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Artigo

VOLATILE COMPOUNDS OF *Nectandra salicina* (LAURACEAE) FROM COSTA RICA AND THEIR CYTOTOXIC ACTIVITY ON CELL LINES

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The chemical composition of the volatiles of *Nectandra salicina* growing wild in Costa Rica was determined by capillary GC/FID and GC/MS. Thirty-seven and forty-two compounds were identified in the leaf and branch oils respectively corresponding to about 92.6 and 86.2% of the total amount of the oils. The major components of the leaf oil were: atractylone (14.6%), viridiflorene (10.1%), α-pinene (9.4%), β-caryophyllene (7.2%), α-humulene (7.0%), δ-cadinene (6.1%), β-pinene (6.0%) and germacrene D (5.8%). The major components of the branch oil were: atractylone (21.1%), germacrene D (10.7%), viridiflorene (7.9%) and 7-epi-α-selinene (5.0%). When the oils were tested on different cell lines, all the LD_{50} values were higher than 150 μg/mL, with values very similar for the leaf and branch oils. Low toxicity could be explained by antagonistic effects among the main compounds present in the oils.

Keywords: *Nectandra salicina*; volatiles; cytotoxicity.

INTRODUCTION

Nectandra is a New World genus constituted by approximately 100 to 150 species, ranging from Florida to Argentina, with the majority of species present in South America.[1] In Costa Rica, this genus is represented by about 20 species. It belongs to the Lauraceae family, which is present with abundance and diversity of species in the Cloud Forests of Costa Rica, together with plants of the Leguminosae (Fabaceae) family.[2] The majority of the species produce relatively small fruits which are of great ecological importance for the sustenance of several mammals such as monkeys and kinkajous, and birds such as quetzals and toucans.[2,3] The Lauraceae family is recognized by the simple, alternate, stiff and aromatic elliptic to obovate leaves, and by the fruits often borne in a cup. Worldwide, this family has a considerable economic value because it is used as a source of timber for construction and furniture, as food (*Persea americana* Mill., Avocado), to obtain flavors for food industry, drinks and perfumery and medicines [*Cinnamomum camphora* (L.) J. Presl., Camphor Laurel].

Nectandra salicina C. K. Allen is a tree (5-10 m tall) from evergreen forests, which is found in both the Caribbean and Pacific slopes of Costa Rica, from about 600 to 1,700 m of elevation. It is commonly known as *ira*, *canelo* and *aguacatillo*[4] and is recognized by its small lustrous narrowly elliptic and acuminated leaves, with the tertiary veins, usually prominent on both surfaces. Inflorescences are small, few-flowered with pink red rachises and puberulent little flowers. Fruits are ellipsoid to globose and borne in shallow cups.[1]

Many members of the *Nectandra* genus have been chemically investigated and they are mainly characterized by the occurrence of alkaloids,[5-10] lignans,[11-13] neolignans,[14-17] tetrahydrofuranoid lignans,[18] norlignans,[19] dehydrodieugenols[20] and γ-lactones.[21,22]

The composition of several essential oils from *N. angustifolia* (syn. *N. falcifolia*),[23-25] *N. coriacea,*[26] *N. elaiophora*[27] and *N. rigida*[28] has been published. However, to the best of our knowledge, only one previous report on the composition of the leaf oil from *N. salicina* appears in the scientific literature.[29]

EXPERIMENTAL

Plant material

Leaves and branches of *Nectandra salicina* C. K. Allen, Lauraceae, growing wild in Costa Rica were collected in June 2001, in Fraijanes, Miramar, Province of Puntarenas, Costa Rica. A voucher specimen was deposited at the Herbarium of the University of Costa Rica (USJ 76991).

Oil isolation

Fresh leaves (1.0 kg) and chipped fresh branches (1.5 kg) were subjected to hydrodistillation for 3 h using a modified Clevenger-type apparatus. The distilled light yellow oils were collected and dried over anhydrous sodium sulfate and stored in a freezer (0-10 °C). Leaf and branch essential oil yields were 0.1% (v/w) and 0.2% (v/w), respectively.

General analytical procedures

GC/FID analysis

The oils of *N. salicina* were analyzed by GC/FID using a Shimadzu GC-17 gas chromatograph. The data were obtained on a 5% phenyl- 95% methylpolysiloxane fused silica capillary column (30 m x 0.25 mm; film thickness 0.25 μm), Heliflex (Alltech) AT-5, with a Shimadzu Class-VP, version 4.3 software. Operating conditions were: carrier gas N_2, flow 1.0 mL/min; oven temperature program: 60-220 °C at 3 °C/min, 220 °C (10 min); sample injection port temperature 250 °C; detector temperature 275 °C; split 1:50.

