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MUTAGENIC AND GENOTOXIC EVALUATION OF MEDICINAL PLANT *EUPHORBIA ROYLEANA* LATEX TO FRESHWATER FISH *CHANNA PUNCTATUS* (BLOCH)

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ABSTRACT

The aim of present study was to evaluate the mutagenicity and genotoxicity induced due to medicinal plant *Euphorbia royleana* crude latex in fresh water fish *Channa punctatus* using micronucleus and single cell gel electrophoresis (SCGE) assays. Two sub-lethal concentration of crude latex were calculated using 24 hrs LC_{50} value and the fish specimens were exposed to these concentrations in a static system. Erythrocytes were sampled at the intervals of 24, 48, 72 and 96 hrs at the rate of five fish per interval for assessment of micronucleus induction and SCGE assay. Significant effects (P<0.05) for both concentration and time of exposure were observed in treated fish. Highest level of micronuclei induction in erythrocytes and DNA damage in the SCGE was at 96 hrs of exposure at all concentrations of crude latex. This study further confirmed that the micronucleus and SCGE assays are useful to determine potential genotoxicity of herbal formulation and might be appropriate as part of monitoring program.

KEYWORDS: ***Channa punctatus*; *Euphorbia royleana*; Genotoxicity; Comet assay; DNA damage; Micronucleus test**

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INTRODUCTION

Medicinal herbs are the local heritage with global importance. The medicinal properties of a plant are due to the presence of certain chemical constituents. These chemical constituents, responsible for the specific physiological action, in the plant, have in many cases been isolated, purified and identified as definite chemical compounds. Quite a large number of plants are known to be of medicinal use remains uninvestigated and this is particularly the case with the Indian flora. The use of plants in curing and healing is as old as man himself [1]. Chemical compounds in plants mediate their effects on the human body through processes identical to those already well understood for the chemical compounds in conventional drugs; thus herbal medicines do not differ greatly from conventional drugs in terms of how they work. This enables herbal medicines to be as effective as conventional medicines, but also gives them the same potential to cause harmful side effects[2,3]. The Euphorbiaceae is the 4th largest family of the angiosperms comprising over 300 genera and about 7500 species. The euphorbiaceae plants are shrubs, trees, herbs or rarely lianas[4]. Many of them are xerophytes and cactoid and most often with milky latex. The family provides food[4,5] and varied medicinal properties used in ethnobotany[6-10]. They are useful in the treatment of ailments such as respiratory infections, venereal diseases, toothache, rheumatism, cough, ulcer and wounds[11]. The genus *Euphorbia* is composed essentially of latex bearing species[12]. Many of them have been the objects of chemical and pharmacological investigation because of the irritant and medicinal properties of their latexes[13]. *Euphorbia royleana* Boiss (Euphorbiceae) is a common thorny succulent species and is a common medicinal plant of India and used extensively as folk medicine and its latex in small doses is a purgative but in large doses it is acrid, counter irritant and emetic and possesses cathartic and antihelimintic properties[14]. Basak *et al*., [15] reported that latex of this plant is irritant to the skin and eye. Various parts of this plant have insecticidal and molluscicidal properties[16-19]. The medicinal as well as the poisonous properties of *E. royleana* latex are due to the presence of several compounds i.e. epitaraxerol, ellagic acid, euphol, taraxerol, sitosterol, m-hydroxy benzoic acid, 7-hydroxy 3,4-benzcoumarin, 7-methoxy-3,4-benzcoumarin, 2',7-dihydroxy3,4-benzcoumarin etc[20,21]. Recently bioactivity investigations on *Euphorbia royleana* have shown anti-inflammatory[22], piscicidal[18] and antiacetylcholinesterase activities[18] as well as immunosuppressive effects[23], Bani *et al*.,[24] have shown that *E. royleana* has analgesic and antipyretic properties in rats and rabbits. There exists a single study which determined the acute toxicity of *E. royleana* for the fish *Channa punctatus*[25]. Although, these medicinal plants were used for many purposes in different countries, whether in crude form or active compounds isolated from them, but without established efficacy, testing its different property and using genotoxic test, it is not good to used in human health remedies. Several ecotoxicological characteristics of air-breathing freshwater food fish *Channa punctatus*, such as its wide distribution in the freshwater environment, noninvasive, availability throughout the years, presence of 32 well-differentiated diploid chromosome numbers, commercial importance, ease of blood collection and acclimatization to laboratory conditions, make this species an excellent test specimen for toxicity studies[26,27]. The effects of genotoxicity are reported to be several-folds on the fitness traits like reproductive success; genetic patterns and subsequent population dynamics in fish have been highlighted during genotoxicity assessment experiments[28-30]. Since there is growing concern over the presence of genotoxins in the aquatic environment, it is important to develop methods for detection of genotoxic effects in aquatic organisms[30-32]. Several studies have shown that the micronucleus (MN) test and single cell gel electrophoresis (SCGE) or Comet assay are two sensitive, rapid and extensively used methods in the detection of mutagenic and genotoxicity of chemicals and xenobiotics under field and laboratory conditions[26,31,33-44]. One of the advantages of MN and SCGE

