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Chemical composition and cytotoxic activity of the essential oil from the leaves of *Casearia lasiophylla*

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Abstract: The essential oil obtained by hydrodistillation from fresh leaves of *Casearia lasiophylla* Eichler, Salicaceae, was analyzed by gas capillary (GC/FID and GC/MS). The cytotoxicity of the leaves essential oil was tested *in vitro* against U251 (glioma), UACC-62 (melanoma), MCF-7 (breast), NC1-ADR/RES (ovarian-resistant), NCI-H460 (lung), PC03 (prostate), OVCAR-3 (ovarian), HT-29 (colon) and K562 (leukemia) human cancer cells and against VERO (no cancer cell). The yield of oil was 0.02%. Fifty two compounds were identified, representing 87.1% of the total of the oil. The main components were identified as germacrene D (18.6%), β-caryophyllene (14.7%), δ-cadinene (6.2%), and α-cadinol (5.4%). The oil exhibited antiproliferative activity against all cell lines (TGI<100 μg/mL), with exception of NCI-H460 cell line (TGI 191.31 μg/mL). The highest activity was observed against UACC-62 (TGI 7.30 μg/mL), and K562 (TGI 7.56 μg/mL) cell lines. The observed activity could be related to high content of germacrene D and β-caryophyllene, compounds known as cytotoxic.

Introduction

Casearia species (Salicaceae) have been used in folk medicine in Brazil against snakebite and for the treatment of skin diseases and ulcers. Several pharmacological activities have been demonstrated to species of this genus, including anti-inflammatory, anti-ulcer and antitumor (Santos et al., 2010; Vieira Jr et al., 2009; Flausino Jr et al., 2009). Previous chemical analyses of *Casearia* have demonstrated the occurrence of clerodane diterpenoids with cytotoxicity against several human cancer cell lines (Santos et al., 2010; Vieira Jr et al., 2009; Silva et al., 2009; Chen et al., 2008; Sertiè et al., 2000).

The essential oils of *Casearia* spp analyzed to date are rich in sesquiterpenes (De Morais et al., 1997; Esteves et al., 2005; Schneider et al., 2006; Sousa et al., 2007; Silva et al., 2008; Stefanello et al., 2010) and one sample showed cytotoxic activity (Silva et al., 2008). *Casearia lasiophylla* Eichler, known as "guaçatunga-graúda" is a tree native from Brazil for which no chemical or pharmacological evaluation has previously been reported. It has been used in folk medicine to treat wounds (Pedroso et al., 2007; Keller & Tressens, 2007). This prompted us to investigate the chemical and biological properties of this plant.

As part of a research project focusing on phytochemical investigation of Brazilian medicinal plants searching for new bioactivity of natural products, in this study, we report the chemical analysis of the essential oil of *C. lasiophylla* by capillary GC/FID and GC/MS, with the identification of fifty-two compounds. Moreover, the cytotoxic activity of this essential oil against human cancer cell lines is also reported.

Materials and Methods

Plant material

The leaves of *Casearia lasiophylla* Eichler,

Salicaceae, were collected in Colombo, Paraná State, Brazil. A voucher specimen (Santos 1250 UPCB) is deposited in the herbarium of Universidade Federal do Paraná.

Isolation and chemical analysis of the essential oil

Fresh leaves were subjected to hydrodistillation for 2 h. The oil was recovered with diethyl ether and dried over anhydrous sodium sulfate. The solvent was removed under vacuum, providing colorless oil with yield (w/w) of 0.02%. The oil was maintained under refrigeration before analysis. The GC analysis was performed on a Shimadzu GC-17A gas chromatograph with FID detector, in a split injector mode. A Durabond-DB5 capillary column (30 m x 0.25 mm, 0.25 µm film thickness) was operated at 60 ºC for 3 min, and then programmed from 60-220 ºC at 5 ºC/min, after which it was kept isothermal at 220 ºC for 5 min. The carrier gas was helium (1 mL/min) and the injector temperature was 250 ºC. The relative percentage of individual components is based on the peak areas obtained by electronic integration without FID response factor correction. The GC/EIMS (70 eV) analysis was performed on a Varian Saturn 2000 GC/ MS spectrometer equipped with a CP-Sil-8CB capillary column (30 m x 0.25 mm, 0.25 µm film thickness) under the same conditions described above. The essential oil components were identified by comparison of their retention indices relative to n-alkanes (Kovat's index) and mass spectra with those found in the literature (Adams, 2007) and stored on the spectrometer database (NIST 1998). In addition, the compounds limonene, eugenol, methyl salicilate, β-caryophyllene, α-humulene and spathulenol were confirmed by comparison with standards.

