Journal of the Chilean Chemical Society
versión On-line ISSN 0717-9707

J. Chil. Chem. Soc. v.48 n.4 Concepción dic. 2003

http://dx.doi.org/10.4067/S0717-97072003000400014

J. Chil. Chem. Soc., 48, N 4 (2003) ISSN 0717-9324

AROMADENDRANE SESQUITERPENOIDS FROM THE ESSENTIAL OIL OF LEAVES OF *DUGUETIA GLABRIUSCULA* – ANNONACEAE

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(Received: March 3, 2003 - Accepted: August 29, 2003)

ABSTRACT

The essential oils obtained from the dried and fresh leaves of *Duguetia glabriuscula* - (Annonaceae) were analyzed by GC/MS and compared. By employing the usual phytochemical workup, five aromadendrane sesquiterpenes were isolated from the essential oil obtained from fresh leaves: alloaromadendran-14β-al, alloaromadendrene, (-)-ledol, viridiflorol, and (+)-spathulenol. Alloaromadendran-14β-oic acid was also obtained, having been generated by the spontaneous oxidation of its corresponding aldehyde. Both essential oils displayed activity against *Artemia salina*.

Keywords: *Duguetia glabriuscula*, Annonaceae; *Artemia salina;* aromadendrane sesquiterpenoids; alloaromadendran-14β-al, alloaromadendran-14β-oic-acid.

RESUMEN

Los aceites esenciales obtenidos de hojas secas y frescas de *Duguetia glabriuscula* (Annonaceae) fueron analizados por CG/EM y comparados. Empleando la fitoquímica usual fueron aislados cinco sesquiterpenos del tipo aromadendrano del aceite esencial de las hojas frescas: *allo*-aromadendran-14β-al, (-)-ledol, viridiflorol, (+)-espatulenol y el ácido *allo*-aromadendran-14β-oico. El ácido *allo*-aromadendran-14β-oico fue también obtenido, habiendo sido generado por la oxidación espontánea de su aldehído correspondiente. Ambos aceites mostraron actividad enfrente *Artemia salina*.

Palabras claves: *Duguetia glabriuscula*, Annonaceae; *Artemia salina;* sesquiterpenos aromadendrano; *allo*-aromadendran-14β-al, ácido *allo*-aromadendran-14β-oico.

INTRODUCTION

Duguetia glabriuscula is a shrub of the Annonaceae family occurring in the state of Mato Grosso do Sul, Brazil. Previous investigations had led to the isolation, from the hexane extract of its dried leaves, of alloaromadendran-10β,14-diol, which showed antibacterial activity[1)], but no activity against *Artemia salina*. From this compound, several derivatives were made by means of chemical transformation[2)] and biotransformation[3)]. The present paper describes the isolation of other aromadendrane-type sesquiterpenes from the essential oil extracted from the leaves of *D. glabriuscula*.

EXPERIMENTAL

General procedures

Mp was determined on a Uniscience 498 apparatus and remains uncorrected. Optical rotation was run in chloroform on a Perkin Elmer 341 instrument. The FT-IR spectrum was measured as chloroform film on a Perkin Elmer 783. The Gas Chromatograph Electron Impact Mass Spectrum (GCEIMS) was obtained on a CG17A/QP 5000 Shimadzu GC/MS instrument. A DB-5 capillary column (30 m × 0.25 mm, with a 0.25-μm-thick film) was used under the following conditions: injector and detector temperatures, 220 ºC and 240 ºC, respectively; column program, 60-240 ºC (3 ºC/min), 240 ºC (15 min); impact energy, 70 eV. The compounds were identified by comparing their mass spectra with those available from the equipments library (Wiley 140), and also by employing their Kovats indices[4)]. All NMR experiments were performed on a DPX-300 Brucker instrument (^{1}H: 300 MHz; ^{13}C: 75 MHz) using $CDCl_3$ as solvent and TMS as the internal standard. Chemical shifts are reported in δ units and coupling constants (*J*) in Hz. 60 G and 60 GF_{254} silica gel (Merck) and 70-230 mesh (Aldrich) were used for TLC and CC, respectively.