assays are that both can be used for the simultaneous assessment of DNA damage in many tissues from the same animal and for the comparison of their responses under identical treatment condition. The SCGE has been considered as sensitive rapid and reliable method of quantitatively measuring DNA damage in eukaryotic and prokaryotic cells[45,46]. It is increasingly being used in testing of substances such as industrial chemicals, biocides, agrochemical, food additives and pharmaceuticals for genotoxicity testing[47]. In the present study, Fish blood is used that favored because it comprises 97% erythrocytes, thus ensuring great homogeneity of cells for SCGE studies. *Euphorbia royleana* are widely used as traditional remedies, and there is very limited knowledge regarding the mutagenic and genotoxic effects of crude latex of *E. royleana*. Very fewer studies using a variety of assays in the past have been done. Hence, hazardous effects of this plant product are a matter of great concern due to its large scale exposure in human's worldwide. In the present study, attempts have been made to evaluate the mutagenic and genotoxic activities of crude latex of *E. royleana* using MN and SCGE assays in erythrocyte of *C. punctatus* exposed *in vivo*.

MATERIALS AND METHODS

(i) Experimental fish specimen and chemical

Freshwater air-breathing fish *C. punctatus* (Bloch; Family: Channidae, order: Perciformes) were caught from nearby ponds and lakes with the help of local fishermen. The specimens had an average (± SD) weight and length of 15.70 ± 1.25gm and 13.06 ± 2.07cm, respectively. Fish specimens were subjected to a prophylactic treatment by bathing twice in 0.05% potassium permanganate ($KMnO_4$) for two min to avoid any dermal infections and fed with fish feed throughout the acclimation processThe bioassays were carried out at specific conditions, as recommended by the American Public Health Association (APHA). The measured values of pH, temperature, dissolved oxygen (DO), and hardness of water were 6.8- 7.0, 26 °C, 7.2 mgL^{-1} and 41mg/$CaCO_3$, respectively.

(ii) Extraction of latex of *Euphorbia royleana*

The plant of *Euphorbia royleana* was collected locally from the Botanical garden of D.D.U. Gorakhpur University, Gorakhpur, (U.P.), India and identified by Plant Taxonomist, Department of Botany, D.D.U. Gorakhpur University, Gorakhpur, U.P. (India). The white, milky latex of *E. royleana* was drained into glass tubes by cutting the stem apices. The latex was centrifuged at 1000 x g for 20 minutes to remove the resin, this resin free latex lyophilized at - 40°C, and the lyophilized powder stored for further use. The freeze-dried powder was mixed with an appropriate volume of distilled water to obtain the desired concentration. The wet weight of one ml latex of *E. royleana* was 1.42 gm and dry weight (lyophilized at -40°C) was 0.620 gm.

