Antiproliferative activity, isolation and identification of active compound from Gaylussacia brasiliensis

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Abstract: Gaylussacia brasiliensis (Spreng.) Meissn., Ericaceae, is used in folk medicine for treatment of several inflammatory processes and as healing agent. The scope of this work was to evaluate the in vitro antiproliferative activity of crude dichloromethane extract (CHD) and to identify the compound(s) responsible for this activity. CHD was evaluated and showed a concentration dependent inhibition on all cells lines. Therefore CHD was submitted to several classical columns chromatography providing the most active fraction (FC), inhibiting all cells line at 25 µg/mL. FC was further fractionated affording isolated compound 2β, 3β-dihydroxy-urs-12-ene-28-oic acid, identified on basis of 2D-NMR experiments and showed concentration-dependent activity and selectivity for kidney and breast cell lines.

Keywords: antiproliferative Gaylussacia brasiliensis medicinal plants

Introduction

Plants have been used as a source of medicine throughout history and continue to serve as the basis for many pharmaceuticals used today (Cragg et al., 2009). Natural products continue to play a major role for the discovery of chemotherapeutic agents or as leads for the development of modern medicines (Newman & Cragg, 2007; Verpoort, 2000; Cragg et al., 1999; Cragg & Newman, 1999; Verpoort, 1998). According to the world health organization (WHO), about three-quarters of the world population relies upon traditional remedies (mainly herbs) for health care of populations (Gilani & Rahman, 2005). Over the last years, the interest in research on natural products has increased focusing on new chemotherapeutic substances and resulting in the discovery of more efficient drugs for many diseases and especially on cancer treatment, playing an important role in the development of chemotherapy (Newman, et al., 2003; Philipson, 2001; Calixto, 2000; Lee, 1999).

The pharmaceutical industry widely implemented biochemical assays and high-throughput screening in the 1990s, and as a result, natural product screening programs have been de-emphasized (Rishton, 2008). During the past fifty years, plants have provided several more clinically used drugs. One of the most important examples is the Catharanthus alkaloids vinblastine and vincristine, currently used for treatment of leukemia, lymphomas and some solid tumors were introduced through the Eli Lilly Company in the 1960s. The NCI collaborative research programme into natural products with anticancer activity was initiated in 1957, and between 1960 and 1986 more than 35000 species were screened against murine tumors and from eleven compounds approved for extensive tumor panel testing, two came into clinical use (Philipson, 2007). According to Walker & Croteau (2001) and Schiff et al. (1979), other important example is the taxoids compounds (docetaxel and paclitaxel), obtained from Taxus genus species (Mattos et al., 2001; Pezzuto, 1997) and the camptothecin derivates (irinotecan and topotecan), obtained from Camptotheca acuminata (Chang, 2000; Wall, 1998; Wall, et al. 1966).

Currently, over a hundred types of cancer are known, differentiated by etiology, natural history therapeutic and procedures. Notwithstanding the
great evolution of basic knowledge concerning this pathology, it has not reflected in the development of efficient techniques of prevention and cure (Verdecchia et al., 2001; Verweij & de Jonge, 2000).

In 1997; Chemical, Biological and Agricultural Research Center at State University of Campinas (Unicamp), with financial support from Fundação de Amparo à Pesquisa do Estado de São Paulo, established a research program in order to identify natural products with potential antineoplastic activity. Therefore, a survey of several plant species from the Brazilian cerrado was carried out (Denny et al., 2008). This region is a hotspot that harbors a great diversity of endemic species and overmore has been significantly impacted and altered by human activities (Myers et al., 2000). This biodiversity provides a great chemical variety encountered among the region, the relatively small amount of biologic knowledge and the fast cerrado extinction areas (Ferri, 1969). Gaylussacia brasiliensis (Spreng) Meissner, Ericaceae, was selected throughout the screening programm. This work evaluated the in vitro antiproliferative activity of extracts and isolated compound from G. brasiliensis, popularly known as “camarinha”. This plant is an arbust, which could be found in the Atlantic Forest (Ilha do Mel, Paranaguá, Paraná State - Brazil) (Corrêa, 1984).

The genus Gaylussacia is known to present antifungal activity, probably due to the presence of phenolic compounds (Cipollini & Stiles, 1992). Other known compounds in the genus include stilbene (Askari et al., 1972) and anthocyanin derivatives (Ballington et al., 1988). An update literature search has shown that the compounds present in this genus have not been evaluated for activity on the in vitro antiproliferative assay.

