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Article

Composition, antioxidant properties, and biological activities of the essential oil extracted from *Ocotea diospyrifolia* (Meisn.) Mez.

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The article has aimed to characterize the essential oil extracted from *Ocotea diospyrifolia* (Meisn.) Mez. leaves, in terms of its chemical composition and antioxidant, hemolytic, and phytotoxic potentials, as well as its toxicity against *Artemia salina*. The major constituents identified by CG-MS were δ-elemene, spathulenol, and β-atlantol. When screened for potential biological activities, the essential oil presented low toxicity against *Artemia salina*, and a capacity of lysing red blood cells. Also, the evaluation of its *in vitro* antioxidant activity, using the phosphomolybdenum method, showed better results when compared to butylhydroxytoluene (BHT) and rutin. In conclusion, the results obtained showed certain *in vitro* toxicity, leading to an interesting target for cytotoxicity evaluations of carcinoma cells.

Keywords: *Ocotea diospyrifolia.* Lauraceae. Spathulenol. Antioxidant. Toxicity.

INTRODUCTION

The essential oils extracted from the *Ocotea* genus have demonstrated potential in the biological and pharmacological activities field. Studies have shown positive results regarding antioxidant, antimicrobial (Bruni *et al*., 2004; Cansian *et al*., 2010; Salleh, Ahmad, 2017), cytotoxic, anti-inflammatory, cardiovascular, molluscicidal, and anti-malarial activities (Salleh, Ahmad, 2017) as well as toxicity against *Artemia salina* (Damasceno *et al*., 2018).

Ocotea diospyrifolia (Meisn.) Mez., a species belonging to this genus and Lauraceae family, is commonly found in Argentina, Paraguay, and Brazil (Marques, 2001). It has a commercial value for the timber industry (Ruschel *et al.,* 2003), and is commonly used for construction (Marques, 2001), similar to the other *Ocotea* species (Duke, Vásquez, 1994). Based on this information, it can be assumed that when processed by the industry, the leaves of the *Ocotea diospyrifolia* might go to waste. Regarding its activities, the larvicide effect of the leaves and stems against ticks has already been reported (Santos *et al*., 2013); as well as the antimicrobial activity of the leaves (Weber *et al*., 2018).

Thus, this article's objective consisted in characterizing the chemical composition of the essential oil, extracted from *Ocotea diospyrifolia* (Meisn.) Mez. leaves, and evaluate its antioxidant, hemolytic, and phytotoxic potential, as well as its toxicity against *Artemia salina*. To our knowledge, this is the first study concerning the essential oil of the *Ocotea diospyrifolia*.

MATERIAL AND METHODS

Plant material

Leaves of the *Ocotea diospyrifolia* (Meisn.) Mez. were collected in Curitiba, Brazil (25° 26' S, 49° 14' W), during winter (July). A voucher specimen was deposited in the herbarium, from the Municipal Botanical Museum of Curitiba, under the registration number MBM 385265. The material collected was dried at room temperature, ground, and carried away for oil extraction.

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Essential oil extraction and characterization

The essential oil was extracted via hydrodistillation, using a Clevenger apparatus, based on the technique described in the Farmacopeia Brasileira (2010). The process lasted six hours, and it was performed with 600g of dried and ground leaves, and 3.5L of distilled water.

Determination of the volatile composition

The oil was further submitted to a GC-MS analysis in a Shimadzu CGMS QP2010 Plus equipment, using the RTX-5MS fused capillary column (30 m x 0.25 mm x 0.25 µm film thickness), with helium (1.0 mL/min) as the carrier gas. The split flow was adjusted to provide a 20:1 ratio. The injector and detector temperatures were adjusted to 250 °C. The programmed oven temperature was 60–280 °C at 5 °C/min.; the electron impact mass spectrometry (EIMS): electron energy was 70 eV; the ion source temperature and the connection parts were at adjusted at 180 °C. Individual components were identified by comparing their arithmetic indices (AI) to a homologous series of C9–C20, and the mass spectra, with those of authentic compounds as stated in Adams' libraries of mass spectral data (Adams, 1995), and a computer data bank, using Wiley 275, NIST 21, NIST 107 (NIST 10).