(iii) Determination of Sub-lethal concentrations

Acute toxicity was conducted to determine the 24 hrs-LC_{50} value of *E. royleana* crude latex with definitive test in static system in laboratory as per standard methods[48]. The range finding test was carried out prior to the definitive test to determine the concentration of the crude latex. Toxicity experiments were performed by the method of Singh and Agarwal[49]. For the test, crude latex was dissolved in 10 ml of 1 % gumacacia solution, filtered and added to the aquarium. A set of 10 specimens were randomly exposed to each of the four concentrations of crude latex viz., 50, 55, 60, 70 mgL^{-1} obtained after range finding test, and the experiment was set in triplicate to obtain the 24 h-LC_{50} value of the test materials for the species. The 24 hrs-LC_{50} value of *E. royleana* crude latex was determined as 61.80 mgL^{-1} for *C. punctatus*. Toxicity data obtained from this study was estimated through POLO plus computer program version 2.0 of Robertson *et al.*,[50]. By using this program lethal concentration (LC values); upper and lower confidence limits and heterogeneity were calculated through probit log analysis method. On the basis of LC_{50} value, 5% and 10% concentrations viz., 3.09 mgL^{-1} and 6.18 mgL^{-1} for crude latex was estimated for use in the *in vivo* experiments.

(iv) *In vivo* exposure experiment

The fish specimens were exposed to the two aforementioned test concentrations of crude latex in a static system. The exposure was continued up to 96h (4 days) and blood sampling was done at the intervals of 24, 48, 72 and 96 h at the rate of five fishes per interval. The specimens maintained in tap water and those exposed to 10 ml of 1% gumacacia simultaneously were considered as the negative and solvent controls, respectively. On each sampling day, the whole blood was collected and immediately processed for MN and CA. Blood was collected by incising the lateral vein using heparinized syringe and was diluted 20-fold. About 0.5 ml of diluted blood was added to an isotonic solution (10 ml) for further dilution[51] in dark and dim light to prevent the occurrence of additional DNA damage. The physico-chemical properties of test water, namely temperature, pH, dissolved oxygen, conductivity and total hardness were analyzed by standard methods[48].

(v) Micronucleus (MN) assay

Peripheral blood samples obtained from the caudal vein were smeared on clean, grease free frosted glass slide. Slides were fixed in methanol for 10 min and left to air-dry at room temperature and finally stained with 6% Giemsa in Sorenson's buffer (pH 6.9) for 20 min. After dehydration through graded alcohol and clearing in xylene, slides were mounted in DPX (distyrene, plasticizer and xylene). From each slide, 1500 erythrocyte cells were scored under a light microscope (Leitz Wetzlar Germany, Type 307 – 083.103, oil immersion lens, 100/1.25). The criteria used for the identification of MN were their size.

(vi)Alkaline single cell gel electrophoresis (SCGE)