Plant material

The leaves of *Duguetia glabriuscula* R. E. Fr. (R. E. Fr.) were collected in the municipality of Jardim, MS, Brazil, and identified by Prof. R. Mello Silva. A voucher specimen (number 4769) has been deposited in the CG-MS-UFMS Herbarium (Campo Grande, MS).

Isolation of mixture and substances from the fresh crude oils

The fresh crude oils were obtained by steam distillation (1.5% and 0.8% v/w, from fresh and dried leaves, respectively). The crude oil from dried leaves was subjected to silica gel column chromatography and monitored by TLC and GC, yielding the compounds alloaromadendran-14β-oic acid, **2**, alloaromadendrene, **3**, (-)-ledol, **4**, (+)-spathulenol, **5**, and, viridiflorol, **6**. The crude oil from fresh leaves was submitted to preparative TLC, yielding alloaromadendran-14β-al, **1**, **4**, **5,** and a mixture of **1** and **2.**

Obtaining alloaromadendran-14β-al, 1, by preparative TLC

Once the presence of the aldehyde signal had been confirmed, the fresh crude oil from fresh leaves (50 mg) was kept in a freezer. After 6 h, a precipitated was separated from the oil and submitted to preparative TLC. Six spots were thus revealed, all of which were extracted by employing the usual workup with chloroform (Merck), and then maintained in a desiccator. The resulting products were stored in flasks under vacuum and kept under refrigeration. The isolation of **1** was confirmed (1.8 mg, Rf 0.35, silica gel preparative TLC, methylene chloride : petroleum ether 1:1, dark violet, ceric sulfate), and this compound was maintained in a N_2 atmosphere for collection of spectral data. This substance was also present in the fresh crude oil from the dried leaves, although in lower concentration. GC/MS, 70 eV, *m/z* (rel. int.) 220 (<1), 202 (10), 55 (100). For ^{1}H and ^{13}C NMR spectra: see Table II.

Alloaromadendran-14β-oic-acid, 2

Compound,(1a*R*,4*S*,4a*S*,7*R*,7aR,7b*S*)-1,1,7-trimethyldecahydro-1*H*-cyclopropa[*e*]azulene-4-carboxylic acid, was confirmed as the main substance (82%) from a fraction of the CC on silica gel of the crude oil from the dried leaves. This same substance was also obtained by preparative TLC of the crude oil from the dried leaves, according to the methodology described below. I.V. ν_{max} (KBr, cm^{-1}) 3410, 2930, 2880, 1700, 1520, 1440, 1380, 1295, 1080; GC/MS, 70 eV, *m/z* (rel. int.) 236 (2), 57 (100). For ^{1}H and ^{13}C NMR spectra: see Table II.

Obtaining alloaromadendran-14β-oic acid, 2, by induced oxidation of the fresh crude oil

Compound **2** was also obtained by induced oxidation of the crude oil, with the following method: 500 mg of fresh crude oil from dried leaves were subjected to aeration at room temperature after the presence of the aldehyde signal had been confirmed. After 48 hours, the signal was absent, and the resulting opaque resin was solubilized in chloroform and extracted with 1% NaOH. The alkaline layer was adjusted to pH 6.0 and then extracted with chloroform. Substance **2** (7.5 mg) was isolated from this chloroform fraction by preparative TLC on silica gel

(eluent $CHCl_3$:MeOH 99:1; detection by aniline/glucose spraying followed by heating, thus yielding a brown spot[5]; citric acid was employed as the standard for acidic substances).

Compound **3** was isolated as the main substance from one of the oil fractions (66%, by GC), and was obtained as oil.

The mixture of viridiflorol, **4**, and ledol, **5** (43% and 57% respectively), obtained from CC on silica gel, was subjected to preparative TLC (silica gel : SiF_{254} : $AgNO_3$ 80:10:10), but the separation failed.

(-)-ledol, **5**, was obtained as a colorless crystal, mp. 102-104 ºC (lit. 103-104 ºC), $[\alpha]_D^{25}$: -6.7 (C = 0.33; lit. -6.3).

(+)-spathulenol, **6**, was obtained as a mixture with ledol from CC on silica gel, and separated by preparative TLC (silica gel : silica F_{254} : $AgNO_3$; 80:10:10); $[\alpha]_D^{25}$: +5.3 ($c = 8 \times 10^{-5}$).

Bioassays

The brine shrimp toxicity (BSL) test was performed as described in the literature[6], with minor modifications.

