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Chemical constituents of the volatile oil from leaves of *Annona coriacea* and *in vitro* antiprotozoal activity

Carlos A. T. Siqueira,[1] Jocimar Oliani,[1] Adilson Sartoratto,[2] Carmen L. Queiroga,[2] Paulo R. H. Moreno,[3] Juliana Q. Reimão,[4] André G. Tempone,[4] Dominique C. H. Fischer*[,1]

[1]Laboratório de Farmacognosia, Departamento de Farmácia, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, Brazil,
[2]Divisão de Fitoquímica, Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas, Universidade Estadual de Campinas, Brazil,
[3]Departamento de Química Fundamental, Instituto de Química, Universidade de São Paulo, Brazil,
[4]Laboratório de Toxinologia Aplicada, Departamento de Parasitologia, Instituto Adolfo Lutz, Brazil.

Abstract: The essential oil of the leaves from *Annona coriacea* Mart., Annonaceae, was extracted by hydrodistillation in a Clevenger apparatus and analyzed by GC/MS and GC/FID. The oil yield was 0.05% m/m. Sixty compounds were identified, in a complex mixture of sesquiterpenes (76.7%), monoterpenes (20.0%) and other constituents (3.3%). Bicyclogermacrene was its major compound (39.8%) followed by other sesquiterpenes. Most of the monoterpenes were in low concentration (<1%). Only β-pinene and pseudolimonene presented the highest level of 1.6%. The volatile oil presented anti-leishmanial and trypanocidal activity against promastigotes of four species of *Leishmania* and trypomastigotes of *Trypanosoma cruzi*, showing to be more active against *Leishmania* (L.) chagasi (IC50 39.93 µg/mL) (95% CI 28.00-56.95 µg/mL).

Article

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Introduction

The alarming socio-economical reality caused by neglected diseases in Brazil and throughout the world, in special leishmaniasis and the Chagas' disease, which have presented high levels of morbidity and mortality, not to mention the limitations and toxicity of today's therapeutics, urges for new drug substances (Newman & Cragg, 2007; Savioli, 2010; Osorio et al., 2008; Carvalho, 2009). Researches on bioactive molecules have revealed potential sources in our flora biodiversity. During the last years the Annonaceae family has been evaluated toward that (Camacho et al., 2003; Carollo et al., 2005).

Annonaceae species are widely distributed around the world (Mello-Silva & Pontes, 2005) and are found in all the continents (Pontes et al., 2004); at a predominant way in tropical zones. This family of plants shows over 135 genera and 2500 species (Smith et al., 2004). In Brazil both tree and shrub specimens are mainly situated in the "cerrado" (Souza & Lorenzi, 2008), the vast tropical savanna ecoregion of the country. Its main economical roles are its edible fruit production, its medicinal proprieties (Villar Del Fresno et al., 1984) and its aromatic oils extracted from several species as *Cananga odorata* Hook Fil. et Thomson ("ylang-ylang"), among other uses (Heywood, 1993; Judd et al., 2002).

The *Annona* genus, with 250 species found in the Brazilian territory (Souza & Lorenzi, 2008; Mello-Silva & Pontes, 2005), has revealed the presence of different classes of active metabolites, like isoquinoline and pyrimidine-ß-carboline alkaloids (Silva et al., 2007a, Costa et al., 2006), acetogenins (Yang et al., 2009), lectins (Coelho et al., 2003) and volatile oils (Ferreira et al., 2009; Boyom et al., 1996), against the protozoaries responsible for endemic tropical diseases.

Annona coriacea (Mart.), popularly known as "marolo", "araticum", "araticum-liso" (Hoehne, 1946; Souza & Lorenzi, 2008), has been used as traditional medicine for treating parasitoses, ulcers and inflammation processes (Sonnet et al., 1971) and is also reported for rheumatism and as anti-helminthic (Cruz, 1985).

The reports of isolated secondary metabolites from this species were of acetogenins (Bermejo et al., 2005; Silva et al., 1998, 1997a,b, 1996, 1995; Yu et al., 1994), diterpenoids (Onan & Mc Phail, 1978; Orsini et al., 1977; Mussini et al.,1973a,b; Ferrari et al., 1971) and pterocarpans (Darko et al., 1983; Nakagawa et al., 1982). In our previous studies, *in vitro* antimalarial (Fischer et al., 2004), anti-leishmanial and trypanocidal (Tempone et al., 2005) activities were found for the crude ethanol extract and for the total alkaloids.

