Pharmaceutical Biology

ISSN: 1388-0209 (Print) 1744-5116 (Online) Journal homepage: https://www.tandfonline.com/loi/iphb20

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To cite this article: Simone dos S. Grecco, Euder Glendes A. Martins, Natália Girola, Carlos R. de Figueiredo, Alisson L. Matsuo, Marisi G. Soares, Bruno de C. Bertoldo, Patricia Sartorelli & João Henrique G. Lago (2015) Chemical composition and *in vitro* cytotoxic effects of the essential oil from *Nectandra leucantha* leaves, Pharmaceutical Biology, 53:1, 133-137, DOI: 10.3109/13880209.2014.912238

To link to this article: https://doi.org/10.3109/13880209.2014.912238

Pharmaceutical Biology

http://informahealthcare.com/phb
ISSN 1388-0209 print/ISSN 1744-5116 online
Editor-in-Chief: John M. Pezzuto
Pharm Biol, 2015; 53(1): 133–137
 DOI: 10.3109/13880209.2014.912238

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ORIGINAL ARTICLE

Chemical composition and *in vitro* cytotoxic effects of the essential oil from *Nectandra leucantha* leaves

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Abstract

Context: Nectandra (Lauraceae) species have been used in folk medicine as an antidiarrheal, analgesic, antifungal, etc., and have many pharmacological proprieties.
Objective: Investigation of the chemical composition and cytotoxicity of essential oil from *Nectandra leucantha* Nees & Mart. leaves. This is the first study involving *N. leucantha* reported in the literature.
Material and methods: The essential oil of *N. leucantha* leaves was obtained by hydrodistillation. Its chemical composition was determined using a combination of GC/FID, GC/MS, and determination of Kovats index (KI). *In vitro* cytotoxic activity was evaluated against six cancer cell lines – murine melanoma (B16F10-Nex2), human glioblastome (U-87), human cervical carcinoma (HeLa), human colon carcinoma (HCT), human breast adenocarcinoma (MCF7), and human cervical tumor (Siha) as well as against one non-tumorigenic cell line – human foreskin fibroblast (HFF).
Results: Thirty-three compounds were identified primarily sesquiterpenes (81.41%), the main compounds being bicyclogermacrene (28.44%), germacrene A (7.34%), spathulenol (5.82%), and globulol (5.25%). Furthermore, monoterpenes were also found in the analyzed oil (12.84%), predominantly α- and β-pinenes (6.59 and 4.57%, respectively). The crude essential oil displayed significant cytotoxic activity against B16F10-Nex2 (IC_{50} 33 ± 1 μg/mL) and U87 (IC_{50} 75.95 ± 0.03 μg/mL) and HeLa (IC_{50} 60 ± 12 μg/mL) cell lines. The main identified compound, bicyclogermacrene, displayed IC_{50} ranging from 3.1 ± 0.2 to 21 ± 6 μg/mL.
Discussion and conclusion: The results indicate that the crude oils from leaves of *N. leucantha* displayed cytotoxic activity being bicyclogermacrene, the main compound identified in the crude oil responsible, at least in part, for this potential.

Keywords

Bicyclogermacrene, cancer cell lines, non-tumorigenic cell line, sesquiterpenes

History

Received 11 December 2013
Revised 26 February 2014
Accepted 2 April 2014
Published online 22 October 2014

Introduction

Essential oils from plant species have usually been used as antimicrobial, analgesic, sedative, anti-inflammatory, spasmolytic, and anesthesic remedies (Bakkali et al., 2008) and are frequently composed by lipophilic compounds. Due this characteristic, these compounds display the ability to pass through the cell wall and cytoplasmic membrane leading the cell to apoptosis (Sikkema et al., 1994), which could explain the ability of crude oils to act as cytotoxic compounds. In view of the ability of essential oils compounds to present several biological activities and to cross the cell wall, the chemical investigation of volatile components from plant species became very relevant, especially those which could be used as antineoplasic agents, which could inhibit the growth and spread of malignant cells. As an example, the essential oil from leaves of *Guatteria friesiana* Erkens & Maas (Annonaceae) showed *in vitro* and *in vivo* cytotoxic activity due to the presence of eudesmol derivatives. The compounds were able to reduce cell proliferation and to induce hepatocellular carcinoma cell to death by caspase-mediated apoptosis pathways (Bomfim et al., 2013).

