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## Mammal Toxicity and Mutagenicity Assessment of the Methanol Extract of the Molluscicidal Plant *Euphorbia schimperiana*

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**Abstract:** In this study, the toxic and mutagenic effects of *Euphorbia schimperiana*-methanol extract were investigated in Swiss albino mice of both sexes. In the course of a toxicological evaluation of this extract and prior to field applications, selected acute toxicity tests (oral, dermal, skin and eye irritation) were firstly determined. Then, three doses of acute oral (maximum, half maximum and double maximum) were chosen for the assessment of mutagenicity using the micronucleus test. While, the tested extract was slightly toxic non-irritant to the skin and moderately irritant to the ocular tissue it significantly increased the frequency of micro-nuclei particularly at the doubly maximum dose in both sexes. In conclusion, the results indicated that the *Euphorbia schimperiana*-methanol extract has slightly toxic effect. But has a potent mutagenic effect in mice.

**Key words:** *Euphorbia schimperiana*, toxicity, mutagenicity, micronucleus, molluscicidal

### INTRODUCTION

During the past decades several important reviews on plant molluscicides have been published (Kloos and McCullough, 1982; Marston and Hostettmann, 1985; Mott, 1987). The introduction of these plants, or their parts as extracts, into the environment requires prior investigation of their possible toxic effects on mammals and certain other non-mollusc groups of invertebrates (Koeman, 1987). Although there has been much research on plant molluscicides, none has been used extensively in an endemic country, nor have there been consistent efforts to ensure adequate supplies of the candidate compounds for laboratory studies (WHO, 1993).

Lambert *et al.* (1991) reported that the extract of *Phytolacca dodecandra* was either slightly toxic or non-toxic, but the extract did, however, prove to be a severe eye irritant. On the other hand, Alard *et al.* (1991) mentioned that *Ambrosia maritima* is virtually non-toxic to aquatic and other non-target organisms. Also, Brackenbury *et al.* (1997) classified the effect of *Apodytes dimidiata* extracts as non-toxic and non-irritating. More recent tests on mice have shown that lethal doses of same plant extract are much higher the LD<sub>50</sub> detected for snails (Mattos *et al.*, 1989). Low toxicity for the skin and eyes of rabbits was also demonstrated by using the same plant extract (Freitas *et al.*, 1991).

In addition, the micronucleus (MN) assay has been commonly used as a predictor of genotoxicity. It involves the enumeration of micronuclei in enucleated

polychromatic erythrocytes (PCEs) obtained from bone marrow samples. MN are formed by condensation of chromosome fragment or whole chromosomes that are not induced in the main nucleus following anaphase (Heddle *et al.*, 1984). Two reports on the genotoxicity (mutagenicity) of other species of *Euphorbia*. Zamith *et al.* (1996) stated that the latex *E. millii* var. *hislopii*, syn. *E. splendens* var. *hislopii* had no effect on frequencies of chromosome aberrations in the bone marrow of male and female rats at a dose of 1000 mg kg<sup>-1</sup>. Latex was also investigated for its capability of inducing gene mutation and chromosome aberrations in V79 cells *in vitro* in the absence or presence of S9 mix. They also found that at concentrations up to 800 mg mL<sup>-1</sup> neither induced gene mutations at HPRT locus nor chromosome aberrations. These results indicated that the latex of *E. millii* is not mutagenic in mammalian cells *in vitro*, or *in vivo* and its use as a molluscicide does not pose a mutagenic hazard for human. The second report concluded that the highly oxygenated diterpene was not mutagenic toward *Salmonella typhimurium* strain TM677 either in the presence or absence of a metabolic activating system (Shamon *et al.*, 1997).

Biologists have been recommended to adhere to the toxicity tests laid down in the minimal data requirements of the Organization of Economic Cooperation and Development (OECD) Guidelines for pre-market chemicals (Lambert *et al.*, 1991; Brackenbury *et al.*, 1997). However, in order to test the applicability of local varieties of *Euphorbia schimperiana* in schistosomiasis intervention

trials where this plant would be introduced experimentally into domestic water bodies, data are needed on *Euphorbia schimperiana* mammal toxicity and mutagenicity. The toxicity tests (oral, dermal, skin irritation and eye irritation) selected in the present study attempted to simulate the most likely routes of exposures that might arise from human-water contact activities. Also the formation of micronuclei was the cytogenetic parameter used to evaluate the possible genotoxic potential of methanol extract of *Euphorbia schimperiana*. Up to now virtually no data were available, however, on the mammalian toxicity and mutagenicity of *Euphorbia schimperiana*, since these data necessary before the plant can be used on a larger scale in the field.

