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In vitro cytotoxic activity of Brazilian Middle West plant extracts

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Abstract: Cytotoxic activity of eight plant extracts, native from the Mid-West of Brazil comprising Cerrado, Pantanal and semideciduous forest, was evaluated for MDA-MB-435, SF-295, and HCT-8 cancer cell strains. A single 100 $\mu g.mL^{-1}$ dose of each extract was employed with 72 h of incubation for all tests. Doxorubicin (1 $\mu g.mL^{-1}$) was used as the positive control and the MTT method was used to detect the activity. Cytotoxicity of distinct polarities was observed in thirty extracts (46%), from different parts of the following species: *Tabebuia heptaphylla* (Vell.) Toledo, Bignoniaceae, *Tapirira guianensis* Aubl., Anacardiaceae, *Myracrodruon urundeuva* Allemão, Anacardiaceae, *Schinus terebinthifolius* Raddi, Anacardiaceae, *Gomphrena elegans* Mart., Amaranthaceae, *Attalea phalerata* Mart. ex Spreng., Arecaceae, *Eugenia uniflora* L., Myrtaceae, and *Annona dioica* A. St.-Hil., Annonaceae. Extracts of at least two tested cell strains were considered to be highly active since their inhibition rate was over 75%.

Introduction

Brazil is one of the few megadiversity countries in the world but investigations of its flora have barely revealed the present phytochemical diversity. The newly discovered molecules have substantial scientific, economic and social interest. Furthermore, they may contribute to the development of interactive disciplines as well as show potential industrial uses for the improvement of health and the quality of life (Braz-Filho, 1999).

In order to carry out this work, the following plant species *Tabebuia heptaphylla* (Vell.) Toledo, Bignoniaceae, *Tapirira guianensis* Aubl., Anacardiaceae, *Myracrodruon urundeuva* Allemão, Anacardiaceae, *Schinus terebinthifolius* Raddi, Anacardiaceae, *Gomphrena elegans* Mart., Amaranthaceae, *Attalea phalerata* Mart. ex Spreng., Arecaceae, *Eugenia uniflora* L., Myrtaceae, and *Annona dioica* A. St.-Hil., Annonaceae, were selected. All species are native to the middle-west region of Brazil and are found in Cerrado, Pantanal and the semideciduous forest biomes in the state of Mato Grosso do Sul. Their selection was mainly based upon the use of these plants in traditional medicine.

People native to the Cerrado and Pantanal regions make use of a large variety of plants for medicinal purposes. A considerable part of their acquired knowledge was received from several indigenous ethnic groups living in this region far before the colonization of South America by the europeans (Maciel et al., 2002; Nunes et al., 2003).

Material and Methods

Plant material and extraction procedure

The vegetal material was collected in March 2007. The voucher specimen was deposited at the CGMS Herbarium of the Federal University of Mato Grosso do Sul-MS, Brazil. *Gomphrena elegans* Mart., Amaranthaceae, was collected in the Sucuri and Baía Bonita rivers in the Bonito district, the MS (21°15′36.4”S

and 56°32´58.1”W) and voucher number CGMS 17715, *Tabebuia heptaphylla* (Vell.) Toledo, Bignoniaceae, voucher number CGMS 118999 and *Attalea phalerata* Mart. ex Spreng., Arecaceae, voucher number CGMS 24772 were collected in the Pantanal region, Miranda-Abobral, subregion, around the Base of Studies of the Pantanal-UFMS (19°34’37”S and 57°00’42”W), *Tapirira guianensis* Aubl., Anacardiaceae, voucher number CGMS 22907, *Myracrodruon urundeuva* Allemão, Anacardiaceae, voucher number CGMS 17659, *Schinus terebinthifolius* Raddi, Anacardiaceae, voucher number CGMS 22945, *Annona dioica* A. St.-Hil., Annonaceae, voucher number CGMS 17980 and *Eugenia uniflora* L., Myrtaceae, voucher number CGMS 25433 were collected in the Biological Reserve of the UFMS (20º30’25.5”S and 54o36’46”W).

