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Artigo

Investigation of Antioxidant Activity, Acute Toxicity and Anticholinesterasic Potential of *Lippia hirta* (Verbenaceae)

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Investigação da Atividade Antioxidante, Toxidade Aguda e do Potencial Anticolinesterásico de *Lippia hirta* (Verbenaceae)

Resumo: O gênero *Lippia*, o segundo maior da família Verbenaceae, possui aproximadamente 200 espécies, das quais aproximadamente 120 espécies encontram-se no Brasil. Uma vez que a espécie *Lippia hirta* não tem sido documentada na literatura, o objetivo deste trabalho foi analisar a atividade antioxidante, a toxicidade aguda e a atividade de inibição da acetilcolinesterase (AChE) por parte do óleo essencial, extrato bruto e frações provenientes desta espécie vegetal. Com relação à atividade antioxidante, avaliada pelo poder redutor, foi possível observar um excelente comportamento antioxidante, especialmente para o óleo essencial (IC_{50}= 6,26 µg.mL^{-1}), e frações butanólica (BuOH, IC_{50}= 7,26 µg.mL^{-1}) e acetato de etila (EtOAc, IC_{50}= 8,09 µg.mL^{-1}), superiores ao padrão quercetina (IC_{50}= 9,43 µg.mL^{-1}) utilizado neste ensaio. Observou-se uma baixa toxicidade para todas as amostras e grande potencial inibitório para a AChE, pela maioria das frações e especialmente para a fração rica em alcaloides (FRA), que foi capaz de inibir a enzima em até 100 % em uma concentração de 0,5 µg.mL^{-1}, o que representa uma atividade de elevada potência. Ainda que a FRA seja uma matriz complexa, o efeito sinérgico é elevado quando comparado ao padrão utilizado no teste, Reminyl-contendo galantamina (IC_{50}=17,8 µmol.L^{-1}). Estes resultados incentivaram o fracionamento cromatográfico de FRA que permitiu a caracterização de três alcaloides, até então não relatados na espécie, dihidrocorinanteol, vincamina and dexoxicordifolina. A baixa toxicidade, a boa atividade antioxidante e o excelente resultado para a inibição da AChE incentivam outras análises para determinação dos compostos ativos nas demais frações, sugerindo o fracionamento bioguiado como uma ferramenta de suma importância na análise fitoquímica e biológica de *Lippia hirta.*

Palavras-chave: *Lippia hirta*; atividade antioxidante; acetilcolinesterase; toxicidade aguda.

Abstract

The genus *Lippia*, the second largest of the Verbenaceae family, has approximately 200 species of which approximately 120 species are found in Brazil. Since *Lippia hirta* has not been documented in the literature, the objective of this work was to analyze the antioxidant activity, acute toxicity and acetylcholinesterase (AChE) inhibition activity of the essential oil, crude extract and fractions from this plant species. In relation to the antioxidant activity, evaluated by the reducing power assay, it was possible to observe a relevant antioxidant behavior, especially for the essential oil (IC_{50}= 6.26 µg.mL^{-1}), and butanol (BuOH, IC_{50}= 7.26 µg.mL^{-1}) and ethyl acetate (EtOAc, IC_{50}= 8.09 µg.mL^{-1}) fractions, higher than the standard quercetin (IC_{50}= 9.43 µg.mL^{-1}) used in this assay. Low toxicity was observed for all samples and a high inhibitory potential for AChE by most fractions and especially for the alkaloid rich fraction (ARF), which was able to inhibit the enzyme by up to 100 % at a concentration of 0.5 µg.mL^{-1}, representing a strong activity. Although ARF is a complex matrix, the synergistic effect is high when compared to the standard used in the test, galantamine (IC_{50} = 17.8 µmol.L^{-1}). These results stimulated the chromatographic fractionation of ARF that allowed the characterization of three alkaloids, hitherto not reported in the species, dihydrocorinanteol, vincamine and deoxycordifoline. The low toxicity, the good antioxidant activity and the excellent result for the inhibition of AChE stimulate other analyzes to determine the active compounds in the other fractions, suggesting the bio-guided fractionation as a tool of paramount importance in the phytochemical and biological analysis of *Lippia hirta*.

Keywords: *Lippia hirta*; antioxidant activity; acetylcholinesterase; acute toxicity.

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Investigation of Antioxidant Activity, Acute Toxicity and Anticholinesterasic Potential of *Lippia hirta* (Verbenaceae)

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1. Introduction

The rational use of medicinal plants for therapeutic purposes may be an important factor in the development of the local

economy of the organs and institutions involved with the health sectors of developing countries.[1] Brazil has the largest biological diversity in the world, with a rich flora, which has attracted interest from international researchers, especially in the medical field.[2] However, despite the rich flora and well-known importance of plant knowledge, there is a need for more Brazilian research on the chemical components and properties of this national flora, given the wide diversity of species.

The knowledge on medicinal plants is often the only resource therapeutic use of communities and ethical groups, and the search for alternative or allied treatments has been increasing considerably, making this a frequent practice among Brazilian society.[3]

Ethnobotany has been the object of study in the world and in Brazil, where the different areas of research try to rescue popular knowledge about vegetables, their uses and especially the medicinal use. The intensification of the ethnobotanical works leads to the knowledge of these species and may serve as an instrument to draw strategies for the use of native species and their potentials.[4] A way to complement phytochemical studies is to associate them with bioassays, for this reason many laboratories of Natural Products have inserted into their routines simple biological assays in order to select and monitor the research of plant extracts in the search for bioactive substances. Among these bioassays, the toxicity of *Artemia salina* Leach, which is a microcrystalline salt water commonly used as food for fish. The simplicity with which it can be handling, rapid testing and low cost its routine use in several studies, besides which, such lethality tests are widely used in analyzes preliminary general toxicity.[5]

