CYTOTOXIC AND ANTITUMOR ACTIVITIES OF VENEZUELAN PLANT EXTRACTS *IN VITRO* AND *IN VIVO*

Josefa Villasmil[1], María Jesus Abad[1], Miriam Arsenak[1], Angel Fernández[2], Marie-Christine Ruiz[2], Beatriz Williams[2], Fabian Michelangeli[2], Fidias Herrera[3], Peter Taylor[1*]

[1] Centro de Medicina Experimental, [2] Centro de Biofísica y Bioquímica, Instituto Venezolano de Investigaciones Científicas, Apartado 21827, Caracas 1020-A, Venezuela, [3] Escuela de Bioanálisis, Universidad Central de Venezuela. ptaylor@ivic.ve

Summary

Venezuela is a country where indigenous and local communities use over 1,500 species of plants for medicinal purposes. Many drugs used in the treatment of cancer are of plant origin. From our databases of botanical collections and ethnobiological usage, we selected 11 species to screen for antitumor activity both *in vitro* and *in vivo*. Ethanolic extracts were screened for activity against a) five tumor cell lines, b) primary tumor growth and metastasis in the B16/BL6 melanoma / C57BL/6 mouse model, and c) NF-κB inhibitory activity in HeLa cells transfected with an NF-κB / luciferase reporter gene plasmid. Three extracts were cytotoxic at <100 μg/ml on more than one cell line. We identified 3 plants with inhibitory activity on the growth of subcutaneous primary tumors when injected i.p. every other day during the course of tumor growth, and 4 which significantly reduced lung metastases after i.v. inoculation of tumor cells. The nuclear factor NF-κB is a promising potential target for antitumor and anti-inflammation therapy. Four plants inhibited NF-κB activity in the HeLa cell reporter gene assay when the cells were activated with TNF-α, without showing an appreciable cytotoxic activity in short-term MTS assays with the same cells. Further studies are being undertaken to investigate the relationship between the antitumor and NF-κB-inhibitory activities shown by these extracts.

Keywords: cancer, cytotoxicity, inflammation, medicinal plants, mouse.

Introduction

Several useful anticancer drugs, such as vinblastine, irinotecan, topotecan, etoposide and paclitaxel have been derived from plants (1, 2), and there is good reason to believe that many more are yet to be discovered. Anticancer drugs derived from plants may act through a variety of mechanisms including a direct effect on tumor cell growth. The terpenoid, paclitaxel, obtained from *Taxus brevifolia*, is known to induce tumor cell death via apoptosis (3, 4) and is one of several plant-derived anticancer drugs, which act directly on tumor cells (5). However, there is also at present interest in controlling tumor growth in other ways. Antiangiogenic drugs may limit growth of tumors by restricting their blood supply; immunomodulatory agents serve to stimulate an existing antitumor immune response, and antiproteolytic drugs inhibit proteases, which are key enzymes in tumor progression and metastasis, allowing cancer cell migration, angiogenesis, and vascular intravasation and extravasation (6). We have previously screened a number of Venezuelan plants for their possible cytotoxic effects on tumor cell lines, and inhibitory effects on protease activity (7).

The role of chronic inflammation in tumor initiation and growth is well established (8, 9). Studies have shown that anti-inflammatory drugs may be effective in cancer therapy and/or prevention (10), and the possible mechanisms of action of anti-inflammatory phytochemicals have been reviewed (11). The transcription factor NF-κB represents an important link between chronic inflammation and cancer (12) and has been suggested as a possible target for the therapy of both (13, 14).

Several of these possible mechanisms of action of plant extracts on tumors are presently under study in our laboratories. The species of plants used in this study were selected on the basis of a variety of ethnopharmacological and chemical properties reported in Venezuela and other neotropical countries. We present results related to the properties of 11 crude extracts from selected plants as potential anticancer agents in four different assays, namely, cytotoxicity against five tumor cell lines, primary tumor growth and metastasis in the B16/BL6 melanoma / C57BL/6 mouse model, and NF-κB inhibitory activity.

