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Chemical constituents of *Ocotea paranaensis* (Lauraceae) essential oil and their antioxidant, anticancer and antimicrobial properties

[Componentes químicos del aceite esencial de *Ocotea paranaensis* (Lauraceae) y sus propiedades antioxidantes, anticancer y antimicrobianas]

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Abstract: The chemical composition of the essential oil obtained from the branches of *Ocotea paranaensis* was studied by gas chromatography/mass spectrometry (GC/MS). Twenty-seven compounds, comprising 94.82% of the total components, were identified. The oil showed relatively high concentration of sesquiterpenes (62.96%), oxygenated sesquiterpenes (33.33%), and diterpene (3.70%). Regarding the major compounds, Z-nerolidol (19.16%), germacrene D (12.92%) and α-bulnesene (8.47%) could be highlighted, which corresponded to 40.55% of the substances that were found. The essential oil from *Ocotea paranaensis* has phosphomolybdenum reducing action and is moderately toxic to the *Artemia salina* (LC_{50} = 147.91 µg/mL). It showed haemolytic potential and moderate activity against *Staphylococcus aureus*, (minimum inhibitory concentration MIC = 250 µg/mL) and *Pseudomonas aeruginosa* (MIC = 500 µg/mL). No satisfactory cytotoxicity results were observed in lineage H460 and HeLa.

Keywords: Lauraceae; Essential oil; Biological properties; Chemical composition.

Resumen: La composición química del aceite esencial obtenido de las ramas de *Ocotea paranaensis* se estudió por cromatografía de gases/espectrometría de masas (CG/MS). Se identificaron veintisiete compuestos, que comprenden el 94,82% de los componentes totales. El aceite se caracterizó por una concentración relativamente alta de sesquiterpenos (62,96%), sesquiterpenos oxigenados (33,33%) y diterpeno (3,70%). En cuanto a los compuestos principales, se destacaron Z-nerolidol (19,16%), germacreno D (12,92%) y α-bulnesene (8,47%), que correspondieron al 40,55% de las sustancias encontradas. El aceite esencial analizado de *Ocotea paranaensis* tiene una buena acción reductora de fosfomolibdeno y es moderadamente tóxico para la *Artemia salina* (LC_{50} = 147,91 µg/mL). Mostró potencial hemolítico y actividad moderada contra *Staphylococcus aureus* (concentración inhibitoria mínima MIC = 250 µg/mL) y *Pseudomonas aeruginosa* (MIC = 500 µg/mL). No se observaron resultados satisfactorios de citotoxicidad en el linaje H460 y HeLa.

Palabras clave: Lauraceae; Aceite esencial; Propiedades biológicas; Composición química.

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INTRODUCTION

The Lauraceae family is predominantly distributed in tropical and subtropical areas of the Americas, Africa, and eastern Asia. It is composed of trees and bush and it is recognized by about 50 genus with estimated 2500–3000 species. Despite its importance and massive diversity, little information is known about the classification, number, and distribution of the species within the family, which may be related to the difficulty of identification (Van Der Werff & Richter, 1996).

From the economic point of view, the family (Lauraceae) presents great importance as some of genus such as *Ocotea*, *Cinnamomum*, *Aniba* and *Nectandra* exhibit various applications. They are used in the manufacturing of culinary products, paper, carpentry and construction. Additionally, they have certain medicinal qualities against various diseases. *Ocotea aciphylla*, used as an anti-rheumatic and depurative; *Ocotea guianensis* used in abscesses; *Laurus nobilis* used as an antispasmodic, against flatulence, wounds and ulcers; *Persea american* used for kidney problems, diuretic and soothing; *Cinnamomum cassia* used in colds (Marques, 2001); *Dehaasia kurzii* and *Litsea liyuyingi* used in leucorrhoea (Hossan *et al*., 2010). Chemically, this family is also rich in metabolites, such as neolignans and lignans, aporphine and benzylisoquinoline alkaloids, flavonoids, sesquiterpenes, and pyrones (Garcez *et al*., 2011).

In Brazil, 22 genus of this family were already found, among them, the genus *Ocotea*, with about 120–160 species, of which 31 species are found in the Parana State, located in the South region of Brazil (Baitello, 2001; Moraes, 2005; Brotto *et al*., 2013). The essential oil from these genus have been investigated by several authors along the years, which have been found to demonstrate anti-inflammatory, acaricidal, antimicrobial, and antioxidant properties (Chaverri & Cicció, 2005; Guerrini *et al*., 2006; Destryana *et al*., 2014; Moraes *et al*., 2017).

Recently, one more species called *Ocotea paranaensis* was discovered in the Atlantic Forest of Parana State and its specific epithet reminds the place where it was first mentioned (Brotto *et al*., 2010).

