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Original Article

Characterization and evaluation of the cytotoxic potential of the essential oil of *Chenopodium ambrosioides*

Ruth T. Degenhardt[a], Ingrid V. Farias[a], Liliane T. Grassi[a], Gilberto C. Franchi Jr.[b], Alexandre E. Nowill[b], Christiane M. da S. Bittencourt[a], Theodoro M. Wagner[a], Marcia M. de Souza[a], Alexandre Bella Cruz[a], Angela Malheiros[a,*]

[a] *Núcleo de Investigações Químico-Farmacêuticas, Programa de Mestrado em Ciências Farmacêuticas, Universidade do Vale do Itajaí, Itajaí, SC, Brazil*
[b] *Centro Integrado de Pesquisas Oncohematológicas da Infância. Universidade Estadual de Campinas, Campinas, SP, Brazil*

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ABSTRACT

The essential oil of *Chenopodium ambrosioides* L., Amaranthaceae, was obtained by steam distillation in a Clevenger apparatus and characterization was performed using chromatographic and spectroscopic assays (GC-FID, GC/MS, ^{1}H NMR). Two major compounds were identified: *p*-cymene (42.32%) and ascaridole (49.77%). The ethanolic extract and hydrolate were fractionated by liquid–liquid partitioning and the compounds were characterized by GC/MS. The essential oil, ethanol extract and fractions by partitioning with dicloromethane, ethyl acetate and butanol were tested in tumor cell lines (K562, NALM6, B15, and RAJI). Significant cytotoxic activity was found for essential oil (IC_{50} = 1.0 μg/ml) for RAJI cells and fraction dicloromethane (IC_{50} = 34.0 μg/ml) and ethanol extract (IC_{50} = 47.0 μg/ml) for K562 cells. The activity of the essential oil of *C. ambrosioides* is probably related to the large amount of ascaridol, since the other major compound, *p*-cymene, is recognized as a potent anti-inflammatory and has low cytotoxic activity.

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Introduction

Chenopodium ambrosioides L., Amaranthaceae, popularly known as "erva-de-santa-maria" or "mastruço" (Kokanova-Nedialkova et al., 2009), has been widely used in folk medicine in the midwest, south and southeast of Brazil and is found mainly in temperate and subtropical countries (Lorenzi and Matos, 2002). The leaves are used as an anthelmintic and vermicide (Alitonou et al., 2012) and this species is also used in the treatment of gastrointestinal, respiratory, vascular, and nervous diseases and to combat diabetes and hypercholesterolemia. Furthermore, it presents sedative, antipyretic, and antirheumatic effects (De Feo and Senatore, 1993). Due to these properties, in 2009, the Brazil's Health Ministry selected the *C. ambrosioides* as one of the plants of interest to the Helth Sistem (Renisus) that can be used as an herbal medicine.

The geographical area where *C. ambrosioides* is obtained, with variable humidity, temperature and general environmental conditions and the degree of evolutionary and genetic variability (the possible existence of chemotypes within the species) are factors that directly influence the chemical composition of the essential oil obtained from this plant (Gobbo-Neto and Lopes, 2007; Chekem et al., 2010). However, despite this variability, the essential oil consists mainly of mono and sesquiterpenes (Kliks, 1985; Cruz et al., 2007).

Various studies have been undertaken to characterize the composition of the essential oil of *C. ambrosioides* by gas chromatography coupled to mass spectrometry (GC/MS). The main compounds found are: (*Z*)-ascaridole, (*E*)-ascaridole, carvacrol, *p*-cymene, α-terpinene and limonene (Cavalli et al., 2004; Jardim et al., 2008; Chekem et al., 2010; Vieira et al., 2011). Studies on the essential oil showed antifungal activity against *Aspergillus fumigatus*, *Aspergillus niger*, *Botryodiplodia theobromae*, *Fusarium oxysporum*, *Sclerotium rolfsii*, *Macrophominapha seolina*, *Cladosporium cladosporioides*, *Helminthosporim oryzae*, and *Pythiumdeba ryanum* at a concentration of 100 g/ml (Matos, 2011). Monzonte et al. (2007) noted that the essential oil of *C. ambrosioides* presents *in vitro* activity against the protozoan *Leishmania donovani*, causing irreversible inhibition of their growth.