Methods

Study area and plant collection

All the plants were collected in the Yutaje area of the Amazonas state. At the time of collection for extraction, voucher samples were taken for firm botanical determination, and are now deposited in Venezuelan and USA herbaria. All collections are covered by legal permits obtained from the competent authority under a “Contrato de Acceso a los Recursos Genéticos” signed between IVIC and MARN (Ministerio de Ambiente y los Recursos Naturales).

Preparation of plant extracts
Plants were collected and extracted fresh in a field laboratory. Depending on the type of plant, different parts were collected (leaves, bark, flower, root, etc).

Species	Family	Part	Species	Family	Part
Byrsonima crassifolia	MALPIGHIACEAE	leaf	*Curatella americana*	DILLENIACEAE	inner bark
Hamelia patens	RUBIACEAE	leaf	*Jacaranda copaia*	BIGNONIACEAE	young leaf
Piper marginatum	PIPERACEAE	leaf	*Psychotria poeppigiana*	RUBIACEAE	flower
Piper marginatum	PIPERACEAE	root	*Tapirira guianensis*	ANACARDIACEAE	inner bark
Uncaria guianensis	RUBIACEAE	bark	*Xylopia aromatica*	ANNONACEAE	young leaf
Vismia cayennensis	CLUSIACEAE	bark			

After grinding in a blender, plant material was macerated in three volumes of 95% ethanol for at least three days. The material was filtered and the alcohol extract was concentrated and dried by rotavaporation and lyophilization. Dried powder was stored at –80^{o}C until use. Stock extract solutions (100 mg/ml) were prepared in 50% ethanol.

Cell lines and animals
The following cell lines, B16/BL6 (murine melanoma), HT-29 (human colon carcinoma), A549 (human lung carcinoma), HeLa (human cervical carcinoma), PANC-1 (human pancreatic carcinoma) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, BRL, USA).
Female C57BL/6 mice (7–9 weeks old), weighing approximately 25 g, were obtained from the Animal Facility of IVIC. All animal experiments were performed according to internationally accepted guidelines for the treatment of animals in research.

Cytotoxicity assay with Crystal Violet
Growth and cytotoxicity of the tumor cells lines in the presence of the plant extracts was assessed using the crystal violet assay. Cells were plated at subconfluence in flat-bottomed 96 well plates and allowed to attach for 24 h. A group of well was then fixed with pure methanol to establish the baseline number of cells at T=0. The cells were then incubated in the presence of different concentrations of the plant extracts for 24 h. The cells were then fixed in 20% methanol and stained with 0.5% crystal violet. After solubilization with 33% acetic acid, the optical density at 562 nm was determined. The results were expressed as a percentage of value at T=0. The values for 50% Growth Inhibition (GI_{50}), Total Growth Inhibition (TGI) and a 50% Cytotoxic Effect (LC_{50}) were calculated by extrapolation.

Mouse models
At day 0, mice were inoculated in the lateral tail vein (i.v.) with 10^5 B16/BL6 cells in 100 µl PBS. The animals were then injected 5 times/ week for 3 weeks, intraperitoneally (i.p.) with 500 µg of the extracts in 100 µl PBS starting on day 0. Control animals received 100 µl PBS. On day 21, the animals were sacrificed with ether; the lungs were removed, placed for 5 min in 3% H_2O_2 in H_2O and fixed in Bouin's solution. The purpose of the H_2O_2 was twofold; to bleach hemorrhages which could be mistaken for metastases, and to inflate the lungs, facilitating the evaluation of metastases under the dissecting microscope.
Primary tumors were induced by the subcutaneous (s.c.) injection of 5 x 10^4 B16/BL6 cells in 100 µl PBS in the hind limb. The mice were treated with the extracts as described above. Tumor size was measured in two dimensions with a vernier gauge.

NF-KB luciferase assay
HeLa cells were transiently transfected with the NF-κB luciferase reporter system (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. Transfected cells were seeded into 96-well plates, allowed to adhere overnight then treated for 1 h with different concentrations of the extracts. The cells were then stimulated for a further 4 h with 25 ng/ml huTNF-α (BD Biosciences, Palo Alto, CA, USA). Luciferase activity was measured using the Steady-Glo assay kit (Promega, Madison, WI, USA), in a 96-well luminometer.

