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Article

Chemical composition, antioxidant and biological activity of *Ocotea bicolor* Vattimo-Gil (LAURACEAE) essential oil

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The essential oil composition of the *Ocotea bicolor*, native plant of Brazil, was studied for the first time. The essential oil of the leaf was obtained by hydrodistillation and analyzed by GC/MS. The analytical procedure revealed a predominance of sesquiterpenes, δ-cadinene (7.39%), β-sesquiphellandrene (6.67%), β-elemene (5.41%) and α-cadinol (5,23%). The essential oil was submitted to brine shrimp toxicity evaluation, antioxidant and antibacterial tests. The antioxidant activity by the formation of phosphomolybdenum complex method presented positive results. The minimum inhibitory concentration (MIC) values were higher than 1000 μg/mL for the microorganisms tested. Toxicity activity revealed LC50 results of 40.10 (μg/mL), being toxic to the organisms in this study.

Keywords: *Ocotea bicolor*/Oil essential/chemical composition. Lauraceae. Antioxidant activity. Biological activity.

INTRODUCTION

The Lauraceae family is composed of approximately 2.750 species, distributed in 54 genera. It is prevalent in tropical regions of America and Asia, along with having a large number of species in Australia and Madagascar, with an insignificant number of species in Africa. The species are predominantly trees and mostly aromatic in nature (Van Der Werff, Ritcher, 1996; Madriñán, 2004).

Ocotea bicolor Vattimo-Gil, native to Brazil is locally known as cinnamon-shit or cinnamon-stinking, justified by the unpleasant odour from their wood (Brotto, Cervi, Santos, 2013). The studies on this species are still incipients, with no information about their chemical characteristics. However, many chemical investigations were carried out for other *Ocotea* species, reporting benzylisoquinoline alkaloids (Takaku, Haber, Setzer, 2007), lignans and neolignans (Silva, Braz-Filho, Gottlieb, 1989) and essential oils composed of monoterpenes, sesquiterpenes and phenylpropanoids (Bruni *et al.*, 2004).

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The *Ocotea* genus is the most studied in the Lauraceae family, was characterized mainly by the presence of antimicrobial, antifungal (Bruni *et al.*, 2004) and anti-inflammatory activity (Chao *et al.*, 2005). In this study, the unpublished description of the chemical composition, the evaluation of the antioxidant, bactericidal and toxic potential of the essential oil of *Ocotea bicolor* Vattimo-Gil leaves are carried out.

MATERIAL AND METHODS

Plant material

The botanical material collection was carried out in the Capão do CIFLOMA region at the Botanical Campus of the Federal University of Paraná, Curitiba, Paraná state, Brazil, in August, 2015. All the plant material was obtained from the same specimen, in a sterile phase. Witness material was identified by the taxonomist Marcelo Leandro Brotto, and deposited in Herbarium of the Department of Botany of the Federal University of Paraná, under registry nº 88118.

This study has been authorized by the Brazilian Institute of the Environment (IBAMA) to access samples of the genetic patrimony for scientific research purposes

without potential economic use, included in Process 02001.001165 / 2013-47.

Essential oil obtaining

The essential oil was obtained from 100g of dried leaves, ground in a knife mil, hydrodistilled for 6 hours using modified Clevenger type apparatus. The essential oil was stored in a sealed amber glass at -18 °C until the analysis. The yield calculation was performed in milliliters (mL/%) of essential oil per 100 g of the drug (Farmacopeia Brasileira, 2010).

Identification of essential oil constituents

The characterization of the chemical constituents present in the essential oil of the *Ocotea bicolor* Vattimo-Gil leaves was carried out at the laboratory of the Chemistry, Department of the Federal University of Paraná, using gas chromatography, composed of a gas chromatograph coupled to a spectrometer Shimadzu® CG-EM-QP 2010 Plus mass equipped with Rtx-5MS capillary column (30 mx 0.25 mm x 0.25 μm). Injector in splitless mode at 250 °C, interface and source of ions at 300 °C. The mass window analyzed was between m/z 40 and m/z 350, using He as drag gas. Injection ramp for analysis with injector temperature at 250º C, column pressure of 20 psi, starting with a temperature of 50 ºC for 5 minutes rising to 200 ºC at a rate of 5 ºC/min. The chemical components of the oil were identified comparing their mass spectra with the reference spectra, and comparing their Kovat indices with those described in the literature (Adams, 2007).