The Verbenaceae family corresponds to a group of plants whose taxonomy is complex. There are many controversies about the circumscription of several genera, species and even the family, distributed throughout the world in tropical and tropical regions, especially in the American continent. Considering what was considered as subfamily Verbenoideae, now considered Verbenaceae family, and excluding the genera *Callicarpa*, *Clerodendron* and *Tetraclea* (now part of the family Lamiaceae), there are reports of about 1000 species belonging to the Verbenaceae family.[6] In the neotropical region, there are 32 genera and 480 species, being Brazil the country who presents the greatest richness of Verbenaceae taxa, with 16 genera and 290 species, of which 191 are endemic, most of which are distributed mainly in the Espinhaço and Central Plateau.[7]

Many species of Verbenaceae are used in popular medicine, in the form of infusion or tea, for the treatment of various human health disorders, such as: some types of cancer and hypertension,[8] fever, diarrhea, gastrointestinal disorders, topical diuretic, expectorant, antirheumatic and anti-inflammatory applications and sexually transmitted diseases.[9] This large biodynamicity observed has encouraged anatomical studies to develop markers that allow distinguishing between species.[10]

Among the genera of Verbenaceae that stand out for the phytochemical potential and for their medicinal use are *Lippia* and *Lantana*.[11] Together, these two genera correspond to approximately 80 % of the representatives of this tribe.[12] The genus *Lippia* has cataloged about 200 species among herbs, shrubs and small trees, which can be found mainly in South and Central America, and in tropical areas of Africa.[13] Extracts and essential oils obtained from *Lippia spp*. have been extensively scientifically tested because of the potential of bioactive principles.[14]

The species *Lippia hirta* has not been documented in the literature, which encourages further studies, since there is great biodynamic activity for the genus. In this work, the toxicity to *Artemia salina* for essential oil, crude extract and fractions was investigated. *Artemia* is a cosmopolitan organism, characterized by a wide array of intrinsic characteristics (i.e. easy rearing, short life-cycle, large offspring production) that make it a good model for testing toxicity in estuarine and hypersaline environments.[15]

Alzheimer's disease (AD) is an age-related progressive neurodegenerative disease, which impairs memory and cognitive function. The therapeutical potential of herbs and marine natural products also contribute to the preventative agents of AD. Preclinical and clinical studies have explored the neuroprotective effect of natural compounds using *in vitro* and *in vivo* models.[16] The structural diversity of known cholinesterase enzyme inhibitors and the possibility of exploring different modes of action have stimulated the phytochemical study of various plant species and microorganisms that may provide new models of anticholinesterase substances. In this sense, several examples of biodiversity have been studied because of their popular use or of ethno-botanical data.[17] Despite a very limited number, there are indications in the literature of anticholinesterase activity of species of the genus *Lippia*.[18]

In this paper in addition to the toxicity test, antioxidant trials and the anticholinesterase potential of essential oil (and its major components separately), crude extract and its fractions were investigated, since anticholinesterase drugs are required in the treatment of neurodegenerative diseases, such as AD, whose treatment. The clinical use of acetylcholinesterase (AChE) inhibitors is a more effective clinical alternative.

2. Experimental

2.1. General

Thin layer chromatography analysis was performed on silica gel plates, using a UV lamp for visualization.

^{1}H, $^{13}C\{^{1}H\}$, DEPT-135, uni and bidimensional NMR (^{1}H-^{1}H COSY, ^{1}H-^{13}C HMQC and ^{1}H-^{13}C HMBC) spectra were acquired on a Varian Oxford AS-400 spectrometer, operating at 9.4 Tesla, observing the ^{1}H and ^{13}C nuclei at 400.13 and 100.13 MHz, respectively. One-bond and long-range ^{1}H-^{13}C correlation from HMQC and HMBC NMR experiments were optimized for an average coupling constant $^{1}J_{(C,H)}$ and $^{LR}J_{(C,H)}$ of 140 and 8 Hz, respectively. All ^{1}H and ^{13}C NMR chemical shifts (δ) are given in ppm related to the TMS signal at 0.00 ppm as an internal reference, and the coupling constants (*J*) in Hz. On the other hand, UV-Vis tests were obtained in a PerkinElmer spectrophotometer with fixed wavelength, in quartz cuvettes with 1 cm of optical path. IR spectra were obtained with the use of KBr (range 4000-400 cm^{-1}) in a FT-16PC equipment.

The melting points were determined using a Microchemical apparatus, with MQA PF-301 hot plate. The spectrum mass spectrometry with electrospray ionization source (ESI-MS) was used as an aid tool for molecular weight determination, consisting of a triple quadrupole trap QTrap 3200of the Applied Biosystems brand.

2.2. Plant material

The aerial parts of *Lippia hirta* were collected in July, 2016, near the Pitangui River, at coordinates UTM 593095 and 7231847, in the region of Campos Gerais, Ponta Grossa, Paraná State, Brazil. Prof. Dr Daniel Barcellos Falkenberg identified the specie by comparison with exsiccates already deposited in the FLOR herbarium of the Federal University of Santa Catarina, in the Department of Botany with the code FLOR 24038.

2.3. Obtaining the crude extract and fractions

The aerial parts of *Lippia hirta* (2.1 kg), after collection and identification, were dried in an air circulating greenhouse, ground and extracted by maceration in 95 % ethanol (3x) at room temperature for seven days. The extract obtained was filtered and

concentrated in a rotatory evaporator under vacuum, yielding 100 g of crude extract (CE).

