CYTOTOXIC EFFECTS OF ARGENTINEAN PLANT EXTRACTS ON TUMOUR AND NORMAL CELL LINES

L. MAMONE[1], G. DI VENOSA[1], J. J. VALLA[2], L. RODRIGUEZ[1], L. GÁNDARA[1], A. BATLLE[1] ✍, M. HEINRICH[3], A. JUARRANZ[4], F. SANZ- RODRIGUEZ[4] AND A. CASAS[1]

1 Centro de Investigaciones sobre Porfirinas y Porfirias (CIPYP). CONICET and Hospital de Clínicas José de San Martín, University of Buenos Aires. Córdoba 2351 1er subsuelo; Ciudad de Buenos Aires, CP1120AAF, Argentina

2 Department of Botany, School of Agronomy, University of Buenos Aires

3 Centre for Pharmacognosy and Phytotherapy, The School of Pharmacy, University of London and Southern Cross Plant Science, Southern Cross Univ. Lismore, NSW, Australia

4 Departamento de Biología, Facultad de Ciencias, Universidad Autónoma de Madrid. Cantoblanco E-28049 Madrid, Spain

Abstract

In the search for possible new anti-cancer agents, we investigated the effects of 75 aqueous and methanol extracts from 41 Argentinean plant species. The effect in cell growth was evaluated in the LM2 mammary adenocarcinoma cells. In a second stage, the highly active selected extracts were assayed in 3 other tumour cell lines: melanoma B16, bladder MB49 and lung A549; and 3 normal cell lines: mammary Hb4a and keratinocytes PAM212 and HaCat. Eight methanol extracts were found to be highly cytotoxic: *Collaea argentina* leaf, *Iochroma australe* leaf, *Ipomoea bonariensis* flower, *Jacaranda mimosifolia* flower, *Solanum amygdalifolium* flower, *Solanum chacoense* leaf, *Solanum sisymbriifolium* flower and *Solanum verbascifolium* flower. However, extract inhibition on cell growth was highly dependent on cell type. In general, except for the highly resistant cell lines, the inhibitory concentrations 50% were in the range of 10-150 µg/ml The eight extracts highly inhibited cell growth in a concentration-dependent manner, and in general the methanol extracts were always more active than the aqueous. Murine cells appear to be more sensitive than human cells to the cytotoxic action of the plant extracts. The human melanoma B16 line was the most resistant to four of the extracts. In terms of selectivity, *S. verbascifolium* was the species which showed most selectivity for tumour cells.

Overall, this is one of the first studies focusing on southern South American native plants and their biological effects. Since some species of 5 genera analyzed have been reported to possess different degrees of alkaloid content, we examined microtubule structures after extract treatments. The eight extracts induced destabilization, condensation and aggregation of microtubules in LM2 cells, although no depolarization, typical of Vinca alkaloids damage was observed. In a near future, antitumour activity of purified fractions of the extracts administered at non-toxic doses will be assayed in transplantable murine tumour models.

INTRODUCTION

The number of higher plant species (angiosperms and gymnosperms) on this planet is estimated to be at least 250,000 (7), with a lower level at 215,000 (13,14) and an upper level as high as 500,000 (63,71). Of these, only about 6% have been screened for biologic activity, and a reported 15% have been evaluated phytochemically (73).

An analysis of the number of chemotherapeutic agents and their sources indicates that over 60% of approved drugs are

derived from natural compounds (12,33,34). The use of natural product derived anticancer compounds in late preclinical development and in early clinical trials was extensively studied (15,20,69,75).

Prominent examples for the success of natural products in current use for the treatment of cancer originally obtained from plants are the Vinca alkaloids from *Catharanthus roseus* (Apocynaceae), the DNA topoisomerase I inhibitor camptothecin from *Camptotheca acuminata* (Nyssaceae), the terpene paclitaxel from *Taxus brevifolia* (Taxaceae) and the lignan podophyllotoxin isolated from *Podophyllum peltatum* (21).

Native American medicinal plants are traditionally used to prevent and treat a variety of diseases, including cancer. These herbal preparations are alleged to have many biological activities, such as stimulation or suppression of immune responses and antiproliferative effects on cancer cells. In Argentina, a few reports based on ethnopharmacological usages of plants for antitumour activity both in cell lines and tumour models have been published (4,5,6,9,22,23, 32,46,51,56,58,60).

Argentina has an abundant and diverse flora ranging from sub-artic to sub-tropical climates; however the medicinal properties of these plants have only been studied to a very limited extend. Therefore, the overall aim of this paper was to investigate a collection of regional plant extracts from Argentina as a source of new antitumour agents. The collection of plants included 75 extracts from 41 species from the North and central regions of the country.

Three major eukaryotic cytoskeletal proteins are actin, tubulin and intermediate filaments. Microtubules are dynamic filamentous cytoskeletal proteins composed of tubulin and are essential for cell transport and division in all eukaryotes (53). Accumulated evidence has shown that microtubule dynamics may play a crucial role in the passage through the metaphase/anaphase checkpoint (77). Mitotic block by drugs at concentrations that suppress microtubule dynamics or alter microtubule mass induces apoptosis (38).

Microtubules are an important therapeutic target in tumour cells. Agents that bind to microtubules have been part of the pharmacopoeia of anticancer therapy for decades. The screening of a range of botanical species and marine organisms has yielded promising new antitubulin agents with novel properties (18,57).

The aim of this work is to analyze the cytotoxicity of a collection of plants native from Argentina, in a panel of tumour and normal cell lines. The species considered most active were studied in regard to their damage to microtubules and induction of cell morphology changes.

MATERIALS AND METHODS

Chemicals
(3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium -bromide) (MTT) was obtained from Sigma-Aldrich (Poole, UK). Vinblastine was a kind gift from Filaxis Argentina. The rest of the chemicals employed were of analytical grade. The primary antibody anti alpha-tubulin was Sigma, USA and FITC-labeled secondary antibody was Alexa-488 from Santa Cruz Biotechnology Inc, USA.

