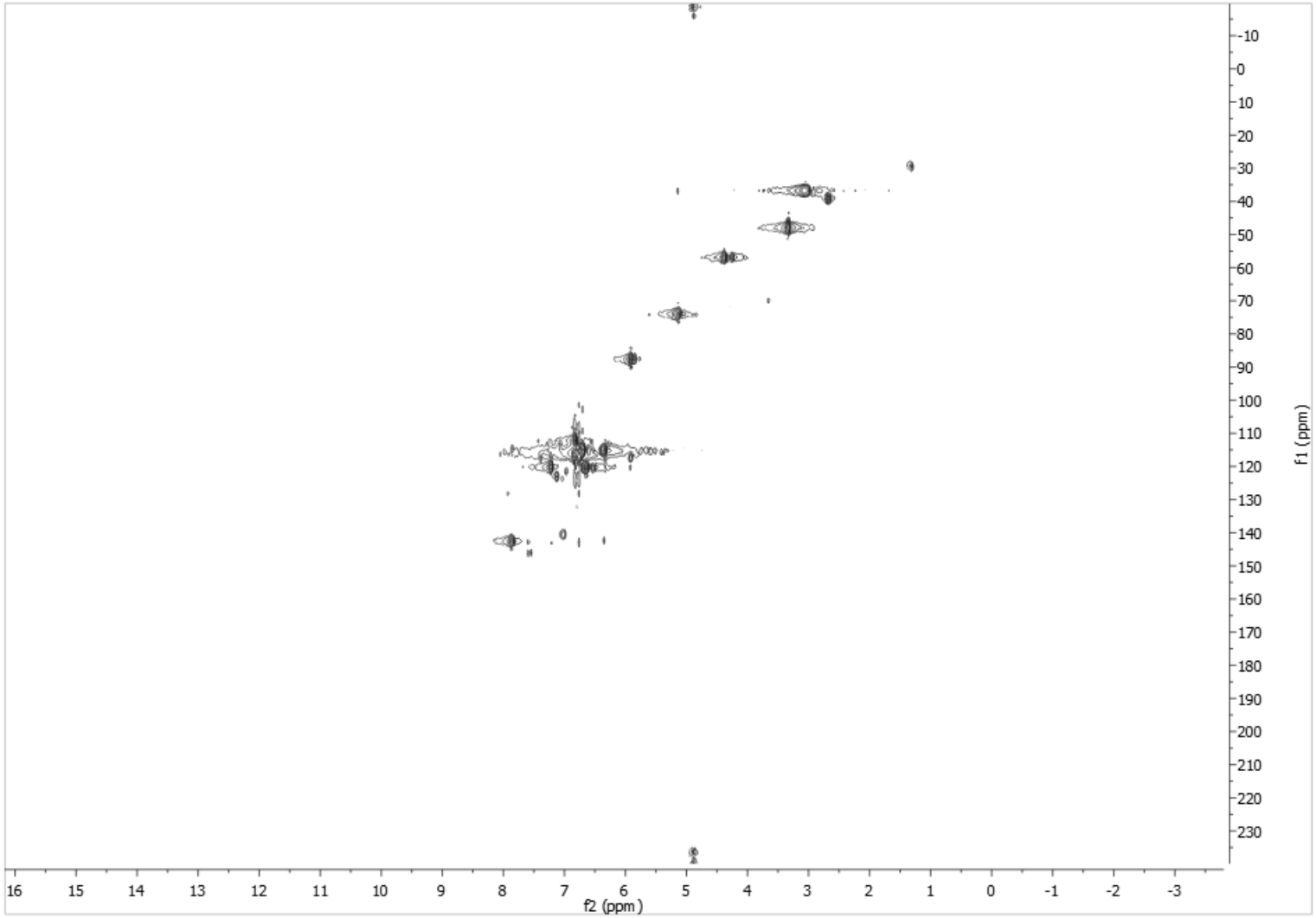
Supplemental figure 3.8.2. Amplification of signals between regions at δ 5.1 and δ 7.9 from ^{1}H NMR spectra (CD_3OD) of **Lithospermic acid A (16)**.



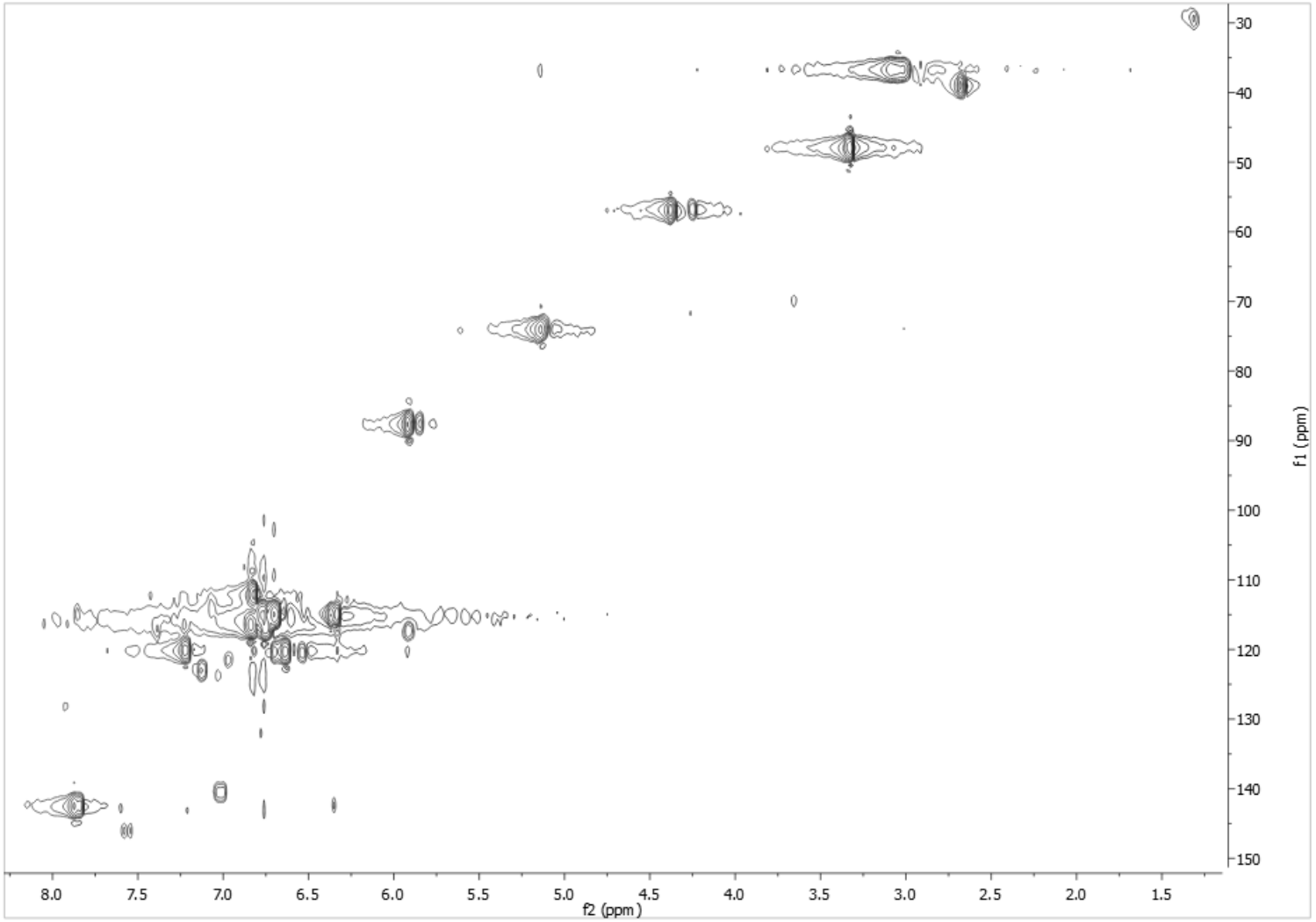
Supplemental figure 3.8.3. ^{13}C NMR spectra (CD_3OD) of **Lithospermic acid A** (**16**).



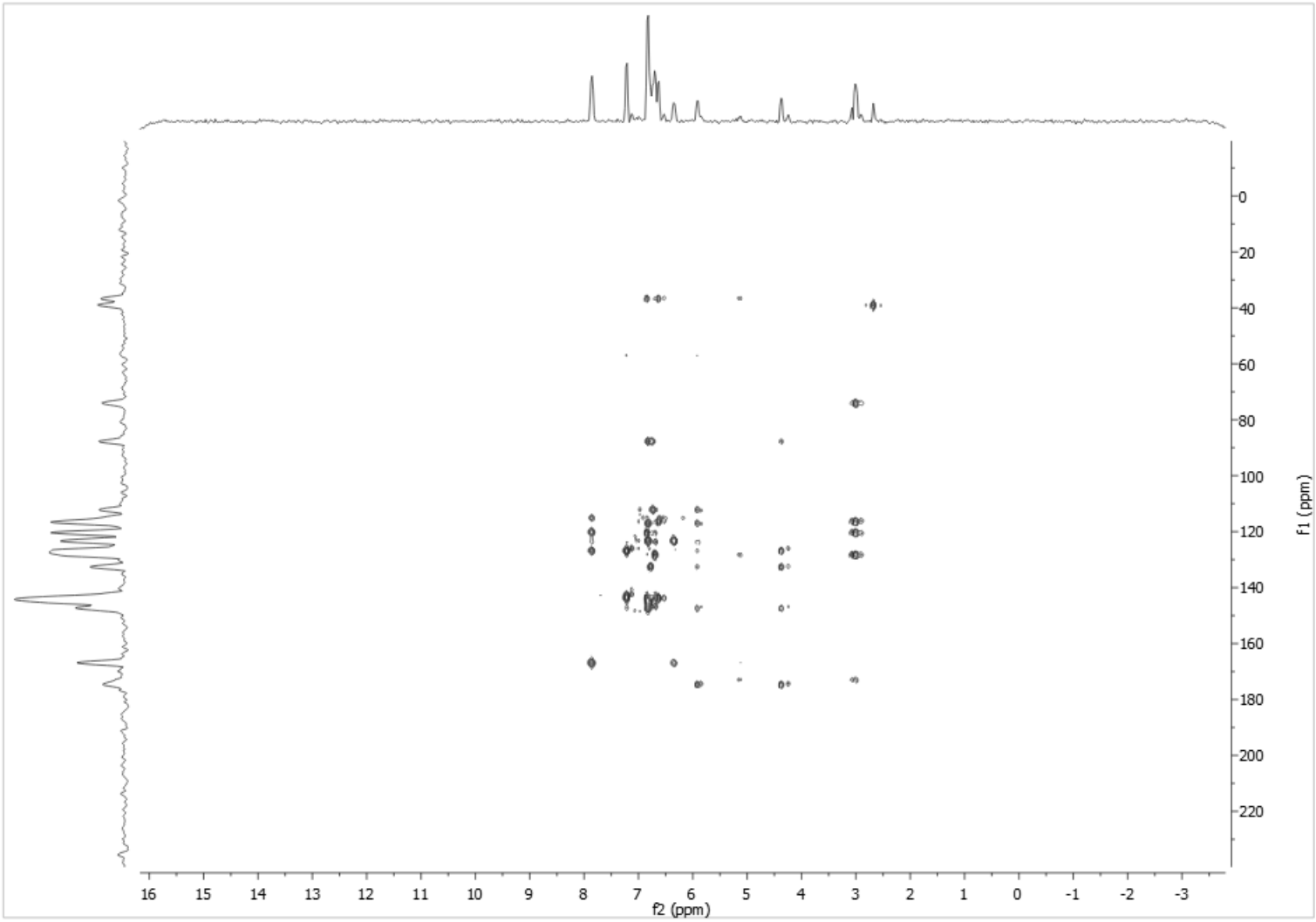
Supplemental figure 3.8.4. Amplification of signals between regions at δ 30 and δ 175 from ^{13}C NMR spectra (CD_3OD) of **Lithospermic acid A (16)**.



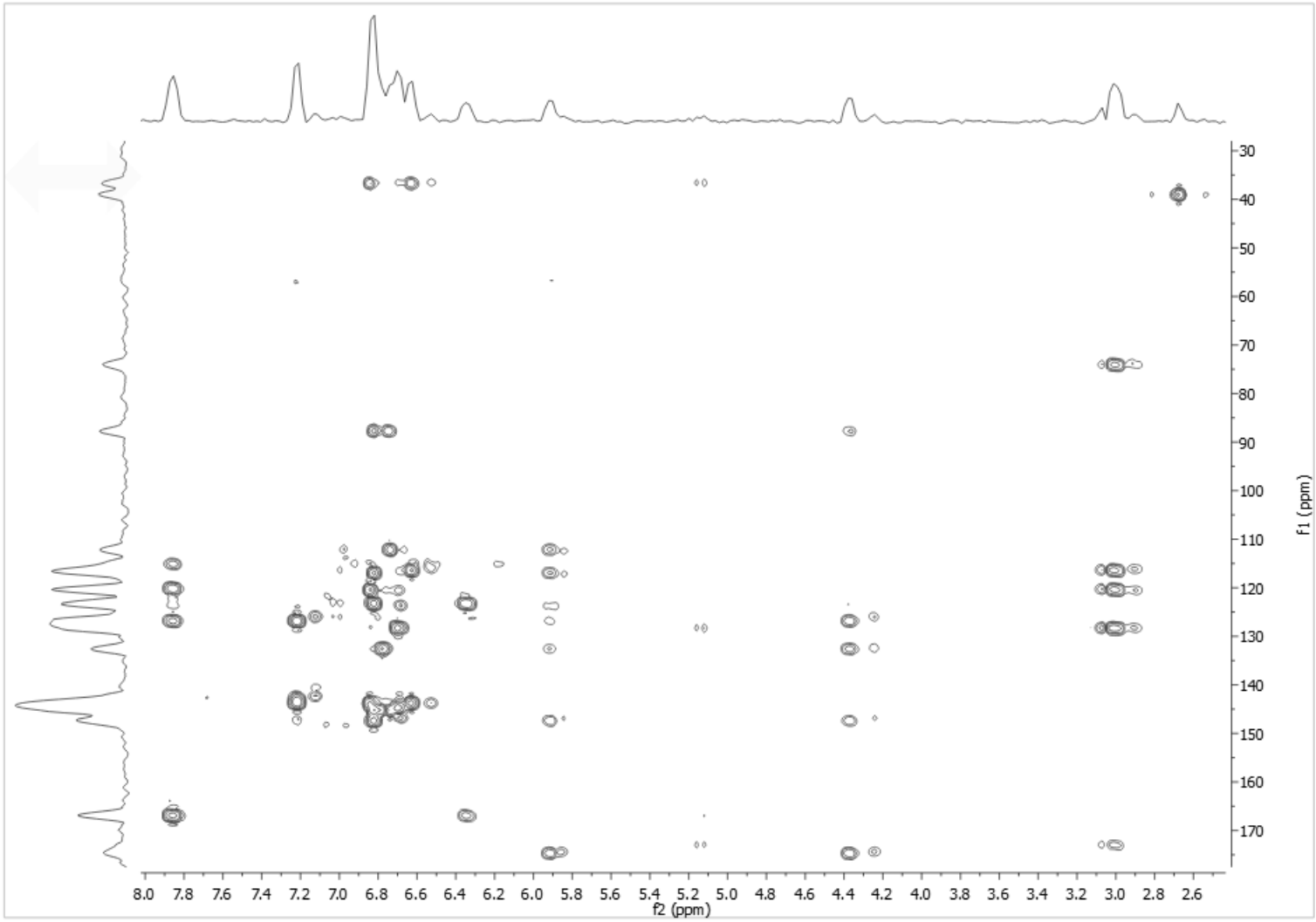
Supplemental figure 3.8.5. HSQC spectra (CD_3OD) of **Lithospermic acid A (16)**.



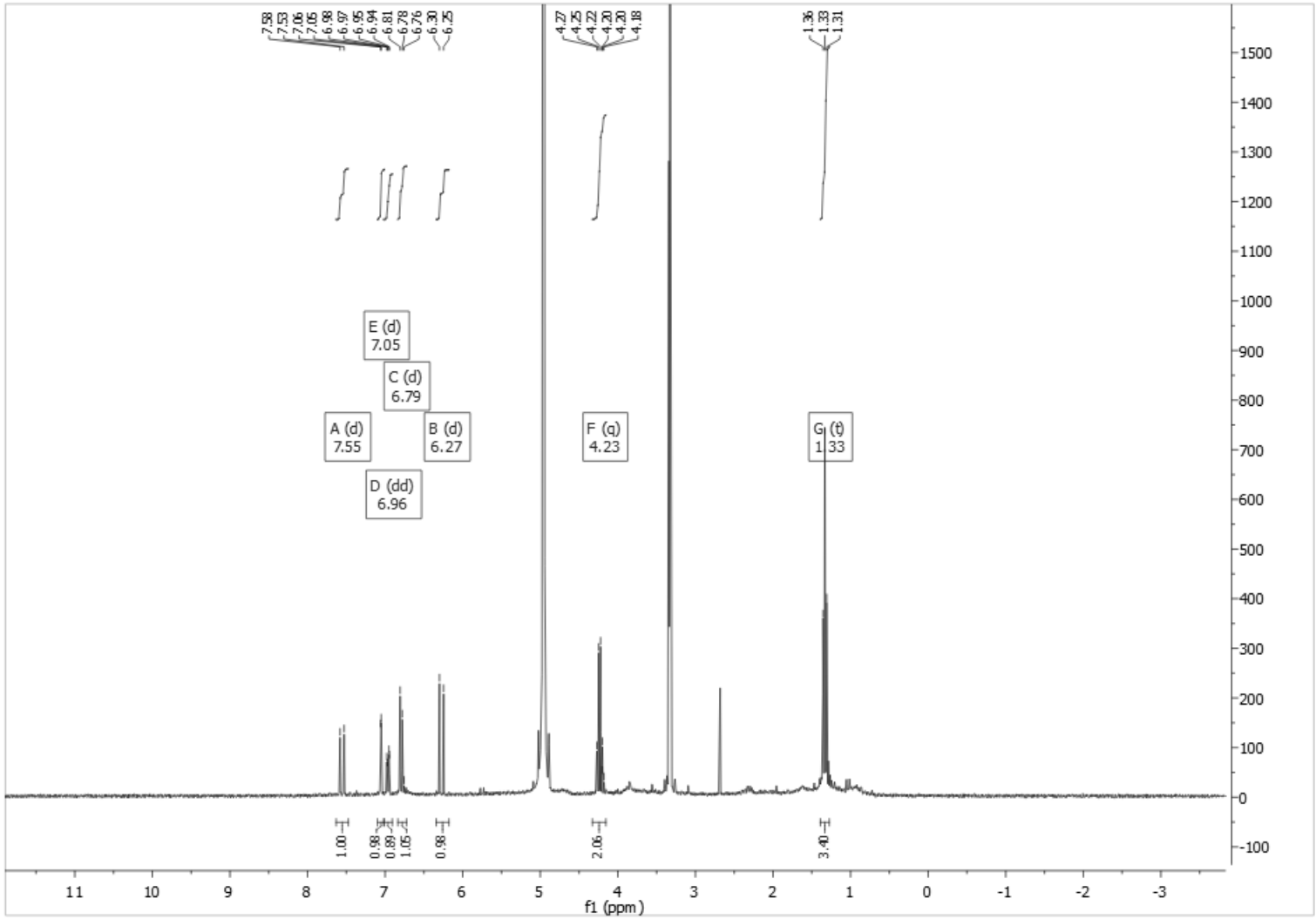
Supplemental figure 3.8.6. Amplification of signals between regions at δ 1.5 and δ 8.0 from HSQC spectra (CD_3OD) of **Lithospermic acid A** (**16**).



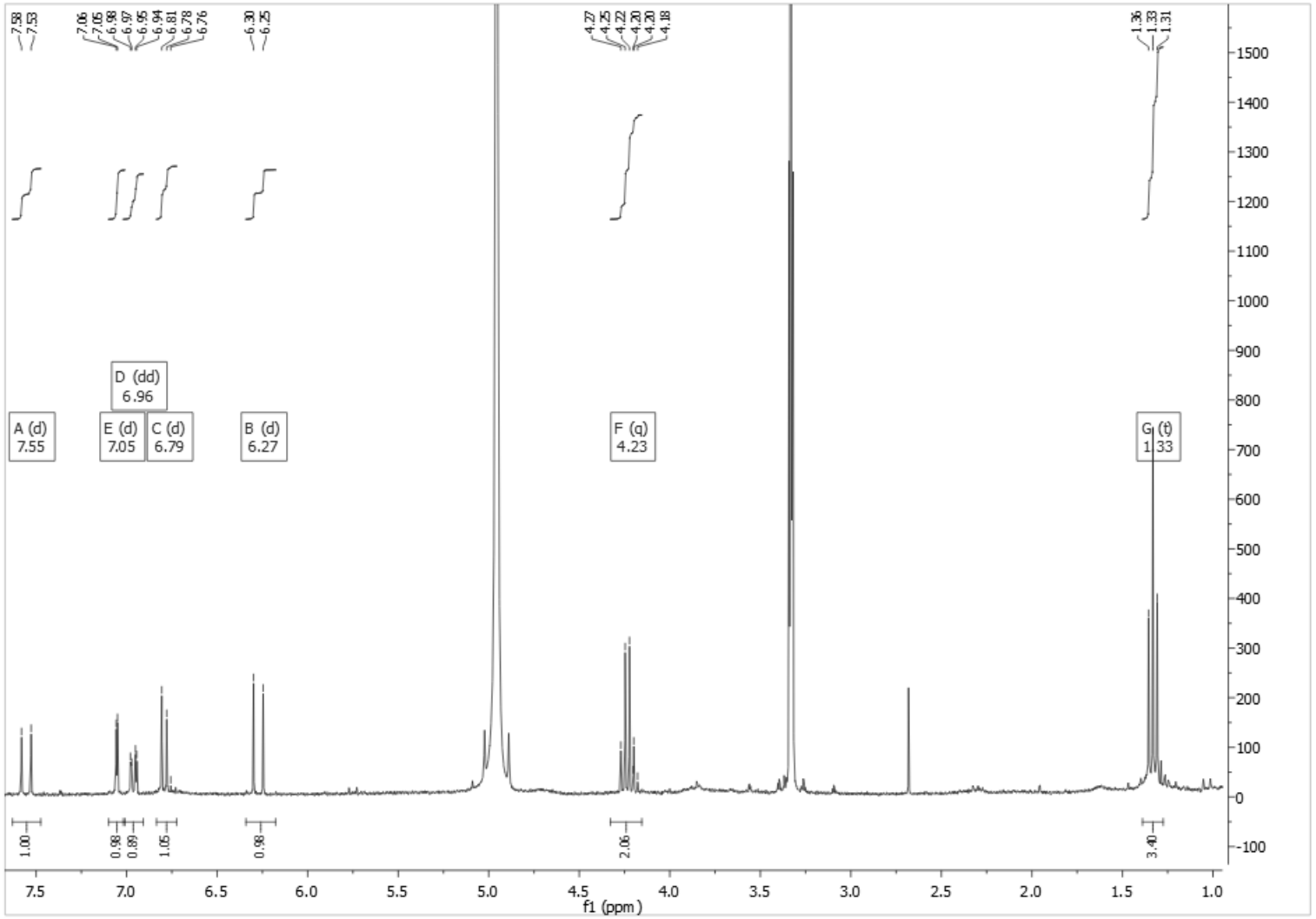
Supplemental figure 3.8.7. HMBC spectra (CD_3OD) of **Lithospermic acid A (16)**.



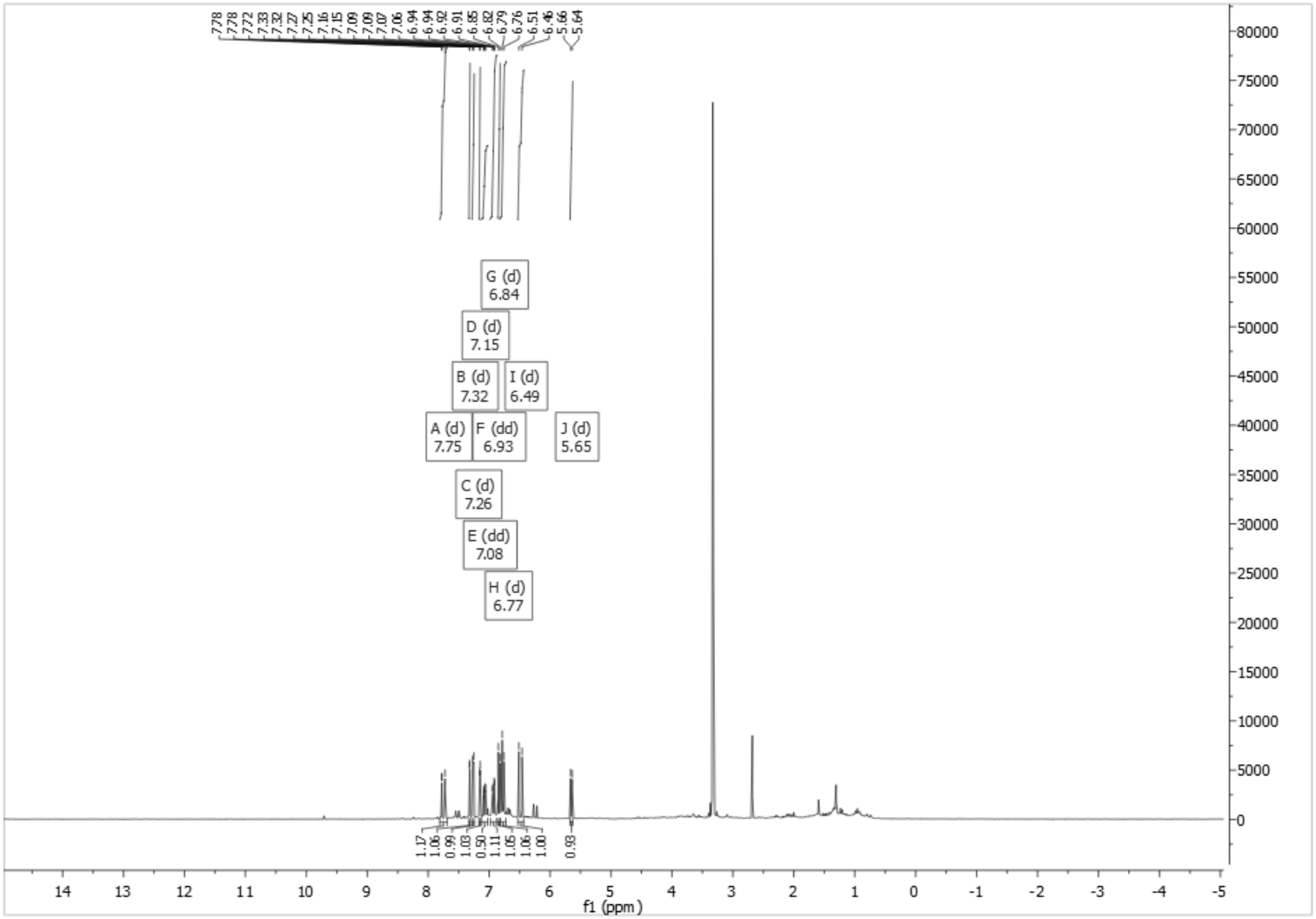
Supplemental figure 3.8.8. Amplification of signals between regions at δ 2.6 and δ 8.0 from HMBC spectra (CD_3OD) of **Lithospermic acid A** (**16**).