Antiproliferative assay

Cell lines: U251 (glioma, CNS), UACC-62 (melanoma), MCF-7 (mama), NCI-ADR/RES (ovarian-resistent), NCI-H460 (lung, no small cells), PC-3 (prostate), OVCAR-3 (ovarian), HT-29 (colon), K562 (leukemia) and VERO cell lines, from American Type Culture Collection (ATCC), were used. The assay was done as described previously (Skehan et al., 1990). Briefly, the cells were distributed in 96-well plates (100 µL cells/well) and exposed to various concentrations of essential oil (0.25, 2.5, 25.0 and 250.0 µg/mL) in DMSO (0.1%) at 37 °C, with 5% of CO_2, for 48 h. The final concentration of DMSO did not affect the cell viability. A 50% trichloroacetic acid solution was added and after incubation for 30 min at 4 °C, the cells were washed and dried. Cell proliferation was determined by spectrophotometric quantification (at 540 nm) of the cellular protein content using sulforhodamine B. The experiments were carried, at least, in triplicate and the concentration necessary to total growth inhibition (TGI) was calculated in µg/mL. Doxorubicin was used as positive control.

Statistical analysis

Triplicates from at least three separate experiments were employed in each of the antiproliferative assays. An exploratory data analysis was performed initially to determine the most appropriate statistical test; the assumptions of equality of variances and normal distribution of errors were also checked. The relative potency was based on the drug concentration that inhibited cell growth by 50% (IC50) [calculated from $[(T\text{-}T0)/(C\text{-}T0)]\times100=50$, which is the drug concentration resulting in a 50% reduction in the net cell number of drug-treated cultures relative to the increase in control cultures during the drug incubation period]. The data were analyzed using ANOVA and the F-test used to determine any difference among the groups. When significant differences were detected, pair wise comparisons were made between all the groups using Tukey's method to adjust for multiple comparisons. Statistical software GraphPad prism 2.01 (GraphPad Software, San Diego, CA) was used to perform the analyses. The level of significance was set at 5%.

Results and Discussion

The oil obtained by hydrodistillation from fresh leaves of *C. lasiophylla* was characterized by high content of cyclic sesquiterpenes (86.4%), mainly hydrocarbons (59.9%). Monoterpenes (limonene), aromatic compounds (mesitylene, methyl salicilate, eugenol), and aliphatic compounds (isopentyl acetate) were found as minor constituents. Altogether sixty-four components were observed, of which fifty two were identified, representing 87.1% of the total of the oil (Table 1). The main constituents were germacrene D (18.6%), *E*-caryophyllene (14.7%), δ-cadinene (6.2%), and α-cadinol (5.4%). The predominance of sesquiterpene hydrocarbons in the leaves essential oil of *Casearia* species has been previously observed (Stefanello et al., 2010; Silva et al., 2008; Sousa et al., 2007; Schneider et al. 2006; Esteves et al., 2005; De Morais et al., 1997). In particular, *E*-caryophyllene was found in all samples as a main component (more than 10%).

The oil exhibited antiproliferative activity for almost all cell lines evaluated, with TGI varying from 7.30 to 55.26 µg/mL, with exception of NCI-H460 cell line, for which the TGI was 191.31 µg/mL. The most significant activity was observed against UACC-62

(TGI 7.30 µg/mL, melanoma) and K562 (TGI 7.56 µg/mL, leukemia). For VERO cell (non-cancer cell-line) the TGI estimated was 65.80 mg/mL, about 8.5 times larger than the TGI of the melanoma and leukemia cell lines, for which the essential oil showed the major bioactivity (Table 2). The coefficients of variation obtained in these analyses were below to 5%. Among the identified sesquiterpenes, germacrene D, *E*-caryophyllene and α-cadinol are recognized as cytotoxic and, could explain the observed activity (He et al., 1997; Silva et al., 2008; Loizzo et al., 2008; Palazzo et al., 2009). The oil of *Casearia sylvestris* also has high content of *E*-caryophyllene (18.1%) and showed similar cytotoxic activity agains HT-29 cell line (Silva et al., 2008).

Table 1. Chemical composition of *Casearia lasiophylla* leaf essential oil.