Cell lines and cell culture
LM2 cell line derived from the murine mammary adenocarcinoma M2 (26), was obtained from Instituto Roffo, Buenos Aires, Argentina, and was cultured in minimum essential Eagle's medium, supplemented with 2 mM L-glutamine and 5% fetal bovine serum.
Hb4a is a clonal, non-transformed, non-tumorigenic line derived from reduction mammoplasty tissue. It was one of a panel of immortal lines developed using an amophotropic retrovirus to transduce the SV40-derived recombinant viral oncogene mutant tsA58-U19 into luminal epithelial cells which had been sorted by FACS using the lumen-specific marker epithelial membrane antigen (31).
Hb4a-Ras was generated by transfecting Hb4a parental cells with the plasmid pEJ containing a 6.6-Kb genomic H-Ras (VAL/12 Ras) sequence (67). Hb4a and Hb4a-Ras was a kind gift of Dr. O´Hare, Univeristy College London.
Both cell lines were maintained in RPMI 1640 medium with 2 mM L-glutamine, supplemented with 10% fetal bovine serum, 5 µg/ml hydrocortisone and 5 µg/ml insulin.
PAM212 The spontaneously transformed murine keratinocyte cell line, PAM212 obtained from American Type Culture Collection (ATCC), USA, was cultured in RPMI-1640 medium containing 2 mM L-glutamine and supplemented with 5% fetal bovine serum.
B16-F10 melanoma cells were obtained from ATCC and maintained in Dulbecco's modified Eagle´s medium F-12 containing 2 mM L-glutamine and supplemented with 10% fetal bovine serum.
A549 human bronchioloalveolar lung carcinoma cells were obtained from the ATCC and cultured in minimum essential Eagle's medium supplemented with 5% fetal bovine serum.
MB49 the mouse bladder cancer cell line MB49 was obtained from the ATCC and cultured in RPMI 1640 medium with 2 mM L-glutamine supplemented with 10% fetal bovine serum.
All cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C and supplemented with 80 µg gentamycin/ml.

Plant material
The species were collected in the Botanical Garden *Lucien Hauman* of the Agronomy School, University of Buenos Aires and were identified by Ing. Agr. Juan José Valla. The samples were taken on Springs and Summers to avoid seasonal variations of the plant components.
Voucher specimens are kept in the Herbarium at the Botanical Garden of the Agronomy School (BAA), University of Buenos Aires. The ethnobotanical information

of the plants assayed is presented in Table 1. Plant nomenclature (scientific names) is largely according to Cabrera and Zardini (10), Hunziker (35), Zuloaga and Morrone (79, 80), and Zuloaga et al. (81). Vernacular names were taken from de la Peña & Pensiero (16).

Extraction procedure

Fresh material (100 to 200 g) was washed with distilled water, dried and homogenized in absolute methanol or water and blended to macerate the plant material during 3 min at high speed. Lower quantities of plants (20-50 g) were blended employing an Ultra Turrax T-50 homogenizer (IKA, Germany). Aqueous mixtures were centrifuged for 10 min and the supernatant decanted and filtered to remove particulate matter and lyophilized. Methanol extracts were filtered and evaporated under reduced pressure using a rotary evaporator and lyophilized afterwards to remove any traces of solvent. The obtained yields were 4-5%, and the resulting powders were stored at -20°C.

Assay for cell viability

Cells in a log-growth phase were incubated with the plant extracts in the 10 –250 µg/ml dose range in complete medium for 24 h. Cell viability was tested in accordance with Mossman (52) based on mitochondrial dehydrogenases activities. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide) solution was added to each well in a concentration of 0.5 mg/ml, and plates were incubated at 37° C for 1 h. The resulting formazan crystals were dissolved by the addition of DMSO and absorbance was read at 560 nm employing an microplate reader (Spectracount, Packard, USA).

In each experiment 4 wells were used to determine the mean OD. The experiment was repeated at least three times for each extract per cell line. The IC_{50} (inhibitory concentration 50%) expressed as µg/ml and SD (standard deviation for 95% confidence), determined as the extract concentration required to reduce the cell viability to half the control value, was analyzed by linear regression from the curve of the cell proliferation vs. the sample concentrations. Coefficient of variation [SD/mean x 100 (%)] was below 12 % in all experiments.

Toluidine blue staining

The cells grown on coverslips were fixed with paraformaldehyde. Afterwards, they were incubated with 0.025 mg/ml Toluidine blue during 1 minute, and subsequently washed with distilled water. The coverslips were mounted on Canadian Balsam and cellular structures were observed under light microscope.

Alpha-tubulin immunostaining

Cell were fixed in cold methanol (-20ºC) for 10 min and hydrated in graded ethanol-PBS solution. Cells were later permeabilised with PBS containing 0.5% Triton X-100 for 30 min and labelled with a 1:2000 dilution of monoclonal anti-alpha-tubulin antibody for 1 h at 37ºC. After washing in PBS, cells were stained with 1:250 dilution of anti-mouse Alexa 488-conjugated antibody (1 h at 37 ºC in darkness), rinsed again in PBS and mounted in ProLong-Gold (Molecular Probes, USA).

Microscopic observation and photography were performed in an Olympus photomicroscope BX51, equipped with a HBO 100 W mercury lamp and the corresponding blue filter (450–490 nm, exciting filter BP 490).

RESULTS

An initial screening for cytotoxicity of 41 plant species and 75 extracts (Table 1) was carried out employing the murine LM2 mammary carcinoma cell line. The species studied belonged to the Acanthaceae, Amaranthaceae, Aristolochiaceae, Asteraceae, Bignoniaceae, Boraginaceae, Bromeliaceae, Commelinaceae, Convolvulaceae, Combretaceae, Ephedraceae, Fabaceae, Malvaceae, Oxalidaceae, Phytolaccaceae, Rhamnaceae, Rosaceae, Solanaceae, Verbenaceae and Vitaceae families, which are highly representative of the central and north regions of Argentina.