RESULTS AND DISCUSSION

The fresh crude oils were obtained by steam distillation of the leaves, yielding 1.5% and 0.8% v/w of fresh and dried leaves, respectively. Both oils had the same strong characteristic odor and light green color. Among the constituents identified (Table I), aromadendrane-type sesquiterpenes predominated (at 61.3% and 59.0%, from fresh and dried leaves, respectively). Alloaromadendrene, viridiflorol, (-)-ledol and (+)-spathulenol were the main compounds found for this class.

Table I. GC/MS analysis of the volatile oils obtained from fresh and dried leaves of Duguetia glabriuscula

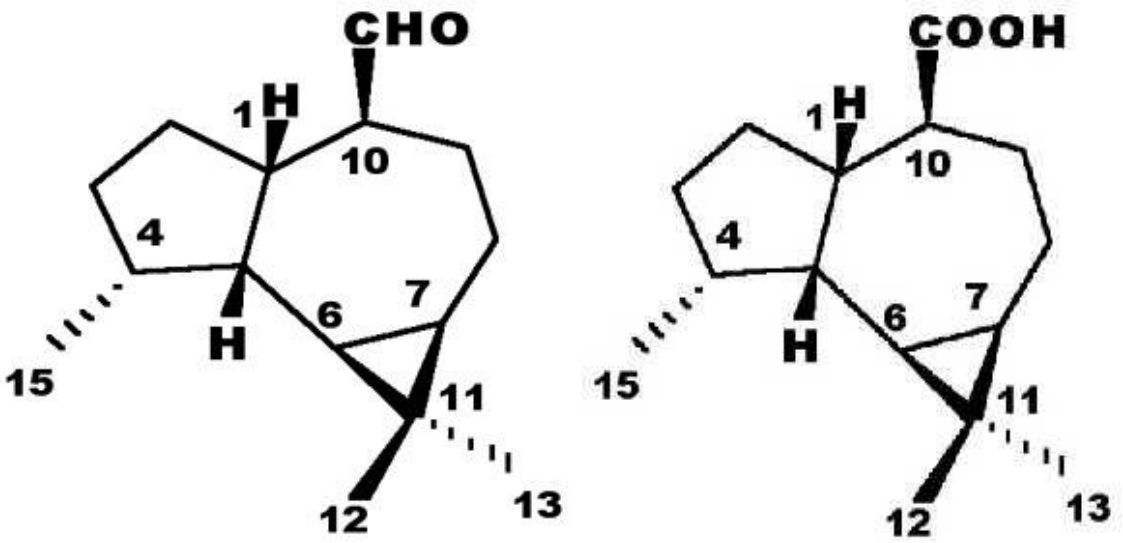
R.time	Substance fresh leaves	%	R. time dried leaves	substance	%
11.80	2-β-pinene	1.88			
32.84	α-gurjunene	3.94	32.85	α-gurjunene	1.88
33.13	α–santalene	7.47	33.16	α–santalene	3.95
33.32	β-caryophyllene	2.40	33.35	*trans*-caryophyllene	2.06
33.75	α-bergamotene	3.74	33.78	α-bergamotene	2.62
			34.23	(+)-aromadendrene	2.19
35.35	alloaromadendrene	22.56	35.44	alloaromadendrene	16.18
35.49	sesquiterpene (MW 204)	3.87	35.61	sesquiterpene (MW 204)	3.10
36.76	Viridiflorene	13.29	36.75	viridiflorene	3.46

			37.45	γ-cadinene	1.80
			37.75	δ-cadinene	3.11
39.84	palustrol	3.63	39.91	palustrol (ledum)	3.98
			39.98	Longipinine oxide	1.47
40.20	(+)-spathulenol	5.77	40.44	(+)-spathulenol	12.09
40.49	sesquiterpene alcohol (MW 220)	1.84	40.61	sesquiterpene alcohol (MW 220)	1.98
40.86	Viridiflorol	3.69	41.02	viridiflorol	4.76
41.38	(-)-ledol	10.64	41.59	(-)-ledol	13.43
42.72	ced-8(15)-en-9- α -ol	2.42	42.63	ced-8(15)-en-9-α -ol	2.50
			43.19	α-copaen-11-ol	1.32
43.54	Aromadendrene epoxide-[II]	1.74	43.66	Aromadendrene epoxide-[II]	2.79
43.72	Farnesol	1.86	43.83	Farnesol	1.78
44.21	alloaromadendran-14β-al*	5.04	44.17	alloaromadendran-14β-al	2.83
			44.33	sesquiterpene(MW 220)	3.26
			51.07	elemodiol-(8-α-11)	1.60
50.94	farnesyl acetate	4.22	51.07	farnesyl acetate	5.86

*According to Rt observed of the **1** obtained from preparative TLC.