The antiprotozoal activity of the volatile oil from another *Annona* species (Costa et al., 2009) motivated this study to identify those constituents from the *A. coriacea* leaves and also to evaluate its potential anti-leishmanial and trypanocidal *in vitro* activities.

Material and Methods

Botanical material

Leaves from *Annona coriacea* Mart., Annonaceae, were collected, in October 2009, in the Estação Ecológica (Nature Reserve) of the Instituto Florestal of São Paulo, at Águas de Santa Bárbara, in São Paulo state, placed 345 km from the capital. The exact collecting site lies at 22º 46' 91" on the South longitude and at 49º 14' 40" on the West latitude, at an altitude of 765 m. Voucher specimens were deposited at the Herbarium (SPF) of the Instituto de Biociências da Universidade de São Paulo (IB-USP), under the denomination "Siqueira-4" and were identified by the Annonaceae specialist Dr. Renato de Mello-Silva.

Standard compounds

As external reference the following standards were acquired from Fluka® (St. Louis, USA), with p.a. purity: (*E*)-caryophyllene and α-humulene (sesquiterpene hydrocarbons), β-pinene (monoterpene hydrocarbon) and globulol (oxygenated sesquiterpene). A homologous series of *n*-alkanes (C_9-C_{20}) was acquired from the same supplier. The standards (0.5 µg/mL) were diluted in *n*-hexane p.a. (Merck®) (1 mL).

Volatile oil extraction

Fresh leaves of *A. coriacea* (260.4 g) were cut and hydrodistillation was carried out in a Clevenger-type apparatus for 4 h, at a temperature of 40 °C. They were previously ground with solid CO_2 in a blender (Ametek®-36BL54) at 18000 rpm for 3 min. The essential oil was separated from the (frozen) water by glass capillary suction, and stored in a glass flask, protected from light and humidity, until analysis. The oil content was determined in analytical balance on a dry weight basis performed in triplicate.

GC/MS and GC/FID analysis of the volatile oil

The GC/MS volatile oil analysis was performed in the Agilent HP-6890 gas chromatograph, using a fused capillary column HP-5 MS (5% phenylmethylpolysiloxane, J&W Scientific) (30m x 0,25mm x 0,25 µm), directly coupled to a HP-5975 selective mass detector.

The oil injected volume was 1.0 µL, with *split* (1:20), at scan mode. The following conditions were used: temperature of injector: 220 °C; temperature of GC detector: 250 °C, programmed column temperature: 60 to 250 °C at 3 °C/min for 60 min, carrier gas: helium (1 mL/min). Electron impact (EI) ionization MS was used at 70 eV. Mass spectra were acquired over a range of 29-400 amu, at 1 scan/s.

The retention indices for all volatile constituents were determined by co-injection of the hydrocarbon standards and the equation of Van Den Dool & Kratz (1963) was used to calculate them. The constituents identification and the comparison of their MS data with external standard data were based on the spectrometry library of the equipment system (NIST 2005, Lib.), retention time, retention indices (RI) (Adams, 2007) and literature data (Adams, 2007; Wiley Service Co.; SciFinder Scholar, USA).

The analysis of the volatile components was carried out on HP5890 equipment under the same conditions described before. The GC system was equipped with a flame ionization detector (FID). The percentage compositions were obtained through the electronic integration of the GC peak areas without taking into account their relative response factors.

In vitro antiprotozoal activity

Parasites

The anti-leishmanial activity was evaluated against promastigotes of *Leishmania* (L.) *chagasi* (MHOM/BR/1972/LD), *Leishmania* (L.) *amazonensis* (WHO/BR/00/LT0016), *Leishmania* (L.) major (MHOM/1L/80/Fredlin) and *Leishmania* (V.) *braziliensis* (MHO/BR/75/M2903), cultivated in M-199 medium and supplemented with 10% calf serum and 0.25% of hemin at 24 °C, without antibiotic addition. And, the trypomastigotes of *Trypanosoma cruzi* (strain Y) were isolated from the supernatant of LLC-MK2 (ATCC CCL 7- Rhesus monkey kidney cells) which had been previously infected with bloodstream trypomastigotes.