The range of extract doses assayed was 10 - 250 µg/ml. Extracts that induced 50 percent of LM2 cell death at concentrations equal or lower than 100 µg/ml were considered to be 'highly toxic'. In all cases, the corresponding aqueous extracts exerted lower cytotoxicity than the methanol ones. The effect in cell growth of the selected extracts was assayed in three tumour cell lines and three normal cell lines, in each case two human and one murine. Eight methanol extracts were identified as highly cytotoxic: *Collaea argentina* (BAA 2664) leaf, *Iochroma australe* (BAA 26662) leaf, *Ipomoea bonariensis* (BAA 26665) flower, *Jacaranda mimosifolia* (BAA 26658) flower, *Solanum amygdalifolium* (BAA 26659) flower, *Solanum chacoense* (BAA 26661) leaf, *Solanum sisymbriifolium* (BAA 26660) flower and *Solanum verbascifolium* (BAA 26663) flower (see Figures 1–2). When both leaf and flower extracts of the same species were toxic in LM2 cells, we chose the most active part of the plant to carry out the cytotoxicity studies in the rest of the cell lines.

In LM2 cells, the extracts that exerted the highest degree of cytotoxicity were: *S. chacoense= I. australe > J. mimosifolia> S. sisymbriifolium> S. verbascifolium> S. amygdalifolium> C. argentina >I. bonariensis*. However, extract inhibition on cell growth was highly dependent on cell type. In general, except for the highly resistant cell lines, the IC_{50} range was 10 -150 µg/ml (Table 2).

All the extracts were cytotoxic for the 7 lines employed in a dose dependant manner (Figure 1-2). The most active extract against all the cell lines tested was *Solanum chacoense* leaf. Overall, the range in IC_{50} values was 5-24 µg/ml, and most of the cell lines except for the highly resistant melanoma B16 cell line, ranged within CI_{50}s of 12-16 µg/ml, which means that the

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Table 1. Ethnobotanical data of studied plants

Species	Family	Vernacular name	Utilised part	Solvent
Amaranthus quitensis Kunth	Amaranthaceae	Yuyo colorado	Flower and seed	Methanol
Aristolochia esperanzae Kuntze	Aristolochiaceae		Flower	Methanol
Abutilon pictum (Hook. & Arn.) Walp.	Malvaceae	Caraguatá	Flower	Methanol
Bromelia balansae Mez	Bromeliaceae	Caraguatá	Fruit	Methanol
Bromelia balansae Mez	Bromeliaceae		Fruit	Water
Brugmansia arborea (L.) Lagerh.	Solanaceae	Floripón	Flower	Methanol
Brugmansia arborea (L.) Lagerh.	Solanaceae	Floripón	Flower	Water
Ceiba speciosa (A. St.-Hil.) Ravenna	Malvaceae	Palo borracho	Flower	Methanol
Ceiba speciosa (A. St.-Hil.) Ravenna	Malvaceae	Palo borracho	Flower	Water
Cissus verticillata (L.) Nicolson & C.E.	Vitaceae	Cortinas del cielo	Root	Methanol
Collaea argentina Griseb	Fabaceae		Flower	Methanol
Collaea argentina Griseb	Fabaceae		Flower	Water
Collaea argentina Griseb	Fabaceae		Leaf	Methanol
Colletia paradoxa (Spreng.) Escal.	Rhamnaceae	curro, crucero, curamanuel	Flower	Methanol
Colletia paradoxa (Spreng.) Escal.	Rhamnaceae	curro, crucero, curamanuel	Stem and leaf	Methanol
Combretum fruticosum (Loefl.) Stuntz	Combretaceae	Cepillo de mano, cepillo	Flower	Methanol
Combretum fruticosum (Loefl.) Stuntz	Combretaceae	Cepillo de mano, cepillo	Flower	Water
Combretum fruticosum (Loefl.) Stuntz	Combretaceae	Cepillo de mano, cepillo	Leaf	Methanol
Combretum fruticosum (Loefl.) Stuntz	Combretaceae	Cepillo de mano, cepillo	Leaf	Water
Commelina erecta L.	Commelinaceae	Flor de Santa Lucía	Flower	Methanol
Cordia trichotoma (Vell.) Arráb. ex Steud.	Boraginaceae	Petiribí, loro negro	Flower	Methanol
Dicliptera tweediana Nees	Acanthaceae	Canario rojo	Flower	Methanol
Ephedra tweediana Fisch. & C.A. Mey.	Ephedraceae	Tramontana, pingo-pingo	Fruit	Methanol
Ephedra tweediana Fisch. & C.A. Mey..	Ephedraceae	Tramontana, pingo-pingo	Stem and	Methanol
Ephedra tweediana Fisch. & C.A. Mey..	Ephedraceae	Tramontana, pingo-pingo	Stem and leaf	Water
Erythrina falcata Benth.	Fabaceae	Seibo de Jujuy	Flower	Methanol
Erythrina falcata Benth.	Fabaceae	Seibo de Jujuy	Flower	Water
Iochroma australe Griseb.	Solanaceae	Perilla, sacha pera	Flower	Methanol
Iochroma australe Griseb.	Solanaceae	Perilla, sacha pera	Flower	Water
Iochroma australe Griseb.	Solanaceae	Perilla, sacha pera	Leaf	Methanol
Iochroma australe Griseb.	Solanaceae	Perilla, sacha pera	Leaf	Water
Ipomoea bonariensis Hook.	Convolvulaceae	Campanilla	Flower	Methanol
Ipomoea bonariensis Hook.	Convolvulaceae	Campanilla	Leaf	Methanol
Ipomoea cairica (L.) Sweet	Convolvulaceae		Flower	Methanol
Jacaranda mimosifolia D. Don	Bignoniaceae	Jacaranda, tarco	Flower	Methanol
Jacaranda mimosifolia D. Don	Bignoniaceae	Jacaranda, tarco	Leaf	Methanol
Jacaranda mimosifolia D. Don	Bignoniaceae	Jacaranda, tarco	Flower	Water
Lantana camara L.	Verbenaceae	Lantana, camará	Flower	Methanol
Lantana camara L.	Verbenaceae	Lantana, camará	Leaf	Methanol
Macfadyena unguis- cati (L.) A.H. Gentry	Bignoniaceae	Uña de gato	Flower	Methanol
Macfadyena unguis- cati (L.) A.H. Gentry	Bignoniaceae	Uña de gato	Flower	Water
Nicotiana glauca Graham	Solanaceae	Palán- palán	Flower	Methanol

