Brazilian J Med Biol Res (1991) 24: 573-582

EVALUATION OF THE GENOTOXIC ACTIVITY AND ACUTE TOXICITY OF *EUPHORBIA SPLENDENS* LATEX, A MOLLUSCICIDE FOR THE CONTROL OF SCHISTOSOMIASIS

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1. The latex of *Euphorbia splendens* var. *hislopii* has a molluscicidal action at low concentration (LD90 less than 1.5 ppm or 1.5 μg/ml) against the vector snails of schistosomiasis.

2. In the present study, the latex *in natura* or after lyophilization was submitted to the Ames test and the chromotest to evaluate genotoxicity, to the Microtox System to determine acute toxicity, and to the Chinese hamster ovary cell assay (CHO) to measure cytotoxicity.

3. The latex had no mutagenic activity in the presence or absence of S9 toward the TA98 and TA100 strains of *Salmonella typhimurium* (Ames test) at concentrations up to 200 μl/plate *(in natura)* and of 200 μg/plate (lyophilized). The lyophilized latex had no genotoxic activity (Chromotest) and no acute toxic effect on *Photobacterium phosphoreum* at concentrations up to 445 μg/ml, whereas the sample *in natura* had a toxic effect with an EC50 of 148,000 μl/l (or ppm). In the CHO/cytotoxicity assay, the lyophilized latex had no cytotoxic effect in quantities up to 200 μg.

4. The latex was found to have no acute toxicity or mutagenic activity at the concentrations of 10 to 12 μg/ml (or ppm) that are being proposed for molluscicidal use in the field.

Key words: toxicity assay, molluscicidal plant, *Euphorbia splendens*, schistosomiasis.

Introduction

Schistosomiasis is one of the most widespread human parasite diseases in the world, with 500 million people estimated to be at risk to contract the infection in 73 countries (Iarotski and Davis, 1981). More recently (Mott, 1989), the disease was found to be prevalent in 76 countries, indicating geographic expansion.

The World Health Organization recommends the simultaneous use of different methods such as chemical treatment of patients, improvement of basic sanitation, health education, and the use of molluscicides against the vector snails of *Schistosoma mansoni* to control the disease. In Brazil, the vector snails are being controlled with imported

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Research supported by UNDP/WORLD BANK/WHO Special Programme for Research and Training in Tropical Diseases and CNPq (No. 30.0074/81). Publication supported by FAPESP.
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synthetic products, at a high cost in addition to their polluting effects on the environment.

An alternative approach is the evaluation of the molluscicidal properties of plant extracts, which should be easier to handle, would not damage the environment and would be less expensive. According to the United States Guidelines, plant-derived molluscicides are considered to be biorational pesticides which differ from standard synthetic products by being species specific, non-toxic and of natural occurrence, thus generating less damage to the environment (Koeman, 1987).

In an attempt to find an effective natural molluscicide, several research groups all over the world have been engaged in this type of investigation, and more than 1100 plant species have been studied since 1930 (Kloos and McCullough, 1987). *Euphorbia splendens* stands out among the plants investigated in Brazil. Its latex has molluscicidal activity at concentrations as low as 0.5 to 4 ppm (Vasconcellos and Schall, 1986) which is present in the plant throughout the year, as well as after 4 months of storage in test tubes in the refrigerator at 10°C (Vasconcellos et al., 1990). The plant has several characteristics favoring its use in endemic regions. It is well adapted to the climate of Brazil, widely distributed throughout the country, easy to grow, and the preparation of the molluscicide activity is done simply by diluting the latex with water.

According to Koeman (1987), the introduction of plant-derived molluscicidal compounds into the environment requires a preliminary investigation of their possible toxic effect on mammals by tests such as the oral acute toxicity test for rats and mutagenic tests such as the Ames test on eukaryotes and mammalian cells, among others.

In this respect, previous data have demonstrated low toxicity of *Euphorbia splendens* latex for the skin and eyes of dogs and mice and for the human skin (Rizzo and Porfirio, 1971), for the skin and eyes of rabbits (Freitas et al., 1990), and extremely higher lethal doses for mice (acute toxicity tests and repeated doses, Mattos et al., 1989) than those for vector snails of schistosomiasis, a fact suggesting an ample safety margin for the use of the product in the field.

