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Studies on the cytotoxicity, antimicrobial and DNA-binding activities of plants used by the Ese'ejas

C. Desmarchelier*[a], E. Mongelli[a], J. Coussio[b], G. Ciccia[a]

[a]*Cátedra de Biotecnología y Microbiología Industrial, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, 1113 Buenos Aires, Argentina*

[b]*Cátedra de Farmacognosia, IQUIMEFA (CONICET), Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, 1113 Buenos Aires, Argentina*

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Abstract

Thirty-nine extracts of 13 plants used traditionally as medicinal by the Ese'ejas were studied in order to determine their cytotoxic effect in the brine shrimp. Infusions showed no toxicity. Those plants that tested positive for methanolic and dichloromethane extracts were assayed for DNA-binding activity. Cytotoxicity was not due to the presence of compounds that interact with DNA. Antimicrobial activity of plants used to treat infectious diseases was also performed for the decoctions. These proved to be active against some of the test microrganisms used in the assay.

Keywords: Ese'ejas; Brine shrimp; Acute toxicity; Cytotoxicity; DNA-binding activity; Antimicrobial activity

1. Introduction

The Ese'eja is a group of Amerindians who occupy the Southwest Amazon rainforest in the area delimited by 10° and 12° south and 66° and 70° west, along the Madre de Dios, Tambopata and Heath rivers in Peru, and the Beni and Madidi rivers in Bolivia. Healing practices of the community shaman involve the use of a hallucinogenic beverage known by the name of *ayahuasca*, a drink made by cooking the bark of a jungle vine, *Banisteriopsis caapi* (Spruce) Morton (Malpighiaceae) (Hissink, 1960; Pages Larraya, 1979). The purpose of taking this beverage is in the diagnosis and healing of illness and the performing of other shamanic tasks such as communicating with the spirits of medicinal plants. Plants are, therefore, especially appreciated not only in Ese'ejas diet, but also for ritual purposes and to prevent diseases.

This paper reports the survey for toxicity of different medicinal plants of the Ese'eja's pharmacopeia using the brine shrimp microwell cytotoxicity bioassay (Solís et al., 1993), the DNA binding activity using the DNA-methyl green assay (DNA-MG) (Burres et al., 1992), and antimicrobial activity using the 'stroke method' (Brantner and Grein, 1994)

* Corresponding author.

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2. Materials and methods

2.1. Plant material

Plants were collected by one of the authors (C.D.) during an ethnobotanical expedition into the Tambopata river area in Madre de Dios, Peru, during the month of January 1994, to collect ethnobotanical information from the Ese'ejas. Field work was carried out in the settlements of Infierno and Filadelphia, and in La Ccollpa. Identification of species was made by Dr. Alberto A. Gurni of the Cátedra de Farmacobotánica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentiña. Voucher specimens are deposited in this institution.

2.2. Preparation of extracts

Infusions were prepared in the locality of La Ccollpa by pouring 100 ml of boiling water over 5 g of powdered dry plant material placed in a stoppered flask. The water used in this preparation was collected from the Tambopata river. The mixture was left standing for 20 min and then filtered.

Decoctions were prepared in our laboratory by boiling 12.5 g of dry plant material in 100 ml of distilled water during 20 min. The mixture was filtered and the resulting solution was sterilized through a 0.22 μm filter ('MSI', Westborough, USA).

Dichloromethane and methanolic extracts used for the brine shrimp microwell cytotoxicity bioassay were prepared in our laboratory. Dry powdered plant material (5 g) was extracted during 24 h at room temperature in a stoppered flask with 50 ml of dichloromethane. The extract was filtered and concentrated under reduced pressure at 40°C. The marc was extracted with methanol in the same conditions as described for the dichloromethane extract. In the case of methanolic extracts used in the DNA-MG, dry plant material (5 g) was extracted during 24 h at room temperature in a stoppered flask with 50 ml methanol. The extract was filtered and concentrated in a Savant SpeedVac System (model SS210 A) at 43°C for 6 h.