The dry CE was divided into two portions of 50 g and applied to the liquid-liquid partition extraction. To the first portion of crude extract CE was added a 10 % aqueous ethanol solution, giving a suspension. The mixture was partitioned using solvents of different polarities, hexane (Hex), ethyl acetate (EtOAc) and butanol (BuOH) yielding the respective fractions soluble in hexane (4.5 g), in ethyl acetate (10 g) and butanol (32 g), respectively. The other part was subjected to the acid-base partition in which the hydroalcoholic suspension is added a 2 % aqueous HCl acid solution, maintaining the pH 2, and then partitioned with ethyl acetate three times, resulting in a fatty acid rich fraction, long chain alcohols and sterols. The pH of the aqueous suspension was raised to approximately pH 10 by the addition of a 10 % ammoniacal solution and then extracted with ethyl acetate, resulting in a fraction rich in nitrogen compounds, termed the alkaloid rich fraction (ARF) (6.5 g).

2.4. Partitioning of the Alkaloid Rich Fraction (ARF)

For the chromatographic separation by column on silica gel ARF 6.0 g aliquot was used. The sample was previously solubilized in ethyl acetate and homogenized in about 10 g of silica gel 60 column. After evaporation of the solvent there was obtained dry pellet composed of ARF adsorbed onto silica. The tablet was carefully applied over a stationary layer of silica (250 g), previously packed in a glass column (h: 40 cm FEØ: 7 cm). Thereafter, the elution was carried out with the following solvents: hexane and acetone saturated with ammonium hydroxide. Elution occurred with a gradual increase of solvent strength, initiated with *n*-hexane 100 %, increasing the eluent strength with stepwise addition of 5 % acetone. 65 fractions of 100 mL were collected (ARF 1 - 65), which were concentrated under reduced pressure with the aid of a rotatory evaporator. The fractions obtained from the elution were analyzed by TLC employing *n*-hexane:acetone:NH_4OH (60:40:1) as eluent. This procedure guided the meeting of fractions according to their similarity of the chromatographic profile.

2.5. Extraction and analysis of essential oil

The essential oil was extracted from the fresh plant material, submitted to hydrodistillation in a Clevenger apparatus for 2 h, according to the methodology described in the Brazilian Pharmacopoeia.[19] The extraction yield was calculated based on the ratio of volume to weight and expressed as a percentage (% v/w).

Analysis of the essential oil was performed by gas-chromatography coupled to mass spectrometry (GC-MS) in an Agilent 6890 hyphenated system equipped with a 5973 series selective detector. Analysis parameters: split inlet 1:100; Carrier gas: He (1 $mL.min^{-1}$); HP5-MS fused silica capillary column (Hewlett Packard, 5 % phenylmethylsiloxane, 30 m x 0.25 mm, film thickness: 0.25 µm); analysis program: 40 °C (Ti) for 4 min, 40-260 °C, 4 °C/min; injector temperature: 220 °C; interface temperature: 250 °C; ionization energy: 70 eV; database: NIST, 1998.

The essential oil components were identified on the basis of the retention index (IR) determined using a calibration curve of a homologous series of *n*-alkanes (C_8-C_{32}) injected under the same chromatographic conditions of the samples and in the models of fragmentation of the mass spectra, both being compared with data from the literature.[20]

2.6. Chromatographic analysis

Considering the behavior against the inhibition test of the AchE enzyme, the ARF was chosen for subsequent separation via column chromatography by adsorption on silica (silica gel 60; 70-230 and 230-400 mesh flash), reverse phase partition chromatography (C18) and or exclusion

chromatography (Sephadex). Solvents for chromatography were of P.A. grade used in increasing order of polarity *n*-hexane, chloroform, dichloromethane, acetone, ethyl acetate, ethanol, methanol, acetic acid, formic acid, ammonium hydroxide and water.

Analytical thin-layer chromatography (TLC) was performed using Merck silica gel 60 F_{254} and a UV lamp (long wavelength 355 nm and short 254 nm) for visualization. They were used as Dragendorff developers and ceric sulfate, by placing the plates chromatography under the incidence of light in suitable dark chambers.

Qualitative analysis of extracts and fractions were obtained in a High-Performance Liquid Chromatography (HPLC) equipment (SHIMADZU, model LC 10AT). The chromatograph consists of a set of binary solvent for analytical scale and for semi-preparative detector (SPD-M10AVP) with 20 µL loop analytical scale and 1 mL semi-preparative scale loop and fractions FRC-10A. The UV-Vis spectra with diode array detector (DAD) were acquired between 200 and 600 nm, peaks were monitored at the maximum wavelength (280 nm) of the constituents of the sample. For the qualitative analysis of extracts and fractions were used Varian C18 chromatographic column (250 x 4.6 mm; 5µm), and eluent system of water 1 % formic acid (A): acetonitrile 1 % formic acid (B); with schedule of time: 0 min B (10 %), 15 min B (16 %), 20 min B (17 %), 23 min B (20), 35 min B (20 %), 40 min B (25 %), 50 min B (30), 60 min B (40), flow 1 mL.min^{-1}, detection at 280 nm. For the semi-preparative analysis, Beckman's C8 column (250 mm x 10 mm; 5 µm) was used, with the same eluent system and the same programming, modifying only the flow (1.5 mL min^{-1}).

2.7. Total polyphenol content

The total phenolic content of each extract was determined by the Folin-Ciocalteu method as described previously.[21] The diluted aqueous solution of extract or fraction (0.5 mL, 1 mg.mL^{-1}) was mixed with Folin-Ciocalteu reagent (0.2 mol.L^{-1}, 2.5 mL). This mixture was incubated at room temperature for 5 min and then sodium carbonate solution (2 mL, 75 g.L^{-1} in distilled water) was added. After 2 h of incubation, the absorbencies were measured at 760 nm against water blank. A standard calibration curve was plotted using gallic acid (0-200 mg.L^{-1}). The results were expressed as mg of gallic acid equivalents (GAE) per 100 mg of dried extract (mg GAE/100 mg). All tests were made in triplicates.

