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In vitro breast cancer cell lethality of Brazilian plant extracts

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In this study we screened the cytotoxicity of 1220 plant extracts obtained from 351 plants belonging to 74 families occurring in the Amazon and Atlantic rain forests against MCF-7 human breast adenocarcinoma cell lines. All extracts were tested at a dose of 100 μg/mL. Only 11 aqueous or organic extracts belonging to the Annonaceae, Apocynaceae, Araceae, Clusiaceae, Flacourtiaceae, Leguminosae, Olacaceae and Violaceae showed marked lethal activity. *Vismia guianensis* and *Annona hypoglauca* extracts showed the greatest lethal activity.

1. Introduction

Breast cancer is one of the most prevalent female health conditions in Brazil. According to the Brazilian public health authority (Brazil 2004), neoplasia was the second most frequent cause of death in the period from 1996 to 2002, and breast cancer accounted for approximately 7% of the cases.

Cytotoxic agents play an important role in the treatment of breast cancer as part of established protocols used to treat advanced diseases that no longer respond to hormone therapy or disseminated tumors that are hormone-receptor negative, or in adjuvant or neoadjuvant settings. The introduction of new drugs to treat breast cancer is essential. Nature is an extremely important source of cytotoxic compounds. Historically, the search for new natural products with antineoplasic activity has led to the identification of paclitaxel, docetaxel (Wall and Wani 1995) and the *Vinca* alkaloids including vincristin and vinblastin (O'Marcaigh and Betcher 1995; Noble 1990).

Approximately 20% of the world's biodiversity can be found in Brazilian territory (Wilson and Peter 1988), particularly in the Amazon and Atlantic rain forests. Due to that great biodiversity and to the fact that 60% of all medicines come from natural sources (Cragg 1997), our team has been screening Amazon plant extracts against different diseases such as prostate cancer (Suffredini et al. 2006) and breast cancer using an *in vitro* methodology which allows us to test a large quantity of extracts as cytotoxic agents.

2. Investigations, results and discussion

The strategy of screening plant extracts for biological activity has been shown to be the fastest way of isolating active compounds. In order to obtain new antitumor drugs, cytotoxic assays are chosen as the first step in selecting active extracts (Horgen et al. 2001; Tempone et al. 2005). The screening procedure was carried out with 1220 extracts obtained from 351 species native to the Amazon and Atlantic rain forests. Due to their species richness (Myers 2000), both places are regarded as biodiversity hotspots and much has been done to preserve the extraordinary remaining areas within the forests. Plants were collected in the period from 1997 to 2001. Cytotoxic activity against the breast cancer cell line was observed for 11 extracts. Results, described as the percentage of cell lethality, are presented in the Table. In addition, an overview of the chemical constituents that are frequent in the genus or family of each active extract is given, as well as reports on the biological activity of extracts or previously isolated compounds.

One of the 44 extracts obtained from Annonaceae species showed inhibitory activity against the MCF-7 breast cancer cell line. The organic extract obtained from the wood of *Annona hypoglauca*, showed significant activity against the cells. The activity may be due to the presence of alkaloids and phenolics in the wood of the plant. Previous reports on *Annona* species show that alkaloids have been isolated and have shown antimicrobial activity (Simeon et al. 1990). Acetogenins (Li et al. 1990) and terpenoids may also play an important role in the extract's cytotoxicity (Fatope 1996).