Koeman (1987) argues that if the active product is based on an aqueous extract of the plant, the water-soluble toxic constituents are less likely to accumulate in substantial amounts in fish, mollusks or other organisms participating in the food chain. This consideration applies to *E. splendens* latex. *Euphorbia splendens* extracts also contain compounds with anti-inflammatory activity (Rao and Sussela, 1982) as well as anticancerigenous compounds (Lee et al., 1982). Lee et al. (1982) have also reported that the plant is used in China as a medication for hepatitis and abdominal edema.

However, Farnsworth et al. (1987) warn that plant species of the family Euphorbiceae with molluscicidal activity very frequently contain phorbol esters which may induce tumors, a fact that precludes the use of these plants. However, these investigators suggest the possibility that some species may have a molluscicidal action, and not necessarily contain high concentrations of phorbol esters. Bibliographic surveys including the NAPRALERT (Farnsworth et al., 1981) have shown that few chemical studies have been conducted on *E. splendens* var. *hislopii*, and although phorbic acid has been found to be present (Nordal et al., 1965), other specific tests should be done to

investigate the possible presence of phorbol esters in this variety. Some of these substances can be cocarcinogenic and it is necessary to study this effect before the use of latex in the field. *Euphorbia milii*, which is the same as *E. splendens*, has been studied by Marston and Hecker (1983, 1984) who identified several diterpenes (miliamines A to I) exhibiting at most an irritant activity, but no carcinogenic activity. Despite these reassuring data, however, further investigation of the *hislopii* variety is needed.

According to Koeman (1987), toxicologic assays with plant derivatives are complicated by the fact that the nature of the active principle is fully or partially unknown. Thus, judicious studies are needed to evaluate the possible toxic properties of the product. On this basis, the objective of the present study was to perform some of the standard tests recommended worldwide for this purpose, using samples of latex *in natura* and lyophilized. The following screening tests were used: the Ames test and the chromotest to evaluate genotoxicity, the Microtox system to evaluate acute toxicity, and the Chinese hamster ovary cell assay (CHO) to evaluate cytotoxicity.

Material and Methods

Plant material and extraction procedure

Latex samples having demonstrable molluscicidal activity were always collected at the same site (Ilha do Governador, Rio de Janeiro) to avoid possible variations due to factors such as soil, climate etc., which affect plant metabolism and active substance concentration, as demonstrated by Lugt (1987). The soil composition was analyzed by the Laboratory of Soil and Fertilizers Analysis of the State Department of Agriculture (Rio de Janeiro) and agronomical aspects of the plant are currently under investigation.

The samples used for the present tests were collected in April 1989. White latex was drained into test tubes after tapping the stem of the plant with a scalpel incision. The tubes were sealed and carried to the laboratory for dilution with water and a part of the latex *in natura* was lyophilized using an Edwards apparatus (Edwards, Brazil).

Reverse mutation assay using Salmonella typhimurium-*Ames test*

The test was performed by the plate-incorporation technique described by Maron and Ames (1983) using strains TA98 and TA100 which are auxotrophic for histidine. The test was performed with and without metabolic activation using a homogenate of rat liver cells after treatment with arochlor 1254 (fraction 59 mix, Hasleton Laboratories of America Inc., Kensington, MD, USA).

Different quantities of latex *in natura* (25, 50, 100, and 200 µl/plate) and of a solution of 1000 µg/ml lyophilized material diluted with distilled water containing 3% ethanol (v/v) (1, 10, 50, 100 and 200 µg/plate) were assayed.

The negative controls used were distilled water for the test on the sample *in natura* and distilled water containing 3% ethanol (v/v) for the test on the lyophilized

sample. Both assays were carried out in triplicate. The positive controls were 5 μg sodium azide (dissolved in water) per plate for TA100 without metabolic activation and 2.5 μg 2-anthramine (dissolved in DMSO) per plate for the TA98 and TA100 *S. typhimurium* strains with metabolic activation.

The data were analyzed using a microcomputer software specially prepared for statistical analysis of the Ames test, called *Salmonel*, which includes an analysis of variance and a linear regression. This program was kindly provided by Dr. Lawrence E. Myers, Research Triangle Institute, Research Triangle Park, NC, USA.

Assay based on the SOS-Chromotest functions

The test was performed in duplicate by the method of Quillardet and Hofnung (1985) using *Escherichia coli* PQ37 without metabolic activation.