2.8. Total flavonoids content

The total flavonoids were estimated as described previously.[22] A diluted methanol solution of extract or fraction (2 mL, 1 mg.mL^{-1}) was mixed with a solution of aluminium trichloride (2 mL, 2 % in methanol). The absorbance was read at 415 nm after 10 min against a blank (2 mL extract, 2 mL methanol). Quercetin was used as reference compound to produce the standard curve, and the results are expressed as mg quercetin equivalent (QE) per 100 mg of dried extract (mgQE/100 mg). Each assay was repeated three times.

2.9. DPPH assay

The assay for the determination of the antioxidant activity using the DPPH free radical was based on the method described by Venzke and colaborators.[23] A 0.004 % DPPH solution in methanol was added to the test solutions in various concentrations, and the absorbances were determined using a UV-VIS (517 nm) spectrophotometer after 30 min. The absorbance at the initial time (A_0) was obtained by measuring the absorbance of a DPPH solution. The analysis was done in triplicate. The values obtained were graphs in the form of % decrease in the DPPH absorbance in concentration of the test solution, where the concentration of concentration required to lower the DPPH

concentration by 50 % (IC_{50}) in the solutions tested.

2.10. Iron Reducing Power (RP)

The RP values were calculated by using the method described previously.[21] For each sample, a graph of absorbance at 700 nm *vs*. increased extract concentrations was constructed to calculate the extract concentration providing 0.5 of absorbance (defined as IC_{50}). RP was expressed as micrograms per milliliter (µg mL^{-1}). Quercetin was analyzed in the same manner as reference.

2.11. Acetylcholinesterase activity

The enzymatic activity was measured using an adaptation of the method described previously.[22] Briefly, 325 µL of 50 $mmol.L^{-1}$ Tris-HCl buffer, pH 8.0, 100 µL of a buffer solution containing the sample at five different concentrations dissolved in EtOH and 25 µL of an AChE solution containing 0.28 $U.mL^{-1}$ (50 $mmol.L^{-1}$ Tris-HCl, pH 8.0 buffer, 0.1 % BSA) were incubated for 15 min. Then, 75 µL of an acetylthiocholine iodide solution (0.023 $mg.mL^{-1}$ in water) and 475 µL DTNB (3 $mmol.L^{-1}$ in Tris-HCl, pH 8.0 buffer, 0.1 $mol.L^{-1}$ NaCl, 0.02 $mol.L^{-1}$ $MgCl_2$) were added, and the final mixture was incubated for another 30 min at room temperature. The absorbance of the mixture was measured at 405 nm. A control mixture containing ethanol instead of the sample was considered to have 100 % AChE activity. The inhibition (%) was calculated as follows: I (%) = 100 ($A_{sample}/A_{control}$) × 100 in which A_{sample} is the absorbance of the vegetal sample and $A_{control}$ is the absorbance without sample. The tests were performed in triplicate, and a blank containing Tris-HCl buffer was used instead of the enzyme solution. The sample concentration with 50 % inhibition (IC_{50}) was determined by plotting the inhibition against the sample solution concentrations. Reminyl-containing galantamine was used as the positive control.

2.12. *Artemia salina* lethality test

The toxic effect of the plant extract and its fractions against *Artemia salina* nauplii was tested according to the method of Sam *et. al*. with minor modifications.[23] Dried *A. salina* eggs were hatched in illuminated artificial seawater at 25 °C. After 48 h incubation, 1000 mL of seawater containing 1 % (v/v) Tween 20 and 7-10 free-swimming nauplii was separately transferred to 24-well flat-bottomed tissue culture plates. Toxicities of the extract and fractions were tested at several concentrations (10-2000 $\mu g.mL^{-1}$). Three replicates were used for each concentration. The culture plates were incubated as described above and the number of dead nauplii were counted after 24 h. Potassium dichromate ($K_2Cr_2O_7$; LC_{50} ~ 20-40 $\mu g.mL^{-1}$) and seawater solution with 1 % (v/v) Tween 20 were used as positive and negative controls, respectively. The lethal concentrations which led to 50 % mortality (LC_{50}) with 95 % confidence intervals were determined using the probit method. LC_{50} values were taken as the measure of toxicity of the extract or fractions.

2.13. Cyclic voltammetry

Voltammetric measurements were performed with a PalmSens3 potentiostat/galvanostat/impedance analyser (PalmSens BV, The Netherlands), operating with data processing software (PSTrace 4.7), and using a conventional three-electrode system: a glassy carbon electrode (GCE, diameter 2.0 mm) as working electrode, a platinum wire as counter electrode and Ag/AgCl (3.0 $mol.L^{-1}$ KCl) as reference electrode. Cyclic voltammetry experiments were performed on ca. 1.0 $mmol.L^{-1}$ of each compound (monoterpenes) in phosphate buffer solution (pH 7.0; 0.1 $mol.L^{-1}$) containing 15 % (v/v) ethanol. Solutions were purged with purified argon gas and the temperature was kept at 25 ± 0.5 °C.

2.14. Statistical analysis

All the experiments were performed in triplicate and data are presented as mean ± SD. Data were analyzed by One-way analysis of variance followed by the Tukey multiple comparison test. A P-value less than 0.05 was used as the criterion for statistical significance.

3. Results and Discussion

3.1. Analysis of essential oil

The constituents of the essential oil of *L. hirta* (Figure 1) were determined by gas-chromatography coupled to mass spectrometry (GC-MS), as shown in Table 1, which revealed carvacrol as the major constituent with a concentration of 68.4 %. Other compounds were also detected, but in lower percentages.