Kinupp (2007) describes this species as also being rich in flavonoids and terpenoids and having very diverse pharmacological activities, including antioxidant and chemopreventive effects against cancer, as well as antimicrobial, anti-inflammatory, and analgesic properties (Cruz et al., 2007; Dembitsky et al., 2008; Grassi, 2011). According to Hmamouchi et al. (2000), the extract

* Corresponding author.
E-mail: angela@univali.br (A. Malheiros).

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obtained from this species presents potential molluscicidal activity against snails transmitting schistosomiasis, *Bulinus truncatus* (LC_{90} = 2.23 mg/l). Patrício et al. (2008), on the other hand, studied the effect of the aqueous crude extract of the leaves of *C. ambrosioides* on skin ulcers induced by *Leishmania amazoniensis* in mice. Intralesional treatment was able to inhibit the progression of the ulcer.

Some authors, cited by Matos (2011), reported that the anti-inflammatory activity of *Chenopodium* is due mainly to ascaridole, which is one of the major components of the essential oil of this plant. This same compound can also exhibit antipyretic effects and has been indicated as being responsible for growth inhibition in different tumor cell lines. Its action is so significant *in vitro* that is a strong candidate for the treatment of cancer (Efferth et al., 2002).

Given the above, the objective of this study was to obtain and characterize the essential oil and fractions from the hydrolate and ethanol extract of leaves of *C. ambrosioides* through chromatographic and spectrometric assays (GC-FID, GC/MS, ^{1}H NMR) and evaluate its cytotoxicity *in vitro* by the MTT method (3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) in different tumor cell lines: myeloid leukemia (K562), acute B lymphoblastic leukemia (NALM6 and B15), and Burkitt's lymphoma (RAJI), since cancer is a major public health problem worldwide (INCA, 2011).

Materials and methods

Plant material

Chenopodium ambrosioides L., Amaranthaceae, was obtained in March 2010 in Cáceres, a city in the state of Mato Grosso, Brazil. Taxonomic identification of the collected material was done by botanist M.Sc. Oscar Benigno Iza by comparison with authentic samples. The exsiccate was deposited in the Herbarium Barbosa Rodrigues (HBR), Itajaí, SC, with the registry number 52802.

Extraction of the essential oil

The essential oil of the leaves of *C. ambrosioides*, which had been dried and weighed (148 g), was obtained by steam distillation in a Clevenger apparatus. The leaves were extracted with 3 l of distilled water in the flask, and the extraction was performed over a period of 4 h. The oil was removed with a micropipette and, to prevent oxidation reactions due to the remaining water, anhydrous sodium sulfate was added. The oil was stored frozen, protected from light.

Hydrolate and ethanolic extract

After the extraction of the essential oil, the water was filtered to yield about 2.75 l of hydrolate. The leaves used for oil extraction (approximately 185 g) were subjected to maceration in ethanol for 7 days. Approximately 1 l each of the hydrolate and ethanolic extract were subjected to liquid–liquid partitioning with immiscible solvents in increasing order of polarity (dichloromethane, ethyl acetate and butanol). For each solvent, two extractions were performed using first 400 ml and then 200 ml of the solvent. After the separation of the phases, anhydrous sodium sulfate was added to the organic phase to remove the remaining water. The obtained fractions were filtered and concentrated in a rotary evaporator at a maximum temperature of 50 °C to obtain a dry residue. Aliquots of these fractions were sent for analysis by GC/MS.

Analysis by chromatographic and spectroscopic assays

The essential oil obtained from the dried leaves of *C. ambrosioides* was analyzed by gas chromatography with a flame ionization detector (GC-FID) and by gas chromatography coupled to mass spectrometry (GC/MS Shimadzu QP2010 S). The analysis by GC-FID, with an Rtx-1 capillary column (30 m × 0.25 mm × 0.10 μm), used helium as the carrier gas (0.8 ml/min); the injector temperature was 180 °C and the detector temperature was 250 °C, 1:50 split, using the following temperature program: 80–200 °C at 20 °C/min, 200–300 °C at 15 °C/min; 300–310 °C at 12 °C/min; FID (310 °C); H_2: 40 ml/min.