Anti-leishmanial activity

The anti-leishmanial activity was determined against promastigotes of L. (L.) *chagasi*, L. (L.) *amazonensis*, L (L.) *major*, L. (V.) *braziliensis* (Sartorelli et al., 2007), using pentamidine as standard drug (100 µg/mL). Promastigotes were counted in a Neubauer hemocytometer and seeded at $1x10^6$ cells/well in 96-well microplates and essential oil was incubated to the highest concentration of 500 µg/mL (based on dry weight) for 24 h at 24 °C. Parasite viability was determined using the MTT assay at 550 nm (Tada et al., 1986).

Trypanocidal activity

Cell culture-derived trypomastigotes from LLC-MK2 cells were counted in a Neubauer hemocytometer and seeded at $1x10^6$ cells/well in 96-well microplates. The essential oil was incubated at the highest concentration of 500 µg/mL for 24 h at 37 °C in a 5% CO_2 humidified incubator with benznidazole as standard drug (100 µg/mL). The trypomastigotes viability was based on the cellular conversion of the soluble tetrazolium salt MTT into the insoluble formazan by mitochondrial enzymes. The formazan extraction was carried out with 10% (v/v) SDS for 18 h at 24 °C (Lane et al., 1996) in a spectrophotometer Multiskan MS (Uniscience) microplate reader.

Statistical analysis

The data obtained represent the mean and standard deviation of duplicate samples from two independent assays. The IC50 values were calculated using sigmoid dose-response curves in Graph Pad Prism 5.0 software, and the 95% confidence intervals (C.I.) are included in parenthesis. The Mann-Whitney test (unpaired two-tailed) was used for significance testing ($p<0.05$).

Results and Discussion

The volatile oil yield from *A. coriacea* leaves was 0.05% m/m, compared to the dry weight of the plant material. This result was in good agreement with that found in other *Annona* species as: *A. densicoma* (0.1% m/m) (Andrade et al., 2007), *A. muricata* (0.0002%, 0.01% m/m) (Kossouoh et al., 2007; Fournier et al., 1999), *A. squamosa* (0.12% m/m) (Garg & Gupta, 2005) and *A. foetida* (0.01% m/m) (Costa et al., 2009).

The essential oil GC/MS analysis identified sixty constituents (Table 1), corresponding to 96.5% of the oil, made up of a complex mixture of sesquiterpenes (76.7%), monoterpenes (20.0%) and other constituents (3.3%). The volatile oil composition from *A. coriacea* showed to be in accordance with the classes of volatile constituents found in the *Annona* genus and in the Annonaceae family (Leboeuf et al., 1982; Lima et al., 2004; Nebié et al. 2005; Andrade et al., 2007; Fournier et al., 1999; Boyom et al., 1996).

The bicyclogermacrene sesquiterpene was the major compound (39.8%) (Table 1). It is the precursor of spathulenol, an oxygenated sesquiterpene that was one of the main constituents found in this oil (4.2%) (Table 1) and could be considered as a chemotaxonomic marker of the Annonaceae family, since its presence is reported in its different genera (Costa et al., 2008). The other main sesquiterpene compounds were: γ-muurolene (7.9%), δ-cadinene (6.0%), (*E*)-caryophyllene (4.9%), spathulenol (4.2%), α-patchoulene (2.7%), sesquisabinene hydrate (2.5%) and α-humulene (2.4%) (Table 1).

The antifungal activity of bicyclogermacrene was previously reported (Silva et al., 2007b). Although there are no records on antiprotozoal action, its high percentage in the *A. coriacea* oil allows inferring a contribution of this compound to the revealed trypanocidal and anti-leishmanial activities. A deeper study should be conducted to confirm it. The isolation for biological testing was not possible due to the low amount of *A. coriacea* oil content.

Two other major sesquiterpenes of the *A. coriacea* oil, δ-cadinene and (*E*)-caryophyllene, also showed to be frequent in the Annonaceae family (Palazzo et al., 2009), as well as β-elemene, a minor constituent (0.7%).

(*E*)-caryophyllene is a marker in Annonaceae (Valter et al., 2008) and showed, together with δ-cadinene, a significant activity against *L. donovani* promastigotes (Zheljazkov et al, 2008) with an IC50 of 19 and 4 µg/mL, respectively, suggesting a probable relation to the anti-leishmanial properties of the *A. coriacea* oil (Table 2).

In the essential oil from *A. foetida*, a similar profile to that of *A. coriacea* was found as it was seen the sesquiterpenes predominance and also in relation to bicyclogermacrene and (*E*)-caryophyllene (Costa et al., 2009).