The sample of latex *in natura* was tested at the doses of 0.02, 0.2 and 2 μl per tube using distilled water as negative control. The lyophilized sample was tested at the doses of 1.25, 2.5, 5, 10 and 20 μg per tube, using distilled water containing 3% ethanol (v/v) as negative control. The positive control consisted of increasing doses (0.31 to 5.0 μg/tube) of mitomycin-C. Larger quantities of the latex could not be tested due to the limitations of the method.

The chromotest is performed in two stages: in the first, the activity of a constitutive enzyme, alkaline phosphatase, is measured, its presence indicating whether acute toxicity is present in the sample; in the second, beta-galactosidase is measured, its induction indicating that the SOS system was stimulated, i.e., that the sample has genotoxic activity.

Acute toxicity assay using Photobacterium phosphoreum - *Microtox system*

The *Photobacterium phosphoreum* (Microtox) assay used to detect acute toxicity in aquatic ecosystems was performed by the method of Bulich (1979), as described in the Beckman Manual (Beckman Instruments, 1982). This assay utilizes lyophilized strains of a luminescent sea bacterium, *Photobacterium phosphoreum* NRRL-B-11177, which is quite sensitive to low concentrations of toxic substances. Under normal conditions, these bacteria continuously produce light that can be quantified with a photometer. Toxic chemical substances interfere with the bacterial metabolism causing a decrease in light production.

The assays were conducted on the latex sample *in natura* and with the lyophilized latex solution prepared at the concentration of 1000 μg/ml (or ppm) in distilled water containing 3% ethanol (v/v). The following concentrations were tested: latex *in natura*, 238,100, 119,000, 59,500, 45,400, 22,700, 11,400 and 5,700 μl/l (or ppm); lyophilized solution, 445, 227, 114, 57, 4.5, 2.25, 1.13 and 0.65 μg/ml (or ppm).

It was impossible to test the same concentration (ppm) of lyophilized latex and latex *in natura* because of the low solubility of the lyophilized material.

Cytotoxicity assay using Chinese hamster ovary cells (CHO)

The cytotoxicity assay using CHO cells was performed by the method described in the EPA Manual (Level 1 Environmental Assessment Biological Tests, 600/8/81-024). CHO cells have been widely used in evaluating pure chemicals, mixtures and environmental samples for cytotoxicity. In this assay, cloned cells are exposed to the test material for a short period of time and the cytotoxic effects are determined by the ability of the material to inhibit cell colony formation after this period. The colonies are then fixed, stained and counted to determine the level of toxicity.

The assay was carried out with the lyophilized latex solution prepared at the concentration of 1000 μg/ml (1000 ppm) in distilled water containing 3% ethanol (v/v) and tested at the concentrations of 60 and 200 μg.

The following controls were used: (a) mercury chloride (10 μg/ml Hg) as positive control, and (b) negative controls consisting of (b1) cloned CHO cell cultures and (b2) diluent (distilled water + 3% v/v) at the concentrations used in the test (60 and 200 μg).

Table 1 - *Ames test for the mutagenic activity of the latex of the molluscicide* Euphorbia splendens in natura.

The test was carried out using strains TA98 and TA100 with and without a metabolic activation system (S9). $\bar{x}$ is the average number of revertants per plate (triplicate assay). MR (mutagenic ratio) is: average number of revertants in test plates/average number of revertants in negative control plates. The negative control was distilled water. DMSO was used as negative control for 2-anthramine present in the positive control. The positive controls were 2.5 μg/plate of 2-anthramine for TA98 and TA100 with S9, and 5 μg/plate of sodium azide for TA100 without S9. ND, Not determined.

Dose (μl)	TA98				TA100			
	-S9		+S9		-S9		+S9	
	$\bar{x}$	MR	$\bar{x}$	MR	$\bar{x}$	MR	$\bar{x}$	MR
Negative control	55.0	-	56.0	-	159.7	-	168.3	-
25	50.0	0.91	62.0	1.11	144.7	0.91	171.3	1.02
50	42.0	0.76	68.0	1.21	153.3	0.96	164.3	0.98
100	41.0	0.75	38.3	0.68	144.0	0.90	141.7	0.84
200	45.0	0.82	35.7	0.64	181.7	1.14	165.7	0.98
DMSO	ND		44.7		ND		139.7	
Positive control	ND		1851	41.41	907.7	5.68	1434	10.26

Results and Discussion

Ames test with Salmonella typhimurium

The results of the assay are reported both as revertants/plate and as mutagenicity ratio (MR), which is the ratio between the number of revertant colonies in the test plate (spontaneous and induced) and the number of revertants in the negative control plate (spontaneous) (Tables 1 and 2).

