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Chemical composition and *in vitro* antiprotozoal activity of the volatile oil from leaves of *Annona crassiflora* Mart. (Annonaceae)

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Abstract

Neglected diseases like leishmaniasis and trypanosomiasis still remain a serious Public Health problem throughout the world with severe socio-economic impacts. There is an urge for new therapeutic alternatives and *Annona* essential oils have showed to be active against some of their protozoan agents, therefore encouraging this study. The volatile oil from the leaves of *A. crassiflora* (Mart.) (Annonaceae) was isolated (0.06% w/ w) in a Clevenger-type apparatus and analysed by GC-MS and GC-FID. Among the 41 identified compounds (83.2 %), sesquiterpenes were predominant (81.7 %), followed by monoterpenes (0.8 %) and other metabolites. The major constituents were α-amorphene (43.6 %), *E*-caryophyllene (17.7 %) and germacrene (5.3 %). The antiprotozoal activity was evaluated against four *Leishmania* species and *T. cruzi,* determining the parasites viability by MTT assay. The oil was mostly active in the promastigotes of *L.* (L.) *infantum chagasi* (IC_{50}: 25.97 µg/ mL), while the activity against *T.cruzi* trypomastigotes (IC_{50}: 5.31 µg/ ml) was nine folds higher than benznidazole and more active than those of the other *Annona* species. These results give support for a further study on the antiprotozoal compounds of *A.crassiflora*.

KEY WORDS: ANNONA CRASSIFLORA, ANNONACEAE, VOLATILE OIL, SESQUITERPENES, α-AMORPHENE, ANTIPROTOZOAL

Introduction

Neglected diseases have affected nearly one billion people in the world. Leishmaniasis and trypanosomiasis are serious health problems in Brazil and other countries of South and Central America, as well as in Asia and Africa, and may lead to severe conditions of high morbidity rates, physical incapacity and disfiguration or, in its most extreme phase, even to death (1).

In general, the usual recommended treatment presents some inconvenient in what concerns duration, parenteral administration, drug toxicity, parasites resistance, high costs, and other factors that influence the adherence of patients to the medication, showing there is a clear and urgent demand for new drugs.

The search for bioactive compounds from plants has been a current strategy to provide new drugs (2). Essential oils are one of the main secondary plant metabolites found in the Annonaceae family and in *Annona* (3,4).

Annona genus has 140 species in tropical regions, among which 33 are found in Brazil [5,6]. The volatile oils from *Annona foetida* Mart., *A. coriacea* Mart., *A. pickelii* (Diels) H. Rainer, *A. salzmannii* A. DC. and *A. vepretorum* Mart. showed *in vitro* antiparasitary activity against different protozoan agents (7-10).

Annona crassiflora Mart. (Annonaceae), popularly known as "araticum" and "marolo", is a native tree of the Brazilian Cerrado's Biome applied as antiparasitary in traditional medicine, but also for healing wounds, controlling louse infestation and treating venereal diseases, among other affections (11,12).

This species contains alkaloids, acetogenins, flavonoids and other phenolic compounds (13-16).

In a previous study, ethanol and total alkaloid extracts, obtained from the leaves, showed anti-*Leishmania* and anti-*Trypanosoma cruzi* activities (17). However, to the best of our knowledge, neither the composition nor the *in vitro* antiprotozoal activity of the volatile oil from *A. crassiflora* has been reported. Therefore, this turned to be the focus of this study.

Methods

Plant material

Leaves of *A. crassiflora* Mart. were collected in August 2009 at the Horto Florestal Andrade Silva, in Avaré, São Paulo state, Brazil. The exact collecting site lies at 22°45' 88" on the South longitude and at 49°11' 20" on the West latitude, at an altitude of 758 m.

A voucher specimen was deposited in the Herbarium of the Instituto de Biociências da Universidade de São Paulo (SPF) under the denomination of "Siqueira 3", after being identified by Dr. Renato de Mello-Silva, an Annonaceae specialist.

Standards, reagents and media

The standard compounds *E*-caryophyllene, β-pinene and globulol, of p.a. purity grades, were acquired from Fluka® (St. Louis, USA). A homologous series of *n*-alkanes (C_9-C_{20}) was obtained from the same supplier.

Pentamidine and benznidazole were purchased, respectively, from Sideron (Brazil) and Sigma (St Louis, MO USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Thiazol blue; MTT), M-199 and RPMI-PR-1640 (without phenol red) media were acquired from Sigma (St Louis, MO USA). The other analytical reagents were purchased from Sigma unless stated otherwise.