Statistical analysis
The unpaired Student's t test with the Welch correction was used to assess the statistical significance of the differences. A confidence level of $P < 0.05$ was considered significant.

Results

Table 1 shows the effects of the extracts on 5 tumor cell lines. Although a degree of growth inhibition was observed for almost all the extracts, except for those from *P marginatum* and *P Poeppigiana*, only *B crassifolia, T guianensis* and *V cayennensis* were cytotoxic for more than two of the cell lines at lower concentrations. Although PANC-1 were the most sensitive cells, and the B16/BL6 cells the most resistant, this pattern was not uniform. Studies with a greater range of cell types are required.

Figure 1 shows the effect of the extracts on primary tumor growth in two separate experiments, carried out to confirm results obtained in preliminary screening experiments with a reduced number of animals. Treatment with the *J copaia* extract delayed tumor growth by up to 40% (day 18, NS). *X aromatica* only inhibited tumor growth to a small degree but did delay the appearance of the tumor by an average of 2 days, although this difference was not significant.

In the second experiment, *X aromatica* was included for comparison, and showed a more pronounced inhibitory effect in this 24 day experiment. Interestingly the leaf but not root extract of P *marginatum* showed a pronounced inhibitory effect on tumor growth. *V cayennensis* and 4 other plants, *H patens*, *T guianensis*, *B crassifolia*, *P poeppigiana* (not shown here for clarity), were not inhibitory. *C americana* showed a consistently stimulatory effect on primary tumor growth.

Table 1. Effect of the plant extracts on tumor cell lines *in vitro*

		B16/BL6			PANC-1			A549			HT-29			HeLa		
	µg/ml	GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}
B crassifolia	Leaf	124	229	>300	16	21	28	29	90	>300	167	>300	>300	24	56	79
C americana	Bark	>300	>300	>300	17	160	>300	>300	>300	>300	>300	>300	>300	>300	>300	>300
H patens	Leaf	149	215	259	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
J copaia	Leaf	>300	>300	>300	36	98	223	287	>300	>300	74	149	228	>300	>300	>300
P marginatum	Leaf	>300	>300	>300	>300	>300	>300	>300	>300	>300	55	92	>300	>300	>300	>300
P marginatum	Root	>300	>300	>300	65	>300	>300	240	>300	>300	298	>300	>300	>300	>300	>300
P poeppigiana	Flower	>300	>300	>300	97	>300	>300	>300	>300	>300	>300	>300	>300	>300	>300	>300
T guianensis	Bark	146	216	>300	13	18	26	28	89	200	138	290	>300	24	48	75
U guianensis	Bark	>300	>300	>300	57	92	190	80	>300	>300	194	>300	>300	48	94	199
V cayennensis	Root	98	149	293	7	11	23	22	66	>300	87	221	291	14	19	25
X aromatica	Leaf	47	92	216	83	179	261	>300	>300	>300	>300	>300	>300	>300	>300	>300

GI_{50} – Growth Inhibition 50%, TGI – Total Growth Inhibition, LC_{50} – Cytotoxic Effect 50%. Cells were cultured for 24 h in the presence of the extracts and the cell density measured by the crystal violet assay. Results are expressed in µg/ml.