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GC/MS analysis

The analyses by GC/MS were performed using a Shimadzu GC-17A gas chromatograph coupled with GCMS-QP5050 apparatus and CLASS 5000 software with Wiley 139 and NIST computer databases. The data were obtained on a 5% phenyl- 95% methylpolysiloxane fused silica capillary column (30 m x 0.25 mm; film thickness 0.25 μm). Operating conditions were: carrier gas He, flow 1.0 mL/min; oven temperature program: 60-220 °C at 3 °C/min; sample injection port temperature 250 °C; detector temperature 260 °C; ionization voltage: 70 eV; ionization current 60 μA; scanning speed 0.5 s over 38-400 amu range; split 1:70.

Identification

Identification of the oils components was performed using the arithmetic retention indices (RI) on DB-5 type column,[30] and by comparison of their mass spectra with those published in the literature[31] or those of our own database. Integration of the total chromatogram, expressed as area percent, has been used to obtain quantitative compositional data.

Cell culture

Mouse macrophage J774, human hepatoma HepG2, human leukemic K562 and mouse myoblastic C2C12 cell lines were obtained from American Type Culture Collection (ATCC). Cells were maintained in Dulbecco essential medium supplemented with 10% fetal bovine serum, 2 mmol/L of glutamine, 100 IU/mL of penicillin and amphotericin B in a 37 °C humidified incubator under an atmosphere of 7% CO_2 in air. For the experiments, adherent cells were cultured in 96-well plates to confluence (15,000 cells/well) and allowed to adhere overnight. Non-adherent cells were also plated at 15,000/well.

Cytotoxicity assay

Various concentrations of essential oils, previously dissolved in 95% ethanol, were added to the plates in 100 μL of fresh medium and incubated for 48 h. After that, 10 μL of [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) (0.5 mg/mL) was added to the culture and, after 2 h at 37 °C, medium was carefully removed and 95% ethanol was added to the wells with the purpose of dissolving formazan crystals. Absorbances were read at 570 nm and results were expressed as viability percentages, using samples incubated with 95% ethanol dissolved in culture medium as 100% viability values. LD_{50} values were calculated with SlideWrite® Plus 6.1 (Advanced Graphics Software, Inc., Carlsbad, CA).

RESULTS AND DISCUSSION

N. salicina leaf and branch oil constituents are listed in Table 1. As it can be observed, 37 components were identified from the leaf, representing 92.6% of the essential oil. Monoterpene hydrocarbons (17.4%), sesquiterpene hydrocarbons (51.1%) and oxygenated sesquiterpenes (22.4%) were the main constituents of the oil, and contained the monoterpenes α-pinene (9.4%), β-pinene (6.0%); the sesquiterpenes viridiflorene (10.1%), β-caryophyllene (7.2%), α-humulene (7.0%), δ-cadinene (6.1%) and germacrene D (5.8%), and the oxygenated sesquiterpene atractylone (14.6%), as a main constituents. From the branch, 42 compounds were identified, representing 86.2% of the oil. This oil is constituted mainly of sesquiterpene hydrocarbons (40.7%) and oxygenated sesquiterpenes (36.2%). The major components of the branch oil were atractylone (21.1%), germacrene D (10.7%), viridiflorene (7.9%) and 7-epi-α-selinene (5.0%).

N. salicina from Costa Rica produced oils which are terpenoid in

Table 1. Percentage composition of the leaf and branch volatiles of *Nectandra salicina*

Compound[a]	RI[b]	Leaf	Branch	Identification Method[d]
(3*Z*)-hexenol	850	0.4		1, 2
(2*E*)-hexenol	854	1.2		1, 2
α-pinene	932	9.4	3.2	1, 2, 3
camphene	944	0.8	1.2	1, 2
β-pinene	974	6.0	1.1	1, 2, 3
myrcene	987	0.4	0.2	1, 2
α-phellandrene	1005	0.3	0.9	1, 2
δ-3-carene	1006		0.3	1, 2
ρ-cymene	1020	0.1	0.3	1, 2
limonene	1025	0.5	0.5	1, 2, 3
bornyl acetate	1284		0.7	1, 2
iso-dihydrocarveol acetate	1324		0.9	1, 2
α-cubebene	1345		1.0	1, 2
α-ylangene	1372		t	1, 2
α-copaene	1374	1.7	1.4	1, 2
β-elemene	1388	1.0	0.7	1, 2
β-longipinene	1405	1.2		1, 2
β-caryophyllene	1416	7.2	2.7	1, 2, 3
β-gurjunene	1433	0.8		1, 2
γ-elemene	1434		0.4	1, 2
α-humulene	1449	7.0	2.5	1, 2
dehydroaromadendrene	1462	0.3	0.3	1, 2
drima-7,9(4)-diene	1469	t[e]	0.4	1, 2
α-amorphene	1481	4.5		1, 2
germacrene D	1485	5.8	10.7	1, 2
β-selinene	1490		4.6	1, 2
viridiflorene	1497	10.1	7.9	1, 2
α-muurolene	1502	0.5	0.4	1, 2
α-bulnesene	1509	t	0.1	1, 2
γ-cadinene	1512	2.9		1, 2, 3
7-epi-α-selinene	1518	1.3	5.0	1, 2
δ-cadinene	1523	6.1	2.3	1, 2
zonarene	1530	0.2	0.2	1, 2
(Z)-nerolidol	1533		0.4	1, 2
α-cadinene	1535	0.5	0.1	1, 2
elemol	1547		0.8	1, 2
spathulenol	1575	1.1	2.3	1, 2
trans-sesquisabinene hydrate	1579	0.9		1, 2
himachalene epoxide	1581		1.2	1, 2
guaiol	1595		0.6	1, 2
humulene epoxide II	1606	0.3	0.7	1, 2
helifolen-12-al D	1617		1.1	1, 2