## MATERIALS AND METHODS

The present study was done in Biology Science Department, Science College, King Abdul Aziz University, Jeddah, Saudi Arabia during 2004-2005.

### Mammal toxicity

**Experimental animals:** Swiss albino mice of both sexes were used for the toxicity tests, they were maintained at the animal house of King Fahd research Centre, King Abdul Aziz University. The weight of male mice ranged from 25 to 35 g, while for female mice it ranged from 23 to 26 g.

**Acute oral LD<sub>50</sub> toxicity tests (OECD, 1981a):** *Euphorbia schimperiana* methanol extract was dissolved in distilled water a single oral administration by gauge was given to five males and five females Swiss albino mice. Animals were initially tested at a maximum required limit does level of 5 g kg<sup>-1</sup> body weight. They were observed for mortality and clinical signs of toxicity for 14 days postdose and then subjected to gross necropsy.

**Acute dermal LD<sub>50</sub> toxicity tests (OECD, 1981a):** The methanol extract of *Euphorbia schimperiana* was applied to the skin of the back of five males and five females Swiss albino mice for a single 24 h exposure. Animals were tested at a maximum required limit dose level of 2 g kg<sup>-1</sup> body weight. They were observed for mortality and clinical signs of toxicity for 14 days postdose, then sacrificed and then subjected to gross necropsy.

**Dermal irritation test (OECD, 1981a):** *Euphorbia schimperiana* methanol extract was moistened with distilled water and applied to the shaven intact skin of the back of five males and females Swiss albino mice using a semi-occlusive patch technique. Four hours later, the

patch was removed and the test site assessed for irritation and on corrosion at 1, 24, 48 and 72 h intervals after patch removal. Animals were sacrificed. No necropsy was necessary.

**Eye irritation test (OECD, 1981a):** Two test groups, each consisting of three Swiss albino mice of both sexes, were used in this trail. The first group received a single concentration of 0.2 g L<sup>-1</sup> and the other group 0.5 g L<sup>-1</sup> in the conjunctival sac of the left eye (both concentrations refer to the amount of dried stem material in one liter of water). The contralateral eye of each animal was left untreated and served as control. Both eyes were examined with an ophthalmoscope and fluorescein before application and then at 1, 24, 48 and 72 h intervals post-application. Higher concentrations were not tested at this stage for ethical reasons.

### Mutagenicity study

**Animals:** Males and females albino mice weighing 20-30 g were used throughout the study as experimental animals.

**Chemicals:** Methanol extract of dry stems of *Euphorbia schimperiana* was used for this study.

**Doses:** Three doses, double maximum does (0.3 g kg<sup>-1</sup>) for males and (0.26 g kg<sup>-1</sup>) for females, maximum does (0.15 g kg<sup>-1</sup>) for males and (0.13 g kg<sup>-1</sup>) for females and half maximum does (0.08 g kg<sup>-1</sup>) for males and (0.06 g kg<sup>-1</sup>) for females were used as mentioned in (Table 2 and 3).

**Treatment:** In the time course study, mice (5 males and 5 females) were orally administrated with each dose. Control animals (five for both sexes) were orally administrated with distilled water alone.

### Micronucleus assay

**Extraction of bone marrow and preparation of the smears:** The preparations were made according to Schmid (1973, 1975) with some modification in fixation and staining based on the method of Heddle *et al.* (1984). The mice were sacrificed by cervical dislocation 24 h after each exposure and both femora were removed and stripped clean of muscles. The proximal end of each femur was carefully shortened with a pair of scissors to obtain a small opening through which bone marrow cells were aspirated into a centrifuge tube using a syringe containing 2 mL of foetal calf serum. The cell suspension was centrifuged for 5 min at 1000 rpm and the supernatant was removed with Pasteur pipette.

The cells in the sediment were carefully mixed using Pasteur pipette. A small drop of the viscous suspension was put on the end of a slide and spread by pulling the material behind a cover glass held at angle 45 degrees. The smears were then air dried.