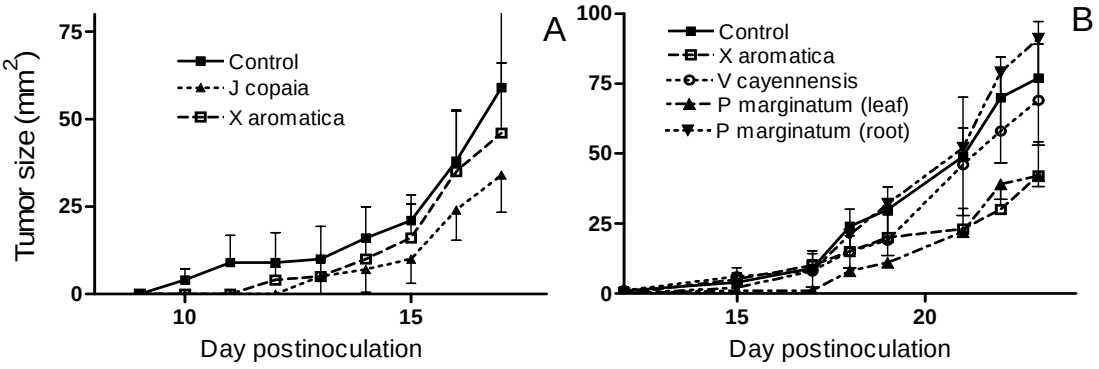

Figure 1. Effect of treatment with plant extracts on primary tumor growth in mice. C57Bl/6 mice were inoculated s.c. with B16/BL6 tumor cells to initiate a primary tumor The animals were then injected i.p. 5 times/ week up to the day of sacrifice, with 500 µg of the extracts in 100 µl PBS starting on day 0. Control animals received 100 µl PBS. Tumor size was measured in two dimensions with a vernier gauge. (mean ± S.E.M., N=6).

Similar results were obtained with the two extracts of *P marginatum* in terms of their effect on the formation of metastases in lung (Fig.2). Treatment with the leaf, but not the root extract showed a significant 65% reduction in the number of lung metastases. *P poeppigiana*, which did not exert any effect on primary tumor growth, inhibited lung metastases by over 50%. *X aromatica* and *J copaia* were also inhibitory to about the same degree.

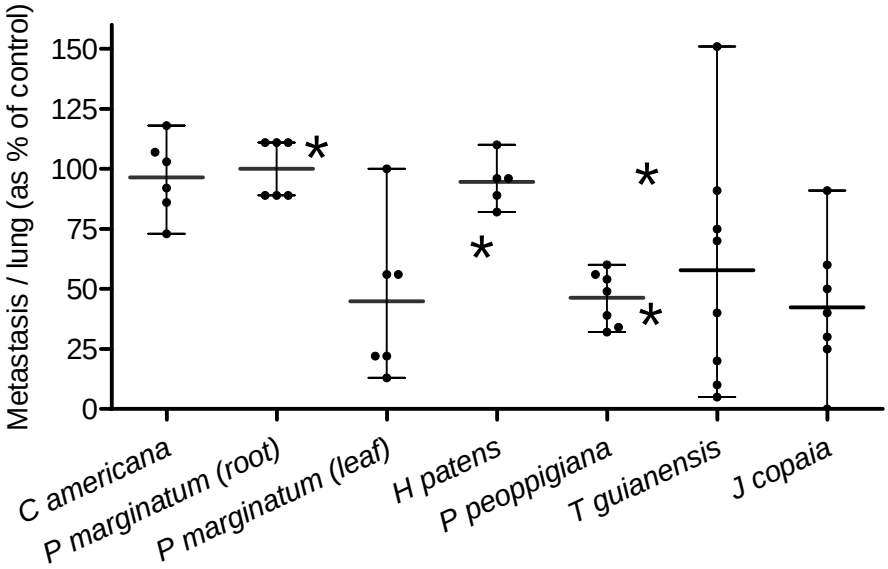

Figure 2. Effect of treatment with plant extracts on metastasis to lung in mice. C57Bl/6 mice were inoculated i.v. with B16/BL6 tumor cells. The animals were then injected i.p. 5 times/ week up to the day of sacrifice (day 21), with 500 µg of the extracts in 100 µl PBS starting on day 0. Control animals received 100 µl PBS. (median ± range, N=6-9) * $P < 0.005$.

As there is powerful evidence in the literature that many anti-inflammatory drugs exert their effect through inhibition of NF-κB, and that his molecule may represent a promising potential target for both antitumor and anti-inflammation therapy, we tested the effect of a selection of plants in an NF-κB reporter system (Fig. 3). Several of the plant extracts showed an inhibitory effect, most notably *J copaia*, *U guianensis* and the two *P marginatum* extracts. In order to ensure that the effects observed were not due to cytotoxic activity, short-term 4 h MTS assays were carried out to ensure cell viability at the extract concentrations used. Significant cytotoxicity (> 15%) was only observed in these assays at extract concentrations above 100 µg/ml. Dexamethasone, an inhibitor, and paclitaxel, which increases NF-κB activity, were included as controls.

