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Evaluation of the antitumor and trypanocidal activities and alkaloid profile in species of Brazilian Cactaceae

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ABSTRACT - Brazilian species of five genus of the family Cactaceae were screened for *in vitro* antitumor activity in a mechanism-based yeast bioassay using DNA repair- or recombination-deficient mutants of the yeast *Saccharomyces cerevisiae*, and in an assay using six different tumor cells. Whereas some species showed to be selectively active against some tumor cells, all of them were inactive in the *S. cerevisiae* assay, thus, demonstrating that the antitumor activity is not related either to the DNA damaging and/or to the topoisomerase I and II inhibition mechanisms. An evaluation for trypanocidal activity in trypomastigote forms of *Trypanosoma cruzi* showed that three species were especially active. The determination of the alkaloid profile of four species through TLC, HT-HRGC, HT-HRGC-MS and HPLC-PAD revealed, in all the species, low concentrations of alkaloids in complex mixtures with no major compounds.

KEYWORDS - Cactaceae; antitumor activity; trypanocidal activity; alkaloids.

INTRODUCTION

The botanical family Cactaceae has around 2000 species growing mainly in arid and semi-arid regions of the American continent. From these *ca.* 400 species occur in Brazil and are found distributed all over the country, some endemic. The Brazilian Cactaceae consists of wild or cultivated species with fruits, flowers, stems and leaves, used as food, cattle forage and in the extraction of natural pigment and with an economic importance as ornamental plants. In spite of the species of Cactaceae have been used all over the world in folk medicine for the treatment of several diseases (1-7) in Brazil this practice has usually been limited and the therapeutic potential of these cacti remains quite unexplored with few reports about their bioactivity and chemical constitution (8, 9). Phytochemical studies of stems, roots, flowers and fruits of Cactaceae species, have led to the isolation and/or detection of triterpenoids (10, 11), triterpenoid glycosides (12), steroids (13), flavonoids (14), flavonoid glycosides (15), alkaloids (16, 17), betalain pigments (7, 18) and others (19, 20). Among these metabolites the alkaloids have assumed a prominent place since mescaline (3,4,5-trimethoxy- ß -phenylethylamine) was determined as the main psychoactive compound from the Mexican cactus peyote (*Lophophora williamsii)* (21). So far, around 160 cactus species have already been examined for their alkaloid content having been found mainly to contain ß-phenethylamine and tetrahydroisoquinoline (16), beside some rarely occurring imidazole type alkaloids (17).

In this paper, as part of our continuing work on the Brazilian Cactaceae, we report the preliminary investigation of the therapeutic potential and chemical constitution of some of Cactaceae species aiming at broadening the knowledge of this plant family and discovering potential therapeutic agents for cancer and

Chagas disease. Seven Brazilian species of five genus of the Cactaceae family were screened in a mechanism-based yeast bioassay using DNA repair- or recombination-deficient mutants of the yeast *Saccharomyces cerevisiae* in order to establish DNA damaging and/or topoisomerase I and II inhibitor agents (22). Four of those species were also assayed *in vitro* on the growing of six different tumor cells. Three of them were selected to be evaluated for the *in vitro* trypanocidal activity in trypomastigote forms of *Trypanosoma cruzi*. The determination of the alkaloid profile of the species was accomplished with the help of tandem techniques, such as high-temperature and high-resolution gas chromatography-mass spectrometry (HT-HRGC-MS) and high-performance liquid chromatography with photodiode-array detector (HPLC-PAD).

MATERIALS AND METHODS

Plant material

Cactus collections were driven by criteria as the abundance of the species, easiness of access, folk use against cancer and the novelty of the chemical study. Sample plants were collected in two different places in the State of Rio de Janeiro, Brazil: Jurujuba, Niterói (December, 1996); on rocks: *Opuntia stricta* (Haw.) Haw. subsp. *reitzii* (Scheinvar) Scheinvar and A. Rodr., *Pereskia aculeata* Mill., *Rhipsalis baccifera* (Mill.) Stearn, *Rhipsalis mesembrianthenoides* Haw. and *Selenicereus rizzinii* Scheinvar; in Grumari beach, Rio de Janeiro (January, 1997); on sand: *Cereus fernambucensis*, *Opuntia monacantha* Haw. and *Pilosocereus arrabidae* (Lem.) Byley & Rowley. Fresh sliced cactus stems were directly freeze-dried and pulverized. Leaves of *P. aculeata* were oven-dried at 60ºC for 24 h.

