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Chemical composition and evaluation of antileishmanial and cytotoxic activities of the essential oil from leaves of *Cryptocarya aschersoniana* Mez. (Lauraceae Juss.)

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ABSTRACT

Leishmaniasis is an endemic disease caused by protozoa of the genus *Leishmania,* which affects around two million people worldwide. One major drawback in the treatment of leishmaniasis is the emergence of resistance to current chemotherapeutics. Medicinal and aromatic plants constitute a major source of natural organic compounds. In this study, the leaf essential oil of *Cryptocarya aschersoniana* was obtained by hydrodistillation in a Clevenger-type apparatus, and the chemical composition was analyzed by GC-MS and GC-FID. The essential oil of these species was predominantly constituted by monoterpene hydrocarbons (48.8%). Limonene (42.3%), linalool (9.7%) and nerolidol (8.6%) were the main constituents in the oil of *C. aschersoniana*. The *in vitro* activity of the oil was evaluated against the promastigote forms of *Leishmania amazonensis*, the causative agent of cutaneous leishmaniasis in humans. The essential oil of *C. aschersoniana* showed high activity against *L. amazonensis* promastigote forms (IC_{50} = 4.46 µg/mL), however, it also demonstrated a relatively high cytotoxicity on mouse peritoneal macrophages (CC_{50} = 7.71 µg/mL). This is the first report of the chemical composition and the leishmanicidal and cytotoxic activities of the leaf essential oil of *C. aschersoniana*.

Key words: *Cryptocarya aschersoniana*, Lauraceae, essential oil, *Leishmania amazonensis*, cytotoxic activity.

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INTRODUCTION

Leishmaniasis comprises a group of infectious diseases caused by parasites belonging to the *Leishmania* genus. This disease is among the six most important tropical diseases, affecting about 12 million people in 98 countries. It displays high endemicity, morbity and mortality, especially in Africa, Medium Orient, Latin America and Australia. In Brazil, leishmaniasis affects populations from 19 States, with predominance of rural transmission (Bastos et al. 2016).

The treatment of leishmaniasis is based on the pentavalent antimonials amphotericin B and pentamidines, which are toxic, expensive, difficult to administrate and can cause resistance in the parasites (Bastos et al. 2016, Estevam et al. 2017). It is clear therefore that the development of new antileishmanial agents has become an urgent matter. In this scenario, a number of papers have recently reported the antileishmanial potential of plant-derived essential oils (Bosquiroli et al. 2015, Oliveira et al. 2014).

Lauraceae is a botanical family known for comprising species of commercial interest due to their essential oils. The family includes approximately 50 genera and 2500 species. Among them, 400 species distributed in 25 genera are found in Brazil, with great incidence in the Amazon region (Yamaguchi et al. 2013). Published studies have described the chemical composition of Lauraceae essential oils as predominantly terpenes (Yamaguchi et al. 2013).

The genus *Cryptocarya* comprises about 350 species distributed mainly in Malaysia and Australia. Twenty-three species occur in South America and, among them, *C. mandioccana, C. moschata, C. saligna* and *C. bothelhensis* have already had the chemical composition of their essential oils previously identified (Telascrea et al. 2008). However, no reports were found in the literature on the chemical composition of the leaf essential oils of *C. aschersoniana* nor their anti-*Leishmania amazonensis* and cytotoxic activities.

The species *C. aschersoniana* is popularly known in Brazil as *canela-nhutinga*, and is an important native species belonging to the ecological group of the shade tolerant climax species. It is a tree of 15 to 25 m of height that is distributed from Minas Gerais to Rio Grande do Sul and stands out mainly for its good quality wood, which favors its indiscriminate exploitation (Bonetti 2016, Tonetti et al. 2016).

Considering the interest in species of the family Lauraceae, the objective of this study was to describe, for the first time, the chemical composition and antileishmanial and cytotoxic activities of the leaf essential oil of *Cryptocarya aschersoniana* grown in the South of Minas Gerais.

MATERIALS AND METHODS

PLANT MATERIAL

Cryptocarya aschersoniana Mez. (Lauraceae) was collected in June 2016, in the municipality of Machado, State of Minas Gerais, Southeastern Brazil (21°41'56"S and 45°52'59"W). The plant material was identified by the botanist Walnir G. F. Júnior. A voucher specimen (GERAES03) was deposited at the Herbário de Machado of the Departamento de Biologia, Instituto Federal de Educação, Ciência e Tecnologia do Sul de Minas Gerais, Brazil.