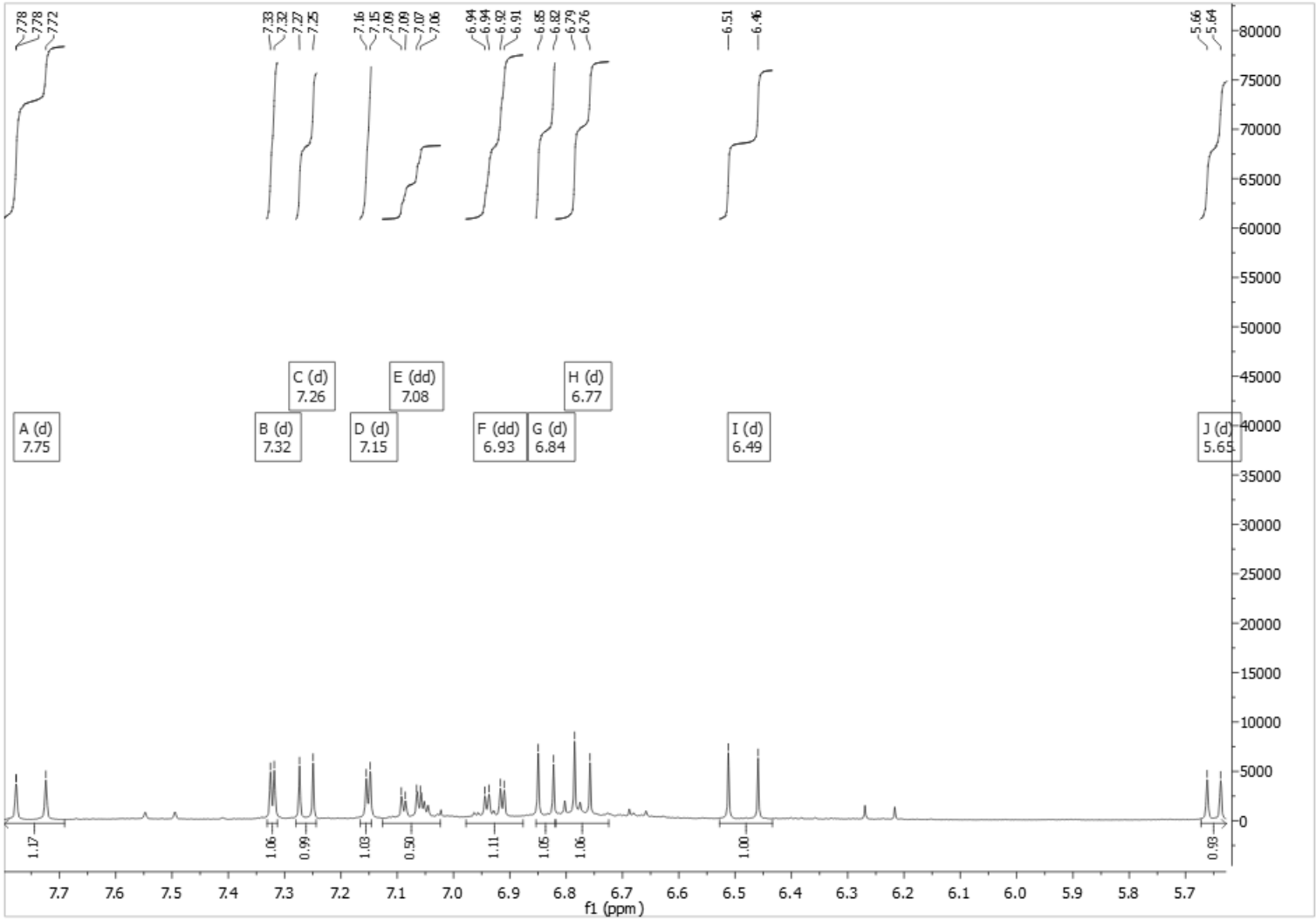
Supplemental figure 3.9.1. ^{1}H NMR spectra (CD_3OD) of **Ethyl caffeate (20)**



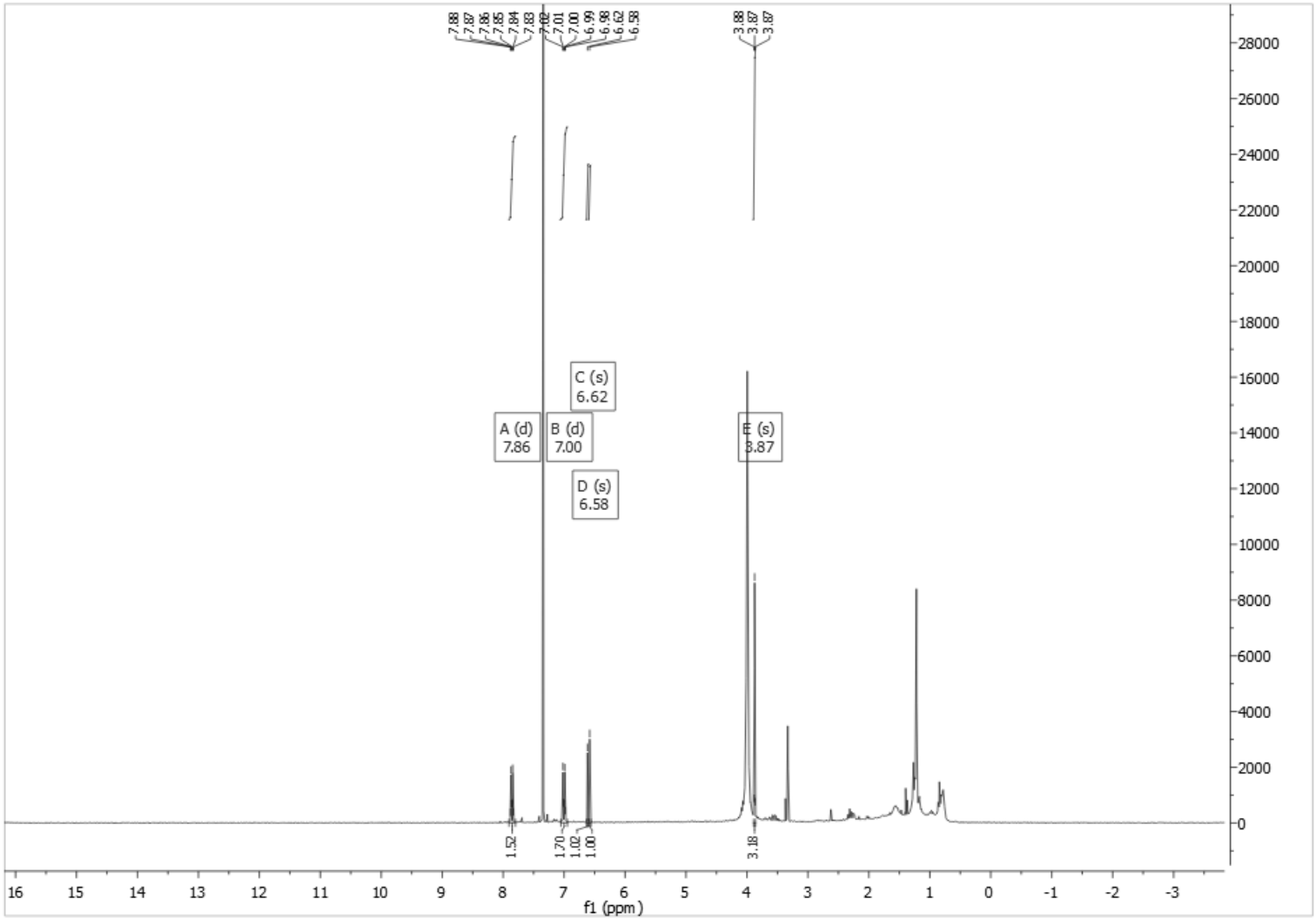
Supplemental figure 3.9.2. Amplification of signals between regions at δ_H 1 and δ_H 7.5 from ^{1}H NMR spectra (CD_3OD) of **Ethyl caffeate (20)**



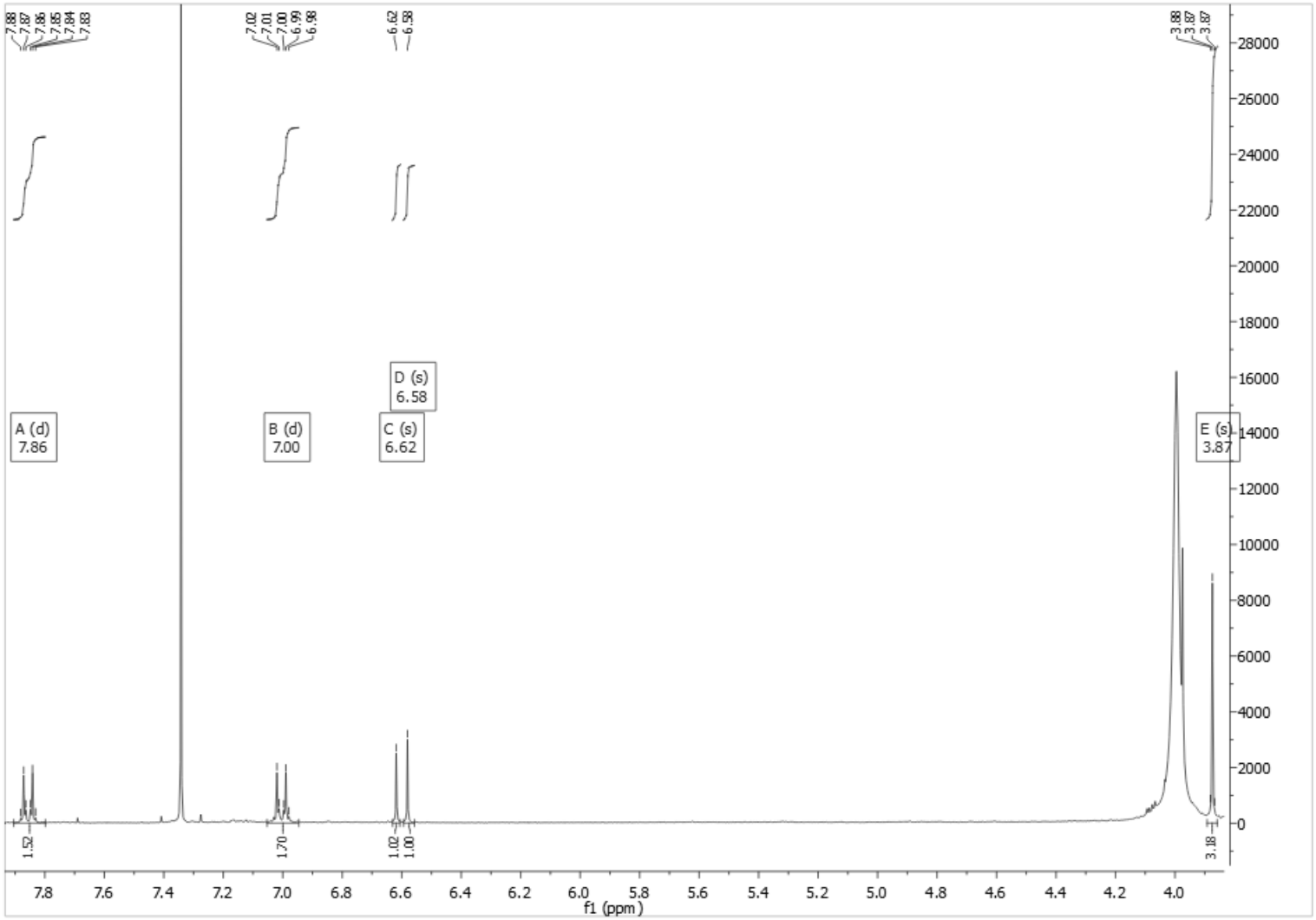
Supplemental figure 3.10.1. A): ^{1}H NMR spectra (CD_3OD) of **Nepetoidin B (24)**



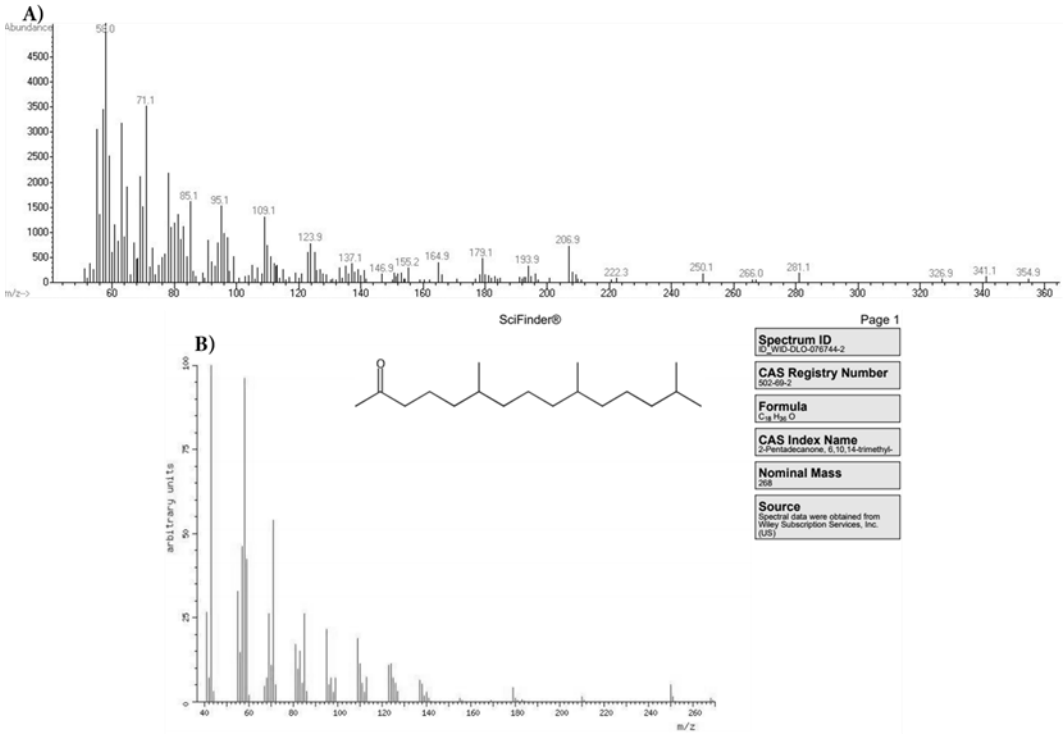
Supplemental figure 3.10.2. Amplification of signals between regions at δ 5.6 and δ 7.8 from ^{1}H NMR spectra (CD_3OD) of **Nepetoidin B (24)**



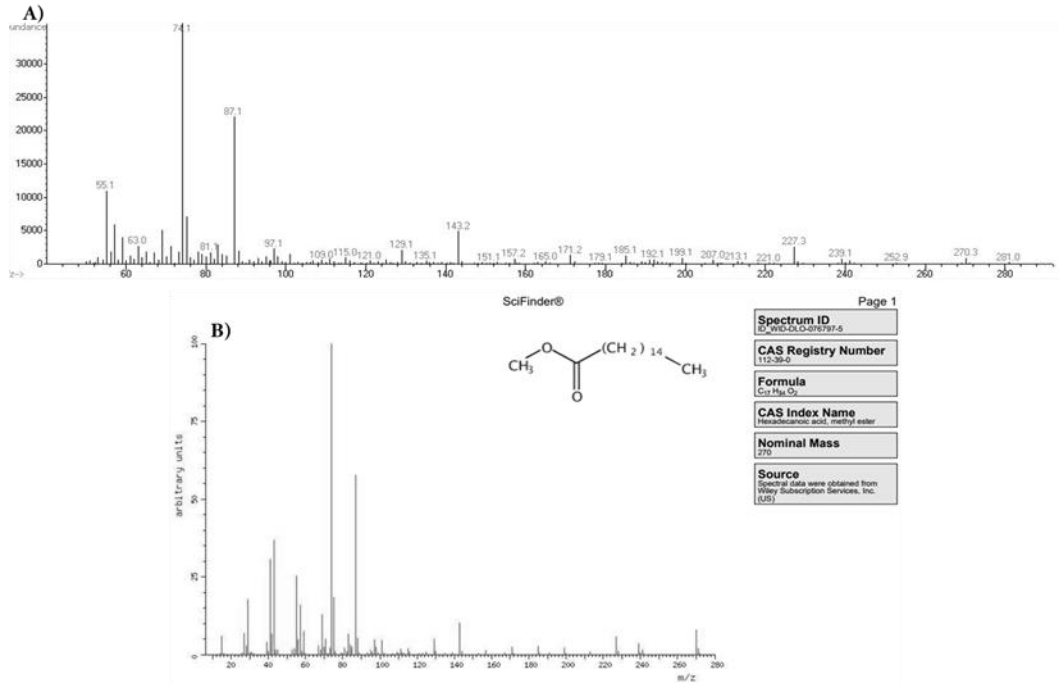
Supplemental figure 3.11.1. ^{1}H NMR spectra ($CDCl_3$) of **Cirsimaritin (25)**



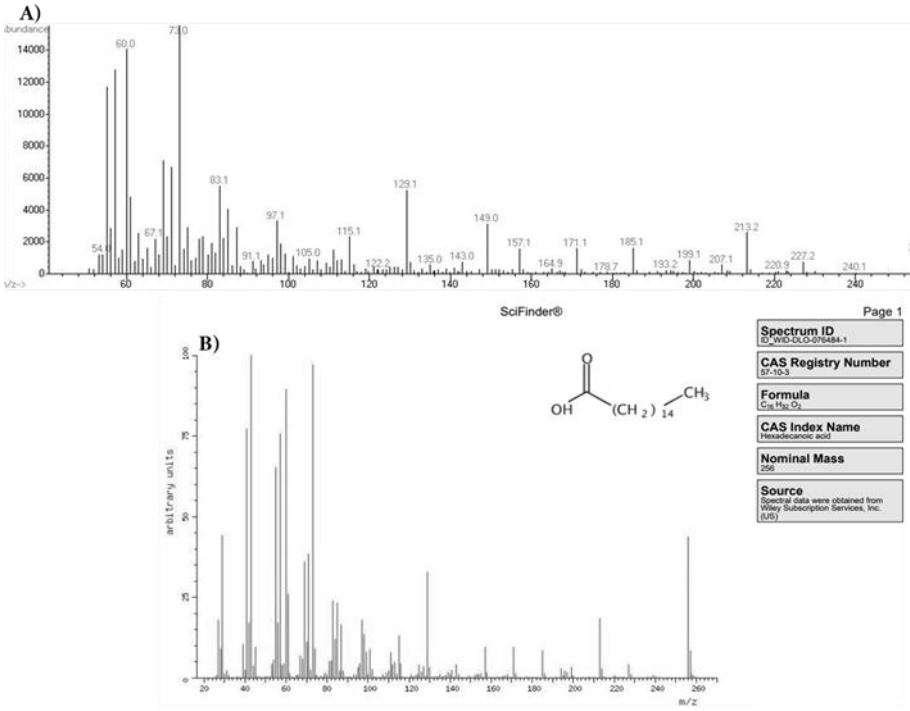
Supplemental figure 3.11.2. Amplification of signals between regions at δ 3.8 and δ 7.9 from ^{1}H NMR spectra ($CDCl_3$) of **Cirsimaritin (25)**



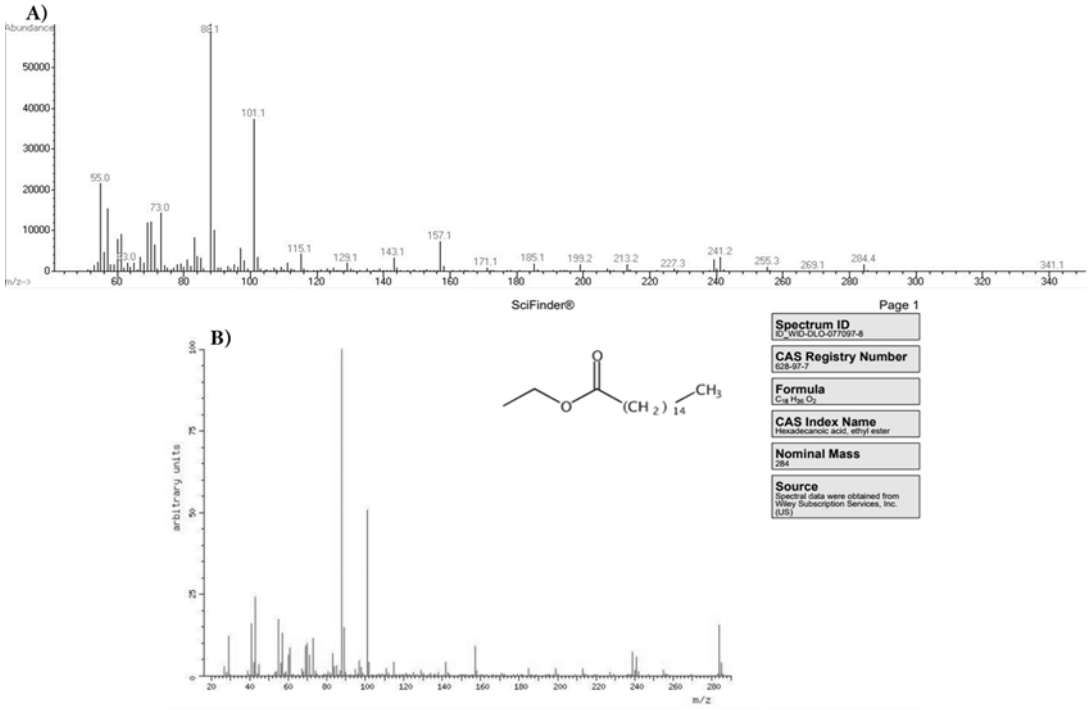
Supplemental figure 3.12.1 2-Pentadecanone, 6,10,14-trimethyl (Hexahydrofarnesyl acetone) **N° 7. A)** Experimental mass spectrum; **B)** Reference mass spectrum (SciFinder®)



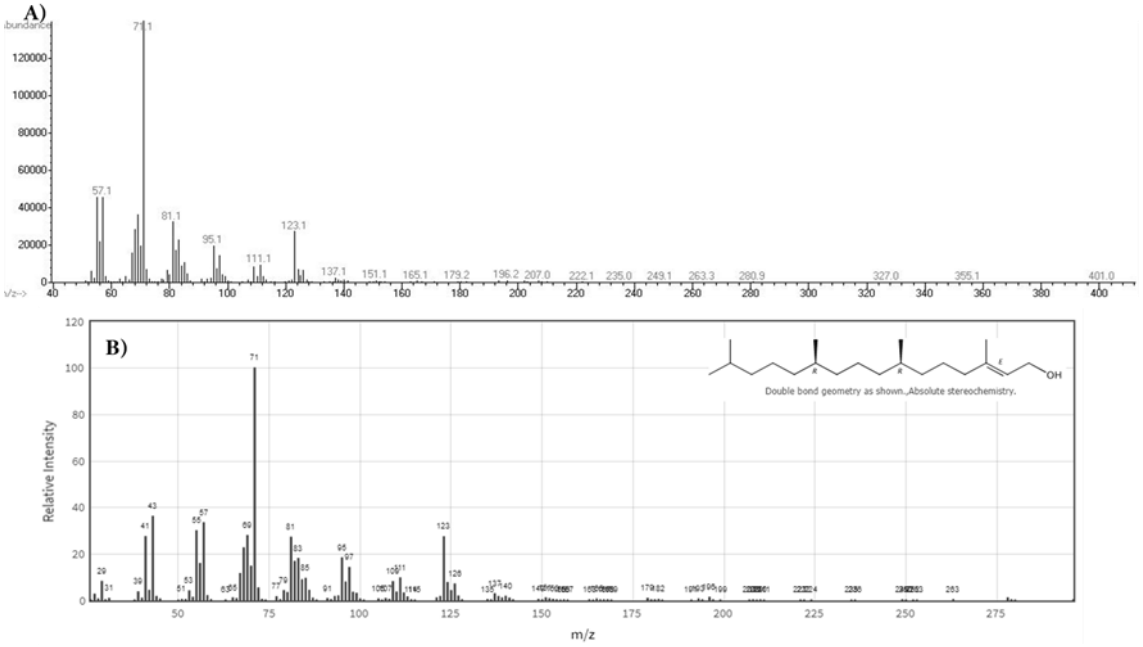
Supplemental figure 3.12.2. n-Hexadecanoic acid methyl ester (Palmitic acid, methyl ester) **N° 9. A)** Experimental mass spectrum; **B)** Reference mass spectrum (SciFinder®)



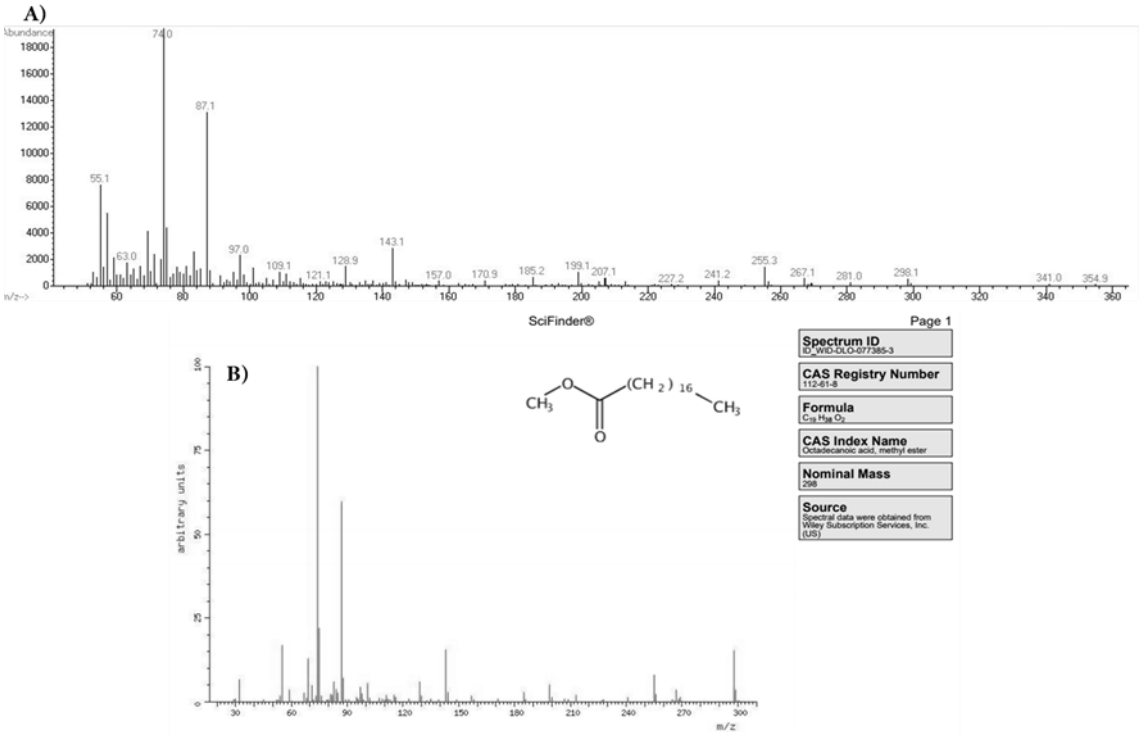
Supplemental figure 3.12.3. n-Hexadecanoic acid (Palmitic acid) **N° 10**. **A)** Experimental mass spectrum; **B)** Reference mass spectrum (SciFinder®)



