Antiproliferative activity of crude extract and fractions obtained from *Aspidosperma tomentosum* Mart.

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ABSTRACT: Antiproliferative activity of crude extract and fractions obtained from *Aspidosperma tomentosum* Mart. The purpose of this study was to evaluate the antiproliferative activity of *Aspidosperma tomentosum* Mart ("peroba do campo") dichloromethane (CHD) and ethanol (CHE) extracts. The in vitro assay was performed using five human cells line: K562 (leukemia), MCF7 (breast), NCIADR (breast expressing the multidrug resistance phenotype), NCI460 (lung) and UACC62 (melanoma), which response was determinate through the Sulforhodamine B assay (SRB). The extracts were tested in concentration between 15.6 and 125 µg/mL, and a concentration dependent inhibition was observed for both crude extracts on MCF7, UACC62, NCIADR and NCI460. However, an important cytocidal activity (46%) was observed for MCF7 when CHD at 125 was tested. Therefore the CHD was submitted to column chromatography on silicagel providing fractions denominated unpolar fraction (UP), terpenic medium polar fraction (TF), alkaloid medium polar fraction (AF) and polar fraction (PF). The best results were observed on MCF7 and NCI460 strains with TF fraction, whereas PF fraction was unspecific, inhibited all the cells lines giving cytotoxicity at 125 mg/mL. Fractions AF and UF did not present a significant inhibition in the tested concentrations. This results suggests that the active principle(s), which is (are) responsible for the antiproliferative activity is (are) found in fraction FT and PF demonstrating a concentration dependent inhibition.

Key words: *Aspidosperma tomentosum* Mart, antiproliferative assay, medicinal plants.

INTRODUCTION

Currently, over a hundred types of cancer are known, differentiated by etiology, natural history and procedures. Notwithstanding the great evolution of basic knowledge concerning this pathology, such progress has not been reflected in the development of efficient techniques of prevention and cure (Hemminki & Mutanen, 2001, Verdecchia, 2001). Low selectivity of chemotherapeutic agents, different levels of toxicity to normal tissues and rapid development of resistance against these drugs are limiting factors to the success of the antineoplastic treatment. Therefore, there is a great need for new and better therapeutic resources, including new chemotherapeutic agents (Verweij & Jonge, 2000; Karsila et al., 2001; Eikesdal et al., 2001).

Over the last years, the interest in research on natural has increased resulting in the discovery of more efficient drugs for cancer treatment (Calixto, 2000; Rates, 2001; Phillipson, 2001). One of the most important examples is the taxoid compound (docetaxel and paclitaxel), obtained from *Taxus* genus species (Walker & Croteau, 2001) and the camptothecin derivatives (irinotecan and topotecan), obtained from *Camptotheca acuminata* (Mattos, et al., 2001).

Another interesting point was to study biodversity coupled to species preservation. Also the lack of modern inexpensive medicines for the needy population tend to increase the run for cheap alternatives, that have rather doubtful efficiency, promising miracles without side effects (Calixto et al., 2001, Taylor & Staden, 2001) and creating the false idea of absolute safety of natural products (Chang, 2000).

In 1997, the Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas (CPQBA) of the Universidade Estadual de Campinas (UNICAMP), with the support of Fundação de Amparo a Pesquisa from the State of São Paulo (FAPESP), began a program in order to identify natural products with potential antineoplastic activity. Therefore, a survey of several plant species from the Brazilian cerrado woods was carried out. This region was selected considering the great chemical variety encountered among this biodiversity. Summed up to the relatively small amount of biologic knowledge and the rapid Cerrado extinction areas aroused our interest in this study.

This work evaluated the in vitro antiproliferative activity of extracts and fractions of *Aspidosperma tomentosum* Mart, popularly known as "peroba do campo". The trunk of this tree has been used as wood and the seeds in art and craft manufactures (Ferri, 1969; Pio Corrêa, 1942). The species belongs to the Apocinaceae family, one of the main sources of antineoplasic substances such as the alkaloids from *Cataranthus roseus* (Schenkel et al., 1999).

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MATERIAL AND METHOD

Plant Material

The aerial parts of *Aspidosperma tomentosum* Mart were collected at “Fazenda Campininha” in Mogi-Guacu (São Paulo State/Brazil), and taxonomically identified. Voucher specimen is deposited at Instituto de Biologia / UNICAMP under registration number UEC-267.

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 3 times with the same solvent. After filtration, the solvent was evaporated under vacuum at 40°C resulting the crude dichloromethane extract (CHD) with 22% yield.

The plant residue was submitted to dynamic maceration with ethanol 70% (700 mL) during 4 h. This procedure was repeated 3 times with the same solvent. After filtration, the plant residue was discarded and the solvent evaporated under vacuum at 55°C resulting the crude hydroalcoholic extract (CHE) with 35% yield.