EXTRACTION OF THE ESSENTIAL OIL

Samples of fresh leaves of *C. aschersoniana* were subjected to hydrodistillation for 2 hours in a Clevenger-type apparatus (Carneiro et al. 2017). For the purpose of analysis, 300 g of plant material was divided into three samples of 100 g each, and 500 mL of distilled water was added to each sample. After manual collection of the essential oil (EO) samples, traces of remaining water in the oil was removed with anhydrous sodium sulfate,

which was followed by filtration. The extraction procedure was done in triplicate. The isolated oil was stored under refrigeration until analyzed and tested. The yields (w/w) were calculated from the weight of the fresh leaves and expressed as the average of triplicate analysis.

IDENTIFICATION OF THE ESSENTIAL OIL CHEMICAL COMPOSITION

Gas chromatography (GC) analyses were performed on a Shimadzu GC2010 Plus gas chromatograph equipped with an AOC-20s autosampler and fitted with FID and a data-handling processor. An Rtx-5 (Restek Co., Bellefonte, PA, USA) fused silica capillary column (30m x 0.25-mm i.d. 0.25 μm film thickness) was employed. The operation conditions were as follows: column temperature programmed to rise from 60 to 240 °C at 3 °C/min and then held at 240 °C for 5 min; carrier gas = He (99.999%), at 1.0 mL/min; injection mode; injection volume, 0.1 μL (split ratio of 1:10); and injector and detector temperatures = 240 and 280 °C, respectively. Components relative concentrations were obtained by peak area normalization (%). The relative areas were the average of triplicate GC-FID analyses.

GC-MS analyses were carried out on a Shimadzu QP2010 Plus (Shimadzu Corporation, Kyoto, Japan) system equipped with an AOC-20i autosampler. The column was a RTX-5MS (Restek Co., Bellefonte, PA, USA) fused silica capillary column (30m x 0.25mm i.d. x 0.25μm film thickness). Electron ionization mode occurred at 70 eV, Helium (99.999 %) was employed as the carrier gas at a constant flow of 1.0 mL/min. The injection volume was 0.1 μL (split ratio of 1:10). The temperatures of the injector and the ion-source temperature were set at 240 and 280 °C, respectively. The oven temperature program was the same as the program used for GC. Mass spectra were taken with a scan interval of 0.5 s, in the mass range from 40 to 600 Da.

The identification of the volatile components from leaves of *C. aschersoniana* (Table I) was based on their retention indices on an Rtx-5MS capillary column under the same operating conditions as in the case of GC relative to a homologous series of *n*-alkanes (C_8-C_{20}); structures were computer-matched with the Wiley 7, NIST 08 and FFNSC 1.2 spectra libraries, and their fragmentation patterns were compared with literature data (Adams 1995).

ANTILEISHMANIAL ASSAY

In order to evaluate leishmanicidal activity, *L. amazonensis* promastigote forms (MHOM/BR/PH8) were maintained in RPMI 1640 (Gibco) culture medium supplemented with 10% fetal bovine serum. Subsequently, about $1x10^6$ parasites were distributed on 96-well plates. The essential oil was previously dissolved in 100% dimethylsulfoxide (DMSO, stock solution 10 $mg.mL^{-1}$ (Synth)) and added to the cultures at concentrations from 1.56 to 50 $\mu g.mL^{-1}$. Amphotericin B was previously dissolved in 100% DMSO at concentration of 1 $mg.mL^{-1}$; afterwards, it was diluted in stock solution 500 $\mu g.mL^{-1}$ in the culture medium (Synth) and added to cultures at concentrations from 0.19 to 3.12 $\mu g.mL^{-1}$. Cultures were incubated at 25 °C in BOD ovens (Quimis) for 24 h and the leishmanicidal activity was determined by growth inhibition of promastigote forms by counting the total number of live promastigotes in the Neubauer chamber (Global Glass, Porto Alegre, BR), considering flagellar motility. RPMI 1640 medium (Gibco) containing 0.5% DMSO (Synth) (highest concentration) was used as negative control and Amphotericin B (Eurofarma, São Paulo, BR) at 1 $\mu g.mL^{-1}$ concentration was used as positive control. Results were expressed as the mean of the lysis percentage relative to the negative control (0.1% DMSO). Two experiments were performed in triplicate. Determination of 50% inhibitory concentration values (IC_{50}) was carried

out by non-linear regression curves of a GraphPad Prism version 5.0 Windows software (GraphPad software, USA). Maintenance of life cycle was approved by the Ethics Committee for Animal Care at the University of Franca, under protocol number 010/14.