Antioxidant activity - 2,2-diphenyl-1-picrylhyrazyl (DPPH•) scavenging assay

The technique used was adapted from the literature (Mensor *et al*., 2001). Methanolic solutions, from the sample and controls (ascorbic acid, BHT and rutin), were prepared at 200 µg/mL. For the essential oil, it was necessary to add Tween 80® (200 µg/mL). In a 96-well microplate, 60 µL of a methanolic DPPH• solution (0.3 mM) was added to 140 µL of the sample/control. The dilution solvent was used as a negative control, and a blank was made to discount the sample's color. The mixture was protected from light for 30 minutes and read using a microplate spectrophotometer at 540 nm.

Antioxidant activity—Formation of the phosphomolybdenum complex assay

Sample and positive controls (ascorbic acid, BHT and rutin) were prepared as described for the DDPH• scavenging assay. The antioxidant activity was measured as published previously (Prieto, Pineda, Aguilar, 1999). Three milliliters of the reagent (sulfuric acid 0.6 M, sodium phosphate 28 mM, ammonium molibdate 4 mM) was added to a tube, followed by 0.3 mL of the sample/control. A blank was prepared with 0.3 mL of a dilution solvent and 3 mL of the reagent. The resultant solution was maintained at 95 °C in a water bath, for 90 minutes, and read in spectrophotometer at 690 nm. The results were expressed as relative antioxidant activity (RAA%), compared to each positive control.

Antioxidant activity—β-carotene bleaching assay

This technique was followed as previously described (Rufino *et al*., 2006), with some adaptations. The controls (ascorbic acid, BHT, and rutin) and essential oil were diluted to 200 µg/mL in methanol; for the essential oil solution, Tween 80® (1:1 w/v), was added. A β-carotene/linoleic acid system was prepared as an emulsion, mixing 40 µL of linoleic acid, 530 mg of Tween 80®, 50 µL of a β-carotene solution (20 mg/mL in chloroform), and 20 mL of oxygenated distilled water. The antioxidant activity was determined in a microplate, where 150 µL of the emulsion was added to 20 µL of the sample/controls. The blank was prepared using 20 µL of methanol instead of the sample. The microplate was read in a spectrophotometer (470 nm), incubated at 50 °C, and read again every 15 minutes for 120 minutes. The protection capacity (%) was calculated as follows:

$$Protection(\%) = \left[1 - \frac{(Abs\,initial_{sample} - Abs\,final_{sample})}{(Abs\,inicial_{blank} - Abs\,final_{blank})}\right] x100$$

where: Abs initial $_{sample}$=sample absorbance at 0 minutes; Abs final $_{sample}$=sample absorbance at 120 minutes; Abs initial $_{blank}$=blank absorbance at 0 minutes; Abs final $_{blank}$=blank absorbance at 120 minutes.

The absorbance decay profile was evaluated at two points of the kinetic study (at 30 and 82.5 minutes), where the F1 and F2 values were calculated (Duarte-Almeida *et al*., 2006):

$$F1 = \frac{tg\ sample\ 30}{tg\ blank\ 30} \qquad F2 = \frac{tg\ sample\ 82.5}{tg\ blank\ 82.5}$$

where, tg sample 30/82.5=sample tangent, when x=30/82.5 minutes; tg blank 30=blank tangent, when x=30/82.5 minutes.