Leaves, stems bark and heartwood collection was performed in plants with natural injury. 100 g of each vegetal material (leaves and stems) was dried under air and cut into small pieces. They were crushed on Wiley type mill and the resulting matter was extracted with ethanol during seven days with occasional stirring, followed by the filtration and concentration of the filtrate by rotaevaporation. The filtrate was placed into desiccators for dehydration. Then, it was submitted to a process of liquid-liquid partition with solvents of increasing polarities: *n*-hexane, dichloromethane or chloroform, ethyl acetate, *n*-butanol or methanol and hydroalcohol or aqueous to obtain the respective fractions. All solvents were of analytical grade.

The choice of partition methods was based on pilot-experiments for each studied species, according to adapted methodology from Cechinel Filho & Yunes (1998).

The following parts were submitted to a partition system with hexane, dichloromethane, ethyl acetate, butanol and hydroalcoholic: stem bark from *Tabebuia heptaphylla*; leaves and stem bark from *Tapirira guianensis*; stem bark of *Myracrodruon urundeuva*; leaves from *Schinus terebinthifolius*; leaves from *Attalea phalerata*; and leaves and stem from *Gomphrena elegans*. By this method, a residue containing more polar compounds, called polar fraction, is usually formed at the end of the partition process. Leaves, heartwood, stem bark, subterranean heartwood and subterranean stem bark from *Annona dioica* were submitted to partition system with hexane, chloroform, methanol and aqueous. In this method, the partition does not produce an insoluble residue. The crude extract of leaves from *Eugenia uniflora* and heartwood

Table 1. Vegetal extracts obtained for testing citotoxic activity against cell strains measured by the MTT assay.

Species (part of the plant)	Extracts (g) Et	Fractions (g)							
		h	D	C	Ea	B	H	M	A
Tabebuia heptaphylla (stem bark)	-	0.320	0.170	-	0.580	0.300	0.015	-	-
Tapirira guianensis (leaves)	2.489	0.873	0.389	-	0.460	0.652	0.065	-	-
Tapirira guianensis (stem bark)	1.470	0.260	0.114	-	0.470	0.735	0.211	-	-
Myracrodruon urundeuva (stem bark)	1.760	0.330	0.387	-	0.354	0.609	0.030	-	-
*Myracrodruon urundeuv*a (heartwood)	0.954	-	-	-	-	-	-	-	-
Schinus terebinthifolius (leaves)	-	0.630	0.743	-	0.840	0.517	0.110	-	-
Attalea phalerata (leaves)	-	0.760	0.700	-	0,400	0.900	0.060	-	-
Eugenia uniflora (stem bark)	0.420	-	-	-	-	-	-	-	-
Gomphrena elegans (leaves)	-	0.090	0.110	-	0.034	0.380	0.256	-	-
Gomphrena elegans (stem)	-	0.102	0.090	-	0.243	0.212	0.045	-	-
Annona dioica (leaves)	-	0.870	-	0.670	-	-	-	0.850	0.097
Annona dioica (heartwood)	-	0.434	-	0.320	-	-	-	0.680	0.045
Annona dioica (stem bark)	-	0.643	-	0.576	-	-	-	0.345	0.064
Annona dioica (heartwood subterranean)	-	0.123	-	0.280	-	-	-	0.290	0.023
Annona dioica (stem bark subterranean)	-	0.230	-	0.245	-	-	-	0.310	0.033

Solvents used in extraction: Et: ethanol, h: *n*-hexane, D: dichloromethane, C: chloroform, Ea: ethyl acetate, B: *n*-butanol, H: hydroalcohol, M: methanol, A: aqueous.

of *Myracrodruon urundeuva* was not submitted to partition. Therefore, only the ethanol extract of this species was tested.

The extracts and fractions obtained from each plant, along with their weight, are shown in Table 1.