Isolation of the volatile oil

Samples of fresh leaves (540 g) were cut and triturated with solid CO_2 in a blender (Ametek®, 36BL54) at 18,000 rpm, for 3 min. Hydrodistillation was carried out in a Clevenger-type apparatus, for 4 h. The volatile oil was separated from the (frozen) water by glass capillary suction and stored under refrigeration (<10 °C), in a glass flask protected from light and humidity, until analysis.

GC-MS and GC-FID analyses

GC-MS analysis of the volatile oil was performed

in Agilent HP-6890 system operating in EI mode at 70 eV, using a fused HP-5 MS capillary column (5% phenylmethylpolysiloxane, 30 m x 0.25 mm i.d. x film thickness 0.25 µm) (J&W Scientific, Folsom, CA) directly coupled to a HP-5975 selective mass detector. The injected oil volume was 1.0 µL, split ratio: 1:20 at scan mode. The temperature was programmed from 60 to 240°C, at 3°C/ min for 60 min, and other conditions were: carrier gas: helium (1 mL/ min), injector temperature: 220°C; GC detector temperature: 250 °C. 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 hydrocarbon standards and the Van den Dool and Kratz (1963) (18) equation through which they were calculated. The identification of constituents and the comparison of their MS data with the external standard data were based on the spectrometry library of the equipment system (NIST 2005, Lib.), retention time, retention indices (RI) (19) and data reported in the literature (19) (Wiley Service Co.; SciFinder Scholar, USA).

The analysis of 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 maintenance

Isolated promastigotes of *Leishmania* (L.) *amazonensis* (WHO/BR/00/LT0016), *L.* (V.) *braziliensis* (MHO/BR/75/M2903), *Leishmania* (L.) *infantum chagasi* (MHOM/BR/1972/LD) and *Leishmania* (L.) *major* (MHOM/ 1L/80/Fredlin) were maintained in M-199 medium, supplemented with 10% calf serum and 0.25% hemin at 24°C. *L.* (L.) *infantum chagasi* (MHOM/BR/1972/LD) was maintained in hamsters (*Mesocricetus auratus*). *T. cruzi* trypomastigotes (Y strain) were maintained in LLC-MK2 (ATCC CCL 7) cells using RPMI-1640 medium supplemented with 2% calf serum at 37°C.

In vitro anti-Leishmania activity

Determination of the 50% inhibitory concentration (IC_{50}) against Leishmania spp. *promastigotes.*

Promastigotes were counted in a Neubauer haemocytometer and seeded at 1 x10^6/ well with a final volume of 100 µL. The volatile oil was solubilised in MeOH and diluted with M-199 medium in 96-well microplates to a maximum concentration of 500 µg/ mL. Controls with MeOH and without the oil were also performed. Pentamidine was used as standard drug at 100 µg/ mL. Two-fold serial dilutions were used over seven concentrations. Each experimental concentration was tested in duplicate. The plate was incubated for 24 h at 24°C and the viability of promastigotes was verified by morphology under light microscopy and MTT assay (20).

Briefly, MTT (5 mg/ mL) was dissolved in PBS, sterilized through 0.22 mm membranes and 20 µL were added per well for 4 h at 24 °C. Promastigotes were incubated without compounds and used as viability control. Formazan extraction was performed using 10% SDS for 18 h (100 µL/ well) at 24°C and the optical density (OD) was determined by a Multiskan MS (Uniscience®) at 570 nm. One hundred percent viability was expressed based on the OD of the control promastigotes after normalization.

In vitro anti-T. cruzi activity

Determination of the 50% inhibitory concentration (IC_{50}) against Trypanosoma cruzi trypomastigotes

The anti-*T. cruzi* assays were performed with free trypomastigotes according to the same conditions applied in the anti-*Leishmania* tests, excepted for the use of RPMI-1640 medium, benznidazole (reference drug) and incubation at 37 °C, in a 5 % CO_2 humidified chamber. The viability of the trypomastigotes was evaluated by MTT assay as described before.

Statistical analysis

Data were expressed as means and standard

deviation values of two independent assays from duplicate samples. The IC_{50} values were calculated using sigmoid dose–response curves in Graph Pad Prism 5.0 software and the 95% confidence intervals (CI) were included in parenthesis. The Mann–Whitney test was applied for significance of differences (*p* values).

