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Research Article

Eupatorium intermedium: Chemical characterization and biological activity

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Abstract

Studies for the knowledge of native species with therapeutic potential gain great relevance and among these, there are researches with essential oils of native species. This study aims to know the yield, chemical characterization and biological activity of the essential oil of the species *Eupatorium intermedium*. The plant material was collected in the Atlantic rainforest of the State of Paraná, Brazil. The essential oil extraction was performed by hydrodistillation of the inflorescences and leaves, in triplicates with 100g of plant material. The yield obtained from the essential oil was 1.02% in the inflorescences and 0.74% in the leaves. Gas chromatography analysis was performed on a Shimadzu GCMS-TQ8040 brand, 90 division chromatographs coupled to a mass spectrometer. The main constituents in flowers were α-pinene (25.88%), β-pinene (39.12%), limonene (12.36%), 5-diene-trans-muurola-4(14) (4, 03%). On the other hands, α-pinene (19.81%), β-pinene (25.93%), limonene (15.03%), 5-diene-trans-muurola-4(14) (10.55%), bicyclogermacrene (9.09%) were found in the leaves. *In vitro* antibacterial assays were performed in triplicate by agar diffusion method. The leaf oil generated a halo of inhibition with an average of 14 mm for Listeria monocytogenes, Escherichia coli (16 mm), Bacillus cereus (16 mm) and Staphylococcus aureus (17 mm). The inflorescence oil generated a halo of inhibition with an average of 16 mm for Listeria monocytogenes, Escherichia coli (13.66 mm), Bacillus cereus (13.66 mm), Staphylococcus aureus (13.33 mm). In addition, the results of enzyme inhibition assays indicated inhibition of α-glucosidase (100%) and acetylcholinesterase (85.4%). These results contribute to the chemical characterization of the species and are also promising concerning the biological activity, being this the first ever report of activity from the essential oil of the species *Eupatorium intermedium* against the enzyme α-glucosidase and acetylcholinesterase.

1. Introduction

Studies with the objective of knowing and studying the therapeutic and technological potential of native species are extremely important for the advancement of science and society. Among these lines of study is the research with essential oils from native species, where, the knowledge of the chemical composition and possible biological activities, related to essential oils from native natural resources, increases the possibility of creating protocols for the sustainable use of biodiversity and the use of this plant with therape-

utic potential.

Volatile essential oils are compounds found in various plants and can be stored in flowers, leaves, or even in the bark of stems, wood, roots, rhizomes, fruits or seeds (4). The chemical composition of essential oils depends on environmental factors, harvest period, the extraction technique and also genetics. When working with essential oils, one should take such factors into consideration [1-2].

Essential oils show biological activities against a wide variety of pathogens and diseases that can affect humans and even other species. The compounds in the oil and their yield vary depending on the species and methods used, however, the main compounds isolated from essential oils are terpenes and their oxygenated derivatives [3].

Being considered a country that harbors an immense biological diversity, Brazil has a large number of flora species in its territory, however, not all plants produce essential oils. Among the family's native to Brazil that stand out in the production and studies with essential oils, the Asteraceae family is rich in number of species and individuals. Due to its great diversity, many of the Asteraceae species are used as medicinal plants [4-5].

In this context, the aim of this work was to characterize the essential oil of dry leaves and inflorescences of *Eupatorium intermedium* as well as to available the enzyme inhibition and antibacterial potential.

2. Materials and methods

2.1 Collection and extraction of plant material

The material was collected under ICMBio authorization number 49770-2, in the city of Palmeira, in the state of Paraná, Brazil. The extraction of the essential oil was performed by the hydrodistillation method, with a Clevenger apparatus. The inflorescences and leaves were used, separated and freshly made in triplicates, placing 100g of the sample in a 2-liter flask. The hydrodistillation process lasts 4 hours, after which the oil is removed, centrifuged and stored [6].

The biomass was obtained from cuts of the aerial parts of the plants. All the obtained material was weighed in triplicates of 10g to calculate the dry mass and in triplicates of 100g to perform the extraction. The evaluation parameters were the biomass moisture content and the extraction yield on a moisture-free basis. The essential oil yield, extracted from aerial biomass, was calculated on a dry matter or moisture-free basis. In the calculation, the equation adapted from [7] was used:

$$To = \frac{Vo}{Ms} \times 100$$

Where:

TO = oil content in % or ml of essential oil in 100 g of biomass; VO = volume of oil obtained; DM= quantity of dry, water-free or moisture-free biomass; and 100 = conversion factor to percentage.