Essential oils from Lauraceae species present also an important economic role, in cookery, papermaking, carpentry, construction, and chemical industry. Additionally, several species of this family are used in folk medicine, mainly due to their biological properties (Marques, 2001). As an example, leaves of *Sassafras albidum* (Nutt.) Ness show analgesic, anti-inflammatory, and anti-thrombotic potential and is source of safrole, a precursor of several bioactive compounds (Barreiro & Fraga, 1999). Other species, *Ocotea indecora* (Shott) Mez, showed sudorific, anti-rheumatic, and anti-syphilitic activities (Marques, 2001).

Among the genus *Nectandra*, several species have been used in folk medicine as an antidiarrheal, analgesic, antifungal, etc. The chemical composition of several

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Nectandra sp. essential oils showed a great diversity of compounds, but with predominance of terpenes. Monoterpenes α- and β-pinenes as well as limonene were the major compounds identified from *Nectandra angustifolia* (Schrad.) Nees & Mart. (Torres et al., 2011), while C_{15} derivatives valerianol and γ-eudesmol were found from *Nectandra coriacea* (Sw.) Griseb. (Pino et al., 2005). *Nectandra salicina* C.K.Allen oil was composed by several compounds such as α- and β-pinenes, atractylone, viridiflorene, β-caryophyllene, α-humulene, δ-cadinene, and germacrene D (Cicció et al., 2009). Otherwise, the oil from *Nectandra megapotamica* Spreng showed the predominance of α-bisabolol (Romoff et al., 2010). Previous work reported also the presence of cytotoxic activity of volatile metabolites in *N. megapotamica* (Apel et al., 2006), *Nectandra rigida* (Kunth) Nees (Le Quense, et al., 1980), and *N. salicina* (Cicció et al., 2009).

Nectandra leucantha Ness & Mart, known as ''canela-seca'' or ''canela-branca'', is a large tree (5–10 m) found in tropical and subtropical areas of America. In Brazil, this species is specially found in Minas Gerais, Rio de Janeiro, São Paulo, Paraná, and Santa Catarina States (Zanon et al., 2009). As a part of a continuous study aiming the identification of compounds with cytotoxic potential in vegetal species from Brazilian plants (Carvalho et al., 2013), this work reports the chemical composition of the essential oil from the leaves of *N. leucantha*, and its cytotoxic activity against tumor and non-tumorigenic cell lineages, for the first time.

Material and methods

General

GC-FID data were obtained on a Shimadzu GC-2010 gas chromatograph equipped with a FID-detector, using a RtX-5 capillary column (5% phenyl, 95% polydimethylsiloxane, 30 m × 0.25 mm × 0.25 μm film thickness, Restek, Anaheim, CA) and an automatic injector (Shimadzu AOC-20i, Tokyo, Japan). These analyses were performed injecting 1.0 μL of a solution at 1.0 mg/mL of essential oil in CH_2Cl_2 in a split mode (1:30), under the following conditions: injector and detector temperatures of 220 °C and 250 °C, respectively; oven programmed temperature from 60 to 240 °C at 3 °C/min, holding 5 min at 240 °C, and employing helium as the carrier gas (1 mL/min). The percentage compositions of the oil samples were computed from the GC peak areas without using correction for response factors, by internal normalization. The sample was analyzed by GC/MS in a Shimadzu GC-17A chromatograph interfaced with a MS-QP-5050A mass spectrometer. The MS operating conditions were an ionization voltage of 70 eV and an ion source temperature of 230 °C with the same conditions described above. ^1H and ^{13}C NMR spectra were measured at 300 and 75 MHz, respectively, on a Bruker model DPX-300 spectrometer with a sample dissolved in $CDCl_3$ containing 1% of TMS (Aldrich, St. Louis, MO).

