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Mikania laevigata: Chemical characterization and selective cytotoxic activity of extracts on tumor cell lines

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ABSTRACT

Cancer is the second major cause of mortality worldwide, losing only to cardiovascular disease. Nowadays, around 50% of antineoplastic drugs were discovered and isolated by indications of plants in folk medicine. In Brazilian flora there are many species of plants which have great therapeutic importance, highlighting the Mikania laevigata (Asteraceae) that has been used for their valuable properties, especially in the respiratory tract. In the present study, the compounds of M. laevigata extracts were characterized by High Resolution Mass Spectrometry (HRMS) and Gas Chromatography with Mass analysis (GC/MS-EI). Therefore, the presence of some compounds with promising biological properties as antitumor activity was detected. Coumarin (1,2-benzopyrone) was previously reported as responsible for some biological activities of this plant species. Here, the extracts were evaluated by their cytotoxic activity against tumor (Hep-2, HeLa) and non tumor (MRC-5) cell lines, presenting significant inhibitory activity of cell growth in all extracts analyzed, chloroform, ethyl acetate, hexane, ethanol, which is related to its chemical composition. From the four different extracts here tested, two of them, hexane and ethanol, presented a clear selectivity against both tumor cells lines investigated. This can be explained by variances and increase of phenolic compounds in the ethanol fraction and an association of molecules with coumarin found in the hexane fraction.

Introduction

The great diversity of vegetal species with therapeutic potential in Brazilian ecosystems provides material for specialized studies to research new drugs against different diseases. Compounds isolated or derivatives from plants have contributed to treatment of different neoplasias. A notorious example concerns to vinblastine and vincristine alkaloids, which were isolated from Catharanthus roseus and used in medical practice as antineoplastic agents (Cragg and Newman 2005).

The use of plants compounds as prototypes of new drugs has a historical and economic importance. Some plants extracts were defined as effective in treating cancer, whose action is attributed to additional or synergistic effect of compounds present in the extract (Li et al. 2000). In consequence, the cytostatic effect observed in tumor cells seems to be more effective than the effect of isolated and biologically active compounds (Vickers 2002).

The *Mikania* genus, Asteraceae family, has a successful therapeutic importance (Rufatto et al. 2012). Mikania laevigata is a subarbustive plant from South America localized in Southeast and South Brazil, from São Paulo to Rio Grande do Sul. It has been initially used for the treatment of respiratory disorders including asthma, bronchitis, chronic lungs diseases and for cough (Santos et al. 2006; Bolina et al. 2009). Its antiulcer, anti-inflammatory, analgesic, antispasmodic and antimicrobial (Bighetti et al. 2005; Suyenaga et al. 2002; Yatsuda et al. 2005) activities also have been investigated, but so far no reports suggested selectivity of extracts against tumor cell lines.

The pharmacological effects of Mikania genus are attributed mainly to the presence of coumarin (1,2-benzopyrone) and derivatives. However, other metabolites showed to produce significant pharmacological effects. Recent studies report the presence of coumarin, triterpenes/steroids, flavonoid glycosides, dihydrocoumarin, o-coumaric acid, kaurenoic acid, cinnamoylgrandifloric acid, stigmasterol, cupressenic acid, isopropiloxi-grandifloric acid, isobutiloxi-grandifloric acid, kaurenol, spathulenol, caryophyllene oxide, syringaldehyde, saponins, tannins (Bolina et al. 2009; Bighetti et al. 2005; Yatsuda et al. 2005; Santos et al. 2006). Ferreira and Oliveira (2010) demonstrated new constituents, which were isolated from the leaves of M. laevigata: taraxerol, lupeol, trans-melilotoside, cis-melilotoside, adenosine, patuletin 3-O-β-D-glucopyranoside, kaempferol 3-O-β-D-glucopyranoside, quercetin 3-O-β-D-glucopyranoside, methyl-3,5-di-O-caffeoyl quinate and 3,3',5-trihydroxy-4',6,7-trimethoxyflavone.

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Few studies have reported cytotoxic activity of isolated compounds or total extracts obtained from the *Mikania* genus. This study aims to characterize the chemical composition and evaluate the cytotoxic activity in a selective manner of different extracts obtained from leaves of *Mikania laevigata* against the tumor cell lines Hep-2 (human laryngeal epidermoid carcinoma cells) and HeLa (human cervical adenocarcinoma), and non tumor MRC-5 (human lung fibroblast) cell line.

Materials and methods

Chemicals

All the reagents were of ultrapure grade. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA). Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were acquired from Hyclone Lab Inc. (USA). Folin-Ciocalteu's phenol reagent (Merck) was used for the determination of total phenols.

Plant material

The leaves of *Mikania laevigata* Sch. Bip. ex Baker were collected in October 2011, in Garibaldi city (29°13'32.28" S and 51°32'11.13" W) located in Rio Grande do Sul state, southern Brazil. The species was identified based on the literature and its voucher specimen deposited in the Herbarium of the University of Caxias do Sul (HUCS 38180).