**Fixation and staining:** The smears were then fixed in absolute methanol for 5 min and stained for 20 min in a 5% solution of Giemsa in 0.01 M phosphate buffer adjusted to pH 6.8 and then mounted in DPX.

**Scoring:** The slides were coded and examined under the microscope at a magnification of 1250 X. One thousand polychromatic erythrocytes PCEs per mouse were scored for the presence of micronuclei. Micronuclei were identified as dark staining rounded bodies in the cytoplasm of PCEs.

Precautions, with regard to scoring and artifacts, were taken as described by Adler (1984) and Sharaf (1992).

**Statistical analysis:** The result of acute oral LD50 toxicity test was statistically analyzed based on Leithfield and Wilcoxon (1949). The result of the mutagenicity study were statistically analyzed using the Tukey test (Daniel, 1999).

## RESULTS

**Mammal toxicity:** According to a review of the Environmental Protection Agency (EPA, 1982) and the World Health Organization (WHO, 2000) protocols and classification schemes, the dry stem methanol extract of *Euphorbia schimperiana* may be categorized as represented in Table 1.

**Acute oral LD<sub>50</sub> toxicity:** At a limit does of 5000 mg kg<sup>-1</sup> body weight, no mortality or clinical signs of toxicity during the 14 days postdose were recorded. But after the experimental animals were subjected to gross necropsy, there were 5/5 males and 0/5 females (for those received maximum dose) that showed a pulmonary hemorrhage with bleeding in the pleura and clotted blood in the lungs within the 14 days postdosing. The conditions of this study yielded LD50 of 54 mg kg<sup>-1</sup> (95% confidence limits: 45- 64.9) and LD90 of 81.8 mg kg<sup>-1</sup> (95% confidence limits: 68- 98) in the males (Fig. 1). Based on this result, the tested subject was classified slightly toxic.

Table 1: Classification of the methanol extract of *Euphorbia schimperiana* Toxicity data according to EPA and WHO schemes

Mammalian toxicity test	Categorization	
	EPA	WHO
Acute oral	III	Slightly toxic
Acute dermal	III	Non-toxic
Dermal irritation	IV	Non-irritation
Eye irritation	II	Moderately irritating

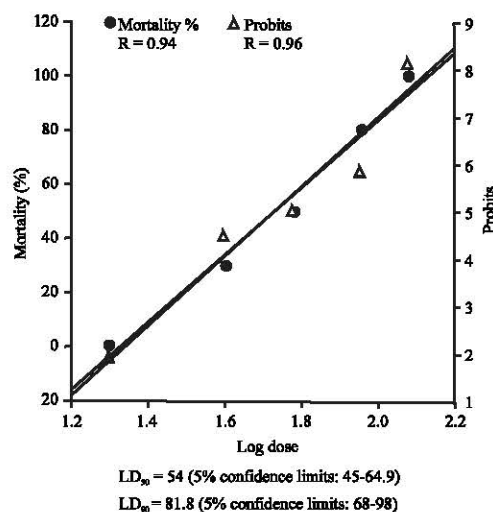


Fig. 1: LD<sub>50</sub> and LD<sub>90</sub> (for 14 days exposure) of the acute oral toxicity test using the methanol extract of dry stems of *Euphorbia schimperiana*

**Acute dermal LD50 toxicity:** No mortality occurred or clinical signs were observed during the 14 days postdosing. After male and female mice were subjected to gross necropsy, no treatment-related gross abnormalities were recorded in the tested animals. Based on these findings, the acute dermal LD50 of *Euphorbia schimperiana* methanol extract in mice is estimated to be greater than 2 g kg<sup>-1</sup>.

**Dermal irritation:** The tested subject did not produce any dermal reversible effects. No erythema or oedema were observed in the shaven skin of the tested animals (both sexes). Thus, *Euphorbia schimperiana* methanol extract is considered to be non-irritant to the skin of mice.

**Eye irritation:** In all eyes of both sexes of animals, the tested subject produced significant irritation progressing to opacity of the cornea at 1, 24, 48 and 72 h postdose. There was no evidence of corrosion in any of the eyes tested. Under the considered of this test, *Euphorbia schimperiana* methanol extract is considered to be moderately irritating to the ocular tissue of mice.