Table 1. Major constituents of essential oil from *Lippia hirta.*

Compound	RI Lit.[a]	RI Calc.[b]	Percentage (%)
Carvacrol	1299	1305	68.4
Carvone	1218	1218	6.3
D-Limonene	1030	1031	7.2
Myrcene	991	997	2.2
α-Pinene	937	935	8.9
α-Terpinene	1017	1017	2.9
γ-Terpinene	1060	1060	2.4
1,4-Cineole	1016	1017	0.6
p-Cymene	1025	1025	0.2
Terpinolene	1088	1088	0.5
Others	-	-	0.4

[a]RI Lit.- Retention Index found in the literature. [b]RI calc.- Retention Index calculated

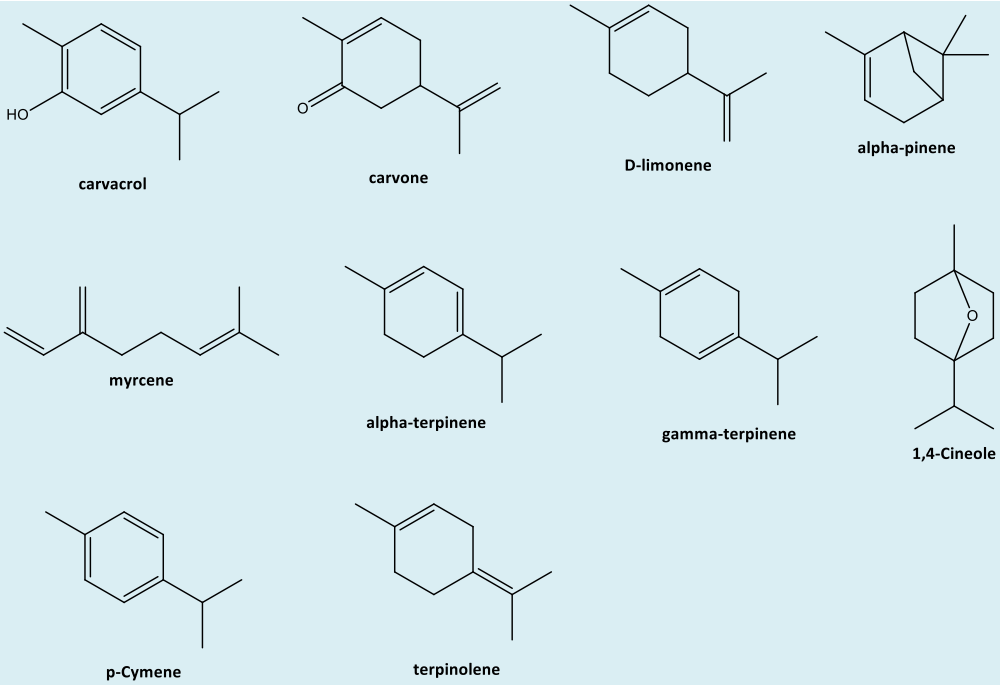

Figure 1. The constituents of the essential oil of *Lippia hirta*

The chemical composition of the essential oil of many species of *Lippia* has been investigated through gas-chromatography and, based on the published data, it is perceived that α-pinene (1), limonene (2), β-caryophyllene (3), *p*-cymene (4), camphor (5), linalool (6), and thymol (8) are the most frequent components. In the genus *Lippia*, the secretion of essential oils has been associated with the presence of trichomes, which are usually of varied forms between plant groups, but usually uniform within the same taxon.[24]

The constituents of the essential oils are very unstable to the presence of light, heat and humidity, consequently the time of harvest of the plant material can influence directly or indirectly in the processes of secondary metabolism that result in quantitative and qualitative variations of the essential oil.

3.2. Phenolic substances, flavonoid content and antioxidant activity

Flavonoids represent one of the most important and diversified phenolic groups among plant products, appearing relatively frequently in species of this genus. They have the ability to modulate enzyme activity and affect the behavior of many cell systems, suggesting that many species may have anti-hepatotoxic, anti-allergic, anti-inflammatory, anti-osteoporotic and even anti-tumor effects.

The contents of phenolic, flavonoids and antioxidant activity (expressed by DPPH and RP) for essential oil, crude extract and corresponding fractions were analyzed. The result is showed in Table 2.

Table 2. Content of phenolics, flavonoids and evaluation of the reducing power of *Lippia hirta*

Extract / fraction	Phenolic[(a)]	Flavonoids[(b)]	Reducing power IC_{50} ($\mu g.mL^{-1}$)
CE	169.87 ± 0.92	8.10 ± 0.02	10.21 ± 0.64
Hex	16.75 ± 0.58	2.90 ± 0.10	23.11 ± 0.02
EtOAc	191.21 ± 5.20	9.94 ± 0.22	8.09 ± 0.06
BuOH	274.46 ± 6.45	13.76 ± 0.01	7.26 ± 1.23
ARF	10.41 ± 0.11	-	27.73 ± 0.83
E. oil	142.32 ± 0.26	-	6.26 ± 6.34
Quercetin	-	-	9.43± 0.11

[(a)] The results were expressed as Gallic Acid Equivalents (GAE)/100 mg of dried extract/fraction.
[(b)] The results were expressed as mg quercetin equivalent (QE)/100 mg of dried extract/fraction (mgQE/100 mg)

We can observe a high concentration of phenolics and flavonoids mainly in the CE and fractions EtOAc and BuOH. Through a regression analysis, 95 % correlation was observed between the values found in the test of reducing power with phenolic content and 92 % between the flavonoid content and the reducing power in the plant samples. The results obtained for the essential oil agree with the composition presented previously, and the value obtained for the test of total phenolics probably is associated to carvacrol, major compound.

Although it was not the objective of this work, the significant antioxidant activity observed for the essential oil and especially for the BuOH and EtOAc fractions highlights this species as promising for the future phytochemical fractionation and isolation of the phenolic compounds of interest.