Table 1. continuing

Compound[a]	RI[b]	Leaf	Branch	Identification Method[d]
1,10-di-epi-cubenol	1617	0.2		1, 2
1-epi-cubenol	1625	0.3	0.8	1, 2
γ-eudesmol	1632		t	1, 2
τ-cadinol	1637	3.2	0.6	1, 2
τ-muurolol	1640		1.7	1, 2
atractylone	1657	14.6	21.1	1, 2
(Z)-α-santalol	1676	0.2	0.7	1, 2
elemol acetate	1679	0.2		1, 2, 3
isobicyclogermacrenal	1729	1.4	4.2	1, 2
Monoterpene hydrocarbons		17.5	7.7	
Oxigenated monoterpenes			1.6	
Sesquiterpene hydrocarbons		51.1	40.7	
Oxigenated sesquiterpenes		22.4	36.2	
Others		1.6		

[a]Compounds listed in order of elution from 5% phenyl- 95% methylpolysiloxane column. [b]RI = Arithmetic retention index (experimental) on 5% phenyl- 95% methylpolysiloxane column on reference to n-alkanes. t = Traces (<0.05%). [d]Method: 1 = Arithmetic retention indices on 5% phenyl- 95% methylpolysiloxane column; 2 = MS spectra; 3 = standard.

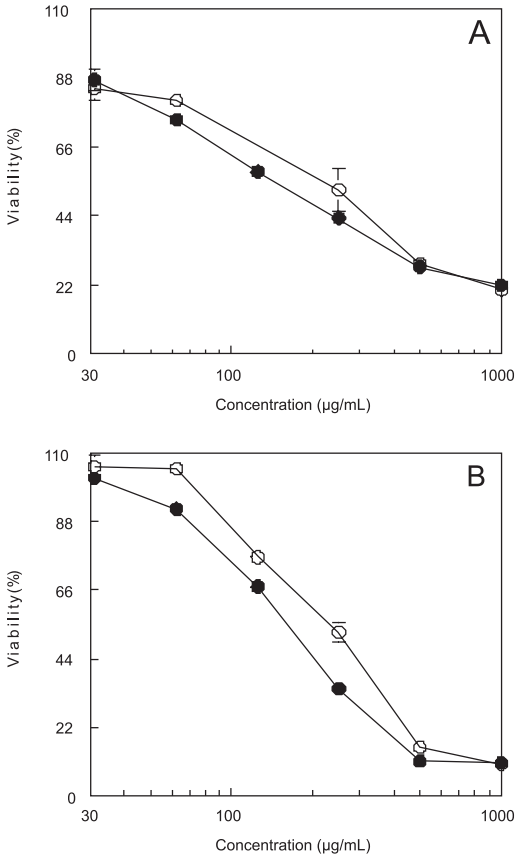

Figure 1. *Cytotoxic activity of N. salicina oils on tumor cell lines. A. Leukemic cell line K562. B. Hepatocarcinoma cell line HepG2. Cells were treated for 48 h with different oil concentrations dissolved in ethanol. • Leaf oil; ○ branch oil. Results are presented as mean ± SE of one representative experiment performed in triplicate*

nature, as well as the oil from leaves of *N. rigida* from Brazil, which contains α- and β-phellandrenes (72.8%)[28] and the oil from the leaf of *N. angustifolia* from Argentina, which contains *p*-menth-1(7),8-diene (25.2%) and terpinolene (20.9%), as main constituents.[25] The major component of both leaf and branch oils of *N. salicina* from Costa Rica was the oxygenated sesquiterpene atractylone (14.6 and 21.1% respectively). In the leaf oil, lesser amounts of the monoterpenes α-pinene (9.4%) and β-pinene (6.0%); and the sesquiterpenes: viridiflorene (10.1%), β-caryophyllene (7.2%), α-humulene (7.0%), δ-cadinene (6.1%) and germacrene D (5.8%) are also present. In the branch oil, together with atractylone, we also found sesquiterpenes germacrene D (10.7%), viridiflorene (7.9%) and 7-epi-α-selinene (5.0%).