CYTOTOXICITY ASSAY

In order to obtain the peritoneal macrophages, BALB/c. mice were intraperitoneally injected with 500 μL of 3% sodium thioglycollate. After 72 hours, the mice peritonea were washed with 5 mL of ice-cold phosphate buffered saline (PBS 1X), and the cells collected during washing were centrifuged at 1000 rpm for 10 minutes at 4 °C. The supernatant was removed and the pellet (cells) was added with 10 ml of RPMI 1640 (Gibco) ice cold medium supplemented with 10% inactivated fetal bovine serum and 1% antibiotic (10,000 U/mL penicillin and 10,000 mg/mL streptomycin).

The cells were counted in a Neubauer's chamber and adjusted to a concentration of 2 x 10^5 cells/mL. The cells were then seeded in a 96-well plate with supplemented RPMI 1640 medium (Gibco).

The cultures were incubated at 37 °C in the presence of 5% CO_2 for 24 and 48 hours, and cell viability was determined by the colorimetric MTT metabolic activity assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)], which assesses the ability of metabolically active cells to reduce MTT by converting their yellow salts to purple formazan crystals.

The essential oil was analyzed at the same concentrations as the assays on promastigote forms and the results were expressed as the mean percent reduction in cell viability versus the negative control (0.1% DMSO). Experiments were performed in triplicate. The 50% cytotoxic concentration (CC_{50}) values were determined by means of non-linear regression curves using GraphPad Prism version 5.0 software for Windows (GraphPad software, USA).

RESULTS AND DISCUSSION

Thirty-four chemical constituents were identified in the leaf essential oil of *C. aschersoniana*, representing 97% of the total compounds. Table I shows these constituents including their respective retention indices, retention time and percentages.

The leaf essential oil of *C. aschersoniana* showed a high yield (w/w on fresh weight basis) of 3.5%, similar to the previously observed yields for the essential oils of other species of the Lauraceae family. For example, yields of 4.9 and 2.5% for *Endlicheria citriodora* essential oils and 1.5% for *Aniba rosaeodora*, which were considered high, are reported in the literature (Yamaguchi et al. 2013).

The analysis of *C. aschersoniana* leaf essential oil showed a complex mixture of monoterpenes and sesquiterpenes, with emphasis on monoterpene hydrocarbons (48.8%), oxygenated monoterpenes (13.4%) and oxygenated sesquiterpenes (26.7%). The major constituents identified were: limonene (42.3%), linalool (9.7%) and nerolidol (8.6%). The chemical composition observed in the present study was similar to the chemical composition already described in the literature for other species belonging to the same genus (Telascrea et al. 2008).

Similarly to what was found in the leaf essential oil of *C. aschersoniana*, the compounds limonene (42.3%), linalool (9.7%) and nerolidol (8.6%) have previously been described as major constituents of the essential oils of three other species of the family Lauraceae; *Litsea helferi*, *Litsea verticillata* and *Persea duthiei*, which exhibited 17.5% of limonene, 23.4% of linalool and 13.2% of nerolidol (Le et al. 2014, Joshi et al. 2009). β-Caryophyllene (0.3%), a common volatile metabolite among the essential oils of the Lauraceae family, was also found in the leaf essential oil of *C. aschersoniana*, but in a smaller quantity.

TABLE I
Chemical composition of the leaf essential oil of *C. aschersoniana* (Lauraceae).