The presence of mutagenic activity in the sample tested is indicated by an MR of 2.0 or more for at least one dose, and by a dose-response effect.

E. splendens latex *in natura* had no mutagenic activity in the presence or absence of S9 toward the TA98 and TA100 *S. typhimurium* strains at concentrations of 25, 50, 100 and 200 µl/plate. The lyophylized latex also had no mutagenic activity in the presence or absence of S9 toward the same strains at concentrations of 1, 10, 50, 100 and 200 µg/plate.

Chromotest (SOS functions)

The results (Table 3) are reported as the ratio (r) between enzymatic beta-galactosidase units (Beta-gal) and enzymatic units measured for alkaline phosphatase

Table 2 - *Ames test for the mutagenic activity of the lyophilized latex of the molluscicide* Euphorbia splendens.

The test was carried out using strains TA98 and TA100 with and without a metabolic activation system (S9). $\bar{x}$ is the average number of revertants per plate (triplicate assay). MR (mutagenic ratio) is: average number of revertants in test plates/average number of revertants in negative control plates. The negative control was distilled water. DMSO was used as negative control for 2-anthramine present in the positive control. The positive controls were 2.5 µg/plate of 2-anthramine for TA98 and TA100 with S9, and 5 µg/plate of sodium azide for TA100 without S9. ND, Not determined.

Dose (µg)	TA98				TA100			
	-S9		+S9		-S9		+S9	
	$\bar{x}$	MR	$\bar{x}$	MR	$\bar{x}$	MR	$\bar{x}$	MR
Negative control	32.3	-	43.0	-	147.7	-	122.7	-
1	33.0	1.02	40.3	0.94	148.0	1.00	126.3	1.03
10	33.0	1.02	39.5	0.92	133.7	0.91	139.7	1.14
50	32.0	0.99	44.3	1.03	128.0	0.87	126.7	1.03
100	35.0	1.08	42.7	0.99	144.0	0.98	133.7	1.09
200	28.0	0.87	50.0	1.16	129.7	0.88	140.7	1.15
Distilled water	ND		ND		146.7		ND	
DMSO	ND		49.0		ND		145.3	
Positive control	ND		1525.0	31.32	888.7	6.06	1798.0	12.37

(AP), as well as an induction factor (IF) that may be defined as follows: IF = r(dose)/r(0), where r(0) is r obtained for the negative control.

A sample is considered to be positive when a dose-response relationship is obtained between the IF values and the doses tested. The data indicate that neither the *E. splendens* latex *in natura* nor the lyophilized preparation showed genotoxic activity on the *E. coli* strain PQ37 when used at concentrations of 0.02, 0.2 and 2 µl/tube and 1.25, 2.5, 5, 10 and 20 µg/tube, respectively.

Table 3 - *Genotoxic activity of latex* in natura *and lyophilized latex of* Euphorbia splendens *assayed by the chromotest method.*

EU, Enzymatic unit; AP, alkaline phosphatase; ß-Gal, ß-galactosidase; r, ratio of ß-Gal/AP activities; IF = r (dose x)/r(0).

Dose	EU		r	IF
	AP	ß-Gal	EU (ß-Gal) / EU (AP)	r (dose) / r (0)
µl	Latex sample *in natura*			
0.00	18.71	5.02	0.27	1.00
0.02	9.96	4.46	0.45	1.67
0.20	12.49	4.26	0.34	1.27
2.00	19.72	5.02	0.25	0.95
µg	Positive control			
0.00	14.27	4.75	0.33	1.00
0.63	7.83	24.07	3.07	9.24
2.50	4.10	14.19	3.46	10.40
5.00	3.62	18.32	5.06	15.20
µg	Lyophilized sample			
0.00	23.75	2.27	0.10	1.00
1.25	22.80	2.80	0.12	1.28
2.50	22.18	2.23	0.10	1.05
5.00	21.30	2.15	0.10	1.06
10.00	22.80	2.15	0.09	0.99
20.00	23.75	2.15	0.09	0.95
µg	Positive control			
0.00	21.88	2.05	0.09	1.00
0.31	15.49	13.76	0.89	9.48
1.25	11.64	12.57	1.08	11.53
5.00	7.59	11.53	1.52	16.21

Acute toxicity with P. phos-phoreum

The results of the assay (Table 4) are reported as EC_{50}, which is a minimum effective sample concentration that causes 50% reduction in the quantity of emitted light by *Photobacterium phosphoreum* at 15°C after 15 min of contact. Toxicity was also expressed as toxic units (TU), reciprocal of sample concentration, to obtain a direct relationship of the toxicity level of the sample.