Extraction

For the pharmacological assays, the species were exhaustively extracted with CH_2Cl_2/MeOH 1:1 (23). The four more abundant species: *O. stricta* subsp. *reitzii* (90.0 g), *P. arrabidae* (100 g), *C. fernambucencis* (84.0 g) and *O. monacantha* (338 g) were exhaustively extracted: the first three with n-hexane for defating, followed by $CHCl_3$/NH_4OH (sonnication and room temperature) for alkaloid extraction (24); the last one, with n-hexane followed by MeOH at room temperature. *P. aculeata* was sequentially and exhaustively extracted with CH_2Cl_2/MeOH 1:1 and H_2O at 60ºC.

Evaluation of haemolytic activity

Sheep blood cells were washed and diluted in Hank's balanced salt solution at 25% concentration. This suspension was plated in 96-well round bottom plate (Corning, NY) in triplicates with saline (negative control), pure water (positive control) or 250 µg/mL of plant extracts diluted in RPMI 1640 culture medium. Deposition of red cells in each well was evaluated after 24h, indicating the absence or presence of haemolytic activity. The same extract concentration was used for the evaluation of trypanocidal and antitumor activities.

Evaluation of trypanocidal activity

The Y strain of *T. cruzi* was used to infect the cell line MK2 (monkey epithelial cell). The cultures were maintained at 37°C in 5% CO_2 with RPMI 1640 medium plus 10% fetal bovine serum (FBS – HyClone, UK). Supernatants were collected and centrifuged at 700 rpm for 15 min; the pellet was discarded and the material centrifuged again at 2,000 rpm for 30 min. Trypomastigotes forms were counted in a Neubauer chamber to adjust the number to 10^6 parasites/mL in RPMI 1640 medium and plated in 96-well flat-bottom plates. Drug-free well (negative control) or 0.2% crystal violet (positive control) were used to compare with the plant extracts. After 24 h and 48 h of incubation, the number of parasites was evaluated by counting in a Neubauer chamber.

Evaluation of antitumor activity through tumor cell growth inhibition

The cell lines (from American Type Culture Collection) SP2/0 (murine myeloma), Neuro-2A (murine neuroblastoma), J774 (murine sarcoma), Ehrlich Carcinoma (murine carcinoma), BW (murine lymphoma) and P3653 (murine myeloma) were cultured at 37°C and 5% CO_2 in RPMI medium plus 10% of FBS. After collection, cell lines were centrifuged at 700 x g for 15 min and cultured 5.0 x 10^5 cells in 96-well flat-bottom plates. Every assay contained a negative (RPMI medium) or positive control (3% Tween 20 – Sigma, USA). The plant extracts were added after 3 h of incubation, and after 20 h the wells were placed in 1.0 mg/mL of MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide – Sigma, USA] solution for 4 h. The supernatants were removed and 100 µL of dimethylsulphoxide (DMSO – Sigma, USA) were added to dilute formazan salt (25). Optical density was measured on a Microelisa Plate Reader, using a reference wavelength of 630 nm and a test wavelength of 490 nm.

Statistical analysis

Statistical differences between the groups and the controls of the bioassays described above were evaluated by t test. Values are expressed as the mean ± S. E. and the level of significance was set at $p<0.001$.

Evaluation of antitumor activity through S. cerevisiae mutant strains growth inhibition

Three S. *cerevisiae* mutant strains were used (26): RAD+, *rad52* and *rad52.top1*. The assay was carried out by introducing the test sample (crude extract) dissolved in a 1:1 v/v mixture of MeOH/DMSO to a 100 µL well in agar plates separately impregnated with normal "wild-type" RAD+ yeast cells (strain derived from a mutation that makes the cell wall of the yeast more permeable to drugs but capable to DNA repair) and with mutant yeast cells and incubating the plates 48 h at 30ºC. If the test sample contains a DNA-damaging agent or a topoisomerase inhibitor then it would inhibit the growth of one or more mutant strains producing a zone of inhibition. A one-dose test of the crude extracts was carried out at the concentration of 2000 µg/mL. Those extracts showing inhibition zone ≥10mm for the mutant strains and <10mm for RAD+ were considered active. The activity of yeast in each plate was tested by the use of camptothecin (positive control) (22), a well known anticancer drug.