Nicotiana glauca Graham	Solanaceae	Palán- palán	Leaf	Methanol
Oxalis latifolia Kunth	Oxalidaceae		Flower	Methanol
Parkinsonia aculeata L.	Fabaceae	Cina cina	Stem and	Methanol
Patagonula americana L.	Boraginaceae	Guayubirá	Flower	Methanol
Pavonia sepium A. St.-Hil.	Malvaceae	Malva del monte	Flower	Methanol
Prunus subcoriacea (Chodat & Hassl.) Koehne	Rosaceae	Persiguero bravo	Flower	Methanol
Prunus subcoriacea (Chodat & Hassl.) Koehne	Rosaceae	Persiguero bravo	Flower	Water
Prunus subcoriacea (Chodat & Hassl.) Koehne	Rosaceae	Persiguero bravo	Leaf	Methanol
Prunus subcoriacea (Chodat & Hassl.) Koehne	Rosaceae	Persiguero bravo	Leaf	Water
Prunus cerasifera Ehrh.	Rosaceae	Ciruelo, mirabolán	Leaf	Methanol
Pseudogynoxys benthamii Cabrera	Asteraceae		Flower	Methanol
Pyrostegia venusta (Ker Gawl.) Miers	Bignoniaceae	Flor de San Juan	Flower	Methanol
Pyrostegia venusta (Ker Gawl.) Miers	Bignoniaceae	Flor de San Juan	Flower	Water
Rivina humilis L.	Phytolaccaceae	Sangre de toro, inchiquil	Fruit	Methanol
Ruellia brittoniana Leonard	Acanthaceae	Petunia mejicana	Flower	Methanol
Ruellia brittoniana Leonard	Acanthaceae	Petunia mejicana	Leaf	Methanol
Ruellia elegans Poir.	Acanthaceae		Flower	Methanol
Scutia buxifolia Reissek	Rhamnaceae	Coronillo	Leaf	Methanol
Scutia buxifolia Reissek	Rhamnaceae	Coronillo	Leaf	Water
Solanum amygdalifolium Steud.	Solanaceae	Jazmin de Córdoba	Flower	Methanol
Solanum amygdalifolium Steud.	Solanaceae	Jazmin de Córdoba	Leaf	Methanol
Solanum chacoense Bitter	Solanaceae	Papa salvaje	Flower	Methanol
Solanum chacoense Bitter	Solanaceae	Papa salvaje	Leaf	Methanol
Solanum sisymbriifolium Lam.	Solanaceae	Espina colorada	Flower	Methanol
Solanum sisymbriifolium Lam.	Solanaceae	Espina colorada	Leaf	Methanol
Solanum verbascifolium L.	Solanaceae	Fumo bravo	Flower	Methanol
Solanum verbascifolium L.	Solanaceae	Fumo bravo	Flower	Water
Solanum verbascifolium L.	Solanaceae	Fumo bravo	Leaf	Methanol
Solanum verbascifolium L.	Solanaceae	Fumo bravo	Leaf	Water
Solidago chilensis Meyen	Asteraceae	Vara de oro	Flower	Methanol
Tabebuia impetiginosa (Mart. ex DC.) Standl.	Bignoniaceae	Lapacho rosado	Flower	Methanol
Tabebuia impetiginosa (Mart. ex DC.) Standl.	Bignoniaceae	Lapacho rosado	Flower	Water
Xanthium cavanillesii Schouw	Asteraceae	Abrojo grande, cadillo	Leaf and fruit	Methanol

Table 2. Cytotoxic activity of plant extracts

|	*C. argentina*	*I. australe*	*S. verbascifolium*	*J. mimosifolia*	*S. chacoense*	*S. amygdalifolium*	*S. sisymbriifolium*	*I. bonariensis*
LM2	90 ± 5	11 ± 8	53 ± 4	22 ± 4	12 ± 4	57 ± 3	30 ± 5	101 ± 4
B16-F10	> 500	61 ± 3	75 ± 6	24 ± 5	24 ± 6	36 ± 3	56 ± 3	147 ± 3
A549	169 ± 3	49 ± 4	71 ± 4	133 ± 2	12 ± 3	70 ± 5	30 ± 3	169 ± 3
MB49	69 ± 4	25 ± 3	122 ± 3	22 ± 5	5 ± 3	45 ± 4	21 ± 5	185 ± 4
PAM212	100 ± 10	16 ± 2	160 ± 4	18 ± 6	16 ± 8	71 ± 4	35 ± 5	72 ± 4
HaCat	219 ± 4	28 ± 5	155 ± 3	68 ± 3	12 ± 6	30 ± 2	30 ± 5	139 ± 3
Hb4a	222 ± 3	25 ± 5	165 ± 4	26 ± 2	13 ± 3	56 ± 3	31 ± 5	159 ± 3

species is highly cytotoxic against cells from different origins.

I. bonariensis (IC$_{50}$ range 72-185 µg/ml), *S. verbascifolium* (53-165 µg/ml) and *C. argentina* (69 to >500 µg/ml) showed the weakest activities. *I. australe* (IC$_{50}$: 11-61), *S. sisymbriifolium* (IC$_{50}$ 21-56) and *S. amygdalifolium* (IC$_{50}$ 30-71) and *J. mimosifolia* (IC$_{50}$ 18- 133) showed intermediate cytotoxicity.

B16 cells were quite resistant to 4 of the extracts: *C. argentina* (IC$_{50}$ >500), *I. australe* (61 µg/ml), *S. chacoense* (IC$_{50}$= 24 µg/ml) and *S. sisymbriifolium* (IC$_{50}$ = 6 µg/ml). The three normal cell lines PAM212, HaCat and Hb4a were the most resistant against *S. verbascifolium* extract, whereas the human normal HaCat and Hb4a cells were the most resistant against *C.*

argentina. The human lung carcinoma cell line A549 was the most resistant to *J. mimosifolia*.

