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Essential oil of *Mitracarpus frigidus* as a potent source of bioactive compounds

RODRIGO L. FABRI[1], ELAINE S. COIMBRA[2], ANA C. ALMEIDA[2], EZEQUIAS P. SIQUEIRA[3], TÂNIA M.A. ALVES[3], CARLOS L. ZANI[3] and ELITA SCIO[1]

[1]Laboratório de Produtos Naturais Bioativos, Departamento de Bioquímica, Instituto de Ciências Biológicas, Universidade Federal de Juiz de Fora, Rua José Lourenço Kelmer, s/n, São Pedro, 36036-900 Juiz de Fora, MG, Brasil
[2]Departamento de Parasitologia, Microbiologia e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Juiz de Fora, Rua José Lourenço Kelmer, s/n, São Pedro, 36036-900 Juiz de Fora, MG, Brasil
[3]Centro de Pesquisa René Rachou – Fiocruz, Avenida Augusto Lima, 1715, Barro Preto, 30190-002 Belo Horizonte, MG, Brasil

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ABSTRACT

In our previous work (Fabri et al. 2009), we showed that different extracts of *Mitracarpus frigidus* had significant antibacterial, antifungal and leishmanicidal activities. In order to increase our knowledge about this species, this work assesses the chemical composition and the *in vitro* biological activity of its essential oil. Thus, the essential oil obtained by hydrodistillation of the aerial parts of *M. frigidus* was analyzed by GC/MS. Among several compounds detected, 11 were identified, being linalool and eugenol acetate the major components. The essential oil exhibited a moderate antibacterial effect against *Staphyloccocus aureus*, *Bacillus cereus*, *Pseudomonas aeruginosa* and *Enterobacter cloacae* (MIC 250 μg/mL). On the other hand, it showed a strong antifungal effect against Cryptoccocus neoformans (MIC 8 μg/mL) and Candida albicans (MIC 63 μg/mL). Expressive activity against *L. major* and *L. amazonensis* promastigote forms with IC_{50} values of 47.2 and 89.7 μg/mL, respectively, were also observed. In addition, the antioxidant activity was investigated through DPPH radical-scavenging and showed a significative activity with IC_{50} of 38 μg/mL. The cytotoxicity against *Artemia salina* was moderate with LC_{50} of 88 μg/mL. The results presented here are the first report on the chemical composition and biological properties of *M. frigidus* essential oil.

Key words: *Mitracarpus frigidus*, essential oil, antimicrobial, antileishmanial, cytotoxicity.

INTRODUCTION

Some *Mitracarpus* species have ethnopharmacological importance as they are used in folk medicine for various purposes. For example, *M. scaber* is widely employed in traditional medicine in West Africa for headaches, toothache, amenorrhea, dyspepsia, hepatic diseases and leprosy. Among those folkloric uses, the juice of the plant is applied topically for the treatment of skin diseases (Dalziel 1936, Kerharo and Adam 1974).

The potential antimicrobial activity of the aerial parts extracts of some *Mitracarpus* species against bacterial and mould strains, like *Staphylococcus aureus*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Candida albicans* and *Cryptococcus neoformans* were already reported (Irob and Daramola 1993,

Correspondence to: Elita Scio
E-mail: elita.scio@ufjf.edu.br

1994, Sanago et al. 1996). Also, in our previous work, we showed that *M. frigidus* extracts had a significant antibacterial, antifungal and leishmanicidal activities (Fabri et al. 2009). So, in order to increase our knowledge about *M. frigidus*, this work deals with the chemical composition and the *in vitro* activity against Gram-positive and Gram-negative bacteria and yeasts strains, promastigote forms of *Leishmania* species and cytotoxicity against *Artemia salina* of the essential oil of this specie. Its *in vitro* antioxidant activity was also determined.

MATERIALS AND METHODS

Plant Material

M. frigidus aerial parts were collected in Juiz de Fora, Minas Gerais, Brazil, in May, 2006. Dr. Tatiana Konno identified the plant. A voucher specimen (CESJ 46076) was deposited at the Herbarium Leopoldo Krieger of the Universidade Federal de Juiz de Fora.