Likewise, in *A. densicoma* (Andrade et al., 2007) and *A. muricata* (Boyom et al., 1996), the sesquiterpenes were the greater part and in the former, the bicyclogermacrene percentage was also high (26.8%).

When compared, *A. senegalensis* (Boyom et al., 1996) presented higher concentration of monoterpenes in their essential oil.

Among the other abundant sesquiterpene constituents, in the essential oil from *A. coriacea*, α-humulene (2.4%) (Table 1) has already been reported

Table 1: Chemical composition (%) of the volatile oil components from leaves of *Annona coriacea* Mart. Annonaceae.

Constituents[a]	Retention time*(min)	RI[b]	RI[c]	%
α-thujene	5.1	933	930	0.7
sabinene	6.0	973	969	tr
β-pinene	6.1	977	979	1.6
myrcene	6.5	992	990	0.2
pseudolimonene	7.6	1000	1004	1.6
β-*cis*-ocimene	7.9	1036	1037	0.2
β-*trans*-ocimene	8.3	1047	1050	0.4
nonanal	10.3	1105	1100	0.1
verbenone	14.0	1199	1205	0.1
β-cyclocitral	14.8	1219	1219	0.1
δ-elemene	19.7	1335	1338	0.2
benzyl butanoate	20.0	1344	1346	0.2
α-cubebene	20.2	1348	1348	tr
cyclosativene	20.8	1362	1371	0.2
α-ylangene	21.3	1373	1375	1.7
β-bourbonene	21.6	1382	1388	1.6
α-isocomene	21.8	1388	1388	0.4
β-elemene	21.9	1390	1390	0.7
β-isocomene	22.6	1406	1408	0.3
(*E*)-caryophyllene	23.0	1417	1419	4.9
α-guaiene	23.4	1426	1439	0.4
gurjunene	23.8	1435	1433	0.5
aromadendrene	24.0	1441	1441	0.2
seychellene	24.2	1447	1446	0.2
α-humulene	24.4	1450	1454	2.4
trans-prenyl limonene	24.4	1452	1459	0.2
α-patchoulene	24.7	1457	1456	2.7
ageratochromene 6-dimethoxy	24.9	1463	1463	0.4
carota-1,4-diene	25.2	1471	1472	0.2
10-β-h-cadina-1,6,4-diene	25.4	1475	1476	0.7
γ-muurolene	25.5	1479	1479	7.9
α-amorphene	25.7	1483	1484	0.1
trans-β-ionone	25.8	1485	1488	0.4
β-selinene	25.9	1489	1490	0.2
bicyclogermacrene	26.3	1497	1500	39.8
aciphyllene	26.5	1503	1501	0.6
δ-amorphene	26.8	1511	1512	0.6
δ-cadinene	27.2	1522	1523	6.0
zonarene	27.5	1529	1529	tr
α-calacorene	27.9	1540	1540	0.1
selina-3,7-11-diene	28.2	1548	1546	tr
eremophila ketone	28.8	1563	1560	0.3
cis-3-hexenyl benzoate	29.0	1569	1566	0.1
spathulenol	29.2	1574	1578	4.2
sesquisabinene hydrate	29.4	1580	1579	2.5
globulol	29.7	1588	1590	1.0

Table 1: Chemical composition (%) of the volatile oil components from leaves of *Annona coriacea* Mart. Annonaceae (cont.).

viridiflorol	29.8	1590	1592	0.3
rosifoliol	30.1	1598	1600	0.8
ethanone	30.4	1605	1607	0.2
β-oplopenone	30.5	1608	1607	0.3
4a(2h)-naphthalenol	31.1	1625	1631	0.6
isocedrenone	31.3	1631	1631	tr
alloaromadendrene epoxide	31.5	1635	1641	0.9
α-*epi*-cadinol	31.6	1638	1640	1.4
hisenol	31.8	1643	1641	0.5
β-eudesmol	31.9	1646	1650	1.1
α-eudesmol	32.0	1649	1653	1.0
α-cadinol	32.1	1651	1654	1.6
α-betulenol	32.6	1666	1667	0.3
khusinol	33.2	1682	1680	0.3

[a]Identification by comparison with GC/MS spectra and RI with the internal NIST library and with Adams (2007), RI[b] (retention index) obtained on a capillary column HP-5MS based on *n*-alkane series and calculated following Van Der Dool e Kratz (1963), RI[c]: according to Adams (2007),*:on HP-5MS, tr: traces (<0.1%)

Table 2. *In vitro* activity of *Annona coriacea* Mart. against promastigotes of *Leishmania* (L.) *amazonensis* (WHO/BR/00/LT0016), *L.* (V.) *braziliensis* (MHO/BR/75/M2903) *L.* (L.) *chagasi* (MHOM/BR/1972/LD) e *L.* (L.) *major* (MHOM/1L/80/Fredlin) and against trypomastigotes of *Trypanosoma cruzi* (strain Y).