2.2 Chemical characterization

Prior to injection each sample was diluted to 1% with dichloromethane (99.9%). Gas chromatography analysis was performed on a Shimadzu brand GCMS-TQ8040, split 90 chromatographs coupled to a mass spectrometer. GC-MS measurements were performed using a Rtx-5MS nonpolar capillary column (5% diphenyl + 95% dimethyl polysiloxane, 30 m × 0.25 mm i.d. × 0.25 μm film thickness), operated under temperature conditions programmed from 60°C to 250 °C at 3°C per minute. The carrier gas was helium with a flow rate of 1.02 mL min-1 and linear velocity of 36.8 cm s^{-1} and the column head pressure was constant at 59 kPa. The injection port was fixed at 250 °C, with an injection volume of 1.0 μL in split mode (1:10 ratio) [8].

After the chromatography procedure, the chromatograms were analyzed in a GC/MS *Postrun Analysis* program, from which the identification of the components present in the essential oil samples was obtained. The data found were compared with the results of the specific bibliography [8-9].

2.3 Antimicrobial assay

The verification of antimicrobial activity was an *in vitro* performed by diffusion, which consists in placing the sample in tanks in contact with the inoculated medium and, after incubation, measuring the inhibition zones [10]. Isolated colonies were cultured in Luria Bertani medium for *Escherichia coli*, *Bacillus cereus*, *Staphylococcus aureus* and *Listeria monocytogenes* incubated with a rotary shaker at 37 °C for 24 hours.

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After this period, a 1:5 dilutions in Luria bertani medium was performed to obtain a suspension containing about 2:10 8 cells/mL, adjusting the turbidity of the bacterial suspension to 0.5 of the McFarland scale. A 100µL aliquot of the dilution was pipetted and distributed on the surface of the solid medium (Tryptone Soy Agar (TSA) for gram-positive bacteria and Luria Bertani Agar (LA) for E. coli) in petri dishes to form the cell mat on the agar gel. After drying the bacterial sample on the plate, wells of 6 mm diameter were drilled with a sterile punch, in this case a glass Pasteur pipette was used. Three wells were drilled, one for the essential oil, the other for the positive control, and one for the negative control. The wells received 100µL of the essential oil, mineral oil was used as a negative control and 100µL of Tetracycline as a positive control.

The assay was performed in triplicate and the plates were incubated at 37°C and after 24 hours, the antibacterial activity was evaluated by measuring the halo of the microbial growth inhibition zone around the wells the results were analyzed by single factor ANOVA test [11].

2.4 Enzymatic inhibition

2.4.1 Glucosidase inhibition assay

Solutions of the samples are prepared in methanol at a concentration of 1 mg mL^{-1}. At the time of the test, each solution is diluted with potassium phosphate buffer (pH 6.8) to 500 µg mL^{-1}. A 50 µL aliquot of alpha-glucosidase solution (1 U mL-1) was premixed with 20 µL of the sample solutions, and 570 µL of potassium phosphate buffer (pH 6.8) (0.1 mol L^{-1}) was added. All tubes were vortexed and incubated in a water bath at 37.5 °C for 20 minutes. After, 100 µL of p-nitrophenyl-a-D-glycopyranoside (pNPG, 1 mmol L^{-1}) is added as substrate and the reaction will start. The tubes are again shaken and the mixture is incubated for 30 min in a water bath at 37.5 °C, followed by the addition of 650 µL of 1 M Na_2CO_3 solution for the termination of the reaction. The amount of p-nitro-phenol formed is measured in a spectrophotometer at a wavelength of 410 nm for the estimation of enzyme activity. An acarbose solution at 50 ug mL^{-1} is used as standard. A negative control using solvent only, instead of the sample, is used. A blank test is performed for each sample, where 20 µL of the sample solution (500 ug mL^{-1}) is added in 570 µL of potassium phosphate buffer (pH 6.8) (0.1 mol L^{-1}) [12]. The inhibitory activity of the alpha-glucosidase enzyme is calculated using the equation:

Inhibitory activity (%) =

$$\frac{\text{Neg C. Abs} - (\text{Sample Abs} - \text{White Abs})}{\text{Neg C. Abs}} \times 100$$

