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Full Length Research Paper

Composition and evaluation of the lethality of *Lippia gracilis* essential oil to adults of *Biomphalaria glabrata* and larvae of *Artemia salina*

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***Lippia gracilis* essential oil (LGEO) was evaluated for its molluscicidal activity against *Biomphalaria glabrata* and toxicity to brine shrimps (*Artemia salina*). *L. gracilis* was collected from the city Tomar do Gerú- Sergipe, Brazil. The LGEO were characterized by gas chromatography-mass spectrometry (GC/MS). The values of LC_{10}, LC_{50} and LC_{90} were respectively 36.9, 62.2 and 82.8 ppm for *B. glabrata* and 19.6, 23.6 and 26.1 ppm for *A. salina*. GC/MS analysis showed a total volatile content of 98.6% in the LGEO. The major components were identified as thymol (24.0%), p-cymene (15.9%), methyl-thymol (11.7%), γ-terpinene (10.9%) and β-caryophyllene (7.8%).**

Key words: Chemical composition, *Lippia* species, molluscicidal activity, Verbenaceae.

INTRODUCTION

Schistosomiasis is a major source of morbidity and mortality in developing countries including Africa, South America, the Caribbean, the Middle East and Asia (Santos et al., 2007). It is caused by the presence of the worm *Schistosoma mansoni* in the liver of the affected person, the fresh-water mollusk *Biomphalaria glabrata* acting as intermediate host (Santos and Sant'Ana, 2001). Two of the most important health problems facing many of the people living in tropical Latin America, including large parts of northern Brazil, are the diseases of schistosomiasis and dengue fever (Luna et al., 2005; Silva et al., 2008; Chantraine et al., 1998). Since a large proportion of the populations living in these areas suffer from varying degrees of poverty, the discovery of plant-derived compounds that could help with the control or eradication of these diseases would be of great value (Mohammed and Chadee, 2007).

The development of the larvae of *S. mansoni* occurs in several snail species belonging to the genus *Biomphalaria*, thus the treatment with molluscicides of water masses serving as transmission sites is considered an important element in an integrated strategy for the control of the disease (Santos et al., 2007; Al-Zanbagi et al., 2001). In this context, our continuing project has the aim of screening plants grown locally in Northeast Brazil for biological activities that could be of value to combat tropical diseases.

The search for efficient natural molluscicide, larvicide or pesticide substances with low environmental toxicity has increased (Lima et al, 2002; Luna et al., 2005; Santos and Sant'Ana, 1999; Santos and Sant'Ana, 2000). Plants essential oils are outstanding candidates, since they are, in some cases, highly active, readily available in tropical countries and economically viable (Lemos et al., 1992). Studies of the essential oil molluscicidal activity have been published

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Abbreviations: LGEO, *Lippia gracilis* essential oil; **GC/MS,** gas chromatography-mass spectrometry; **LC_{10},** 10% lethal concentration; **LC_{50},** 50% lethal concentration; **LC_{90},** 90% lethal concentration.

in the literature. Plants and products, such as, latex of *Euphorbia conspícua* (LC_{90} = 4.87 µg/mL) (Santos et al., 2007), *Xylopia langsdorffiana* (LC_{90} = 5.6 µg/mL) (Tavares et al., 2007), *Ocotea bracteosa* (LC_{90} = 8.3 µg/mL) (Coutinho et al., 2007), have been tested and are potent molluscicides.

The genus *Lippia* (Verbenaceae) includes approximately 200 species of herbs, shrubs and small trees. These species are mainly distributed throughout the South and Central America countries and Tropical Africa territories (Terblanché and Kornelius, 1996). *Lippia gracilis* H.B.K. (Verbenaceae), known in Brazil by the name "alecrim-da-chapada", is an herb commonly found in Northeastern Brazil vegetation (Marreto et al., 2008).

Most of them are traditionally utilized as gastrointestinal and respiratory remedies (Pascual et al., 2001). Some *Lippia* species have shown antimalarial (Gasquet et al., 1993), antiviral (Abad et al., 1995) and cytostatic activities (Anderson et al., 1991).

The aim of the present study was to explore the molluscicidal activities of the essential oil from *L. gracilis* using *B. glabrata* as the target snail. This species grows abundantly at Sergipe, Paraíba and Pernambuco States in Northeastern Brazil and the occurrence of bioactive compounds in the oil of other *Lippia* encouraged us to perform the present study. In order to determine the toxicity of the oil towards non-target aquatic species, the brine shrimp (*Artemia salina*) were employed as a model assay system since they provide a convenient in-house pre-screening for general toxicity (Meyer et al., 1982).