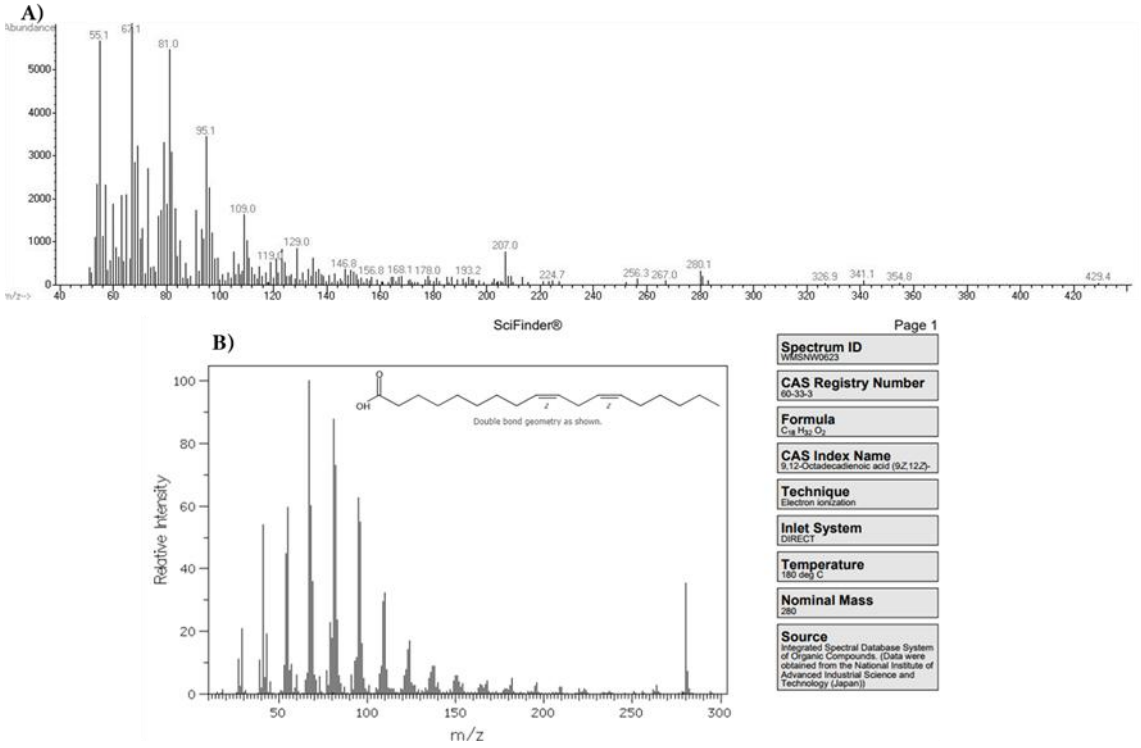
Supplemental figure 3.12.4. n-Hexadecanoic acid, ethyl ester (Palmitic acid, ethyl ester) **N° 11. A)** Experimental mass spectrum; **B)** Reference mass spectrum (SciFinder®)



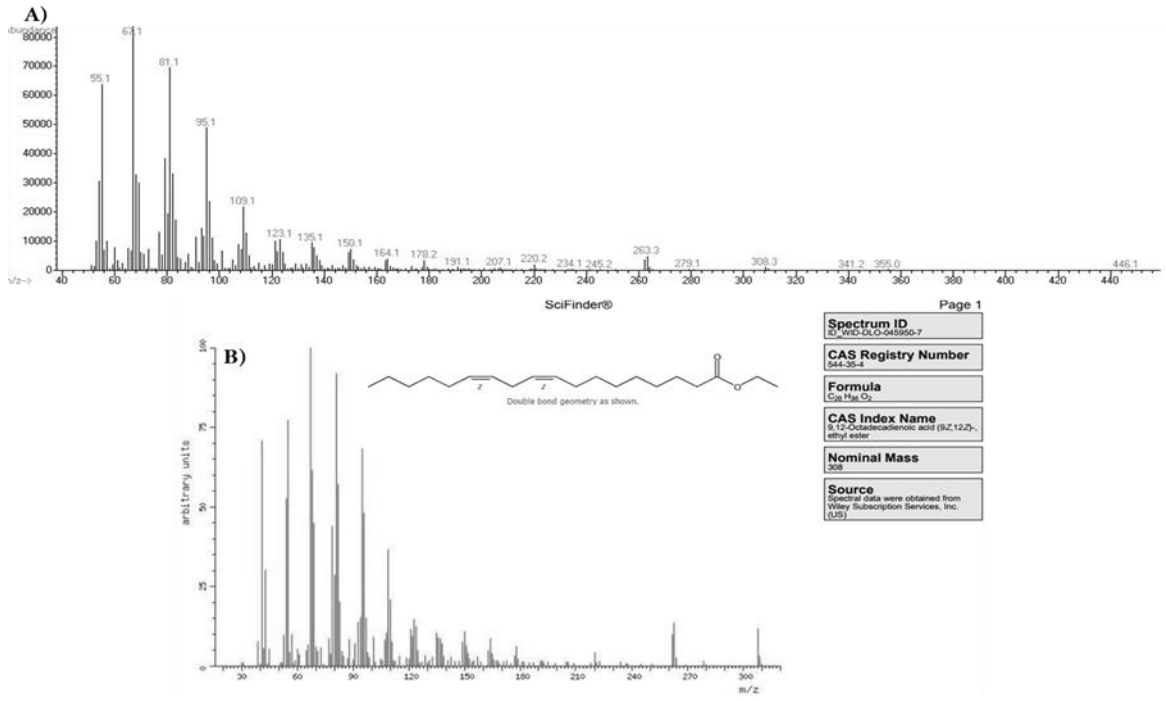
Supplemental figure 3.12.5. 3,7,11,15-Tetramethylhexadec-2-en-1-ol (Phytol) -> Phytol derivative **N° 15. A)** Experimental mass spectrum; **B)** Reference mass spectrum NIST Mass Spectrometry Data Center



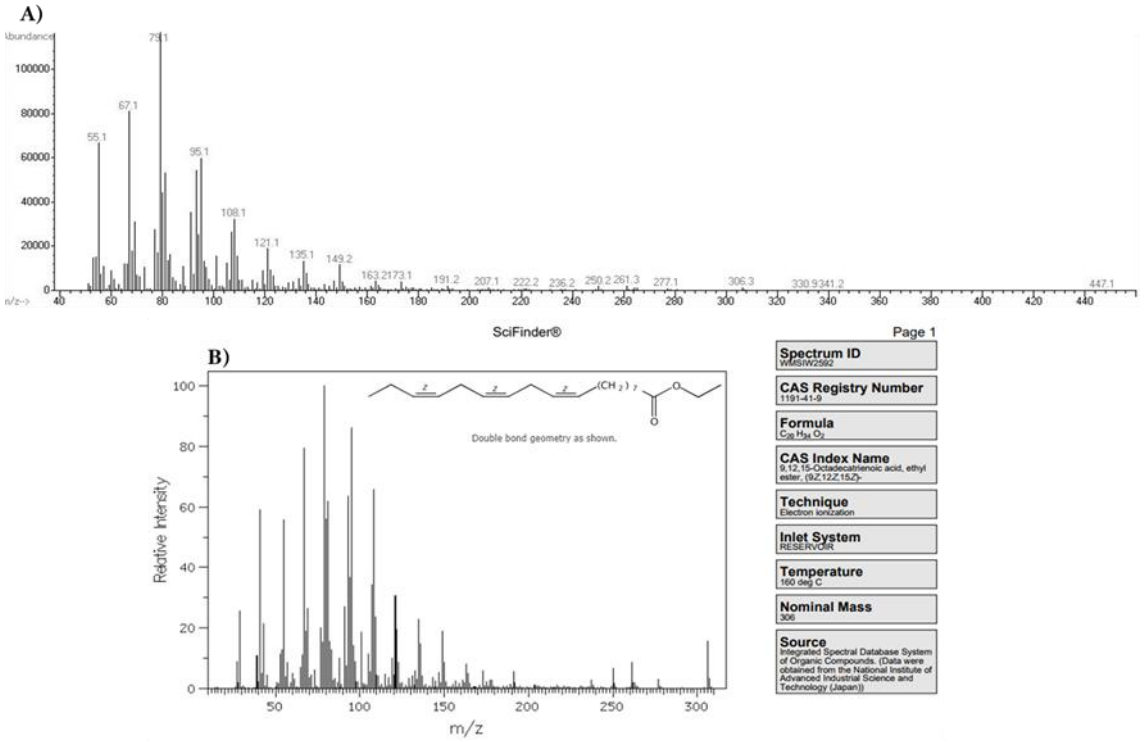
Supplemental figure 3.12.6. n-Octadecanoic acid, methyl ester (Methyl stearate) **N° 16. A)** Experimental mass spectrum; **B)** Reference mass spectrum (SciFinder®)



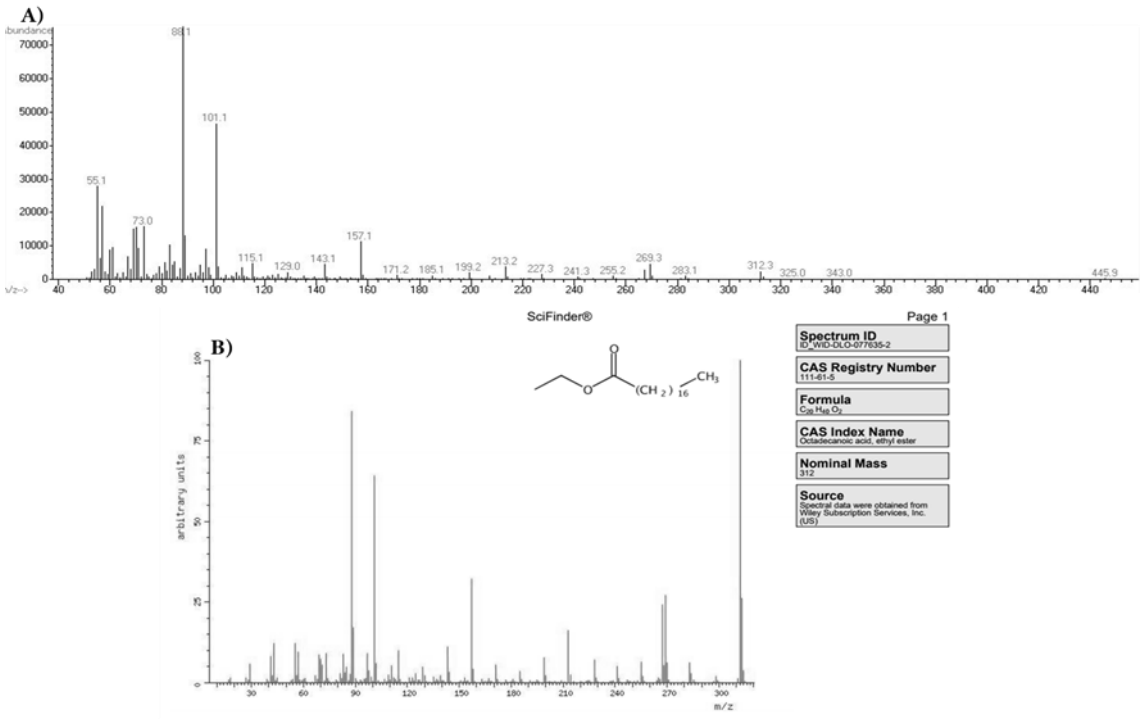
Supplemental figure 3.12.7. 9,12-Octadecadienoic acid (*Z*,*Z*)- (Linoleic acid) **N° 17. A)** Experimental mass spectrum; **B)** Reference mass spectrum (SciFinder®)



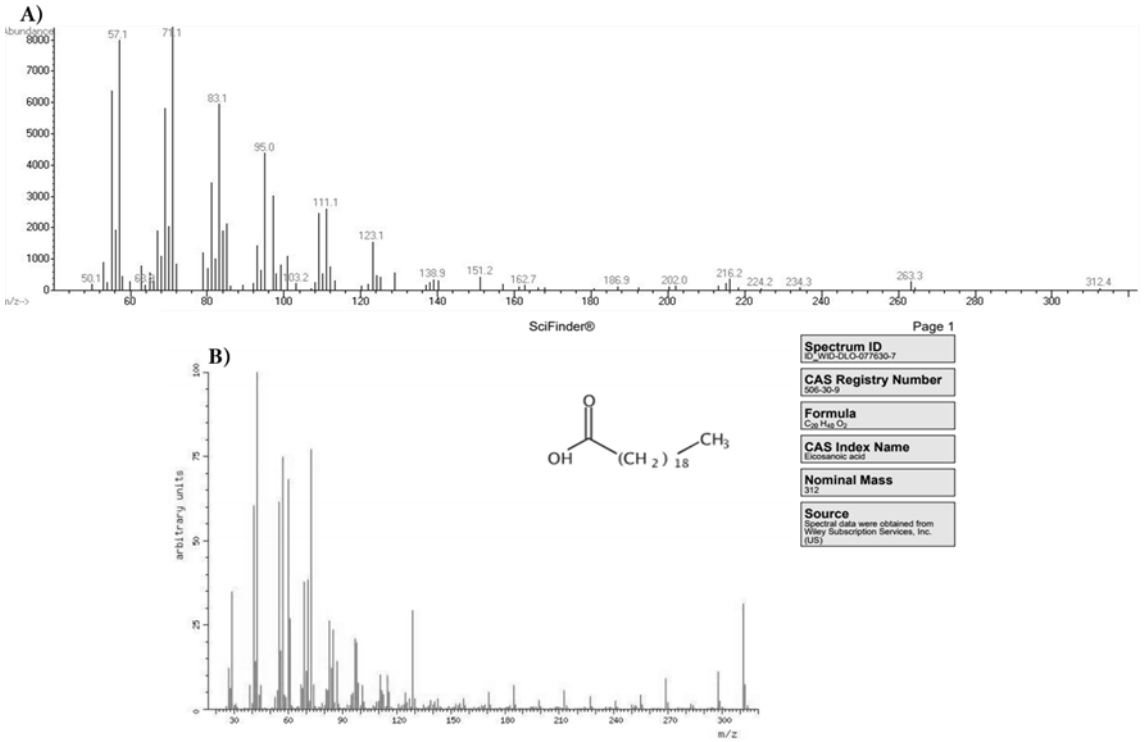
Supplemental figure 3.12.8. 9,12-Octadecadienoic acid (*Z*,*Z*)-, ethyl Ester (Linoleic acid ethyl ester) **N° 19. A)** Experimental mass spectrum; **B)** Reference mass spectrum (SciFinder®)



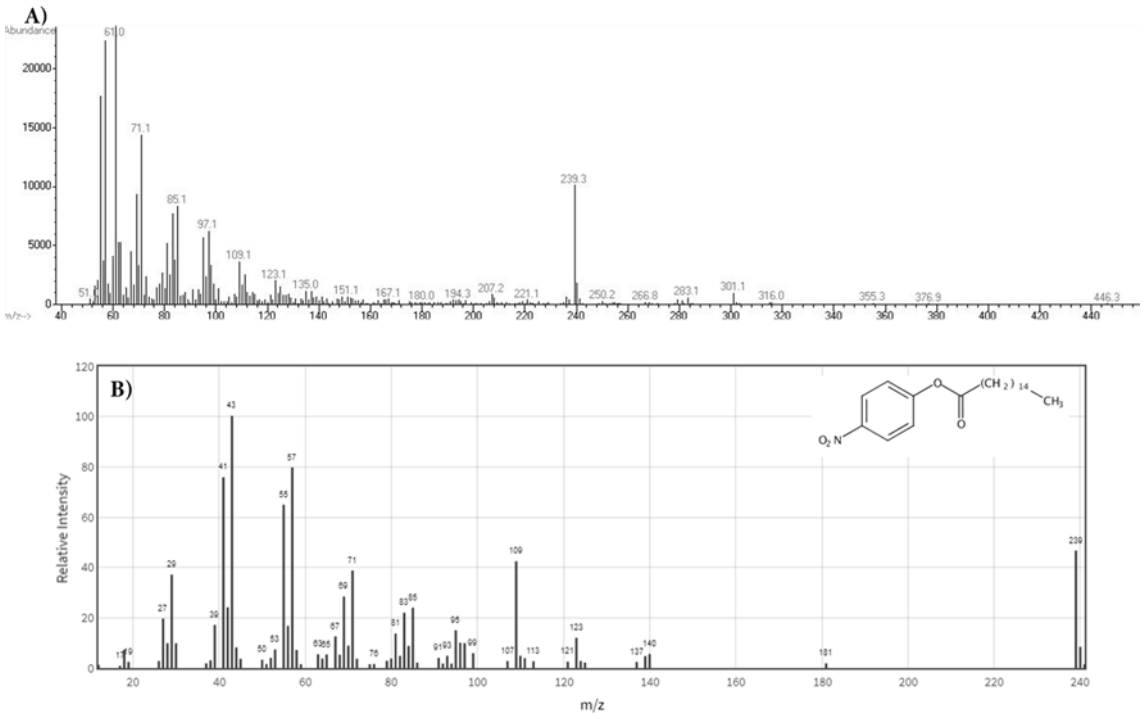
Supplemental figure 3.12.9. Ethyl 9,12,15-octadecatrienoate (Linolenic acid, ethyl Ester) **N° 20**. **A)** Experimental mass spectrum; **B)** Reference mass spectrum (SciFinder®)



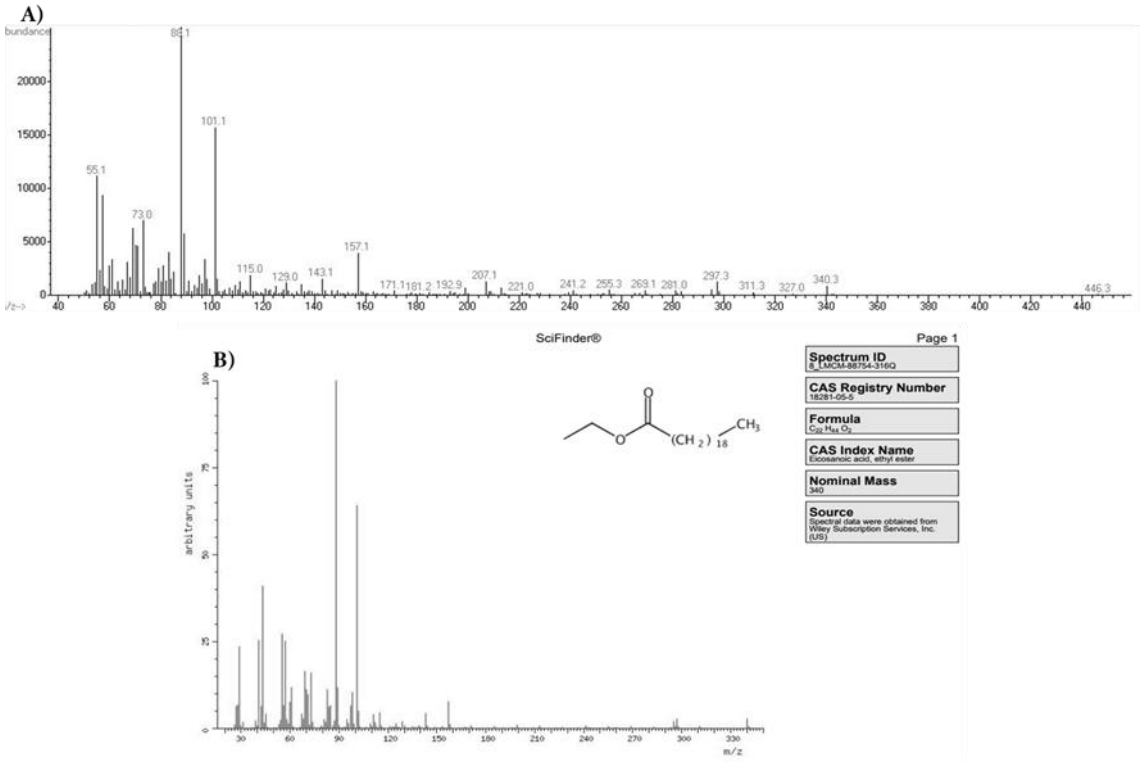
Supplemental figure 3.12.10. Octadecanoic acid, ethyl Ester (Stearic acid, ethyl ester) **N° 22**. **A)** Experimental mass spectrum; **B)** Reference mass spectrum (SciFinder®)



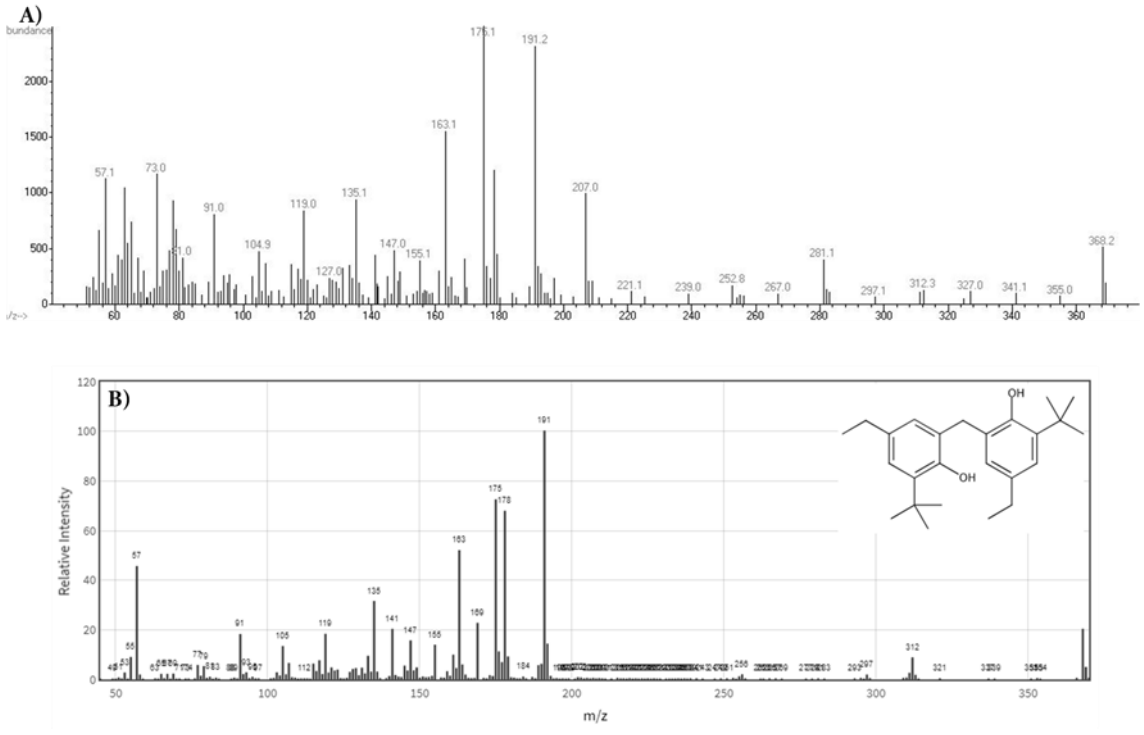
Supplemental figure 3.12.11. n-Eicosanoic acid (Arachidic acid) **N° 23. A)** Experimental mass spectrum; **B)** Reference mass spectrum (SciFinder®)



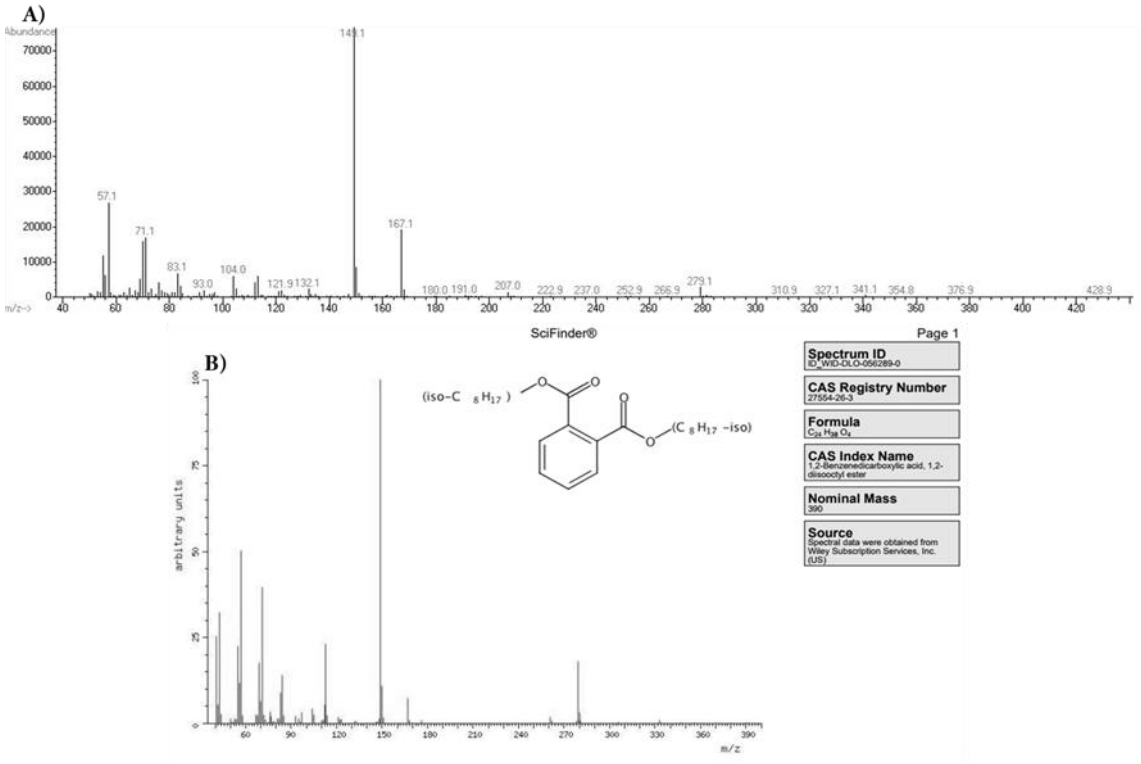
Supplemental figure 3.12.12. Hexadecanoic acid, 4-nitrophenyl ester (4-nitrophenyl palmitate) **N° 24. A)** Experimental mass spectrum; **B)** Reference mass spectrum (NIST Mass Spectrometry Data Center)