2.3. Brine shrimp microwell cytotoxicity bioassay

Infusions were tested at the concentration levels of 2.5%, 1.25% and 0.25% (g of dry plant material/100 ml of artificial sea water). Brine shrimp eggs (*Artemia salina*), purchased at a pet shop ('S & S', Buenos Aires, Argentina), were hatched in artificial sea water prepared from sea salt ('Marinemix', Wiegandt, Germany) and water from the Tambopata river (3.8 g of sea salt/100 ml of water) at room temperature. Dichlorometane and methanolic extracts were tested in our laboratory at concentration levels of 1000, 100 and 10 μg/ml, under the same conditions as above, except that in this case, artificial sea water was prepared using distilled water. In both cases, plant extract solutions were tested by dispensing 100 μl in six replicates in wells of 96-well microplates ('Kartell', Milan, Italy). The organic extracts were dissolved previously in dimethyl sulfoxide (DMSO) in the proportion of 500 μl/10 ml of artificial sea water. After 48 h, the phototrophic nauplii were collected. A suspension of 10–15 nauplii (100 μl) was added to each well and the covered microplate was incubated for 24 h at room temperature. After this period, the number of dead nauplii in each well was counted using an E. Lentz Wetzlar binocular microscope (×10). Finally, 100 μl of methanol were added to each well and after 30 min the total number of shrimps in each well was counted. Berberine chloride ('Sigma', St. Louis, USA) was used as a positive control. Finney's (1971) statistical method of probit analysis was used to calculate the concentration of the extract that would kill 50% of the brine shrimps within the 24 h exposure (LC_{50}) and the corresponding 95% confidence interval.

2.4. DNA-methyl green bioassay

Sample solutions containing 1000, 100 and 10 μg of the corresponding methanolic extract per ml of ethanol were used. Twenty μl of samples solutions to be tested were dispensed in triplicate into wells of 96-well microplate ('Kartell', Milano, Italy) and the solvent was removed under vacuum. Twenty mg of DNA methyl green ('Sigma', St. Louis, USA) were suspended in 100 ml of 0.05 M Tris-ClH buffer, pH 7.5, containing 7.5 mM $MgSO_4$, and stirred at 37°C during 24 h. Two hundred μl of DNA-methyl green reagent were added to each well. The initial absorbance of each sample was read at 655 nm using a Biorad Microplate Model

450 Reader ('Biorad', Richmond, USA). Samples were incubated in the dark at room temperature. After 24 h, the final absorbance of samples was measured as described above. The decrease in absorbance was calculated as the percentage of the untreated DNA-MG absorbance value. Doxorubicine hydrochloride ('Sigma', St. Louis, USA) was used as a positive control. The concentration that causes a decrease of 50% of the initial absorbance (IC_{50}) and the corresponding 95% confidence interval were determined as above.

2.5. Antimicrobial activity

The extracts were tested by means of the 'stroke method' (Brantner and Grein, 1994). Four ml of the corresponding decoction was mixed with 16 ml of trypticase soy agar (TSA) ('Difco', Detroit, USA). This mixture was poured into a Petri plastic dish (8 cm diameter) under aseptic conditions and allowed to cool for 30 min at room temperature. For inoculation of the microorganisms, strokes of the cultures were applied to the surface of the agar medium. The microorganisms used were: *Staphylococcus aureus* (ATCC 13709), *Escherichia coli* (ATCC 25922), *Salmonella gallinarum* (ATCC 9184), *Klebsiella pneumoniae* (ATCC 10031), *Candida albicans* (ATCC 10231), *Pseudomonas aeruginosa* (ATCC 27853) and *Mycobacterium gordonae* (strain M3 from the culture collection of the Department of Clinical Biochemistry at the Hospital de Clínicas of Buenos Aires). These test organisms were maintained on TSA and recovered for testing by growth in presterilized trypticase soy broth ('Difco', Detroit, USA) flasks at 37°C for 24 h in a New Brunswick incubator shaker at 230 rev./min, except for *M. gordonae*, which was grown for 48 h. The microorganisms were diluted with sterile saline solution (4.25 g sodium chloride/500 ml distilled water) in order to give suspensions of about 10^6–10^7 cells/ml. Positive and negative controls were streaked first and last in each test series. The two positive control plates contained 10 μg/ml of streptomycin sulfate ('Rontag', Buenos Aires, Argentina) and the two negative controls were plates containing TSA but no plant extract. The plates were incubated at 37°C for 24 h, after which the results were rated by unaided eye as: ++, strong inhibition (no growth); +, partial inhibition (less growth than normal); −, no inhibition (full growth), in comparison with the growth observed in the negative controls.