DPPH

The sequestering potential of Radical DPPH (2,2-diphenyl-1-picrylhydrazyl) was determined using ascorbic acid and rutin as standard, by adapted technique (Mensor *et al.*, 2001; Santos *et al.*, 2007; Nascimento *et al.*, 2011). Methanolic solutions of the sample were prepared from a stock solution (1 mg /mL) at concentrations ranging from 2 μ /mL to 500 μg / mL. 96-well microplates with round bottoms were used, with 71 μL of the sample and 29 μL of the DPPH solution (0.3 mM). The specific blank of each sample was determined using 71 μL of the sample and 29μL of methanol, and the negative control 71 μL of methanol and 29 μL of DPPH.

Absorbance readings were taken after thirty minutes of incubation under light in the Multiskan FC spectrophotometer, Thermo Scientific® at wavelength 518 nm. The percentage of inhibition was obtained through the equation: % Inhibition = 100 - [(Absorbance of sample - white absorbance)/absorbance of standard] X 100. From the percentages of inhibition of DPPH, by linear regression the IC50 was calculated, that is, the concentration required to exert 50% of the antioxidant activity. The IC50 results were compared according to Tukey's statistical method ($p < 0.05$).

Formation of the Phosphomolybdenum Complex method

The antioxidant activity through the phosphomolybdenum complex reduction method was performed using the standard solutions of ascorbic acid and rutin, that were prepared at the concentration of 200 μg / mL in methanol and 0.5% DMSO, as well as the samples (Prieto, Pineda, Aguilar, 1999). Aliquots of 0.3 mL were added to 3 mL of the phosphomolybdenum reagent (0.1 M tribasic sodium phosphate (28 mL), 0.03 M ammonium tetrahydrate molybdate solution (12 mL), 3 M sulfuric acid (20 mL) and water until complete 100 mL). The tubes were closed and brought to the thermostated bath at 95 ºC for 90 min. After cooling, the absorbances were obtained in 96-well microplates with round bottoms by reading in the Multiskan FC spectrophotometer, Thermo Scientific® at wavelength 695 nm. The antioxidant capacity of the samples was expressed in relative antioxidant activity (AAR%), in relation to the standards, using the equation: AAR% = [(Sample Absorbance - Absorbance of White)/(Absorbance of Standard - Absorbance of White)] X 100 The variance of the obtained results was evaluated by the ANOVA test and the difference between the means verified by the test (t) by Scott and Knott ($p < 0.05$).

Minimum inhibitory concentration (MIC)

The antimicrobial activity was determined in vitro, with modifications from the original method, using the broth microdilution method to determine the minimum inhibitory concentration (MIC) (CLSI, 2012).

The sample was solubilized in 0.5% of Polysorbate 80 and tested in concentrations between 1000 and 7.8 μg / mL with the following microorganisms: *Escherichia coli* (ATC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATC 25923), *Enterobacter aerogenes* (ATCC 13048), *Klebsiella pneumoniae* (ATCC 700603), *Staphylococcus epidermidis* (ATCC 12228) and *Salmonella typhimurium* (ATCC 14028). The bacterial suspensions were prepared in saline solution at a concentration of 1.0 x 108 CFU / mL, which corresponds to the 0.5 scale of Mac Farland. 10 μLof the bacterial strain was inoculated resulting in a final concentration of 104 CF

/mL. The negative control of the inhibitory activity of the diluents, ethanol and DMSO, were performed adding 100 µL of 10% ethanol solution and 2% DMSO in 100 µL of MHB and 10 µL of the bacterial inocula. For the sterility control, 100 µL of MHB and 100 µL of the extract and fraction were used. The positive control was prepared with 100 µL of MHB and 5 µL of the bacterial inocula. The microplates were closed and maintained in bacteriological oven at 35 ºC for a period of 16 to 20 h. After incubation, was added 20 µl of aqueous 2,3,5-triphenyltetrazolyl chloride (TTC) solution at 0.5%, and the microplates were reincubated for 3 h at 35 °C. Then, the results reading was taken for the results. Wells with bacterial growth showed red coloration. The minimum inhibitory concentration was considered from the well with no color.