Lethality against brine shrimp

The evaluation of *in vitro* toxicity against *Artemia salina* was carried out as described previously (Meyer *et al.,* 1982). The essential oil was diluted in methanol, and

Tween 80® was added to facilitate the solubilization (same concentration as the sample). Quinidine sulfate was used as a positive control. Both the sample and the control were prepared in the following concentrations: 10, 50, 100, 250, 500, 750, and 1000 µg/mL. Eggs of *Artemia salina* were hatched in an artificial marine salt solution (38g/L; pH 8–9; constant aeration; 48 hours). Ten nauplii were put in contact with the sample/control solution for 24 hours, and the number of alive and dead *Artemia salina* were counted. The results were analyzed, using the Probit statistical method, to calculate the LC_{50}.

Hemolytic activity

The methodology used for evaluating hemolytic activity was adapted from the literature (Banerjee *et al*., 2008). The essential oil was diluted to the concentrations of 100, 250, 500, 750 and 1000 µg/mL in phosphate buffered saline (PBS) of pH 7.4; 1% methanol (v/v), and 0.5% dimethylsulfoxide (v/v) were also added. Saponine was used as the standard and prepared in the same concentrations using 1% methanol in PBS. Three milliliters of defibrinated sheep blood was centrifuged in a falcon tube for 5 minutes (3000rpm). The supernatant was discarded, and the remaining tube material was washed with 5ml of PBS and centrifuged at 3000 rpm for 5 minutes. The procedure was repeated thrice, or until the supernatant was clean. The resultant red blood cells were diluted to 2% (w/v) in PBS. For the hemolytic activity measurement, 200 µL of sample/standard was added to an Eppendorf, followed by the 200 µL suspension of red blood cells. A blank was used to discount the sample's color (200 L sample/positive control + 200 µL PBS), and the negative control was composed of 200 µL RBC + 200 µL of solvent. All the samples and the standard were measured based on two positive controls; potable water and a triton (10 µg/mL in PBS). The Eppendorf tubes were incubated at 37 °C for 3 hours and centrifuged for 5 minutes at 3000 rpm. Around 150 µL of the supernatant was transferred to a microplate, which was read in a spectrophotometer at 540 nm.

The results were expressed as a percentage of hemolysis, assuming 100% of the activity for absorbance was obtained utilizing the triton and potable water.

Phytotoxic activity

For the allelopathic activity (adapted from Silva, Overbeck, Soares, 2014), the essential oil was diluted in methanol and Tween 80® (1:1 w/w with the sample) to 0.001, 0.01, 0.1 and 1% (w/v). A filter paper was inserted in a Gerbox, with 5 mL of distilled water and 20 *Lactuca sativa* cv. Grand Rapids seeds (Feltrin®), were disposed in four quadrants. Two filter papers were placed in the lid, with 3mL of the sample. Distilled water was used as a negative control, and methanol + Tween 80® was used to guarantee that the solvent had no effect. The Gerboxes were wrapped in a plastic film and placed in an incubator (20 ± 5 °C) for 7 days. For each concentration, two Gerboxes were prepared, for germination and growth evaluation. The germination samples were analyzed daily; the germinated seeds were counted and taken from the box. Growth was observed only on the seventh day, when the radicle and hypocotyl were measured using a ruler.

Statistical analyses

The results obtained, with the antioxidant and hemolytic activities, were submitted to a variance (ANOVA), and mean comparison (Tukey) analyses. The results from the phytotoxic assay were analyzed utilizing the Scott-Knot test; the lethality against *Artemia salina* was evaluated using the Probit method, to calculate the LC_{50}. All the statistical analyses were conducted with a significance level of 95%.

RESULTS AND DISCUSSION

The extraction of the essential oil from the leaves of *Ocotea diospyrifolia* (Meisn.) Mez. had a yield of 0.21% (v/w), and the chemical composition has been provided in Table I.

A total of 26 compounds were identified (86.53% of the sample). The essential oil was predominantly composed of sesquiterpenes (85.7%), and mostly hydrocarbons (55.91%). Oxygenated sesquiterpenes comprised 29.79% of the sample. A similar profile was found for the leaves of the *Ocotea brenesii* Standl., 54.3% of which was composed of hydrocarbon sesquiterpenes and 29.9% of oxygenated sesquiterpenes (Chaverri, Cicció, 2005).