The alkaline single cell gel electrophoresis or comet assay (CA) was performed as a three-layer procedure[52] with slight modifications[53]. The blood was suspended in chilled phosphate buffered saline (PBS). Viability of the erythrocytes was evaluated by the trypan blue exclusion test method[54] and tissue samples showing cell viability exceeding 84% were further processed for comet assay. In brief, about 15µl of cell suspension (approx 20,000 cells) was mixed with 85µl of 0.5% low melting point agarose (LMPA) and layered on one end of a frosted glass slide, coated with a layer of 200 µl of 1% normal agarose. It was covered with a third layer of 100 µl LMPA. After solidification of the gel, the slides were immersed in lysing solution (2.5 M NaCl, 100 mM Na_2-EDTA, 10 Mm Tris, pH 10, with 10% DMSO and 1% triton X- 100, added fresh) overnight at 4 ºC. After lysis, the slides were placed side by side in a horizontal electrophoresis unit containing fresh cold alkaline electrophoresis buffer (300 mM NaOH, 1mM Na_2-EDTA and 0.2% DMSO, pH 13.5) and left in the solution for 20 min at 4 ºC for DNA unwinding and conversion of alkali-labile sites to single-strand breaks. Alkaline electrophoresis was performed using the same alkaline electrophoresis buffer for 20 min at 15 V (0.8 V/cm) and 300mA at 4 ºC. The slides were then washed three times for 5 min with neutralization buffer (0.4 M Tris, pH 7.5) to remove the excess alkali. The slides were stained with 75 µl ethidium bromide (20 µg/ml). Two slides per specimen were prepared and 25 cells per slide (250 cells per concentration) were scored randomly and analyzed using an image analysis system (Komet – 5.5 Kinetic Imaging, UK) attached to florescent microscope (Leica) equipped with appropriate filters. The parameter selected for quantification of DNA damage was percent tail DNA (%tail DNA = 100-%head DNA) as determined by the software.

(vii) Statistical analysis

Statistical analysis was performed with the SPSS 10.1 computer program (SPPS Inc, Chicago, IL, USA).The results were expressed as mean ± SE. The one-way analysis of variance (ANOVA) was employed to compare the mean differences in % tail DNA damage. The percentage MN was compared between concentrations within duration using Mann-Whitney test. A *p* values less than 0.05 was considered statistically significant.

RESULTS

(i) Physico-chemical properties of the test water

The temperature of test water varied from 18.5 to 25.60 ºC and pH ranged from 7.1 to 8.2. The dissolved oxygen (DO) ranged from 6.0 to 8.2 mgL^{-1}. The conductivity of the water ranged from 250 to 303 μMcm^{-1} while total hardness ranged from 166-185 mgL^{-1} during the experimental period, respectively.

(ii) 24 h-LC_{50}values

Acute toxicity bioassays, of different lethal concentrations at different exposures are listed in Table-1. The LC_{50} values (with 95% confidence limits) of different concentration of *E. royleana* crude latex (Table 1) were found to be 61.80 (58.91-65.98), 58.19 (55.23-61.36), 55.17 (51.23-58.18) and 52.09 mgL^{-1} (47.17-54.96) for 24, 48, 72 and 96 h respectively in *C. punctatus*. Toxicity of *E. royleana* crude latex was time and dose dependent in the fresh water fish *C. punctatus*. There was a significant negative correlation between LC_{50} values and exposure periods.

Table 1

Lethal concentrations of E. royleana crude latex (mgL^{-1}) (95% confidence intervals) depending on exposure time for C .punctatus (n=10 in three replications)

Tested materials	Lethal concentration	Exposure time (h)			
		24 hrs	48 hrs	72 hrs	96 hrs
E. royleana crude latex	LC_{10}	48.08 (41.30-51.67)	45.02 (37.31-49.00)	41.58 (31.47-46.43)	39.87 (29.20-44.92)
	LC_{50}	61.80 (58.91-65.98)	58.19 (55.23-61.36)	55.17 (51.23-58.18)	52.09 (47.17-54.96)
	LC_{90}	79.42 (72.25-98.00)	75.20 (69.02-91.04)	73.21 (67.05-90.65)	68.05 (63.26-80.98)

(iii) Induction of micronuclei

There was significant induction of MN in the fish specimens upon exposure to different concentration of *E. royleana* crude latex than the control and solvent control group. Increase in the concentration of the *E. royleana* crude latex resulted in higher induction of MN. The highest MN frequency observed in 10% concentration for 96 h in *E. royleana* crude latex. The lowest concentration of *E. royleana* crude latex treated fish specimen induced MN frequency of 0.068% in blood erythrocyte at 5% in 24 hrs which was significantly increased to 0.1358% in 10% for 96 h (Table 2).