The fresh crude oil obtained from fresh leaves showed toxicity (LD_{50} = 1.6, with a 0.5- to 2.3-μg/mL confidence interval) when submitted to the BSL test. In order to isolate and identify its bioactive compounds, an activity-guided fractionation was started. Preliminary data from the ^1H NMR spectrum of the fresh crude oil not only suggested the presence of aromadendrane-type sesquiterpenes[7], but also showed an additional signal for an aldehyde proton at δ 9.60 (*d*, *J* 2.8 Hz). However, difficulties in isolating and identifying pure bioactive constituents led to the use of another methodology[8]. Preliminary chromatographic separation of the fresh volatile oil on silica gel column yielded several fractions. Some of them, mostly containing one or two compounds (as confirmed by GC monitoring), were then subjected to preparative TLC, yielding the following substances:

alloaromadendrene[7], **3**, (-)-ledol[9], **4**, (+)-spathulenol[10], **5**, and viridiflorol[11], **6** (this one in mixture with **4**), in addition to a new compound, alloaromandendran-14β-oic acid, **2**.



The FT-IR of **2** showed a broad band at 3.410-2.600 cm^{-1} and a narrow one at 1.700 cm^{-1}, and its GC/MS revealed a molecular ion at *m/z* 236. The ^{1}H NMR spectrum (Table II) contained signals at δ 0.26 (dd, *J* 11.4, 9.0 Hz, H-6) and δ 0.56 (ddd, *J* 11.4, 9.0, 5.4 Hz, H-7), indicating a cyclopropane ring, δ 0.92 (d, *J* 6.9 Hz), δ 0.95, and 1.01 (s, CH_3), corresponding to three methyl groups. Two additional hydrogen signals were observed at δ 2.29 (1H, m) and δ2.81 (br td, *J* 11.7, 4.2 Hz).

The ^{13}C NMR spectrum (PND/DEPT 135º) of **2** (Table II) showed the presence of 15 carbon signals. Fourteen of these corresponded to three methyl, four methylene, six methine, and one quaternary carbon at δ 17.28, whereas one signal (at δ 182.04) was attributed to a carboxylic carbon. Connectivities were confirmed by COSY 45º and HMBC experiments, as shown in Table II. From the information gathered, it was possible to propose that **2** was an aromadendrene-type sesquiterpene compound with an acid carbonyl at C-14. The NOESY experiment allowed the α orientation of H-10 to be confirmed, based on its observed correlations with α-H-6 and α-H-7. Additional interaction was found between H-10 and H-1, which could be explained by vicinal system interaction caused by twists in the 7-membered ring. No interaction, however, was observed between H-1 and α-H-6/α-H-7.

Because no compounds with an aldehyde function resulted from the purification of the essential oil constituents, it was hypothesized that oxidation might have occurred during their isolation[7]. In order to confirm this hypothesis, a sample of fresh oil was stored for a long period, after which it was possible to verify the disappearance of the aldehyde proton signal from the ^{1}H NMR spectrum.

Preparative TLC of the fresh essential oil yielded 1.8 mg of aldehyde aromadendrane sesquiterpene (not totally pure), which was maintained in a N_2 atmosphere for collection of ^{1}H NMR, ^{13}C NMR, and COSY spectral data.

The ^{1}H NMR spectrum of **1** (Table II) contained signals for the hydrogens of the cyclopropane group at δ 0.29 (dd, *J* 11.5, 9.0 Hz, H-6) and δ 0.58 (ddd, *J* 12.2, 9.0, 4.8 Hz, H-7), and for three methyl groups at δ 0.94 (d, *J* 6.7 Hz, CH_3-15), δ 0.98 and 1.02 (s, CH_3-12 and 13, respectively). Furthermore, three other signals were observed at δ 2.24 (1H, m), δ 2.71(1H, m), and δ 9.60 (1H, d, *J* 2.8 Hz, aldehyde proton).