The three major constituents that were discovered were the δ-elemene (25.93%), spathulenol (11.4%), and β-atlantol (15.35%). In previous studies, the δ-elemene demonstrated containing antitumor activity against cervical (Wang *et al*., 2006) and colorectal carcinomas (Xie *et al*., 2009). It is interesting to notice that its isomer, β-elemene, is also present in a considerable amount (9.49%) and together they make up 35% of the essential oil. The β isomer was also evaluated regarding its anti-cancer properties. Studies showed that the β-elemene inhibited the metastasis of mammalian carcinoma cells

TABLE I - Composition of the essential oil of *Ocotea diospyrifolia* (Meisn.) Mez. leaves extracted via hydrodistillation

AI	Literature AI	Component	[%]	Classification
928	932	α-Pinene	0.2	Bicyclic monoterpene
967	974	β-Pinene	0.37	Bicyclic monoterpene
1019	1024	Limonene	0.26	Bicyclic monoterpene
1330	1335	**δ-Elemene**	**25.93**	Monocyclic sesquiterpene
1360	1374	Isoledene	1.03	Bicyclic sesquiterpene
1363	1373	α-Ylangene	0.49	Bicyclic sesquiterpene
1368	1374	α-Copaene	1.00	Bicyclic sesquiterpene
1381	1389	β-Elemene	9.49	Monocyclic sesquiterpene
1407	1408	(*Z*)-Caryophylenne	1.08	Bicyclic sesquiterpene
1417	1430	β-Copaene	0.26	Bicyclic sesquiterpene
1421	1434	γ-Elemene	2.86	Monocyclic sesquiterpene
1427	1439	Aromadendrene	0.54	Tricyclic sesquiterpene
1440	1452	α-Humulene	0.92	Monocyclic sesquiterpene
1463	1458	allo-Aromadendrene	1.92	Tricyclic sesquiterpene
1466	1478	γ-Muurolene	1.70	Bicyclic sesquiterpene
1471	-	NI	0.53	-
1482	1500	Bicyclogermacrene	2.19	Bicyclic sesquiterpene
1486	1500	α-Muurolene	1.26	Bicyclic sesquiterpene
1497	1495	γ-amorphene	0.39	Bicyclic sesquiterpene
1508	1511	δ-Amorphene	0.49	Bicyclic sesquiterpene
1526	1546	Elemol	0.54	Monocyclic sesquiterpene alcohol
1540	1559	Germacrene B	4.36	Monocyclic sesquiterpene
1553	1577	**Spathulenol**	**11.4**	Bicyclic sesquiterpene alcohol
1564	-	NI	0.67	-
1569	-	NI	0.75	-
1573	-	NI	0.99	-
1596	-	NI	0.49	-
1602	1608	**β-Atlantol**	**15.35**	Monocyclic sesquiterpene alcohol
1611	-	NI	1.00	-
1620	-	NI	0.72	-
1626	1639	allo-Aromadendrene epoxide	0.84	Oxygenated tetracyclic sesquiterpene
1631	-	NI	0.59	-
1635	1668	*trans*-Calamenen-10-ol	0.54	Bicyclic sesquiterpene alcohol
1677	1668	14-hydroxy-9-epi-(E)-Caryophyllene	1.12	Bicyclic sesquiterpene alcohol
1753	-	NI	1.55	-
1807	-	NI	0.43	-
Total			92.45	-

AI: Arithmetical index; NI: Not identified

in vitro (Zhang *et al*., 2013), and had a synergistic effect along with etoposide against lung carcinoma (Zhang *et al*., 2011).

Spathulenol, the second major substance, was seen to be capable in increasing the susceptibility of multi-resistant lymphoma to chemotherapies, by inhibiting the efflux pump of cells (Martins *et al*., 2010).