RT (min)	*Compounds*	RI_{exp}	RI_{lit}	*RA %*
6.32	Hex-3(*Z*)-en-1-ol	858	857	1.5
9.09	α-Pinene	935	939	0.6
10.94	β-Pinene	979	980	0.9
13.21	*p*-Cymene	1030	1029	4.6
13.34	Limonene	1033	1034	**42.3**
13.45	Eucalyptol	1035	1035	0.8
14.75	γ-Terpinene	1063	1062	0.4
16.20	*trans*-Linalool oxide	1095	1093	1.8
17.04	Linalool	1112	1110	**9.7**
20.36	*trans*-Pyranoid linalool oxide	1179	1178[a]	0.3
20.49	Terpinen-4-ol	1181	1180	0.3
26.07	Limonene dioxide	1302	1300[b]	0.5
29.44	α-Copaene	1378	1376	0.5
29.52	β-Elemene	1379	1379	0.7
30.31	β-Cubebene	1398	1397	1.0
31.01	β-Caryophyllene	1414	1415	0.3
32.12	Aromadendrene	1441	1439	0.7
33.67	Gemacrene D	1479	1480	0.6
33.87	α-Amorphene	1485	1485[c]	1.3
34.09	β-Selinene	1490	1489	0.5
34.25	δ-Selinene	1494	1495[d]	0.4
34.64	Viridiflorene	1504	1505	1.0
35.17	γ-Cadinene	1518	1518	0.4
35.53	δ-Cadinene	1527	1525	0.8
36.01	Hedycaryol	1539	1538[e]	0.5
37.30	Nerolidol	1573	1572	**8.6**
37.72	Spathulenol	1583	1584	6.6
37.88	Isoaromadendrene epoxide	1588	1585	0.8
38.53	Guaiol	1605	1604	2.5
38.78	Globulol	1612	1610	0.5
39.34	δ-Cadinol	1627	1628[f]	1.2
39.65	Cubenol	1636	1637	0.4
40.64	α-Cadinol	1663	1663	2.5
41.11	Bulnesol	1676	1675	1.5
	Monotepene hydrocarbons			48.8
	Oxygenated monoterpenes			13.4
	Sesquiterpene hydrocarbons			8.2
	Oxygenated sesquiterpenes			25.1
	Others			1.5
	Total			97.0

RT: Retention time; RI_{exp}**:** Retention index determined relative to *n*-alkanes (C_8–C_{20}) on the Rtx-5MS column; RI_{lit}**:** Retention index from literature (Adams 1995); ***RA %*****:** relative area (peak area relative to the total peak area in the GC-FID chromatogram), average of three replicates. [a] from Boulanger and Crouzet (2000); [b] from Hognadóttir and Rouseff (2003); [c] from Karioti et al. (2003); [d] from Albuquerque et al. (2004); [e] from Bin Ahmad and Bin Jantan (2003); [f] from Hamm et al. (2005).

Linalool (9.7%), an important constituent found in essential oils of several species of Lauraceae, deserves special attention. This open-chain tertiary monoterpene alcohol has been successfully applied as sedative, anticonvulsant and also has wide application in the fragrance and flavor industry (Monteiro et al. 2005).

The leishmanicidal potential of essential oils has been well studied (Cardoso et al. 2015), and the leaf essential oil of *C. aschersoniana* displayed high leishmanicidal activity when tested against *L. amazonensis* promastigote forms. Increase in parasite lysis was observed with increase in essential oil concentration, with IC_{50} = 4.46 µg/mL (Table II). The leaf essential oil of *C. aschersoniana* inhibited parasite growth in a concentration/dose-dependent manner. As positive control was used amphotericin B (IC_{50} = 1.88 µg/mL), an antifungal with broad spectrum of action that is used as second-line drug against leishmaniasis and that show a high toxicity in the host (Bastos et al. 2016, Fernández-García et al. 2017).

Regarding the leishmanicidal activity (IC_{50} values), the literature describes that samples having IC_{50} < 10 µg/mL are considered highly active, (IC_{50} > 10 < 50 µg/mL) active, (IC_{50} > 50 < 100 µg/mL) moderately active, and (IC_{50} > 100 µg/mL) inactive (de Lima et al. 2012).

The high leishmanicidal activity of the essential oil from leaves of *C. aschersoniana* may be related to the presence of the chemical components limonene (42.3%), linalool (9.7%) and nerolidol (8.6%), which are the major constituents in the essential oil studied and with anti-*Leishmania* activity already known (Arruda et al. 2005, Graebin et al. 2010, Camargo and Vasconcelos 2014). Limonene has also been previously reported as the compound responsible for the leishmanicidal activity exhibited by the essential oils of *Citrus limonia* and *Citrus latifolia* (Estevam et al. 2016). The leishmanicidal potential of the linalool was already reported in the literature against promastigote and amastigote forms of *L. amazonensis* (LD_{50} of 4.3 ng/mL and 15.5 ng/mL, respectively) (Camargo and Vasconcelos 2014). Nerolidol, in turn, showed promising leishmanicidal activity against the promastigote forms of *Leishmania amazonensis*, *L. braziliensis*, and *L. chagasi* (Arruda et al. 2005). The treatment of macrophages infected by *L. amazonensis* with 100 µM of nerolidol resulted in 95% reduction in infection rates (Arruda et al. 2005). However, further studies should be addressed to verify the occurrence of possible synergistic and/or additive effects between these compounds.