**Mutagenicity study:** Table 2 and 3 show the number, mean and standard error of micronucleated PCES in bone marrow cells after orally administrated of male and females mice, respectively with maximum (max), half maximum (half-max) and double maximum (double-max) doses of dry stems methanol extract of *Euphorbia schimperiana*.

The sample with maximal increase was observed after double-max does in both males and females. In this dose, the number of micronucleated PCES reached more than 4 times (in males) and more than 4 times (in females) than those of controls.

Table 2: Incidence of micronucleated polychromatic erythrocytes (MNPCEs) in bone marrow of male albino mice after exposure to three doses of dry stems methanol extract of *Euphorbia schimperiana*

Group	Dose	No. of animals	No. of scored	No. of MNPCE	MNPCE (%)	Mean	Standard error
Control	0.0	5	5000	26	0.0052	5.2	1.02
Max-dose	0.15	5	5000	87	0.0174	17.4	2.14
Half max-dose	0.08	5	5000	38	0.0076	7.6	0.51
Double max-dose	0.3	5	5000	103	0.0206	20.6	3.09

Table 3: Incidence of micronucleated polychromatic erythrocytes (MNPCEs) in bone marrow of female albino mice after exposure to three doses of dry stems methanol extract of *Euphorbia schimperiana*

Group	Dose	No. of animals	No. of scored	No. of MNPCEs	MNPCEs (%)	Mean	Standard error
Control	0	5	5000	22	0.0044	4.4	0.75
Max-dose	0.13	5	5000	52	0.0104	10.4	1.47
Half max-dose	0.06	5	5000	36	0.0072	7.2	1.02
Double max-dose	0.26	5	5000	66	0.0132	13.2	3.38

Table 4: Comparison of the incidence of micronucleated polychromatic Erythrocytes (MNPCEs) between males and females

Group (male-female)	T-value	p-value
Control	0.6325	0.5447
Max dose	2.7	0.0271
Half max	0.3508	0.7348
Double max	1.615	0.145

The analysis of variance of the data reveals that there was a highly significant difference between the four doses for males whereas it was only significant in females.

To compare the incidence of PCES of each group in males with its counter part in females. (t) test for comparing two independent group is used. Table 4 shows the mean of number of MNPCEs differs significantly (at  $\alpha = 0.05$ ) between males and females only when using maximum dose. For other doses, the average number of MNPCEs in males and females are not significantly different from each other.

## DISCUSSION

The toxicity studies identified under (OECD, 1981b) were selected to address the possible adverse effects that might be predicted following exposure to *Euphorbia schimperiana* methanol extract. For the toxicity testing we did not use the methanol extract of dry stems of *Euphorbia schimperiana* as a stock solution, but as a crude extract, thus, the doses for these studies represent a (worst case scenario) in which the active principle constitutes a major percentage of the dose.

Only a few plant molluscicides have been studied beyond their target molluscicidal effects. Lemma and Yau (1974), Squire *et al.* (1989), Lambert *et al.* (1991), Clark (1994), Geerts *et al.* (1994) and Brackenbury (unpublished data) have investigated the effects of extracts of various plant species on non-target organisms such as Daphnia, snails, mosquito larvae, earthworms, fish and birds in an attempt to predict hazards to non-target biota. However, few have been subjected to mammal or long-term toxicological studies (WHO, 1993;

Brackenbury *et al.*, 1997). *Phytolacca dodecandra*, *Ambrosia maritima*, *Apodytes dimidiata* and *Euphorbia splendens* are the only molluscicidal plants, known to the authors, to have undergone mammal and mutagenic testing. *Euphorbia schimperiana* underwent similar toxicity assessment in the present study in an attempt to identify possible human health hazards that might arise from its use. This toxicological data when compared with that of *Phytolacca dodecandra* (Lambert *et al.*, 1991), *Ambrosia maritima* (Alard *et al.*, 1991), *Apodytes dimidiata* (Brackenbury *et al.*, 1997) and *Euphorbia splendens* (Freitas *et al.*, 1991) suggests that *Euphorbia schimperiana* is less toxic to mammals. These results show there are slight toxic signs for males only after the oral administration of a single dose of 5 g *Euphorbia schimperiana* methanol extract per kg body weight within 14 days postdosing which resemble the result of the same toxicity test done for the *Phytolacca dodecandra* by Lambert *et al.* (1991), who found that at a limit dose of 5000 mg kg<sup>-1</sup>, 4/5 males and 5/5 females died within 3 days of dosing and differ from that of Alard *et al.* (1991) which showed that *Ambrosia maritima* has no toxic signs neither after oral administration of 5 g kg<sup>-1</sup> of dried leaves of the plant as a powder or as a methanolic extract.