The cell line most susceptible to each extract was also quite variable, and some of the extracts were more cytotoxic against tumour while others were against normal cell lines. In general, murine cells appear to be more sensitive to the extracts. The murine adenocarcinoma LM2 was the cell line which was mostly affected by *I. australe* and *S. verbascifolium* exracts, whereas the murine lung adenocarcinoma MB49 cells were the most affected by *C. argentina*, *S. sisymbriifolium* and *S. chacoense*. The murine normal keratinocytes PAM212 was the line most susceptible to *J. mimosifolia* and *I. bonariensis* damage. The human cell line of normal keratinocytes HaCat was the most responsive to *S. amygdalifolium* damage.
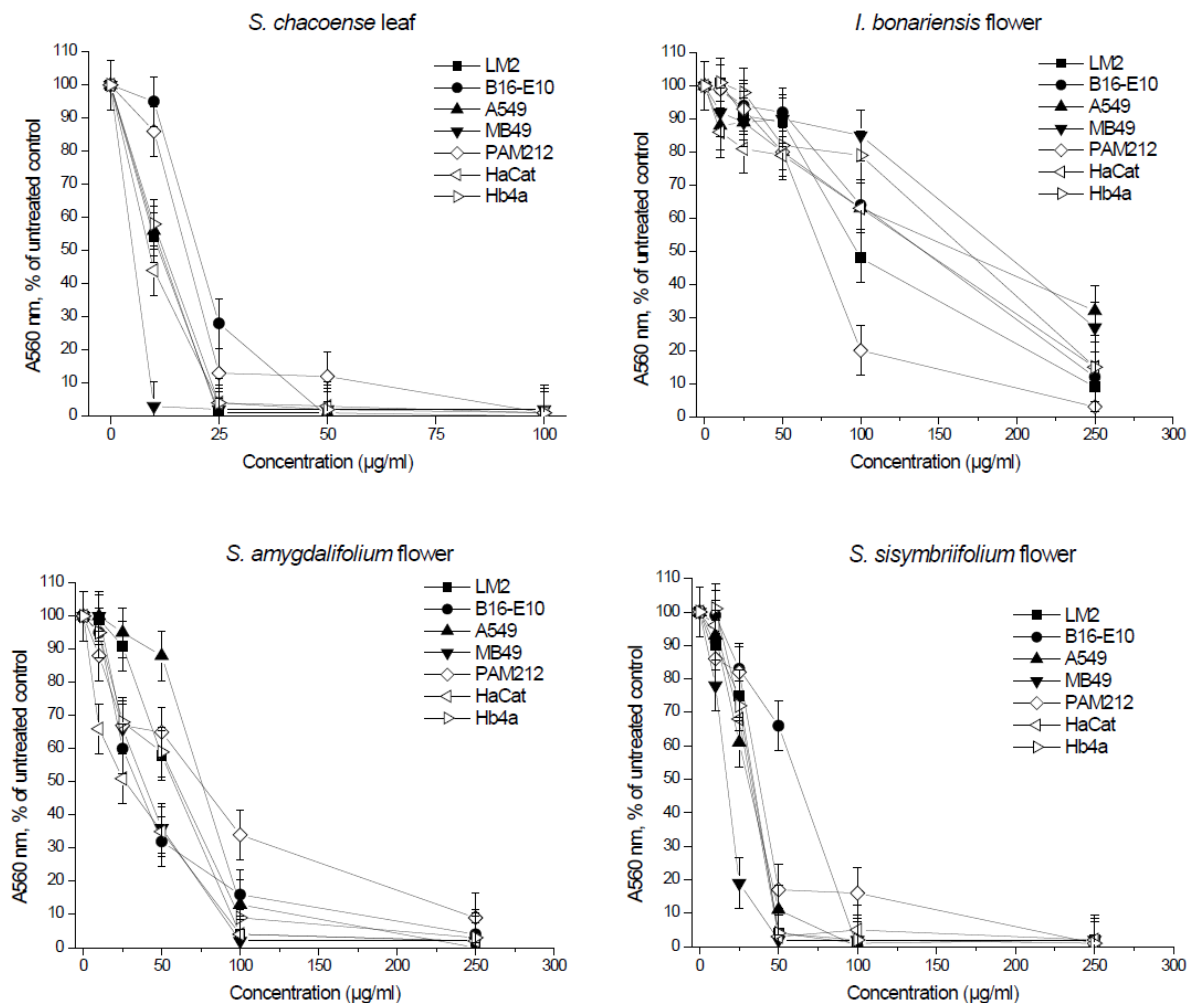
Figure 1. *In vitro* cytotoxicity of *S. chacoense* leaf, *I. bonariensis* flower, *S. amygdalifolium* flower and *S. sisymbriifolium* flower extracts against normal and tumour cell lines
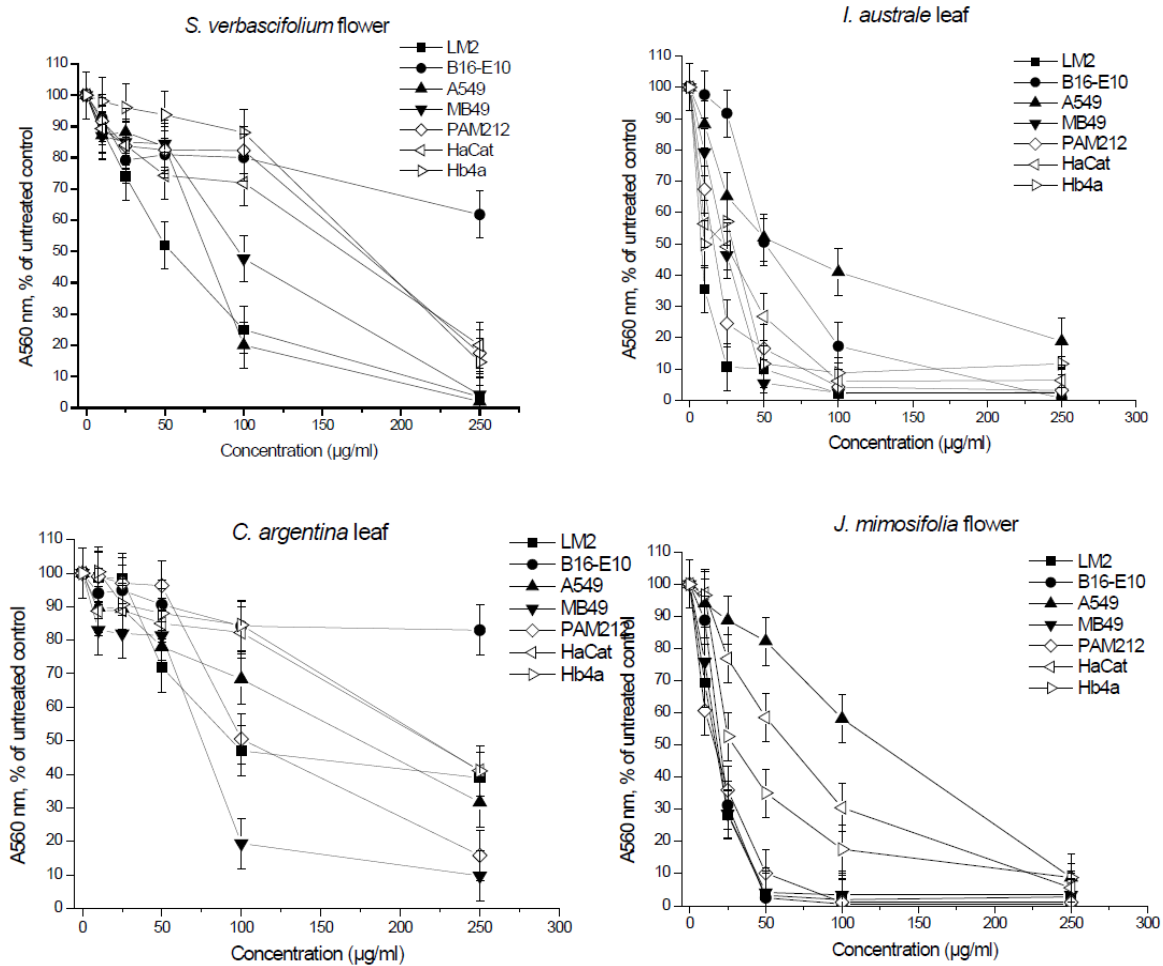
Figure 2. *In vitro* cytotoxicity of *S. verbascifolium* flower*, I. australe* leaf*, J. mimosifolia* flower and *C. argentina* leaf extracts against normal and tumour cell lines