This present study aimed to evaluate the chemical composition of the essential oil from branches of *Ocotea paranaensis*, as well as its antioxidant and biological properties, among them, its haemolytic, antimicrobial and anticancer properties and its toxicity in brine shrimp.

MATERIALS AND METHODS

Plant material

The plant material (branches) were obtained from mature trees in the State Park of Lauraceae, Parana, Brazil, in September 2017, at the following coordinate: Zone 22J, East: 731234.389; North: 7249262.709. The species was identified for comparison by the taxonomist Marcelo Leandro Brotto, under the number MBM 409884. This study was approved by the Brazilian Institute of the Environment (IBAMA) and permission was received to access samples of the genetic patrimony for scientific research purposes and to use and disseminate the results obtained from the botanical material of *Ocotea paranaensis*, included in Process 02001.001165/2013-47.

Essential oil extraction

Essential oil (EO) was extracted and obtained from the dried branches of *Ocotea paranaensis* and the steam distillation process was carried out, using modified Clevenger (Brasil, 2010). Subsequently, the essential oil was separated from the emulsion through decantation using a separating funnel and dried with anhydrous sodium sulfate.

The dried plant material was ground (600 g) in a knife mill and distilled into the water at 100°C for 6 hours. The collected oil was stored in an amber bottle in a freezer at -18°C for later use. The yield was calculated in milliliters of essential oil per 100 g of the dried plant material (mL%) (Brasil, 2010).

Chemical analysis: Gas chromatography-mass spectrometry (GC/MS)

The analysis was performed using a Shimadzu CGMS-QP2010 Plus system equipped with a quadrupole mass detector with a Rtx-5MS (Crossband 5% diphenyl/95% dimethylpolysiloxane) low-bleeding column (30 m × 0.25 mm × 0.25 μm), where helium was the carrier gas at a flow rate of 1.02 mL/min. Splitless injection of 1 μL of the sample was carried out at an initial oven temperature of 60°C. The injector and detector temperatures were adjusted to 250°C. The programmed oven temperature was 60–250°C at 3°C/min.; EIMS: electron energy, 70 eV; ion source temperature and connection parts at 180°C.

Peak identification

Individual components were identified by comparing retention indices (RIs) and mass spectra with those of authentic compounds given in the Adams Libraries of mass spectral data and from a computer database

using Wiley 275, NIST 21, NIST 10733 (NIST, 1998; Adams, 2017).

Antioxidant properties

Reduction of Radical DPPH• (2,2-diphenyl-1-picrylhydrazyl)

The antioxidant property was evaluated by reducing the DPPH radical according to the adapted methodology (Mensor *et al*., 2001). Initially, a sample solution of 200 μg/mL was prepared, which was solubilized in methanol with DMSO added to the essential oil in a 1:1 (w/w) ratio. Ascorbic acid, butylated hydroxytoluene (BHT), and rutine - each with a concentration of 200 μg/mL - all solubilized in methanol were used as positive control.

In 96-well flat-bottom microplate, 142 μL of the sample and 58 μL of the 0.3 mM DPPH solution were added. It was kept for 30 minutes in the dark at 25°C; then, absorbance readings were performed on a Thermo Scientific® Multiscan FC spectrophotometer at 540 nm.

To eliminate the influence of the staining of the extracts, a blank was prepared for each sample. The blank of the sample was determined using 142 μL of the sample and 58 μL of methanol and the negative control 142 μL of methanol and 58 μL of DPPH•. All assays were performed in triplicate. The ability of essential oil to reduce the DPPH• moiety was calculated from the following equation: % Inhibition of oxidation of DPPH• = 100 – [(sample absorbance - white absorbance) / (control absorbance - white absorbance)] x 100. The statistical analysis of the results that were obtained was carried out by the ANOVA test and the comparison between the means was verified by the Tukey test, with a 95% level of significance. The Sisvar software was used to perform these analyses.

Formation of the Phosphomolybdenum Complex

The antioxidant property was evaluated by reduction of the phosphomolybdenum complex according to the adapted methodology (Prieto *et al*., 1999). For the performance of this assay, the essential oil and standards (ascorbic acid, BHT, and rutin) solutions were prepared at the concentration of 200 μg/mL. The essential oil was diluted in methanol and DMSO (1:1), while the positive controls were diluted only in methanol. The reagent was prepared at the time of use, which consisted of a 0.6 $mol.L^{-1}$ aqueous sulfuric acid solution, 28 $mmol.L^{-1}$ sodium phosphate, and 4 $mmol.L^{-1}$ ammonium molybdate.

In triplicate, 3 mL of reagent and 0.3 mL of the essential oil and standard were added to a test tube. The tubes were capped and taken to a water bath for 90 min at 95°C. After cooling, the samples were transferred to 96-well microplates for absorbance readings on a Multiscan FC Thermo Scientific® spectrophotometer at a wavelength of 690 nm.

