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Cytotoxicity screening of endemic plants from Guayana highlands

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Abstract. A chemical-ecology approach has been used to screen plants growing in Guyana Highlands as an indicator of production of biologically active secondary metabolites. Extracts of leaves from 19 species, most of them endemic in this area, and collected at the top of Roraima Tepui (2,723 m) were screened *in vitro* at different concentrations for their potential cytotoxic activity against three tumour cell lines: HT29 (colon), A549 (lung) and MDA-MB-231 (breast). MTT (tetrazolium blue) colorimetric assay was employed as cytotoxicity test. Extracts of nine species caused less than 30% growth in at least one cell line. From these species, high cytotoxic activity was detected in *Casearia sylvestris* var. *lingua* and *Ledotamnus sessiliflorus* extracts; medium activity was found in *Cyathea* sp. Two other species, *Cyrilla racemiflora* and *Heliamphora minor* showed lower but significant cytotoxicity. Further cytotoxicity-directed fractionation of these extracts would be advisable to isolate and identify the active principles of these plants.

INTRODUCTION

Despite recent additions to the armoury of chemotherapeutic agents for cancer treatment, the results of chemotherapy still remain unsatisfactory, so the development of new strategies for research is needed. One of them, chemical-ecology approach has proven to be an efficient and rapid tool for natural drug discovery. This strategy has been used to screen plants according to their plant growth habitat as an indicator of the potential production of biologically active secondary metabolites. For instance, intense UV-B radiation in tropical mountains may induce the production of these metabolites, affecting their composition profiles (Bassman, 2004).

Tepuis are isolated table mountains in Guayana Highlands that host a highly endemic flora of new bioactive compounds. Indigenous and local communities inhabiting these highlands use more than 1 500 species of plants for medicinal purposes, but rarely employ plants growing at the wild top of the tepuis. Few of them have been tested for cytotoxicity (Villasmil *et al.*, 2006).

Plants inhabiting the summit of these plateaus are separated by the surrounding lowlands by vertical cliffs reaching altitudes from 1 500 to 2 700 m, and must survive in an environment with limited nutrients supply and cope with extremes of radiation and temperature. This floristic province, called Pantepui, hosts some of the highest rates of plant endemism in northern South America (Berry *et al.*, 1995). Of the 2 322 species of vascular plants belonging to 630 genera, 766 are endemics to this province. Thus, novel adaptations of its plants make them very interesting for screening studies of new biological activities of medical interest.

Among surveyed species, it is remarkable *Casearia sylvestris*, which is known as a medicinal plant in South America, called as "guacatonga", that has traditional uses, such as antiseptic, anaesthetic, anti-ulcer and snakebite venoms antidote. For *C. sylvestris*, new cytotoxic clerodane diterpenes, named casearins, have

already been isolated and described (Itokawa *et al.*, 1990). Another potential antitumor clerodane diterpenes, called casearvestrins, have later been identified (Oberlies *et al.*, 2002). Research on casearins is ongoing and might be able to develop these chemicals into new effective chemotherapeutic agents in the future. Nevertheless, although the pharmacological properties of this plant have been previously studied, is not yet known properties differentials that could exist among different varieties of this plant.

MATERIALS AND METHODS

Screening criteria

Leaf samples were high on priority collected from endemic species growing at Mount Roraima (Guyana, 5º14'N; 60º45'W) in June 2006. Due to the high endemism and high protection of most of these plants, great care was taken and only the minimum amounts required were harvested.

Plants endemic to Pantepui province were *Drosera roraimae* Mag. & Laudon (Droseraceae), *Epidendrum secundum* Oliver (Orchidaceae), *Heliamphora nutans* Gleason (Sarraceniaceae), *Shefflera rugosum* Elmer (Araliaceae), *Stegolepis guianensis* Klotzsch ex Korn (Rapataceae).

Plants endemic to Guayana region: *Brocchinia tatei* Schult and *Brocchinia reducta* Baker (Bromeliaceae), *Orectante sceptrum* Mag. (Xirydaceae). *Ledothamnus sessiliflorus* N.E.Br. (Ericaceae).

Other plants collected: *Befaria imthurnii* N.E. Brown (Ericaceae), *Bonnetia roraimae* Oliver (Theaceae), *Cyrilla racemiflora* L. (Cyrilliaceae), *Tillandsia turneri* Baker (Bromeliaceae), *Cyathea caracasana* Domin. (Cyatheaceae), *Casearia sylvestris* var. *lingua* Sw. (Flacourtiaceae). Roots of *Curatella americana* L. (Dilleniaceae) and leaves of *C. sylvestris* Sw. var. *lingua* (Camb.) Eichl. were selected due to known medicinal uses indicated by our native guides.