2.4.2 Anticholinesterase assay

The anticholinesterase activity was determined using Ellman's *in vitro* spectroscopic method according to [13]. Samples were diluted in methanol at a concentration of 1 mg mL-1. In each test tube, 325µL Tris-HCl buffer was added to maintain the pH of the medium at 8. To a 100µL aliquot of sample, 20µl of acetylcholinesterase enzyme solution diluted in Tris-HCl buffer containing 0.1% Bovine Serum Albumin (0.25 U mL-1) was added. This mixture was incubated at room temperature for 15 minutes. Then, 70µL of acetylcholine iodide solution (0.021 mg mL-1) and 470µL of Ellman's Reagent (5,5-dithiobis (2-nitrobenzoic acid) prepared in Tris HCl added 0.1M NaCl and 0.02M $MgCl_2.6H_2O$) were added. After homogenization, the test tubes were incubated away from light for 25 minutes. Then 1000 ul of Tris-HCl buffer solution was added and the absorbance of the solution was measured in a spectrophotometer at a wavelength of 405 nm.

As a positive control, a neostigmine hydrochloride solution (100 µg ml^{-1}) was used; and as a negative control, the samples were replaced only by the solvent used for dilution. The acetylcholinesterase inhibitory activity of the enzyme was calculated by the equation:

Inhibitory Activity (%) =

$$\frac{100 - (\text{Sample Abs} - \text{White Abs})}{\text{Average Negative C. Abs}} \times 100$$

3. Results and discussion

3.1 Essential oil yield and chemical composition

The essential oil yield was (1.02%) in the inflorescences and (0.74%) in the leaves. The majority constituents of *Eupatorium intermedium* flowers were, α-pinene (25.88%), β- pinene (39.12%), limonene (12.36%), 5-diene-trans- muurola-4(14) (4.03%), as can be seen in Table 1. The essential oil of the

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Table 1. Chemical composition of the essential oil of *E. intermedium* inflorescences (≥1%).

Name	%	IK	IR	IR literature
α-Pineno	25.88	939	946	932
Sabineno	2.74	975	987	969
β- Pineno	39.12	979	994	974
Mirceno	2.76	990	1005	988
α-Felandreno	1.19	1002	1021	1002
ρ-Cimeno	3.67	1024	1039	1020
Limoneno	12.36	1029	1045	1024
(*E*)-β-Ocimeno	2.95	1050	1062	1044
(*E*)- Cariofileno	1.51	1419	1443	1417
5-Diene-trans-Muurola-4(14)	4.03	1493	1507	1493
Biciclogermacreno	2.71	1500	1522	1500
Espatulenol	1.08	1578	1604	1577

IK: Kovats index. IR: Calculated retention index. IR Literature: retention index from literature.

inflorescences showed 12 chemical compounds with area greater than 1%, with β- pinene and α- pinene as the majorities. Fig. 1 shows the chromatogram generated for the oil from the flowers of the species *E. intermedium.*

The majority constituents of *Eupatorium intermedium* leaves were, α-pinene (19.81%), β- pinene (25.93%), limonene (15.03%), 5-diene-trans- muurola-4(14) (10.55%), bicyclogermacrene (9.09%), as can be seen in Table 2. Fig. 2 shows the chromatogram generated for the oil from the leaves of the species *Eupatorium intermedium.*

Table 2. Chemical composition of the essential oil of *E. intermedium* leaves (≥1%).

Name	%	IK	IR	IR literature
α-Pinene	19.81	939	946	932
Sabinene	2.40	975	988	969
β- Pineno	25.93	979	994	974
Mirceno	3.38	990	1005	988
ρ-Cimeno	1.47	1024	1039	1020
Limoneno	15.03	1029	1046	1024
(E)-β-Ocimeno	3.01	1050	1062	1044
(E)- Cariofileno	3.16	1419	1444	1417
5-Diene-trans-Muurola-p4(14)	10.55	1493	1507	1493
Biciclogermacreno	9.09	1500	1522	1500
Espatulenol	2.93	1578	1605	1577
Globulol	1.40	1590	1623	1590
α-Cadinol	1.84	1654	1687	1652

IK: Kovats Index. lR: Calculated retention index. IR Literature: retention index from literature.

Figure 1. GC chromatogram essential oil derived from flowers of *Eupatorium intermedium*.