To evaluate the antioxidant capacity of essential oil, the relative antioxidant activity (AAR%) was calculated, taking into account the positive standards, ascorbic acid, BHT, and rutin, from the following equation: AAR (%) = [(sample absorbance - white absorbance)/(standard absorbance - white absorbance)] x 100. The statistical analysis of the results was carried out by the ANOVA test and the comparison between the means was verified by the Tukey test, with a 95% level of significance. The Sisvar software was used to perform these analyses.

Biological properties

Toxic activity in Artemia salina

The evaluation of toxicity against *Artemia salina* was done by following the methodology (Meyer *et al*., 1982; Martinez *et al*., 1997) with some adaptations.

Initially, artificial seawater was prepared by dissolving 38 g of sea salt (Blue Treasure®) in 1 L of distilled water and adjusting its pH to 9.0 with Na_2CO_3 (Meyer *et al*., 1982). Saline brine shrimp eggs (200 mg/400 mL) hatched in artificial seawater for 48 hours under continuous aeration while being exposed to constant light under controlled temperature (27 to 30°C) to pH 8–9. The negative control (white) was a saline solution and the positive control quinidine sulfate.

The essential oil was prepared in triplicate with different concentrations (10, 100, 250, 500, and 1000 μg/mL) and solubilized in polysorbate 80 in 1:1 (w/w) ratio in methanol; the solvent evaporated by 24 hours at 37°C and the volume of artificial seawater filled up to 5 mL.

To evaluate the toxicity, 10 larvae of *Artemia saline* were transferred to glass vials containing the essential oil and controls. Subsequently, the flasks were incubated at room temperature for 24 hours. After this period, it was possible to count dead and alive larvae. As a negative control, the larvae were evaluated against methanol solvent plus polysorbate 80 at a ratio of 1:1 (w/w); the positive control quinidine sulfate was used at the same concentrations that were prepared for the samples. Their preparation was performed as described for the essential oil.

The results are expressed as LC_{50} (lethal

concentration for 50% of the individuals) and 95% confidence intervals. For the calculation, the statistical program Probitos was applied using the IBM SPSS Statistics® software.

Haemolytic activity in vitro

The evaluation of haemolytic activity was performed, based on the methodology described by Benerjee *et al*. (2008), with adaptations. Defibrinated ram blood (Newprov®) was prepared to obtain a diluted erythrocyte pope with phosphate-saline buffer (PBS) to 2% (w/v) (Banerjee *et al*., 2008).

For the haemolysis test, the saponin standard and the essential oil were prepared at concentrations of 1000, 750, 500, 250, and 100 µg/mL. Preparation of the 1000 µg/mL saponin solution was performed in 10% methanol and PBS. 0.5% (v/v) DMSO was added only for the preparation of the essential oil. As a negative control, 200 µL PBS was used in 200 µL 2% erythrocyte solution; negative controls of the solvents were performed with PBS in 10% methanol and PBS with 0.5% (v/v) DMSO. As positive control water, 200 µL of potable water was used in 200 µL of 2% erythrocyte solution. As positive control, Triton was used as 200 µL of 1% triton in 200 µL of 2% erythrocyte solution. The assay was performed on Eppendorf and incubated for 3 hours at 37°C controlled temperature. After incubation, the solutions were centrifuged at 3000 rpm for 5 minutes.

Haemolysis was assessed by reading the displayed absorbance of the supernatant in a Multiscan FC Thermo Scientific® microplate reader adjusted to 540 nm. For the quantification of haemolysis, the value was calculated as a percentage, considering the value of 100% the absorbance read in the total haemolysis tube and using 1% triton and potable water as reference.

Using linear regression, the concentration that is required to achieve 50% haemolytic activity was determined. The statistical analysis of the results was conducted using the ANOVA test and the comparison between the means was verified by the Tukey test, with a 95% level of significance. The Sisvar software was used to perform these analyses.

Antimicrobial activity

Antimicrobial activity was assessed by determining Minimum Inhibitory Concentration (MIC) using the microdilution method according to the CLSI (2008), which was standardized and validated by Veiga *et al*. (2019). Standard strains of the bacteria *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 9027), *Escherichia coli* (ATCC 8738), and *Candida albicans* (ATCC 10231) were used.

Initially, the inoculum was prepared in 0.9% saline. Subsequently, turbidity was standardized to 0.5 Mc Farland using the DEN-1 Biosan® densitometer. The broth microdilution technique was performed on sterile 96-well U-bottom plastic microplates. All samples were tested in triplicate by placing only one microorganism per plate.