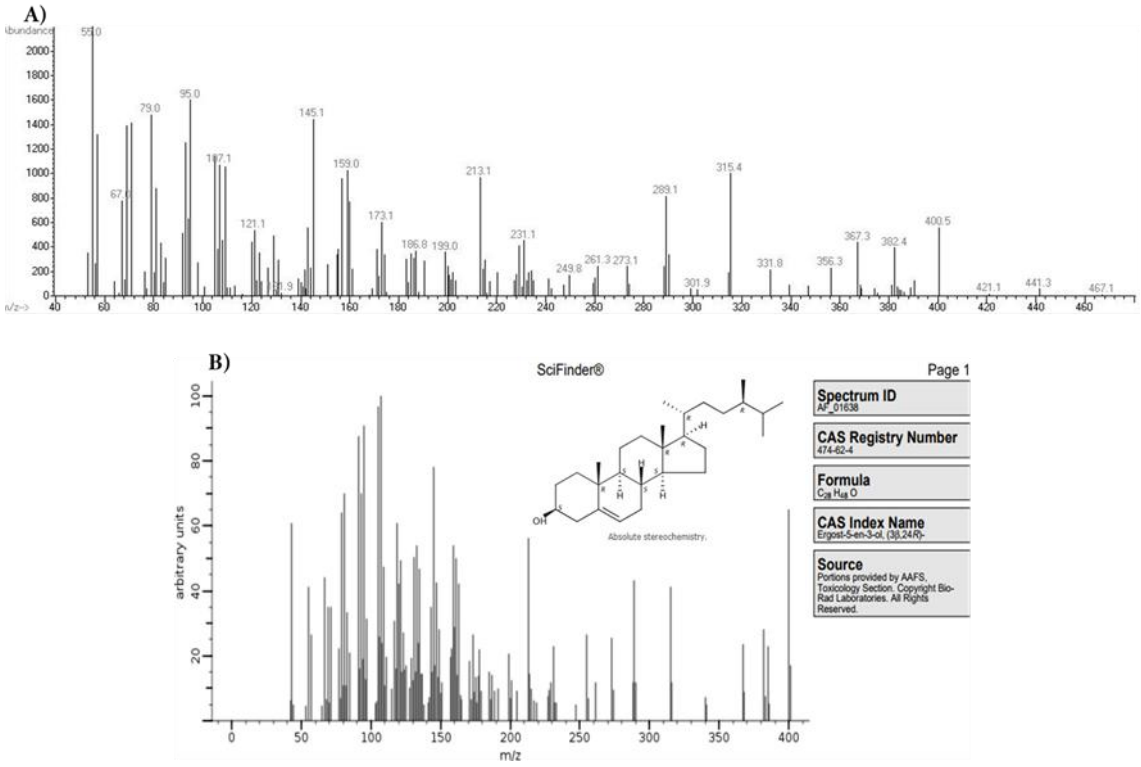
Supplemental figure 3.12.13. Eicosanoic acid, ethyl ester (Arachidic acid, ethyl Ester). **N° 25. A)** Experimental mass spectrum; **B)** Reference mass spectrum (SciFinder®)



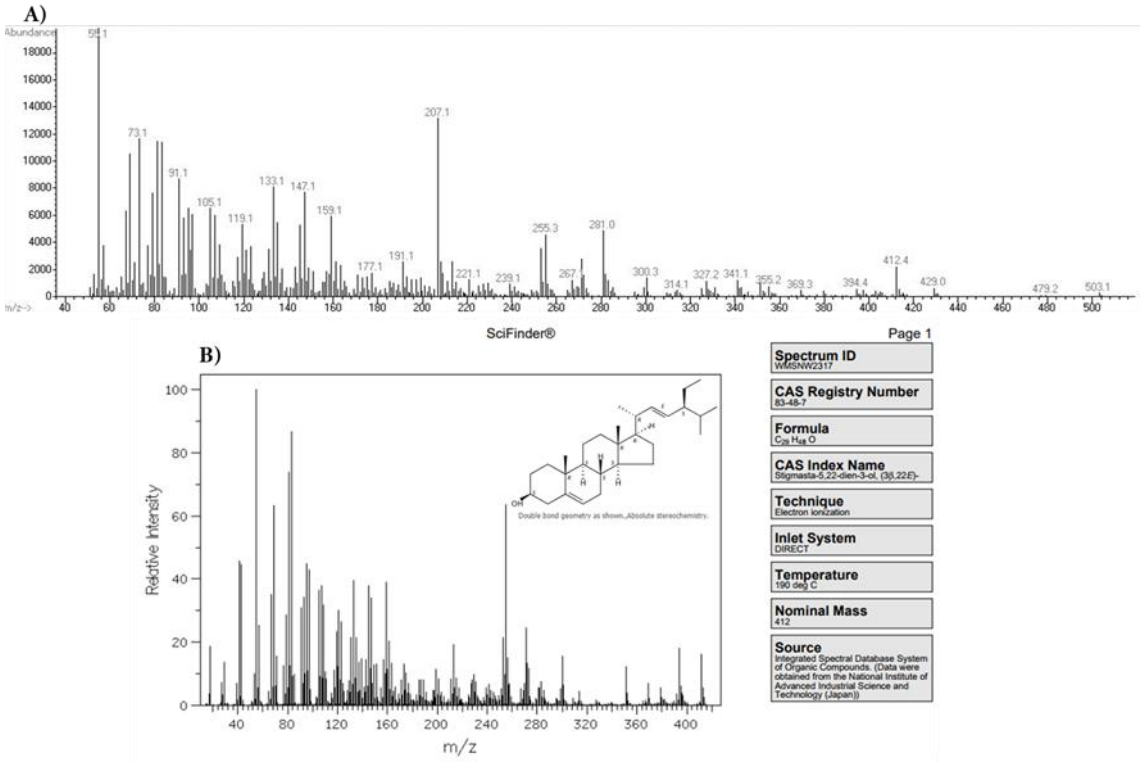
Supplemental figure 3.12.14. Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-ethyl- N° 28. A) Experimental mass spectrum; **B)** Reference mass spectrum (NIST Mass Spectrometry Data Center)



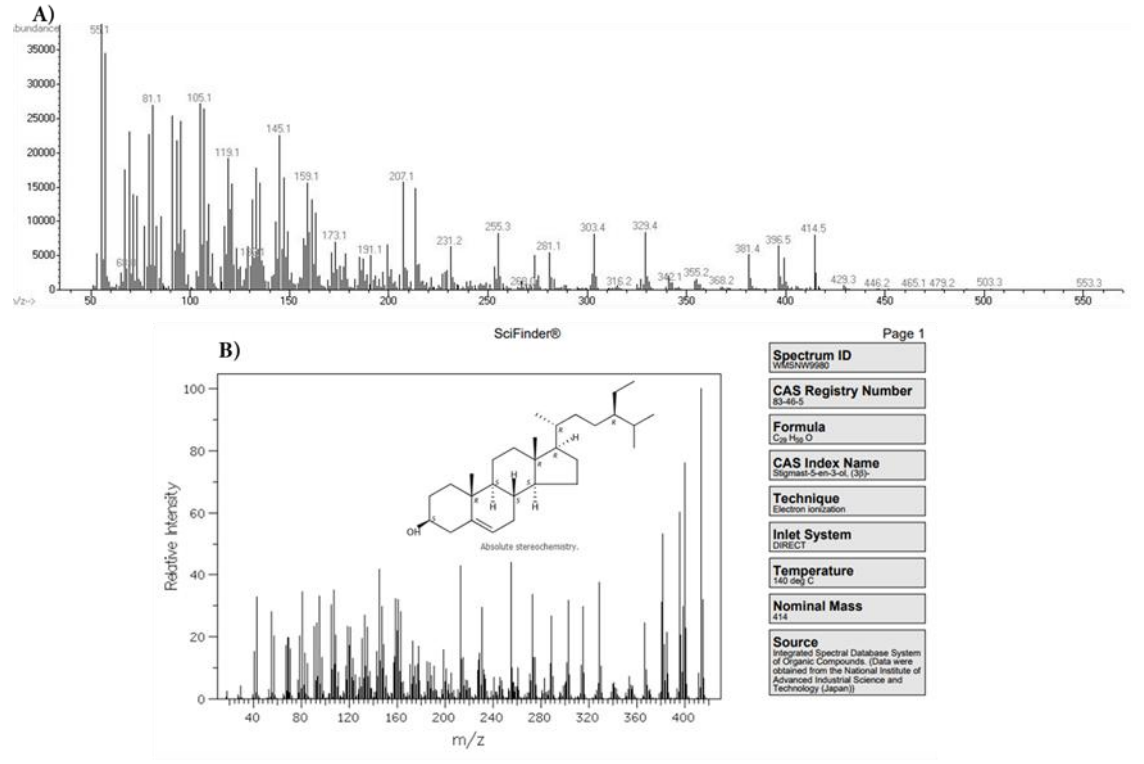
Supplemental figure 3.12.15. 1,2-Benzenedicarboxylic acid, diisooctyl ester (Phthalic acid, diisooctyl ester) **N° 29. A)** Experimental mass spectrum; **B)** Reference mass spectrum (SciFinder®)



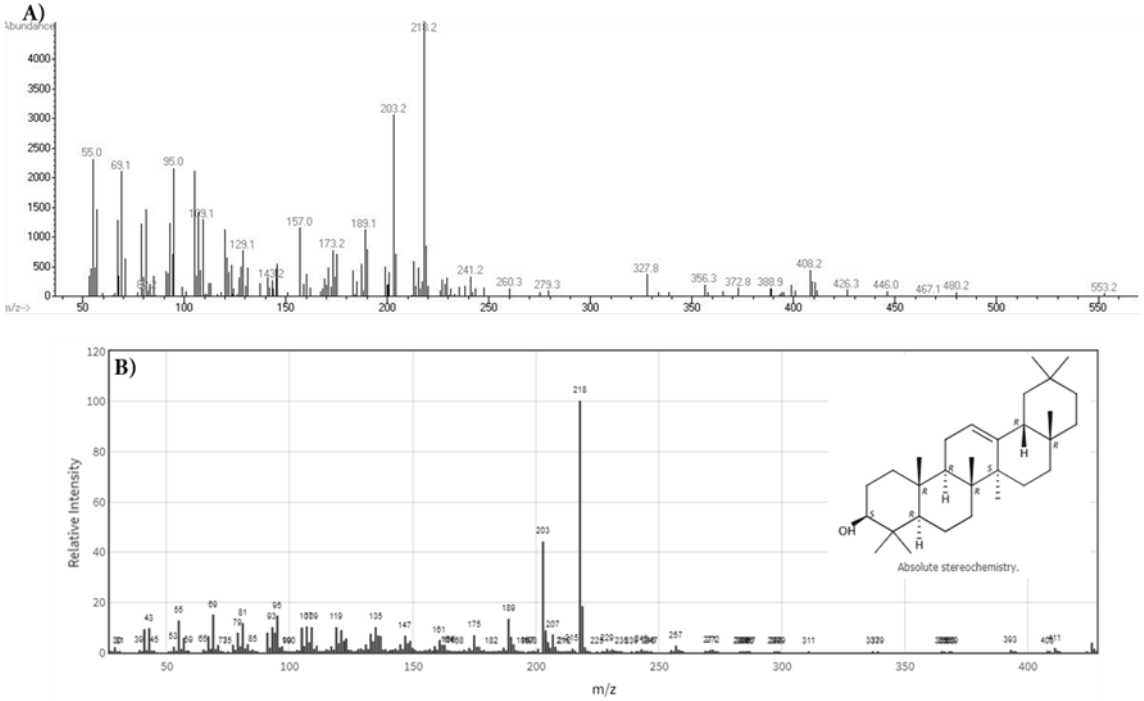
Supplemental figure 3.12.16. 24α-Methyl-5-cholesten-3β-ol (Campesterol) **N° 32. A)** Experimental mass spectrum; **B)** Reference mass spectrum (SciFinder®)



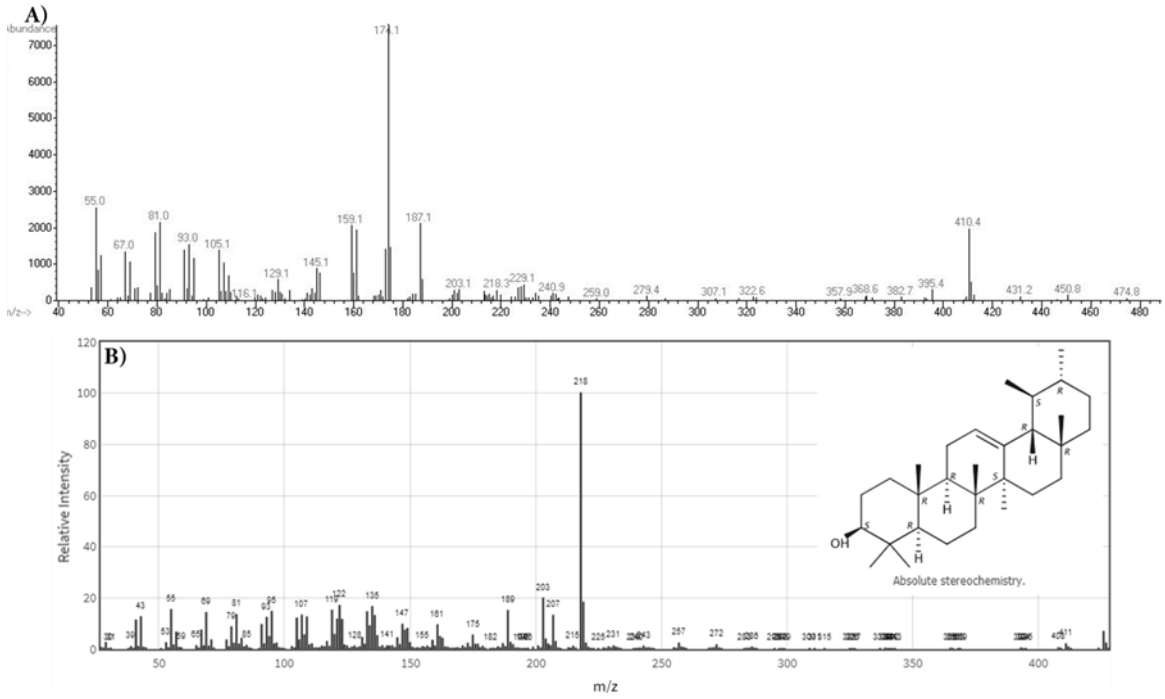
Supplemental figure 3.12.17. Stigmasta-5,22-dien-3β-ol (Stigmasterol) **N° 33. A)** Experimental mass spectrum; **B)** Reference mass spectrum (SciFinder®)



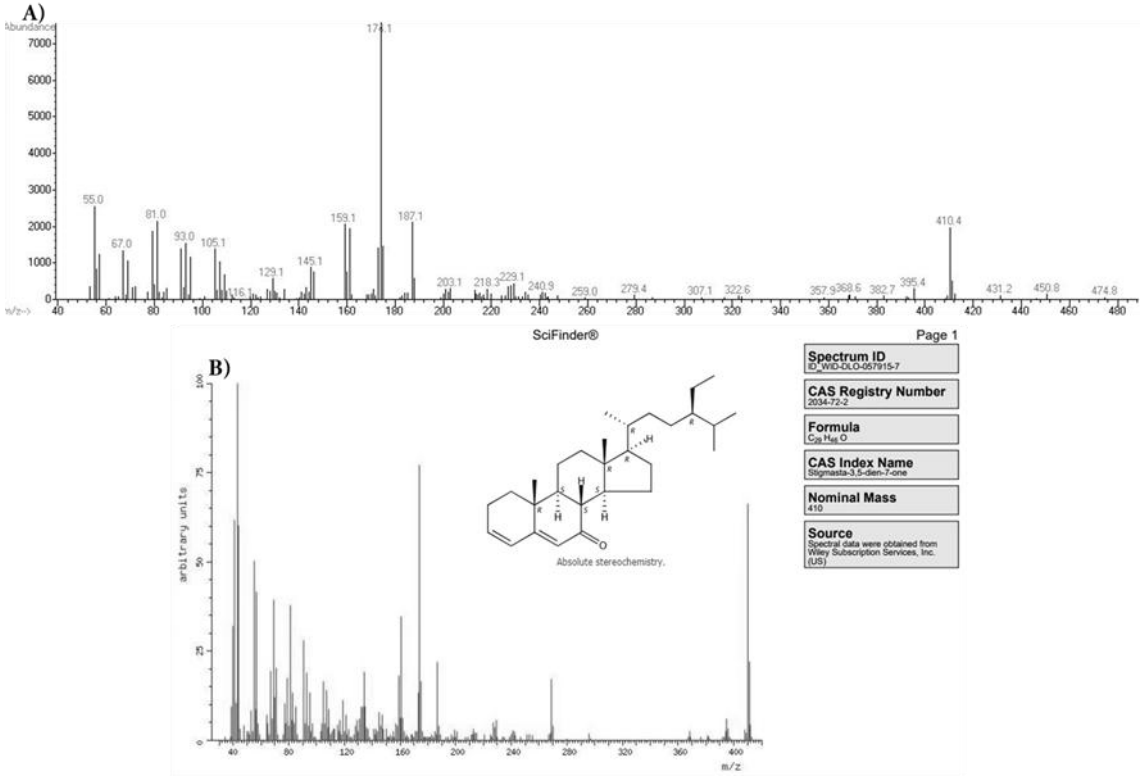
Supplemental figure 3.12.18. Stigmast-5-en-3β-ol (β-sitosterol) **N° 34. A)** Experimental mass spectrum; **B)** Reference mass spectrum (SciFinder®)



Supplemental figure 3.12.19. Olean-12-en-3β-ol (β-amyrin) **N° 35. A)** Experimental mass spectrum; **B)** Reference mass spectrum (NIST Mass Spectrometry Data Center)



Supplemental figure 3.12.20. Urs-12-en-3β-ol (α-amyrin) **N° 36**. **A)** Experimental mass spectrum; **B)** Reference mass spectrum (NIST Mass Spectrometry Data Center)



Supplemental figure 3.12.21. **Stigmasta-3,5-dien-7-ona** (Tremulone) **N° 37. A)** Experimental mass spectrum; **B)** Reference mass spectrum (SciFinder®)

CHAPTER IV

Antioxidant, Anti-acetylcholinesterase and cytotoxic potential of *Hyptis* spp.

Oxidation, reactive oxygen species (ROS), what does these mean and which are their consequences?

A free radical can be defined as any molecular species that contains an unpaired electron in an atomic orbital, presenting consequently a high reactivity in the attempt to capture an electron from other atoms and reach their electrochemical stability. Once the free radical can subtract the electron it needs (reduction), the stable molecule that loses an electron (oxidation) becomes, in turn, a free radical by being left with an unpaired electron, thus initiating a cascade reaction. Because these reactive species do not have specific receptors, they have indiscriminate aggression ability on living cells and tissues (Saavedra, et al., 2010; Lobo et al., 2010; Phaniendra et al., 2015).

Electron transfer is one of the most fundamental chemical processes for cell survival. The side effect of this dependence is the production of free radicals and other reactive oxygen species (ROS) that can cause oxidative damage ROS can be divided into 2 groups: free radicals and nonradicals this last is created when 2 free radicals share their unpaired electrons. Free radicals are also atoms or molecules produced continuously during metabolic processes and act as mediators for the transfer of electrons in various biochemical reactions, performing functions relevant to metabolism and are the result of the imbalance between the generation of ROS and antioxidant defense system which results in what is known as oxidative stress (Birben, et al., 2012; Bae et al, 1999; Manian et al., 2008). The main sources of free radicals are the cytoplasmic organelles as mitochondria, lysosomes and peroxisomes that metabolize oxygen, nitrogen and chlorine, generating a large number of metabolites (Shami and Moreira, 2004; Saavedra, et al., 2010).

Free radicals have different roles in the body and are involved in energy production, phagocytosis, and regulation of cell growth, intercellular signaling and synthesis of important biological substances. However, its excess has deleterious effects, such as damage to DNA, proteins and cellular organelles, causing changes in cellular structure and functions. ROS are hugely implicated in the pathogenesis of various diseases, such as cancer

and gene mutation, diabetes, heart and blood vessel disorders, muscle degeneration, neurodegenerative disorders, such as Parkinson's and Alzheimer's disease (Džinić and Dencher, 2018; Shahrbandy and Hosseinzadeh, 2007).

As a product of our metabolism, different types of free radicals are generated, such as superoxide anion, peroxide anion, perhydroxyl radical, hydroxyl radical and under hypoxic conditions, the mitochondrial respiratory chain also produces nitric oxide (NO), which can generate reactive nitrogen species (RNS). RNS can further generate other reactive species, for example, nitric oxide and peroxynitrite radical, reactive aldehydes—malondialdehyde and 4-hydroxynonenal—by inducing excessive lipid peroxidation (Hussain et al., 2003; Poyton et al., 2009; Saavedra, et al., 2010). Targets of free radicals include all kinds of molecules in the body. Among them, lipids, nucleic acids, and proteins are the major targets and modification of these molecules can increase the risk of mutagenesis and homeostatic disruption (Lobo et al., 2010).

Free radicals can also be generated by factors such as environmental contamination, exposure to ionizing radiation, smoke, drugs, chemical additives in processed foods and some xenobiotics such as pesticides, herbicides and fungicides (Saavedra, et al., 2010).

Antioxidant defense system

The antioxidant defense system has the function of inhibiting and/or reducing the damage caused by the deleterious action of free radicals or non-radical reactive species. These actions can be achieved through different mechanisms of action: preventing the formation of free radicals or non-radical species (prevention systems), preventing the action of these ROS (sweep systems) or, further, favoring the repair and reconstitution of structures biological systems (repair systems) (Clarkson and Thompson, 2000; Koury and Donangelo, 2003).

Under normal physiological conditions, the human body neutralizes ROS by means of several antioxidant mechanisms that involve the production of enzymatic antioxidants (such as superoxide dimutase, catalase and glutathione peroxidase) or non-enzymatic (ascorbic acid, glutathione, carotenoids and flavonoids) to prevent the oxidative damage (Saavedra, et al., 2010).

In addition to the endogenous antioxidants, there are also exogenous antioxidants, which can be found in products of natural origin, such as plants, which contain several antioxidants such as polyphenols, which may play an important role in the sequestration and neutralization of free radicals. Among the range of antioxidants, polyphenols constitute

a huge and complex category of compounds. Phenylpropanoids, flavonoids, lignans and proanthocyanidins (also called condensed tannins) may be included in this class of substances. The interest in flavonoid as antioxidants has increased considerably in recent years due to its high capacity to quenching free radicals, which are associated with the development of various diseases (Souza et al., 2008).

But, what is an antioxidant? Antioxidant is defined as any substance which at lower concentration than those of the oxidizable substrate is capable of effectively retarding or inhibiting oxidation. These substances can act directly, neutralizing the action of free radicals and non-radical species, or indirectly, participating in the enzymatic systems with such capacity (Halliwell and Whiterman, 2004).

Antioxidant substances can present different protective properties and act in several stages of the oxidative process, functioning by different mechanisms and are therefore classified in two main categories: primary and secondary antioxidants. Primary antioxidants promote the removal or inactivation of free radicals formed during the initiation or propagation of the reaction, by donating hydrogen atoms to these molecules, interrupting the chain reaction (by primary antioxidant). Secondary antioxidant mechanisms involve removal of ROS initiators by quenching chain-initiating catalyst (Dubey, 2014).

Antioxidants have different mechanisms of action including electron donation, metal ion chelation, co-antioxidants, gene expression regulation, metal ion bonding (valence change), ROS inactivation, conversion of hydroperoxides to non-radical species, or absorption of UV radiation (Maisuthisakul et al., 2007; Krinsky, 1992 *apub* Lobo et al., 2010).

Natural antioxidants may effectively retard the oxidation of lipids and other compounds both *in vivo* and *in vitro*, therefore they may be useful food additives and inhibitors of reactive oxygen species in living cells and due to their natural origin, antioxidants obtained from plants are of greater benefit in comparison to synthetic ones which were found to have undesirable secondary effects. The antioxidant properties of plant extracts have been attributed mainly to their polyphenol contents (Atoui et al., 2005; Šliumpaitė et al., 2013).

Why are antioxidant compounds important against inhibition of acetylcholinesterase?

As written in previous paragraphs, ROS are the underlying cause of oxidative stress and are hugely implicated in the pathogenesis of various diseases, such as Alzheimer's disease (AD) (Džinić and Dencher, 2018).

AD is the most common form of neurodegenerative disease, characterized by memory dysfunction. The notable biochemical change is cholinergic loss in the brain caused by the reduction of acetylcholine levels. Specifically, a reduction in the amount of acetylcholine released from cholinergic synapses in Alzheimer's patients has been identified (Özbek et al., 2017; Loizzo et al., 2009), thus cholinesterase inhibition has been one of the mainstays for treatment of AD and is a promising strategy for dementia therapy. Therefore, a treatment method has been developed to increase or protect the amount of acetylcholine by inhibiting acetylcholinesterase (AChE) (Gupta et al., 2009; Mathew and Subramanian, 2014).

Several studies found the oxidative stress to be dramatically overexpressed in neurodegenerative disorders, for this reason oxidative stress might have a key role in AD pathogenesis and disease progression and it has been proposed as a novel candidate biomarker, especially in prodromal stage (asymptomatic stage) of the disease. Recent studies have been demostrated that the oxygen concentration is an important factor that modulates cellular parameters and cell survival/death of neuronal like human neuroblastoma cells. To date, it is not clear whether oxidative stress, in AD, is a cause or a consequence of the main neuropathological mechanisms involved and how it interacts with them (Džinić and Dencher, 2018; Vergallo, et al., 2018; Di Domenico, et al., 2016; Chang, et al., 2014).

Brain metabolism is diminished in AD and key mitochondrial enzymes are reduced in brain. Several reports suggest diminished antioxidant capacity in cells from AD patients. The antioxidant defenses of cells from familial AD patients are lower than those from normal subjects (Gibson and Huang, 2005). Gibson and Huang (2005) concluded that mitochondrial dysfunction and oxidative stress occur in neurodegenerative diseases. Compared to controls of their study, bombesin releasable calcium stores (BRCS) are exaggerated in fibroblasts from patients with AD and in fibroblasts from a young control treated with H_2O_2. They hypothesized that alterations in oxidative stress underlie the exaggeration in BRCS in AD, and appropriate antioxidants may be useful in treating this abnormality, and those that lead to DNA damage.

Nowadays, most of the drugs approved and licensed for AD can cause undesirable side effects and they are largely ineffective for treating severe AD cases. Therefore, it is compulsory to search for new anti-AD drug candidates. Recent studies are focusing on their replacement with naturally occurring antioxidants derived from medicinal plants (Gupta et

al., 2017). Furthermore, Gupta and colleagues (2017) concluded that high phenolic content from ethanolic extracts of *Syzygium aromaticum* (L.) Merr. & L. M. Perry can be correlated with its high free radical scavenging and high inhibitory activity against AChE. Supporting and confirming that antioxidant compounds are an excellent source of drugs for the treatment of AD.