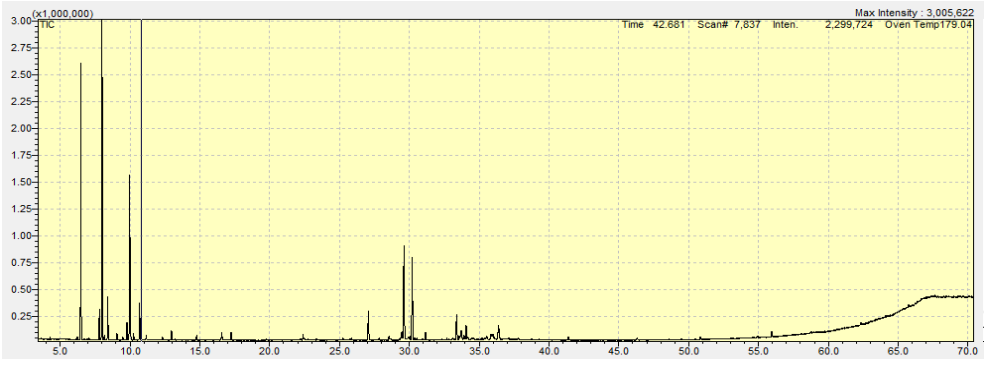

Figure 2. GC chromatogram essential oil derived from leaves of *Eupatorium intermedium*.

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The essential oil of the leaves showed 13 chemical compounds with concentration higher than 1%, with β-pinene, α-pinene, limonene and 5-diene-trans-muurola-4(14) as the majorities. And it did not indicate the presence of α-phellandrene which was found in the essential oil of the inflorescences. On the other hand, it indicated the presence of globulol (1.40%) and α-cadinol (1.84%) which were not found in the inflorescences volatile portion.

In a study conducted on three species of the genus *Eupatorium*, one of which was the species *Eupatorium intermedium*, they obtained the same majority compounds for both the essential oil of the flower and the leaf. The majority compounds were α-pinene, β-pinene and limonene [14]. A study on the extraction and evaluation of essential oil obtained from dried flowers of *Eupatorium intermedium*, hydrodistillation achieved a maximum yield of 1.01% essential oil and the compounds found in the oil were similar to those in this article, being α-pinene, sabinene, β-pinene, limonene [15].

A study conducted with the species *Eupatorium glabratum*, also from the Asteraceae family, chemically characterized the oil which was composed mainly of monoterpenes, being α-pinene (29.5%) and α-phellandrene (19.6%) [16].

A study conducted in 2007 the essential oil obtained from leaves and flowers of *Eupatorium polystachyum* DC. (Asteraceae) collected in the state of Rio Grande do Sul, Brazil. The yield of the essential oil, in relation to the fresh material, was 1.6 and 0.9% for the leaves and flowers, respectively. Both oils are composed exclusively of terpenic compounds. The leaf oil contains a large number of monoterpenes (66.4%); on the other hand, the inflorescence oil contains comparable amounts of mono- and sesquiterpenes (51.8 and 47.7%, respectively). The same compounds are in the majority in both oils: β-pinene (14.7 and 9.8%), β-myrcene (15.3 and 10.8%) and limonene (22.8 and 20.5%) among monoterpenes and β-caryophyllene (10.4 and 15.4%), germacrene D (7.2 and 9.4%) and cyclogermacrene (12.0 and 19.2%) among sesquiterpenes [17].

The antibacterial activities of the essential oil were performed *in vitro*, in triplicate by the agar diffusion method using four bacterial strains *Escherichia coli, Bacillus cereus, Staphylococcus aureus, Listeria monocytogenes*. The results of the inhibition halos are shown in millimeters and are arranged in Table 3.

Table 3. Results of the antibacterial assays with essential oil from *E. intermedium* inflorescences

Bacterium	Halo with oil	Halo with positive control
Listeria mono-cytogenes	16 mm	27 mm
Escherichia coli	13.66 mm	28 mm
Bacillus cereus	13.66 mm	26 mm
Staphylococcus aureus	13.33 mm	25.33 mm

Average of the inhibition halos values found in triplicates for the four bacterial strains and the positive control.

The oil from the inflorescences generated a halo of inhibition with an average of 16 mm for *Listeria monocytogenes*, *Escherichia coli* (13.66 mm), *Bacillus cereus* (13.66 mm), *Staphylococcus aureus* (13.33 mm), Table 3. The leaf oil generated a halo of inhibition averaging 14mm for *Listeria monocytogenes*, *Escherichia coli* (16 mm), *Bacillus cereus* (16 mm) and *Staphylococcus aureus* (17 mm), as can be seen in Table 4.

Table 4. Results of the antibacterial assays with essential oil from *E. intermedium* leaves.

