

Full Length Research Paper

Biological screening of extracts of Brazilian Asteraceae plants

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Accepted 28 June, 2013

Natural products are a very productive source of leads for the development of medicines. Seven Brazilian Asteraceae adult plants were randomly chosen. The current study was designed to evaluate the antiprotozoal and cytotoxic activities *in vitro* of 21 extracts obtained. Phytochemical properties of the most active extracts also were checked. Cytotoxic activity was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method against human tumor cell lines (HCT-116, OVCAR 8 and SF-295). The antiprotozoal activity was evaluated against *Trichomonas vaginalis*, *Entamoeba histolytica* and *Giardia lamblia*. None of the extracts showed antiprotozoal activity. However, 76% of the extracts displayed moderate to high *in vitro* cytotoxic activities against human cancer cell which suggests that they are a promising source of anticancer compound, since none of the extracts showed hemolytic activity. Terpenoids, flavonoids, saponins, tannins, besides other compound classes, were identified and may be responsible for their antitumor activity. Cytotoxic assays indicate the anticancer potential of Asteraceae species from Brazil.

Key words: Drug prospecting, Antiprotozoal agents, Cytotoxicity, Drug screening assays, Brazilian plant.

INTRODUCTION

Currently, there still persists many difficulties and challenges in cancer therapy such as drug resistance, toxicity and low specificity of drugs (Mesquita et al., 2009). Plants have a long history of use in the cancer treatment. Active constituents of *Catharanthus roseus* (L.) G. Don (Apocynaceae), *Angelica gigas* Nakai (Apiaceae), *Podophyllum peltatum* L. (Berberidaceae), *Taxus brevifolia* Nutt. (Taxaceae), *Ochrosia elliptica*

Labill. (Apocynaceae), and *Camptotheca acuminata* Decne. (Cornaceae) have been used in the treatment of advanced stages of various malignancies (Patel et al., 2009). Over 50% of drugs used in clinical trials for anticancer activity were isolated from natural sources or are related to them (Majumdar, 2012). The three anaerobic protozoa, *Entamoeba histolytica*, *Giardia lamblia* and *Trichomonas vaginalis* are highly prevalent

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human-infective parasites with a worldwide prevalence (Cantillo-Ciau et al., 2010). The most effective and commonly used drug in the treatment of these three protozoans is metronidazole. However, this substance has unpleasant side effects such as a metallic taste, headache, dry mouth, urticaria, pruritus, and dark-colored urine (Pérez et al., 2012). Due to these undesired side effects and taking into account the possibility of the development of resistant strains of the *T. vaginalis*, *E. histolytica*, and *G. lamblia* against metronidazole, there is a clear need for new, effective, and safer antiprotozoal agents.

Natural products, especially of plant origin, represent an excellent starting point for research. In traditional medicine there are also several plants used to treat vaginitis (Girón et al., 1988) and amoebic dysentery (Bautista et al., 2011). Amaral et al. (2006) described 153 plant species from 69 families that were evaluated for their giardicidal activity. It was found that the majority of extracts and fractions obtained from plant species employed in popular medicine for the treatment of diarrhea and dysentery exhibited *in vitro* giardicidal activity, and these were mainly from species belonging to the Asteraceae family.

Asteraceae is the largest family of angiosperms and it comprises 1535 genera and about 23 thousand species distributed in 3 subfamilies and 17 tribes (Bremer, 1994). The plants of the Asteraceae family are very common in the open formations of Brazil, mainly in the *cerrado*, where the family is well represented by approximately 250 genera and 2000 species (Guimarães et al., 2012). Asteraceae species have been used in the Brazilian folk medicine for several therapeutic purposes. For example, species of the genus *Lychnophora*, popularly known as "arnica", are widely used in Brazilian folk medicine as anti-inflammatory, to treat bruise, pain, rheumatism and for insect bites (Ferrari et al., 2012). Species of the genus *Mikania*, known as "guaco", they are widely used in Brazil in the formulation of syrups for the treatment of the respiratory system (Guimarães et al., 2012). Among the native plants of Brazil, species of genus *Baccharis*, popularly known as "carqueja", has been used as diuretic, tonic, digestive, protective and stimulate of the liver, antianemic, anti-rheumatic, obesity control, diabetes, hepatitis and gastroenteritis (Morais and Castanha, 2011).