Hydrodistillation of Volatile Oils

Essential oil was obtained by means of hydrodistillation (3 h) of the dried plant material using a Clevenger type apparatus. Oil was dried and stored at 4°C until tested and analyzed.

Collection of Volatiles by Solid Phase Micro-Extration (SPME)

Fifty microliters from the 20 mg/mL solutions were transferred to a 2 mL glass vial and the solvent was removed under vacuum (speedVac®, SC250 model, ThermoSavant, U.S.A) for 18 h, 30°C and 10 millibar. The vial was closed with a cap sealed Teflon coated septum (Supelco, U.S.A) and placed in a heat block adjusted to 90°C. A SPME fiber (PDMS/DVB™ 65 μm, SUPELCO, U.S.A) was inserted with a manual holder through the septum and left in the headspace for 30 min. Immediately after that, the holder with the saturated fiber was analyzed by means of injection on gas chromatography (GC) injection port. Before use, the fiber was preconditioned at 230°C during 30 min in the GC injector port.

Gas Chromatography/Mass Spectrometry Analysis

Gas Chromatography/Mass Spectrometry (GC-MS) analyses were performed on a Shimadzu QP-5050A (SHIMADZU, JP) instrument, equipped with a PTE™-5 column (30 m, 0.25 mm, 0.25 μm, Supelco, USA), using helium as the carrier gas. The following conditions were employed for all analysis: helium at 22.3 mL/min; injector temperature maintained at 230°C; the oven at 80°C during 3 minutes and then heated to 300°C at 7°C/min, holding for 5 min at 300°C. The split valve was closed during the first minute of injection and then opened, with a 1:10 ratio. The mass detector was set to scan from 50 to 500 *m*/*z*, at a rate of 2 scans per second. Data acquisition and handling was done via CLASS 5000 Shimadzu software.

Analysis of the Raw Data using AMDIS Software (Automated Mass Spectral Deconvolution and Identification System)

Raw data files were analyzed by Automated Mass Deconvolution and Identification System software (AMDIS), version 2.1, supplied by National Institute of Standards and Technology (NIST, USA). Retention Index (RI) in the range of 900 to 3,000 was generated from the analysis of a standard mixture containing hydrocarbons C9 to C30 (Fluka, U.S.A).Elucidation of the compounds was done by means of the NIST MS Search 2.0 Program (NIST/EPA/NIH Mass Spectral Library, version 2002) and on the basis of comparison of retention indices determined according Van Den Dool and Kratz (1963) for each constituent, as well as, previously described by Adams (2007).

Microbial Strains

The sample was evaluated against a panel of microorganisms, including *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 15442), *Salmonella enterica* sorovar Typhimurium (ATCC 13311), *Shigella sonnei* (ATCC 11060), *Klebsiella pneumoniae* (ATCC 13866), *Escherichia coli* (ATCC 10536), *Bacillus cereus* (ATCC 11778), *Micrococcus luteus* (ATCC 10054), *Enterococcus faecalis* (ATCC 51299), *Enterobacter cloacae* (ATCC 10699), *Streptococcus pyogenes* (ATCC 10096), *Candida albicans* (ATCC 18804) and *Cryptococcus neoformans* (ATCC 32608). Bacterial strains were cultured overnight at 37°C in Mueller Hinton agar (MHA). Yeasts were cultured for 48 h at 30°C in Sabouraud dextrose agar (SDA).