Brine shrimp lethality assay

The toxicity assay employs the larvae of the brine shrimp *Artemia salina* Leach was performed using a saline solution containing 30 g L^{-1} of sea salt (Meyer *et al.*, 1982) prepared with pH adjustment to 9 with $Na_2 CO_3$. This solution was used in the hatching of *Artemia salina* cysts and in the preparation of dilutions. The cysts were placed to hatch in the saline solution for 48 hours, under continuous aeration and exposure to daylight. The temperature was controlled between 27 and 30 °C and the pH between 8–9. In the first hour of the process the recipient was kept under illumination (20 W). After hatching of the cysts, 10 larvae of *Artemia salina* were transferred to tubes containing saline solution and added with the following samples: Essential oil diluted in 0,5% of Polysorbate 80 and saline solution tested at concentrations of 10, 100 and 1000 µg/mL, followed by a positive control prepared with saline solution and sodium dodecylsulfate (SDS), and negative control with saline solution and Polysorbate 80. Whole assay was performed in triplicate. The tubes were incubated in oven (27-30 °C) for 24 hours, for later counting of nauplii. The results were submitted to statistical treatment using the PROBIT method, which provided LC50 values (Lethal concentration to 50% of individuals) with 95% reliability. The degree of toxicity was classified as: low toxicity: LC50> 500 µg/mL; moderate toxicity: LC50 between 100 µg/mL to 500 µg/mL and high toxicity: LC50 <100 µg/mL (Amarante *et al.*, 2011).

RESULTS AND DISCUSSION

The analysis of the essential oil of *O. bicolor* leaves allowed the recognition of the sesquiterpenes predominance (48.21%), followed by alcohols, ketones and aldehydes (31.97%) and in lesser amount, aromatic sesquiterpenes (0.56%) and diterpenes (1.14%). It was possible to identify 38 constituents totaling 81.88%, whose retention times, retention indices and percentages are summarized in Table I.

TABLE I - Chemical compounds present in the essential oil of *Ocotea bicolor* determined by CG / MS

Compounds	TR	IRc	IRt	(%)
Sesquiterpenes				
α -cubebene	22.523	1354	1345	1.78
α -Ylangene	23.696	1381	1373	2.48
β -bourbonene	24.100	1390	1387	0.92
β-elemene	24.425	1397	1389	5.41
E- caryophyllene	25.615	1426	1417	2.86
β-copaene	26.006	1435	1430	0.16
Trans-muurola-3,5-diene	26.921	1457	1451	1.24
α-humulene	27.073	1460	1454	2.90
Allo-aromadendrene	27.380	1468	1458	0.53
Dauca-5,8-diene	27.901	1480	1471	0.78
Germacrene D	28.053	1484	1484	2.21
α-cubenene	28.238	1488	1478	2.48
Bicyclogermacrene	28.902	1504	1500	1.09
α-murolene	29.034	1507	1500	1.92
E,E-α-farnesene	29.257	1513	1505	0.66
β-sesquiphellandrene	29.657	1523	1521	6.67

TABLE I - Chemical compounds present in the essential oil of *Ocotea bicolor* determined by CG / MS (cont.)

Compounds	TR	IRc	IRt	(%)
δ-cadinene	30.042	1532	1522	7.39
γ-cuprenene	30.391	1541	1532	4.87
α-cadinene	30.573	1545	1537	0.51
Alcohols, ketones and aldehydes				
Spathulenol	31.613	1571	1577	1.90
Viridiflorol	32.136	1584	1592	1.53
Thujopsan-2-α-ol	32.513	1593	1586	1.28
Globulol	32.836	1602	1590	1.26
Epi-cedrol	33.383	1616	1618	2.48
Isolongifolan-7-α-ol	33.778	1626	1618	1.32
Cis-cadin-4-en-7-ol	34.230	1638	1635	0.65
Methyl Z-jasmonate	34.620	1648	1648	2.66
Epi-α-muurolol	34.806	1653	1640	4.50
α-muurolol	34.975	1657	1644	1.81
α-cadinol	35.330	1667	1652	5.23
Epi-β-Bisabolol	35.558	1673	1670	3.39
Trans-Calamenen-10-ol	35.824	1679	1668	0.18
Z-γ-Atlantone	36.729	1703	1694	2.56
Eremofilone	38.198	1743	1734	0.66
Aromatic sesquiterpene				
α-corocalene	34.003	1632	1622	0.56
α-calacorene	30.829	1552	1544	1.35
Diterpenes				
Ent-rosa-5,15-diene	45.150	1939	1933	0.27
Kaurene	48.799	2043	2042	0.87
Total (identified)				81.88
Unidentified				
	25.187	1415	--	0.56
	26.569	1448	--	0.50
	28.451	1493	--	0.36
	28.761	1500	--	4.73
	29.861	1528	--	0.56
	31.047	1557	--	0.49
	31.372	1565	--	4.23
	32.255	1587	--	1.44
	32.969	1605	--	0.53
	33.262	1613	--	1.79
	34.323	1640	--	0.22
	36.285	1692	--	2.20
	38.693	1757	--	1.09
Total (Unidentified)				18,7