When compared to other plants from the Ocotea genus, the essential oil from the leaves of *Ocotea diospyrifolia* exhibited a different composition. According to the literature, from the ten Ocotea species evaluated (*O. floribunda*, *O. holdrigdeana*, *O. meziana*, *O. sinuata*, *O. tonduzii*, *O. valeriana*, *O. veraguensis*, *O. whitei*, *Ocotea* new species "los llanos", *Ocotea* new species "small leaf"), nine substances were found to be commonly present in the essential oils (Takaku, Haber, Setzer, 2007). Among these compounds, only four of them were found in the oil analyzed in this study: α and β-pinene, β-elemene, and α-humuleno.

Regarding the antioxidant activity, the results from the DPPH• scavenging and the phosphomolybdenum complex formation assay have been summarized in Table II.

In the DPPH• scavenging assay, the essential oil inhibition capacity (10.22%) was far below the values found for ascorbic acid, BHT, and rutin. On the other hand, the phosphomolybdenum complex formation demonstrated better antioxidant activity for the oil, when compared to BHT and rutin. The variance in these results might be explained by the differences in the mechanisms, evaluated by each method, as well as the difference in the solubility of the molecules, during the reaction. Both assays were performed in a polar media, but, while the DPPH• scavenging occured at room temperature, in the phosphomolybdenum test, the samples were submitted to a water bath at 90 °C. The high temperature in the second assay could contribute to the solubilization of more apolar compounds.

A similar response profile was observed by Damasceno *et al*. (2018) for the essential oil of *Ocotea bicolor* leaves. This species presented low activity in the DPPH assay (IC_{50} > 500 μg/mL), but an antioxidant capacity comparable to the ascorbic acid (102.5%) in the phosphomolybdenum method (Damasceno *et al*., 2018). In contrast, the essential oil from *Ocotea quixos* and *Ocotea bofo* calyces inhibited 52% and 64.4% of the DPPH oxidation, respectively (Bruni *et al*., 2003; Guerrini *et al*., 2006).

In the β-carotene bleaching assay, at the end of 120 minutes of reaction, the essential oil was unable to prevent the lipid peroxidation. Also, based on the values calculated for F1 and F2, the sample didn't interfere with the speed of the reaction. F1 indicates the sample's ability to interfere in the formation of peroxide radicals, either by accelerating it or slowing it down, and F2 concerns the second stage of oxidation.

When tested for the antioxidant capacity, using the same mechanism, *Ocotea bofo* could inhibit 75.8% of the β-carotene degradation (Guerrini *et al*., 2006).

The essential oil tested can be characterized as a complex mixture, composed of substances with various functional groups and polarity. For samples such as this, the varying results are expected, depending on the test employed (Sacchetti *et al*., 2005) Sesquiterpenes hydrocarbons, the major class found in the essential oil tested, has low antioxidant activity in the TBARS and ABAP assays (Ruberto, Baratta, 2000). This fact might explain the results obtained in the DPPH and β-carotene bleaching experiments. However, the results from the phosphomolybdenum formation complex indicate that the antioxidant activity should be further explored, using different methodologies.

The preliminary *in vitro* toxicity test showed a low activity against *Artemia salina* (Table II), according to the stratification system by Amarante *et al*. (2011). The toxicity against brine shrimp has already been described for other *Ocotea* species: *O. praetermissa*-IC_{50}: 31.6 μg/mL, *O. endresiana*-IC_{50}: 6.9 μg/mL (Agius *et al*., 2007), *O. notate*-IC_{50}: 2.3 7μg/mL (Garrett *et al*., 2010), *O. bicolor*-IC_{50}: 40.10 μg/mL (Damasceno *et al*., 2018).