3. Results and discussion

The brine shrimp cytotoxicity bioassay was performed to determine the toxicity of 39 extracts

Table 1
Ethnobotanical data of studied plants

Species/family/voucher[a]	Common name	Part used[b]	Uses
Abuta grandifolia (Mart.) Sandwith (Menispermaceae) 533	Abuta	S	Tuberculosis and other lung diseases
Aspidosperma excelsum Benth. (Apocynaceae) 534	Remo caspi	B	Toothache
Cyperus articulatus L. (Cyperaceae) 559	Piri piri	R	Dysentery and other severe intestinal infections
Clavariadelphus sp. (Clavariaceae) 539	Hongo	WP	Earache
Dracontium sp. (Araceae) 542	Sacha jergon	R	Snakebite
Erythrina ulei Harms. (Fabaceae) 543	Amazizo	B	Stingray wounds
Euterpe edulis Mart. (Palmae) 544	Palmera huassai	R	Anemia treatment
Gentianella alborsea (Gilg.) Fabris (Gentianaceae) 557	Hercampure	AP	Digestive, hepatoprotective
Gnaphalium spicatum Lam. (Compositae) 558	Keto keto	AP	Pneumonia
Jatropha macrantha Muell. Arg. (Euphorbiaceae) 548	Huanarpo	R	Blood depurative
Petiveria alliaceae L. (Phytolacaceae) 550	Mucura	L	Colds
Piper angustifolium R. et P. (Piperaceae) 552	Matico	L	A cure-all
Triplaris americana L. (Polygonaceae) 556	Tangarana	B	A cure-all

[a]Voucher specimens were collected by Cristian Desmarchelier
[b]Parts used: AP, aerial parts; L, leaves; S, stem; B, bark; R, root

prepared from 13 plants used as medicinals by this indigenous group. In Table 1 we summarize the ethnobotanical data of the plants studied, according to local information.

Infusions, prepared with Tambopata river water according to local use, were assayed in the field testing the initial concentration of 2.5 g of dry plant material per 100 ml of artificial sea water. Solutions of 1.25% and 0.25%, prepared from this original solution, were also tested. None of the thirteen infusions studied showed toxicity at this concentration level (LC_{50} > 2.5%). Taking into account that the ethnopharmacologically relevant aspect of this test is the possible relationship between active brine shrimp assay and possible human acute toxicity to the plant (Calleja and Persoone, 1992; Noamesi et al., 1994), these results suggest the probable innocuousness of these infusions.

In order to search for antitumor activity, toxicity to the brine shrimp of methanolic and dichloromethane extracts was determined in our laboratory, based on the good correlation with cytotoxicity in cancer cell lines such as KB, P-388, L5178Y and L1210 (McLaughlin, 1992; De Rosa et al, 1994). It is interesting to point out that the plants under study do not have a recorded popular use in treatment of cancer, mainly due to the fact that the disease signs and symptoms are hardly recognized by the Ese'ejas, as has been reported for several traditional health systems (Souza Brito and Souza Brito, 1993).

The results obtained are shown in Table 2. Six plants showed a strong concentration-related toxic activity in this bioassay for both extracts: *Abuta grandifolia*, *Aspidosperma excelsum*, *Cyperus articulatus*, *Erytnrina ulei*, *Jatropha macrantha* and *Piper angustifolium*. In addition, *Gentianella alborosea* and *Petiveria alliacea* tested positive only for dichloromethane extracts. To see if cytotoxicity was due to the presence of compounds that interact with DNA, a colorimetric microassay was carried out with those plants which were positive for both extracts. In this case, methanolic extracts were prepared directly from the plant material. No decrease of the initial absorbance of DNA-MG complex was observed in any of the six extracts in comparison with the reference compound, doxorubicine. Work is in progress to test the cytotoxicity observed in other systems and to establish the mechanisms by which this biological action is exerted.