Serial Dilution Assay for Determination of the Minimal Inhibitory Concentration (MIC)

The minimal inhibitory concentration (MIC) was determined by using broth microdilution techniques for bacterial and yeasts (NCCLS 2002 Perez et al. 1990). MIC values were determined in RPMI 1640 (Sigma) buffered to a pH 7.0 with MOPS (Sigma) for yeasts and Mueller Hinton broth (MHB) for bacteria. Yeasts were cultured at 30°C for 48 h in SDA and bacteria were cultured overnight at 37°C for 24 h in MHA. Sample stock solutions were two-fold diluted from 5,000 to 2.5 μg/mL (final volume = 80 μL) and a final DMSO concentration ≤ 1%. Then, 100 μL of RPMI or MHB were added onto microplates. Finally, 20 μL of 10^6 CFU/mL (according to McFarland turbidity standards) of standardized yeasts and bacterial suspensions were inoculated onto microplates and the test was performed in a volume of 200 μL. Plates were incubated at 30°C for 48 h for yeasts and 37°C for 24 h for bacteria. The same tests were performed simultaneously for growth control (RPMI + yeast and MHB + bacteria) and sterility control (RPMI or MHB + essential oil). The test was performed in duplicate. The MIC values were calculated as the highest dilution showing complete inhibition of tested strain.

DPPH Radical Scavenging Assay

The free radical scavenging activity of sample solutions in methanol was determined based on their ability to react with stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals (Govidarajan et al. 2003). The essential oil at various concentrations (7.8 to 250 μg/mL) was added to a 152 μM solution of DPPH in methanol. After incubation at 37°C for 30 min, the absorbance of each solution was determined at 517 nm. The antioxidant activity of essential oil was expressed as IC_{50} (inhibitory concentration), which was defined as the concentration (in μg/mL) of sample required to inhibit the formation of DPPH radicals by 50 %. α-Tocopherol and rutin were used as positive control.

Antileishmanial Assay

Three species of *Leishmania* were used in this study: *L. chagasi* (MHOM/Br/74/PP75), *L. amazonensis* (MHOM/Br/75/Josefa) and *L. major* (MRHO/SU/59/P). Anti-leishmanial activity was determined by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method based on tetrazolium salt reduction by mitochondrial dehydrogenases (Braga et al. 2007). Briefly, promastigotes of *L. amazonensis* and *L. major* were cultured in Warren's medium (brain heart infusion plus hemin and folic acid) and promastigotes of *L. chagasi* were maintained in Medium 199, both supplemented with 10 % fetal bovine serum at 24°C. The screening was performed in 96-well microtiter plates maintained at 24°C. The essential oil solution in DMSO was added in a serial solution in the wells (11 to 108 μg/mL). Then a parasite suspension from a logarithmic phase

culture was suspended to yield 2 million cells/mL (*L. amazonensis* or *L. major*) or 3 million cells/mL (*L. chagasi*) after Neubauer chamber counting. Controls with DMSO and without plants samples were performed. All the tests were performed in triplicates. The viability of promastigotes was assayed after a three day incubation period with addition of MTT. The reaction was stopped with HCl in isopropyl alcohol and the optical densities were evaluated in a spectrophotometer at 570 nm (Multiskan MS microplate reader, LabSystems Oy, Helsink, Finland). The results were expressed as the concentrations inhibiting parasite growth by 50% (IC_{50}) and the percentage of inhibition of parasite growth. Amphotericin B was used as the standard drug.

Cytotoxicity Assay

Brine shrimp lethality bioassay (Meyer et al. 1982) was carried out to investigate the cytotoxicity of the essential oil. Brine shrimp (*Artemia salina* Leach) eggs were hatched in a beaker filled with sea water under constant aeration. After 48 h, the nauplii were collected by pipette and were counted macroscopically in the stem of the pipette against a lighted background. Solutions of the essential oil were made in seawater containing 1 % DMSO, at varying concentrations (10 to 1,000 μg/mL) and incubated in triplicate vials with 10 brine shrimp larvae. After 24 h of incubation, the nauplii were examined against a lighted background, with a magnifying glass and the number of survivors in each vial were counted and noted. Both positive (thymol) and negative (sea water containing 1 % DMSO) control assays were carried out in order to verify the susceptibility of A. salina under assay conditions employed.