Results and Discussion

Volatile oil analysis

The leaves revealed low contents of volatile oil (0.06 % w/ w in dry matter). Other species from this genus also presented little values of oil yielding, as seen for the leaves of *A. foetida* (0.01 %), *A. muricata* (0.01 %), *A. pickelli* (0.3 %) and *A. salzmannii* (0.04 %) (7,9,21). The highest amount of oil extracted from leaves was reported in *A. reticulata* (1.42 % w/ w) (22).

Forty one constituents were identified, corresponding to 83.2 % of the oil components. Most of them showed percentages lower than 1.0 and six had concentrations lower than 0.1 %. Percentages and retention indexes are showed in Table 1.

See Table 1.

Sesquiterpenes (81.7%) were the majority, followed by monoterpenes (0.8 %), aromatic compounds (0.4 %) and other constituents (0.3 %). A similar predominance of sesquiterpenes was previously seen in the oil from leaves some other species such as: *A. cherimolia*, *A. coriacea*, *A. densicoma*, *A. foetida*, *A. muricata*, *A. reticulata*, *A. senegalensis*, *A. squamosa*, *A. pickelli, A. salzmannii* and *A. vepretorum* (4,7-10,21-27).

The main constituents of *A. crassiflora* volatile oil were the sesquiterpene hydrocarbons α-amorphene (43.6 %), *E*-caryophyllene (17.7 %) and β-germacrene (5.3 %). The other sesquiterpenes were found in concentrations of 2.2% or lower.

The high percentage of α-amorphene is a remarkable character in this species when compared with the other studied ones, showing to be a probable chemical marker. It was also a component in the oil from leaves *A. coriacea* (0.1 %), *A. cherimolia* (2.2%) and *A. muricata* (0.2 %), nevertheless, in considerable lower amounts (8,23,27).

Besides, *E*-caryophyllene, a frequent compound in the Annonaceae family (4), has also been reported in the essential oil of different organs from eleven other species of *Annona* (7,8,21-23,25-30), in general, with a lower concentration than in *A. crassiflora*, excepted for *A. muricata* (40 %), *A. squamosa* (22.9 %), *A. pickelii* (27.8 %) and *A. salzmanii* (21.4 %) (9,21,26). Therefore, the high frequency of its occurrence in the genus, leads it to be considered a taxonomic marker (7).

Moreover, ß-germacrene (5.3 %) was also identified in leaves from *A. cuneata* (0.4 %), *A. densicoma* (1.4 %), *A. foetida* (0.8 %), *A. salzmanii* (2.7 %) and *A. senegalensis* (0.6 %) however, in smaller percentage when compared to *A. crassiflora* (7,9,21,24).

Furthermore, results clearly showed that the minority of the constituents were composed of oxygenated compounds (3.9 %), among which sesquiterpenes were predominant (3.2 %), most of them unsaturated cyclic alcohols such as spathulenol. Unless presenting low concentration (0.7%), its occurrence in all those species corroborate to the chemotaxonomic significance, since it was considered a marker for the Annonaceae family (31).

Concerning the minority of monoterpenes, two hydrocarbons were recorded: ß-pinene and α-thujene, which were also in small amounts in other *Annona* (8,21-24,27,32) while, to the best of our knowledge, the oxygenated isobornyl isobutanoate has not been identified in the species or in the genus.

Antiprotozoal activity

The essential oil was active against the four species of *Leishmania* and *T. cruzi*, as seen in Table 2.

see Table 2.

The promastigotes of *Leishmania* (L.) *infantum chagasi* were the most sensitive to this oil (IC_{50}:

25.97 µg/ mL) among the tested *Leishmania* species. A similar profile was found in a previous work, when tested with the oil of *A. coriacea* (8), although it showed to be less active (IC_{50}: 39.93 µg/ mL) than this. Nevertheless, *A. foetida* presented a closer level of activity (IC_{50}: 27.20 µg/ mL) to that of *A. crassiflora*, against the same parasite (7).

In contrast, the volatile oil against *T. cruzi* was almost 9 times more effective than benznidazole, the reference compound, in the same test conditions.

Comparatively to the oil from other species, the anti-*T.cruzi* activity of *A. crassiflora* was significantly higher in a ratio of, respectively, 31.7, 5.1, 16.9, and 6 times for *A. coriacea*, *A. pickelii*, *A. salzmannii* and *A. vepretorum* (7-10).

To the best of our knowledge, there is no reported data on the antiprotozoal activity of α-amorphene and β-germacrene, however a study showed that *E*-caryophyllene was effective against promastigotes of *L. amazonensis* (IC_{50}: 96 µM) (33).