The data show that *E. splendens* latex *in natura* had an acute toxic effect on *P. phosphoreum* culture with an EC_{50} of 148,000 µl/l or ppm, a value more than 10,000 times

greater than the concentration proposed for field application.

The lyophilized latex solution had no acute effect on *P. phosphoreum* at any of the concentrations tested.

Table 4 - *Acute toxicity test with* P. phosphoreum *for the latex of the molluscicide* Euphorbia splendens.

EC_{50}, Effective concentration of the sample that causes 50% reduction in the quantity of light emitted by *Photobacterium phosphoreum* at 15°C; TU, toxic unit.

Sample	Acute toxicity test Microtox system			
	pH	EC_{50} (at 15 min)	TU	Toxicity
Latex of *Euphorbia splendens* (*in natura*) liquid	6.0	148,000 µl/l or ppm	6.8	Toxic
Latex of *Euphorbia splendens* (lyophilized)	6.0	Nontoxic	<1	Nontoxic

CHO cytotoxicity assay

The results of this assay (Table 5) are reported as percent cell survival or relative plating efficiency (RPE). Cytotoxicity was classified as RPE < 50% (toxic) and RPE > 50% (nontoxic). A sample of lyophilized latex of *E. splendens* was tested on the basis of these criteria (pH = 6.0). Under the assay conditions the lyophilized latex solution had no cytotoxic effect on a sensitive CHO culture at the concentrations tested (60 and 200 µg) resulting in 93.8 and 92 RPE, respectively.

The results show that latex *in natura* and lyophilized latex at the concentrations proposed for field application had no acute toxicity for *Photobacterium phosphoreum* and for CHO cells, although the latex *in natura* had shown toxicity for *P. phosphoreum* at higher concentrations.

Although the active substance has not yet been identified, the intention of using the latex *in natura* in the simplest and least expensive manner of application by dilution with water or by the use of macerated plant material inside sacks (for slow release) led us to study the toxicity of unprocessed latex.

Table 5 - *Cytotoxicity test with Chinese hamster ovary cell cultures for the latex of the molluscicide* Euphorbia splendens.

RPE, Relative plating efficiency.

Sample	Cytotoxicity test			
	pH	Concentrations	RPE	Toxicity
Latex of *Euphorbia splendens* (lyophilized)	6.0	200 µl	92	Nontoxic
		60 µl	93.8	Nontoxic

In addition to the data reported, we have carried out other toxicological evaluations such as the determination of acute toxicity and repeated doses in mice, and the evaluation of irritability (skin and eyes) in rab-

bits, which have produced encouraging results (Mattos et al., 1989; Freitas et al., 1990).

The active principle in the latex is also being studied and a highly active fraction against snails (LD90 = 0.008 ppm) has been identified and has been found to be lethal for fish at 7 times this concentration (LD90 = 0.052 ppm) (Zani et al., 1989).

Marston and Helker (1983, 1984) have provided important evidence that the diterpenes identified in *Euphorbia milii*, the same species as *E. splendens*, are not tumor promoters but, at most, only skin irritants.

In relation to the variety *hislopii*, Zani et al. are also investigating the presence of possible irritant and cocarcinogenic diterpene esters derived from phorbol or ingenol in the latex since these substances are present in several Euphorbiaceae. This study will be conducted using different geographic samples obtained in different seasons.

As demonstrated by Furstenberger and Hecker (1986), there may be chemical races of plants from distant places with qualitative and quantitative differences in irritant lattice constituents. These authors pointed out that some irritant constituents of the latex of *Euphorbia tirucalli* collected in Madagascar or in South Africa, such as the tigliane- and ingenane-type diterpene esters, are not present in the latices from *E. tirucalli* plants grown in greenhouses in Heidelberg.

The present study is the first step in a systematic survey of the toxicological properties of the latex of *Euphorbia splendens*. The data obtained until now justify undertaking the more complex biological assays in animals that are required before this material can be tested for the control of schistosomiasis in open field tests.

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Received March 4, 1991
Accepted June 12, 1991