Aiming to explore the rich Brazilian biodiversity, we initiated a bioprospection of plants from the Asteraceae family occurring in the state of Minas Gerais, Brazil, by screening plant extracts for cytotoxic and antiprotozoal activities.

MATERIALS AND METHODS

Plant material

Seven plants belonging to the Asteraceae family were collected in Ouro Preto-MG, Brazil (April 2010 to April 2012), and were identi-

fied by comparison with voucher specimens present in the herbarium, previously identified. Voucher specimens for each plant collected were deposited at the Herbarium José Badini, Universidade Federal de Ouro Preto-UFOP (Table 1).

Extract preparation

Approximately 4 g of the powdered aerial plant material of each specimen was extracted at room temperature by maceration with hexane (100 ml, 3 consecutive extractions over 24 h) followed by extraction using ethyl acetate (100 ml, 3 consecutive extractions over 24 h) and ethanol (100 ml, 3 consecutive extractions over 24 h). The colored solution from each of the plant material was filtered and finally concentrated by vacuum evaporation. The concentrated extract obtained was preserved for further use.

Antiprotozoal activity

E. histolytica, strain HM1:IMSS (ATCC 30459), *T. vaginalis*, strain JT, were maintained in YI-S medium (Diamond et al., 1995) and *G. lamblia*, strain Portland (ATCC 30888), was grown in Diamond's modified TYI-S-33 medium (Diamond et al., 1978). All protozoans were placed in individual vials containing axenic trophozoites cultures (2.4×10^5 *E. histolytica*, 6×10^4 *T. vaginalis* and 1.2×10^5 *G. lamblia* inoculums) in log growth phase.

The extracts were dissolved in 1 ml of dimethylsulfoxide (DMSO). Aliquots of 300 μ l of each solution were diluted in 5 ml of culture medium and added in the glass tubes (13 x 100 mm) containing trophozoites, reaching a final concentration test of 17 μ g/ml in a final volume of 6 ml.

The vials were incubated for 48 h at 37°C. All assays were performed in triplicate and repeated twice. Three vials were used as negative control (inoculum + medium) and three as positive control (Metronidazole, Sigma-Aldrich®). Protozoans viability was qualitatively measured using an inverted microscope (Nikon TMS), to observe trophozoites motility and adherence by comparing with the positive and negative controls.

Cytotoxic assay

The antiproliferative potential of the seed extracts was evaluated by the MTT assay (Mosmann 1983) against 3 human tumor cell lines: HCT-116 (colorectal carcinoma), OVCA8 (ovarian) and SF-295 (glioblastoma), all obtained from the National Cancer Institute (Bethesda, MD, USA). All cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin, at 37°C with 5% CO₂. Tumor cell growth was quantified by the ability of living cells to reduce the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoliumbromide (MTT) to a purple formazan product. Briefly, cells were plated in 96-well plates (0.7×10^5 cells/ml) and extracts (50 μ g/ml) were added to each well. After 72 h of incubation, the supernatant was replaced by fresh medium containing MTT (0.5 mg/mL), the formazan product was dissolved in 150 μ l DMSO and absorbance was measured at 595 nm (DTX 880 Multimode Detector, Beckman Coulter). Doxorubicin (0.3 μ g/ml, Sigma Aldrich) was used as positive control. The results are summarized in Table 1.

Hemolytic test

The hemolytic test was performed in 96-well plates following the method described by Berlinck et al. (1996). Each well received 50 ml of 0.85% NaCl solution containing 10 mM CaCl₂. The first well

Table 1. Tumor cell proliferation inhibition (%) of crude extracts of seven plant species belonging to *Brazilian Asteraceae* family determined by MTT assay after 72 h of incubation at the concentration of 50 µg/ml.