Table 2

Induction of MNi in Blood of fishes exposed to different concentrations of E. royleana crude latex

Exposure time	concentration	No. of fishes observed	No. of cells observed	MN% frequencies ±SE
24h	Control	5	10248	0.0096 ± 0.009^{a1}
	Solvent Control	5	10251	0.0192 ± 0.011^{a1}
	5%	5	10268	0.068 ± 0.012^{b1}
	10%	5	10272	0.078 ± 0.012^{b1}
48h	Control	5	10283	0.0194 ± 0.012^{a1}
	Solvent Control	5	10263	0.0292 ± 0.012^{a1}
	5%	5	10253	0.0876 ± 0.013^{b12}
	10%	5	10311	0.1176 ± 0.012^{b12}
72h	Control	5	10331	0.0292 ± 0.012^{a1}
	Solvent Control	5	10271	0.0382 ± 0.027^{a1}
	5%	5	10294	0.0868 ± 0.018^{a12}
	10%	5	10283	0.1214 ± 0.019^{a12}
96h	Control	5	10279	0.0288 ± 0.019^{a1}
	Solvent Control	5	10338	0.0386 ± 0.019^{a1}
	5%	5	10227	0.1176 ± 0.012^{b2}
	10%	5	10271	0.1358 ± 0.019^{b2}

The values with different alphabet (lowercase) superscript differ significantly ($p<0.05$) between concentrations within sampling time. Values with numeric superscript indicate significant ($p<0.05$) difference between sampling times and within concentration.

The observed MN varied from cell to cell. In some cells, MN was found attached to the cell wall or boundary while others were unattached and located near the main nucleus. The incidence of the micronucleated erythrocytes of the both tested materials indicated positive dose response effects over the entire dose range tested (Fig. 1).

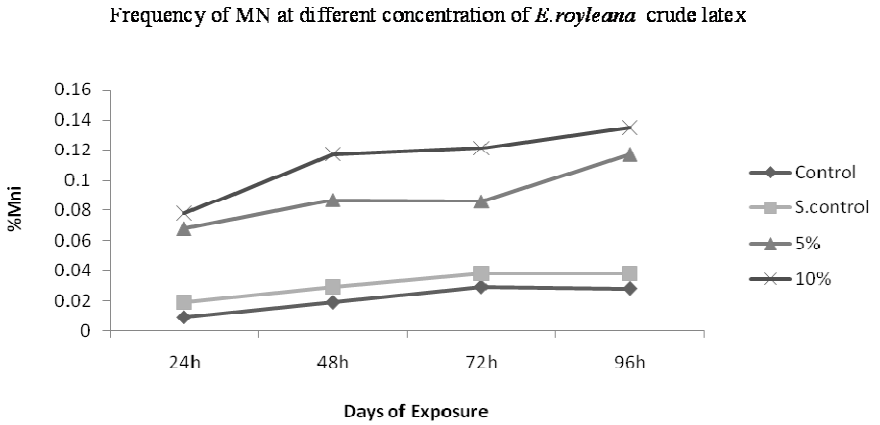
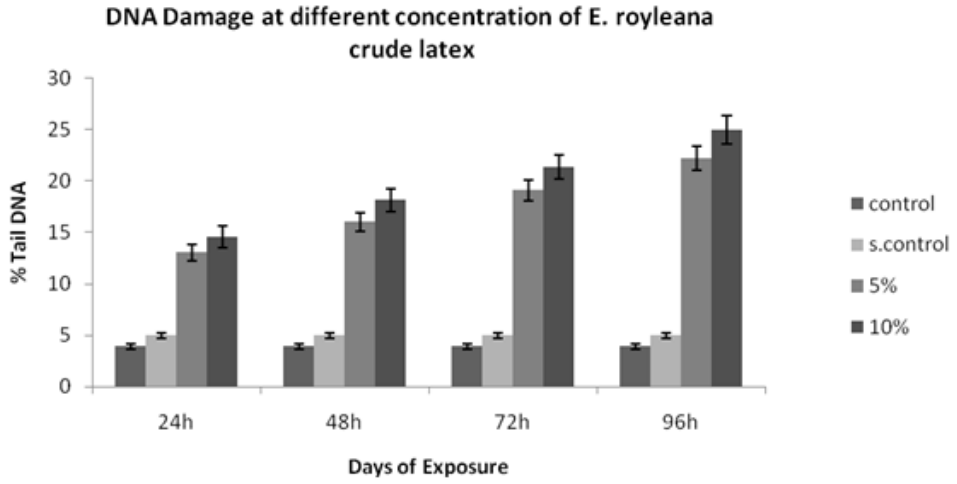