Plant material

Leaves of *N. leucantha* were collected in December 2012, at Atlantic Forest region (Parque Ecológico do Pereque, Cubatão/SP). The botanical identification was made by Prof. MSc. Euder Glendes Andrade Martins (USP, São Paulo/SP) and the voucher specimen (EM357) was deposited at Herbarium of CEPEMA, Universidade de São Paulo – SP, Brazil.

Essential oils extraction procedures

The fresh leaves of *N. leucantha* (61 g) were extracted by a steam hydrodistillation in a Clevenger type apparatus for 4 h. The distillate was extracted with CH_2Cl_2 and after solvent evaporation under reduced pressure, yielded 140 mg of crude essential oil (yield 0.23%). Chemical analyses of the obtained crude oil were immediately performed.

Identification of oil components

The volatile compounds were analyzed by GC-FID and GC-MS, and the identification of the individual compounds was performed by comparison of retention indexes (determined relatively to the retention times of a series of *n*-alkanes) and comparison of recorded mass spectra with those available in the database (Adams, 2009).

Chromatographic separation procedures

A part of the crude essential oil (100 mg) from leaves of *N. leucantha* was subjected to column chromatography on silica gel soaked with $AgNO_3$ (15%) and eluted with CH_2Cl_2 and CH_2Cl_2–MeOH (95:5 and 9:1) affording 30 fractions, which were analyzed by FID-GC and pooled in to 13 groups (A–M). This procedure afforded pure bicyclogermacrene at groups D (10 mg) and E (9 mg).

Bicyclogermacrene: ^1H NMR (δ, $CDCl_3$, 300 MHz): 4.83 (dd, $J = 11.2$ and 5.1 Hz, H-1), 4.35 (d, $J = 11.5$ Hz, H-5), 1.66 (s, CH_3-14), 1.48 (s, CH_3-15), 1.09 (s, CH_3-12), 1.03 (s, CH_3-13), 0.62 (ddd, $J = 12.2$, 9.2, and 3.1 Hz, H-7). ^{13}C NMR (δ, $CDCl_3$, 75 MHz): 140.8 (C-4), 128.0 (C-1), 126.5 (C-5), 124.9 (C-10), 41.2 (C-8), 37.2 (C-2), 30.1 (C-6), 29.3 (C-7), 26.9 (C-9), 26.8 (C-12), 26.0 (C-3), 20.9 (C-15), 19.9 (C-11), 16.6 (C-14), 15.4 (C-13). EIMS (70 eV): *m/z* (rel. int.): 204 (33), 189 (12), 161 (50), 147 (32), 133 (30), 121 (100), 105 (67), 93 (84), 79 (50), 67 (34), 55 (60), 41 (90).

Cell lines

The murine melanoma subline B16F10-Nex2 was derived from the original B16F10 cell line obtained from the Ludwig Institute for Cancer Research (São Paulo, Brazil). This subline is characterized by low immunogenicity and moderate virulence. Human glioblastome (U-87), human colon carcinoma (HCT), human breast adenocarcinoma (MCF7), human cervical (Siha), and human foreskin fibroblast (HFF) cell lines were obtained from the Ludwig Institute for Cancer Research. Human cervical carcinoma (HeLa) was acquired from Dr. Hugo Pequeno Monteiro, UNIFESP. Cells were cultured in a humidified atmosphere containing 5% CO_2, at 37 °C, in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (Hepes) (Sigma, St. Louis, MO), 24 mM sodium bicarbonate (Sigma, St. Louis, MO), 40 mg/L gentamycin

(Schering-Plough, São Paulo, Brazil), pH 7.2, and 10% fetal calf serum (Invitrogen, Carlsbad, CA).