Statistical Analysis

The IC_{50} for antioxidant activity was calculated by Grafit 5. The IC_{50} for leishmanicidal activity and cytotoxicity were calculated by Probit analysis. Both were expressed as mean ± standard error (SE). The inhibition percentages for leishmanicidal activity were analyzed by Graph Pad Prisma 4. Statistical differences between the treatments and the control were evaluated by ANOVA test.

RESULTS

Chemical Composition

Water-distillation of the dried aerial parts of *M. frigidus* yielded 0.01 % (v/w) of an orange oil. Chemical analysis of the oil's components resulted in the identification of 12 known components (Table I). Linalool (29.29%) and eugenol acetate (15.85%) were the major constituents of the essential oil, followed by 5-hydroxy-isobornyl isobutyrate (8.41%), 5-methyl-1-undecene (7.69%) and methyl salicylate (6.55%).

Antimicrobial Activity

The essential oil of *M. frigidus* exhibited a moderate antibacterial effect against *Staphyloccocus aureus*, *Bacillus cereus*, *Pseudomonas aeruginosa* and *Enterobacter cloacae* (MIC 250 μg/mL) (Table II). On the other hand, the oil showed a strong antifungal effect against *Cryptoccocus neoformans* (MIC 8 μg/mL) and *Candida albicans* (MIC 63 μg/mL). It was considered that if the extracts displayed a MIC less than 100 μg/mL, the antimicrobial activity was good; from 100 to 500 μg/mL, moderate; from 500 to 1,000 μg/mL, weak; over 1,000 μg/mL the extract was considered inactive (Holetz et al. 2002).

Antileishmanial Activity

The effect of the essential oil of *M. frigidus* on the viability of promastigotes of *L. major*, *L. amazonensis* and *L. chagasi* was tested. The essential oil showed expressive activity against *L. major* and *L. amazonensis* promastigote forms with IC_{50} values of 47.2 ± 4.0 and 89.7 ± 8.6 μg/mL, respectively. Those forms were susceptible to the essential oil

TABLE I
Chemical constituents, retention index and relative concentration of compounds of essential oil of *Mitracarpus frigidus* analyzed by GC-MS.

No	Compound	Retention time (min)	% relative	Retention index	Molecular Mass
1	7-Octen-4-ol	4.07	5.03	850.8	128
2	5-Hydroxy-isobornyl isobutyrate	5.0	8.41	1033.9	276
3	5-methyl-1-Undecene	6.39	7.69	1092.1	168
4	Linalool	6.53	29.29	1098.9	154
5	*β*-Phenylethyl alcohol	6.85	3.79	1113.5	122
6	Cucumber aldehyde	7.63	3.55	1149.4	138
7	*trans*-2-Nonenal	7.77	4.83	1155.6	140
8	Methyl salicylate	8.61	6.55	1194.3	152
9	8-Isobutyryloxy-isobornyl isobutyrate	9.38	4.66	1230.1	281
10	Not known	9.87	5.99	1253.0	341
11	Eugenol acetate	12.04	15.85	1357.6	206
12	Damascenone	12.58	4.36	1384.7	190

TABLE II
Antimicrobial activity of *Mitracarpus frigidus* essential oil.

Test microorganisms	MIC (μg/mL)[a)]	S[b)]
Bacteria		
Staphylococcus aureus	250	62.5[c)]
Escherichia coli	500	15.6[c)]
Salmonella typhimurium	1,000	1.0[c)]
Shigella sonnei	1,000	1.0[c)]
Klebsiella pneumoniae	500	1.0[c)]
Bacillus cereus	250	3.9 [c)]
Pseudomonas aeruginosa	250	15.6[c)]
Enterobacter cloacae	250	31.3[c)]
Enterococcus faecalis	1,000	31.3[c)]
Streptococcus pyogenes	500	15.6[c)]
Yeasts		
Candida albicans	63	0.08[d)]
Cryptococcus neoformans	8	0.04[d)]

[a)] Minimum inhibitory concentration.