We did not find any correlations between cell doubling time length and sensitivity to any of the extracts.

Methanol extract from *S. chacoense* and *I. australe* flower had prominent cytotoxic effects, although at a higher concentration compared to the extract of the leaves (not shown) In LM2 cells CI_{50}s of flower extracts of the above mentioned plants were 1.2 and 1.3 times higher than the leaf extracts respectively. *J. mimosifolia* and *S. amygdalifolium* methanolic extract from leaves had slightly lower cytotoxic effect as compared to the flower extract.

S. verbascifolium and *I. bonariensis* methanolic extracts from leaves only had moderate effects on cell cytotoxicity (not shown). In LM2 cells CI_{50}s of flower extracts were 3 to 4 times higher than the leave extracts. *S. sisymbriifolium* leaves and *C. argentina* flower showed no effect at all (data not shown).

Analysis of cell morphology and microtubules was performed employing CI_{75}s data, since we aimed at inducing sublethal damage (Figure 3). Toluidine blue staining revealed that all extracts affected LM2 cells morphology at different levels. The extracts that affected the nuclei most strongly were *S. verbascifolium, C. argentina, S. chacoense, I. bonariensis* and *S. amygdalifolium.* The above mentioned extracts induced nuclear condensation together with cell shrinkage. In the case of *I. bonariensis*, at earlier stages of cell damage, the cytoplasm was almost lost, but nuclei remained quite conserved, and when the cell damage progressed, the nuclei became tightly condensed. At the cytoplasmatic level, *I. australe* induced spindle cell shape in most of the surviving cell population, with consequent stretching of nuclei. *S. amygdalifolium* induced a star-like shape of cells, whereas *J. mimosifolia* induced dramatic changes at the cytoplasm level, leading to

considerable reduction of the cytoplasm content, and appearance of large prolongations. Intracytoplasmatic vacuoles were also a consequence of the treatment of LM2 cells with *S. sisymbriifolium, S. chacoense* and *C. argentina* extracts.

In control cells, microtubules labelled with anti α-tubulin specific antibody are organized in a network around the nucleus and distributed uniformly in the cytoplasm (Fig 4B). All the plant extract used in this study, at all concentrations and time points tested, induced changes in the organization of microtubules, which correlates to the extract-induced morphology changes. After plant extract treatments, destabilization, condensation and aggregation of microtubules became evident.

Microtubules are no longer distributed uniformly in the cytoplasm but they concentrate at different intracellular sites; in some cases at the cell periphery (Fig. 4B).