Cytotoxicity assay *in vitro*

Crude extracts were obtained percolated in iso-PrOH from 1g air-dried leafs and stored at 4ºC. Before use, extracts were evaporated and diluted in culture medium according to required final testing concentrations. A three tumour cell lines panel was used to determine dosage-dependent activity of every sample: HT29 (colon), A-549 (lung) and MDA-MB-231 (breast) (ATCC, Manassas, VA, USA). Cells were grown in RPMI 1640 medium (Sigma) supplemented with 5% FBS and 2 mM L-glutamine. For cytotoxicity assay, cells were inoculated in a volume of 100 µL per well into 96 well microtiter plates at cell densities according to doubling times (Monks *et al.*, 1991), and incubated at 37ºC, 5% CO_2, 95% air and humidified atmosphere for 24 h prior to addition of 100 µL of plant extracts diluted in culture medium. Four final extract concentrations (5, 10, 25 and 50 µg/mL) were tested 6 times for every sample. After an additional 48 h incubation, tetrazolium blue colorimetric assay (MTT, Sigma), measuring absorbance at 540 nm, was employed as test for quantification of cell proliferation and viability (Twentyman & Luscombe, 1987). Sample effectiveness was assessed by two calculated response parameters: GI_{50} to determine the concentration for 50% growth inhibition, and LC_{50} for 50% tumour killing activity. These are interpolated values from the dose-response curves. In case these parameters could not be determined by interpolation of the data, "default values" were assigned, corresponding to the highest (50 µg/ml) or lowest (5 µg/ml) tested concentration, as previously reported (Boyd *et al.*, 1992). Only samples showing less than 32% net growth in at least one cell line, in the range of concentrations tested, or more than 50% cell killing, were considered for GI_{50}s or LC_{50}s determination, respectively. Taxol was used as a positive control, while DMSO was used as the negative (vehicle) control.

Statistical analysis

For all experiments, linear regression analysis was performed and multiple range tests and Duncan's test was used for individual comparisons between concentrations of each extracts. A P value < 0.05 was considered statistically significant.

RESULTS

Growth inhibition activity

Extracts of nine species caused less than 30% growth in at least one cell line. Estimated concentrations resulting in 50% growth inhibition (GI_{50}) for these species against the three cell lines are shown in Fig. 1. The highest inhibition activities (GI_{50}s<25 µg/ml) were shown by the four species with the highest associated cytotoxic activity (Fig. 2). Antiproliferative activity of these samples was mostly exhibited against lung tumour cells, with GI_{50}s of 5 µg/ml for *C. sylvestris* var. *lingua*, 6.4 µg/ml for *L. sessiliflorus*, and 6.3 µg/ml for the tree fern *C. caracasana*, the most active extracts. These four species, were also the most active against breast cells, ranging from <5 µg/ml in *C. sylvestris* var. *lingua* to 14.5 µg/ml in *H. nutans*; also against colon cells, with GI_{50}s ranging from <5 µg/ml for *C. sylvestris* var. *lingua* to 23.5 µg/ml for *L. sessiliflorus*. *C. sylvestris* var. *lingua* was by far the most active antitumour extract, with GI_{50}s below the lowest concentrations tested against the three cell lines (<5 µg/ml).

Cytotoxic activity

Significant cytotoxic activity (LC_{50}<50 µg/ml in at least one cell line) was detected in four species (Fig. 2). *L. sessiliflorus* extract showed medium cytotoxic activity against lung and breast lines, with LC_{50}s at 27.9 µg/ml and 36.3 µg/ml, respectively. Medium activity was also found in *Cyathea* sp. against colon cells (LC_{50} at 42.7 µg/ml) and *H. minor* against breast and lung cells (LC50s at 40.6 and 43.7 µg/ml respectively). Other species, *C. racemiflora*, *C. americana* and *B. roraimae*, showed lower but significant cytotoxicity at 50 µg/ml dose, with LC_{50}s >50 µg/ml (data not shown).

The most cytotoxic plant against the three cell lines was *C. sylvestris* var. *lingua* (Table 1), with the highest activity (81% cell killing) against colon cells, at the lowest concentration tested (5 µg/ml), and a LC50 value below this concentration. This species was also the most cytotoxic against lung cells (24% cell killing at 5 µg/ml, LC_{50} of 8.3 µg/ml) and breast cells (53% cell killing at 5 µg/ml, LC50 <5 µg/ml). At higher concentrations (15 and 50 µg/ml), no significant differences in cytotoxic activity against the three lines were exhibited by this plant.