[b)] Standard antimicrobial agents: [c)] Chloramphenicol; [d)] Amphotericin B.

at 108 μg/mL with 97% and 86% cell inhibition, respectively (Figure 1). Interestingly, *L. major* presented the greatest dose-dependent relationship. On *L. chagasi*, the percentage of inhibition did not varied significantly between the concentrations used. The percentage of inhibition presented by reference drug Amphotericin B at 10 μg/mL was 60 %, 90% and 86% for *L. major*, *L. amazonensis* and *L. chagasi* promastigotes, respectively.

Antioxidant Activity

The antioxidant activity of the essential oil of *M. frigidus* was investigated through DDPH radical-scavenging and showed a moderate activity with IC_{50} of 38 ± 8 μg/mL. The reference controls α-tocopherol and rutin presented IC_{50} of 0.2 ± 0.1 and 3 ± 1.8 μg/mL, respectively.

Cytotoxicity

The cytotoxicity against *Artemia salina* was moderate with LC_{50} 88 ± 10 μg/mL. The reference control thymol showed LC_{50} of 1.4 ± 0.7 μg/mL.

DISCUSSION

The essential oil exhibited a moderate antibacterial and a strong antifungal effect against *Cryptoccocus neoformans* and Candida albicans. Also, it showed expressive activity against *Leishmania major* and *L. amazonensis* promastigote forms.

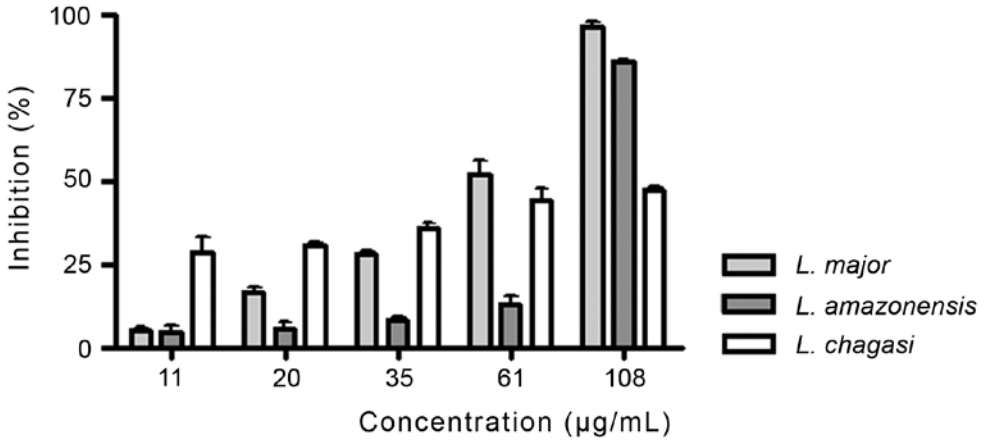

Figure 1 - Effect of the essential oil from *M. frigidus* on growth of *L. major*, *L. amazonensis* and *L. chagasi* promastigote forms. Parasites were treated with 11 to 108 µg/mL essential oil. Each bar represents the mean ± standard deviation of three different experiments.

Linalool and eugenol acetate were the major constituents of the essential oil. Linalool is a monoterpene commonly found in the essential oils of some aromatic plants. It is also obtained as a by-product in the industrial synthesis of vitamin E (Ohashi et al. 1997). Its biological activity, including antioxidant, antimicrobial, anti-inflammatory, anesthetic and antitumor was already reported (Ghelardini et al. 1999, Mazzanti et al. 1998, Letizia et al. 2003, Dadasoglu et al. 2011). Also, the ability to inhibit the development of the mosquito larvae of *Aedes aegypt* has been attributed to linalool (Gottlieb et al. 1981). Eugenol acetate is a derivative of eugenol, which is employed as antimicrobial, anti-inflammatory, anesthetic, anti-septic, antioxidant, repellent agent, and in cosmetics and condiments (Lahlou 2004).