In vitro cytotoxic activity

The essential oil obtained from the leaves of *N. leucantha* as well as pure bicyclogermacrene were re-suspended in dimethylsulfoxide (DMSO) at a final concentration of 10 mg/mL, and diluted in RPMI medium supplemented with 10% fetal calf serum. Gradient concentrations of these materials (100–0 µg/mL) were incubated with 1×10^4 cells in a 96-well plate with 5% CO_2 at 37 °C. After 24 h of incubation, the cell viability was assessed using the Cell Proliferation Kit I (MTT) (Sigma, St. Louis, MO), an MTT-based colorimetric assay as previously described (Mosmann, 1983). Readings were made in a plate reader at 570 nm with a reference of 650 nm. All experiments were performed in triplicates using cisplatin (Sigma, St. Louis, MO) and DMSO 1% as positive and negative controls, respectively.

Results and discussion

The crude essential oil was obtained from the fresh leaves of *N. leucantha* by hydrodistillation using a Clevenger apparatus. Its respective yield, calculated based on the fresh weight of the leaves, was 0.23%. The identification of the individual compounds was achieved by interpretation of mass spectra and also by calculation of their respective Kovats indexes, determined relative to the retention times of a series of *n*-alkanes (Adams, 2009).

The chemical investigation of the crude oil showed to be composed by 33 derivatives, corresponding to 92.83% of the total identified volatiles (Table 1). The oils showed to be composed predominantly by sesquiterpenes (81.41%), in which hydrocarbon derivatives were the majority (58.78%). Among the major compounds, the sesquiterpene bicyclogermacrene was predominate, with 28.44%, followed by its isomer germacrene A (7.34%). However, oxygenated derivatives were also detected in great amounts, such as spathulenol (5.82%), globulol (5.25%), and intermediol (4.17%). Hydrocarbon monoterpenes were identified in reduced part in this essential oil (12.62%), being α- and β-pinenes (6.59 and 4.57%) the most abundant derivatives (Figure 1).

The crude oil was fractionated on a silica gel column soaked with $AgNO_3$ in order to isolate the main derivative (Gupta & Dev, 1963; Lago et al., 2002). This procedure afforded 13 groups (A–M), in which groups D and E were composed of pure bicyclogermacrene, their structure was confirmed by analysis of MS data with those reported in the literature (Adams, 2009).

Based on some evidence that essential oils showed *in vitro* cytotoxicity effects (Suffness & Pezzuto, 1991), the obtained crude oil from leaves of *N. leucantha* and purified bicyclogermacrene were evaluated against seven different cell lineages. Dose–response curves were generated and IC_{50} values were calculated for this active oil/compound against a murine melanoma cell line (B16F10-Nex2), a panel of five different human tumor cell lines (U87, HeLa, HCT, MCF7, and Siha) and a non-tumorigenic human cell line (HFF), as shown in Table 2.

Table 1. Chemical composition of essential oil from leaves of *N. leucantha*.

KI[a]	Constituent	Relative amount (%)
939	α-Pinene	6.59
979	β-Pinene	4.57
990	Myrcene	0.34
1029	Limonene	0.63
1037	(*Z*)-β-Ocimene	0.49
1116	exo-Fenchol	0.22
1376	α-Copaene	0.35
1408	β-Isocomene	3.62
1408	(*Z*)-Caryophyllene	0.37
1414	β-Funebrene	1.11
1452	α-Humulene	0.35
1460	allo-Aromadendrene	0.33
1477	β-Chamigrene	0.98
1480	NI	0.43
1481	Germacrene D	3.89
1490	β-Selinene	3.36
1493	*cis*-β-Guaiene	2.50
1500	Bicyclogermacrene	28.44
1509	Germacrene A	7.34
1513	γ-Cadinene	0.26
1523	δ-Cadinene	0.99
1533	NI	0.19
1546	Selina-3,7(11)-diene	0.30
1557	*trans*-Dauca-4(11),7-diene	0.29
1561	Germacrene B	4.30
1569	Longipinanol	0.88
1572	NI	0.81
1575	NI	0.53
1578	Spathulenol	5.82
1590	Globulol	5.25
1592	Viridiflorol	2.35
1596	NI	0.35
1600	Rosifoliol	1.26
1611	NI	1.15
1628	NI	1.92
1640	τ-Cadinol	0.72
1652	Cedr-8-(15)-en-10-ol	0.91
1654	α-Cadinol	0.59
1659	NI	0.37
1666	Intermediol	4.17
1686	Germacra-4(15),5,10(14)-trien-1α-ol	0.68
	Hydrocarbon monoterpenes	*12.62*
	Oxygenated monoterpene	*0.22*
	Hydrocarbon sesquiterpenes	*58.78*
	Oxygenated sesquiterpenes	*22.63*
	Not identified compounds (NI)	*5.75*
	Total	100.00%

[a]KI, Kovats Index.