Antioxidant potential in Lamiaceae

Studies on free radicals and the development of new methods for evaluating antioxidant activity have increased considerably in recent years. The discovery of the deleterious effect of free radicals on cells and their relationship to certain diseases, acting as a cause or aggravating factor, has stimulated the search for new substances capable of preventing or minimizing oxidative damage to living cells. Medicinal plants provide a very rich source of metabolites to discover new bioactive molecules. Species from Lamiaceae have been traditionally used as drugs because of their antioxidant, antimicrobial, antihypertensive, antiinflammatory, antitumor, anti-Alzheimer and gastroprotective properties. There is a correlation between content and type of phenolic compounds found in plant extracts and their biological activity (Valant-Vetschera et al., 2003; Matkowski and Piotrowska, 2006; Birkett et al., 2010; Gupta et al., 2017).

Some *Hyptis* species have been shown to possess antioxidant activities *in vitro* as *Hyptis crenata* Pohl ex Benth. which exhibited strong antioxidant activity when assayed by DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging method (Rebelo et al., 2009); *Hyptis rhomboidea* Mart. et Gal., *Hyptis conferta* Pohl ex Benth., and *Hyptis dilatata* Benth. also showed antioxidant activity when tested by DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)) radical scavenging methods (Li et al., 2014; Tafurt-García et al., 2015); *Hyptis fasciculata* Benth. exhibited activity protecting yeast cells against the toxic effect of ROS produced by hydrogen peroxide and menadione stress. This cellular protection was correlated with a decrease in oxidative stress markers, such as the levels of ROS, protein carbonylation and lipid peroxidation (Silva et al., 2009).

Regarding the evaluation of cytotoxicity were made because according to McGaw and colleagues (2014) cytotoxicity studies are a useful initial step in determining the potential toxicity of a test substance, including plant extracts or biologically active compounds isolated from plants this because minimal to no toxicity is essential for the successful development of a pharmaceutical or cosmetic preparation. In investigations like those

presented in this thesis by the type of biological activities that are tested it is important to identify substances with promising biological activity and negligible cytotoxicity.

The aim of the present chapter was investigating *Hyptis radicans* (Pohl) Harley & J.F.B. Pastore and *Hyptis multibracteata* Benth. in terms of their antioxidant, anti-acetylcholinesterase and cytotoxic potential.

Material and Methods

Material and methods including plant material, extraction and fractionation (plant material and sample preparation), chemical analyzes and isolation of bioactive compounds and identification by Nuclear Magnetic Resonance (NMR) were described in chapter III.

Free radical scavenging activity determination using DPPH• (2,2-Diphenyl-1-picrylhydrazyl)

Free radical scavenging activities of all samples were determined according to the DPPH radical method by Furlan and colleagues (2015). DPPH• solution in methanol (0.2 mM) was freshly prepared and 200 µL were mixed with 20 µL of each extract in various concentrations (1000 -1.95 µg mL^{-1}). Reaction mixture was incubated for 20 min at room temperature and in the dark. The decrease in absorbance was measured at 515 nm by a microplate reader. As positive control, Trolox (6–200 µg mL^{-1}) and quercetin (7.5–120 µg mL^{-1}) were used. Methanol was used as negative control. All the reactions were performed in triplicate.

Free radical scavenging activity determination using ABTS• (2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)

Scavenging activity of the ABTS radical followed the protocol modified by Santos and colleagues (2016). The ABTS solution (3.84 mg mL^{-1}) was prepared using ultrapure water and a solution of 2.6 mM potassium persulfate. For the formation of ABTS radical, the two solutions above were mixed in a ratio of 1:1. Mixture was maintained in the dark at room temperature for 12–16 h. At the time of the test, 1 mL of the ABTS• previously prepared was diluted in 30 mL of methanol. To each 20 µL of each extract in various concentrations (1000 -1.95 µg mL^{-1}) and controls were added 280 µL of the diluted solution of ABTS•. Microplate was incubated for 2 h at room temperature in the dark. The absorbance was detected at 734 nm by a microplate reader. Trolox (12–250 mg mL^{-1}) and quercetin (15–120 mg mL^{-1}) were

used as positive controls. Methanol was used as negative control. All the reactions were performed in triplicate.

Ferric-reducing antioxidant potential (FRAP)

Ferric-reducing power of samples was determined according to a modified protocol from Furlan and colleagues (2015). FRAP solution was prepared on the day of analysis by mixing 25 mL of acetate buffer (0.3 M, pH 3.6), 2.5 mL of 10 mM TPTZ and 2.5 mL of 20 mM ferric chloride. FRAP solution (265 µL) was mixed with 10 µL of each extract in various concentrations (1000 -1.95 µg mL^{-1}) and 25 µL of ultrapure water. After incubation of 30 min at 37°C, the absorbance at 595 nm was detected by a microplate reader. Trolox (6–200 mg mL^{-1}) and quercetin (7.2–180 mg mL^{-1}) were used as positive control. Methanol was used as negative control. All the reactions were performed in triplicate.

Metal chelating assay

Ferric chelating activity was performed according to Santos and colleagues (2016), by mixing 10 µL of each extract in various concentrations (1000-1.95 µg mL^{-1}) with 130 µL of 10% acetate buffer, 10 µL of 1 mM ferrous ammonium sulfate, and 10 µL of 6.1 mM ferrozine solution (after 5 min). Microplate was kept at room temperature for 10 min, and absorbance at 562 nm was detected by a microplate reader. A methanolic solution of gallic acid (5–100 mg mL^{-1}) was used as a positive control and methanol as negative control. All the reactions were performed in triplicate.

Determination of oxygen radical absorbance capacity (ORAC)

ORAC assay was determined according to a modified protocol by Santos and colleagues (2016). Sodium fluorescein was dissolved in phosphate buffer solution (PBS) (75 mM, pH 7.0) to obtain a stock solution of 4.0 µM. The fluorescein working solution (8 nM) was freshly prepared in PBS, and 150 µL of this was mixed with 25 µL of each extract in various concentrations (1000-1.95 µg mL^{-1}) (in PBS) at different concentrations. The reaction mixtures were incubated for 30 min at 37°C, and 25 µL of 75 mM AAPH (2,2′-Azobis(2-methylpropionamidine) dihydrochloride) were added to the wells. As positive controls, a PBS Trolox solution was plotted (6.25–50 mM) and PBS as negative control. A blank with PBS was run with each assay. The fluorescence (excitation = 485 nm; emission = 520 nm) was registered 120 times with a delay of 60 seconds between repeats using a microplate reader. The antioxidant capacity was based on the calculation of the area under the curve (AUC), using the following formula:

(AUC)=1+ f1/fo+ f2/fo+ f3/fo+... fi/fo, (AUC)=1+ f1/fo+ f2/fo+ f3/fo+... fi/fo,

Where f_0 is the initial fluorescence reading at 0 min, and f_1 is the fluorescence reading at time 1. The net AUC was obtained by subtracting the AUC of the blank from the AUC of the sample. The final ORAC values were calculated by using a regression equation between Trolox concentrations and the net AUC. All the reactions were performed in triplicate.

Inhibition of lipid peroxidation assay (TBARS)

A modified thiobarbituric acid-reactive species (TBARS) assay was used to measure the lipid peroxide formed, using egg-yolk homogenates as lipid-rich media (Dorman et al., 1995; Upadhyay et al., 2014). 125 µL of egg homogenate (egg-yolk 10% in KCl 1.15% v/v, homogenized for 30 s, followed by ultrasonication for a further 5 min.) and 25 µL of each extract in various concentrations (1000 -1.95 µg ml^{-1}) were mixed and then 100 µL of ultrapure water were added. Finally, to induce lipid peroxidation 12,5 µL $FeSO_4$ (0.07 M) was added to the above mixture and incubated for 30 min. Thereafter 370 µL of 20% of Trichloroacetic acid (TCA) and 370 µL of 0.8% TBA (w/v) (prepared in 1.1% sodium dodecyl sulphate SDS) were added, homogenated, and heated in a boiling water bath for 15 to 30 min. After cooling, 800 µL of 1-butanol was added to each sample and centrifuged at 3000 rpm for 10 min. 300 µL of the organic upper layer (supernatant) were then taken and put into a 96 wells microplate for absorbance detection at 532 nm by a microplate reader. For positive control BHT (1000-1.95 µg mL^{-1}) and as negative control, for both 25 µL of ultrapure water was used in place of the extract.

Percentage inhibition was calculated as [Ac – As/Ac × 100]. Ac is the absorbance of the negative control and As is the absorbance of the test samples/the absorbance of the positive control. All the reactions were performed in triplicate.

Nonsite-Specific Hydroxyl Radical-Mediated 2-Deoxy-D-ribose Degradation

The ability of the extract to inhibit nonsite-specific hydroxyl radical-mediated peroxidation was carried out essentially as described by Halliwell and colleagues (1987) and Dorman and colleagues (2004) adapted for microplate reader. The reaction mixture contained 15 µL of each extract in various concentrations (1000-1.95 µg ml^{-1}) dissolved in KH_2PO_4-KOH buffer (50 mM, pH 7.3), 18 µL of 30 mM 2-deoxy-D-ribose in KH_2PO_4-KOH buffer, 36 µL of a premixed 9 µL of 1 mM $FeCl_3$, 9 µL of 1.04 mM EDTA, 9 µL of 1.0 mM H_2O_2, and 9 µL of 1.0 mM aqueous ascorbic acid (1:1:1:1 v/v, all solutions were made in ultrapure sonicated water). Ascorbic acid and $FeCl_3$ solutions were made inmediately before the test.

Microplates were incubated at 37°C for 60 min. Thereafter, 80 µL of 20%TCA were added to each well followed by 80 µL of 1.0% TBA (TBA, sonicated and warmed). The microplates were heated at 80°C for 1 hour. The extent of oxidation was estimated from the absorbance at 532 nm. Butylated hydroxytoluene best known as BHT (1000-1.95 µg mL^{-1}) was used as positive control and a negative control were used KH_2PO_4-KOH buffer (50 mM, pH 7.3).

Percentage of inhibition was calculated as [*A*c – *A*s/*A*c × 100]. *A*c is the absorbance of the negative control and *A*s is the absorbance of the test samples/ the absorbance of the positive control. All the reactions were performed in triplicate.

Site-Specific Hydroxyl Radical-Mediated 2-Deoxy-D-ribose Degradation.

The ability of the extracts to inhibit site-specific hydroxyl radical-mediated peroxidation was carried out as described in the nonsite-specific hydroxyl radical-mediated peroxidation inhibition procedure, except that EDTA was replaced by buffer (Dorman et al., 2004). All the reactions were performed in triplicate.

For DPPH, ABTS, FRAP, ORAC and Metal chelating antioxidant assays, sample antioxidant potential was expressed as milligram per gram of dry extract equivalents to positive control (Trolox and quercetin) and effective concentration of each sample to achieve 50% of the antioxidant activity (EC_{50} µg mL^{-1}) were calculated for all assays.

Acetylcholinesterase inhibitory activity

Acetylcholinesterase (AChE) inihibitory activity was measured using a 96-well microplate assay (Mathew and Subramanian, 2014) based on Ellman and colleagues method (1961). The further enzyme-dilution was done in 0.1% of bovine serum albumin (BSA) in buffer. DTNB was dissolved in the buffer containing 0.1 M NaCl and 0.02 M $MgCl_2$. Acetylthiocholine iodide (ATCI) was dissolved in ultrapure water. In the 96-well plates, 100 µL of 5 mM DTNB, 40 µL of buffer (50 mM Tris–HCl pH 8.0), 20 µL of each extract in various concentrations (1000-1.95 µg/ml) dissolved in buffer containing not more than 10% methanol were added to the wells and 20 µL of 0.26 U/mL of AChE. The plate was incubated for 15 min (25°C) and then the absorbance was measured at 412 nm and the readings were used as negative control. The enzymatic reaction was initiated by the addition of 20 µL of 18 mM ATCI and the hydrolysis of acetylthiocholine was monitored by reading the absorbance every 5 min during 20 min. Neostigmine (2000-31.2 ng mL^{-1}) was used as positive control and buffer (50 mM Tris–HCl pH 8.0) as negative control. All the reactions were performed in triplicate. The percentage inhibition was calculated as follows:

$$\% \text{ inhibition} = (E\ S) / E \times 100$$

Where; E is the activity of the enzyme without extract and S is the activity of enzyme with the extract. IC_{50} value was calculated using the regression curve between the % inhibition and the different concentrations of each plant extract.

Cytotoxic activity

The procedure used was adapted by Dr. Caroline Fernandes da Silva (University of São Paulo) from Mosmann, 1983.

The supplemented DMEM culture medium (Dulbecco's Modified Essential Medium, Life Tecnologies) was supplemented only when it was used, adding 10 μg mL^{-1} gentamicin and 10% heat-inactivated fetal bovine serum (Gibco), was heated to 37 ° C in a water bath. In a 15 mL sterile falcon tube, 9 mL of culture medium (performed inside the laminar flow) was added. RAW 264.7 Cell Lines (macrophagues) were thawed in a water bath at 37 °C (not more than 2 min) and the entire contents of the thawed vial were transferred to the tube containing the culture medium and centrifuged for 5 min to precipitate the cells. The liquid medium was discarded from the tube and the cells were resuspended in 1 mL of supplemented DMEM medium (homogenize well, all in the flow). The cells were transferred to a sterile culture bottle containing about 30 ml of supplemented DMEM medium. The bottle was placed in the CO_2 incubator (the lid was opened slightly but not removed from the bottle). The culture was left in the incubator until reaching a confluence of 80% (about two or three days).

For the colorimetric MTT (tetrazolium) assay the cell culture was washed twice with PBS 1x buffer (diluted from PBS 10x, 2g de KCl, 80g de NaCl, 14,4g de Na_2HPO_4 e 2,4g de KH_2PO_4. Dissolve in ultrapure water and make up to 1L in volumetric flask, autoclave for 20 min at 120 °C), and discarding the buffer in the vessel with sodium hypochlorite. Add 3 mL of trypsin to the culture bottle. Add 3 mL of supplemented DMEM culture medium.

In an autoclaved 0.2 mL tube, make a 1:100 cell aliquot and place 10 μL of that dilution in the neubauer chamber to count the cells under the microscope, 0.5x105 cells in each well. In a 96 well plate, place 200 μL of supplemented DMEM medium and add $0.5x10^5$ cells per well. Maintain the plate for 24 hours in the oven at 37ºC and 5% CO_2 atmosphere. After this period, discard the old medium and add a new one together with the sample (10 μL at 100 μg mL^{-1}). For the macrophages were used, the toxicity of different volumes of 10% DMSO (0.2, 1, 2, 4, 8, 16 and 20 μL) was tested for a total of 200 μL of DMEM per well. Cytotoxicity results demonstrate that with 1% DMSO per culture (ie 20 μl of 10% DMSO in 200 μl total), the cells

have viability of 86.62 ± 5.09%. From these results it was standardized that the cytotoxicity of the extracts would be tested containing at most 1% DMSO, so that the viability of the cells was equal to or greater than 80. At the same time a control, containing the same volume of DMSO 10% used for elution of extracts. After assembling the plate with the extract, keep the macrophages in the oven at 37ºC and 5% CO2 for 24 h. After this period, add 20 µL of MTT (3- (4,5-Dimethylthiazol-2yl) -2,5-diphenyl Tetrazolium Bromide (Sigma, 5 mg ml^{-1} solution prepared in PBS), following further incubation at 37 ° C and 5% CO_2 for 3 h, then discard the supernatant and disperse the cells with 100 µL of DMSO. Analysis the plate in a spectrophotometer at 595 nm.

Results and discussions

Antioxidant activity results

Antioxidant capacity mesured by antiradicalar capacity (DPPH, ABTS, and ORAC), ferric reducing power (FRAP), and the capacity of metal chelating of extracts from *Hyptis radicans* and *Hyptis multibracteata* is shown in table 4.3. Results were expressed as mg g^{-1} of trolox or quercetin (DPPH, ABTS, FRAP), or as µM $µg^{-1}$ of trolox for ORAC, and mg g^{-1} of gallic acid for metal chelating assay.

The use of two standards in some assays is based on the fact that most of the studies show their results only in relation to trolox antioxidant capacity and this makes comparisons among species easier, but as *Hyptis* extracts contain flavonoids and other phenolic compounds in their composition, quercetin was also used as a standard. Furthermore, quercetin is also recognized as a potent antioxidant substance (Baghel, et al., 2012).

Ethanol extract (**EE**), ethyl acetate phase (**EAP**), and methanol phase (**HMP**) showed similar results for DPPH, ABTS and FRAP assays for both species. **EAP** seems to be the sample with highest content of compounds equivalent to trolox or quercetin (Table 4.1). Furthermore, for DPPH, ABTS, FRAP, and ORAC assays the phase that showed smaller quantity of antioxidant substances was hexane phase (**HP**), for both species, presenting less than half of the amount of antioxidant compounds per gram of dry extract when compared to ohter samples. Metal chelating activity was similar among samples, being **EAP** from *H. multibracteata* the less activity sample for this assay. Similar result was observed for **HMP** from *H. radicans* (Table 4.1).

EC_{50} values for all antioxidant assays (DPPH, ABTS, FRAP, ORAC, metal chelating, lipid peroxidation - TBARS, site-specific and nonsite-specific OH• mediated 2-Deoxy-D-ribose degradation – Deoxi SS and Deoxi NSS, respectively) are shown in Table 4.2.

Santos and collaborators (2018) observed, when comparing the ethanolic extracts of seven *Hyptis* species that the extracts with higher activity of DPPH quenching were *H. lappulacea* (EC_{50} 12.33 μg mL^{-1}); for ABTS assay, *H. radicans* was the most active sample (6.01 μg mL^{-1}); for FRAP assay, *H. lacustris* was the most active sample (5.35 μg mL^{-1}); and for ORAC assay, *H. lappulacea* showed to be more active than other species (0.56 μg mL^{-1}). Only the ethanolic extract were tested by DPPH, ABTS, FRAP, and ORAC assays, showing different EC_{50}, and consequently, different antioxidant potential (Santos et al., 2018). *H. multibracteata* was one of the less active species in all assays, while *H. radicans* is among the three most active species in all assays.

Several methods are used to measure the antioxidant activity of extracts and solutions of pure substances, being the most commonly used those involving chromogen compounds of a radical nature which stimulates reductive oxygen species, due to their ease, speed, and sensitivity. The presence of antioxidants leads to the disappearance of the radical chromogens and this can be monitored by colorimetric reactions. The most widely used methods are ABTS and DPPH (Ali et al., 2008). The present study performed more four antioxidant assays than Santos and colleagues (2018) in order to better evaluate the antioxidant potential of each sample.

Table 4.1. Contents of antioxidant substances equivalents to trolox, quercetin, and galic acid per gram of dry extract for DPPH, ABTS, FRAP, ORAC, and Metal chelating assays. Ethanol crude extract (**EE**), hexane phase (**HP**), ethyl acetate phase (**EAP**), hydromethanol phase (**HMP**); H. rad - *Hyptis radicans*; H. mul - *Hyptis multibracteata*.

Species	Sample	DPPH[a]		ABTS[a]		FRAP[a]		ORAC[b]	MCh[a]
		Trolox	Quercetin	Trolox	Quercetin	Trolox	Quercetin	Trolox	Gallic acid
H. rad	EE	350.14±13.67	231.60 ± 14.16	495.54 ± 7.47	303.70 ± 0.42	852.76 ± 14.11	274.52 ± 14.80	2648.30±370.70	220.57 ± 32.45
	EAP	463.51 ± 18.83	248.03 ± 16.80	504.77 ± 12.25	304.46 ± 6.75	940.70 ± 4.79	269.97 ± 13.17	3656.30±901	260.01 ± 47.31
	HP	44.32 ±13.88	-	96.56 ± 13.47	20.04 ± 1.75	84.45 ± 2.63	24.48 ± 1.98	322.22±252.40	233.06 ± 39.49
	HMP	407.56 ± 10.81	231.64 ± 10.36	508.87 ± 7.62	312.13 ± 17.33	690.90 ± 11.35	193.25 ± 11.93	2352.53±202.52	186.68 ± 29.50
H. mul	EE	214.35 ± 4.99	107.93±13.96	247.51 ± 11.10	143.03 ±3.24	357.59 ± 6.27	102.28 ± 0.93	906.05±286.03	257.79 ± 45.54
	EAP	271.51 ± 19.55	122.81±7.68	251.97 ± 8.99	149.97 ±7.15	393.83 ±10.76	112.78 ± 3.32	1727.18±80.66	187.19 ± 31.22
	HP	58.68 ± 12.77	-	47.59 ± 7.62	18.31 ±1.35	46.98 ± 3.31	13.96 ±3.64	-	223.43 ± 44.66
	HMP	240.41 ± 8.05	134.82±8.08	264.56 ± 19.03	150.85 ±5.49	355.54 ± 8.34	103.63 ± 2.29	926.32±175.80	212.81 ± 25.87

a: DPPH, ABTS, FRAP and Metal chelating (MCh) activities are expressed in milligrams equivalent of antioxidant substances to standard substance per gram of dry extract (mg E g^{-1}). **b**: ORAC activity is expressed as µMol of trolox equivalents per gram of extract (µMol TE g^{-1}). -: uncalculated values.

Table 4.2. Effective concentration of **EE**, **HP**, **EAP** and **HMP**, from of *H. radicans* and *H. multibracteata* to achieve 50% antioxidant activity (EC_{50} in μg mL^{-1}) for antioxidant assays: sequestration of DPPH and ABTS, FRAP, ORAC, metal chelator assay (MCh), lipid peroxidation (TBARS), Site-Specific (S-Spe) and nonsite-Specific (NS-Spe) OH• mediated 2-Deoxy-D-ribose degradation.

Species	Samples	DPPH	ABTS	FRAP	ORAC	MCh	TBARS	S-Spe	NS-Spe
H. rad	**EE**	37.61	6.01	6.01	2.68	55.86	59.98	0.73	112.94
	EAP	32.12	5.04	12.76	2.98	42.36	40.46	3.35	75.08
	HP	432.68	75.41	193.80	8.77	432.68	100.29	10.24	201.88
	HMP	36.51	7.65	10.97	2.78	79.75	53.68	0.32	140.41
H. mult	**EE**	72.19	23.17	17.60	38.09	52.01	61.22	0.49	97.74
	EAP	69.55	39.93	16.30	26.04	74.06	330.40	1.86	154.92
	HP	-	151.11	144.56	38.97	155.47	277.01	5.93	-
	HMP	65.01	46.56	17.37	40.40	55.65	421.30	0.45	162.58
Standards	**Trolox**	13.36	9.41	10.71	1.47	-	-	-	-
	Quercetin	8.18	2.72	3.43	0.44	-	-	-	-
	Rosmarinic Acid	7.51*	2.22*	0.89*	0.12*	-	-	12.93	196.38
	BHT	-	-	-	-	-	1.25	7.42	48.67
	Gallic acid	-	-	-	-	6.6	-	-	-

-: Not tested; **BHT**: *butylated hydroxytoluene*; **D** *: Santos and colleagues (2018).

DPPH, ABTS, and ORAC were used to assess and compare the scavenging activity of extracts and phases from *H. radicans* and *H. multibracteata*. DPPH radical-scavenging assay measures the ability of an antioxidant to react with DPPH radicals in *in vitro* experiment. When a solution of DPPH• is mixed with a substance that can donate a hydrogen atom, the reduced form of the radical is generated accompanied by loss of colour (Murillo et al., 2012; Ali et al., 2008). ABTS is based on the inhibition by antioxidants of the absorbance of the radical cation ABTS. The experiment is carried out using a decolorization assay, which involves the generation of the ABTS chromophore by the oxidation of ABTS• with potassium persulphate (Ali et al., 2008). ORAC assay uses an oxidizable xanthene dye fluorecein and AAPH as a peroxyl radical generator. It is a method that takes free radical action to completion and uses an area-under-curve (AUC) technique for quantitation and thus,

combines both inhibition percentage and the length of inhibition time of the free radical action by antioxidants into a single quantity (Ali et al., 2008).

Results of DPPH• reduction by extracts of *Ginkgo biloba* in a study from Silva and collaborators (2009) showed EC_{50} of 26.6 μg mL^{-1}. *G. biloba* extract is used as standard due to its well-established antioxidant activity. In this study, *H. radicans* showed an outstanding DPPH• scavenging activity; except **HP**, all phases and **EE** showed EC_{50} near to 30 μg mL^{-1}, very close in activity to *Gynko biloba* L.. *H. radicans* also showed antioxidant activity similar to *Condea undulate* (EC_{50} 35 μg mL^{-1}), one of the well-studied *Hyptis* species (Barth *et al*., 1991), known as *H. fasciculata* before Harley and Pastore (2012). As well as, *H. radicans* showed EC50 for ABTS and ORAC assays closer to commercial antiantioxants used as standards when compared to *H. multibracteata* (Table 4.2).

For the ORAC test **EE** from *H. radicans* showed EC_{50} (2.68 μg mL^{-1}) below only *H. multibracteata* (30.09 μg mL^{-1}), in comparison with the species of the Santos study. Values of EC50 for **EE**, **EAP** and **HMP** are very similar to the EC50 of trolox (1.47 μg mL^{-1}).

Another antioxidant mechanism is the metal chelating active of a sample, since an excess of free irons has been implicated in the induction and formation of free radicals in biological systems (Wong et al., 2014). In the metal chelating assay, ferrozine forms complexes with ferrous iron, yielding a red colour that can be quantify by spectrophotometry. However, in the presence of chelating agents, the formation of the complex is disrupted, which leads to a decrease in the red colour. Measurement of colour reduction gives an estimation of the binding ability of the coexisting chelator (Adjimani and Asare, 2015). Extracts and phases from *H. radicans* and *H. multibracteata* were tested by the metal chelating assay, being **EAP** of *H. radicans* the sample showing the best EC_{50} (42.36 μg mL^{-1}). Once more, even being the more active sample, EC_{50} of **EAP** is 6 times greater than the EC_{50} of gallic acid, evidencing EAP as 6 times less active than gallic acid.

As DPPH, ABTS, and ORAC, the non-site specific OH•-mediated 2-deoxy-D-ribose is an antiradicalar assay. Nonsite-specific assay indicates samples that are capable of scavenging hydroxyl radicals, but by the protection of carbohydrates from oxidation. Site-specific OH•-mediated 2-deoxy-D-ribose differs from nonsite-specific once it does not use EDTA as chelator agent. In this assay, when EDTA is absent, metal ions bind to 2-deoxy-D-ribose and hydroxyl radicals are formed from the oxidation of the carbohydrate. Site-specific assay indicates samples capable of preventing OH• formation by chelating and deactivating metal ions (Dorman et al., 2004). *Hyptis* samples were very efficient in preveting

carbohydrate oxidation (nonsite-specific assay), being more active than the commercial antioxidant BHT. However, as seen in metal chelator assay, *Hyptis* samples does not show high antioxidant activity by chelating metal (Table 4.2).