TR = retention time (minutes), IRt = retention index (Adams, 2007), IRc = retention index calculated, % = percentage of component.

The major chemical constituents in order of abundance were: δ-cadinene (7.39%), β-sesquiphellandrene (6.67%), β-elemene (5.41%), sequiterpenes and α-cadinol alcohol (5, 23%) (Figure 1). δ-cadinene is among the major components of essential oils of other species of *O. genus*, such as *O. gomezii*, *O. morae* (Chaverri, Díaz, Cicció, 2011), *O. bracteosa* (Takaku, Haber, Setzer, 2007), *O. brenesii* (Chaverri, Cicció, 2005), *O. valerianae* and a new one called *Ocotea "small leaf"* (Takaku, Haber, Setzer, 2007). All the mentioned species presented predominance of sesquiterpenos in the composition of their essential oils.

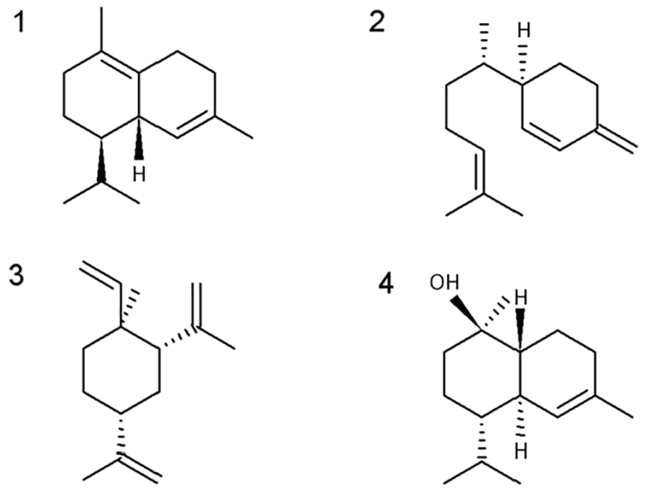

FIGURE 1 - Majority components of *Ocotea bicolor* essential oil. (**1**) δ-cadinene, (**2**) β-sesquiphellandrene, (**3**) β- elemene, (**4**) α-cadinol.

Coming from the cyclization of nerolidine pyrophosphate, δ-cadinene presents a cadinane skeleton, which the precursor is the farnesyl pyrophosphate (Ruiz-Reyes, Suarez, 2015). Among the activities reported in the literature for δ-cadinene can be mentioned the antitumor activity (Wright *et al.*, 2007), antimicrobial activity (Skaltsa *et al.*, 2003) and anti-cariogenic activity (Muroi, Kubo, 1993).

The yield of 3.2% of the *O. bicolor* essential oil was considerably higher when compared to other species of the same genus: *O. odoriferous* 1.2% (Cansian *et al.*, 2010), *O. ceanothifolia* 0.11%, *O. leucoxylon* 0.13%, *O. minor* 0.11% (Yamaguchi, 2011), *O. nigrescens*, 0.23% and *O. splendens*, 0.35% (Alcântara, 2009). Environmental factors to which the plants species are subjected to, such as light, temperature and humidity, have significant influence on the emission of volatile compounds, altering their yield (Dudareva, Pichersky, Gershenzon, 2004).

The antioxidant activity results are described in Table II. The *O. bicolor* essential oil presented better results than rutin standard (102.5%), deducing that this species has considerable amount of phosphomolybdenum reductive action. This assay is based on the reduction of molybdenum VI in molybdenum V, in the presence of certain antioxidant substances, resulting in a green complex formation. Furthermore, it has the advantage of evaluating the antioxidant capacity of both lipophilic and hydrophilic components (Prieto, Pineda, Aguilar, 1999).