Previous information described in the literature (Suffness & Pezzuto, 1991) indicated that values of IC_{50} lower than 30 µg/mL are characteristic of cytotoxic essential oils. Based on this information, the oil from leaves of *N. leucantha* showed moderate activity against B16F10-Nex2 (IC_{50} 33 ± 1 µg/mL), U87 (IC_{50} 75.95 ± 0.03 µg/mL), and HeLa (IC_{50} 60 ± 12 µg/mL) cell lineages. These data also indicated that the oil is more active than the positive control cisplatin (Table 2) but presented no selectivity towards human tumor over healthy cells since IC_{50} determined with HFF was 86 ± 7 µg/mL. Nevertheless, as previous studies showed that the bicyclogermacrene presents cytotoxic activity against B16F10-Nex2 and HCT cell lineages (Silva et al., 2013), this isolated compound was also evaluated against human lineages U87 (glioblastome), HeLa (cervical carcinoma), MCF7 (breast adenocarcinoma), Siha (cervical tumor), and HFF

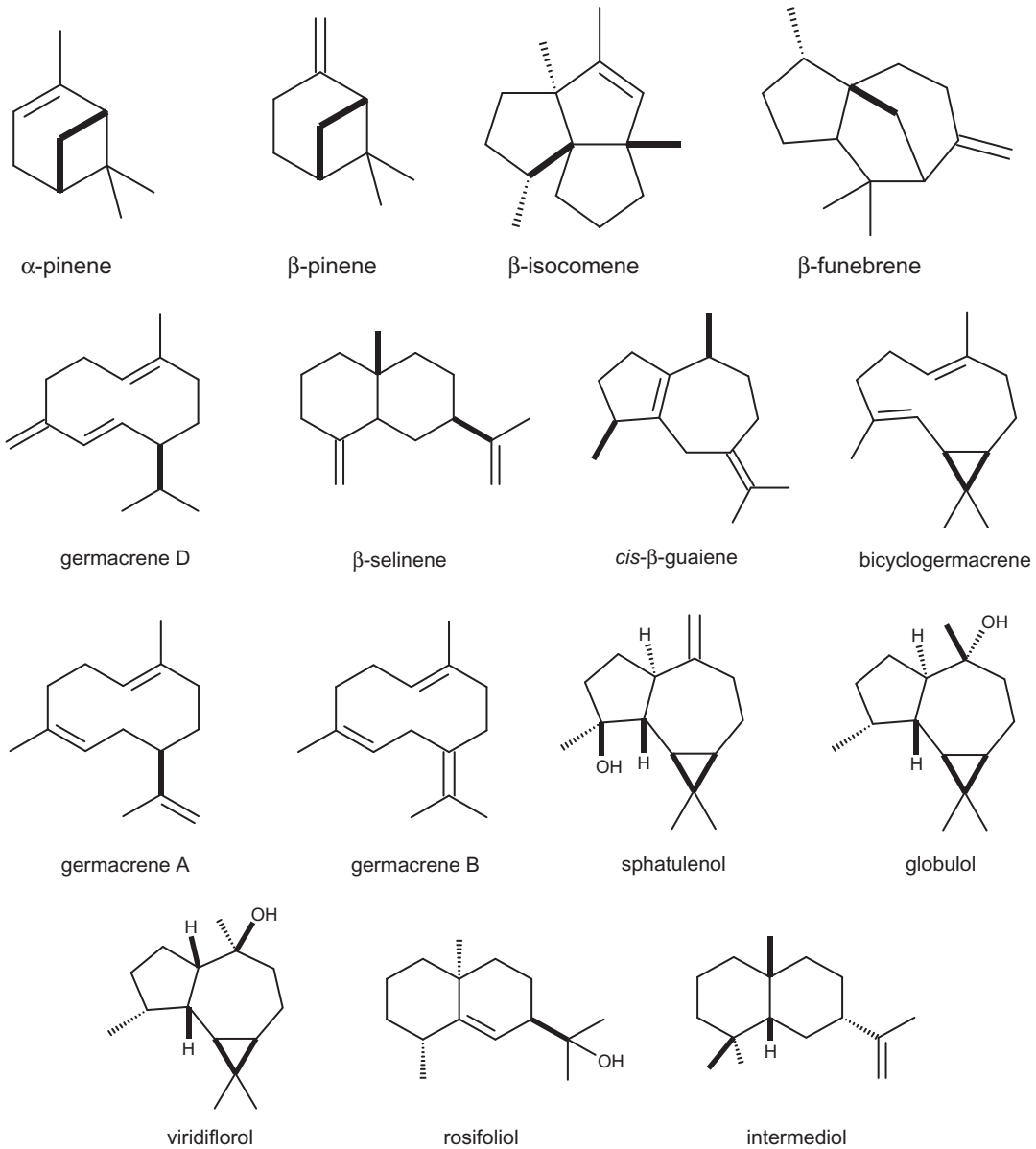

Figure 1. Structures of main compounds identified in the essential oil from leaves of *N. leucantha* (Lauraceae).

Table 2. Cytotoxic effects of essential oil from leaves of *N. leucantha*, bicyclogermacrene, and positive control cisplatin against different tumor cell lines.

	IC_{50} (μg/mL)		
Cell lines[a]	Essential oil	Bicyclogermacrene	Cisplatin
B16F10-Nex2	33 ± 1	3.1 ± 0.2	53 ± 4
U87	75.95 ± 0.03	6.7 ± 0.4	45 ± 6
HeLa	60 ± 12	12.4 ± 0.3	20 ± 1
HCT	194.9 ± 0.1	4.0 ± 0.2	> 50
MCF7	193.79 ± 0.03	19 ± 4	nd
Siha	180 ± 6	21 ± 6	nd
HFF	86 ± 7	2.4 ± 0.3	nd

[a]B16F10-Nex2, murine melanoma; U87, human glioblastome; HeLa, human cervical carcinoma; HCT, human colon carcinoma; MCF7, human breast adenocarcinoma; Siha, human cervical tumor cell; HFF, non-tumorigenic human cell; nd, not determined.