The Apocynaceae family includes *Catharanthus roseus*, the plant from which vincristin and vinblastin were isolated. This plant and its natural products have been studied for more than half a century, due to the importance of the *Vinca* alkaloids to medicine. For this reason, screening of plants belonging to the Apocynaceae family is focused on the possible occurrence of indole alkaloids, so our group tested 97 extracts obtained from 21 different species, some of them recollected for comparison purposes. One of the 97 extracts showed activity in the breast carcinoma cell line assay. Two collections of *Macoubea sprucei* were made in January 1999 and April 1999. The aqueous extract obtained from the wood of the plant collected in April showed activity against the cancer cell line. Antifungal activity, anti-inflammatory activity and MAO inhibitory activity were detected in plumericin and isoplumericin. Previous studies have reported the occurrence of

Table: List of plant extracts that showed lethal activity against breast cancer cell line MCF-7 at a dose of 100 μg/mL

Extract number	Collect number	Organs	Family	Species	% GI	Collect date
64/A	PS98	WS, FR	Clusiaceae	*Vismia guianensis*	−80.73	Apr/97
63/O	PS98	WS, FR	Clusiaceae	*Vismia guianensis*	−38.03	Apr/97
1109/O	AAO3577	WD	Annonaceae	*Annona hypoglauca*	−32.77	Mar/00
205/O	IBS26	WD	Leguminosae	*Hymenaeae courbaril*	−27.77	Jun/98
832/A	AAO3423	RT	Araceae	*Philodendron solimoensis*	−24,09	Jul/99
206/A	IBS26	WD	Leguminosae	*Hymenaeae courbaril*	−23.11	Jun/98
812/A	AAO3412	AO	Flacoutiaceae	*Homalium racemosum*	−19.22	Jul/99
284/A	PS80	AO	Violaceae	*Amphirrox* sp.	−18.35	Apr/97
827/O	AAO3481	WD	Leguminosae	*Pentaclethra macroloba*	−15.62	Sep/99
84/A	PS408	WS	Olacaceae	*Chaunochiton loranthoides*	−15.01	Dec/97
698/A	AAO3402	WD	Apocynaceae	*Macoubea sprucei*	−14.89	Apr/99

Results are related to analysis done with a single dose of 100 μg/mL of plant extract; % CL = percentage of cell lethality (mean of six measurements); LF = leaf; ST = stem; FR = fruit; WD = wood, AO = aerial organs; RT = roots; O = organic extract; A = aqueous extract

indole alkaloids in *Macoubea* species, such as vincadifformine and vincadine (Anderson et al. 1985).

Two species of Araceae were screened in the assay, and only the aqueous extract obtained from the roots of *Philodendron solimoensis* showed activity. Although Amazon communities use several species of *Philodendron* (Duke and Vasquez 1994), there have not been many studies of their pharmacological activity or phytochemistry. Several plants from South and Central America have been screened for trypanocidal and trichomonicidal activities, and an extract obtained from a species of *Philodendron* was considered to be active (Muelas-Serrano 2000).

Two out of 60 extracts from the Clusiaceae showed cytotoxicity. Aqueous and organic extracts from the stem and fruits of *Vismia guianensis* showed very good activity in the assay. Anthraquinones with antiprotozoal activity have been isolated from *V. orientalis* (Mbwambo 2000), and cytotoxic compounds have been found in three species of *Vismia* (Hussein et al. 2003).

Thirty extracts from Flacourtiaceae species were analyzed, but activity was found only in the aqueous extract obtained from the aerial organs of *Homalium racemosum*. Cochinolide and its beta-glucopyranoside, together with tremulacinol, benzoic acid, tremulacin and tremuloidin have been isolated from *Homalium* and showed a mild antivirus activity (Ishikawa et al. 2004; Ishikawa 1998). Vacciniin and other benzenoid glucosides have been isolated from another species of *Homalium* (Ekabo 1993).

The Leguminosae were the largest group of plants subjected to the assay, and 3 out of 198 extracts showed activity against the MCF-7 cell line. *Hymeneaea courbaril* was collected in 1998 and organic and aqueous extracts made from the wood showed activity in the assay. *H. courbaril* is a traditional plant widely used in Brazil, where it is known as "*jatobá*". Diterpenoids (Abdel-Kader 2002; Nogueira 2001) and xyloglucans and oligossacharides (Lima-Nishimura 2003) have been previously isolated from the species. The organic extract obtained from the wood of an unidentified Leguminosae also showed activity.