Once the constituents of the essential oil were determined, experiments involving cyclic voltammetry with the monoterpenes were carried out, in order to correlate with the observed data for the antioxidant activity of the essential oil. Since an antioxidant compound can act as reduction agent, and it has a tendency to be easily oxidized on inert electrodes, such as carbon.[25]

The electrochemical behavior of monoterpenes provides important information about their antioxidant activities. The species with the low oxidation potential has demonstrated significant antioxidant activity, whereas compounds with high oxidation potential acted as prooxidants.[25] The onset oxidation potentials (E_{onset}) have been used to compare the antioxidant strength of compounds, low oxidation potentials are associated with a greater facility of a given molecule for the electrodonation and, thus, low values of oxidation potential are associated with the high antioxidant power.[26-27] The results obtained in the electrochemical studies together with the DPPH assay are showed in Table 3.

Table 3 DDPH results and electrochemical parameters of the voltammograms recorded for the monoterpenes studied

Compound	E_{onset} (V)[a]	DPPH (μg.mL^{-1})
Myrcene	N.D.[b]	450.27 ± 0.84
γ-Terpinene	N.D	300.12 ± 0.99
Carvone	1.56 ± 0.01	228.43 ± 1.29
α-Terpinene	0.77 ± 0.01	113.65 ± 1.56
D-Limonene	0.71 ± 0.02	105.98 ± 1.26
α-Pinene	0.67 ± 0.02	96.43 ± 0.23
Carvacrol	0.41 ± 0.01	60.32 ± 0.22
Quercetin	0.07 ± 0.01	10.25 ± 0.12

[a] E_{onset}: onset oxidation peak potential versus Ag/AgCl at 30 mV s^{-1}.
[b] not detected under the experimental conditions tested.

Figure 2 shows the correlation obtained between the E_{onset} values and DPPH assay of the most significant compounds of the study. The regression equation obtained was DPPH = 0.22 + 146.47 E_{onset} (Adj.R-Square 0.9996). From these results it follows that the excellent correlation existing between the E_{onset} and spectrophotometric assay indicates that the voltammetric method provide important information about antioxidant activity of these monoterpenes.

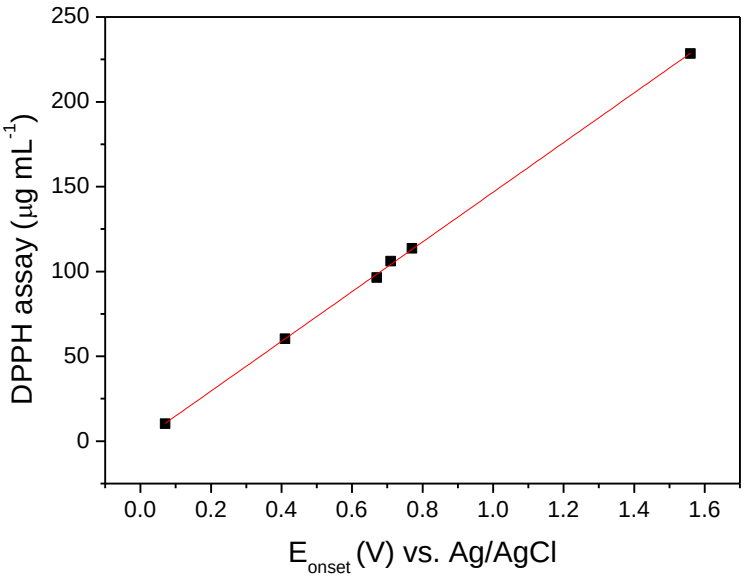

Figure 2. DPPH assay and onset oxidation potentials correlation for monoterpenes studies

The difference in antioxidant activity observed for the monoterpenes studied is attributed to the difference at the chemical structures. The positions of double bonds in the terpenes derivatives affect the oxidation thermodynamics via the oxidation potential.

Therefore, the determining factor is the conjugation of double bonds that facilitates the oxidation process by lowering the E_{onset}.[27] A proposed mechanism for the electro-oxidation of monoterpenes involves an electron transfer followed by a deprotonation of the cation radical.[27]

For carvacrol, that is the compound that presented better antioxidant activity, the addition of a hydroxyl–group on benzene makes the electro-oxidation mechanism more favorable, a fact that can be observed by the decrease of the oxidation potential. The formation of a phenoxenium cation *via* phenoxy radical that subsequently evolves through other chemical processes such as loss of a proton.[28,29]

3.3. Acetylcholinesterase activity and acute toxicity

The hypothesis of the use of AChE inhibitors has been successful and applied in the prophylactic and therapeutic treatment of AD, thus verifying a great improvement in the life of the patients. The search for substances with a low degree of toxicity has been intensifying more and more, investigating plants used in traditional medicine as source of obtaining inhibitory compounds of AChE.[30] These inhibitors of plant origin bind to the enzyme in a reversible manner by weak intermolecular bonds, thus allowing the recovery of the active enzyme center.

The assay for the detection of compounds that inhibit the AChE enzyme activity is based on the measurement of the production of thiocholine, when the substrate acetylthiocholine is hydrolyzed by AChE. This reaction is followed by the appearance of a yellow color measured at 405 nm due to the 5-thio-2-nitrobenzoate anion obtained from the reaction of the Ellman reagent (5,5'-dithiobis-2-nitrobenzoic acid) with the thiocholine.

Plant extracts tested at a concentration of 0.1 $mg.mL^{-1}$ having percentages of inhibition greater than 50 % have their anticholinesterase potential described as high and percentages of inhibition between 50 and 15 % are described as having low to moderate activity. In this sense, plant samples exhibiting inhibition values greater than or equal to 50 % were used to investigate the inhibitory concentration (IC_{50}).