The sample was prepared at 1 mg/mL in 5% DMSO distilled water and serially diluted in Muller Hinton Broth (MHB - Merck, Darmstadt, Germany) for bacteria and Sabouraud Dextrose broth for yeast. Concentrations ranged from 500 µg/mL–3.9 µg/mL. 10 µL of the previously prepared bacterial suspensions were inoculated into all wells except the blank. Negative control was performed by adding 100 µL of the sample diluent solution to 100 µL of broth and 10 µL of inoculum. In the white wells of the sample, 100 µL of broth and 100 µL of the sample that was to be tested (essential oil) were used. To demonstrate that the technique is effective and to confirm the lack of resistance of microorganisms to the drugs that were used, positive control with 100 µL broth, 100 µL antimicrobial solution (chloramphenicol 250 µg/mL for bacteria and ketoconazole 500 µg/mL for yeast) and 10 µL of the inoculum was performed.

The microplate incubation was performed in a bacteriological oven at a constant temperature of 35ºC for 22 hours. Then, 20 µL of 0.125% 2,3,5-triphenyl tetrazolium chloride developer (TTC - Merck, Darmstadt, Germany) was added and the microplates were incubated again for two hours at 35°C. The development of red/pink coloration was interpreted as cell growth, as TTC (colorless) is reduced by the metabolism of microorganisms, thereby becoming a compound with coloration. Results were classified as good antibacterial activity (up to 100 µg/mL), moderate activity (between 100–500 µg/mL), weak activity (between 500–1000 µg/mL) and inactive (greater than 1000 µg/mL) (Ayres *et al*., 2008; Santos *et al*., 2008).

To evaluate the Minimum Fungicidal Concentration (CFM) and the Minimum Bactericidal Concentration (CBM), the technique of Santurio et al. (2007) was used with modifications. In duplicate, 100 µL of a specimen that was positive for antimicrobial activity was transferred to plates containing TSA agar (bacteria) and Sabouraud agar (yeast). Afterward, the plates were incubated for 22 hours at 35ºC. Lack of

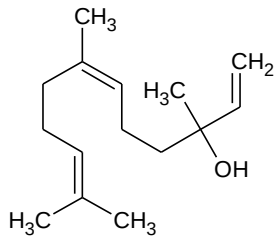
growth indicated the bactericidal action of the tested sample and colony growth indicated the bacteriostatic action of the tested sample (Santurio *et al*., 2007).

Antiproliferative activity

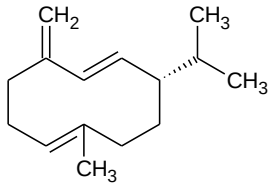
H460 (lung cancer) and HeLa (human uterine carcinoma) cell lines were grown in medium DMEM (*Dulbecco's Modified Eagle Medium*) high glucose, supplemented with 10% of fetal bovine serum and 1% of antibiotic. Cells were grown in a cell culture incubator with controlled humid atmosphere which contained 5% of CO_2 at 37°C. Cell viability was assessed by reducing MTT tetrazolium salt bromide [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] (yellow) to formazan (purple), which was quantified by spectrophotometry (Mosmann, 1983).

In brief, cells were plated in 96-well plates at $1.0x10^4$ cells/well for H460 cells and $1.5x10^4$ cells/well for HeLa cells. Cells were kept in a cell culture incubator for 24 hours for adhesion. Then, the cells were treated with the essential oil at 10 and 50 µg/mL, respectively, for 72 hours. The control condition corresponding to 100% of the viable cells was performed with culture medium containing 0.1% of DMSO (v/v). The results were expressed as a percentage of viable cells that was relative to the control condition. After treatment, the supernatant was removed. 100 µL of [3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide] (MTT) (0.5 mg/mL) was added, which was incubated for 4 hours in the dark at 37°C. Then, the MTT solution was removed and the formazan crystals were eluted with 100 µL of DMSO/ethanol solution (1:1). Absorbances were read on a Thermo Scientific® Multiscan FC spectrophotometer at 570 nm. The assays were performed as independent replicates and the graphs showing the mean ± SD results were created using the GraphPad Prism 5 software.

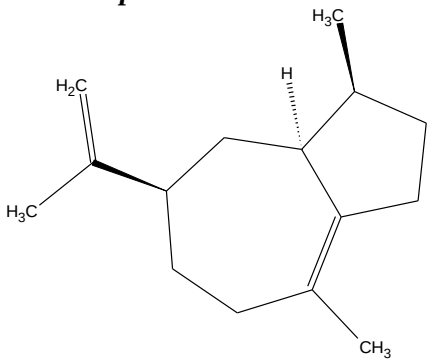
Figure No. 1
Abundant compounds identified in essential oil of *Ocotea paranaensis*



***Z*-nerolidol**



Germacrene-D



α-bulnesene

TABLE No. 1
Identification of compounds in essential oil from *Ocotea paranaensis* by GC-MS

