Revista Brasileira de Farmacognosia
Brazilian Journal of Pharmacognosy
15(4): 294-297, Out./Dez. 2005
Received 02/23/05. Accepted 08/29/05

Artigo

Biological activity of the aqueous extract of *Lychnophora pinaster* Mart.

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ABSTRACT: Lyophilized aqueous extract (LAE) from *Lychnophora pinaster* Mart (Asteraceae) aerial parts was evaluated in the search of possible biological activities. LAE exhibited trypanocidal activity (113.62 µg/mL), but could not inhibit 5-lipoxygenase *in vitro* (17% of inhibition). LAE chemical characterization by HPLC with UV-Diode Array Detector showed the presence of caffeic acid, isochlorogenic acid, vitexin, isovitexin and quercetin, in comparison with authentic samples.

Keywords: *Lychnophora pinaster*, trypanocidal activity, 5-lipoxygenase assay.

INTRODUCTION

Lychnophora species (Asteraceae) grow in Brazilian Savanah (Cerrado), in the State of Minas Gerais, Bahia and Goiás (Semir, 1991) and Distrito Federal. Almost all *Lychnophora* species are named "arnica" and used in folk medicine as anti-inflammatory and anti-rheumatism remedies (Cerqueira et al., 1987; Saúde et al., 2002) as well as antimicrobial agent (Borella et al., 1998). Commercial exploitation of "arnica-da-serra" has resulted in a devastating decline of the plant population and the Brazilian Botanical Society has included this plant species in the list of Brazilian plants that are likely to become extinct (Santos et al., 2004). On the other hand, *Lychnophora* species have been the target of several studies about their chemical composition and/or biological activities.

The ethyl acetate extract from aerial parts of *L. salicifolia* showed trypanocidal activity against trypomastigote forms of *Trypanosoma cruzi*. Such activity was due to the flavonoid quercetin-7,3',4'-trimethyl ether and lychnopholic acid (Jordão et al., 2004). Aerial parts ethanol extract from *L. villosissima* and isolated 15-deoxygoyasenzolide (Chiari et al., 1991), *L. passerina* ethanol extract and isolated goyazensolide and *L. trichocarpha* and isolated lychnopholide and eremantholide showed *in vitro* trypanocidal activity against trypomastigote forms of *T. cruzi* (Oliveira et al., 1996; Chiari et al., 1996). The ethyl acetate extract from the aerial parts of *L. granmongolense* also exhibited trypanocidal (trypomastigote forms of *T. cruzi*) activity (Grael et al., 2000).

The dichloromethane extract from the roots (50 and 100 mg/kg), methylcubecin (40 mg/kg), cubecin (40 mg/kg) (Borsato et al., 2000), 3,5-di-*O*-[*E*]-caffeoylquinic acid, 4,5-di-*O*-[*E*]-caffeoylquinic acid (Santos et al., 2005), aqueous extract from leaves and stems (Cerqueira et al. 1987) of *L. ericoides*, presented analgesic activity *in vivo*. Flavonoids isolated from the ethanol extracts of the leaves and stems of *L. affinis*, demonstrated activity in cell cultures of human nasopharyngeal carcinoma (9KB) (Le Quesne et al., 1976), as well as the sesquiterpene lactones isolated from *L. trichocarpha* presented anti-tumor activity against several type cells (Saúde, 1994). Acetyl lychnopholic acid isolated from the ethanol extract of *L. salicifolia* leaves and stems (Miguel et al., 1996) and the sesquiterpenes from *L. trichocarpha* presented antimicrobial activity (100µg/mL) (Saúde et al., 2002).

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ISSN 0102-695X

Lychnophora pinaster Mart, commonly known as "arnica", "arnica-da-serra" or "candeia" is used in infusions, in baths, or macerated in "cachaça" (sugar-cane spirit) or ethanol as anti-flogistic, anti-rheumatic, and analgesic. In previous work, a phytochemical study of the hexane and ethanol extracts from the aerial parts of *L. pinaster* led to isolation of the *E*-isomer of lychnophoric acid (named lychnophoic acid), quercetin, 15-deoxygoyazensolide, lupeol, a mixture of α- and β-amyrin, friedelin and fat acid esters mixture (Oliveira et al., 1996; Silveira et al., 2005) and saturated hydrocarbons (Duarte et al., 1999). *Lychnophora pinaster* hexane and ethanol extracts and isolated *E*-isomer of lychnophoric acid showed *in vitro* trypanocidal activity against trypomastigote forms of *T. cruzi* (Silveira et al., 1993; Oliveira et al., 1996). In a continuing effort to known more about this species, assays were carried out to detect biological activity of the aqueous extract from aerial parts of *L. pinaster*.