The fractions obtained from the hydrolate and ethanol extract and the essential oil were analyzed by GC/MS with an injector temperature of 250 °C; the temperature program was 80–200 °C at 20 °C/min; 200–300 °C at 15 °C/min, with other conditions equal to those of GC-FID. The identification of the chemical composition of the essential oil and fractions was performed by comparing the mass spectra obtained with the data available in the library (NIST version 8.0).

The ^{1}H NMR spectra were obtained on a Bruker AC-300 MHz 300F. Spectra were obtained in deuterated chloroform (purity 99.8% + 0.05% TMS) obtained from Cambridge Isotope Laboratories Inc., with tetramethylsilane as the internal reference (TMS). Chemical shifts were recorded in dimensionless values δ (ppm) indicating the sign as singlet (s), doublet (d), triplet (t), *etc.*

^{1}H NMR ($CDCl_3$, 300 MHz) essential oil consisting mainly of ascaridole and *p*-cymene. Ascaridole δ: 1.03 (d, *J* = 6.9, H9, H10), 1.39 (s, H7), 1.52 (d, *J* = 9.0, H5), 1.91 (sept, *J* = 6.9, H8), 2.07 (d, *J* = 9.0, H6), 6.43 (d, *J* = 8.7, H3), 6.51 (d, *J* = 8.7, H2). *p*-Cymene δ: 1.26 (d, *J* = 6.9, H9, H10), 2.34 (s, H7), 2.90 (n, *J* = 6.9, H8), 7.16 (s, H3, H4, H5, H6).

The essential oil, ethanol extract and dichloromethane, ethyl acetate and butanol fractions from the extract were evaluated for cytotoxicity using K562 (myeloid leukemia), Nalm6 and B15 (acute B lymphoblastic leukemia), and RAJI (Burkitt's lymphoma) cells. Cell viability was assessed by the MTT (3 (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, which is based on a method using a dye, *i.e.* a tetrazolium salt soluble that is in water which is converted to purple formazan after reduction by mitochondrial dehydrogenases in viable cells (Mosmann, 1983).

Cells were grown in appropriate plastic dishes with DMEM (Dulbecco's Modified Eagle's Minimum Essential Medium), supplemented with fetal calf serum (FBS) 10% inactivated for 1 h at 56 °C, 10 mM of ethanesulfonic acid hydroxyethyl piperazine (HEPES), 1.5 g/l of sodium bicarbonate, 1% of penicillin G (100 U/ml), 100 mg/ml of streptomycin, 50 μg/ml of amphotericin B in an incubator humidified at 37 °C with 5% CO_2 emissions. Before the experiments, the number of viable cells was determined by the trypan blue exclusion method, with counts performed in a Neubauer chamber.

Cells were plated using epMotion® 5070 equipment (Eppendorf, Vaudaux, Schonenbuch, Switzerland) which distributed 2×10^4 cells per well in 96-well plates, which were incubated with the test substances at different concentrations (0.01, 0.1, 1, 10, 100 and 1000 mg/ml) for 48 h at 37 °C in 5% CO_2. DMSO was used for solubilization of the test substances in a maximum concentration of 0.1% well/treatment; this concentration is not cytotoxic to the cells. Doxorubicin at the same concentrations tested was used as a positive standard.

After the treatment period, the medium was removed and 100 μl of the MTT solution was added at 0.5 mg/ml in the culture medium and incubated for 4 h. After that time, the medium was removed and the formazan precipitate was dissolved in 100 μl DMSO/well and reading was performed on a microplate reader at 540 nm (Bio-Tek Power Wave XS). The optical density obtained in the control group, *i.e.* untreated cells (incubated with growth medium only), was regarded as 100% viable cells in order to establish concentration *vs.* response curves, and therefore the IC_{50} (concentration inhibiting 50% growth of cell), according to

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