Lipid peroxidation constitutes a chain reaction of the polyunsaturated fatty acids of cell membranes, generating free radicals that can alter membrane´s permeability, fluidity and integrity (França et al., 2013). In this process, the major reactive aldehyde resulting from the peroxidation of biological membranes is malondialdehyde (MDA) (Vaca et al., 1988), and it can be used as an indicator of tissue damage (Ohkawa et al., 1979). TBARS assay monitors the reaction between MDA and thiobarbituric acid (TBA), which results in a red colored product when MDA is procuded, *i.e.*, sample tested was not efficient in minimize lipid peroxidation. Comparing *H. radicans* to *H. multibracteata*, the first species showed to be more effective to prevent lipid peroxidation (**EAP** EC_{50} 40.46 μg mL^{-1}), but it is 40 times less effective than BHT, a commercial antioxidant.

HP from *H. radicans*, showed the highest EC_{50} in all assays, which means it was the less active sample as antioxidant, making this phase less interesting for fractionation and isolation of substances. On the other hand, **EE**, **EAP** and **HMP** from *H. radicans* showed promising activities in comparison to commercial antioxidants. It is important to point out that in this study we are comparing EC_{50} values of an isolated substance (commercial antioxidant) and extracts and phases, composed by a mixture of constituents. For *H. multibracteata*, it was observed the same, **HP** seems to be the less active sample in the majority of antioxidant assays.

EC_{50} is currently the most important result used in biological activities; it allows obtaining a dose-response curve using different concentrations of a tested sample. It also allows to compare different samples with standards compounds, as in this study: trolox, rosmarinic acid, quercetin, gallic acid, BHT or another commercial antioxidant.

Samples with the high antioxidant activity (higher amounts of antioxidant substances and with best values of EC_{50}) were **EE**, **EAP** and **HMP** from *H. radicans*; they were also the ones that presented the highest levels of phenolic compounds, especially flavonoids and chlorogenic acid derivatives (Table 3.2, Chapter III), contrasting with. **HP,** for both species, which showed less amounts of phenolic compounds (Table 3.2, Chapter III) and were samples with the lowest antioxidant activity.

Phenolic compounds are substances widely distributed in nature; more than 8,000 phenolic compounds have already been detected in plants. This large and complex group is

part of the constituents of a variety of vegetables, fruits and industrialized products. These compounds are involved in the antioxidant activity since they are able to chelate metal ions involved in reactive oxygen species (ROS) generation, or to scavenge free radicals and form stable intermediate structures, thus limiting free radical initiation or propagation. Not only for their ability to donate hydrogen or electrons, but also because of their stable intermediate radicals, which prevent the oxidation of various food ingredients, particularly lipids (Brand-Williams et al., 1995; Zheng and Wang, 2001; Moon and Shibamoto, 2009).

The term 'phenolics' can be refered to compounds that possess an aromatic ring bearing one hydroxyl group, whereas 'polyphenols' are compounds that can have one or more aromatic rings bearing more than one hydroxyl group. However, these two terms are often used interchangeably in most literature. The antioxidant activity as well as other biological functions of polyphenols has been largely attributed to the aromatic feature and highly conjugated system with multiple hydroxyl groups. The activity depends on the number and position of them in the molecule, that make these compounds good electron or hydrogen atom donors, neutralizing free radicals and another ROS (Tsao, 2010; Zhang and Tsao, 2016)

The antioxidant activity of phenolic acids and flavonoids is related to their ability to homolytically release hydrogen from the O-H bond and form stable radicals either by resonance effect or by hyperconjugation. The hydroxylation and/or methoxylation patterns influence the antioxidant potential of these molecules and can also retard oxidative reactions in biological systems (Simões et al., 2001; Quideau et al., 2011).

In plants, phenolic compounds cover a range of substances, from simple molecules to those with a high degree of polymerization, such as tannins and lignins. Gallic acid and cinnamic acid derivatives, such as caffeic, synaptic, and ferulic acid, have proven antioxidant activity inhibiting lipid peroxidation (Soares, 2002).

It is very common to find studies reporting the presence of phenolic derivatives in Lamiaceae species, especially cinnamic and chlorogenic acid derivatives, but also other phenolic acids. As examples, *Lavandula angustifolia* Miller (lavender) showed caffeic acid and rosmarinic acid as major constituents; for *Stachys officinalis* (L.) Trev. (Bruconic) were reported caffeic acid, rosmarinic and chlorogenic acid; *Orthosiphon spicatus* Bak. showed rosmarinic acid and caffeic acid derivatives, as well as glycolic and benzoic acids (Cunha et al., 2006). In *Hypenia salzmannii* (Benth.) Harley, some phenolic derivatives already reported were *p*-methoxycinnamic acid (Messana et al., 1990), some glycosylated

phenylpropanoids, chlorogenic acid, protochatechuic acid, hydroquinone and thymohydroquinone (Pedersen, 2000). Among phenolic compounds, despite rosmarinic acid, cinnamic and chlorogenic acid derivatives, lignoids, such as brevipolides, are also consider as very frequent class of substances described among *Hyptis* spp. (Falcão and Menezes, 2003; Table 2.1, Chapter II). *H. radicans* and *H. multibracteata* studied in the present study showed similar chemical composition regarding their phenolic constitution corroborating previous studies within Lamiaceae and *Hyptis* (Table 3.3, Chapter III).

It is important to take into consideration the rosmarinic acid, that is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid commonly found in species from Boraginaceae and Lamiaceae (especially from the Nepetoideae subfamily), but less frequently in some other families (Petersen and Simmonds, 2003). The presence of rosmarinic acid confers antioxidant action, reducing numerous deleterious events to the organism, such as the formation of reactive oxygen species, lipid peroxidation and DNA fragmentation. This compound and its derivatives showed many notable biological and pharmacological activities, such as antioxidant (Izzo and Capasso, 2007; Erkan et al., 2008).

H. radicans and *H. multibracteata* analyzed in the present study presented rosmaric acid (**15**) as one of their phenolic constituents, but it is not the major one. Due to this fact, contents of rosmarinic acid were quantified separately from other cinnamic and chlorogenic acid derivatives (Table 3.2, Chapter III). *H. radicans* **EE** showed the highest rosmarinic acid content (17.64 mg ρ-CE g^{-1}), but this substance was not detected in **EAP** phase, leading us to conclude that the high antioxidant activity observed for **EAP** is not related to rosmarinic acid content. In other words, other phenolic compounds may be responsible for this biological activity. In **EAP** from *H. radicans* was detected the highest content of flavonoids, followed by chlorogenic acid derivatives (Table 3.2, Chapter III).

Regarding major constituents of *H. radicans* **EE** and **EAP** (Table 3.3, Chapter III), lithospermic acid A (**16**) a rosmarinic acid derivative is the major constituent of **EE** (20.59 mg g^{-1}), followed by rosmarinic acid (17.64 mg g^{-1}), while **EAP** is composed mainly by lithospermic acid A (14.37 mg g^{-1}), followed by cirsimaritin (**25**) (13.37 mg g^{-1}), an apigenin derivative. **HMP** from *H radicans* was the sample showing the highest content of lithospermic acid A (37.86 mg g^{-1}), followed by rosmarinic acid (8.27 mg g^{-1}). Comparing to *H. radicans*, the content of rosmarinic acid in *H. multibracteata* was 4 times lower (3.89 mg g^{-1}), and lithospermic acid A and cirsimaritin were not detected, considering **EE** (Table 3.3, Chapter III). These results point out to rosmarinic acid and lithospermic acid A as important

substances to confer the antioxidant activity observed in *H. radicans*. Differences in relative amounts of these compounds may be responsible for the minimal differences in EC_{50} observed for the **EE** and **HMP**.

According to the literature, polyphenols such as rosmarinic acid and flavonoids exert *in vivo* cytoprotection effects by increasing the production of endogenous prostaglandins, reducing histamine secretion, inhibiting the development of *Heliobacter pylori*, and reducing the formation of oxygen free radicals (Alimi et al., 2010, Awaad et al., 2013, Yesilada et al., 2014). These molecules are able to increase the activity of antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, and glutathione reductase, to chelate iron and copper ions, to inhibit the Fenton reaction. These actions are important to interfer in the electron transport and oxy-reduction reactions, as well as reducing lipid radicals (Harborne and Williams, 2000; Zheng and Wang, 2001). The biological activities are mainly correlated, as already mentioned, with the presence of electron donating hydroxyl groups and double bonds presents in phenolic chemical structure (de Lira Mota et al., 2009) as observed and already reported for rutin and quercetin, two flavonols (Pietta, 2000).

In the case of flavonoids, the antioxidant activity is directly related to the free radical capture capacity and the chemical structure of these compounds. Generally, this activity depends on the substitution of hydroxyl groups and the aromatic rings, where by resonance there is proton donation and electron stabilization (Sharififar et al., 2009). It was verified that the mechanisms involved in the antioxidant activity of flavonoids are related to their chemical structure in two ways, by the ability of donation of protons and stabilization by displacement of electrons due to their structure; and by chelation of free radicals produced naturally by our body in the oxidative process (Pourmorad et al., 2006).

For non-flavonoids, such as hydroxycinnamic and hydroxybenzoic acid derivatives, longer distance separating the carbonyl group and the aromatic ring of a phenolic acid seemed to increase the antioxidant activity. Hydroxyl groups on benzoic ring at the *ortho*-position and/or *para*-position can lead to elevated antioxidant activity compared to other positions and unsubstituted phenol (Göçer and Gülçin, 2011; Zhang and Tsao, 2016).

In order to evaluate the antioxidant activity of a plant extract and pure substances, the concomitant use of more than one method is recommended. It is important due to the complexity in the composition of a plant extract and can provide information regarding classes of substances responsible for the expression of the activity and the mechanisms involved in the antioxidante activity (Bozin et al., 2006).

The high concentration of rosmarinic acid in *H. radicans* is relevant, since Tepe (2008) established for several species of *Salvia* a strong correlation between the concentration of rosmarinic acid and the antioxidant potential presented by them. Also, the author suggested a relationship with rosmarinic acid presence and the astringent, anti-inflammatory, antibacterial and antiviral activities, verified in the majority of species. Rosmarinic acid together with flavonoids present in the aqueous extract of *Salvia officinalis* showed antiulcerogenic activity that probably is a result of the high antioxidant activity of this extract (Roman Junior et al. 2015). It is also important the presence of litospermic acid A, because it is one of the most popular polyphenol in several medicinal plants, such as *Tournefortia sarmentosa* Lam., *Salvia miltiorrhiza Bge*. (Danshen), and *Origanum vulgare L.* It is considered to be the major active constituent of *T. sarmentosa*, corresponding to about 0.6% w/w in the dried herb and showing *in vitro* antioxidant activity (Liu et al., 2008; Lin et al., 2003; Lin et al., 2002). Recently Zhang and colleagues (2018) corroborated this last information in *Salvia miltiorrhiza* Bge. (Lamiaceae) correlating by bivariate analyses that caffeic acid, danshensu (salvianic acid A), rosmarinic acid, lithospermic acid A, and salvianolic acid B were the major contributors to antioxidant activity of this species. These studies corroborated the results of the present work where rosmarinic acid and lithospermic acid A have an important role in antioxidant activity observed.

PCA statistical analysis method

To analyze correlations between the antioxidant activity and sample chemical composition a principal component analysis (PCA) was performed (Figure 4.1). For this analysis were used the contents of phenolic classes in each sample (Table 3.2, Chapter III) and their biological activity.

Principal component analysis (PCA) summarized 56% of data variability within the first two axes (Figure 4.1). It showed the chemical composition as a determinant variable for the separation of samples along axis 1. **EAP** from *H. radicans* was placed on the positive superior side of axis 1, being the sample with the highest levels of flavonoids and also showing high antioxidant activity in ABTS, metal chelating, TBARS, and site-specific hydroxyl radical-mediated 2-Deoxy-D-ribose degradation. **EE** and **HMP** from *H. radicans* also showed high antioxidant potential. In this case both sample were placed at the same positive side of axis 1, but at the negative side of axis 2, being characterized by higher amounts of chlorogenic acid derivatives and the presence of rosmarinic acid. This composition is correlated, using PCA analyse, to higher active in DPPH, ORAC, FRAP and

nonsite-specific hydroxyl radical assays. On the negative side of axis 1 were placed samples that showed poor antioxidant potential, as **HP**, from both species, and all other phases from *H. multibracteata.* **EE** from *H. multibracteata* was the sample showing the highest levels of Nepetoidin A and B, and the highest amount of flavonoid among *H. multibracteata* samples, which probably influenced its antioxidant activity.

PCA corroborated results where samples presenting higher contents of flavonoids and rosmarinic acid are more efficient as antioxidants.

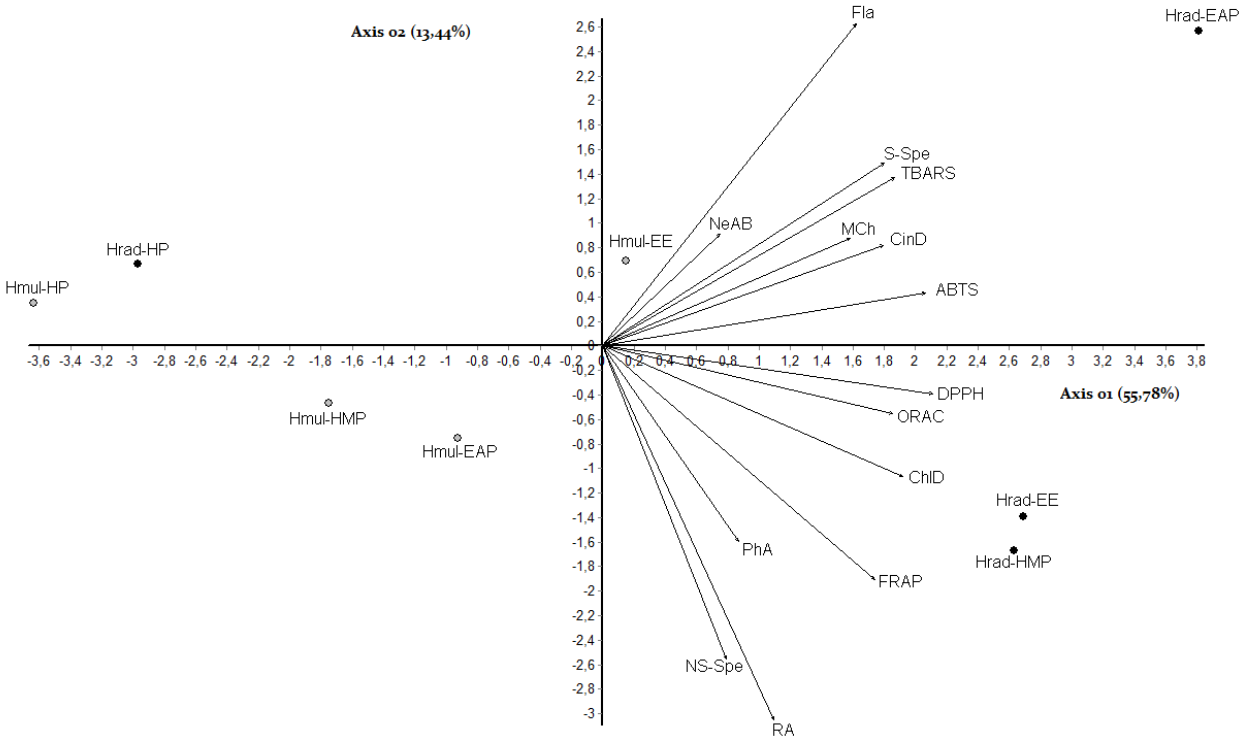


Figure 4.1. Principal component analyses (PCA) using 13 variables (**Fla**: flavonoids; **NeAB**: nepetoidins A and B*;* **CinD**: cinnamic acid derivatives; **ChlD**: chlorogenic acid derivatives; **PhA**: phenolics acids; **RA**: rosmarinic acid; **S-Spe** and **NS-Spe**: site-specific and nonsite-specific hydroxyl radical-mediated 2-Deoxy-D-ribose degradation; **TBARS**: thiobarbituric acid reactive substances; **MCh**: metal chelating assay; **ABTS**: 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid scavenging assay; **DPPH**: 2,2-Diphenyl-1-picrylhydrazyl scavenging assay; **ORAC**: oxygen radical absorbance capacity; **FRAP**: ferric-reducing antioxidant potential) evaluated in four samples of *H. radicans (***Hrad-EE**: crude ethanol extract; **Hrad-HP**: hexane phase; **Hrad-EAP**: ethyl acetate phase; **Hrad-HMP**: hydromethanol phase), and four samples of *H. multibracteata* (**Hmul-EE**: crude ethanol extract; **Hmul-HP**: hexane phase; **Hmul-EAP**: ethyl acetate phase; **Hmul-HMP**: hydromethanol phase). **a)** Percentage of variance explained by PCA, and percentage of expected variance estimated by the broken-stick test; and **b)** correlation coefficients between variables and axes 1 and 2.

Flavonoids have been widely studied for their antioxidant properties since they are able of preventing or delaying the rate of oxidation, which takes place in autoxidisable materials and are involved in the antioxidant activity; or by the hability to chelate metal ions

involved in reactive oxygen species (ROS); or by generating or scavenging free radicals while forming stable intermediate structures, thus limiting free radical initiation or propagation (Zheng and Wang, 2001; Tarnawski et al., 2006; Moon and Shibamoto, 2009; Boulila, et al., 2015).

Harmful effects of disturbed antioxidant–prooxidant balance can be largely prevented by intake of antioxidant supplements or antioxidant containing foods and for that reason studies with the objective of find new sources of natural antioxidants are important (Charles, 2012; Halliwell and Gutteridge, 2015).

Salvia miltiorrhiza Bunge is considered as one of the most important plant in traditional Chinese medicine, being mentioned in the Chinese Pharmacopoeia (2010). Roots and rhizome of *S. miltiorrhiza* include a group of bioactive compounds that comprises numerous polyphenols in which *i.e.* rosmarinic acid, caffeic acid, salvianolic acid, and litospermic are included (Ożarowski et al., 2017; Zhong et al., 2009; Xu et al., 2007). Considering this information and comparing with the present results, it can be concluded that *H. radicans* can be a potencial candidate as fitotherapic.

Cytotoxicity assay (MTT)

MTT assay is the best-known method to determine the mitochondrial dehydrogenase activity in the living cells. Cytotoxicity assays together with cell viability studies are used for drug screening of chemicals.

Overall, it can be stated that **HP** from *H. multibracteata* induced the death of more than 80% of cells (Table 4.3), *i.e.* this species may contain a range of phytochemical constituents with anticancer potential (Dongre et al., 2008). Synergistic effects of different bioactive compounds in **HP** may be responsible for cytotoxicity of this phase (Arullappan et al., 2014). The anticancer activity of **HP** on studied cells is explicit, and the MTT assay suggests a mitochondrial relation. HPLC-DAD was used for the determination of phenolics compounds, and the major class of substances in this sample are flavonoids. According to some studies this class have been shown a biochemical and pharmacological potential including antioxidant activities, modulation of carcinogen metabolism, and inhibition of cell proliferation (Ahmad et al., 1997; Fujiki et al., 1999; Lin et al., 1999).

HP was the phase with the largest amount of substances called steroids (table 3.8, Chapter III). Recently Biradi and Hullatti (2017) have shown that the steroid stigmasterol

has cytotoxic activity when tested against A549 and HepG2 cells and when tested by Brine Shrimp Lethality (BSL) bioassay against *Artemia salina* Lich.

The most abundant substance in **HP** is β-sitosterol or 24-ethylcholest-5-en-3 beta-ol **(34)** with 20.46 % of relative abundance (table 3.7, Chapter III). The cytotoxic activity of β-sitosterol have been proved on the Caco-2 cells using the MTT cell viability assay with significant cytotoxic activity with an IC_{50} of 54 μg mL^{-1} (Maiyoa et al., 2016).

The cytotoxic activity is an indicator of the possible applicability of the extracts in further *in-vitro* and *in-vivo* studies related with antitumoral activities.

Table 4.3. Percent cell inhibition of *H. radicans and H. multibracteata* extracts and phases in a concentration of 100 μg mL^{-1} on RAW 264.7 Cell Lines (macrophagues).

Sample	*H. radicans*				*H. multibracteata*			
	EE	**HP**	**EAP**	**HMP**	**EE**	**HP**	**EAP**	**HMP**
Cell inhibition (%)	-	-	-	-	10.13	83.35	-	-

-: No cell inhibition

Cytotoxicity studies are a useful initial step in determining the potential toxicity of a test substance, including plant extracts or biologically active compounds isolated from plants (McGaw et al., 2014). Minimal to no toxicity is essential for the successful development of a pharmaceutical or cosmetic preparation. In investigations like those presented in this thesis by the type of biological activities that are tested it is important to identify substances with promising biological activity and negligible cytotoxicity, in this case, only **HP** from *H. multibracteata* showed cytotoxicity effect.

Biological activity of isolated compounds

Antioxidants, whether natural or synthetic, have high oxidative stability due to their molecular structure and, therefore, play a fundamental role in the prevention of oxidation resulting from the action of reactive species as free radicals (Andrade-Wartha, 2007).

Studies of identification of chemical structure are always necessary to demonstrate which substances in an extract are responsible for the observed biological activity. In the present study isolated substances were tested against four antioxidant assays: DPPH, ORAC, and the two Deoxy D-Ribose degradation assays. These assays have different mechanism of action and are currently used to access the antioxidant potential of plant extracts as

mentioned above. In table 4.4, are shown the EC_{50} (μg mL^{-1}) for the isolated compounds from both *Hyptis* species. These compunds were also screened for the acethylcholinesterase (AChE) inhibition assay. For information about identification of constituents, please see Table 3.3, Chapter III.

During the present study, it was possible to isolate 15 susbtances, from both species, but only those substances with sufficient mass were selected for test. From *H. multibracteata*, 5 subtances were isolated: caffeic acid (**6**), rutin (**11**), rosmarinic acid (**15**), ethyl caffeate (**20**) and nepetoidin B (**24**); from *H. radicans* were isolated the remaining 11 compounds: two unidentified flavanones (**7** and **13**), apigenin derivative (**8**), lithospermic acid A (**16**), chlorogenic acid derivative (**17**), cinnamic acid derivative (**18**), two unidentified flavonoids (**19** and **21**), apigenin (**23**), cirsimaritin (**25**) and an unknown substance (NI, **26**), the numbers between parentheses correspond to the identification number shown in Table 3.3, Chapter III. Rosmarinic acid (**15**), nepetoidin B (**24**) and cirsimaritin (**25**) are common compounds reported for Lamiaceae, especially for Nepetoideae, while lithospermic acid A (**16**) is the major substance from *H. radicans*. These four constituents were selected to be tested to better correlate the sample chemical compostion and its biological activity.

Among the 8 substances tested, lithospermic acid A prove to be the most antioxidant constituent, especially in ORAC and in site-specific hydroxyl radical-mediated 2-Deoxy-D-ribose degradation and could be related to the high antioxidant activity of **EAP** and **HMP** from *H. radicans*. This substance showed to be more active than the commercial antioxidants trolox (ORAC and DPPH) and BHT (nonsite-specific hydroxyl radical-mediated 2-Deoxy-D-ribose), as well as, this compound is more active than rosmarinic acid (**15**). As expected, isolated substances from *H. radicans*, although also found in *H. multibracteata* but in smaller contents, showed higher antioxidant activity, suggesting their importance to the superior antioxidant activity of *H. radicans* compared to *H. multibracteata*.

Regarding the anti-cholinesterase activity, nepetoidin B (**24**) was the most active substance with EC_{50} value of 52.73 μg mL^{-1}, although this EC_{50} is higher than the standard, Neostigmine (33.84 ng mL^{-1}).

Table 4.4. Effective concentration of isolated substances from *H. radicans* and *H. multibracteata* to achieve 50% of antioxidant (EC_{50} μg mL^{-1}) and anti-acetylcholinesterase (EC_{50} ng mL^{-1}) activities tested by the methods of sequestration of DPPH, ORAC, Site-Specific (S-Spe), nonsite-Specific (NS-Spe) OH• mediated 2-Deoxy-D-ribose degradation, and anti-acetylcholinesterase enzyme assay (AChE).

Species	Samples	DPPH	ORAC	S-Spe	NS-Spe	AChE
H. radicans	**Flavonoide (19)**	30.52	4.09	2.31	120.25	NA
	Lithospermic acid A (16)	21.83	0.51	0.90	166.12	345.75
	Cinnamic acid der. (18)	44.37	1.52	8.64	114.91	149.22
	Flavonoide (21)	50.88	1.15	1.62	236.83	NA
	Cirsimaritin (25)	21.77	2.27	6.74	116.86	NA
	NI (26)	27.87	5.76	5.73	72.22	122.63
H. multibracteata	**Nepetoidin B (24)**	21.38	14.66	115.4	55.90	52.73
	Rosmarinic Acid (15)	7.51	1.98	12.93	196.38	240.66
Standards	**Trolox**	13.36	1.47	-	-	
	Quercetin	8.18	0.44	-	-	
	BHT	-	-	7.42	48.67	
	Neostigmine	-	-	-		33.84*

- : Not tested, **NA**: No activity; **BHT**: butylated hydroxytoluene. *: EC_{50} value of Neostigmine it was obtained by the MSc. Fernanda Anselmo.

Caffeic acid and its related compounds such as rosmarinic acid, chlorogenic acid, and lithospermic acid A are well-known for their diverse biological actions, including antioxidant effects. Rosmarinic acid is now recognized to be one of the promising food-functional polyphenols. With respect to its chemical structure, rosmarinic acid has two catechol moieties. Catechol is an important sub-structure for the potent antioxidant activity of phenolic antioxidants. In rosmarinic acid, one catechol structure exists in the caffeoyl moiety and the other is in the 2-oxyphenylpropanoyl moiety, plus it also has four hydroxyl groups (Moosavi et al., 2017; Chen and Ho, 1997).

As mentioned before, the antioxidant activity of hidroxicinamic acids is largely influenced by the number of hydroxy groups present on the aromatic ring. The phenoxy radical that is formed when hidroxicinamic acid molecule is oxidized by ROS can be stabilized by the adjacent electron-donating hydroxy groups. Furthermore, molecules with

ortho-dihydroxy or 4- hydroxy-3-methoxyl groups possess higher antioxidant activity than those bearing no such functionalities (Razzaghi-Asl et al., 2013; Cheng et al., 2007).

According to Razzaghi-Asl and colleagues (2013), most of the investigations concluded that the presence of *ortho*-dihydroxy phenyl group (catechol moiety) is significant to the antioxidant activity, while, the presence of three hydroxy groups does not necessarily improve this activity. Taguchi and colleagues (2017) also concluded that compounds without catechol type aromatic ring did not exhibit significant antioxidant activity, as it was showed by the low inhibition percentages even in very high concentrations on DPPH assay and they explained the significant radical scavenging activity of the tested compounds by the presence of catechol phenolic hydroxyls that are important in the scavenging action of ROS species. Two critical factors have been proposed emphasizing the antiradical activity of hydroxicinnamic acids containing catechol moiety, dissociation energy of OH bond and ionization potential. The OH bond dissociation energy is a function of the presence of the electron-donating hydroxy groups at the *ortho* position. This substitution pattern lowers the OH bond energy and enhances the rate of H-atom abstraction from the molecule and hence generates of ortho-semiquinone radical anion or *ortho*-hydroxy phenoxy radical. This species is more readily oxidized to a final *ortho*-quinone product (Razzaghi-Asl et al., 2013; Siquet et al., 2006; Son and Lewis, 2002). The great antioxidant activity of Rosmarinic acid, lithospermic acid A and nepetoidin B can be related to the *ortho*-dihydroxy phenyl groups (catechol moieties) in their structure, this three substances posses two of these groups.