The dichloromethane crude extract was submitted to treatment with a methanolic/HCl (95:5) solution (V/V), leaving to stand overnight. The crude extract was concentrates to 25% the original volume. The suspension was washed with hexane to remove non-alkaloids. The acid layer was adjusted to pH 9.0 with NH₄OH and partitioned against CH₂Cl₂. The combined layers were washed with water and concentrated in vacuum, yielding 0.1% crude alkaloid fraction (AF). The remaining non-alkaloid fraction was fractioned by dry column chromatography on silicagel 60 (Merck 7734) with a chloroform:methanolic (95:5) solution (V/V) as solvent mixture providing three fractions, denominated unpolar fraction (UP), terpenic medium polar fraction (TF), and polar fraction (PF). These fractions were submitted to gas chromatography analysis and biological assays.

Chromatographic analysis

Gas Chromatography / Mass Spectrometry analysis (GC/MS): The GC/MS analyses 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 0.25mm x 0.25 mm). Temperature programs 150°C (2min)-(5°C/min.) – 240°C – (10°C/min)-300°C (34 min). Injector 280°C and detector 300°C. Helium was used as carrier gas (0.7 bar, 1 mL/min). The MS were taken at 70eV. Scanning speed was 0.84 scans/s from 40 to 550. Sample volume was 1 µL.

Biological Activities

Human tumor cell lines UACC62 (melanoma), MCF-7 (breast), NCI 460 (lung, nonsmall cells), K562 (leukemia) and NCI-ADR (breast expressing fenotipe multiple drugs resistence) were kindly provided by National Cancer Institute (NCI). Stock cultures were grown in a medium containing 5 mL of RPMI 1640 (GIBCO BRL, Life Technologies) and supplemented with 5% of fetal bovine serum. Gentamicine (50µg/mL) was added to the experimental cultures. Cells in 96-well plates (100 µL cells/well) were exposed to various concentrations of samples in DMSO (15.6, 31.3, 62.5 and 125 µg/mL) at 37°C, 5% of CO₂ in air for 48h. The final concentration of DMSO did not affect the cell viability. Then, a 50% of trichloroacetic acid solution was added and after incubation for 30 min at 4°C, washing and drying, the cell proliferation was determined by spectrophotometric quantification (540 nm) of the cellular protein content using sulforhodamine B assay described by Skehan et al., 1985.

Statistical analysis

The absorbance mean were calculated using the Excel® software (Microsoft, USA) and the standart error of the mean were calculated for 4 wells in triplicated samplings. The results presented here refer to a representative experiment since all assays were run in triplicated and the average standard error was always < 5%. The data were submitted to one way variance analysis with critical range at p < 0.05 and afterwards to Duncan’s test with the same critical range.

RESULT AND DISCUSSION

In the last fifty years, research on anticancer drugs introduced new drugs in therapy, but most of these drugs are only useful in rapid growth tumors, such as leukemia and lymphomas. The lack of drugs for solid tumors probably is consequence of murine leukemia screening models used (Johson, 1999; Gragg, 1994). Therefore, there is an evident needing of screening models for drugs oriented for solid tumors with methodology using human cancer cells (Gragg & Newman, 1999; Boyd, 1989).

The in vitro screening methodology recommended by the National Cancer Institute (USA), employed in this work, allow the evaluation of drugs in various types of neoplastic cells and the discovery of new specific agents. Other advantages are the quickness, efficiency and reproducibility of the method (Monks et al., 1991).

The selection of active substances was carried
throughout the analysis of cell growth percentages, considering those extracts and/or fractions which growth percentage lower than 50% (negative values meaning cell death) and/or selective activity on a type of cell line, with concentration-dependent. The positive control used was the chemotherapeutic doxorubicin (FIGURE 1). In the graph, the line presented on point zero (0) of the coordinate axis, representing the quantity of cells at the moment of addition of test substances. The data presented in the upper part of this line indicate cytostatic activity and those below this line refer to the cytocidal activity. This last activity is considered more interesting, since it provides the elimination of the tumor nodule, besides reducing chances of resistant cells formation to chemotherapy substances (Gottesman, 1993).

In this study the plant was initially extracted with dichloromethane and sequentially with 70% ethanol. The dichloromethane extract concentrates the less polar compounds, whereas in the ethanol extract polar compounds are the main components (Fellows, 1992). These crude extracts inhibited, in a concentration-dependent way, the growth of the MCF7, UACC62, NCIADR and NCI460 lines. Comparatively to CHE, the CHD presented the largest antiproliferative activity in these lines, with cytocidal activity of 46% for the MCF7 line, at 125 µg/mL (TABLE 1). It was observed that in this assay the crude extracts did not significantly inhibit the growth of the K562 line, suggesting specificity activity for solid tumors. The antiproliferative activity of CHD was confirmed through the concentration-dependent assay and, so, it was selected for phytochemical fractionating.

**TABLE 1.** Percentage of growth at 125 mg/mL for crude extracts obtained from *Aspidosperma tomentosum* incubated with tumoral cell lines for 48 h.