However, the antioxidant potential of the oil, determined based on the sequestering activity of DPPH·, demonstrated a different profile. This test measures the capacity that a specific substance has in sequester the DPPH·,which is capable of accepting a hydrogen radical to become a stable diamagnetic molecule diphenyl-picrylhydrazine. Thus, the simultaneous change in coloration from violet to pale yellow occurs with consequent disappearance of the absorption, can be monitored by decreasing of the absorbance. From the obtained results, the percentage of DPPH is determined for the remaining oil in the reaction medium (Alves *et al.*, 2010; Molyneux, 2004).

The result of the antioxidant activity from this method demonstrated that concentrations above 500 μg/mL were necessary to reduce 50% of DPPH present in the reaction medium. The discrepancy between the results obtained in both the techniques applied may be associated with the mechanisms involved in this method and the hydro/lipophilicity of the antioxidant substances present in the essential oil.

Studies with *Beilschmiedia* genus (LAURACEAE) demonstrated antioxidant activity in *Beilschmiedia kunstleri*, *Beilschmiedia maingayi* and *Beilschmiedia penangiana*, which present a predominance of sesquiterpenes in the essential oil composition. The DPPH method showed the best result for Beilschmiedia maingayi (IC 50 84.7 μg/mL) (Salleh *et al.*, 2016).

TABLE II - Antioxidant activity of *Ocotea bicolor* essential oil obtained by phosphomolybdenum, DPPH

Sample	PHOSPHOMOLYBDENUM		DPPH
	AA in relation to rutin (%)	AA in relation to ascorbic acid (%)	IC_{50} (μg/mL)
EO	29.21± 2.01	102.5± 7.07	>500

AA= Antioxidant activity, EO= essential oil. Results are expressed as mean ± standard deviation (n = 3)

However, in another study with *Beilschmiedia madang* also with sesquiterpenes predominance, the antioxidant activity by DPPH method was insignificant with IC50 = 263.9 μg/mL in leaf oil and 212.0 μg/mL for barley oil (Salleh, Ahmad, Yen, 2015).

O. bicolor essential oil showed MIC values >1000 μg/mL for Gram-positive and Gram-negative bacteria tested. Sesquiterpenes as δ-cadinene, α humulene and caryophyllene had their bactericidal activity proven (Muroi, Kubo, 1993; Skaltsa *et al.*, 2003; Sylvestre *et al.*, 2006; Veiga Junior, Pinto, 2002). However, when these substances are tested together, this activity may be reduced by competitive inhibition among them or enhanced because of the synergistic effect.

The brine shrimp bioassay correlates with cytotoxicity on 9Kb and 9PS cells (leukemia), confirming that it is a useful tool for the preliminary determination of antitumor activity (Meyer *et al.*, 1982; Mclaughlin, Rogers, Anderson, 1998). The *O. bicolor* essential oil had positive toxicity in this assay, demonstrating LC50 of 40.10 (μg/mL).

The toxic potential of essential oils from species of Lauraceae to *Artemia salina* is well documented in the literature. Studies with *Licaria canella* and *Aniba canelilla* showed LC50 of 5.25 (μg/mL) and 68.37 (μg/mL) respectively (Silva *et al.*, 2009), *O. notata*, presented LC50 of 2.37 37 μg/mL (Garret *et al.*, 2007) and *Beilschmiedia brenesii*, *Cinnamomum paratriplinerve* and *Ocotea endresiana* (LC50 = 9.05, 8.07 and 6.93 μg/mL, respectively) (Agius *et al.*, 2007).

In addition, the β-elemene and α-cadinol present in the essential oil as the major components showed pronounced antitumor activity in a variety of cell lines (Tao *et al.*, 2006; Wang *et al.*, 2005; He *et al.*, 1997; Sylvestre *et al.*, 2006).

The mixture of other components found in this oil, such as α-humulene and caryophyllene are also reported in the literature as antitumor agents, which may increase the toxicity of the essential oil by synergism (Sylvestre *et al.*, 2006; Veiga Junior, Pinto, 2002).

Although there is no accentuated bactericidal activity in the *O. bicolor* essential oil leaves, the positivity for the antioxidant activity and pronounced toxicity can be directed towards vivo studies where these characteristics can be tested.

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