CAS	RT	IA	IAt	Compounds	[ ] %	Classification
20307-84-0	20.160	1336	1335	δ-Elemene	0.58	Sesquiterpene
17699-14-8	20.665	1348	1348	α-Cubebene	5.43	Sesquiterpene
14912-44-8	21.575	1370	1373	α-Ylangene	0.51	Sesquiterpene
3856-25-5	21.755	1375	1374	α-Copaene	1.04	Sesquiterpene
18252-44-3	22.350	1389	1430	β-Copaene	1.81	Sesquiterpene
515-13-9	22.425	1391	1389	β-Elemene	1.97	Sesquiterpene
87-44-5	23.535	1418	1417	E-Caryophyllene	5.02	Sesquiterpene
29873-99-2	24.110	1432	1434	γ-Elemene	0.6	Sesquiterpene
6753-98-6	24.905	1452	1452	α-Humulene	1.7	Sesquiterpene
30021-74-0	25.855	1476	1478	γ-Muurolene	0.71	Sesquiterpene
23986-74-5	26.025	1480	1484	Germacrene D	12.92	Sesquiterpene
-	26.450	1490		-	0.95	
21747-46-6	26.605	1494	1496	Viridiflorene	0.65	Sesquiterpene
99529-78-9	26.650	1495	1498	β-Alaskene	2.78	Sesquiterpene

31983-22-9	26.805	1499	1500	α-Muurolene	0.61	Sesquiterpene
39029-41-9	27.335	1513	1513	γ-Cadinene	1.35	Sesquiterpene
483-76-1	27.705	1523	1522	δ-Cadinene	4.97	Sesquiterpene
-	28.605	1546		NI	1.1	
3691-11-0	28.990	1556	1509	α-Bulnesene	8.47	Sesquiterpene
142-50-7	29.260	1563	1531	Z-Nerolidol	19.16	Oxygenated sesquiterpene
6750-60-3	29.780	1577	1577	Spathulenol	5.4	Oxygenated sesquiterpene
1139-30-6	29.980	1582	1582	Caryophylene oxide	4.71	Oxygenated sesquiterpene
489-86-1	30.560	1598	1600	Guaiol	1.26	Oxygenated sesquiterpene
5937-11-1	31.710	1629	1638	α-epi-Cadinol	3.99	Oxygenated sesquiterpene
481-34-5	32.210	1642	1652	α-Cadinol	3.56	Oxygenated sesquiterpene
-	32.365	1647		NI	0.74	
28400-11-5	32.660	1655	1636	β-Acorenol	2.77	Oxygenated sesquiterpene
22451-73-6	33.130	1668	1670	Bulnesol	1.44	Oxygenated sesquiterpene
1209-91-2	33.550	1679	1676	Mustakone	0.4	Oxygenated sesquiterpene
-	36.615	1766		NI	0.56	
34424-57-2	45.420	2036	2042	Kaurene	1.01	Diterpene
Identified					94.82	Identified
Diterpene					3.70	Diterpene
Oxygenated sesquiterpenes					33.33	Oxygenated sesquiterpenes
Sesquiterpenes					62.96	Sesquiterpenes
Unidentified					3.35	Unidentified

RT = retention time (minutes), IAt = theoretical arithmetic index (Adams, 2017), IA = calculated arithmetic index, [ ] % = component percentage. NI = Not identified.

RESULTS AND DISCUSSION

Ocotea paranaensis essential oil obtained through Clevenger hydrodistillation was light green and its yield was 0.1%, calculated in milliliters of essential oil per 100 g of dried plant material (mL%) (Brasil, 2010).

The yield presented by *Ocotea paranaensis* is an expected value for essential oils of this genus, as already noted in *Ocotea ceanothifolia* (0.07%), *Ocotea leucoxylon* (0.05%), *Ocotea minor* (0.04%) (Yamaguchi, 2011), and *Ocotea porous* (0.08%) (Brito, 2009). Yields with high essential oil content were reported in *Ocotea duckei*, which presented a yield of 1.0% for branch oil (Barbosa-Filho *et al.*, 2008).

The different yields in species of the same genus may be related to geographical and environmental factors, such as temperature, light intensity, stage of development, humidity (Sangwan *et al*., 2001; Destryana *et al*., 2014;), as well as the plant material that was used and harvest time (Barbosa-Filho *et al*., 2008). The results of GC/MS analyses are listed in Table No. 1.

A total of 27 compounds (94.82%) were identified, of which sesquiterpenes (62.96%) were predominant, followed by oxygenated sesquiterpenes (33.33%) and diterpene (3.70%). The predominance of sesquiterpenes has already been evidenced in other Ocotea species (Takaku *et al*., 2007; Damasceno *et al*., 2017). Regarding the majorities, Z-nerolidol (19.16%), germacrene D (12.92%), and α-bulnesene (8.47%) can be highlighted, corresponding to 40.55% of the compounds found (Figure No. 1). No compounds below 0.4% were identified, which represented 3.35% of the sample analyzed.