Mikania extracts

The extracts were obtained by maceration under sonication of the crushed fresh leaves (50 g), at room temperature, for 20 min, using the following solvents: hexane, chloroform, ethyl acetate, ethanol and ethanol/water (1:1, v/v). After extraction, the mixture was filtered and the solvent evaporated. The five dried extracts obtained were dissolved in 50% ethanol.

Chemical composition of the extracts

For chemical analysis by High Resolution Mass Spectrometry (HRMS), the extracts of M. laevigata were diluted in specific solutions according with the analysis mode (positive or negative). In positive mode, 0.1 ml of each extract was diluted in 1 ml of a solution of chromatographic grade acetonitrile/deionized water (1:1, v/v) and 0.1% formic acid. In the negative mode, 0.1 ml of each extract was diluted into 1 ml of a solution of chromatographic grade acetonitrile/deionized water (1:1, v/v) and 0.1% ammonium hydroxide. The solutions were infused directly into the ESI source by means of a syringe pump (Harvard Apparatus) at a flow rate of 10 μl min^{-1}. ESI(+)-MS, ESI(-)-MS and tandem ESI-MS/MS were acquired using a hybrid high-resolution and high accuracy (5 μl/l) Orbitrap mass spectrometer (Thermo Fisher Scientific) with the conditions: capillary and cone voltages were set to +3500 V and +40 V, respectively, with a de-solvation temperature of 100 °C. For MS/MS, the energy for the collision induced dissociations (CID) was optimized for each component. Diagnostic ions in the Mikania extracts were identified by the comparison of their MS/MS dissociation patterns with compounds identified in previous studies. For data acquisition and processing, Xcalibur software (Thermo Fisher Scientific) was used. The data were collected in the *m*/*z* range of 50–700 at the speed of two scans per second, providing the resolution of 50,000 (FWHM) at *m*/*z* 200. No important ions were observed below *m*/*z* 50 or above *m*/*z* 450, therefore ESI(+)-MS and ESI(-)-MS data is shown in the *m*/*z* 50–450 range.

In addition, extracts of M. laevigata were evaluated qualitatively and quantitatively by Gas Chromatograph coupled with Mass Spectrometer with ionization by Electron Impact (GC/MS-EI) in Hewlett Packard 6890/MSD 5973, equipped with HP-Chemstation software and Wiley 275 library spectra. Analyses were performed on a column HP-5MS 5% phenylmethylsiloxane (30 m × 250 μm), 0.25 μm thick film (Hewlett Packard, California, USA) with the following temperature program: 40 °C (4 min), 310 °C at 8 °C/min (35 min), 280 °C interface, splitless, carrier gas He (43 cm/s), acquisition mass range 45–550 and 1 μl injection.

Phenolic compounds

The quantitation of phenolic compounds was performed by the Folin-Ciocalteu colorimetric method, which involves the reduction of the reagent by the phenolic compounds of the samples with concomitant formation of a blue complex. The total phenolics content of each extract was quantified using a standard curve prepared with gallic acid (5–120 μg/ml; Chem. Service, Inc., USA) and expressed as μg gallic acid equivalents (GAE)/mL of extract.

Cytotoxic assay

Tumor (Hep-2, HeLa) and non tumor (MRC-5) cell lines were cultured in DMEM supplemented with antibiotics and 10% Fetal Bovine Serum (FBS) at 5% CO_2 and 37 °C. For the assessment of the cytotoxic activities of hexane, chloroform, ethyl acetate, ethanol and ethanol/water Mikania extracts, cells were seeded in 96-well flat-bottomed microplates at a density of 7×10^4 cells/ml in 10% FBS DMEM. After cell attachment, serial dilutions (10–800 μg/ml) of the extracts in culture medium were added and incubated for 1 h, subsequently incubated without extract for 24 h. Cell proliferation was determined by the Tetrazolium salt method (MTT) (Denizot and Lang 1986). At the end of the incubation period, following incubation with MTT solution for 2 h, the media was removed, the formazan crystals were solubilized in 100 μl dimethyl sulfoxide (DMSO)/well and the absorbance values were determined at 540 nm. At least three independent experiments were taken for each experimental cell line and IC_{50} (dose causing 50% cell death) calculated using mean and standard deviation.

Statistical analysis

Results were expressed as mean ± standard deviation obtained from three independent experiments. Statistical significance was evaluated using analysis of variance ANOVA and Tukey test. *P*-values less than 0.05 were considered significant by the SPSS 20.0 program.

Results and discussion

The genus Mikania is among the list of best-selling natural products in the world because it's biological activity. Different classes of compounds were previously isolated from Mikania, which can be associated to the pharmacological potential observed and related to the plant. Different compounds were search in extracts using a variation of solvent polarity (hexane, chloroform, ethyl acetate, ethanol and ethanol/water (1:1)). We selected High Resolution Mass Spectrometry (HRMS) and Gas Chromatography with Mass (GC/MS-EI) to evaluate the complex constituents. Some differences between the techniques are: HRMS detects volatile and nonvolatile compounds, confirms the chemical structure by its molecular ion and tandem MS/MS; the GC/MS-EI is able to analyze only volatile compounds giving fragmentation pathways dates. They are complementary techniques. The literature reports different information about compounds identification using GC/MS-EI and HRMS.