MATERIAL AND METHODS

Plant material

Aerial parts from *Lychnophora pinaster* Mart. were collected at Serra da Moeda (State of Minas Gerais, Brazil), in May 1994, and identified by Professor Julio A. Lombardi. A voucher species was deposited at Herbarium of the Departamento de Botânica, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brasil (BHCB 19520). The plant material was dried at room temperature and powdered by knife mill. The aqueous extract was obtained by infusion method, using 200g of powdered plant material and 2 L of distillated water at 70 °C. After filtration, the extract was submitted to lyophilization method (8.2% yield).

Brine shrimp lethality test (BST)

The assay was performed basically according to simplified Meyer's method (Meyer et al., 1982). Briefly, brine shrimp *Artemia salina* L. encysted eggs (Maramar) were incubated in artificial seawater at 28 °C. Samples were solved in 20 mL of artificial seawater. Serial dilutions (triplicate) were prepared in the same solution. Metanauplii (10 units) were added to each set of tubes containing samples and the cultures further incubated for 24 h. Controls containing only artificial seawater were included on set of experiment. Lapachol was used as reference standard. LC_{50} (after 24 h) was calculated by Probit analysis.

In vitro trypanocidal activity

The assay was performed according Chiari et al. (1996): Albino mice infected with the Y strain of *T. cruzi* were used to provide trypomastigotes. The lyophilized extract was suspended in dimethyl sulfoxide (DMSO) (0.2 mL) plus Krebs-Ringer-glucose (1.8 mL) and mixed with infected blood (0.2 mL). A parasite density of 2 x 10^5/0.1 mL was calculated for each flat-bottomed test tube (4.0 mL, 56 x 13 mm); control tubes with DMSO and gentian violet (0.125 mg/mL) were run in parallel. After incubation at 4 °C for 24 h the suspensions were examined microscopically.

5-Lipoxygenase assay

5-Lipoxigenase assay was performed according to a previously described method (Kuhl et al., 1984; Duarte et al., 2000). Briefly, porcine polynuclear lymphocytes (PMNL) were isolated by dextran sedimentation and the remaining erythrocytes removed by cell lyses. The PMNL fraction was treated with eicosatetranoic acid, a lipoxygenase inhibitor, Ca^{2+} and Ca^{2+}-ionophore A23187 (Boehringer Mannheim) as inductor. After addition of crude aqueous extract in 0.05 mL ethanol and 0.01 mL 9 μM (0.1 μCi) 1-[^{14}C]arachidonic acid, the mixture was incubated for 5 min at 37 °C, followed by metabolite extraction with ethyl acetate. The solvent was then evaporated and the metabolites dissolved in ethanol before HPLC separation. HPLC fractionation was carried out on a reversed-phase column coupled to a β-scintillation detector. An estimate of 5-lipoxygenase inhibition was obtained by measuring the radioactivity of the metabolite [^{14}C]-5-hydroxy-eicosatetraenoic acid produced by different amounts of extract. The uninhibited activity was determined in the presence of controls containing ethanol. The HPLC apparatus consisted of a Merck Hitachi L-6200 Intelligent Pump coupled to a Merck Hitachi AS-2000 auto sampler and Berthold HPLC radioactivity monitor LB 506 C-1. The analysis was carried out using a LiChroCART 4 RP-18 (5 mm) Merck column and LiChroCART 4-4 RP-18 (5 μm) precolumn. The mobile phase was composed of acetonitrile and water containing 1% (v/v) 0.1 M phosphoric acid. The stepwise gradient started with 36% acetonitrile for 12 min then 80% acetonitrile for 15 min at a flow rate of 1.0 mL/min (final time run: 27 min) .

Chemical constituents

HPLC analysis of crude aqueous extract from L. pinaster:

The analysis was carried out as previously described (Duarte et al., 2000).