One of nine extracts was active in tests of Olacaceae species. *Chaunochiton loranthoides* was collected in December 1997 and January 1999. The aqueous extract obtained from the stems of the plant collected in 1997 showed activity. No previous reports on the phytochemistry or pharmacology of *Chaunochiton* have been found.

Only one of 11 plant extracts showed activity in the assay out of the Violaceae. The aqueous extract obtained from the aerial organs of *Amphirrox* sp. showed a significant cytotoxic activity against the breast cancer cell line. No papers have been found relating to phytochemical or pharmacological studies with the genus, but studies related to the family indicate the presence of cyclic peptides (Trabi et al. 2004).

Thus, the initial evaluation of the cytotoxicity of 1220 plant extracts resulted in 11 active aqueous or organic extracts (0.9% yield) belonging to different species of plants. LD_{50} measures will be obtained for the selected active extracts, and the active compounds will be further isolated by bioguided-fractionation.

3. Experimental

3.1. Plant collection and extract preparation

Plants were collected in the Amazon rain forest, in a region near Manaus, AM and in the Atlantic Forest, near Iguape, SP. Plants were identified in the field and in the laboratory, with the aid of general keys for identification. Voucher specimens were deposited in the Herbarium of UNIP (Universidade Paulista, São Paulo, SP).

During the experiments, 1220 organic and aqueous extracts were obtained from 351 plants belonging to 74 different families. Different organs of the plants were collected according to the biomass available of each individual or population, depending on their species and habits, i.e., trees, herbaceous plants, lianas, epiphytes, or shrubs. Each plant material was dried and ground before being submitted to 24-h maceration with methanol:dichloromethane (1 : 1), then dried and submitted to a further 24-h maceration with water, resulting in two extracts, concentrating non-polar and polar substances respectively. Further information on the technique can be found elsewhere (Younes 2000).

3.2. Cell culture technique

The MCF-7 tumor cell line (estrogen receptor positive breast adenocarcinoma) was cultivated in tissue-culture flasks (Coastar), supplemented with RPMI-1640 plus 5% fetal bovine serum (both Cambras) and 1% glutamine (Sigma), and was kept in an incubator (Forma) at 37 °C with 5% CO_2 and 100% relative humidity. Cells were passaged weekly (Trypsin-EDTA, Cambras). Cell densities were obtained with a haemocytometer chamber, using the trypan blue exclusion method. Tests were carried out in 96-well microplates, and a density of 10,000 cells per well was used for the screening experiment. Cells were incubated for 24 h before the drug/extract was added, and the drug/extract remained in contact with the cells for 48 h in the microculture assay. After that, end points were obtained by the sulforhodamine B (SRB) assay (Monks et al. 1991).

Doxorubicin (DOXO; Sigma) and 5-fluorouracil (5-FU; Sigma) were used as standard drugs in the assays. DOXO concentration in the test was 2.5×10^{-5} M, and 5-FU concentration was 1.86×10^{-5} M. Extracts were tested at a single dose of 100 μg/mL, and a percentage of cell lethality <15 was considered selective in the assays, when compared to cells without treatment.

3.3. SRB assay

Viable cells were fixed in the 96 well microplates with cold trichloroacetic acid (TCA) solution (50 μL/well of 50% TCA). Microplates were washed with water five times until non-viable cells were totally removed. Plates were left to air-dry for 24 h. One hundred μL of SRB/well was added, and the dye was left to react for 10 min. After that period, plates were washed five times with 1% acetic acid until unbound SRB was completely removed. Plates were left to air-dry for 24 h. The stain was resuspended with 100 μL of Tris-

ma Buffer. The amount of viable cells was measured by obtaining the optical densities of the wells in a microplate spectrophotometer reader (Biotek 408x) at 515 nm. The percentage of cell lethality was obtained from the formula $100 \times [(T\text{-}T0)/(C\text{-}T0)]$, which is the comparison between the control (untreated cells) and test (cells treated with drug/extract) cell growth and time zero growth (which is the cell growth until addition of extract).

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