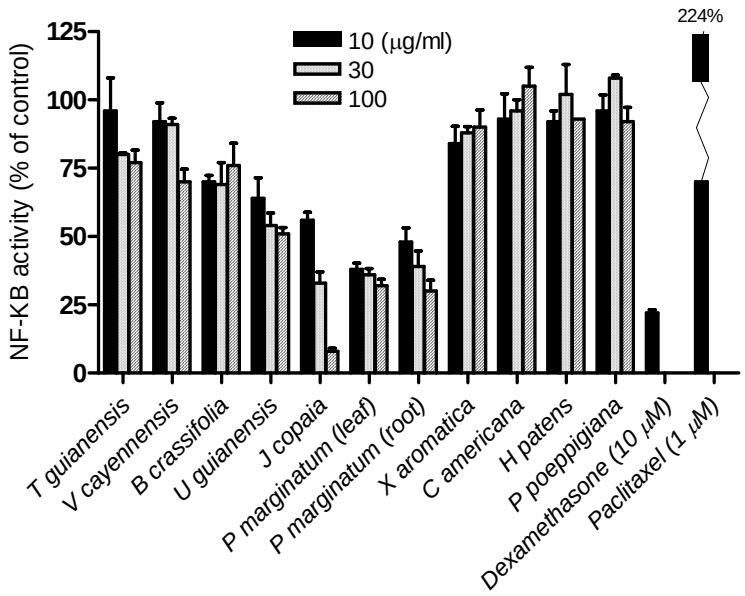

Figure 3. Effect of plant extracts on NF-κB activity. HeLa cells, transfected with the NF-κB luciferase reporter system, were treated with the plant extracts for 1 h, then stimulated with huTNF-α for a further 4 h. NF-κB activity was then assessed using luciferin as substrate (mean ± S.E.M., N=6).

Discussion

Most of the species tested here have not been reported in ethnobotanical surveys to be used for the treatment of tumors, although many are reported to have an anti-inflammatory action. However, other species of the same genera may have been reported to show antitumor activity (15, 16). Some of these species, or other species of the same genera, are cytotoxic for cancer cells *in vitro*, or contain chemical compounds with known antitumor or anti-inflammatory activity. We have discussed previously the biological activities shown by these plants in a previous paper (7).

The results obtained from the *in vivo* mouse tumor models indicate that *J copaia*, *X aromatica* and the leaf extract of *P marginatum* show promise as antitumor agents, requiring further study and identification of the active compounds. *X aromatica* is known to contain acetigenins with activity against three solid tumour cell lines (17). However the antimetastatic activity of *T guianensis* which was not paralleled in the primary tumor model illustrate the problems involved in choosing an appropriate model when screening for activity. *X aromatica* is cytotoxic for B16/BL6 cells *in vitro* and inhibits primary tumor growth, whereas the *P marginatum* leaf is inhibitory in the two *in vivo* models but is not cytotoxic. *P poeppigiana* is not cytotoxic, does not

inhibit primary tumor growth, but does inhibit metastasis, suggesting other possible targets are available to inhibit the process of metastasis.

As far as we are aware, this is the first report of anti-inflammatory activity in a *J copaia* extract, although jacarandic acid, present in other members of the genus inhibit prostaglandin synthesis (18). The lack of cytotoxicity in the short-term assays indicate that this activity is not due to a general detrimental effect of the extracts on the HeLa cells. *U guianensis* has been reported to inhibit NF-κB activity (19). Both extracts from *P marginatum* showed anti-NF-κB activity, but only the leaf was active in the *in vivo* tumor models, again suggesting that multiple mechanisms may be available to target in antitumor therapy, but also that such crude plant extracts may contain several compounds acting synergistically.

Further studies are being undertaken to investigate the relationship between the antitumor and NF-κB inhibitory activities shown by these extracts.

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