Cytotoxicity studies

The extracts cytotoxicity was tested against HCT-8 (human colon carcinoma), SF-295 (glioblastoma) and MDA-MB-435 (melanome) tumor cell strains (National Cancer Institute, Bethesda, MD, USA). Cells were cultured in RPMI-1640 medium, supplemented with 10% fetal calf serum, 2 mM glutamine, 100 µg.mL-1 streptomycin and 100 U.mL^{-1} penicillin at 37 ºC with 5% CO_2. For experiments, cells were plated in 96-well plates (10^5 cells/well for adherent cells or 0.3 x 106 cells/well for suspended cells in 100 µL of medium). After 24 h, the extracts at 100 µg.mL^{-1}, or in serial dilution dissolved in DMSO (1%) were added to each well and incubated for three days (72 h). Control groups received the same amount of DMSO. Doxorubicin (0.01-0.58 µg.mL^{-1}) was used as positive control. Growth of tumoral cells was quantified by the ability of living cells to reduce the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to a blue formazan product (Mosmann, 1983; Skehan et al., 1990; Berridge et al., 1996). At the end of 72 h incubation, the medium in each well was replaced by fresh medium (200 µL) containing 0.5 mg.mL^{-1} of MTT. Three hours later, the formazan product of MTT reduction was dissolved in DMSO, and absorbance was measured using a multi-plate reader (DTX 880 Multimode Detector, Beckman Coulter Inc., Fullerton, CA, USA). Drug effect was quantified as the percentage of control absorbance of reduced dye at 590 nm (Zani et al., 1991; Da Silva et al., 2003; Correia et al., 2003). In our screening program to the discovery and development of potential anticancer natural compounds, we adopted the criteria of the American National Cancer Institute to consider a crude extract promising for further purification based on the IC50 values lower than 30 µg.mL^{-1} (Suffness & Pezzuto, 1990).

In this context, we first assessed the extracts in a single concentration of 100 µg.mL^{-1} to select those that inhibited cell growth in at least 75%. The percentage of cell growth (%G) was calculated by comparing the absorbance of test samples with the control (100%), zero-time (0%) and the cytotoxic standard etoposide (-100%). The percentage of cell lethality was obtained by the formula [100 × (T−T0)/(C−T0)], where T = test, T0 = cell growth before addition of the extracts. The fractions were classified as possessing no activity (blank space), low activity (up to 50% growth inhibition), moderate activity (between 51% and 75% growth inhibition) and high activity (over 75% growth inhibition) for each cell strain tested. Through this approach, the number of "false positive" extracts assessed were reduced.

The experiments were assessed according to the mean±SEM of percentage inhibition of cell growth using the Graphpad program (Intuitive Software for Scince, San Diego, CA).

Results and Discussion

The extracts activities are shown in Table 2. The tested cell strains were selected due to their resistance to different drugs. Samples were classified according to their cytotoxic potency as: no activity, low activity (growth inhibition ranging from 1 to 50%), moderate activity (growth inhibition ranging from 51 to 75%) and high activity (over 75%).

All analyzed species showed cytotoxic activity. Among the 65 tested extracts from eight plant species, thirty showed considerable cytotoxicity, corresponding to 46% of all extracts as follows: *Tabebuia heptaphylla* (Vell.) Toledo, Bignoniaceae: dichloromethane, ethyl acetate and *n*-butanol of the stem bark; *Tapirira guianensis* Aubl., Anacardiaceae: all fractions of the leaves, with the exception of n-hexane; *Tapirira guianensis*: *n*-hexane and hydroalcohol of the stem bark; *Myracroduon urundeuva* Allemão, Anacardiaceae: ethyl acetate, *n*-butanol, hydroalcohol and ethanol of the stem bark; *Myracroduon urundeuva*: ethanol of heartwood; *Schinus. terebinthifolius* Raddi, Anacardiaceae: ethyl acetate of the leaves; *Gomphrena elegans* Mart., Amaranthaceae: *n*-hexane and *n*-butanol of the leaves; *Attalea phalerata* Mart. ex Spreng., Arecaceae: *n*-hexane, dichlorometane, ethyl acetate and hydroalcohol of leaves; *Eugenia uniflora* L., Myrtaceae: ethanol of stem bark; *Annona dioica* A. St.-Hil., Annonaceae: *n*-hexane, chloroform, methanol and aqueous of the heartwood; *Annona dioica*: *n*-hexane, chloroform, methanol and aqueous of the Stem bark; *Annona dioica*: *n*-hexane, chloroform, methanol and aqueous of the heartwood subterranean; *Annona dioica: n*-hexane, chloroform, methanol and aqueous of the stem bark subterranean; *Annona dioica*: *n*-hexane, chloroform, methanol and aqueous of leaves, presented high cytotoxic activity (over 75%).