The ^{13}C NMR spectra (PND/DEPT 135º) of **2** showed the presence of 15 carbon signals corresponding to three methyl, four methylene, seven methine (one at δ 205.86, attributed to an aldehyde carbonyl carbon), and one quaternary carbon (at δ 17.60). The main hydrogen nucleus correlations observed in the COSY 45º experiment are shown in Table II. The signals of the ^{13}C NMR spectra of compounds **1** and **2** were found to be similar, with main differences in the signals of carbons 1, 10, and 9. These features are compatible with the presence of the aldehyde (**1**) and acid (**2**) functional groups at C-14 of aromadendrane sesquiterpenes.

Table II. ^{13}C NMR (75 MHz), ^{1}H NMR (300 MHz) spectral data and mean correlations observed in COSY, HMBC, and NOESY experiments for compounds **1** and **2** in $CDCl_3$, employing TMS as the internal standard.

	1			2				
C/H	δ_C (DEPT)	δ_H (m, *J* in Hz)	COSY 45º	$^a\delta_C$ (DEPT)	$^a\delta_H$ (m, *J* in Hz)	COSY 45º	HMBC	NOESY
1	42.71 (CH)	2.24 (m)	H-10	44.39 (CH)	2.29 (m)	H-10	H-10	H-5, H-1
2	30.34 (CH_2)	*		31.26 (CH_2)	*			
3	31.35 (CH_2)	*		30.63 (CH_2)	*			
4	37.85 (CH)	*		38.07 (CH)	*			
5	41.70 (CH)	*		41.24 (CH)	*		H-15	
6	22.83 (CH)	0.29 (dd, 11.5, 9.0)	H-7, H-5	22.66[b] (CH)	0.26 (dd, 11.4, 9.0)	H-7		H-7, H-10
7	22.83 (CH)	0.58 (ddd, 12.2, 9.0, 4.8)	H-6	22.50[b] (CH)	0.56 (ddd, 11.4, 9.0, 5.4)	H-6		H-6, H-10
8	19.85 (CH_2)	*		19.98 (CH_2)	*			
9	24.97 (CH_2)	*		28.91 (CH_2)	*		H-10	
10	54.51 (CH)	2.71 (m)	H-1, H-9	47.14 (CH)	2.81 (td, 11.7, 4.2)	H-1	H-9	H-6, H-7, H-1
11	17.60 (C)	*	-	17.28 (C)	-			
12	28.80 (CH_3)	1.02		15.25 (CH_3)	1.01 (s)			

13	15.31 (CH_3)	0.98		28.71 (CH_3)	0.95 (s)	
14	205.86 (CH)	9.60 (d, 2.8)	H-10	182.04 (C)		H-10
15	15.76 (CH_3)	0.94 (d, 6.7)		15.72 (CH_3)	0.92 (d, 6.9)	

[a] ^{1}H and ^{13}C assignments were based on an HMQC experiment. [b] Signals are interchangeable within column.

The correlation between these compounds was confirmed by auto-oxidation of **1** into **2**. The mixture of **1** and **2** (1.9 mg) was obtained through preparative TLC and, after being confirmed by ^{1}H and ^{13}C NMR spectra, was air-dried and maintained in the resonance tube at room temperature for 48 h. It was then again solubilized and its ^{1}H and ^{13}C NMR spectra confirmed the presence of **2** alone.

Compounds **4** and **5**, both pure, were each tested, but did not show any activity[14]. Only the crude oil could be reliably tested against *Artemia salina*.

Although substance **1** has been recently described as a side product of reactions that involve aromadendrane-type sesquiterpenes[15], it had never been described, to our knowledge, as a natural product before our investigation. This also applies to its artifact **2**.

ACKNOWLEDGEMENTS

We are grateful to Maria C. Guerra and João R. Fabri (technicians of the Pharmacognosy Laboratory of UFMS) for their technical support during the experimental part of this investigation. This work was financially supported by CPq-PROPP-UFMS and FUNDECT-MS, Brazil (process 0117/99). A student fellowship was provided by the PIBIC-CNPq-UFMS Program.

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