Cryptoccocus neoformans and *Candida albicans* are opportunistic pathogens commonly associated with disease in immunocompromised hosts. *C. neoformans* causes systemic disease, and cryptococcal infection is usually acquired by inhalation of fungal cells and can be limited to the lung or disseminate to the central nervous system, causing meningoencephalitis (Rodrigues et al. 1999). *C. albicans* can cause local and systemic infection and it is found normally in the buccal and vaginal regions (Zhang and Lewis 1997). The lipophylicity of the essential oil constituents could explain their antimicrobial activity, a characteristic that allows the partition of these compounds in lipids of cell membrane and mitochondria, increasing their permeability and leading to leakage of cellular contents (Cowan 1999). According to other authors, essential oil constituents can also act on cellular proteins located in cytoplasm membranes, including the ATPases, by their accumulation in the lipid double layer and the consequent destruction of lipid-protein interaction (Ultee et al. 2002, Burt 2004). Alternatively, a direct interaction of lipophylic compounds may occur with the hydrophobic portions of proteins (Juven et al. 1994, Sikkema et al. 1995). However, due to the large number of different chemical groups present in essential oils, it is likely that its antimicrobial activity is not related to a specific mechanism of action (Carson et al. 2002, Kalemba and Kunicka 2003, Skadamis and Nychas 2001).

Leishmaniasis is a chronic disease that can assume different fatal clinical forms ranging from selfhealing cutaneous to progressive mucocutaneous infection, and potentially visceral leishmaniasis (Tripathi et al. 2007). According to the World

Health Organization (2004), leishmaniasis currently threatens 350 million people around the world and it is estimated that 2 million new cases occur each year. A common feature of volatiles compounds is their hydrophobic nature. Several studies addressing the action mode of such compounds usually point to cell membranes as the primary target as the essential oils, in general, have a passive entry through the membrane, leading to an increase of membrane permeability (Bakkali et al. 2008).

Antioxidant activity possibly proceeds from the presence of phenolic compounds such as methyl salicylate in the oil. The brine shrimp lethality assay is based on the ability to kill laboratory-cultured *Artemia salina* nauplii brine shrimp and it is considered to be one of the most useful tool for the preliminary assessment of general toxicity (Maclaughlin 1991). LC_{50} values < 250 μg/mL are considered significant for plant samples and had the potential for futher investigation (Rieser et al. 1996).

The results presented here are the first report on the chemical composition and biological properties of the *Mitracarpus* essential oil.

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RESUMO

Em nosso trabalho anterior (Fabri et al. 2009), mostramos que diferentes extratos de *Mitracarpus frigidus* apresentaram atividades antibacteriana, antifúngica e leishmanicida significativas. Com o objetivo de aprofundar o conhecimento sobre essa espécie, esse trabalho objetiva identificar os constituintes químicos e avaliar as atividades biológicas *in vitro* de seu óleo essencial. Dessa forma, o óleo essencial das partes aéreas de *M.frigidus* foi obtido por hidrodestilação e analisado por CG/EM. Entre os compostos detectados, 11 foram identificados, sendo linalol e o acetato de eugenol os componentes majoritários. O óleo essencial de *M. frigidus* exibiu moderada atividade antibacteriana contra *Staphyloccocus aureus*, *Bacillus cereus*, *Pseudomonas aeruginosa* e *Enterobacter cloacae* (CIM 250 μg/mL). Por outro lado, apresentou forte efeito antifúngico contra Cryptoccocus neoformans (CIM 8 μg/mL) e *Candida albicans* (CIM 63 μg/mL). Expressiva atividade contra as formas promastigostas de *Leishmania major* e *Leishmania amazonensis* com valores CI_{50} de 47,2 e 89,7 μg/mL, respectivamente, também foi observada. Além disso, a atividade antioxidante foi investigada através do ensaio com o radical DPPH e este apresentou uma significativa atividade com CI_{50} de 38 μg/mL. A citotoxidade contra *Artemia salina* foi moderada com CL_{50} de 88 μg/mL. Os resultados aqui apresentados são o primeiro relato sobre a composição química e propriedades biológicas do óleo essencial *M. frigidus*.

Palavras-chave: *Mitracarpus frigidus*, óleo essencial, antimicrobiano, leishmanicida, citotoxidade.

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