Vinblastine treatment, used as a control of mitotic arrest, induces the formation of non-microtubule polymers resulting in metaphase arrests (Figure 4A). These results suggest that although plant extracts alter the organization of cell microtubules, do not produce a depolarization, as it is observed after Vinca alkaloids treatment. In *I. australe*-exposed LM2 cells, long dendritic-like extensions of tubulin are shown. Overall, we cannot discard that purified fractions of these extracts can lead to a higher degree of microtubule damage.
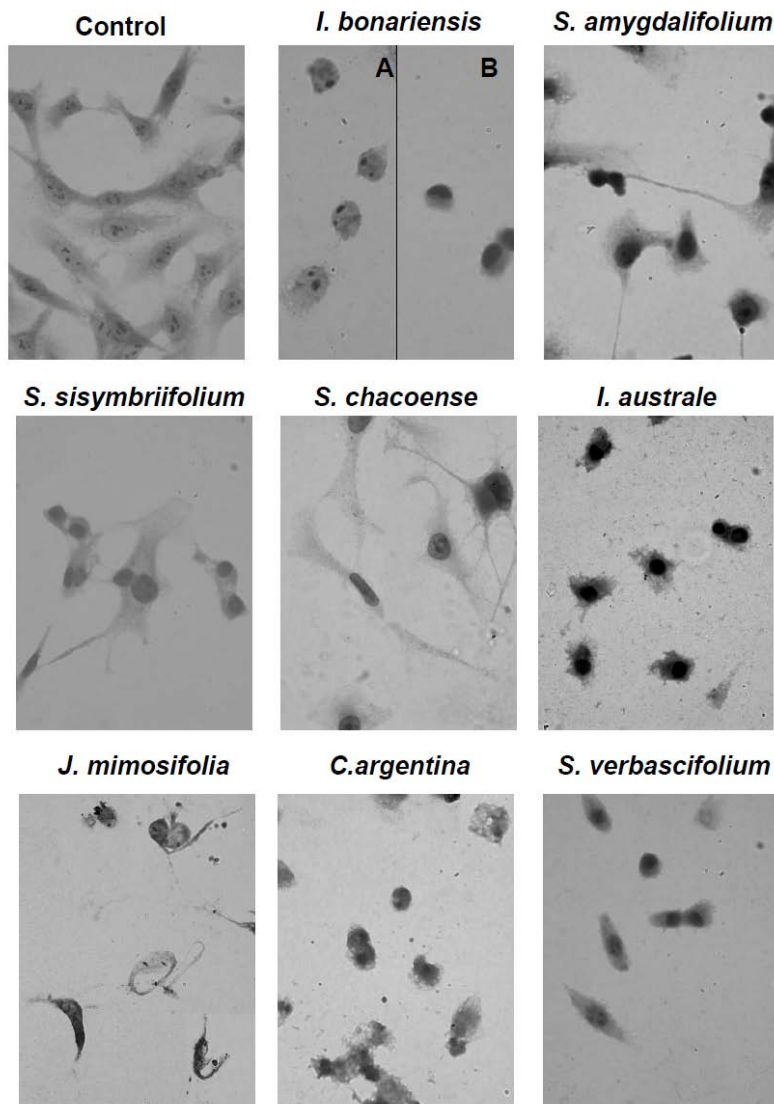
Figure 3. Toluidine blue staining of LM2 cells treated with the active plant extracts
Cells were exposed to CI$_{75}$ concentrations of plant extracts during 24 h and stained with Toluidine blue. Control: cells without treatment. In micrographs of *I. bonariensis* we show A) an earlier stage of damage, B) later stage of damage at the same time point. Magnification: 100x.
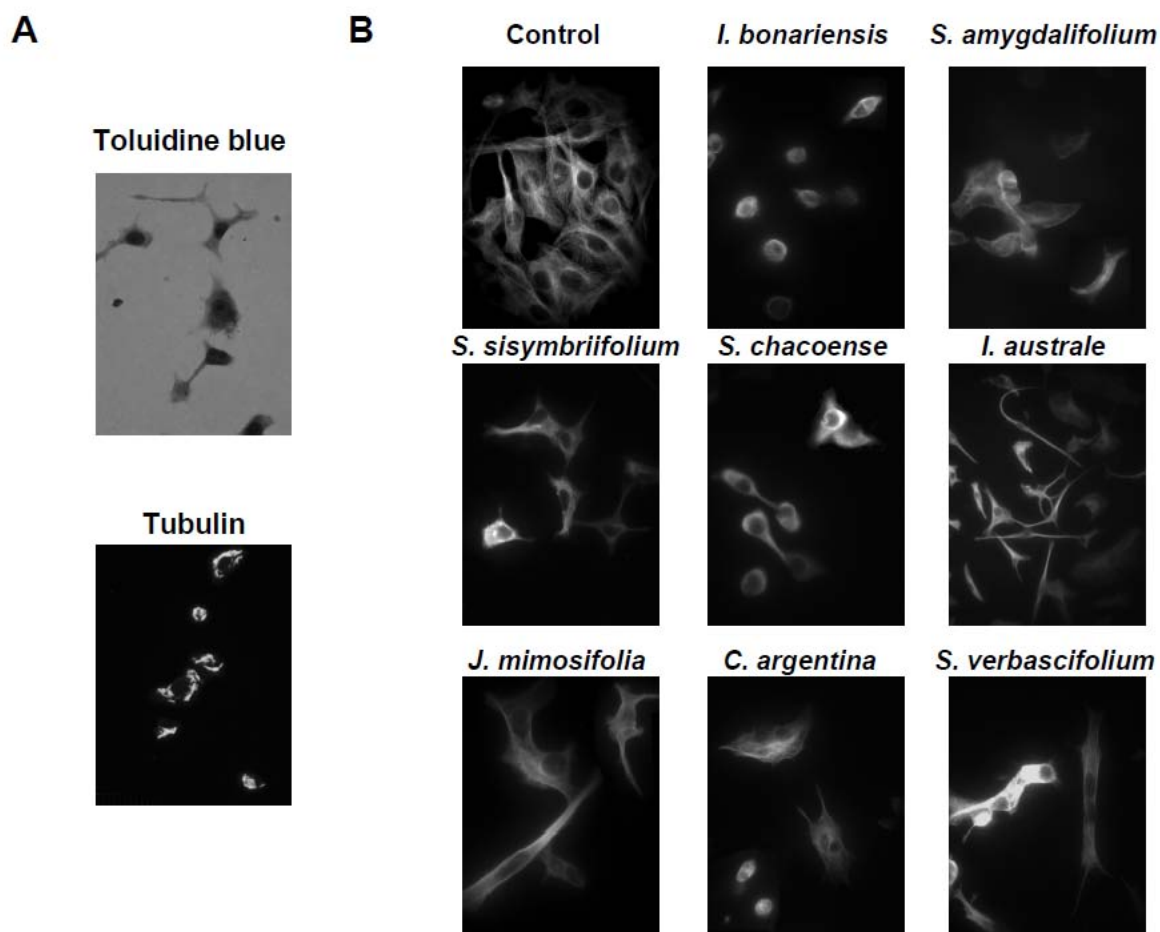
Figure 4. Alpha-tubulin immunostaining of LM2 cells treated with the active plant extracts and vinblastine.
A) Cells were exposed to a CI$_{75}$ concentration of Vinblastine (0.05 µg/ml) during 24 h. Staining was carried out with Toluidine blue, and immunostaining with anti alpha-tubulin antibody. B) Cells were exposed to CI$_{75}$ concentrations of plant extracts during 24 h and alpha-tubulin was immunostained. Control: cells without treatment. Magnification: 100x.