Although the essential oil toxicity against *Artemia salina* is considered low, it indicates the presence of molecules capable of interacting with biological systems, causing some type of alteration. Thus, toxic results against

TABLE II - Results from the DPPH• scavenging, phosphomolybdenum complex formations, and brine shrimp lethality assays

DPPH• scavenging (%)	Phosphomolybdenum complex			Brine shrimp lethality (IC_{50} – μg/mL)
	AAR ascorbic acid (%)	AAR BHT (%)	AAR rutin (%)	
10.22 ± 0.40	89.17 ± 5.74	134.10 ± 8.63	284.62 ± 18.30	602.81 (424.09 – 744.06)

Note: (AAR) Antioxidant activity related (to)

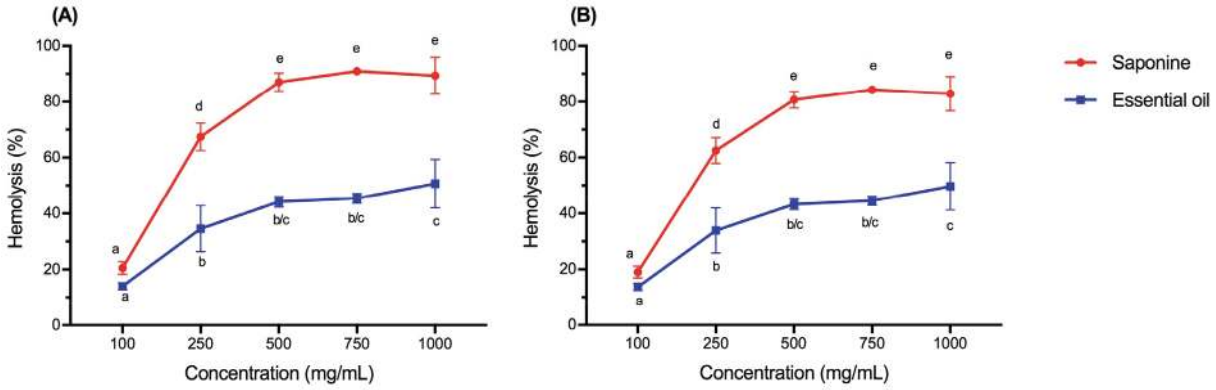

FIGURE 1 - Results obtained in the hemolysis assay when compared to (A) triton 0.1% (w/v) and (B) potable water. Note: Results followed by the same letter, in the same graph, belong to the same statistical group.

Artemia salina can be used as a screening process for biologically active samples (Amarante *et al*., 2011). A study performed using plants from the Asteraceae family showed a possible correlation between the *Artemia salina* results and anti-*Trypanosoma cruzi* activity (Zani *et al*. 1995).

Therefore, the ability to interact with red blood cells membranes, causing hemolysis, is a cytotoxicity indicator (Sharma, Sharma, 2001). Hence, the hemolysis assay results obtained (Figure 1) corroborate with the *Artemia salina* lethality test. The hemolysis caused by the oil reached a maximum of 50%, probably due to the limitation of its solubility in the solution.

TABLE III - Results from the phytotoxicity in *Lactuca sativa* var. and Grand Rapids TBR seeds

Sample	Concentration (% m/v)	Hypocotyl (mm)
		Average ± SD (mm)
Control water	-	29.87 ± 7.43 [b]
Control Tween 80®	-	29.90 ± 9.84 [b]
Essential oil	0.001	32.15 ± 9.73 [b]
	0.01	30.35 ± 10.32 [b]
	0.1	29.75 ± 2.86 [b]
	1	16.00 ± 6.21 [a]

Note: Results followed by the same letter belong to the same statistical group.

The phytotoxicity results showed no interference with the germination of *Lactuca sativa* seeds and the growth of its radicle. The hypocotyl growth was affected by only the tested 1% (w/v) concentration (Table III). The phytotoxicity of *Ocotea quixos* was also evaluated by Rolli *et al*. (2014), and its essential oils delayed the germination of *Solanum lycopersicum* seeds and inhibited its root growth.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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