Figure 1
***E. royleana* crude latex concentration response relationship of MN Frequency in the erythrocytes of *C. punctatus* for multiple sampling times**

(iv) DNA Damage

The DNA damage measured as % tail DNA in the erythrocytes of the control and treatment groups indicated that the fish specimens exposed to different concentrations of *E. royleana* crude latex, exhibited significantly higher DNA damage ($P< 0.05$) in their blood cell erythrocytes as compared to the control sample and solvent control sample. The DNA damage in *E. royleana* crude latex was found to be dose and time dependent. The lowest DNA damage was observed at 24 hrs and there was gradual non-linear increase in the DNA damage in all concentration of *E. royleana* crude latex with progression of the experiment and the highest DNA damage was observed on 96 hrs for all treatment groups (Fig. 2).

Figure 2



Highest level of DNA damage was reported at 5% of the LC_{50} of *E. royleana* crude latex (22.19%) for 96 hrs followed by the damage induced by 10% of the LC_{50} (24.92%) for 96 hrs (Table 3).

Table 3
Mean ± SE % tail DNA in erythrocytes of C. punctatus exposed to different concentrations of E. royleana crude latex

E. royleana crude latex	24h	48h	72h	96h
Control	3.91(±0.28)^{a1}	3.91(±0.28)^{a1}	3.91(±0.28)^{a1}	3.91(±0.28)^{a1}
Solvent control	4.95(±0.28)^{a1}	4.95(±0.28)^{a1}	4.95(±0.28)^{a1}	4.95(±0.28)^{a1}
5%	13.01(±0.83)^{a2}	15.99(±0.94)^{b2}	19.05(±1.04)^{c2}	22.19(±1.15)^{d2}
10%	14.55(±1.03)^{a2}	18.14(±1.12)^{b2}	21.35(±1.14)^{c2}	24.92(±1.38)^{d2}

Value with different lowercase alphabet superscript differ significantly (P<0.05) between durations within concentration. Value with different numeric superscript differ significantly (P<0.05) between concentrations within duration.