HP1090 Chromatograph coupled to a UV-diode-array detector and HP3392A integrator, with HP79994A software HPLC Chem-Station was used. The analysis was carried out in a LiChroCART 125-4 RP-18 (5 μm) Merck Column linked to LIChroCART 4-4 RP-18 (5 μm) pre-column. Authentic samples used as standard, crude extract and partially purified fractions were dissolved in methanol (1 mg/mL) and filtered through a Millex-HV 0.45

Table 1. Phenolic compounds of the aqueous extract from the aerial parts of *Lychnophora pinaster* by HPLC-DAD analysis

Compound	Standard RT (min)	Sample RT (min)
Caffeic acid	11.32	11.31
Vitexin + isovitexin	16.82	16.81
Quercetin	19.11	19.09
Isochlorogenic acid	21.90	21.92

Mobile phase: acetonitrile and H_2O containing 1.0% (v/v) 0.1 M phosphoric acid. Elution of sample and standard substances was performed by stepwise increase in acetonitrile as follows: 1min - 5%; 20min – 20%; 30min – 30%; 40min – 80%; 50min – 95% at 1mL/min flow rate (time run: 50 min).

μm filter (Millipore) before applying to the column. The retention time and UV spectra from standard compounds, obtained at the same conditions than crude extracts and fractions, were used to compare with samples. The mobile phase was composed of acetonitrile and water contained 1% (v/v) 0.1M phosphoric acid. Elution of sample and standard compounds was performed by stepwise increase in acetonitrile as follows: 1 min – 5%; 20 min - 20%; 30 min - 30%; 40 min – 80%; 50 min – 95% at 1 mL/min flow rate (time run: 50 min).

Fractionation of crude aqueous extract by sephadex LH-20 column using bi-phase technique:

The bi-phase technique sephadex LH-20 column was performed according personal communication of Dr Christina Dunstan (LM- University, Munich). Briefly, a glass column was filled with sephadex LH-20 (Pharmacia), previously in 24 h contact with upper phase of an ethyl acetate: isopropanol: water mixture (10:2:2). *Lychnophora pinaster* extract (200 mg) was dissolved in the 5 mL of lower phase of the described mixture and applied to column. The elution was carried out using the lower phase and the fractionation was monitored by TLC. Eight fraction groups were obtained and analyzed by TLC and HPLC (at the same conditions described to the crude extract).

RESULTS AND DISCUSSION

A comparison of retention times and on-line UV spectra of *L. pinaster* crude aqueous extract and fractions with authentic samples, led to characterization of the phenolic compounds showed at Table 1. Only the peaks that show retention time and on-line UV spectra similar to that showed by standards were considered. The vitexine and isovitexine presented same retention time and UV spectra. By using sephadex LH-20 bi-phase separation, it was possible to obtain a fraction containing both flavonoids. Because it was not possible to isolate the vitexine from isovitexine using usual chromatograph techniques, the mixture was submitted to peracetylation reaction and usual work-up followed by silica gel column purification, afforded vitexine and isovitexine acetylated derivatives. The identity was confirmed through their NMR spectra.

Meyer and cols. (1982) classified crude extracts into toxic (LC_{50} < 1000 ppm) and non-toxic (LC_{50} > 1000 ppm), according to the levels required to attain the LC_{50} in the BST assay. Based on this classification, crude aqueous extract from *L. pinaster* can be considered non-toxic. BST assay present a good correlation with several others biological assays (De Rosa et al., 1994; Ojala et al., 1999). However, during our experiments with *Lychnophora* extracts (Duarte, 1993; Duarte, 1999), we could not find correlation between trypanocidal activity and toxicity to *A. salina* larvae, despite this correlation was suggested by literature (Zani et al., 1995). This fact may corroborate the idea that BST assay can be used only for pre-screening purpose.

Chiari et al. (1991) consider an extract or pure substance as active when remove 100% of the trypomastigote form in blood samples from animals previously infected with the parasite. Based on the Chiari classification, the crude extract showed a good activity (113.62 μg/mL killed 100% of parasites). However, at this concentration the sample caused lyses of the blood cells. The lyses maybe can be explained by the presence of saponins. Even though we were not able to isolate saponins from *L. pinaster* extracts, this class of compounds was detected in the aerial parts of *L. pinaster* during a preliminary pharmacognostic assay (Duarte, 1993)

Despite aerial parts aqueous extract (infusion) is used in folk medicine to alleviate pain and inflammation caused by contusions and rheumatism, in the 5-lipoxygenase assay aqueous extract (1.0mg/mL) inhibited only 17.3% of enzyme activity. The weak inhibitory effect to 5-lipoxygenase may be due to the low lipophilicity of the constituents present in the aqueous extract (Panthong et al., 1994).

We consider the fractionation of mixtures by the sephadex LH-20 technique used here (bi-phase) can be a non-expensive and useful tool to the phytochemical work.

ACKNOWLEDGEMENTS

Financial support from CNPq, CAPES, FAPEMIG and KFA.

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