Material and Methods

General experimental procedures

The physical and spectral data (1H-NMR, 13C-NMR) were recorded at 11.75 Tesla (500 MHz for 1H and 125 MHz for 13C) using INOVA 500 spectrometer, tetramethylsilylane was used as internal standard. Optical rotation on Polarimeter-Lep A, with sodium lamp; IR spectra recorded on Bomen MB series Hartmann & Braun-Michelson instrument TLC was performed on precoated aluminum sheets (Merck 5554). Compounds were visualized by spraying with 4-anisaldehyde-H2SO4-acetic acid (0.5:1:50) followed by heating at 110-120 °C to yield blue, pink or purple spots. For column chromatography, Si 60 (70-230 mesh and 230-400 mesh, Merck) All solvents were analytical grade and redistilled before use.

Gas chromatography/mass spectrometry analysis (GC/MS) were carried out using a HP-5890/5970 system equipped with a J&W Scientific CP-SIL 24 CB fused capillary column (30 m x 0.25 mm x 0.25 m). Temperature program: 150 °C (2 min); (5 °C/min); 240 °C; (10 °C/min); 300 °C (34 min). Injector temperature: 280 °C; detector temperature: 300 °C. Helium was used as carrier gas (0.7 bar, 1 mL/min). The MS were taken at 70 eV. Scanning speed was 0.84 scans.s⁻¹, from 40 to 550 atomic mass unit. Sample volume was 1 µL.

Plant material

The aerial parts of Gaylussacia brasiliensis (Spreng) Meissner, Ericaceae, were collected at “Reserva Biológica e Estação Experimental de Mogi Guaçu” in São Paulo State, Brazil (22°15’07.18”S; 47°10’13.37”W) by botanist Maria do Carmo Estanislau do Amaral and Volker Bittrich. Voucher specimen is deposited at Instituto de Biologia, Unicamp, under registration number UEC-266.

Crude extract and fractions preparation

The material was grinded to a fine powder prior to use. The powder (500 g) was submitted to dynamic maceration with dichloromethane (700 mL) during 4 h. This procedure was repeated three times with the same powder. After filtration, the solvent evaporated under vacuum at 40 °C resulted in the crude dichloromethane extract (CHD) with 9.6% yield.

The crude extract was pre-purified by dry column chromatography on silicagel 60 (Merck 7734) with chloroform/methanol 5% providing differences five fractions. The active Fraction C was further fractionated (10 g) on successive column chromatography using Silicagel (Merck 7734) (5 x 60 cm) with hexane/CH3Cl mixtures, affording compound 2β,3β-dihydroxy-urs-12-ene-28-oic acid.

Cell culture

The experiments were performed using the following human cancer cell lines: MCF-7 (breast), NCI-ADR (ovarian expressing the multidrug resistance phenotype), NCI-460 (lung), UACC-62 (melanoma), 786-0 (renal), OVCAR-03 (ovarian), PC-03 (prostate) and HT-29 (colon). The National Cancer Institute, Frederick MA/USA, kindly donated these cell lines; and stock cultures were kept in liquid nitrogen.

Cells were cultured in 25 cm² flasks (Nunc Brand Products) containing 5 mL of RPMI 1640 (Gibco BRL, Life Technologies.) with 5% fetal bovine serum (Gibco BRL, Life Technologies). The cells are used up to twenty serial passages, afterwards they are discarded.
and new flasks are unfrozen for use.

**Biological assays**

All the adherent cell lines were detached from the culture flasks by addition of 0.5 mL of trypsin (Nutricell Nutrientes Celulares). Thereafter, trypsin was inactivated by addition of 5 mL of 5% serum in RPMI 1640 medium. Cells were separated into single-cell suspensions by a gentle pipetting action. After counting, the cells were diluted into appropriate seeding densities and inoculated onto 96-wells microtiter plates (Nunc Brand Products). Cells plating volume was 100 μL per well. Seeding densities varied among the cell lines as follows: 6.5 x 10^4 (MCF - 7); 5.0 x 10^4 (NCI - ADR); 4.0 x 10^4 (NCI - 460); 3.0 x 10^4 (UACC62); 5.0 x 10^4 (786 - 0); 6.5 x 10^4 (OVCAR - 03); 4.5 x 10^4 (PC - 03) and 5.0 x 10^4 (HT - 29) cells per mL. Microtiter plates containing cells were pre incubated for 24 h at 37 ºC in order to allow stabilization before the addition (100 μL) of the test substance (crude extract, fractions and drugs). The plates were incubated with the test substance for 48 h at 37 °C and 5% CO₂. The positive controls of these experiments were DOX (doxorubicin) and TAM (tamoxifen) both from Sigma Chemical Company. These agents were tested at five 10-fold concentrations, starting from with maximum concentration of 10⁻⁴ Mol for DOX and 5 10⁻⁶ Mol for TAM in RPMI/FBS/gentamicin (Chabner, 1993).