A previous report on the leaf volatile composition of *N. salicina*[29] (from the Species Collection at South Coast Research and Experiment Station, UC, Riverside, USA), had indicated that the main constituents were indene (30.1%) and the furanocoumarin methoxsalen (24.5%). In our study, which characterizes the leaf oil of the plants growing in Costa Rica, the composition differs both qualitative and quantitatively from that previous report. We were unable to find any evidence of the presence of indene or methoxsalen in the leaf or branch volatiles of this wild tree. On the other hand, these results could be identifying a different chemotype of *N. salicina*, rich in atractylone, or could be reflecting edaphic and climate factors (ecotypes), since plants were not grown in the same environments.

When both volatiles were tested on four different cell lines: leukemic and hepatoma cells and two non-tumor cells (macrophages and myoblasts), low toxicity was observed. There was a little variation in the LD_{50} values on tumor cells, which were around 175 μg/mL for the branch oil whereas for the leaf oil, values were over 230 μg/mL (Figure 1). Both volatiles gave almost identical LD_{50} values on non-tumor cells C2C12 and J774 (from 125 to 200 μg/mL) (Figure 2).

Almost no cytotoxic effect was previously observed at the concentration of 100 μg/mL with *Nectandra membranacea* on the hepatoma cell line HepG2,[32] the same cell line tested in our study. Some studies showed that leaf oils from *Ocotea veraguensis*, *O. whitei* and *Persea americana*, other members of the Lauraceae family, have no cytotoxic effect on mammary ductal carcinoma (MDA-MB-435) and ovarian adenocarcinoma (OVCAR-5), but they show toxicity at concentrations of 100 μg/mL in other mammary adenocarcinoma (MCF7, MDA-MB-468, MDA-MB-231) and malignant melanoma UACC-257.[33]

In an interesting study, Wright *et al.*[34] previously showed that essential oil components such as β-caryophyllene and α-humulene present antagonistic effects when combined with α-pinene. Since these are some of the main compounds present in *N. salicina* volatiles, it could partially explain the low cytotoxic activities observed in this study.

Also, even though it has been shown that atractylone has antiproliferative activity on leukemia cell lines (HL-60 and P-388) and normal peripheral blood mononuclear cells and is able to trigger apoptosis,[35] its presence seems insufficient to induce high toxicity on the cells tested here. However, different atractylone concentrations present in *N. salicina* leaf and branch volatiles could partially explain differences in LD_{50} values observed between both samples.

This is the first report in the literature that shows the composition and cytotoxic characterization of the essential oils obtained from leaves and branches of *N. salicina*. It seems clear that, as other members of the Lauraceae family, these oils have low toxicity, possibly due to antagonistic effects induced by their main compounds, since some of these compounds (α- and β-pinene, β-caryophyllene, α-humulene), when tested individually, are able to induce high toxicity on tumor cells.[34]

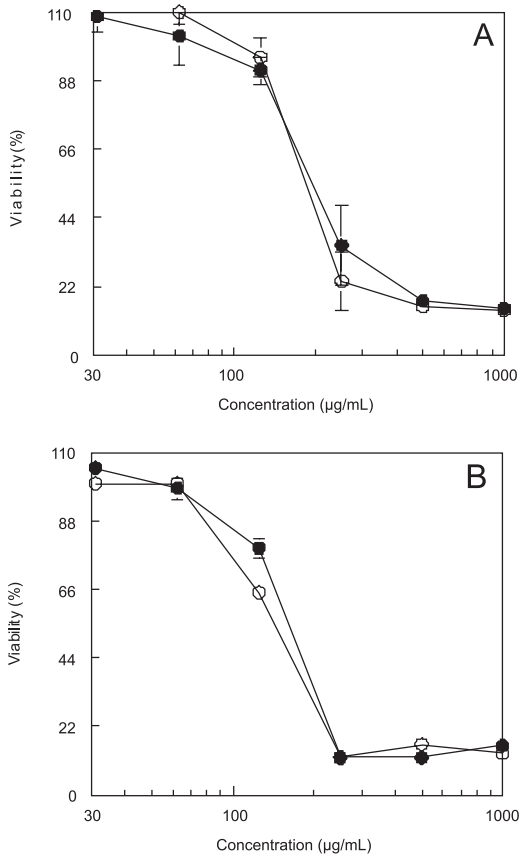

***Figure 2**. Cytotoxic activity of N. salicina oils on non-tumor cell lines. A. Macrophage cell line J774. B. Myoblastic cell line C2C12. Cells were treated for 48 h with different oil concentrations dissolved in ethanol. • Leaf oil; ○ branch oil. Results are presented as mean ± SE of one representative experiment performed in triplicate*

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