In addition, a relationship between the degree of toxicity and the lethal dose, LD_{50}, presented by extracts of plants on larvae of *A. salina*, since then, it is considered non-toxic when values for LD_{50} are above 1000 $\mu g.mL^{-1}$.

The results observed for inhibition of the AChE enzyme and acute toxicity using *A. salina* are shown in Table 4.

Table 4: Percent inhibition of acetylcholinesterase enzyme and acute toxicity to crude extract, essential oil and fractions from *Lippia hirta.*

Extract / fraction	Percent inhibition of AchE (0.1 $mg.mL^{-1}$)	Inhibition of AchE IC_{50} ($\mu g.mL^{-1}$)	Acute toxicity LD_{50} ($\mu g.mL^{-1}$)
CE	64.8	43.2	1232.6
Hex	25.3	-	1441.7
EtOAc	99.1	54.9	1320.8
BuOH	70.7	49.3	1285.6
ARF	100.0	0.15	1580.1
E. oil	48.6	-	1233.08
Reminyl®	100.0		-

The data presented in Table 4 shows that the crude extract and the EtOAc, BuOH and ARF fractions showed a percentage of inhibition above 50 %, for the concentration of 0.1 mg.mL^{-1}. Thus, new concentrations for these samples were tested, in order to determine the inhibitory concentration (IC_{50}) expressed in µg.mL^{-1}.

From the data of IC_{50} it is possible to perceive the high capacity to inhibit the AchE, with absolute prominence for the ARF. A number of AChE and butirilcolinesterase (BChE) inhibitors have been isolated from various natural sources, as extensively described in the literature.[31] It is noteworthy that most of the compounds have demonstrated only *in vitro* activity; few of them have been tested on animal models, which is imperative to prove their capacity to cross the blood–brain barrier and exert beneficial effects in the brain. Among these natural products, alkaloids are the most promising candidates for use in the treatment of AD, due to their complex nitrogen-containing structures.[32] In fact, one of the binding sites of AChE involves the interaction of the positively-charged nitrogen, even though other binding site exists, in order to allow the inhibition by non-alkaloid compounds, mainly terpenes, xanthones and coumarin.[33]

In addition, we can also observe in Table 4 that CE and the fractions showed no toxicity. LD_{50} values greater than 1000 ppm are free of toxicity, although this is a simple and not very specific in vitro assay. These values obtained by both tests encourage new researches, since an activity of inhibition of the AChE associated with a low toxicity was verified in the fractions of *Lippia hirta* which is important in the search of new drugs.

In this work it was observed that ARF was able to inhibit the enzyme up to 100 % at a concentration of 0.5 µg.mL^{-1}, which represents an activity high power. Although ARF is a complex matrix, the synergistic effect when compared to the standard used in the test, galantamine (IC_{50} = 17.8 µmol.L^{-1}). Given the excellent results for the inhibition of the AChE, the chromatographic fractionation was chosen to identify the main constituents of the fraction. Chromatographic separation of the ARF resulted in 65 fractions, which were pooled according to their similarities by thin layer chromatography analysis. Through the chromatographic fractionation, 3 alkaloids were obtained, being: dihydrocorinanteol (1), vincamine (2) and deoxycordifoline (3) (Figure 3), whose characterization data are below:

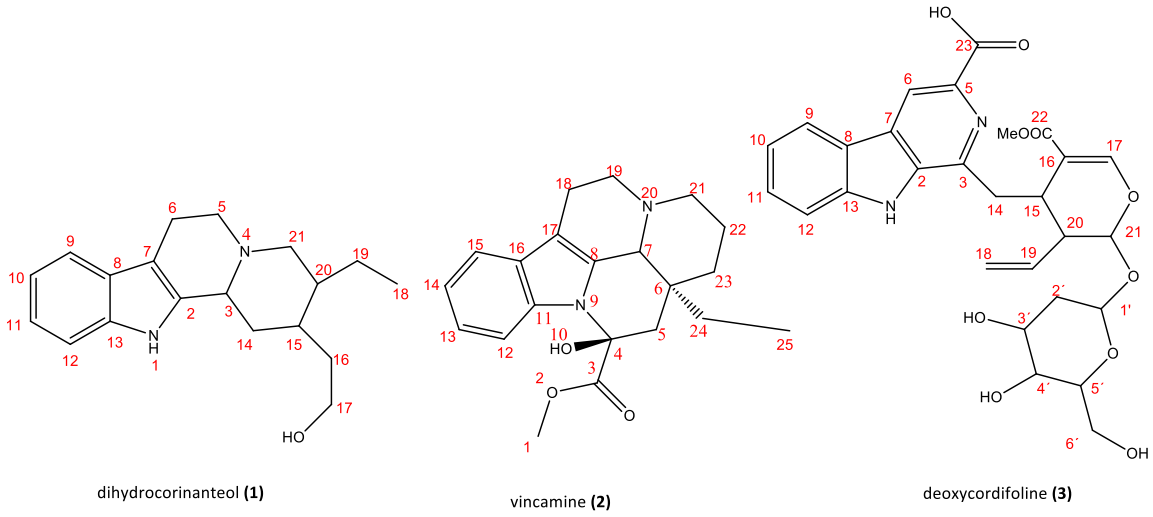

Figure 3. Chemical structures of the isolated alkaloids of *Lippia hirta*

Dihydrocorinanteol (1): (2-(3-ethyl-1,2,3,4,6,7,12,12b-octahydroindolo[2,3-a]quinolizin-2-yl)etanol): colorless crystals, m.p. 173-175ºC. IR (ATR) ν / cm $^{-1}$ 3410 (OH),