When the composition of essential oils of the genus Ocotea was evaluated, a wide variety of chemical constituents were observed. In the analysis of essential oils from ten species of Ocotea (Lauraceae), Monteverde and Costa Rica, the primary common constituents were α-pinene, β-pinene, β-caryophyllene, and germacrene-D (Takaku *et al*., 2007). Germacrene-D in Ocotea caudata is one of the predominant substances of essential oils obtained from branches and leaves (Da Silva *et al*., 2017). Several oils that have germacrene-D in their composition are reported to possess antimicrobial properties (Sousa *et al*., 2008; Goren *et al*., 2011).

Additionally, the presence of nerolidol in *Ocotea tonduzii*, *Ocotea valeriana*, *Ocotea varaguensis*, and *Ocotea whitei* was also evidenced (Takaku *et al*., 2007). Nerolidol is an oxygenated sesquiterpene that is present in various floral-scented plants and is widely used as a fragrance in cosmetics and toiletries (Lapczynski et al., 2008). It has a variety of biological properties, such as antioxidant,

antibacterial, anti-biofilm, antifungal, and antitumor (Brehm-Stecher & Johnson, 2003; Chan *et al*., 2016). The presence of α-bulnesene has already been evidenced in other genus of the Lauraceae family. It is a sesquiterpene that inhibits platelet aggregation (Hsu *et al*., 2006). In *Nectandra salicina*, twigs and leaves were analyzed, which presented 0.1% and <0.05% α-bulnesene, respectively (Cicció *et al*., 2009).

The potential for antioxidants in natural products is widely evaluated to discover the mechanisms involved that contribute to a wide variety of biological properties. Compounds that exhibit this activity can protect biological systems from harmful effects caused by the excess formation of reactive oxygen species (Alam *et al*., 2013; López-Alarcón & Denicola, 2013).

The activity of antioxidants in reducing the DPPH• radical that was obtained for oil essential *Ocotea paranaensis* is described in Table No. 2. The DPPH• molecule is characterized as a stable free radical having an unpaired electron, which causes violet staining. When this free radical comes into contact with a substrate (HA) that can donate a hydrogen atom due to oxidoreduction, the formation of a reduced colorless form occurs (Mensor *et al*., 2001; Alam *et al*., 2013). From the absorbance readings obtained for the analyzed sample, the ability to reduce the DPPH• radical was calculated. From preliminary tests, it was found that the IC_{50} of the essential oil would be above 1000 μg/mL, so it was decided to perform the test only at a concentration of 200 μg/mL. Thus, the analyzed sample presented low antioxidant activity against DPPH•, when compared to controls that are tested at the same concentration.

Samples with more polar characteristics are expected to have higher antioxidant activity, as they have more hydroxyl groups. Besides, the structural conformation of compounds that interact with the reaction site also contributes to the development of the reaction *(Brand-Williams et al., 1995; Mensor et al*., 2001). Such arguments justify the results that were obtained, because the sample has a nonpolar character and a larger amount of non-hydroxyl constituents.

Table No. 2
Antioxidant activity of *Ocotea paranaensis* essential oil

Method		Essential oil (200 μg/mL)	Ascorbic acid (200 μg/mL)	BHT (200 μg/mL)	Rutin (200 μg/mL)
		Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
DPPH• scavenging (%)		15.54 ± 0.89[a]	99.42 ± 0.38[c]	95.68 ± 0.25[b]	95.97 ± 0.25[b]
Phosphomolybdenum complex AAR (%)	Related to Ascorbic acid	74.95 ± 1.42[a]	100[b]	-	-
	Related to BHT	175.96 ± 3.33[b]	-	100[a]	-
	Related to Rutin	406.49 ± 7.71[b]	-	-	100[a]

(1) AAR% (Percent relative antioxidant activity), SD (standard deviation); Means followed by equal letters do not differ according to the Tukey test (p<0.05).

However, the antioxidant potential based on the reduction of the phosphomolybdenum complex showed a different profile (Table No. 2). The best result was observed in comparison with the rutin standard (406.49%), followed by BHT (175.96%), which in turn demonstrated that the essential oil has phosphomolybdenum reducing action. The principle of this essay is based on the reduction of molybdenum VI to molybdenum V when exposed to lipophilic or hydrophilic substances with antioxidant properties, which results in a green-colored phosphomolybdenum complex (Prieto *et al*., 1999).

In the evaluation of toxicity against *Artemia salina*, the *Ocotea paranaensis* essential oil presented an LC_{50} of 147.91 μg/mL (Table No. 3), whereas the quinidine control presented an LC_{50} of 116.01 μg/mL. Thus, it can be suggested that the essential oil is moderately bioactive against *Artemia salina*, since the LC_{50} value obtained for the essential oil is between 100 and 500 μg/mL (Amarante *et al*., 2011), which allows correlation with other potential biological activities.