Component [a]	RI [b]	Percentage (%)
Isopentyl acetate	879	0.3
NI (*m/z* 91, 105, 120)	892	Tr
NI (*m/z* 91, 105, 120)	957	Tr
Mesitylene	994	0.1
Limonene [c]	1027	0.1
Methyl salicilate [c]	1192	0.1
NI (*m/z* 73, 149)	1299	Tr
NI (*m/z* 77, 93, 105, 121)	1329	Tr
δ-Elemene	1332	0.9
α-Cubebene	1344	0.1
Eugenol [c]	1353	0.1
α-Copaene	1373	0.4
β-Bourbonene	1380	0.1
β-Cubebene	1385	0.2
β-Elemene	1387	1.5
E-Caryophyllene [c]	1417	14.7
β-Copaene	1427	2.2
α-Guaiene	1432	2.6
α-Humulene [c]	1452	2.8
allo-Aromadendrene	1457	0.4
cis-Muurola-4(14), 5-diene	1470	Tr
γ-Gurjunene	1473	1.1
Germacrene D	1479	18.6
β-Selinene	1484	0.7
cis-β-Guaiene	1488	0.8
Bicyclogermacrene	1493	1.8
α-Muurolene	1496	1.0
trans-β-Guaiene	1499	1.0
γ-Cadinene	1511	0.5
δ-Cadinene	1516	6.2
Zonarene	1521	0.4
trans-Cadina-1,4-diene	1530	0.3
α-Cadinene	1534	0.2
Germacrene B	1556	2.4
Maaliol	1567	0.6
Spathulenol [c]	1575	1.6
Caryophyllene oxide	1578	1.7
Globulol	1585	Tr
NI (*m/z* 135)	1587	Tr
Salvial-4(14)-en-1-one	1590	0.1
Carotol	1592	2.5
Cubeban-11-ol	1594	0.8
Guaiol	1600	0.3
5-*epi*-7-*epi*-α-Eudesmol	1605	0.6
Humulene epoxide II	1607	0.1
NI (*m/z* 69, 161, 179)	1613	Tr
NI (*m/z* 79, 91, 105, 123)	1616	Tr
Junenol	1619	1.6
10-*epi*-γ-Eudesmol	1622	0.8
1-*epi*-Cubenol	1626	2.8
NI (*m/z* 91, 105, 119)	1628	Tr
cis-Cadin-4-en-7-ol	1633	0.6
epi-α-Cadinol	1641	1.8
epi-α-Muurolol	1644	4.8
α-Muurolol	1647	0.1
α-Cadinol	1655	5.4
Selin-11-en-4α-ol	1659	Tr
NI (*m/z* 123)	1672	Tr
Khusilol	1680	Tr
2,3-Dihydrofarnesol	1689	Tr
Eudesm-7(11)-en-4-ol	1696	0.3
NI (*m/z* 71, 81, 95, 123)	2109	1.0
NI (*m/z* 73, 221, 355, 429)	2262	7.6
NI (*m/z* 73, 221, 355, 429)	2576	4.3
Total identified (%)		87.1

NI: not identified; tr: trace (<0.05%); [a]Tentative identification based on computer matching of the mass spectra of peaks with NIST 98 library and published data (Adams, 2007), besides comparison of retention indices on published data (Adams, 2007). [b]RI Retention index relative to *n*-alkanes on a DB-5 column. [c]Identification based on retention time of authentic compounds on the DB-5 column.

Despite the cytotoxic activity of the essential oil of *C. lasyophylla* is lower than that of the positive control (doxorubicin), the present results reveal the antitumor potential of this species. The presence of significant amounts of unidentified compounds in the oil that could be contributing to bioactivity points

out the need of phytochemical studies in this species, aiming at the isolation and identification of its secondary metabolites.

Table 2. Antiproliferative activity of *Casearia lasiophylla* leaves essential oil.

Cell lines	TGI (μg/mL)*	
	Essential oil	Doxorubicin
U251	27.39	0.12
UACC-62	7.30	<0.025
MCF-7	40.72	0.25
NCI-ADR/RES	43.32	2.26
NCI-H460	191.31	1.17
PC-3	36.97	0.40
OVCAR-3	29.49	0.28
HT-29	55.26	3.04
K562	7.56	0.15
VERO	65.80	6.12

*TGI: Total growth inhibition - concentration that inhibited cell growth by 100%. The coefficients of variation obtained in these analyses were below to 5%. Cell lines: U251 (glioma); UACC-62 (melanoma); MCF7 (breast); NCI-ADR/RES (ovarian-resistent); NCI-H460 (lung); PC03 (prostate); OVCAR-3 (ovarian); HT-29 (colon); K562 (leukemia); VERO (no cancer cell).

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