**Solubilization and dilution of test substance**

For initial screening, the crude extracts were tested at 250 μg/mL. If antiproliferative activity was detected, the test substance was retested at four concentrations (0.25; 2.5; 25; 250 μg/mL), and each concentration was studied in triplicate wells. All samples were initially solubilized in dimethyl sulfoxide (Sigma Chemical Company) at 400 times the desired drugs concentration eliciting 50% inhibition) were determinate by non linear regression analysis. The values for mean±average standard error of data from replicated wells were calculated, from this the background optical measurements are subtracted from appropriated control well values and the appropriate drug-blank measurements. Cellular responses were calculated for growth stimulation, test substance effect, and growth inhibition. Three measurements were run at: a time zero (T0) value of SRB protein content at the beginning of test substance incubation, control value (C) at the end of the test substance incubation, and a set of test substance-treated test values (T) at the end of the test substance incubation period. If T was greater than or equal to T0 (cytostatic effect), the calculation was 100 x [(T-T0)/(C-T0)]. If T was less than T0, cell killing (cytotoxic effect) occurred and was calculated from 100 x [(T-T0)/T0]. The IC₅₀ values (drugs concentration eliciting 50% inhibition) were determine by non linear regression analysis.

This results presented here refer to a representative experiment since all assays were run in triplicates the average standard error was always lower than 5%.

**Results and Discussion**

The SRB assay was performed according to the assay described by Skekan et al. (1990). Briefly, the cells were fixed by means of protein precipitation with 50% trichloroacetic acid (TCA) (Sigma Chemical Company) at 4 °C (50 μL/well, final concentration 10%) for 1 h. The supernatant was then discarded and the plates were washed five times with tap water. The cells were stained for 30 min with 0.4% the SRB (Sigma Chemical Company), dissolved in 1% acetic acid (50 μL/well) (Sigma Chemical Company) and subsequently washed four times with 1% acetic acid to remove unbound stain. The plates were dried and bound protein stain was solubilized with 150 μL of 10mM Trizma buffer (Sigma Chemical Company). The optical density was read on an automated spectrophotometer plate reader (Molecular Devices Versa Max Microplate Reader) at 540 nm.

**Data calculations**

The optical density data were calculating according to Excel® program (Microsoft Office Package) and the values for mean±average standard error of data from replicated wells were calculated, from this the background optical measurements are subtracted from appropriated control well values and the appropriate drug-blank measurements. Cellular responses were calculated for growth stimulation, test substance effect, and growth inhibition. Three measurements were run at: a time zero (T0) value of SRB protein content at the beginning of test substance incubation, control value (C) at the end of the test substance incubation, and a set of test substance-treated test values (T) at the end of the test substance incubation period. If T was greater than or equal to T0 (cytostatic effect), the calculation was 100 x [(T-T0)/(C-T0)]. If T was less than T0, cell killing (cytotoxic effect) occurred and was calculated from 100 x [(T-T0)/T0]. The IC₅₀ values (drugs concentration eliciting 50% inhibition) were determinate by non linear regression analysis.

This results presented here refer to a representative experiment since all assays were run in triplicates the average standard error was always lower than 5%.

Sulfurhodamine B (SRB) is an aminoxantine with a bright pink color that has two sulfonic groups. Since it is an anionic dye in weak acid solution it is capable of bonding to protein’s aminoacids basic terminals cells fixed with trichloroacetic acid. Therefore this non-clonogenic methodology permits a high sensitive protein with a straight relationship to cell culture (Johnson, 1990; Skekan et al., 1990).

Our phytochemistry studies were biomonitoried throughout the cell growth percentages analysis on the antiproliferative assay. The criteria selection for those extract and/or fractions was growth percentage lower than 50% (negative values meaning cell death) and/or selective activity on a type of cell line, with a profile of concentration-dependent antiproliferative activity. The positive controls were the chemotherapeutical doxorubicin...
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In this study, aerial parts from Gaylussacia brasiliensis (Spreng) Meissner were initially extracted with dichloromethane and sequentially with 70% ethanol. The crude extracts were tested at concentrations from 0.25 to 250 μg/mL during 48 h. The crude dichloromethane extract (CHD) presented the highest antiproliferative activity, inhibiting all cell lines with cellular death for OVCAR-03 (ovarian), PC-03 (prostate) and 786-0 (renal) lines in a concentration-dependent relationship, whereas DOX showed no selectivity in its antiproliferative action (Table 1).