Flavonoids can prevent injury caused by free radicals by diverse mechanisms like direct scavenging reactive oxygen species, for example by hydrogen atom donation and inactivation of free radicals, and metal chelating activity (Ferrali et al., 1997; Nijveldt et al., 2001; Procházková et al., 2011).

Ferrali and colleagues (1997) afirm that specific flavonoids are known to chelate iron and copper, thereby removing a causal factor for the development of free radicals. Pietta (2000) proposed that the binding sites for trace metals in the molecule of flavonoids are the catechol moiety in the B ring, the 3-hydroxyl and 4-oxo groups in the heterocyclic C ring, and the 4-oxo and 5-hydroxyl groups between the C and A rings (Figure 4.2), concluding that the major contribution to metal chelation activity is due, again, to the catechol moiety.

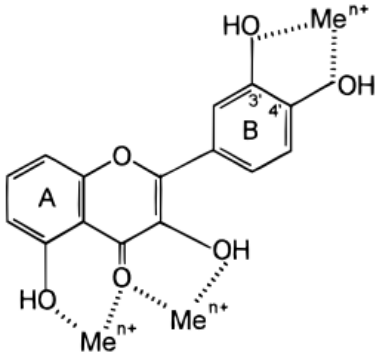


Figure 4.2. Binding sites for trace metal (Image: Pietta, 2000).

This information is important because in the test of site-specific hydroxyl radical-mediated 2-Deoxy-D-ribose degradation the absence of EDTA, a portion of the iron ions is complexed by 2-deoxy-D-ribose. The hydroxyl radicals arise close to the 2-deoxy-D-ribose molecule. Accordingly, compounds with ligand properties compete for iron ions with the 2-deoxy-D-ribose molecules and thus decrease 2-deoxy-D-ribose degradation that is caused by iron-catalyzed hydroxyl radical attack, so using this assay, we are measuring the sample metal chelating capacity (Gutteridge, 1984; Aruoma et al., 1987; Chobot, 2010).

DPPH, ORAC and nonsite-specific hydroxyl radical-mediated 2-Deoxy-D-ribose degradation have basically the same fundaments. In the last one the complex of iron with EDTA avoids complex formation with the tested substance, 2-deoxy-D-ribose or ascorbic acid, but does not prevent the participation of the iron in the Fenton reaction. Redox active scavengers inhibit efficiently 2-deoxy-D-ribose degradation by hydroxyl radicals, which are formed during the phase where iron ions were complexed by EDTA (Gutteridge, 1984; Aruoma et al., 1987; Buettner, 1988; Chobot, 2010).

Figure 4.3 shows the structure of cirsimaritin and its possible mechanism of action described by Banjarnahor and Artanti, 2014. According to Santos and Mira (2004) the B-ring hydroxyl structure is the most important part of the flavonoid structure for scavenging oxygen and nitrogen reactive species. Hydroxyl groups on this nucleus donate hydrogen and an electron to hydroxyl, peroxyl, and peroxynitrite radicals, stabilizing them and giving rise to a relatively stable flavonoid radical (Banjarnahor and Artanti, 2014). Cirsimaritin has one hydroxyl group on its B ring, another in A ring, and two *O*-methylation in the same ring. According to Procázková and colleagues (2011) and Moalin and colleagues (2011) the antioxidant active of flavanoids is given by an *ortho*-dihydroxy structre (cathecol) in B ring, which is important for electron delocalization; a doulbe bond C2-C3 conjugated with a 4-oxo

function in C ring, which provides electron delocalization from B ring; and hydroxyl groups at positions 3 and 5, which provides hydrogen bonding to oxo group. Thus, is expected that cirsimaritine, a flavone, presents low antioxidant activity; also because *O*-Hydroxyl groups were considered to be highly powerful electron-donors when compared with *O*-methoxy groups due to the weakening effect on the electron-withdrawing activity of the carboxyl substituent (Farhoosh, et al., 2016).

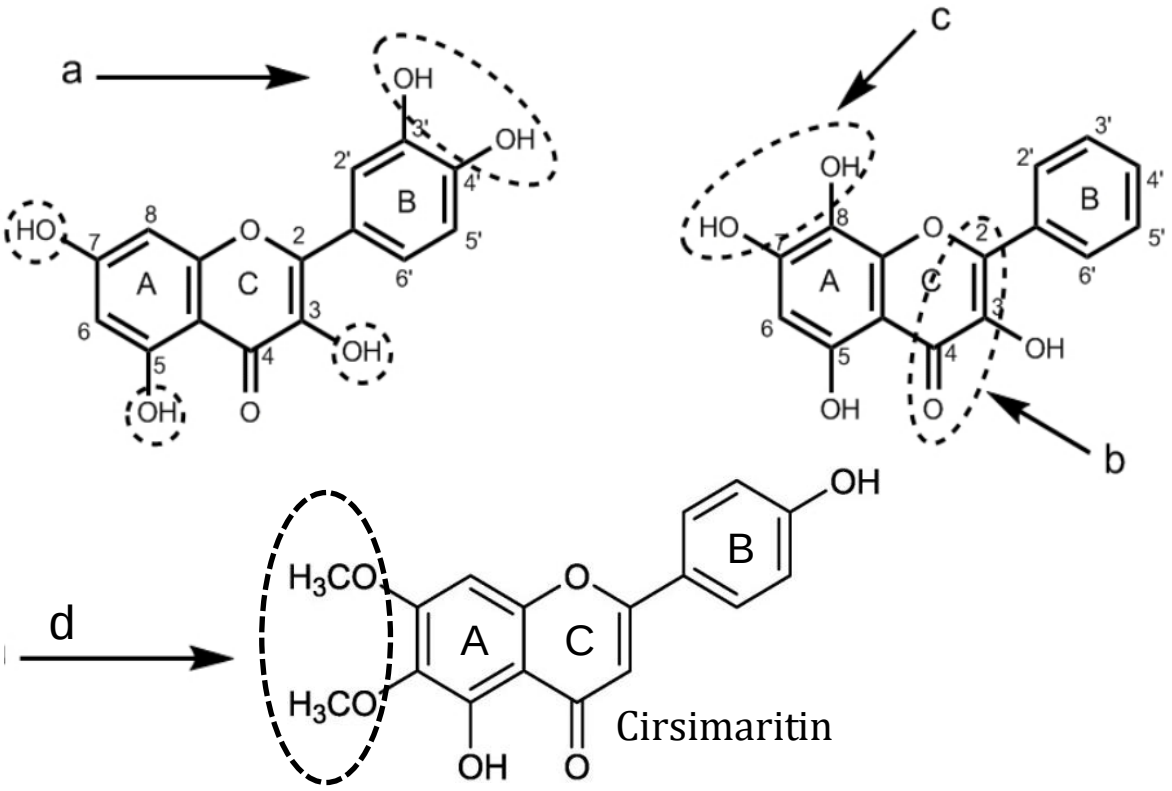


Figure 4.3. Antioxidant structure-activity relationships including: *ortho*-dihydroxy arrangement in the B ring **(a)**; C2-C3 unsaturated bond combined with C4 carbonyl group in the C skeleton **(b)** and hydroxyl groups **(c)**; *O*-methylation **(d);** this last structure belongs to Cirsimaritin (**25**). Adapted from Banjarnahor and Artanti (2014).

However, Cirsimatitin showed antioxidant activity close to trolox and BHT (Table 4.3). Comparng to quercetin, a flavonol that presents an *ortho*-dihydroxy arrangement on its B ring and three more hydroxyl gropus (C_7 and C_5 of A ring, and C_3), cirsimaritin is less active, but the *O*-methylation arrangement in the A ring is a part of its structure that could present an excellent *in vivo* activity. The obstruction of the hydroxyl group by methylation discharge the effect of metabolizing enzymes, and subsequently improves the antioxidant activity, this is due to the greater hepatic metabolic stability but also to improve intestinal absorption than that of unmethylated compounds (Borges Bubols et al., 2013; Banjarnahor and Artanti, 2014).

Four cinamic derivates were isoletd and tested in this study: rosmarinic acid (**15**), lithospermic acid A (**16**), cinnamic acid derivative (**18**), and nepetoidin B (**24**). Chlorogenic acids are a family of esters formed between certain trans cinnamic acids and quinic acid (Clifford, 2003); while rosmarinic acid and nepetoidin B are also caffeic acid ester, as chlorogenic acids, being rosmarinic acid an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid and nepetoidin B an ester of cafeic acid and 3,4-dihydroxyphenyl)ethenyl, according to Adomako-Bonsu and colleagues (2017).

Conclusions

The present research provides, for the first time, a comprehensive report on the antioxidant and cytotoxic activities of Hyptis species. **EAP** from *H. radicans* was the sample that presented the highest levels of total phenolic content, especially flavonoids, being also the sample with the high antioxidant activity with promising EC_{50}: DPPH (32.12 μg mL^{-1}), ABTS (5.04 μg mL^{-1}), Metal chelator assay (42.36 μg mL^{-1}), TBARS (40.46 μg mL^{-1}) and nonsite-Specific Hydroxyl Radical-Mediated 2-Deoxy-D-ribose Degradation *(*NS-Spe) with a EC_{50} of 75.08 μg mL^{-1}. **EE** from *H. radicans* showed the high antioxidant activity for FRAP and ORAC with EC50 of 6.01 and 2.68 μg mL^{-1}, respectively and has the highest amount of rosmarinic acid (17.64 mg g^{-1}). **HMP** from *H. radicans* showed the high antioxidant activity in Site-Specific Hydroxyl Radical-Mediated 2-Deoxy-D-ribose Degradation (S-Spe) assay with EC50 of 0.32 μg mL^{-1} and has the highest content of chlorogenic acid derivatives.

Lithospermic acid A isolated from *H. radicans* and rosmarinic acid and nepetoidin B from *H. multibracteata*, were substances with better antioxidant activity. Nepetoidin B isolated from *H. multibracteata* had the best EC_{50} (52.73 μg mL^{-1}) for anti-acetylcholinesterase activity. Regarding the results of cytotoxicity, **HP** from *H. multibracteata* induced the death of more than 80% of RAW 264.7 Cell Lines.

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CHAPTER V

Anti-HIV-1 and antibacterial potential of *Hyptis radicans* (Pohl) Harley & J.F.B. Pastore and *Hyptis multibracteata* Benth. (Lamiaceae).

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Abstract

It is urgent to develop a more effective treatment to overcome the problem of drug resistance in patients with HIV and bacterial infections, it leads to increased risk of disease progression, to increased medical costs, prolonged hospital stays and increased mortality. Flavonoids and other phenolics are groups of natural bioactive compounds widely distributed in edible plants and are well documented to possess antiviral and antimicrobial activities. *Hyptis* (Lamiaceae) is known to be used in Brazilian folk medicine to treat various diseases. The aim of this study is to evaluate antibacterial and anti-HIV-1 activities of *Hyptis radicans* and *Hyptis multibracteata* and correlate these data with the amount of phenolic compounds. Antibacterial activity was evaluated against two Gram-negative bacteria, *Pseudomonas aeruginosa* and *Escherichia coli*, and a Gram-positive *Bacillus subtilis*, *in vitro*. *H. multibracteata* phases were more effective on inhibiting *B. subtillis* with MIC_{50} 23.6 μg mL^{-1} and 12.13 μg mL^{-1} for HP and EAP, respectively. HP also presented activity against *P. aeruginosa* with MIC_{50} of 37.55 μg mL^{-1}. Crude ethanol extract and hydromethanol phase from *H. radicans* showed moderate anti-HIV-1 activity (MIC_{50} 159 μg mL^{-1}; MIC_{50} 180 μg mL^{-1}), but contents of total phenolic compounds are not the main sample feature to define anti-HIV activity, but there is correlation between Rosmarinic acid and anti-HIV1 activity of *H. radicans*. There was no correlation between phenolic content and antibacterial activity of *H. multibracteata*. This study provides the first evidence of the anti-HIV-1 and antibacterial activity for these two species of *Hyptis*.

Keywords

Phenolics; flavonoids; rosmarinic acid; microdilution method; anti-RT.

Abbreviations

HIV, Human Immunodeficiency Virus; EE, crude ethanol extract; HMP, hydromethanol phase; HP, hexane phase; EAP, ethyl acetate phase; $MIC_{50,}$ minimal inhibitory concentration; AIDS, acquired immune deficiency syndrome; WHO, World Health Organization; HPLC, high-performance liquid chromatography; DAD, diode array detector; *ρ*-CE, milligrams of *ρ*-coumaric acid equivalent; LE, milligrams of luteolin equivalent; RT-HIV-1, human immunodeficiency virus-1 reverse transcriptase enzyme; DMSO, dimethyl sulfoxide; LB, Luria Bertani; PCA, Principal component analysis; QSAR, *Quantitative structure–activity relationship*; ATP, adenosine triphosphate.

Introduction

According to Ruhfel *et al.* (2014) there are about 270 000 to 450 000 spermatophytes (gymnosperms and angiosperms) on our planet. Only 15% of these species were chemically studied, and only 6% have already been studied regarding their biological activities. These estimations are important when considering the vast potential of bioactive constituents in nature that have not been identified yet. This fact attracts the attention of the pharmaceutical industry that sees plants as an important source for new bioactive molecules for drug discovery. An outstanding example is the anti-malaria activity of artemisinin, a drug isolated from *Artemisia annua* L., which was discovered by Youyou Tu, who was awarded for this work with the Nobel Prize for Physiology and Medicine in 2015 (Nobel Prize, 2015).

Nowadays, more than 36 million people are living with HIV/AIDS, of which an estimated 17.5 million receive an antiretroviral therapy, in the world. Resistance to drugs by HIV is due to the ability of this virus to mutate and reproduce in the presence of antiretrovirals (WHO, 2016).

Another type of drug resistance is bacterial resistance to antibiotics, resulting in higher medical costs, longer hospital stays and higher mortality (WHO, 2016). The accelerated emergence of drug-resistant bacterial strains caused by the extensive use of traditional antibiotics is a current major threat (Davies and Davies, 2010). Therefore, the development of a more effective treatment for overcoming drug resistance (antibiotics and antiretrovirals) is urgently needed.

Plants have chemical mechanisms to control pathogens attacks and these compounds may have activity against mammalian viruses as well as against other

microorganism attacks (Matsuse *et al*., 1998; Kang *et al*., 2005). Therefore, a possible role for natural products in the fight against transmission of the human immunodeficiency virus or the associated acquired immune deficiency syndrome and bacterial resistance is routinely investigated (Andrae-Marobela *et al*., 2013; Balouiri *et al*., 2016).

Chemical substances widely distributed in edible plants are well documented to possess antiviral and antimicrobial activities, being flavonoids and other phenolics, one of the natural bioactive compounds groups (Dzialo *et al*., 2016). In addition, studies on Lamiaceae have provided great source of evidence to treat viral and microbial ailments (Van Wyk *et al*., 2011).

Phytochemical, biological and pharmacological interest of *Hyptis* Jacq. occurred in 1952 when *Hyptis suaveolens* (L.) Poit., precursor in the studies with this genus, had its chemical composition of volatile oil studied, since it was used for the treatment of various infections (Nayak and Guha, 1952). Another species reported on the literature are *Hyptis oblongifolia* Benth. and *Hyptis pectinata* (L.) Kuntze (Pereda-Miranda and Delgado, 1990; Pereda-Miranda *et al*., 1993), *Hyptis spicigera* Lam. (Kini *et al*., 1993; Pereda-Miranda and Delgado, 1990), *Hyptis ovalifolia* Pohl. (Souza *et al*., 2003), and *Hyptis martiusii* Benth. (Caldas *et al*., 2014).

Hyptis species are also known to be used in folk medicine for the treatment of various diseases, such as influenza and constipation (*Hyptis fruticosa* Salzm. ex Benth); respiratory diseases (*Hyptis macrostachys* Benth); stomach and intestinal disorders and bactericidal (*Hyptis martiusii* Benth); colic and liver diseases (*Hyptis pectinata*); nasal and atrial disorders (*Hyptis umbrosa* Salzm. ex Benth) and to combat fever (*Hyptis suaveolens* (Agra *et al*., 2008; Coutinho *et al*., 2008).

However, Harley and Pastore (2012), based on molecular data, proposed a major revision for Hyptidinae, recognizing 12 genera and the monophyly for *Hyptis*. Due to this new circumscription, some chemically important species, such as *Hyptis suaveolens*, *H. oblongifolia, H. pectinata, Hyptis spicigera, H. ovalifolia, H. macrostachys, H. fruticosa, H. martiusii, H. umbrosa* and more, were placed into other genera.

After the new circumscription, *Hyptis* comprises about 148 species included in 10 sections (Harley and Pastore, 2012), distributed in tropical and subtropical regions from North America to Caribbean and southward to Argentina and Peru (Harley and Pastore, 2012). *Hyptis* species are used in folk medicine to treat various diseases, such as *Hyptis*

atrorubens Poit. for flu and sore throat (Grenand *et al*., 2004), and *Hyptis capitata* Jacq. for fever and asthma (Lee *et al*., 1988).

Traditional uses and the great diversity of species occurring in Brazilian biomes turn *Hyptis* a promising source for studies of bioactive natural products. This study is aimed to explore antibacterial and anti-HIV-1 activities of extracts and phases from aerial parts of *H. radicans* (Pohl) Harley & J.F.B. Pastore, a common species from Central Brazilian Savannah (Cerrado) and Atlantic Rain Forest, and *H. multibracteata* Benth, a Brazilian endemic species restricted to the Atlantic Rain Forest. In addition, we correlate these data with the amounts of phenolic compounds. This study provides the first evidence of the anti-HIV-1 and antibacterial activity for both species.

Material and methods

Plant Material

Individuals from the same population of *Hyptis radicans* and *Hyptis multibracteata* were collected in the morning, near the Biological Reserve of Alto da Serra de Paranapiacaba in the municipality of Santo André, São Paulo. Aerial parts were stored for identification and voucher specimens (Silva-Luz 295 and Silva-Luz 294) were deposited at SPF Herbarium (University of São Paulo, Brazil).

Extraction and Fractionation (plant material and sample preparation)

H. radicans and *H. multibracteata* were dried at 40°C, powdered and macerated in 70% ethanol for 7 days at room temperature in the dark. Crude ethanol extracts (**EE**) were concentrated under reduced pressure below 50°C using a rotary evaporator and freeze-dried. Crude ethanol extract (2 g each) were dissolved in 100 mL of 50% methanol and were fractionated by partition with the same volume of hexane and ethyl acetate for four times, originating three phases: hexane (**HP**), ethyl acetate (**EAP**), and hydromethanol phase (**HMP**). All phases were concentrated under reduced pressure below 50°C by a rotary evaporator and freeze-dried.

Phenolic content

All freeze-dried samples (**EE**, **HP**, **EAP** and **HMP**) of both species were dissolved in MeOH (2 mg mL^{-1}) and analyzed by HPLC (1260 Agilent Technologies) using a DAD and a Zorbax-C18 column (150 × 4.6 mm, 3.5 µm) at 40°C. Solvents used were 0.1% acetic acid (AcOH) and acetonitrile (CH_3CN), starting with 15% of CH_3CN (0–20 min), increasing to 100% (20–25 min); isocratic (5 min); decreasing to 15% (30–32 min); isocratic (3 min). Solvent

flow rate was 1.5 mL min^{-1} (0–25 min), 1.0 mL min^{-1} (25–26 min), 1.5 mL min^{-1} (26–35 min); injection volume of 3 µL, and detection at 352 and 280 nm (Santos *et al*. 2016). *ρ*-Coumaric acid (Sigma-Aldrich) (0.005 to 0.400 µg mL^{-1}) was used to quantify phenolics acids, chlorogenic *and cinnamic acid derivatives.* Luteolin (INDOFINE Chemical Company, Inc.) at concentrations from 0.005 to 0.400 µg mL^{-1} was used to quantify flavonoids. Results are expressed as *ρ*-CE or LE per gram of dry sample (mg g^{-1}).

HIV-1 reverse transcriptase (RT) inhibitory bioassay

The inhibitory activity of the extracts and phases over the RT-HIV-1 was analyzed using the reverse transcriptase assay (Roche®), according to the manufacturer's instructions. Samples were dissolved to reach concentrations of 100 to 1000 µg mL^{-1} in DMSO 10% prepared using diethylpyrocarbonate (DEPC). Three controls were used: 10% DMSO instead of samples (S) with no addition of RT (Blank -B); 10% DMSO instead of samples with the addition of RT (negative control - NC); and foscarnet sodium hexahydrate (Sigma-Aldrich) with the addition of RT (positive control - PC; 0.075 to 3 µg mL^{-1}). The percentage of reserve transcriptase inhibition was calculated using the formula: {[(AbsNC – AbsB) – (AbsS – AbsB)] / (AbsCN – AbsB)} × 100, in which Abs = absorbance at 405 nm after subtraction of the absorbance at 490 nm. MIC_{50} values were calculated by using a regression equation between the concentration and the percentage of activity of each sample.

Antimicrobial assay

Antibacterial assay was adapted from the protocol proposed by Carneiro *et al*. (2011). Antimicrobial activity was evaluated against two Gram-negative bacteria, *Pseudomonas aeruginosa* (ATCC® 10145 Vitroids™) and *Escherichia coli* (MG1655), and a Gram-positive *Bacillus subtilis* (PY79), using the broth microdilution method in 96-wells microplate. For each microorganism, a culture stock was prepared on glycerol 50% and preserved at -80°C. Bacteria was transferred into Petri dishes containing Luria Bertani (LB) solid broth and incubated at 35°C for 24 h. After growth on the solid broth, an isolated colony was removed and inoculated into 5 mL of LB liquid broth and incubated for 16 h (*P. aeruginosa* and *E. coli)* or 18 h (*B. subtilis*) to originate the pre-inoculum. All bacteria were incubated at 35°C under constant agitation of 120 rpm. After incubation, 1 mL from pre-inoculum was diluted in 40 mL of LB liquid broth and this solution was incubated for 2 h under constant agitation of 120 rpm to reach 1 x 10^8 cells mL^{-1} or an absorbance of 0.1. From each final inoculum, 180 µL were placed on each plate well and added 20 µL of each sample diluted in DMSO 4% at

nine different concentrations (100 µg mL^{-1} to 2 mg mL^{-1}); Gentamicin was used at nine different concentrations (3.9 to 1,000 µg mL^{-1} in DMSO 4%) as positive control, and DMSO 4% was used as negative control (NC). All samples and controls were assayed in triplicate. Microplates were incubated for 24 h and the optical density was read at 595 nm using a Synergy H^1 equipment (BioTek, Inc.) to calculate the percentage of inhibition of bacterial growth. 2 µL of each well were inoculated in a new microplate containing LB solid broth to test the bactericidal effect of extracts and phases. As an indicator of cell viability, 20 µL of 0.01% Resazurin (7-hydroxy-10-oxidophenoxazin-10-ium-3-one) also known as diazo-resorcinol, were added to each plate well with LB liquid broth and the mixture was incubated for 1 h at 35°C under constant agitation of 120 rpm.

Antibacterial activity in percentage was calculated using the formula: [(AbsNC – AbsS)/ (AbsCN)] × 100. Antimicrobial activity was expressed as the minimum concentration of the sample to inhibit 50% of bacterial growth (MIC_{50}). The MIC_{50} values were calculated using a regression equation between the sample concentration and the percentage of bacteria growth inhibition.

Statistical analysis

All data were obtained in triplicate (n = 3) for antibacterial test and duplicate (n=2) for anti-HIV activity. The results were expressed as mean ± SD (standard deviation) and the software for data processing and statistical analyzes was R: The R project for statistical computing (R-3.5.0). In order to verify the correlation between phenolic contents and biological activities, a PCA analysis was performed using FITOPAC2 software (Shepherd 2011).

Results and discussions

Extraction yield

Yields of crude ethanol extract (**EE**) of *H. radicans* and *H. multibracteata* were 14.37% and 3.93%, respectively. *H. radicans* hydromethanol phase (**HMP**) showed the highest yield (52%), while *H. multibracteata* hexane phase (**HP**) yielding 49% (Table 1).

Results point out to a less polar composition of *H. multibracteata* due to the low yield of **EE**, and the highest yield of its **HP**. Moreover, comparing the total phenolic content of the ethanolic extracts (Table 1), it was observed higher phenolic contents in *H. radicans* (64.3 mg g^{-1}) than in *H. multibracteata* (15.53 mg g^{-1}).

Table 5.1. Yield (%) of **EE** and phases (**HP**, **EAP** and **HMP**) of *H. radicans* and *H. multibracteata*.

Sample	*H. radicans*	*H. multibracteata*
EE	14.4	3.9
HP	27.0	49.0
EAP	14.0	10.0
HMP	52.0	40.0

Phenolic content

The most common phenolic acids in plant tissues are hydroxycinnamic acid derivatives. This broad class includes caffeic acids, chlorogenic acids, *o*-, *m*- and *p*-coumaric acids, ferulic acids, and sinapic acids (Castelluccio et al., 1995; Prasad et al., 2011). The combination of caffeic acid and quinic acid results in a broad class of caffeic acid esters named chlorogenic acids. Rosmarinic acid is an ester of caffeic acid as well, but it has 3,4-dihydroxyphenyl lactic acid in its structure (Petersen, 2013). Chlorogenic acid**,** or *trans*-5-*O*-caffeoyl-D-quinate, and caffeic acid, are common constituents of coffee and some fruits, which are the most important sources of these compounds among plants, but they are also frequently reported as constituents of Lamiaceae species, as *H radicans* and *H. multibracteata* (Basílio et al., 2006; Garambone and Rosa 2008).

Rosmarinic acid is a major constituent of Lamiaceae, especially of Nepetoideae. It cannot be considered as a taxonomic marker for this subfamily due to its occurrence in other 39 families as Boraginaceae and in some monocotyledons families (Kim et al., 2015). For *Hyptis* species *sensu* Harley and Pastore (2012), rosmarinic acid was reported for *H. capitata* (Almtorp et al., 1991), *H. brevipes* and *H. lanceolata* (Pedersen, 2000), and *H. atrorubens* (Abedini et al., 2013). More recently Santos and colleagues (2018), reported the presence of rosmarinic acid in crude ethanol extract of *H. campestris*, *H. comaroides*, *H. lacustris*, *H. lappulaceae*, *H. meridionalis*, *H. radicans,* and *H. multibracteata*.