DISCUSSION

The nine samples which caused reduced net growth of at least one of the cell lines tested to less than 30% can be considered worthy of further study (Fig. 1). Although three lines pre-screen has been shown to efficiently remove many of the inactive samples from unnecessary and costly full scale evaluation (Herrera & Taylor, 2006), a broader concentration range, and more complete cell panel assay, such as NCI's 60-cell line assay (Monks *et al.*, 1991) would be a next advisable step in order to confirm and characterise the antiproliferative activities detected in these samples. According to our pre-screening data, this condition was fulfilled in more than half (9/16) of the species tested (Fig. 1). Additionally, one quarter of total species showed significant tumor killing activity (Fig. 2).

Table 1. Cytotoxic activity (% net growth) of *Casearia sylvestris* var. *lingua* extracts against three tumour cell lines

Cell lines	Extract concentration (µg/ml)			
	5	10	25	50
HT29 (colon)	-53 ± 4.9	-74 ± 7.7	-89 ±	-94 ± 8.7
A549 (lung)	-24 ± 2.2	-48 ± 3.9	-90 ± 8.9	-93 ± 7.7
MDA-MB-231 (breast)	-77 ± 6.9	-80 ± 7.9	-85 ± 8.3	-87 ± 7.5

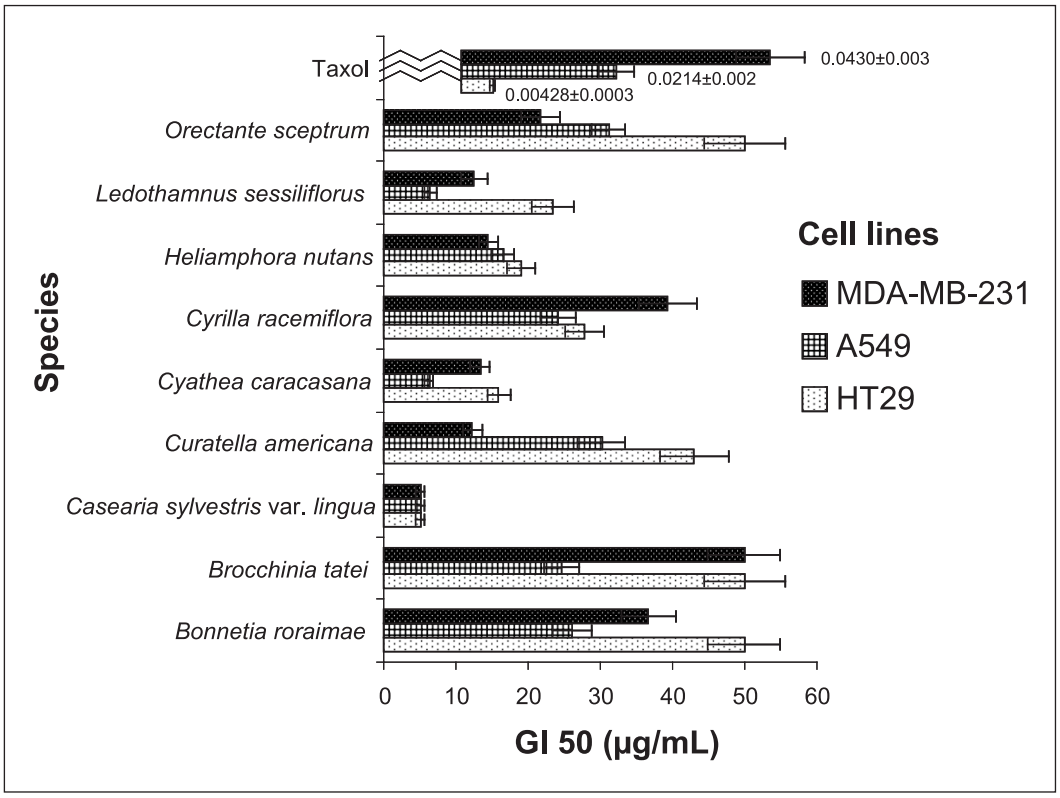

Figure 1. Antiproliferative activity of the most active plant extracts against three tumour cell lines. Calculated GI50 concentrations.