Determination of the alkaloid profiles

High-temperature and high-resolution gas chromatography analyses (HT-HRGC) (27) were made in a HP5890-II gas chromatograph using a 15 m x 0.30 mm i.d. OV-1701OH column, with a cold on-column injection, flame ionization detector (FID) and nitrogen and phosphorous detector (NPD) at 350ºC, with H_2 as the carrier gas at 50 cm^3/s, sample volumes from 0.2 to 5.0 µL and temperatures varying from 40ºC to 350ºC, with a 25ºC/min rate. High-temperature and high-resolution gas chromatography-mass spectrometry analyses (HT-HRGC-MS) were made in a HP5972MSD gas chromatograph at the same conditions described above, using He as a carrier gas. The thin layer chromatography analyses (TLC) were made using pre-coated silica-gel $60F_{254}$ (Merck), the solvent systems: a-Et_2O/MeOH/NH_4OH 16:3:1; b-MeOH/NH_4OH 9:1 and, to visualize the spots: UV irradiation (254 and 326 nm) followed by spraying with fluorescamine 0.02% in acetone and observation under UV light and subsequent overspraying with dansyl chloride 0.05% in acetone, followed by observation under UV light (28). The analyses were made using three ß-phenethylamine alkaloid standards: dopamine, tyramine and mescaline. High-performance liquid chromatography analysis (HPLC) were made in a Shimadzu LC10 liquid chromatograph with a SPDM 10A VP photodiode-array detector, LC10AD VP pumps, SIL10AD VP injector and a SCL 10 VP system controller. The separation was performed isocratically at 25ºC on a Shimpack MRC-ODS 250 x 6 mm, 15 µm column, mobile phase CH_3CN/H_2O (108:892), containing 5.0 mL of H_3PO_4 85 % and 0.28 mL of hexylamine per 1000 mL, flow-rate of 1.0 mL/min (29). The analyses were made using two ß-phenethylamine alkaloid standards: dopamine and tyramine. Methoxamine was used as an internal standard.

For the isolation of the alkaloids, the $CHCl_3$ extracts of *C. fernambucensis* (735 mg), *O. stricta* subsp. *reitzii* (1.78 g) and *P. arrabidae* (312 mg) were treated overnight with 1 N HCl (150, 200 and 250 mL respectively), followed by $CHCl_3$ extraction. The aqueous fractions were neutralized with NH_4OH, and then extracted with $CHCl_3$ yielding alkaloid rich organic fractions. Instead of employing the classic acid/base partition method, the MeOH extract of *O. monacantha* (21.0 g) was submitted to a cationic resin (30) treatment, to afford a fraction containing acid and/or neutral compounds (20.7 g) (eluted with MeOH/H_2O) and a fraction containing basic compounds (75.4 mg) (eluted with MeOH/NH_4OH).

RESULTS

The extracts of the species *Opuntia monacantha*, *O. stricta* subsp. *reitzii*, *Pereskia aculeata*, *Pilosocereus arrabidae*, *Rhipsalis baccifera*, *R. mesembrian thenoides* and *Selenicereus rizzinii*, when evaluated for antitumor activity in the *Saccharomyces cerevisiae* mutant strains, showed inhibition zones ≤10mm in all used strains (*rad52*, *rad52top1* and RAD^+).

In the assay for trypanocidal activity in trypomastigote forms of the Y strain of *Trypanosoma cruzi*, the species *O. stricta* subsp. *reitzii* and *P. aculeata* (aqueous extract) were responsible for a percentage of parasitic death of 100% in the period of 24 h. *P. arrabidae* and *P. aculeata* (CH_2Cl_2/MeOH extract) killed 100% and 90% the parasites, respectively in the period of 48 h (Table 1). As also shown in Table 1, *O. stricta* subsp. *reitzii* caused a remarkable inhibition on the growth of the tumor cell lines SP2/0 (88.5%), Ehrlich Carcinoma (36.0%), BW (88.1%) and P3653 (88.4%). The species *O. monacantha* affected the growth of cells J774 (43.6%), SP2/0 (91.0%), Ehrlich Carcinoma (76.9%), BW (90.1%) and P3653 (81.2%), whereas the aqueous extract of *P. aculeata* inhibited the growth of strains J774 (49.4%), SP2/0 (49.9%) BW (72.4%) and P3653 (58.7%) and the organic extract of the same species inhibited in 46.2% the cell proliferation of the Ehrlich Carcinoma. Finally, the species *P. arrabidae* had a selective effect on the growth of SP2/0 (68.8%) and BW (42.2%) cell lines.

The acid-base partitioning of *C. fernambucensis* (23.5 mg), *O. stricta* subsp. *reitzii* (612 mg) and *P. arrabidae*

Table 1. Results of trypanocidal and antitumor activity in the tumor cell assay of the studied Brazilian cactus crude extracts (data in inhibition percentage).