By evaluating the families or the selected species one can clearly observe that they have medicinal properties.

The species *Tabebuia heptaphylla* is popularly known as "piúva" and is native to the Pantanal region. In traditional medicine, including indigenous people, the bark extract is used against cancer, as a stomach depurative and a bactericide

Table 2. Cytotoxic activity of fractions from vegetal extracts against tumor cell lines measured by the MTT assay*.

Sample	*Lethality (%)		
	MDA-MB-435	HCT-8	SF-295
Stem bark of *Tabebuia heptaphylla*			
n-hexane	62.80	69.12	53.60
dichloromethane	97.80	100.00	100.00
ethyl acetate	59.72	100.00	98.00
n-butanol	99.45	78.91	76.37
hydroalcohol	89.87	61.19	48.65
Leaves of *Tapirira guianensis*			
n-hexane	46.51	48.14	49.17
dichloromethane	86.13	88.00	79.67
ethyl acetate	100.00	100.00	100.00
n-butanol	97.36	99.88	100.00
hydroalcohol	97.69	96.27	99.13
ethanol	64.78	76.69	86.36
Stem bark of *Tapirira guianensis*			
n-hexane	77.11	77.11	77.11
dichloromethane	36.72	36.72	36.72
ethyl acetate	31.32	31.32	31.32
n-butanol	37.93	37.93	37.93
hydroalcohol	100.00	100.00	100.00
ethanol	41.12	41.12	41.12
Heartwood of *Myracrodruon urundeuva*			
ethanol	95.60	95.60	95.60
Stem bark of *Myracrodruon urundeuva*			
n-hexane	94.28	49.42	60.12
dichloromethane	99.45	58.75	56.21
ethyl acetate	96.81	93.71	75.24
n-butanol	94.28	100.00	94.27
hydroalcohol	82.83	97.90	90.79
ethanol	98.24	97.67	89.83
Leaves of *Schinus terebinthifolius*			
n-hexane	36.61	28.33	19.28
dichloromethane	43.76	84.62	35.70
ethyl acetate	94.83	97.44	100.00
n-butanol	28.35	14.00	34.31
hydroalcohol	30.38	54.14	27.27
leaves of *Gomphrena elegans*			
n-hexane	96.35	100.00	96.35
dichloromethane	19.17	13.80	19.17
ethyl acetate	10.04	0.91	10.04
n-butanol	97.39	99.68	97.39
hydroalcohol	10.43	18.24	10.43
Stem of *Gomphrena elegans*			
n-hexane	24.38	24.38	24.38
dichloromethane	9.78	9.78	9.78
ethyl acetate	16.43	16.43	16.43
n-butanol	18.38	18.38	18.38

hydroalcohol	19.95	19.95	19.95
Leaves of *Attalea phalerata*			
n-hexane	100.00	100.00	100.00
dichloromethane	100.00	100.00	100.00
ethyl acetate	94.72	81.35	65.77
n-butanol	73.81	56.88	57.95
hydroalcohol	100.00	100.00	100.00
Stem bark of *Eugenia uniflora*			
ethanol	95.60	84.85	75.24
Heattwood of *Annona dioica*			
n-hexane	43.19	35.74	52.87
chloroform	100.00	95.63	100.00
methanol	54.89	26.58	55.27
aqueous	4.50	7.49	1.00
Stem bark of *Annona dioica*			
n-hexane	53.57	53.57	53.57
chloroform	100.00	100.00	100.00
methanol	4.76	4.76	4.76
aqueous	3.64	3.64	3.64
Heartwood subterranean of *Annona dioica*			
n-hexane	63.29	58.59	47.93
chloroform	100.00	100.00	100.00
methanol	92.46	82.84	80.73
aqueous	29.83	2.54	9.80
Stem bark subterranean of *Annona dioica*			
n-hexane	91.53	91.53	91.53
chloroform	100.00	100.00	100.00
methanol	38.69	38.69	38.69
aqueous	29.23	29.23	29.23
Leaves of *Annona dioica*			
n-hexane	39.88	35.74	44.53
chloroform	96.89	89.44	80.40
methanol	36.38	41.60	38.33
aqueous	13.89	10.33	11.93