(non-tumorigenic). IC_{50} values determined to bicyclogermacrene ranged from 3.1 ± 0.2 to 21 ± 6 μg/mL, suggesting that the occurrence of this compound as the main derivative (28.44%) which could be associated to the cytotoxic activity detected in the crude oil. However, other compounds, such as α- and β-pinenes, could also be related to this potential since previous studies described that α-pinene exhibited IC_{50} values from 15.0 ± 0.1 to 30.7 ± 0.3 μg/mL, and β-pinene presented IC_{50} ranging from 20.9 ± 0.4 to 80.2 ± 0.2 μg/mL against murine melanoma cell line (B16F10-Nex2) and human melanoma (A2058), breast adenocarcinoma (MCF7), leukemia (human leukemia (HL-60)), and cervical carcinoma (HeLa) cell lines. Studies also showed that these monoterpenes are responsible to induce apoptosis as well as confer antimetastatic protection in a melanoma *in vivo* model (Matsuo et al., 2011; Santana et al., 2012). Previous studies showed that the volatile oil from *N. megapotamica* displayed cytotoxic activity against prostate carcinoma and multiple myeloma (65.5% and 76.2% of lethality, respectively) (Apel

et al., 2006). Therefore, the chemical investigation as well as cytotoxic potential of the essential oil from the leaves of *N. leucantha* is reported here for the first time. These results provide preliminary data for the selection of new compounds with promising cytotoxic activity that are of interest for further investigations about their mechanism of action.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper. The authors are grateful to UFABC, CAPES, FAPEMIG, FINEP, FAPESP, and CNPq for the financial support.

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