TABLE No. 3
Determination of the LC_{50} of the essential oil of *Ocotea paranaensis* by the bioassay in *Artemia salina*

SAMPLE	MORTALITY *Artemia* test 24h					LC_{50} (µg.mL^{-1})	CI 95% (µg.mL^{-1})
Concentration (µg.mL^{-1})	10	100	250	500	1000		
Essential oil	0	7	25	30	30	147,91	120,063177,66
Methanol	0	0	0	0	0	>1000	-
Quinidine	0	10	30	30	30	116,01	94,39-139,70

LC_{50} = Lethal concentration to 50%, CI 95% = 95% confidence interval

The toxic potential of oils extracted from the genus *Ocotea* can be observed in other species, such as *Ocotea notata*, which presented an LC_{50} of 2.37 µg/mL (Garrett *et al*., 2007), and as observed in *Ocotea nutans*, which presented an LC_{50} of 71.70 µg/mL (Betim *et al*., 2019). Based on the results shown in Table No. 3, we can say that the essential oil from the *Ocotea paranaensis* branches showed haemolytic activity (to potable water and 1% triton respectively: IC_{50} 313.30 µg/mL; IC_{50} 294.62 µg/mL). Saponin was used as a positive control of haemolytic action evaluation, as it produces alterations in the erythrocyte membrane and, consequently, leads to the rupture and release of hemoglobin (Manaargadoo-catin *et al*., 2016).

Table No. 4
Evaluation of the essential oil haemolytic activity of the *Ocotea paranaensis*

Sample	Concentration µg/mL	% Haemolysis			
		Triton 1%		Potable water	
		Mean ± SD	Test Tukey[1]	Mean ± SD	TestTukey[1]
Saponin	100	21,21 ± 1,08	a3 a4	20,50 ± 1,05	a4 a5
	250	69,82 ± 3,35	a8	67,48 ± 3,24	a9
	500	85,53 ± 0,76	a9	82,67 ± 0,74	a10
	750	88,18 ± 3,17	a9	85,22 ± 3,06	a10
	1000	89,24 ± 1,99	a9	86,25 ± 1,91	a10
Essential oil	100	35,74 ± 0,87	a5 a6	34,54 ± 0,84	a6 a7
	250	42,56 ± 0,75	a6 a7	41,13 ± 0,72	a7 a8
	500	66,83 ± 2,56	a8	64,59 ± 2,47	a9
	750	92,7 ± 1,07	a9	89,59 ± 1,03	a10 a11
	1000	113,35 ± 5,73	a11	115,2 ± 9,89	a12

(1) Means followed by equal letters do not differ according to the Tukey test (p<0.05)

Within *in vitro* haemolysis of medicinal plants, samples are widely used for preliminary toxicity studies (Pequeno & Soto-Blanco, 2006), which may be related to erythrocytic membrane instability concerning plant extracts (Sharma & Sharma, 2001). As a consequence of elevated hemoglobin in the plasma due to red cell lysis, detrimental effects can be evidenced mainly in the kidneys and cardiovascular system (Carvalho *et al.*, 2007).

Analysis of essential oils from other genus of the Lauraceae family, such as *Cinnamomum zeylanicum*, reports the osmotic fragility of red blood cells when exposed to the oil that is obtained from the barks, possibly due to morphological and structural alterations of erythrocytes (Barros *et al*., 2016).

Regarding antimicrobial activity (Table No. 5), the oil exhibited higher activity against *Staphylococcus aureus* with MIC of 250 µg/mL. It

also showed activity against *Pseudomonas aeruginosa* with a MIC of 500 µg/mL. The bacteriostatic effect was observed for both microorganisms. For *Candida albicans* and *Escherichia coli*, there was no activity until the highest concentration tested, 500 µg/mL.

Table No. 5
Minimum inhibitory concentration (MIC) (µg/mL) of the essential oil of *Ocotea paranaensis*

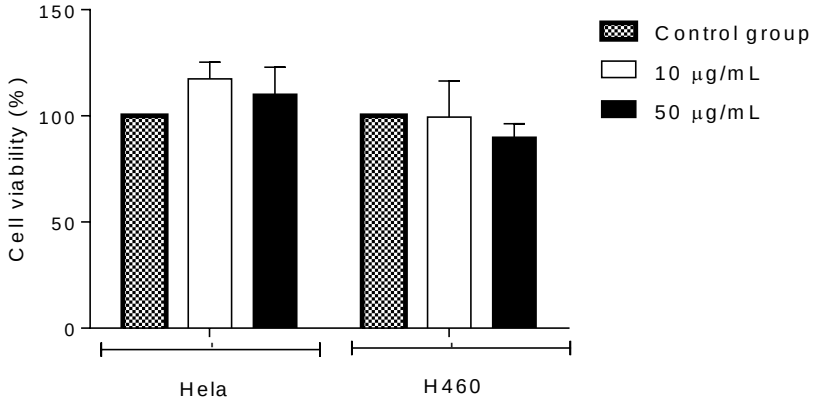
Microorganism	MIC (µg/mL)
Candida albicans	>500
Pseudomonas aeruginosa	500
Staphylococcus aureus	250
Escherichia coli	>500