<table>
<thead>
<tr>
<th>Cells line</th>
<th>K 562(^a)</th>
<th>MCF 7(^b)</th>
<th>NCI ADR(^c)</th>
<th>UACC 62(^d)</th>
<th>NCI 460(^e)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Groups</td>
<td>(% growth ± se)</td>
<td></td>
<td></td>
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<td>------------</td>
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<tr>
<td>CHD</td>
<td>70 ± 0.047(^f)</td>
<td>-46 ± 0.002(^g)</td>
<td>-1 ± 0.005(^h)</td>
<td>6 ± 0.018(^i)</td>
<td>2 ± 0.018(^i)</td>
</tr>
<tr>
<td>CHE</td>
<td>97 ± 0.186</td>
<td>-18 ± 0.022(^j)</td>
<td>19 ± 0.173(^k)</td>
<td>13 ± 0.034(^l)</td>
<td>31 ± 0.065(^m)</td>
</tr>
</tbody>
</table>

The experiments were done in triplicated: ANOVA: \(^a\) K-562: \(F_{(10,30)} = 35.38; \(^b\) MCF 7: \(F_{(10,30)} = 293.05; \(^c\) NCI-ADR: \(F_{(10,30)} = 102.69; \(^d\) UACC-62: \(F_{(10,30)} = 102.69; \(^e\) NCI-460: \(F_{(10,30)} = 144.76; p<0.001, Duncan’s test:* p<0.05 and **p<0.001.}
The study of Apocinaceae species used in traditional medicine has been described for indole alkaloid content with an important role in medicinal purposes (Omino, 1993). Therefore the CHD was fractioned allowing to obtain a crude alkaloid fraction (AF), an unpolar fraction (UF), a terpenic medium polar fraction (TF), and a polar fraction (PF). The TF fraction showed best results for MCF7, NCI460 and K562 but did not significantly inhibit the growth of the UACC and NCIADR suggesting specificity activity for some solid tumors (FIGURE 2).

![Figure 2](image1)

**FIGURE 2.** Percent Growth in dose response curve Terpenic Fraction (TF) incubated with tumoral cell lines for 48 h. Growth between 0% and 5% indicated a cytostatic effect while growth < 0% indicated a cytocidal effect. The experiments were done in triplicated. ANOVA: K-562: $F_{(5,30)} = 58.42$; MCF 7: $F_{(5,31)} = 292.78$; NCI-ADR: $F_{(5,31)} = 196.15$; UACC-62: $F_{(5,31)} = 221.48$; NCI-460: $F_{(5,31)} = 906.38$; p<0.001, Duncan’s test: *p<0.001.

The analysis of this fraction by CG/MS revealed three main compounds with m/z 426. Comparison with the equipment’s data base indicated a 99% match with lupeol, α-amyrin and β-amyrin, respectively. Whereas PF fraction was unspecific, inhibiting all the cells lines giving cytotoxicity at 15 μg/mL (FIGURE 3).

![Figure 3](image2)

**FIGURE 3.** Percent growth in dose response curve of Polar Fraction (PF) incubated with tumoral cell lines for 48 h. Growth between 0% and 5% indicated a cytostatic effect while growth < 0% indicated a cytocidal effect. The experiments were done in triplicated. ANOVA: K-562: $F_{(5,100)} = 40.55$; MCF 7: $F_{(5,100)} = 624.27$; NCI-ADR: $F_{(5,100)} = 20.12$; UACC-62: $F_{(5,100)} = 359.88$; NCI-460: $F_{(5,100)} = 50.06$; p<0.001, Duncan’s test: *p<0.001.
Since indolic alkaloids isolated from a species of Apocinaceae genus, such as Olavicine, showed an antitumor activity (Scrispsema et al., 1999), the isolation of an alkaloid enriched fraction was prompted. However, the AF fraction wasn’t able to promote the inhibition of cell proliferation, demonstrating that this fraction is not involved in the plant’s antiproliferative activity. Fraction UF did not present a significant inhibition in the tested concentrations.

The results suggest that the active principle(s), responsible for the antiproliferative activity, are found in fractions FT and PF, which demonstrated inhibition profiles dependent of the concentrations.

CONCLUSION

The dichloromethane and methanol crude extract of dried leaves of *Aspidosperma tomentosum* Mart., displayed antiproliferative activity against the cancer cells tested in a concentration-dependent way for the cell lines herein screened. The dichloromethane extract presented a higher inhibition for these lines and the best results was observed in FT. This information suggests that the active principle(s) which is (are) responsible for the antiproliferative activity is (are) concentrated in this fraction.

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REFERENCE


PIO CORRÊA, M. Dicionário de Plantas Úteis do Brasil e das Exóticas Cultivadas. 1942. 


VERDECCHIA, A. et al. Incidence and prevalence of all cancerous disease in Italy: trends and implication. *European Journal of Cancer*, v.37,