On the other hand, among the thirteen plants studied above, three plants with folk use related to the treatment of microbial infections were selected to perform the antimicrobial test: *Abuta grandifolia*, which is used for the treatment of tuberculosis; *Cyperus articulatus*, employed in the treatment of severe intestinal infections, and

Table 2
LC_{50} and 95% confidence interval (μg/ml) of dichloromethane, methanolic tested at 10, 100 and 1000 μg/ml obtained in the BSA

Plant	Methanolic extract	Dichloromethane extract
Abuta grandifolia	368 (890–195)	69 (107–43)
Aspidosperma excelsum	764 (1678–452)	85 (121–58)
Cyperus articulatus	69 (106–43)	33 (51–20)
Clavariadelphus sp.	>1000	>1000
Dracontium sp.	>1000	>1000
Erythrina ulei	84 (144–48)	68 (111–41)
Euterpe edulis	>1000	>1000
Gentianella alborosea	>1000	373 (516–276)
Gnaphalium spicatum	>1000	>1000
Jatropha macrantha	667 (1239–443)	149 (242–96)
Petiveria alliaceae	> 1000	499 (803–325)
Piper angustifolium	719 (1330–445)	220 (318–157)
Triplaris americana	> 1000	>1000

Table 3
Results of the microbiological assay

Microbial species	Plant		
	Abuta grandifolia	*Cyperus articulatus*	*Gnaphalium spicatum*
Escherichia coli	–[a]	–	–
Staphyloccus aureus	–	++	+
Mycobacterium gordonae	+	–	–
Candida albicans	–	–	–
Pseudomonas aeruginosa	+	+	–
Klebsiella pneumoniae	–	–	–
Salmonella gallinarum	–	–	–

[a]–, no inhibition; +, partial inhibition; ++, full inhibition.

Gnaphalium spicatum, used for pneumonia. The plant decoctions were assayed according to the traditional use. Several Gram-negative bacterias and one species of Gram-positive bacteria, yeast and mycobacteria were selected as the test microorganisms. All these species were chosen based on their clinical importance in cases of infection. The results obtained are presented in Table 3. As it can be seen, all of the decoctions studied showed antimicrobial activity against some of the microrganisms used in the assay. The decoctions of *Abuta grandifolia* and *Cyperus articulatus* partially inhibited the growth of *Pseudomonas aeruginosa*. This fact is interesting, since this organism is one of the most resistant of the species tested (Stickler and King, 1992). *Staphylococcus aureus* was fully inhibited by the decoction of *C. articulatus* and partially inhibited by the decoction of *Gnaphalium spicatum*. The decoction of *Abuta grandifolia* also showed partial inhibition against the *Mycobacterium gordonae*. Based on this promising result, which suggests a correlation between the biological activity studied and its indigenous claim, further studies will be carried out to determine the actual degree of activity against pathogenic species of *Mycobacterium*. None of the decoctions tested showed effect against *Escherichia coli*, *Salmonella gallinarum*, *Klebsiella pneumoniae* and *Candida albicans*.

4. Conclusions

The history of drug discovery implies that the ethnobotanical approach is the most productive of the plant-surveying methods, and recent findings confirm that impression (Cox and Balick, 1994). The search for new active chemical compounds in high biological diversity regions has become a challenge to the modern pharmaceutical industry. However, rainforests of the world and societies that have lived in them for many generations are disappearing at an alarming rate. The loss of both genes and knowledge has led to a vital need to compile and study the information provided by the shamans and healers of these traditional cultures.

The running of field bioassays, such as those described in this paper, has been demonstrated to be useful for supporting ethnobotanical information (Soejarto et al., 1991; Beloz, 1992) and for searching for other pharmacological activities. The authors hope that these results will contribute to encouraging further research seeking new drug prototypes from the tropical rainforests, and to saving their botanical and cultural resources.

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