Species	*T. cruzi*	CITO[a]	J774	SP2/0	NEURO-2A	EHRLICH	BW	P3653
Opuntia stricta subsp. *reitzii*	100/24h	Neg.	NA	88.5	NA	36.0	88.1	88.4
Opuntia monacantha	NA	Neg.	43.6	91.0	CGI	76.9	90.1	81.2
Pereskia aculeata (CH_2Cl_2/MeOH)	90/48h	Neg.	Neg.	Neg.	NA	46.2	Neg.	Neg.
Pereskia aculeata (H_2O)	100/24h	Neg.	49.4	49.9	Neg.	Neg.	72.4	58.7
Pilosocereus arrabidae	100/48h	Neg.	Neg.	68.8	CGI	Neg.	42.2	Neg.

[a]*Cytotoxicity on erythrocytes; NA-Not assayed; Neg.-Negative; CGI-Cellular Growth Inducer; J774-murine sarcoma; SP2/0-murine myeloma; Neuro-2A-murine neuroblastoma; Ehrlich Carcinoma-murine carcinoma; BW-murine lymphoma; P3653-murine myeloma; Concentration of extracts: 250µg/mL; P<0.001.*

(10.3 mg) as well as the cationic resin treatment of *O. monacantha* (75.4 mg) extracts led to low yield alkaloidal fractions. The presence of alkaloids in these fractions was confirmed by TLC using different solvents and visualization systems.

The submission of these basic fractions of *P. arrabidae* and *O. monacantha* to HT-HRGC using NP and FI detectors showed them to be complex mixtures with no major compounds. The preliminary analysis through mass fragmentography of the ions obtained by HT-HRGC-MS indicated the possible presence of ß-phenethylamine, tetrahydroisoquinoline and imidazole alkaloids. Submission to HPLC of the basic fractions of *O. stricta* subsp. *reitzii* and *C. fernambucensis*, also indicated complex mixtures, while the comparison of the UV spectra, obtained by the photodiode-array detector of the authentic standards, to those of the analyzed compounds suggested the presence of ß-phenethylamine alkaloids.

DISCUSSION

Chagas disease (American trypanosomiase) is a protozoan parasitic disease threatening millions of people in Latin America. It is among the most neglected diseases in the world and the current chemotherapy is expensive and suffers from undesirable side effects and low efficacy in many cases. Throughout Latin America the transmission of *Trypanossoma cruzi*, its etiological agent, has been steadily reduced through a series of multinational initiatives that caused a consequent decline of the rate of new infections (31). However the drugs available for the already infected people besides their limited therapeutic value must be used in early stage of the disease. On the other hand cancer is a disease which has no geographic, national or social boundaries with dreadful mortality rate. Thus, the search for effective new tripanocidal and antitumor agents is still attractive and has involved several different scientific fields. The natural products approach has been a great source of lead compounds (26, 32-35).

All the tested extracts proved to be very active against the trypomastigote forms [the form responsible for the dissemination of the infection (32)] of the Y strain of *T. cruzi*.

The *in vitro* tumor cell assays indicated that all the cactus species were significant and selective inhibitors of cellular proliferation. Furthermore, the CH_2Cl_2/MeOH extract of *P. aculeata* showed to be selectively active against the Ehrlich Carcinoma. The two *Opuntia* species and the aqueous extract of *P. aculeata* presented the higher rates of inhibition. On the other hand, all the extracts assayed were not able to inhibit the growth of the *S. cerevisiae* mutant strains, what means that the antitumor activity of *O. stricta* subsp. *reitzii, O. monacantha, P. aculeata* and *P. arrabidae* is not related to the DNA damaging and/or topoisomerase I and II inhibition mechanisms.

CONCLUSION

The present work has revealed the promising pharmacological results of the species *O. stricta* subsp. *reitzii* and *P. aculeata* (aqueous extract) for both antitumor and tripanocidal activities and *O. monacantha* for antitumor activity, encouraging further and more detailed analysis of these Cactaceae species. This is the first report about trypanocidal activity screening of cactus species. *P. aculeata* known in Brazil as *ora-pro-nobis* has edible leaves and its popular use against cancer has already been described (36).

The use of HT-HRGC-MS with more modern capillary columns than the ones usually used in the alkaloid analysis of Cactaceae, seemed to suggest the presence of new substances never before isolated in the species. Nevertheless, the complexity of the alkaloid fractions, allied to the low yield they presented has limited the capacity of isolating and purifying any substances in this study. Therefore, the chemical reevaluation of these species, using larger quantities of vegetable samples, followed by the introduction of new extraction methods, will be the focus of our future studies.

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