Subsequently, CHD was pre-purified by dry column chromatography over silica-gel affording five different fractions, from which fraction C (FC) was most active, inhibiting all cell lines at 25 μg/mL. Fraction C obtained from CHD presented antiproliferative activity in all cell lines studied and was more potent than CHD. The IC50 values for all cell lines are listed in Table 1.

Table 1. Efficacies of CHD (dichloromethane extract), FC (fraction C), isolated compound from Gaylussacia brasiliensis (Spreng) Meissner and positive controls (DOX: doxorubicin and TAM: tamoxifen) against human tumoral cell lines, assessed by the Sulforhodamine B assay.

<table>
<thead>
<tr>
<th>Cancer cell lines</th>
<th>IC50 (µg/mL) values of samples*</th>
<th>CHD</th>
<th>FC</th>
<th>isolated compound</th>
<th>DOX</th>
<th>TAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>UACC-62 (melanoma)</td>
<td>324.8</td>
<td>144.5</td>
<td>180.2</td>
<td>2.39</td>
<td>13.6</td>
<td></td>
</tr>
<tr>
<td>MCF-7 (breast)</td>
<td>ND</td>
<td>12.6</td>
<td>44.5</td>
<td>1.33</td>
<td>4.2</td>
<td></td>
</tr>
<tr>
<td>NCI-ADR*</td>
<td>306.6</td>
<td>0.3</td>
<td>6.2</td>
<td>43.9</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td>786-0 (renal)</td>
<td>441.1</td>
<td>10</td>
<td>10.6</td>
<td>0.006</td>
<td>4.7</td>
<td></td>
</tr>
<tr>
<td>NCI-460 (lung)</td>
<td>803.6</td>
<td>0.6</td>
<td>25.3</td>
<td>1.19</td>
<td>3.3</td>
<td></td>
</tr>
<tr>
<td>PC-03 (prostate)</td>
<td>321.9</td>
<td>21.9</td>
<td>56.2</td>
<td>27.54</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>OVCAR-03 (ovarian)</td>
<td>372.9</td>
<td>0.5</td>
<td>7.9</td>
<td>1.8</td>
<td>4.9</td>
<td></td>
</tr>
<tr>
<td>HT-29 (colon)</td>
<td>ND</td>
<td>11.4</td>
<td>121.3</td>
<td>ND</td>
<td>0.01</td>
<td></td>
</tr>
</tbody>
</table>

IC50 values (concentration eliciting 50% inhibition) were determined from non linear regression analysis; ND: not be determined. The dose range tested was 0.25 a 250 μg/mL. *Ovarian expressing the multidrug resistance phenotype.

The isolated compound showed antitumoral activity with a concentration-dependent relationship, with cellular selectivity for 786-0 and MCF-7 cancer lines; the lowest IC50 was for NCI-ADR line with 6 μg/mL (Table 1). The similarity of 2β,3β-dihydroxy-urs-12-ene-28-oic acid to steroidal compound’s activity was demonstrated by the inhibition of hormone-dependent lines (ovarian, prostate and breast). This compound presented citotoxity for all cell lines from 70 μg/mL concentrations, in a straight concentration-dependent relationship, such as the positive control tamoxifen (Table 1). This result may suggest a possible connection of hormonal receptors and intracellular signalization with the pharmacological mechanism.

Some sterols such as the glucocorticoids are used in chemotherapy in order to prevent the proliferation of lymphocytes. Additional examples are estrogens (fosfestrol) prescribed for prostate cancer and progestogens (megestrol and medroxiprogesterone) for endometrium cancer (uterus). As hormonal antagonists used in chemotherapy there is the goserelin, which acts in inhibition of gonadotrofines’ liberation in treatment of breast and prostate cancers, the tamoxifen (antiestrogen) for breast cancer and the flutamide used for prostate cancer (Calabresi & Chabner, 2001).

Despite the high chemical purity of isolated compound, fraction C obtained from CHD presented was more potent, suggesting the presence of other compounds involved in the antiproliferative activity. Nowadays, studies on its mechanisms of action studies are being investigated and the bioassay guided isolation of other active components present in this fraction is also being carried out.

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