*Results are represented by means of three replicates.

(Hoehne, 1939; Pott & Pott, 1994). Although we have not found any report on phytochemical analysis of the leaves and fruits, the isolation of the lignans cycloolivil and secoisolariciresinol, three lapachones, two naphthofuranes and one naphthalene derivative from the stem bark of this species have been reported previously (Schmeda-Hirschmanna & Papastergiou, 2003). In the Bignoniaceae family, naphthoquinones is the principal group of secondary metabolites. There are reports dealing with the following activities for these compounds: antimalarial (Pérez et al., 1997), antitumor (Ueda et al., 1994), anticancer (Abbott et al., 1967), antifungal (Guiraud et al., 1988), antibacterial and anti-inflammatory (Zani et al., 1991). Among the naphthoquinones, studies performed with lapachol and beta-lapachone showed important *in vitro* antitumor activity and they were thus clinically tested by NCI (USA) (Da Silva et al., 2003).

Tapirira guianenses is popularly known as "tatapiririca" or "cedroí". People use different parts of the plant to treat diarrhoea and syphilis. The aqueous extract of the bark presents a uterus-stimulant effect (Correia et al., 2003). No published phytochemical study was found for this species.

In brazilian traditional medicine, *Myracrodruon urundeuva* has achieved popularity and a reputation. It is still largely used in the states of Mato Grosso do Sul and Mato Grosso by indigenous and riverside people.

Properties such as anti-inflammatory and wound healing in gynecological illnesses are attributed to the plant. Its leaves are utilized to prepare plaster casts for broken bones, and as bactericidal and first-aid medicine for snake bites. In addition, the plant is also used as an antidiarrhoeic, astringent and antisyphilis (Martins et al., 2000). Souza et al. (2006) evaluated the anti-inflammatory and antiulcer properties of the tannin-enriched fraction isolated from the stem bark of *M. urundeuva*. They disclosed that these activities are partly due to its antioxidant action, known to be linked to the presence of polyphenols, including tannins (Morais et al., 2005). Botelho et al. (2007) examined the effect of topical herbal gel from *Lippia sidoides* 0.5% (v/w) and *M. urundeuva* 5% (w/w) in experimental periodontal disease in rats showing potential as a treatment for reducing tissue lesions (Souza et al., 2006). The chalcone dimers urundeuvina A and B were isolated from the inner bark of a specimen of *M. urundeuva* (Botelho et al., 2007) together with the antioxidant compounds cicloeucalenol and cicloeucalenona (Bandeira et al., 1994).

Fruits of *Schinus terebinthifolius* are spices used to increase flavor in food worldwide (Viana et al., 2003). In folk medicine, bark, leaves and fruits are used as antidiarrhoeic, astringent, anti-inflammatory, depurative, diuretic and febrifuge. The essential oils are used to treat respiratory distress (Ceruks et al., 2007). Moreover, triterpenes with an inhibitory effect on phospholipase A2 were isolated from the plant and its extracts showed antibacterial, antifungical and antiradicalar activities (Degáspari et al., 2005; Jain et al., 1995).

It is worth mentioning that species of Anacardiaceae family are known for presenting compounds with alkyl and alkenyl groups attached to catechol, resorcinol and phenol rings. These compounds, named as phenolic lipids, show nematicidal (Velászquez et al., 2003), antioxidant (Valcic et al., 2002), antifungical, and cytotoxic activities (Queiroz et al., 2003; Davis et al., 1997; Correia et al., 2001).