In addition, Grayer and colleagues (2003) identified other caffeic acid esters known as nepetoidin A and nepetoidin B in extracts of *H. ramose* and *H. lanceolata.* Nepetoidins were also reported by Tsai and Lee (2014) as constituents of *H. rhomboidea*. Grayer and colleagues (2003) analyzed 166 species from 78 Lamiaceae genera and correlated families. They observed the presence of nepetoidins in 110 of 116 Nepetoideae species. Falcão and colleagues (2013) also observed the presence of these compounds in the vast majority of the species

belonging to Nepetoideae and their absence in other subfamilies. The authors proposed these two nepetoidins as chemotaxonomic markers for Nepetoideae.

Based on the previous information, phenolic classes here analyzed were established using the UV-VIS absorption spectra (240 – 600 nm) as follows: phenolic acids - one absorption band in range of 250-285 nm; flavonoids - two absorption bands between 240-285 nm and 300-550 nm; chlorogenic acid derivatives - one band ranging from 315 nm to 335 nm with a shoulder near to 300 nm; cinnamic acids derivatives - one band in range of 280-320 nm. Rosmarinic acid and nepetoidins were quantified as two subclasses due to their importance as chemical features of Lamiaceae (both were identified using co-chromatography with authentic substances).

Comparing total phenolic content of **EE** (table 2), were observed higher phenolic content in *H. radicans* (53.03 mg g^{-1}) than *H. multibracteata* (33.66 mg g^{-1} **EAP** from *H. radicans* was the phase showing the highest amounts of these compounds (63.82 ρ-CE g^{-1}). **HMP** from *H. multibracteata* showed 163.64 times less amount of total phenolic compounds (0.39 ρ-CE g^{-1}) when compared to *H. radicans*.

Flavonoid content ranged from 1.74 to 33.29 mg g^{-1} for *H. radicans*, and 3.10 to 11.87 mg g^{-1} for *H. multibracteata.* **EAP** from *H. radicans* showed the highest contents of flavonoids 33.29 mg g^{-1} and for *H. multibracteata,* **EE** showed the highest value, 11.87 mg g^{-1}. Chlorogenic acid derivatives ranged from 21.97 to 41.18 mg g^{-1} (table 3.2) for *H. radicans,* while *H. multibracteata* **EE** and **EAP** showed values near to 9 mg g^{-1}. On the other hand, cinnamic acid derivatives were detected only in **EAP** and **HMP** from *H. radicans* (2.02 and 1.75 mg g^{-1}, respectively).

As expected, nepetoidins were detected in both species (**EE** and **EAP**), since they are considered as chemotaxonomic markers of Nepetoideae (Grayer and colleagues 2003). *H. multibracteata* showed higher amount of nepetoidins in **EE** (6.95 mg g^{-1}). Regarding the content of rosmarinic acid, **EE** from *H. radicans* showed the highest amount of this compound (17.64 mg g^{-1}) and **EE** from *H. mutibracteata* showed the smallest amount of rosmarinic acid (3.89 mg g^{-1}).

According to Falcão and Menezes (2003), terpenes, such as labdane-type diterpenes and triterpenes, are the main chemical constituents reported to *Hyptis*. Flavonoids seem to be less important, being flavones, especially apigenin derivatives, the main flavonoid class found in *Hyptis*. Among phenolic compounds, rosmarinic acid, cinnamic and chlorogenic acid derivatives are always described as major phenolic constituents in *Hyptis*, as well as,

lignans, such as brevipolides (Falcão and Menezes, 2003; Mohapatra et al., 2015). Present results corroborate previous reports.

Table 5.2. Content of phenolic compounds (mg g^{-1}) of **EE** and phases of *H. radicans* and *H. multibracteata* analyzed by HPLC-DAD detected in 280 nm: **HP**, **EAP** and **HMP**.

Phenolic classes	*H. radicans*				*H. multibracteata*			
	EE	**EAP**	**HP**	**HMP**	**EE**	**EAP**	**HP**	**HMP**
Phenolic acids[a]	0.50	0.81	-	1.61	0.69	2.21	-	0.39
Flavonoids[b]	9.74	33.29	1.74	8.99	11.87	10.73	3.10	-
Chlorogenic acid derivatives[a]	21.97	25.55	-	41.18	9.15	9.25	-	-
Cinnamic acid derivatives[a]	-	2.02	-	1.75	-	-	-	-
Nepetoidin A and B[b]	3.18	2.15	-	-	6.95	1.45	-	-
Rosmarinic acid[a]	17.64	-	-	8.27	3.89	8.08	-	-
NI					1.11		0.64	
Total phenolic content	53.03	63.82	1.74	61.80	33.66	34.70	3.75	0.39

-: Not detected; [a] milligrams equivalents of *ρ*-coumaric acid per gram of dry mass (mg *ρ*-CE g^{-1}); [b] milligrams equivalents of luteolin per gram of dry mass (mg LE g^{-1}); NI: Not identified

Anti-HIV-1 capacity

Some samples from *Hyptis* spp. analyzed in this study demonstrated a moderate inhibitory activity and are worthy of further investigation, especially **EE** (MIC_{50} 159 μg mL^{-1}) and **HMP** (MIC_{50} 180 μg mL^{-1}) from *H. radicans* (table 3).

Table 5.3. Minimal inhibitory Concentration (μg mL^{-1}) of **EE** and phases of *H. radicans* and *H. multibracteata* to inhibit 50% of Reverse Transcriptase activity (HIV1). **HP**, **EAP** and **HMP**.

Samples	*H. radicans*	*H. multibracteata*
EE	159	1100
HP	890	2040
EAP	260	540
HMP	180	320
Foscarnet (μg mL^{-1})	0,51	

The discovery of new substances that have new mechanisms of action and less toxicity remains of great interest and in this search has been given special importance to plant products because some have been identified as possible anti-HIV drugs. Treatment of HIV usually involves a therapy combining different drugs that target different stages of the viral replication cycle, a procedure that overcomes the development of virus resistance due to its high mutation rate (Wichner et al., 2017 *apud* Maes et al., 2012).

Geraniin and corilagin, both ellagitannins, are polyphenols that reportedly inhibit HIV-1 RT (Notka et al., 2003). Several phenolic compounds have been highlighted as exhibiting anti-HIV properties. Flavonoids as baicalin, quercetin, chrysin, and epigallocatechin, were reported as inhibiting all three enzymes essentials to ensure the virus life cycle, reverse transcriptase, protease and integrase (Kumar and Pandey, 2013).

Olivero-Verbel and Pacheco-Londoño (2002) investigated the flavonoid structure and its anti-HIV and cytotoxicity activity using Quantitative Structure-Activity Relationship (QSAR). They found that both cytotoxicity and the anti-HIV potential are dependent on electronic parameters such as the atomic charges in C3, the presence of the carbonyl group and the dipolar moment of the flavonoid. However, Kumar and Pandey (2013) report flavonoids and their antiviral activity, being flavones, as apigenin and luteolin, active against Herpes and Auzesky virus, probably by inhibiting viral polymerase or by binding viral nucleic acid and affecting the virus life cycle.

H. radicans seems to be more active than *H. multibracteata*. The positive control (foscarnet) inhibited 98% of the HIV-1 RT activity at 3 μg mL^{-1} (MIC_{50} 0.51 μg mL^{-1}). Moreover, **HMP** of *H. multibracteata* presented anti-HIV-1 RT activity almost twice lower than **HMP** of *H. radicans* (MIC_{50} 320 and 180 μg mL^{-1}, respectively). However, **HMP** of *H. radicans* has MIC_{50} tree hundred times higher than the positive control foscarnet, a synthetic antiretroviral drug.

EE of *H. radicans* was the sample with the highest amount of rosmarinic acid (RA) and was also the sample with the highest anti-HIV activity. RA is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid which was isolated for the first time from *Rosmarinus officinalis* L. leaves and is widely distributed in Lamiaceae herbs as well as the Boraginaceae, Rubiaceae, Apiaceae, and Araliaceae families (Jeong et al., 2018; Shekarchi et al., 2012). RA has been reported to have *in vitro* biological activities such as antiviral properties including anti-HIV-1 and antibacterial (Hooker et al., 2001), however, as antiviral, rosmarinic acid was previous described as having a weak inhibitory activity for HIV (Dubois et al., 2008).

Rohn and collaborators (2002) demonstrated that phenolic substances that are able to form quinones (*p*-benzoquinone, chlorogenic acid, gallic acid, caffeic acid, ferulic acid and quinic acid) react with proteins/enzymes influencing their physicochemical properties and as a consequence their in vitro enzymatic activity. The activity of the enzyme decreased depending on the concentration and on the number and position of hydroxyl groups of the phenolic compounds applied. The reaction occurs at lysine side chains, at the indole ring of the tryptophan residues, and at the free thiol groups of cystein side chains of the enzymes. McCue and collaborators (2004) corroborated the activity of rosmarinic acid inhibiting the activity of a porcine pancreatic amylase and they observed that the activity of á-amylase decreased depending on the RA content in the herbal extract. The results of the present study match with these reports.

Geuenich and colleagues (2008) studied aqueous extracts from three Lamiaceae plants: lemon balm (*Melissa officinalis* L.), peppermint (*Mentha* × *piperita* L.), and sage (*Salvia officinalis* L.) and observed a potent activity of all three species against HIV-1 infection. Crude plant extract from *Hyptis lantanifolia* Poit. has also been reported with anti-HIV-1 RT activity (Matsuse et al., 1998) showing a MIC_{50} of 7 μg mL^{-1}. These studies suggest Lamiaceae as a promising source of substances presenting potential antiretroviral activity. Isolating constituents from *H. radicans* could enhance the anti-HIV observed for **EE** and **HMP**, both showing MIC_{50} under 200 μg mL^{-1}.

Antibacterial potential

Multidrug-resistant Gram-negative bacteria pose the greatest risk to public health because they are extended-spectrum beta-lactamase producing bacteria, and for this reason, *Escherichia coli* and *Pseudomonas aeruginosa* (both Gram-negative bacteria), were used in this study (Medina and Pieper, 2016). *Bacillus subtilis* was the third bacterium tested in the present study. It is a laboratory model organism that has been extensively studied for over a century, being considered the Gram-positive bacterium model (Gallegos-Monterrosa et al., 2016).

Antibacterial activity of extract and phases from *H. radicans* and *H. multibracteata* was evaluated by determining the MIC_{50} using a microdilution broth method. *H. multibracteata* phases were more effective on inhibiting the bacterial growth of *B. subtillis,* Gram-positive bacteria. MIC_{50} of **HP**, sample that presented flavonoids as principal constituents, and **EAP**, sample showing the highest content of phenolic acids, were 23.6 μg

mL^{-1} and 12.13 μg mL^{-1}, respectively. For *H. radicans,* **EAP**, sample that presented the highest levels of flavonoids (tables 2 and 4) showed MIC_{50} of 140.91 μg mL^{-1} against *B. subtillis*.

Hyptis species were less effective against Gram-negative bacteria, *P. aeruginosa* and *E. coli,* excepted **HP** from *H. multibracteata* that showed MIC_{50} of 37.55 μg mL^{-1} against *P. aeruginosa*, being the third most active sample.

Table 5.4. Bacteriostatic effect (MIC_{50} μg mL^{-1}) of **EE** and phases (**HP**, **EAP** and **HMP)** of *H. radicans* (*H. rad*) and *H. multibracteata* (*H. mul*) against *Bacillus subtilis, Pseudomonas aeruginosa* and *Escherichia coli*.

	B. subtilis		***P. aeruginosa***		***E. coli***	
Sample	***H. rad***	***H. mul***	***H. rad***	***H. mul***	***Hrad***	***H. mul***
EE	159.41 ± 4,79	-	-	-	-	-
HP	-	23.6 ± 3.01	-	37.55 ± 3.66	-	642.97 ± 98.42
EAP	140.91 ± 4.12	12.13 ± 2.09	195.32 ± 5.48	-	-	946.05± 38.94
HMP	-	-	-	-	-	-
Gentamicin	12.18		14.30		0.4	

-: Inactive sample, ±: Standard deviation.

Some authors consider a high antibacterial active extract those which present MIC_{50} < 100 μg mL^{-1}, while for isolated compounds, MIC_{50} of 10 μg mL^{-1} indicates highly antibacterial substances (Rios and Recio, 2005). **HP**, as a phase composed by a mixture of substances showed to have potential antibacterial activity, especially against *P. aeruginosa* and *B. subtilis* (Table 4).

Antibacterial activity of *Hyptis* seems to be related to the chemical composition of the sample, but apparently it is not directly related to their phenolic content (Table 2). The most active sample was **HP** of *H. multibracteata* and **EAP** from *H. radicans* (*B. subtilis*), being flavonoids, its main phenolic class detected.

Studies indicate phenolic substances, in particular flavonoids, as having higher antimicrobial activity against Gram-negative bacteria. Wu et al. (2013b) suggest that among flavonoids, flavones exert greater effect on Gram-negative bacteria. When testing different flavonoids against *E. coli*, Wu et al. (2013a) found that flavonols are more effective in inhibiting the activity of DNA gyrase, being kaempferol the most active substance, followed by quercetin, chrysin (flavone), galangin, luteolin (flavone), some isoflavones, and as less

active, the flavonol myricetin. As a mechanism of action, the authors reported a probable interaction of the flavonoid presenting a 5-OH with the ATP binding site, important to antibacterial activity. Moreover, the presence of additional hydroxyl groups at C7 and C4' may enhances the antibacterial action.

Similarly, Wu et al. (2013b) tested flavonoid interaction with *E. coli* membrane. Isoflavones were less active to alter the membrane permeability compared to other flavonoids, when using QSAR *in silico* model. Kaempferol, once more, was the most active flavonoid to increase the rigidity of the bacterial membrane, followed by chrysin, quercetin, luteolin, and baicalein. Hydroxyl group at C3 seems to be a decisive feature for decreasing the permeability of the bacterial membrane.

Both *Hyptis* species showed flavones, specially apigenin derivatives, as the main flavonoid type, characterized by their UV-Vis spectra presenting two absorption bands: band I near to 336 nm and band II around 270 nm. Apigenin is one of the most common flavones found in plant species and has gained particular interest in recent years as a beneficial agent and health promoting, due to its low intrinsic toxicity (Lin et al., 2008; Odonbayar et al., 2017).

Extracts major constituents are often associated with the biological activities of natural products (Ait-Ouazzou et al., 2012). However, it is possible that the activity of the major components is modulated by the presence of minor constituents (Bakkali et al., 2008) Resulting in a synergistic action among the constituents. Considering rosmarinic acid, nepetoidins and chlorogenic acid derivatives as cinnamic acid derivatives, the cinnamic skeleton was the major chemical structure found in *Hyptis* samples.

Cinnamic acid derivatives are often reported as potential antimicrobial substances (Guzman, 2014). Currently, some infections are difficult to treat, for example, drug-resistant tuberculosis, infections caused by methicillin-resistant *Staphylococcus aureus*, *Klebsiella* and *E. coli* pan-resistant strains. The cinnamic skeleton has been considered a potential model for the development of new drugs, although its mechanism of action has not been completely identified yet (Guzman, 2014).

H. multibracteata showed lower yield of its ethanol extract (3.93 %) when compared to *H. radicans* (14.37 %). In addition, **HP** was the phase with the highest yield among *H. multibracteata* phases. Also, this phase showed the lower contents of phenolic compounds, suggesting, for this species, that the lesser polar constituents might be responsible for the antibacterial activity observed.

Since the 1970s, almost all antibiotics approved for use in humans are molecules based on sulfonamides and β-lactam, with few exceptions, such as linezolid (an oxazolidinone), daptomycin (a lipopeptide) and retapamulin (a pleuromutilin derivative) (Butler and Cooper, 2011). The number of bacteria resistant to multiple drugs is increasing, so the lack of new antibiotics in the market is a challenge. According to Butler and Cooper (2011), there are few drugs in clinical trials that offer significant benefits targeting Gram-negative bacteria over existing drugs. In the United States, a quarter to 50% of the drugs were developed from natural products, specially from plants, but few are used as antimicrobials due to the different structures that they have and, consequently, presenting different activities and non-specific mechanisms of action (Hintz et al., 2015).

PCA analysis for antibacterial and anti-HIV activities

Principal component analysis (PCA) summarized 60% of the variability of the data within the first two axes (Fig.1). It was applied to analyze if chemical composition of the samples could be correlated to their biological activities.

On the positive side of the axis 1 are the samples with higher amounts of chlorogenic acid derivatives (Hrad-EAP and Hrad-HMP), while on the negative side are the samples with high content of rosmarinic acid and Nepetoidin A and B that are Hrad-EE and Hmul-EE, respectively. Contents of chlorogenic acid derivatives (ChlD) and phenolic acids (PhA) were the variables that most influenced this distribution, showing the highest correlation values with the axis 1 (r= 0.4147 and 0.4087, respectively). The content of phenolic acids (PhA) and the activity against *B. subtilis* (B. sub) (r= 0.4309 and 0.5992, respectively) were the variables that had strong correlation with axis 2. PCA analysis points out *H. radicans* as more active on anti-HIV assay, especially **EE, EAP** and **HMP** samples. These three samples are very similar on their composition and in their total phenolic content, but they are very different in the proportion of rosmarinic acid. These results show that contents of total phenolic compounds are not the main sample feature to define anti-HIV activity, but probably the proportion among rosmarinic acid and other phenolics constituents in the sample is important to define its biological activity. On the other hand, antibacterial activity seems to be more related to other classes of constituents than phenolic compounds. Samples presenting the highest antibacterial activity were those with less content of total phenolic compounds.Results showed that the simple correlation between total phenolic contents and biological activity of a plant extract is not direct. Composition and the relative proportion among constituents are more relevant to determine the biological activity of a plant extract.

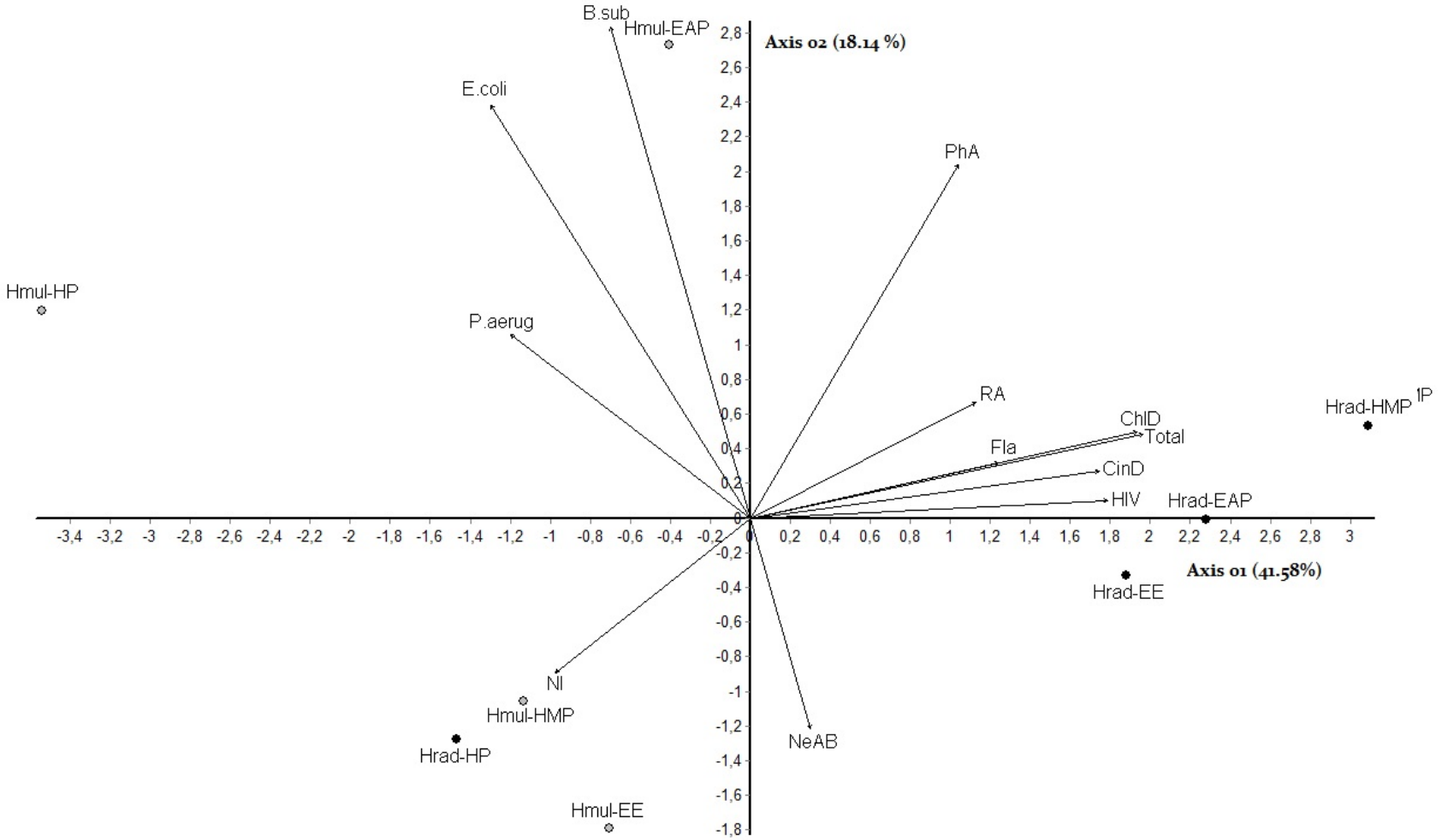

Figure 5.1. Principal component analyses (PCA) using 12 variables (**PhA**: phenolics acids; **RA**: rosmarinic acid; **NeAB**: nepetoidins A and B; **CinD**: cinnamic acid derivatives; **ChlD**: chlorogenic acid derivatives; **Fla**: flavonoids; **NI:** no identified compounds; **Total:** total phenolic content; **E. coli**: *Escherichia coli;* **B. sub**: *Bacillus subtilis;* **P. aerug**: *Pseudomonas aeruginosa;* **HIV**: inhibit of Reverse Transcriptase activity); evaluated in four samples of *H. radicans (***Hrad-EE**: crude ethanol extract; **Hrad-HP**: hexane phase; **Hrad-EAP**: ethyl acetate phase; **Hrad-HMP**: hydromethanol phase), and four samples of *H. multibracteata* (**Hmul-EE**: crude ethanol extract; **Hmul-HP**: hexane phase; **Hmul-EAP**: ethyl acetate phase; **Hmul-HMP**: hydromethanol phase). **a)** Percentage of variance explained by PCA, and percentage of expected variance estimated by the broken-stick test; and **b)** correlation coefficients between variables and axes 1 and 2.

Conclusions

Natural products remain a source of novel compounds for drug discovery due to their lower toxicity (Park et al., 2009). The majority of drugs on the market are plant-derived (Newman and Cragg, 2016) and the area of infectious diseases is largely dependent on natural products and their structures for sources of better treatment.

The anti-HIV-1 and antibacterial results from the present study lend support for further investigation of the bioactive constituents of *H. radicans* and *H. multibracteata* to validate the use of these plants in traditional medicine as antiviral and/or as antibacterial.

EE and **HMP** of *H. radicans* showed anti-HIV-1 activity but contents of total phenolic compound are not the main sample feature to define anti-HIV activity but there is a correlation between the presence of rosmarinic acid and their anti-HIV activity. **HP** of *H. multibracteata* was the sample with better antibacterial activity but there was no correlation between phenolic contents and its activity. The simple correlation between total phenolic contents and biological activity of a plant extract is not direct, but probably the proportion among rosmarinic acid and other phenolics in the sample is important to define its biological activity.

Further identification of both *Hyptis* species constituents will provide a better understand of which phenolic compound could be influencing the biological activity observed; as well as will help to further establishing their mechanism of action.

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Final considerations

Natural products remain a source of novel compounds for drug discovery due to their lower toxicity (Park et al., 2009). Most drugs on the market are plant-derived (Newman and Cragg, 2016) and the area of infectious diseases is largely dependent on natural products and their structures for sources of better treatment.

Related to *Hyptis* species, although a considerable number of papers founded in databases: 879 in SciFinder, 528 in Web of Science and 96 in SciELO, only 20% of *Hyptis sensu* Harley and Pastore (2012) have been studied. Most species were studied regarding their volatile oil composition; remaning poorly explored the polar constituents.

Furthermore, based on the articles published, it was possible to notice that these species are characterized by the presence of substances with promising pharmacological potential, mainly antimicrobial, antifungal, cytotoxic, anti-inflammatory, and anti-HIV, pointing to a great relevance of *Hyptis* to bioprospecting studies.

This study corroborated rosmarinic acid, chlorogenic acids, and nepetoidins as common constituents of Nepetoideae. Furthermore, the results corroborate the presence of these constituents also in *Hyptis* species. Lithospermic acid A and cirsimaritin were described for the first time in this study for *Hyptis*, both found in *H. radicans*. Fatty acids and triterpenes are the most abundant kind of apolar substances in *H. radicans* and *H. multibracteata*. This differs from what is most reported in the literature; first because the majority of reports focused on volatile oils and in this study, we analyzed also the apolar constituents of aerial parts extracts.

The present research also provides, for the first time, a comprehensive report on the antioxidant and cytotoxic activities of *Hyptis* species. **EAP** from *H. radicans* was the sample that presented the highest levels of total phenolic content, especially flavonoids, being also the sample with the high antioxidant activity with promising EC_{50}: DPPH (32.12 μg mL^{-1}), ABTS (5.04 μg mL^{-1}), Metal chelator assay (42.36 μg mL^{-1}), TBARS (40.46 μg mL^{-1}) and nonsite-Specific Hydroxyl Radical-Mediated 2-Deoxy-D-ribose Degradation *(*NS-Spe) with a EC_{50} of 75.08 μg mL^{-1}. **EE** from *H. radicans* showed the high antioxidant activity for FRAP and ORAC with EC50 of 6.01 and 2.68 μg mL^{-1}, respectively and has the highest amount of rosmarinic acid (17.64 mg g^{-1}). **HMP** from *H. radicans* showed the high antioxidant activity

in Site-Specific Hydroxyl Radical-Mediated 2-Deoxy-D-ribose Degradation (S-Spe) assay with EC50 of 0.32 μg mL^{-1} and has the highest content of chlorogenic acid derivatives.

Lithospermic acid A isolated from *H. radicans* and rosmarinic acid and nepetoidin B from *H. multibracteata*, were substances with better antioxidant activity. Nepetoidin B isolated from *H. multibracteata* had the best EC_{50} (52.73 μg mL^{-1}) for anti-acetylcholinesterase activity. Regarding the results of cytotoxicity, **HP** from *H. multibracteata* induced the death of more than 80% of RAW 264.7 Cell Lines turning **HP** as an interesting phase as promising cytotoxic agent.

The anti-HIV-1 and antibacterial results from the present study lend support for further investigation of the bioactive constituents of *H. radicans* and *H. multibracteata* to validate the use of these plants in traditional medicine as antiviral and/or as antibacterial.
EE and **HMP** of *H. radicans* showed anti-HIV-1 activity but contents of total phenolic compound are not the main sample feature to define anti-HIV activity but there is a correlation between the presence of rosmarinic acid and their anti-HIV activity. **HP** of *H. multibracteata* was the sample with better antibacterial activity but there was no correlation between phenolic contents and its activity. With the great results on biological activity it can be concluded that *H. radicans* is a promising phytotherapic.