MATERIALS AND METHODS

Plant material

L. gracilis (Verbenaceae) was collected in March from the City Tomar do Gerú-Sergipe, Brazil (11 19' 1,1" S; 37 55' 14,9" W; 205 (m)). The voucher specimen's # 9208 has been deposited in the Herbarium of the Department of Biology, Federal University of Sergipe, Brazil.

Isolation of the essential oil

Prior to water distillation, leaves were dried at 40°C in a forced air oven (Marconi MA 037) for 48 h and pulverized using a mill. The essential oil from leaves (1 kg) was subjected to water distillation for 3 h using a Clevenger–type apparatus. The oil was extracted from the distillation water with Et_2O, dried over anhydrous sodium sulfate, filtered and reduced the *ca*. 1.5 mL at room temperature under reduced pressure on a rotatory evaporator. The oil was kept at -4°C until GC analysis.

Gas chromatography/ mass spectrometry

Oil sample analysis was performed on a Shimadzu QP5050A (Shimadzu Corporation, Kyoto, Japan) system comprising a AOC - 20i autosampler, the gas chromatograph has interfaced to a mass spectrometer (GC/MS) instrument employing the following conditions: column J&W Scientific DB-5MS fused silica capillary column (30 cm x 0.25 mm i.d, composed of 5% phenylmethylpolysiloxane), operating in electron impact mode at 70 eV; helium (99.999%) was used as carrier gas at a constant flow of 1.2 mL min^{-1} and an injection volume of 0.5 µL was employed (split ratio of 1:100) injector temperature 250°C; ion-source temperature 280°C. The oven temperature was programmed from 50°C (isothermal for 2 min), with an increase of 4°C/min to 200°C, then 10°C/min to 300°C, ending with a 10 min at 300°C. Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 40 to 550 Da.

Gas-chromatography (GC-FID)

Gas chromatography analysis was performed by flame ionization gas chromatography (FID), using a Shimadzu GC-17A (Shimadzu Corporation, Kyoto, Japan) equipment, under the following operational conditions: capillary ZB-5MS column (5% dimethylpolysiloxane) fused silica capillary column (30 m x 0.25 mm i.d. x 0.25 µm film thickness) from Phenomenex (Torrance, CA, USA), under same conditions GC-MS. The relative proportions of the essential oil constituents were expressed as percentages obtained by peak area normalization, without using correcting factors. Compounds concentrations were calculated from the GC peak areas and they were arranged in order of GC elution.

Identification of essential oil constituents

Identification of individual components of the essential oil was performed by computerized matching of the acquired mass spectra with those stored in NIST21 and NIST107 mass spectral library of the GC-MS data system. Relative retention index (RRI) for all compounds were determined according to the Vandendool and Kratz (1963) for each constituent as previously described (Adams, 2007).

Assay of molluscicidal activity

A population of adult *B. glabrata* snails was collected from City São Cristóvão-Sergipe, Brazil and maintained according to established procedures (Santos and Sant'Ana, 1999). Stock solutions of samples were prepared by dissolving the test material in 70 mL of dechlorinated water containing 0.1% (v/v) DMSO. The lethality against adult snails was determined (four replicates for each sample) as previously described (Santos and Sant'Ana, 2000). Ten adult snails were exposed to each test solution. The results were expressed as 24 h-LC_{50}, considering the exposition time, but the number of deaths was counted after 72 h (21). Both positive (niclosamide at 3 ppm) and negative [dechlorinated water containing 0.1% (v/v) DMSO] controls were included in the assay.

Brine shrimp lethality test

The brine shrimp lethality test (BST) was performed using the method of Meyer et al. (1982) and McLaughlin et al. (1991). The brine shrimp eggs were hatched in artificial sea water (34 g sea salt per litre of water) and, after an average of 2 days from hatching, the shrimp larvae were used for experimental bioassay. Stock solutions of samples were prepared by dissolving the test material in 10 mL of artificial seawater containing 1% (v/v) DMSO (three replicates for each sample) and negative [seawater containing 0.1% (v/v) DMSO] control was included in each bioassay. Ten shrimps were exposed to each test solution. The time of exposition of the target organisms was of 24 h. After this time, survivors were counted and the percentage of deaths at each concentration was recorded.

Table 1. Essential oil composition from the leaves of *L. gracilis* characterized by GC/MS.