Some species from the Amaranthaceae family are being investigated for their varied biological properties. Antitumor activity against melanomas was detected in *Pfaffia paniculata* (Takemoto et al., 1983), and this property was accredited to the presence of anabolic agents such as β-ecdysterone and ecdysteroids glycoside. Phytochemical studies with in the genera *Amaranthus* account for the presence of saponins (Takemoto et al., 1983; Nishimoto et al., 1984; Oleszek et al., 1999), sapogenins (Escudero et al., 1998), rutin (Xaziev et al., 1992), steroids (Ologunde et al., 1992) and hydroxycinnamic esters of isocitric acid (Strack et al., 1987). Several works describe the antitumor activity of the genera *Gomphrena*, possibly associated to the production of saponins and phytoecdisteroids (Pomilio et al., 1994; Sarker et al., 1996; Young et al., 1997; Savchenko et al., 1998). *Gomphrena elegans* Mart. var. elegans is a herbaceous species frequently found in locations called "carandazais", "espinheirais" (thorn scrub), "vazantes" (drainage channels), ciliar vegetation, wetlands and clayed soil. Its distribution is restricted to tropical and subtropical South America as a hygrophytic plant in fields near rivers Sucuri and Baía Bonita (Bonito-MS) (Prance & Schaller, 1982). No phytochemical study was found for this species.

The Palmae family has special economical and ecological importance. Millions of animal species make use of palm trees not only as a source of food, but also for protection and reproduction area (Vormisto et al., 2004). A considerable number of people in the tropics depend on these plants for their daily survival. Acuri (*Attalea phalerata*), coconut palm (*Cocos nucifera*), palm kernel (*Elaeis guienensis*), date palm (*Phoenix dactyliphera*), açai (*Euterpe oleraceae*), pejibaye (*Bactris gasipaes*), babassu (*Attalea speciosa*), piassava (*Attalea funifera*) and the carnauba (*Copernicia prunifera*) are some examples (Vormisto et al., 2004). The limited chemical studies of this plant family are focused on the investigation of *Cocos nucifera* biological activity. Alviano et al. (2004), detected analgesic and free radical scavenging properties in the aqueous extract of stem bark fibers. In addition, we also detected effects such as leishmanicide, antioxidant and antiviral (Mendonça et al., 2004; Kirszberg et al., 2003; Esquenazi et al., 2002) when analyzing the same extract. The *Attalea phalerata* (acuri) is a palm tree from the Pantanal region known as "acurizais" (Pott & Pott, 1994). In some areas, the fruit seems to be a key source of energy for animals (Terborgh, 1986; Guedes & Harper, 1995). It was selected because of its abundance and lack of available phytochemical data even though there is no literature register on popular medicinal use for this species.

Another promising source of bioactive compounds is the Annonaceaea family comprising about 120 genera and over 2.300 species. From a phytochemistry point of view, this family is worth studying due to several types of structural compounds classes that can be found such as: alkaloids, amides, diterpenes, steroids, flavonoids and acetogenins (Pontes et al., 2004). The use of Annonaceae plants in folk medicine has been widely reported associated with varied biological activities such as antimicrobial, antiemetic, pesticide, abortive, antitumor, cytotoxic, anorexic and antimalarial (Leboeuf et al., 1982). Many of these activities are assigned to acetogenins that have been a target of intense investigation in the last years (Rupprecht et al., 1990).

Therefore, as a part of our research program

on bioactive plants native to the brazilian mid-west region, this study aimed to perform a preliminary investigation of not fully studied vegetal species extracts in order to obtain a scaffold for more detailed chemical analysis in the future.

The good results of cytotoxic activity in wild *Tapirira guianensis, Attalea phalerata* and *Myracrodruon urundeuva* front cell lines tested (MDA-MB-435, HCT-8 and SF-295); clearly indicate the importance of continuing phytochemical studies aimed at identify the active compounds present in extracts.

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