The *in vitro* cytotoxic activity of the *C. aschersoniana* leaf essential oil and the drug amphotericin B against peritoneal macrophages is shown in Table III.

This is the first report of the cytotoxic activity of the leaf essential oil of *C. aschersoniana.* The oil evaluated in this study showed high toxicity to

TABLE II
Leishmanicidal activity of the leaf essential oil of *C. aschersoniana* against *L. amazonensis* promastigote forms.

	% of lysis ± S.D /Concentration (µg.mL^{-1})						IC_{50} (µg/mL)
	100	**50**	**25**	**12.5**	**6.25**	**3.12**	
EOCA	100 ± 0.00	100 ± 0.00	97.86 ± 1.88	72.68 ± 1.48	52.96 ± 1.88	45.60 ± 1.48	4.46
	50	25	12.5	6.25	3.12	1.56	
Amph. B	100 ± 0.00	99.98 ± 0.10	96.15 ± 0.54	85.84 ± 0.24	80.78 ± 0.29	75.5 ± 0.57	1.88

EOCA: Leaf essential oil of *Cryptocarya aschersoniana*. **Negative Control:** RPMI Medium + 0.1% DMSO. **Amph. B:** Amphotericin B.

TABLE III
Cytotoxicity of the leaf essential oil of *C. aschersoniana* and Amphotericin B.

	Concentrations (µg/mL) ± Standard Deviation					CC_{50} (µg/mL)
	50	25	12.5	6.25	3.12	
EOCA	84.53 ± 2.68	84.32 ± 4.24	80.70 ± 7.80	36.62 ± 6.13	13.86 ± 3.85	7.71
Amph. B	52.54 ± 3.04	51.44 ± 1.90	50.64 ± 2.21	19.76 ± 0.08	9.73 ± 0.38	51.86

EOCA: Leaf essential oil of *Cryptocarya aschersoniana*. **Amph. B:** Amphotericin B. **Positive control:** 25.0% DMSO; **Negative control:** 0.1% DMSO.

mouse peritoneal macrophages (CC_{50} = 7.71 µg/mL), while the drug amphotericin B was less toxic (CC_{50} = 51.86 µg/mL). Toxicity levels are reported in the literature as highly toxic CC_{50} < 10 µg/mL, toxic (10 < CC_{50} < 100 µg/mL), moderately toxic (100 < CC_{50} < 1000 µg/mL), and nontoxic (CC_{50} > 1000 µg/mL) (de Lima et al. 2012).

Evaluation of cytotoxicity is important because it allows us to understand the biological mechanism that produces the cytotoxic effect and the mechanism of action of different substances in their interaction with tissues. However, it is recognized that the use of cell cultures is not physiological and does not replicate the actual architecture of the living tissue in which the underlying cells could repair the aggressions suffered. Thus, the occurrence of *in vitro* cytotoxic effect does not guarantee that the evaluated sample is toxic when applied *in vivo* (Marreiro et al. 2014).

In summary, the strong anti-*Leishmania amazonensis* activity and cytotoxicity observed for the leaf essential oil of *C. aschersoniana* can be explained by the fact that most of the leaf essential oil contains a large number of compounds that have no specific cellular targets. Essential oils have non-polar character and can easily cross the cell walls and cytoplasmic membranes (Estevam et al. 2018). Thus, essential oil components cross the membrane, cause the cytoplasm to coagulate, denature proteins, disrupt metabolic pathways such as biosynthesis of various lipids, and ultimately lead to cell death through necrosis and apoptosis (Raut and Karuppayil 2014).

The results in this study show that the leaf essential oil of *C. aschersoniana* has strong anti-*Leishmania amazonensis* activity and high toxicity against mouse peritoneal macrophages. Previous studies on the leishmanicidal potential of some of the chemical constituents identified in the essential oil of *C. aschersoniana* also corroborate the potential observed in the present investigation. However, further *in vivo* studies are needed to confirm and evaluate the potential use of the leaf essential oil of *C. aschersoniana* as a leishmanicidal agent.

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