DISCUSSION

Eight methanol plant extracts were considered to be highly active against four tumour and three normal cell lines. The selected plants included 4 species from the genus *Solanum* (Solanaceae family), known for possessing a diverse range of alkaloids.

Solanum chacoense, which showed the highest cytotoxicity to all seven cell lines, has been reported to possess a high content of leptine glycoalkaloids (47), which confers insecticidal properties against a range of insects.

The main toxic compound of *S. verbascifolium* is known to be the glycoalkaloid, solasodine. In addition, solaverbascine (2), solasodiene, solafloridine and tomatidenol have also been identified in *S. verbascifolium* (17,72). In a study carried out with Vietnamese medicinal plants *Solanum verbascifolium* exhibited high potent cytotoxic activity against different tumour cell lines (70). However, remarkable variation in the steroidal alkaloid content of the plant was found in different geographical regions.

In Paraguay the root of *Solanum sisymbriifolium* has been used as a traditional medicine possessing diuretic and antihypertensive properties, and it was reported that extracts from this plant exerted hypotensive effects in rats. Nuatigenosido was isolated from the extract as one of the prospective active compounds (36). However, no antitumour effects have been described to date for this plant.

Some other species from the genus *Solanum* have been used in Chinese medicine in the treatment of cancer. Total alkaloid isolated from *S. nigrum* interfered structure and function of tumour cell membrane, disturbed the synthesis of DNA and RNA, and changed the cell cycle

distribution (3). Solamargine, a steroidal glycoalkaloid isolated from *S. incanum*, has been shown to induce apoptosis in several cancer cell lines (42). At the immunological level, *S. lyratum* extracts have shown to induce immunomodulatory activity in tumour-bearing mice (45).

In general, species of genus *Solanum* are known for possessing a diverse range of alkaloids, which can be moderately to strongly cytotoxic to humans and animals. However, there are no reports neither in the content nor in the bioactivity of the Solanaceaes studied in the present work *Iochroma australe, Solanum amygdalifolium* and *Solanum sisymbriifolium.*

Some data indicates that the leaves and root bark of *Jacaranda mimosifolia* contain iridoids, triterpenes, quinones, flavones, fatty acids, acetosides, as well as traces of alkaloids (11,27,50,54,61,62). However, there are no data either on the components of the flowers, or reports on anticancer activities of *J. mimosifolia*.

The biological activities and cytotoxicity of pure compounds from other *Jacaranda* species are mainly related to the treatment of protozoa and parasites-caused diseases as well as of skin illnesses and infections (25). In addition, Jacaranone, the most important constituent isolated from species of the genus *Jacaranda* is known to possess anti-cancer activity (25,30).

There are no reports on the chemical content of *Collaea* genus or on the antitumour activity of their species. However, at the familiy level, the Fabaceaes usually have a varying degree of alkaloid content. Proanthocyanidins and flavonoids are usually present in the species belonging to this family (48,55,59), some of them possessing antitumour activity (37,40,49).

Ipomoea species have been shown to have antitumour activity both *in vitro* and *in vivo* (29,39,43). Many types of alkaloids, including ergot alkaloids have been isolated from some of these species (1,24,68,74), and some of them have been responsible for their antitumoural activity, mainly related to immunomodulation and regulation of angiogenesis (28). However, there are no specific reports neither on the phytochemistry nor on the biological activity of *I. bonariensis*.

In general, except for extract resistant cell lines, the IC_{50} range of the eight extracts was 10-150 µg/ml. This range is within the cytotoxic range reported for crude extracts of plants of several families having antitumour effects (19,21,64,65,76) and even for purified compounds derived from plants (41,66).

In the present screening, in general, murine cells appear to be more sensitive to the cytotoxic action of the plant extracts. The human melanoma B16 was the most resistant to the extracts of *C. argentina, I. australe, S. chacoense* and *S. sisymbriifolium*. This is not surprising, since melanomas are usually most resistant to conventional antineoplastic treatments. The human lung carcinoma cell line A549 was the most resistant to *J. mimosifolia*. The only extract which induced mild toxicity in both normal murine and human cell lines was *S. verbascifolium,* and it was highly toxic against all the tumour lines, inducing CI_{50}s in the 50-70 µg/ml range in the human tumour lines.

On the hypotheses that the eight active extracts could have a variable but significant content of alkaloids, we employed the Vinca alkaloid vinblastine as a control of inhibition of microtubules depolarisation (78). Some species of the genus *Solanum*, such as *S. berthaultii* have been found to bind and interfere in the bundling of both microtubules and F-actin (8,44). Our eight active extracts induced in LM2 cells destabilization, condensation and aggregation of microtubules, although no depolarization of microtubules, typical of Vinca alkaloids damage.

Overall, this is one of the first studies focusing on southern South American native plants and their biological effects. Specifically, eight methanol extracts from Argentinean plants were considered as highly active against four tumour and three normal cell lines. Although some species of 5 genera analyzed have been reported to possess different degrees of alkaloid content, to date, there are almost no reports on the composition of the species employed in this study. Destabilization, condensation and aggregation of microtubules were observed, although no depolarization of microtubules as it is induced by Vinca alkaloids.

In terms of selectivity, *S. verbascifolium* was the species which showed most selectivity for tumour cells. Our future goal is to isolate the bioactive compounds mainly of *S. verbascifolium* for its direct use as drug. Antitumour activity of purified fractions of the extracts administered at non-toxic doses will be assayed in transplantable murine tumour models, aiming to produce entities of high antitumour activity and low toxicity. Purified fractions will be obtained employin different solvents so that optimal extraction of the active compound can be obtained.

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