DISCUSSION

Fishes are often used as sentinel organism for ecotoxicological studies because they play a number of roles in the trophic web, accumulate toxic substances and respond to low concentration of mutagens[55,56]. Therefore, the importance of uses of fish as bio-indicators of the effects of pollution in increasing and can permit early detection of aquatic environmental problems[35,57]. If any medicinal plants become toxic to fish and through food chain it enters to human beings, making it unsafe and questionable for medicinal use. The result shows that LC_{50} at 24 hrs of *E. royleana* crude latex was determined as 61.80 mg/l, which indicated that it is very toxic to *C. punctatus*, in a static system and on the basis of LC_{50} value, 5% and 10% concentrations viz. 3.09 mgL^{-1} and 6.18 mgL^{-1} was determined. It is clear from the present study, that *E. royleana* crude latex significantly increases the frequency of MNi and comet assay after exposure to sub lethal doses in time and dose dependent manner. There is very less data available on the effect of plant product on MNi and comet assay of fish. But several reports are available on genotoxic effect of synthetic pesticides viz. Profenofos, which is one of the most commonly used broad-spectrum organophosphate. Profenofos is commonly used in India for pest control in mango, banana, cotton, and pineapple agriculture[58-60]. Pandey *et al.*,[30] reported that organophosphate pesticide profenofos induces acute toxicity (96-h LC_{50} 2.675 μgl^{-1}) to freshwater fish, *C. punctatus* (Bloch), in a static bioassay. Another broad spectrum organophosphate pesticide, Chlorpyrifos used heavily throughout the world for agriculture and domestic purposes. Ali *et al*.,[41] estimated the LC_{50}-96 h of CPF in a semi-static system in the fish *C. punctatus* and significant effects for both the concentrations and time of exposure were observed in treated fish indicating that MN and comet assays as sensitive and rapid methods to detect mutagenicity and genotoxicity of CPF and other pollutants in fishes. Carbosulfan belongs to the benzofuranyl methyl carbamate group of pesticide and has been widely used in agriculture for broad spectrum control of insect pests of crops and was recently proposed for treatment against pyrethroid- resistant mosquitoes. Nwani *et al*.[61] conducted studies on the mutagenic and genotoxic effect of carbosulfan in fresh water fish *C. punctatus* using micronucleus (MN) test and comet assay. The 96 hrs LC_{50}, estimated by probit analysis in a semi-static bioassay experiment, was 0.268 mg l^{-1}. The MN induction and DNA damage was highest on 96 h at all the concentrations in the peripheral blood and gill cells respectively. Pandey *et al.,*[27] conducted acute toxicity tests to determine the lethal toxicity of an organophosphorus pesticide, malathion, to air-breathing teleost, *C. punctatus* (Bloch) and to study their behavior. The results indicated that malathion is toxic and dose and time dependent increase in mortality rate were also observed in response to test chemicals. Tiwari *et al*.,[21] isolated a compound known as cycloart-24-en-3β-ol

from latex of *E. royleana* which have a significant effect on the predatory fish *C. punctatus*, this compound shift fish respiratory pathway, inhibit energy production and act upon neuro-enzyme acetylcholinesterase (AChE) activity. The mechanism which causes the genotoxic effect in case of organophospahate pesticide cannot be ruled out in case plant *E. royleana*. *E. royleana* is classified as a medicinal plant and its latex is slightly toxic. The stem latex has been shown to anti-inflammatory[22], as well as immunosuppressive effects[23]. However, because of genetic variation in response to drugs by different species, it is difficult to directly translate the results of this study to other animal species or to man[62]. This has been recognized as a limitation in this study. Never the less, in view of the above finding, patients receiving larger doses, or under-going prolonged medication with latex extracts of this plant, should have an immune system evaluation regularly. Finding of this study indicates that the latex of *E. royleana* at higher dose have potent piscicidal activity. So the plants latex cannot be used directly in water bodies or for medicinal used, without studies on their structure activity relationship on non-target organisms. It could be concluded that *E. royleana* contains various bioactive compounds including such with strong cytotoxic activity and could be recommended as a plant of phytopharmaceutical importance. Use of herbal formulation derived from medicinal plants for therapeutic purposes, initially considered not to have any side effect or any toxic insult, now seems to be not safe completely. These formulations are likely to contain quite large number of chemical ingredient, several of them may have adverse effect at one or the other level on consumers. The present experiment clearly reveals the existence of toxic ingredients in the crude latex of *E royleana*. No doubt herbal formulations have curative effect but in view of the presence of certain element of toxic nature it is highly advisable to identify such toxic substances present in the herbal formulation and their elimination to make these herbal formulations as safe as possible.

CONCLUSION

The present study exhibit extensive clastogenic and DNA damaging properties of the herbal crude extract of *E. royleana*, particularly at its higher doses. The formulations, however, have marked medicinal value and therefore, its discontinuation cannot be suggested. Rather attenuation of its toxic potential is needed for making the formulation more effective with no side effects. For this purpose, the characterization of different components and herbal formulation of this crude latex is required to be done so that the identified toxic components can be removed before it is used as medicinal formulation.

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