RT (min)[a]	Compounds[b]	(%)	RRI[c]
6.817	α-Thujene	1.8	924
7.042	α-Pinene	1.2	931
7.567	Camphene	0.5	946
8.317	Sabinene	0.6	971
8.483	β-Pinene	0.3	976
8.875	Myrcene	3.9	988
9.458	α-Phelandrene	0.7	1005
9.833	α-Terpinene	2.3	1016
10.108	p-Cymene	15.9	1023
10.275	Limonene	0.7	1028
10.400	1,8-Cineole	4.7	1031
10.900	(E)- β Ocimene	0.1	1045
11.333	γ- Terpinene	10.9	1057
12.892	Linalool	1.1	1099
14.650	Camphor	0.1	1147
15.892	Terpinen-4-ol	0.9	1180
16.442	α-Terpineol	0.8	1195
17.667	Methyl thymol	11.7	1229
19.883	Thymol	24.0	1290
20.183	Carvacrol	4.9	1298
21.800	Thymol acetate	0.1	1344
24.350	β- Caryophyllene	7.8	1418
24.792	α-trans-Bergamotene	0.2	1432
24.976	Aromadendrene	0.5	1437
25.542	α- Humulene	0.5	1454
26.692	Viridiflorene	0.6	1490
26.850	Bicyclogermacrene	1.8	1494
Total		98.6	

[a]Retention time, [b]compounds listed in order of elution and [c]relative retention index calculated applying Vandendool and Kratz (1963). Column DB-5 MS relative to C_8 to C_{18} *n*-alkanes.

RESULTS AND DISCUSSION

The essential oil of *L. gracilis* was obtained in 2.8% yield. Twenty seven compounds, representing 98.6% of the essential oil have been identified; their retention indices and percentage composition, listed in order of elution in the DB-5MS column, are given in Table 1. The major components of *L. gracilis* essential oil were identified as thymol (24.0%), p-cymene (15.9%), methyltymol (11.7%), γ-terpinene (10.9%) and β-caryophylene (7.8%). Other minor constituents were found to be, carvacrol (4.9%), 1,8-cineole (4.7%) and myrcene (3.9%). So, the oil was constituted by monoterpene (87.2%) and sesquiterpene (11.4%) fraction. Some species showed variable oil composition, these differences often separate them into several chemotypes. Silva et al. (2008) demonstrated that the major components in *L. gracilis* essential oil were identified as carvacrol (44.43%), o-cymene (9.42%), c-terpinene (9.16%) and b-caryophyllene (8.83%), which accounted for 80.25% of monoterpenes and 18.14% of sesquiterpenes.

The results of molluscicidal activity of some monoterpenoids against the adult *B. alexandrina* snails are presented by Radwan et al. (2008). All monoterpenoids exhibited marked potency in killing the snail-vector of schistosomiasis. The results showed that thymol was the most effective followed by β-citronellol and carvacrol, with menthol being the least effective. Their LC_{50} values were 13.92, 20.74, 21.85 and 101.59 mg/ml, respectively (Radwan et al., 2008).

In 1981, Craveiro and collaborators related that the essential oils of Brazilian *Lippia* species and the residual waters from the steam distillation of *L. aristata* Schau. and *Lippia aff. sidoides* Cham. had also molluscicidal activity against *B. glabrata*, the most important host of *S. mansoni* in Brazil (Craveiro et al., 1981). Chifundera et al. (1993) also showed that substances such as saponins, terpenoids and steroids as well as flavonoids are presently

known to be molluscicidal agents.

Carvalho et al. (2003) found that the essential oil of *Lippia sidoides* was toxic against *A. aegypti* larvae. The predominance of thymol in *L. sidoides* essential oil, with concentrations ranging from 50.57 to 22.37% (Sousa et al., 2002), was believed to be determinant for its activity. Although thymol was present in the essential oil of *L. gracilis*, its proportion was considerably lower than *L. sidoides* (3.83%). However, the most abundant compound in the essential oil of *L. gracilis*, carvacrol, was found to be active against our strain of *A. aegypti*.

In this work, the essential oil from *L. gracilis* leaves exhibited potent molluscicidal activity against *B. glabrata* with LC_{10} = 36.9, LC_{50} = 62.2 and LC_{90} = 82.8 ppm. The values of LC_{10}, LC_{50} and LC_{90} were respectively 19.6, 23.6 and 26.1 ppm, for *A. salina*. The brine shrimp, *A. salina* Leach, is a crustacean belonging to the subclass Branchiopoda. The brine shrimp toxicity test is considered to be the first screening that would be used in assessing the bioactivity of any given natural sample (Luna et al., 2005).

Conclusion

In conclusion, there are few studies about the pharmacological activity of this specie. The essential oil from *L. gracilis* showed sufficient activity against *B. glabrata* snails to warrant its consideration as a potential alternative molluscicide. Since the essential oil from *L. gracilis* showed activity against both adult snails and the cercaria of *S. mansoni*, it may be possible to employ the material, or a fraction derived from, in an integrated pest management program. In the present study, the brine shrimp toxicity assay was used to assess toxicity to non-target aquatic species. The *L. gracilis* essential oil, which contains mainly thymol, showed significant toxic activities to brine shrimps.

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