Besides, two derivatives of an oxygenated caryophyllene-type structure compound presented a significant anti-*T. cruzi* activity, killing 100 % of the trypomastigotes bloodstream forms at concentrations of 5.6 and 6.5 µg/ mL (34), characterizing the potential of those compounds as a source of new bioactive analogs.

Moreover, some well-known natural-occurring oxygenated terpenes, when tested isolated demonstrated an anti-*Leishmania* activity, such as the sesquiterpenes artemisinin, nerolidol (35,36) and the monoterpene alcohol linalool (37).

Parallel to this, the hydrocarbon monoterpene β-pinene presented a moderated anti-*Trypanosoma* action against *T. brucei* (IC_{50}: 54.8 µg/ mL), when isolated (38).

Therefore, as oxygenated terpenes seem to play an important role in the antiprotozoal activity and non-oxygenated compounds were prevalent in the analysed volatile oil, investigations are required, mainly in what concerns its potential and high anti-*T. cruzi* activity. Likewise, its mechanism of action and a possible synergism among those compounds has to be studied.

Furthermore, a survey on the seasonal oil yielding is required to overcome the limitation of its low content that hindered the isolation of α-amorphene and to carry out deeper antiprotozoal tests.

Conclusion

This was the first study to report the anti-*Leishmania* and *Anti-T. cruzi* activities as well as the chemical composition of the volatile oil from leaves of *A. crassiflora* which shows to be a potential species for further studies in search of new antiprotozoal compounds.

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Compound[a]	**RI**[b]	%
α-Thujene	933	0.2
β-Pinene	977	0.6
δ-Elemene	1336	0.8
α-Cubebene	1347	tr
α-Ylangene	1373	0.6
β-Bourbonene	1382	1.8
β-Cubebene	1388	0.6
β-Elemene	1390	0.7
β-Isocomene	1406	0.2
E-caryophyllene	1417	17.7
β-copaene	1426	0.6
Isobornyl isobutanoate	1431	0.2
Trans-α-Bergamotene	1435	tr
α-Guaiene	1441	0.1
Spirolepechinene	1450	2.2
α-Patchoulene	1457	0,4
Allo-aromadendrene	1460	tr
Cis-cadina-1,6,4-diene	1463	0.2
α-Amorphene	1482	43.6
Aristolochene	1484	tr
Eudesmadiene	1489	0.2
Byciclogermacrene	1496	0.2
γ-Amorphene	1498	0.3
Premnaspirodiene	1502	0.9
α-Cuprenene	1504	0.2
δ-Amorphene	1511	0.2
β-Curcumene	1518	0.4
7-epi-α-Selinene	1521	1.4
Naphthalene	1535	0.1
β-Germacrene	1554	5.3
Spathulenol	1574	0.7
Trans-Sesquisabinene hydrate	1579	0.8
Globulol	1588	0.2
Rosifoliol	1598	0.1
4a(2H)-Naphthalenol	1625	0.1
1,7-diepi-α-Cedrenal	1634	tr
Epi-α-Cadinol	1639	0.5
Hinesol	1643	0.1
1-α-Cadinol	1651	0.7
Khusinol	1682	tr
Phenylmethyl ester	1766	0.3

Table 1: Chemical composition of the volatile oil from leaves of *A. crassiflora* Mart. [a]Compounds are listed in the elution sequence time on HP-5 MS capillary column. [b]Retention indices calculated on HP-5 MS capillary column, relative to C_9-C_{20} *n*-alkanes series. tr: traces (<0.1%)

Annona crassiflora	IC_{50} (µg/ mL) (95% CI)				
	L. (L.) *amazonensis*	*L.* (V.) *braziliensis*	*L.* (L.) *infantum chagasi*	*L.* (L.) *major*	*T. cruzi*
	promastigotes				trypomastigotes
Volatile oil*	39.19 (36.08 - 42.56)	31.69 (26.75 - 37.53)	25.97 (20.87 - 32.32)	28.62 (23.37 -35.06)	5.31 (3.99 - 7.05)
Reference 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	nd	nd	nd	nd	45.02 (29.31 - 68.42)

Table 2: *In vitro* activity of the volatile oil from leaves of *Annona crassiflora* Mart. against four species of *Leishmania* (promastigotes) and *Trypanosoma cruzi* (trypomastigotes). IC_{50}: 50% inhibitory concentration; 95% CI: 95% Confidence Interval, * Volatile oil= 500 µg/ mL, ** Reference drug= 100 µg/ mL, nd= not determined.

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