Species (Voucher no.)	Extract	Cell proliferation inhibition (%)*		
		HCT-116	OVCAR 8	SF-295
<i>Stevia urticifolia</i> Thunb. (OUPR 24049)	H	63.0 ± 0.5	61.2 ± 0.3	55.7 ± 1.1
	EtAc	97.8 ± 7.4	97.9 ± 0.1	69.6 ± 4.0
	Et	5.2 ± 0.2	4.9 ± 1.4	41.8 ± 1.1
<i>Vernonia polyanthes</i> Less. (OUPR 26355)	H	17.6 ± 0.9	10.9 ± 1.9	53.5 ± 2.6
	EtAc	99.4 ± 1.2	92.5 ± 2.7	82.8 ± 0.8
	Et	15.9 ± 2.1	-2.1 ± 2.2	45.6 ± 1.2
<i>Vernonia crotonoides</i> (DC.) Sch.Bip. (OUPR 25896)	H	55.8 ± 5.6	32.6 ± 13.0	36.9 ± 5.2
	EtAc	100.7 ± 0.8	98.6 ± 0.7	89.6 ± 2.8
	Et	7.1 ± 1.1	4.5 ± 0.8	53.1 ± 0.9
<i>Moquinia racemosa</i> DC. (OUPR 26602)	H	58.1 ± 0.5	51.9 ± 2.1 97.8	55.7 ± 1.2
	EtAc	98.3 ± 1.7	0.6	96.9 ± 0.4
	Et	23.3 ± 0.0	5.8 ± 0.3	52.3 ± 0.4
<i>Mutisia campanulata</i> Less (OUPR 26754)	H	47.4 ± 0.5	49.1 ± 0.6	58.3 ± 0.1
	EtAc	3.3 ± 0.9	6.4 ± 0.3	45.3 ± 0.2
	Et	24.2 ± 4.2	5.4 ± 7.1	49.6 ± 0.8
<i>Acanthospermum australe</i> (Loefl.) Kuntze (OUPR 25895)	H	48.7 ± 0.1	75.3 ± 0.7	54.5 ± 6.5
	EtAc	96.5 ± 5.6	96.4 ± 0.0	85.4 ± 0.4
	Et	12.0 ± 2.8	2.2 ± 4.0	42.5 ± 3.0
<i>Calea fruticosa</i> (Gardner) Urbatsch, Zlotzky & Pruski (OUPR 26290)	H	72.7 ± 3.2	95.3 ± 0.5	45.2 ± 0.3
	EtAc	99.4 ± 0.2	96.7 ± 1.0	96.2 ± 2.4
	Et	95.7 ± 19.5	95.6 ± 19.4	61.2 ± 10.9

H, hexane; EtAc, ethyl acetate; Et, ethanol. *Results are expressed as mean ± standard error mean (S.E.M.) from two independent experiments for colorectal carcinoma (HCT-116), ovarian (OVCAR 8), and glioblastoma (SF-295) human cancer cells. All cell lines were plated with RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin, at 37°C with 5% CO₂. Doxorubicin (0.3 µg/ml) was used as positive control. High activity: > 75%; moderated activity: 50 to 75%; low activity: < 50 %.

was the negative control that contained only the vehicle (1% DMSO), and in the second well 50 ml of test substance that was diluted in half was added. The extracts were tested at concentrations ranging from 1.56 a 200 µM. The last well received 50 ml of 0.2% triton X-100 (in 0.85% saline) to obtain 100% hemolysis. Then, each well received 50 ml of a 2% suspension of mouse or human erythrocytes in 0.85% saline containing 10 mM CaCl₂. After incubation at room temperature for 1 h, and centrifugation, the supernatant was removed and the liberated hemoglobin was measured spectroscopically as absorbance at 540 nm.