The composition of *Ocotea paranaensis* oil suggests its antimicrobial potential. Nerolidol, its main constituent, has antibacterial and antifungal properties (Chan *et al*., 2016). Another constituent that also has antimicrobial properties is germacrene-D (Sousa *et al*., 2008; Goren *et al*., 2011).

When compared to other species of the same genus, the essential oil presented higher activity than that found in *Ocotea nutans*, which in turn showed MIC of 1000 µg/mL against *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Betim *et al*., 2019). The antimicrobial properties of other species from genus *Ocotea* have good results for different microorganisms. *Ocotea quixos* demonstrated excellent results with flower and leaf calyx oils against *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Candida albicans* (Chaverri *et al*., 2011). In *Ocotea bofo*, the results obtained from bacterial strains showed a higher sensitivity to *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis*, while *Pseudomonas aeruginosa* was not affected by the oil (Guerrini *et al*., 2006). Essential oils from other genus of the Lauraceae family, such as *Nectandra megapotamica*, have also proved to be active against *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Apel *et al*., 2006).

Given the variety of compounds that make up the essential oil, the mechanism of action in different microorganisms may involve multiple targets in the bacterial cell. The hydrophobicity of the oils makes them permeable to the cell membrane, thereby resulting in the extravasation of the cell contents. Gram-negative bacteria are less sensitive to volatile oils than Gram-positive bacteria (Pintore *et al*., 2002) and may be related to the presence of lipopolysaccharides in the membrane, which would hinder the diffusion of hydrophobic compounds present in volatile oils (Burt, 2004; Apel *et al*., 2006).

Figure No. 2
Antiproliferative activity of *Ocotea paranaensis* essential oil



In order to further investigate the biological activities of this essential oil, the test for anticancer activity was carried out using two different cancer cells. The human lung cancer (H460) and a human uterine carcinoma (HeLa) cell lines were exposed to the essential oil for 72 h at 10 and 50 µg/mL. As shown in figure 2, any cytotoxic effect was observed, even in higher concentrations. Despite some studies proposing that oil concentrations between 10–50 µg/mL can inhibit cell growth, oils present a very low cytotoxic effect (Sylvestre *et al*., 2006; Niksic *et al*., 2019).The *in vitro* cytotoxicity of essential oils is related to the specific components that are present in its composition (Niksic *et al*., 2019). The low cytotoxic effect against H460 cells was reported for *Ocotea gomezii* (LD_{50} of 160 µg/mL for leaves; 119 µg/mL for bark and 414 µg/mL for wood) and *Ocotea morae* (LD_{50} of 353 µg/mL for leaves; 139 µg/mL for bark and 218 µg/mL for wood), which have different compounds in the essential oils of leaves, bark, and wood (Chaverri *et al*., 2011). Moreover, a low cytotoxicity was also reported by the Lauraceae family. The essential oil of *Nectandra leucantha* leaves showed a LD_{50} of 60 µg/mL against HeLa cells (Grecco *et al*., 2015).

Terpenoids in essential oils prevent the proliferation of tumor cells by necrosis or apoptosis induction. Also, they may have antioxidant potential, which reduces oxidative stress that is characteristic of the development of malignant tumors (Raut & Karuppayil, 2014). Germacrene-D, one of the main substances found in *Ocotea paranaensis* branch oil, presents cytotoxicity against leukemia tumor cells (HL-60) and shows lower selectivity concerning healthy cells. When present in mixtures, they are usually associated with observed cytotoxic effects (Silva *et al*., 2013). However, as it is a complex combination of chemical constituents, each substance can modulate and contribute to the effects of other compounds. As a consequence of synergism, the study of isolated substances may not match studies performed with mixtures containing a certain known activity compound (Wright *et al*., 2007).

CONCLUSION

The chemical characterization of the essential oil of *Ocotea paranaensis* branches allowed us to identify a total of 27 compounds (94.82.10%), including *Z*-Nerolidol (19.16%), Germacrene D (12.92%), and α-Bulnesene (8.47%), which corresponds to 40.55% of the compounds found.

Regarding its properties, the analyzed sample showed antioxidant potential, toxicity to brine shrimp, hemolytic and antimicrobial potential. These results direct further studies to explore other activities and the use of this essential oil.

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