3258 (NH), 1452 (NH), 1622 (C-C sp^2), 740 (disubstituted 1,2-aromatic ring). ^{1}H NMR (400 MHz): δ 7.96 (NH), 7.50 (*d*, *J* = 7.5 Hz, H9) and 7.31 (*d*, *J* = 7.5 Hz, H12), 7.10 (*dd*, *J* = 7.5 and 1.5 Hz, H10) 7.14 (*dd*, *J* = 7.5 and 1.5 Hz, H11). ^{13}C NMR (100 MHz) δ 10.9 (C18), 21.6 (C6), 23.4 (19), 35.2 (C14), 35.4 (C16), 37.2 (C15), 41.6 (C20), 53.0 (C5), 59.8 (C3), 60.1 (C21), 60.3 (C17), 107.7 (C7), 110.8 (C12), 118.0 (C9), 119.2 (C10), 121.1 (C11), 127.3 (C8), 135.0 (C2), 136.1 (C13). ESI-EM: m/z 299,15 $[M+1]^+$, calculated for $C_{19}H_{26}N_2O$, 298.20.

Vincamine (2): (12S,13aS)-methyl 12-hydroxy-13a-methyl-2,3,41,5,6,12,13,13a-octahydro-1H-indolo[3,2,1-de]pyrido[3,2,1-ij][1,5]naphthyridine-12-carboxylate). White crystals: mp 230-232 °C. IR (ATR) ν / cm $^{-1}$ 3400-2400, 1748 cm^{-1}; ^{1}H NMR (400 MHz) δ 0.91 (*t*, *J* = 8.0 Hz, CH_3), 1.30-1.80 (*m*; H23, H22 and H24; 5H), 2.10 (*d*, *J* = 8.0 Hz, H5), 2.24 (*d*, *J* = 8.0 Hz, H5), 2.27 (*m*, H24), 2.42-2.70 (*m*, H21, H18; 3H), 2.90-3.10 (*m*, H21), 3.20-3.40 (*m*, H19, 2H), 3.82 (*s*, OCH_3), 3.92 (*s*, H7), 4.64 (*s*, H10), 7.08-7.18 (*m*; H13, H14 and H15; 3H), 7.45- 7.52 (*m*, H12); ^{13}C NMR (100 MHz) δ 7.6 (C25), 16.0 (C6), 16.8 (C18), 20.8 (C22), 25.1 (C23), 28.9 (C24), 44.4 (C21), 44.6 (C5), 50.9 (C19), 54.2 (C1), 59.1 (C7), 81.9 (C4), 105.9 (C17), 110.3 (C12), 118.4 (C15), 120.2 (C14), 121.6 (C13), 129.0 (C16), 131.4 (C8), 134.4 (C11), 174.4 (C3). Anal. Calcd for $C_{21}H_{26}N_2O_3$: C, 71.16; H, 7.33; N, 7.90. Found: C, 71.05; H, 7.40; N, 7.80.

Deoxycordifoline (3): 1-((5-carboxy-2-((4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)-3-vinyl-3,4-dihydro-2H-pyran-4-yl)methyl)-9H-pyrido[3,4-b]indole-3-carboxylic acid. m.p. 178-180° C. IR (ATR) ν / cm $^{-1}$ 2750-3600 (OH, NH and CH aromatic), 1671 (C=O), 1630 (C=C). ^{1}H NMR (400 MHz) δ 3.67 (*d*, *J* = 7.0 Hz, H6´), 3.98 (*dd*, *J* = 7.0 and 1.5 Hz, H6´), 3.39 (*m,* H5´, H3´), 3.22 (*m*, H4´), 3.19 (*d*, *J* = 7.0 Hz, H2´), 4.78 (*d*, *J* = 7.0 Hz, H1´), 3.22 (*s*, OCH_3), 5.86 (*d*, *J* = 7.5 Hz, H21), 2.62 (*dd*, *J* = 7.5 and 1.5 Hz, H20), 5.67 (*ddd*, *J* = 7.5; 7.5 and 1.5 Hz, H19), 4.67 (*d*, *J* = 7.5 Hz, H18), 7.60 (*s*, H17), 3.67 (*m*, H15), 3.27 (*m*, H14), 3.43 (*m*, 7.59 (*d*, *J* = 7.5 Hz, H12), 7.56 (*t*, *J* = 7.5 Hz, H11), 7.28 (*t*, *J* = 7.5 Hz, H10), 8.19 (*d*, *J* = 7.5 Hz, H9), 8.69 (*s*, H6). ^{13}C NMR (100 MHz) δ 30.9 (C15), 33.1 (C14) 43.2 (C20), 50.7 (MeO), 61.1 (C6´), 70.0 (C2´), 73.1 (C3´), 76.7 (C4´), 77.4 (C5´), 95.6 (C21), 98.6 (C1´), 109.6 (C16), 112.3 (C12), 115.4 (C6), 118.9 (C18), 120.1 (C10), 121.3 (C8), 122.1 (C9), 127.2 (C7), 128.4 (C11), 134.2 (C19), 136.4 (C2), 140.8 (C13), 143.5 (C3), 152.0 (C17), 166.6 (C23), 166.9 (C22). ESI-EM: m/z, 571 $[M+H]^+$, 409 [M-Glu+ H]$^+$.

The data obtained agree with those published in the literature for these compounds, although not documented for the genus *Lippia*.[34]

4. Conclusion

Based on the results obtained in this work, it is possible to highlight the potential of *Lippia hirta* in the treatment of neurodegenerative diseases. More specific tests for determination of toxic potential *in vivo* and other tests will be applied to understand the therapeutic potential of fractions and compounds isolated from this plant species. Further future research will be carried out in order to make possible a better understanding of the antioxidant and anticholinesterase mechanism of the compounds present in the polar fractions, especially ethyl acetate and butanol. These investigations are in line with the idea that bioguided fractionation is a strategy of great relevance in the area of chemistry of natural products.

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