Bacterium	Halo with oil	Halo with positive control
Listeria monocytogenes	14 mm	26.33 mm
Escherichia coli	16 mm	28 mm
Bacillus cereus	16 mm	25.33 mm
Staphylococcus aureus	17 mm	26 mm

Average of the inhibition halos values found in triplicates for the four bacterial strains and the positive control.

Compounds with a zone of inhibition halo below 12 mm cannot be classified as antibacterial agents. However, due to the fact that the zone of growth inhibition is greatly influenced by the diffusion velocity of the substances in the agar, which is hydrophilic in nature, and the essential oil is viscous and of low polarity, which hinders the diffusion of the oil in this medium, then any halo value obtained, however small, provides support for classifying such oil as an antibacterial agent [18-19].

The antibacterial effectiveness of *Eupatorium intermedium* EO flower against four bacterial strains (*Listeria monocytogenes, Staphylococcus aureus, Escherichia coli* and *Salmonella typhimurium*) was studied. The results showed that *Eupatorium*

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intermedium EO flower had a potent antibacterial ability against two-gram positive bacteria (*Listeria monocytogenes, Staphylococcus aureus*) with the inhibition zone diameter of 11.09 and 13.07 mm, respectively [15].

A study using the oil of *Eupatorium triplinerve* Vahl, verified the antibacterial activity of the essential oil against ten bacteria (including Gram positive and Gram negative). The oil showed moderate antibacterial activity against all tested pathogens. The largest zone of inhibition was recorded against *Salamella typhae* (21 mm) followed by *Shigella sonnei* (18 mm) [20].

For the enzyme inhibition assays, we have performed with two target enzymes, α-glucosidase and acetylcholinesterase, using the oil from *E. intermedium* flowers. The results are shown in Table 5.

Table 5. Results of the enzymatic evaluation of α-glucosidase and acetylcholinesterase using the essential oil of *E. intermedium* flowers

Essential oil	α-glycosidase	SD	Acetylcholi-nesterase	SD
E. intermedium flowers	100.00%	± 0.00	85.41%	±0.51

M= Mean; SD= Standard Deviation.

Many molecular targets, such as enzymes, have been studied in order to find drugs and herbs with enzyme inhibition mechanisms, for beneficial use by the body and cure related diseases. A sample capable of inhibiting at least 50% of enzyme activity at the concentration tested is considered promising. Enzymatic tests performed with the essential oil of *Eupatorium intermedium* reached an average of 100% in inhibiting α-glycosidase and 85.4% in inhibiting of acetylcholinesterase enzyme [21].

Since in this oil the monoterpenes α- and β-pinene are the majority compounds in all seasons and the oil showed low sesquiterpene content, it was possible to associate this property with its mostly monoterpene constituents. The statement is based on the fact that the compound α-pinene has been described in previous studies as an acetylcholinesterase inhibitor [22].

Enzyme inhibition assays with species native to the Atlantic Forest Biome, were performed with commercial enzymes employing spectrophotometric methods. It was found that for the enzyme α-glucosidase none of the samples tested showed significant inhibitory activity. Among the samples tested for acetylcholinesterase, the bark oil of *Drymis angustifolia*, rich in monoterpenes α-pinene and β-pinene, was the most active in inhibiting acetylcholinesterase, resulting in a 73% inhibition [21]. Studies with native species are gaining more and more space and the essential oil of *Eupatorium intermedium* showed positive results in the inhibition of the enzymes α-glucosidase and acetylcholinesterase.

4. Conclusions

In this paper, we demonstrate that the essential oil of the species *E. intermedium* extracted by hydrodistillation, had a good yield and a chemical composition composed mostly of monoterpenes, being α-pinene and β-pinene the main constituents. The antibacterial tests show that the oil is able to act against the bacteria tested, showing halos of up to 17 mm, and the enzymatic tests show a high inhibition power of the enzymes α-glucosidase and acetylcholinesterase. The next steps to be tested with this essential oil is to verify if it has changes in yield, chemical composition and biological activity when collected in other seasons of the year. Also, given the results, isolate the components to study them separately.

Authors' contributions

Cleiton de Oliveira and Wanderlei do Amaral did the experimental procedure and the collected the inflorescences and leaves. Camila Confortin carried out the isolation and characterization of volatile compounds, besides she wrote the manuscript. Luiz Everson da Silva supervised, rearranged, completed and corrected the manuscript. Michele Debiasi Alberton and Ana Helena Loos Moritz carried out the biological assays.

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Conflicts of interest

The authors declare that they have no conflict of interests.

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