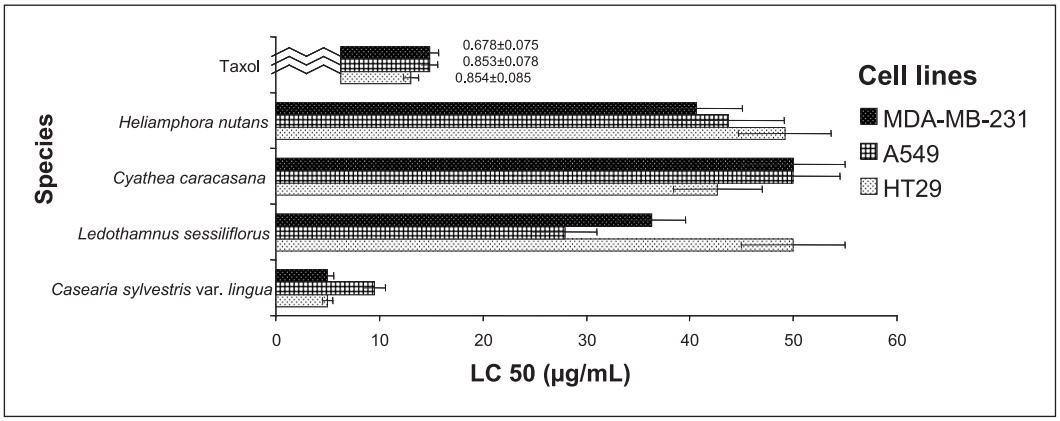

Figure 2. Cytotoxic activity of the most active extracts against three tumour cell lines. Calculated LC50 concentrations.

Although for *C. sylvestris* several antitumor compounds have been previously reported, the variety *lingua* collected here had never been essayed for cytotoxicity. Some other known antitumor agents, such as sesquiterpenes ±-humulene, ^{2}-elemene, ^{2}-caryophyllene were detected by us in preliminary GC MS carried on this sample (data not shown). The relative influence of these chemicals, as well as casearins, in the activity detected is undetermined until now.

Some known cytotoxic agents, such as triterpenoids and xanthones, have already been isolated from other species of the genera *Cyathea* (Arai *et al.*, 1995) and *Bonnetia* (De Oliveira *et al.*, 1990) respectively. However, although these or related compounds might be responsible for part, if not all of the activity detected in the samples collected in Roraima, at the moment we have no concluding evidences supporting this assumption. No cytotoxic compounds have been reported from the three other species with cytotoxic activity cited in this work: *L. sessiliflorus*, *H. nutans* and *C. racemiflora.* Flavonol-glycoside avicularin and gallic acid have been isolated from ethanolic extract of the leaves of *C. americana*, a medicinal plant from the low lands of Guayan Shield (Elazizi *et al.*, 1980), but the roots of this plant, locally used against malaria according to our native guides, had never been tested for antitumor activity.

Little is known about the active compounds or possible synergistic effects that might be responsible for the measured activity in these plants. Some novel active chemicals yet to be discovered might be responsible for part of cytotoxicity shown by some extracts. On the other hand, characterisations of secondary metabolite profiles in response to UV-B radiation are rare and might be the key to some cytotoxic activities detected (Caldwell *et al.*, 2007). Unfortunately, we have not had the resources, nor the amount of samples needed to complete the isolation and identification of these agents by cytotoxicity-guided fractionation.

The most promising species for further research are *C. sylvestris* var. *lingua*, *Cyathea* spp., *L. sessiliflorus* and *H. nutans.* Cytotoxicity-directed fractionation of these extracts would be then advisable in order to isolate and identify the main active principles involved. Once the *in vitro* cell growth patterns for these compounds are determined in 60-cell panel, antitumor mechanisms of action might be characterized by means of the COMPARE pattern-recognition algorithm (Paull *et al.*, 1989), or possible unknown mechanisms detected.

In particular, *C. sylvestris* var. *lingua*, the most potent cytotoxic extract, deserves to be retested in a broader cell lines panel, and lower concentration ranges to determine more accurate LC50s and GI50s values than the default values shown in this study.

According to our pre-screening data, endemic plants inhabiting Guayana Tepuis can be considered as promising candidates for cytotoxicity screening programs. Further research and better conservation strategies of this truly unique and pristine ecosystem are worthy to be promoted. For instance, activity of other parts of the plants, and variations due to stages of development and environmental factors remain undetermined. Nevertheless, in order to continue phytochemical and pharmacological studies on these plants, collections of higher, even massive, amounts of plant material would need to be collected for the next steps, and this can be a handicap as some of these are endemics and highly protected species, growing in specific radiation and other tropical mountains conditions, so that the potential for cultivation of the plants of interest would need to be assessed.

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