Phytochemical screening

Chemical tests were carried on the most active extracts (that exhibited high *in vitro* cytotoxic activities, cell growth inhibition between 75 to 100%) to identify the phytoconstituents, that is, alkaloids, flavonoids, saponins, tannins, and terpenoids, as per the standard procedure (Edeoga et al., 2005; Egwaikhide and Gimba,

2007; Abalaka et al., 2011). The results are summarized in Table 2.

Statistical analysis

The analysis of cell proliferation (*in vitro* cytotoxic assays) and hemolytic potential were determined by non-linear regression using the Graphpad program (Intuitive Software for Science, San Diego, CA).

RESULTS

Table 1 summarizes the cytotoxic activities displayed by the Asteraceae plant extracts that were evaluated in this research. Of the 21 extracts tested, the analyses by MTT assay showed that 16 (76%) displayed moderate to high *in vitro* cytotoxic activities against human cancer cells. Ethyl acetate extracts were the most active against the 3

Table 2. Phytochemical analysis of the most active extracts.

Species	Extract	Terpenoid	Flavonoid	Saponin	Alkaloid	Tannin
<i>S. urticifolia</i>	EtAc	+	+	+	–	–
<i>V. polyanthes</i>	EtAc	+	+	–	–	–
<i>V. crotonoides</i>	EtAc	+	+	–	–	–
<i>M. racemosa</i>	EtAc	+	+	+	–	–
<i>A. australe</i>	H	+	+	–	–	–
<i>A. australe</i>	EtAc	+	+	–	–	–
<i>C. fruticosa</i>	H	+	–	–	–	–
<i>C. fruticosa</i>	EtAc	+	+	–	–	–
<i>C. fruticosa</i>	Et	–	+	+	–	+

H, Hexane; EtAc, ethyl acetate; Et, ethanol. Key: + = present ; – = absent

human tumor cell lines when compared to those from hexane or ethanol. *Calea fruticosa* (Gardner) Urbatsch, Zlotzky and Pruski was the only plant species that revealed promising *in vitro* cytotoxic action with their hexane, ethyl acetate and ethanol extracts. All extracts were found to be ineffective against all the tested protozoa. The results of phytochemical screening of the most active extracts are shown in Table 2. Alkaloids were not present in any extract. The presence of tannins was observed only in *C. fruticosa* (ethanolic extract). The presence of terpenoids has been detected in all extracts except for the ethanolic extract of *C. fruticosa*. Flavonoids were also observed in all extracts except for the hexanic extract of *C. fruticosa*. Only *Stevia urticifolia* Thunb. (ethyl acetate extract), *Moquinia racemosa* DC. (ethyl acetate extract) and *C. fruticosa* (ethanolic extract) showed the presence of saponins.

DISCUSSION

The 16 extracts that showed moderate to high *in vitro* cytotoxic activities against human cancer cells were considered promising anticancer compound sources. Researches for antineoplastic compounds have demonstrated the great pharmacological relevance of the plant extracts (Ferreira et al., 2011). According to the American National Cancer Institute, the limit to be considered a promising crude extract for further purification is a value lower than 50 µg/ml and cell proliferation inhibition is higher than 90% (Suffness and Pezzuto, 1990; Ferreira et al., 2011). In relation to the cytotoxic or antitumor activity of these plant species, rare findings are available. For example, *Acanthospermum australe* extracts were capable to increase the survival of Ehrlich ascites tumor-bearing mice and stimulated myelopoiesis, which can influence on antitumor immune responses (Mirandola et al., 2002).