No toxic effects could be observed after applying a single, 24 h exposure of *Euphorbia schimperiana* methanol extract to the mice skin and no mortality occurred during the 14 days postdosing. These results are convenient with that recorded by Lambert *et al.* (1991) who recorded that transient, minimal erythema was produced at the exposure site of *Phytolacca dodecandra*, but no clinical signs were related to dosing and treatment-related gross abnormalities were observed in any animals at necropsy. Also, the present finding resembles that obtained by Brackenbury *et al.* (1997) who classified *Apodytes dimidiata* as non-toxic however, it showed a slight erythema and oedema after 6 days postdose.

Dermal irritation test is one of the mammal toxicity tests. In this test no irritation recorded by applying the moistened methanol extract of *Euphorbia schimperiana*

to the shaven intact skin of mice for a maximum period of 72 h. This result is different from that of Lambert *et al.* (1991) who found that *Phytolacca dodecandra* produced slight, reversible erythema in the test animals. Also, it differs from that Freitas *et al.* (1991) who demonstrated low toxicity for the skin of rabbits by using the latex of *Euphorbia splendens*.

The result of eye irritation confirm that of Lambert *et al.* (1991) and Freitas *et al.* (1991) where both of them demonstrated significant and low toxicity for the eyes of the tested animals respectively. Also, it differs from that of Brackenbury *et al.* (1997) who found that the aqueous extract of *Apodytes dimidiata* were evaluated as being non-irritating to eyes.

Except for the eye irritation, all mammalian acute toxicity tests are characterized as either slightly toxic or non irritating. In the case of eye irritation classification of moderately irritating, eye protection is indicated for whomever handles *Euphorbia schimperiana* methanol extract.

The main objective of the mitogenicity (genotoxicity) test was the identification of clastogenic and/or spindle toxicity (aneugenic) effects of the dry stems methanol extract of *Euphorbia schimperiana*, which can achieved by using micronucleus test.

The present study indicated that the extract induced a highly significant difference between the four doses for males and only significantly different for females in the incidence of micronuclei in polychromatic erythrocytes in bone marrow cells. Since the appearance of micronuclei is related to loss of chromosome segment as a result of chromosome breaks or chromosomal non-disjunction, the extract appears to be potent clastogen and/or aneugen in males than in females.

From the literature review, it is clear that there is a paucity of information on dry stem methanol extract of *Euphorbia schimperiana* genotoxic activates from direct experimental testing. Zamith *et al.* (1996) reported that latex of *Euphorbia millii* neither induced HPRT Locus nor chromosome aberrations in V79 Chinese hamster lung cells. Al-Zanabagi *et al.* (2000) reported that the molluscicidal activity of dry stem methanol extract of *Euphorbia schimperiana* was found to be due to terpenoids and phenolic with LD50 of 5.7 ppm. Shamon *et al.* (1997) found that the highly oxygenated diterpens was not mutagenic toward *salmonella typhimurium* strain TM677 either in the presence or absence of a metabolic activity system. The previous reports are contradicted with our results. On the other hand, phenolic compound (quercetin) induced significant increase of micronuclei in human lymphocytes *in vitro* in

the absence and in the presence of S9 (Caria *et al.*, 1995). Therefore, the mutagenic potential of *Euphorbia schimperiana* extract observed in the present study may be attributed to the phenolic part.

Since it is a preliminary study and the results recorded some hazards of *Euphorbia schimperiana*-methanol extract to human health from both toxicological and mutagenical point of views. There fore, it important to carry out more investigations using various type of test system under different conditions to evaluate the toxic and mutagenic potential of this extract in order to be used in a large scale as natural molluscicide for replacing a chemical one which is the main objective of this study.

Then ,the way is probably clear to test this extract in a preliminary field trial if prepared as recommended and keeping strong precautions during the application.

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