	IC50 (μg/mL) (95% C.I.) promastigotes				
	L.(L.) *amazonensis*	*L.*(V.) *braziliensis*	*L.*(L.) *chagasi*	*L.*(L.) *major*	trypomastigotes *T. cruzi*
volatile oil*	160.20 (117.70-218.10)	261.20 (179.50-380.10)	39.93 (28.00-56.95)	305.20 (189.20-492.30)	168.50 (99.10-286.60)
standard drug					
pentamidine**	0.16 (0.15-0.16)	0.06 (0.05-0.06)	0.22 (0.17-0.27)	0.16 (0.15-0.18)	-
benznidazole**	-	-	-	-	45.02 (29.31-68.42)

IC50: inhibitory concentration of 50% parasites; 95% C.I.= confidence interval, *up to 500 μg/mL, **100 μg/mL.

in *A. senegalensis* (Nebié et al., 2005; Lima et al., 2004)

In what concerns the monoterpene constituents of the oil, the majority presented a percentage lower than 1% and were non-oxygenated (91.7%). The most abundant compounds were β-pinene (1.6%) and pseudolimonene (1.6%) (Table 1).

In contrast, although the sesquiterpenes of the volatile oil from *A. foetida* had been the main compounds similarly to that from *A. coriacea*, monoterpenes were not in its composition (Costa et al., 2009).

The trypanocidal activity against *T. cruzi* trypomastigotes was ascribed before to the oxygenated monoterpenes of the volatile oil from *Cymbopogum citratus* (Santoro et al., 2007). And, although they are in lower proportion in the *A. coriacea* oil, it is likely that they are related to the activity (IC50 168.50 μg/mL) (95% C.I. 99.10-286.60 μg/mL) of its other constituents, in a synergic behavior (Table 2). The same authors related this activity to the process of rupture of the cell wall, with the extravasations of the cytoplasm and of the nucleus of the parasite.

Likewise, researches have attributed to the oxygenated sesquiterpenes the activity against different species of *Leishmania* (Arruda et al., 2005). In *A. coriacea*, these constituents represent 39.1% of the sesquiterpene compounds found in its oil. Possibly, also a synergic action with the oxygenated monoterpenes caused the leishmanicidal action verified against the four species of *Leishmania*, in this study (Table 2), even considering the lower percentage related to monoterpenes. However more investigations are

required to know better the real mechanisms of action of the essential oils.

L. (L.) *chagasi* was the most sensible specie to the volatile oil from *A. coriacea* (IC50 39.93 µg/mL) (95% C.I.: 28.00-56.95 µg/mL) (Table 2). A similar behavior was observed against the same protozoa with the *A. foetida* oil (IC50 27.2 µg/mL) (Costa et al., 2009). Meanwhile, against the other two same *Leishmania* species, the volatile oil from *A. foetida* was more active than this of *A. coriacea*. Therefore, against *L.* (V.) *braziliensis* and *L* (L.) *amazonensis* parasites, the *A. coriacea* and *A. foetida* IC50 were, respectively: 261.20 (95% C.I.: 179.50-380.10 µg/mL); 9.9 µg/mL and 160.20 (95% C.I.: 117.70-218.10 µg /mL); 16.2 µg/mL.

This work is the first report of the volatile oil analysis from *A. coriacea* and contributed to improve the knowledge about this species and its genus, still little has been known in this respect. The chemosystematic markers of the Annonaceae family and of the genus were found in this *Annona* for the first time, being of importance for future researches. Since its antiprotozoal activity was confirmed, the species can be seen as a potential source of antiprotozoal molecules to be searched in further studies.

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***Correspondence**

Dominique C. H. Fischer
Laboratório de Farmacognosia, Departamento de Farmácia, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo
Av. Professor Lineu Prestes 580, 05508-000 São Paulo-SP, Brazil
domi@usp.br
Tel.+55 11 3091 3658
Fax: +55 11 3815 4418.