Studies indicate that some plant substances like polyphenols, epicatechins, steryl glycosides and triterpenoid saponins cause damage to red cell membranes and

produce hemolysis (Costa-Lotufu et al., 2002). The mechanical stability of erythrocyte membrane a good indicator of insults by vegetal substances (Sharma and Sharma, 2001; Santos et al., 2010). Then, hemolysis detection is an useful and cheap technique which can displays the effect of increasing concentrations and can to be sigmoidally related to the logarithm contact time, emphasizing the membrane stability as a biological complex to maintain its structure under stress conditions, such as oxidation, hipotonicity, pH changes, heat and in presence of osmotic active solutes (Van Ginkel and Sevanian, 1994; Sharma and Sharma, 2001; Freitas et al., 2008). Herein, none of the extracts tested caused hemolysis even at the highest concentration (200 µg/ml), suggesting that the mechanism of cytotoxicity is probably related to a more specific pathway. Guo and Gao (2013) described the antiproliferative effects of SPV (total saponin extract from *Patrinia villosa*) and FPV (total flavonoid extract from *P. villosa*) on four cancer cell lines and concluded that the mechanisms involved in cancer chemoprevention by FPV and SPV extracts were cell cycle arrest and induction of apoptosis. Targeting cell cycle and apoptotic pathways has emerged as an attractive approach for treatment of cancer (Aslantürk and Çelik, 2013).

Vernonia polyanthes Less., known as assa-peixe, have been studied and their pharmacological properties which include antinociceptive and anti-inflammatory (Temponi et al., 2012), antibacterial (Silva et al., 2012), antifungal and leishmanicidal (Braga et al., 2007), have been established. Fixed acids, alkaloids, aminoacids, coumarins, steroids, triterpenes, anthraquinones, flavonoids, saponins and tannins were detected in infusions of *V. polyanthes* and may be responsible for their pharmacological effects (Temponi et al., 2012). *Acanthospermum australe* (Loefl.) Kuntze is an annual shrub widely distributed in South America. In Brazil, where it is popularly known as “carrapichinho” or “carrapicho-de-carneiro”. Its aerial parts are used in folk medicine as a tonic, diaphoretic, eupeptic, vermifuge, antidiarrheal, antimalarial, antigonorrhoeal, febrifuge, and antianemic

(Lorenzi and Matos, 2002). Previous phytochemical investigations of *A. australe* have led to the isolation of germacranolides, melampolides, diterpene lactones and 6-methoxyflavonoids (Bohlmann et al., 1979, 1981; Matsunaga et al., 1996). Antiviral (Rocha Martins et al., 2011) and antitumor properties (Mirandola et al., 2002) were already described for this species of plant. *Vernonia crotonoides* (DC.) Sch.Bip. is a synonym of *Eremanthus crotonoides* (DC.) Sch.Bip. (Robinson, 1999). Bohlmann et al. (1982) revealed that germacrene D, bicyclogermacrene, α -humulene, caryophyllene, lupeol and its acetate derivative, taraxasterol and its acetate, stigmasterol, and sesquiterpene lactones are present in aerial parts of *E. crotonoides*. Lobo et al. (2012) evaluated the antiproliferative effects of extracts and sesquiterpene lactone from *E. crotonoides* against two brain tumor cell lines. Dichloromethane fraction was cytotoxic to both glioblastoma multiforme cell lines. Centratharin alone was also evaluated against both U251 and U87-MG cells, which showed IC₅₀ values comparable with those obtained for the commercial anticancer drug doxorubicin.

To our knowledge, no further research was carried out with the species *S. urticifolia*, *M. racemosa*, *Mutisia campanulata* Less, and *C. fruticosa*.

In this study, the evaluation of the extracts (hexane, ethyl acetate and ethanol) against *E. histolytica*, *T. vaginalis* and *G. lamblia*, was carried out. However, none of the extracts showed antiprotozoal activity.

The Asteraceae plant species tested showed important activity against human tumor cell lines examined. These findings are the base for further studies to isolate (guided by biological assays) and elucidate, the structure of the bioactive compounds assessed from these plants.

Our results are a contribution to a better understanding of the Brazilian biodiversity, which indicate that these natural sources may become an important source for therapeutic agents.

ACKNOWLEDGEMENT

We are grateful to Universidade Federal de Ouro Preto (UFOP) and to Brazilian agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa de Minas Gerais (FAPEMIG), Fundação de Amparo à Pesquisa do Estado do Piauí (FAPEPI) and Fundação Cearense de Amparo à Pesquisa (FUNCAP) for financial support.

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