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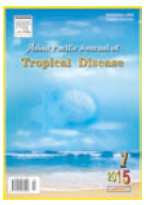

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Estimation of genotoxic and mutagenic potential of latex and methanolic leaves extract of *Euphorbia helioscopia* by comet assay and Ames test

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ABSTRACT

Objective: To investigate the genotoxic and mutagenic potentials of *Euphorbia helioscopia* (Family: Euphobiaceae) latex and methanolic leaves extract.
Methods: Comet assay was adopted to investigate genotoxicity potential and Maron and Ames protocol was followed to evaluate mutagenicity of standardized methanolic leaves extract and latex in concentrations of 10, 5, 2.5, 1.25, 0.625 μg/mL and 1 000, 200, 40, 8, 1.6 μg/mL respectively.
Results: Latex did not damage DNA in lymphocytes and could not produce revertant of *Salmonella typhimurium* (TA98) at any of the experimental concentrations. While, methanolic leaves extract showed 190.00 ± 0.51 revertants in *Salmonella typhimurium* (TA98) plate incubated with extract at 10 μg/mL and also damaged lymphocytes DNA at the same concentration. There was statistically significant difference, $P < 0.05$, at 10 μg/mL/plate concentration with reference to negative control. Pearson correlation was 0.948 showing high consistency between results of comet assay and Ames test.
Conclusion: It was concluded that latex showed no mutagenic and genotoxic potentials at concentrations ranging from 1.6-1 000 μg/mL. Whereas, mutagenic and genotoxic potential threshold level of methanol leaves extract appeared at 10 μg/mL concentration, while remaining used concentrations were devoid of any toxicity.

1. Introduction

Natural products are being consumed from ancient time for the treatment and prevention of various ailments on the basis of knowledge of experienced old persons of the population. Consumption of the herbal medicinal products in the developed countries along with developing countries has been increased from previous few decades[1]. In modern health care system, patient safety is of prime importance. The therapeutic weightage of the drug should be higher than its toxicity in particular disease condition. Genotoxicity and mutagenicity assays were recommended to ensure the quality and safety of natural therapeutic compounds by national and international regulatory agencies[2].

Though medicinal importance of plants has been proved with scientific research but care is needed while selecting the right plant for right indication. Proper authentication and identification of plants is needed to limit their side effects[3,4]. The use of plant based medicines in the treatment of different ailments can be found 5000 years ago[5,6]. More than hundred allopathic medicines are being obtained from plant extracts[7,8]. Digitoxin

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and quinidine are life-saving drugs in cardiac patients, morphine is narcotic analgesic drug used in severely sick patients, colchicine used in gout, physostigmine and pilocarpine used to treat glaucoma, L-hyoscyamine is antispasmodic and taxol (paclitaxel) is anticancer drug, all these key drugs were obtained from plant source[9,10].

Traditionally, leaves and stems of *Euphorbia helioscopia* L. (*E. helioscopia*) are used for vermifuge and febrifuge action, roosted pepper mixed with seed are used in cholera, oil obtained from seeds used in constipation, and roots are used as anthelmintic[11,12]. A number of researchers had explored different pharmacological activities of plant extracts such as insulin secretagogue[13], antibacterial, antifungal, antiviral, phytotoxiciy[14-16], vasodepressor[17], anticancer[18], allelopathic[19], antioxidant[14,20,21], anti-allergic and anti-asthmatic[22], breast cancer resistant protein and P-glycoprotein (ABCB1 and ABCG2) [23], cytotoxic[24] and molluscicidal action[25].

Most of the plant based products possessed genotoxic potential[26,27]. Nonetheless to date, no data are available that can rule out the genotoxic potential of the *E. helioscopia*. This study was planned to probe into genotoxic and mutagenic potential of latex and methanolic leaves extract of *E. helioscopia* by comet assay and Ames test respectively to evaluate the safety level.

2. Materials and methods

2.1. Plant collection

E. helioscopia (Family: Euphorbiaceae) was collected from suburbs of Lahore, Pakistan in the months of February and March. After identification and authentication by a taxonomist of Botany Department, Government College University, Lahore, Pakistan, a voucher specimen (1501) was deposited to the herbarium. Leaves and stem were separated and dried under shade then ground to fine powder separately which were later on used in extraction. The latex was collected in dried bottles by cutting the leafy part from the stem.

2.2. Preparation of extract

The pulverized material from both parts of the plant was extracted separately at room temperature by maceration in water and ethanol as solvents. Then both the materials were extracted sequentially using solvents (petroleum ether, chloroform, and methanol) in the order of increasing polarity by Soxhlet apparatus. The solvents were removed from the extracts on rotary evaporator at 40 ℃.

Latex and methanolic leaves extract were selected in the present research based on their promising *in vitro* antioxidant activities in our previous study[28].

2.3. Standardization of extract and latex

All the extracts and latex have been standardized by applying following analytical techniques: high performance liquid chromatography, UV and Fourier transform infrared spectroscopy fingerprints[29,30]. Quercetin, myricetin, and kaempferol were used as biomarkers in high performance liquid chromatography.

2.4. Preparation of test samples solutions

Phosphate buffer saline was used as vehicle in the preparation of working solutions of test samples. Latex was used in fivefold serial dilutions ranging from 1 000-1.6 μg/mL and similarly methanolic leaves extract concentrations ranged from 10-0.625 μg/mL. The concentrations were selected on the base of our study on cytotoxicity[31].

2.5. Genotoxicity assay

In genotoxicity assay, comet is formed when nucleated cell got damage as a result of exposure to chemicals/toxins[32]. Genotoxic potential of latex (1 000, 200, 40, 8, 1.6 μg/mL) and methanolic leaves extract (10, 5, 2.5, 1.25, 0.625 μg/mL) was determined by comet assay following the protocol of Singh *et al*.,[33]. Briefly, normal melting point agarose gel (0.75%) was poured (80 μL) on clean glass slides (n = 36) and allowed to solidify for 45 min. Lymphocytes, separated from blood of sheep, were poured (100 μL) into 96-well plate and incubated with selected concentrations for 2 h at 37 ℃. The suspension pipetted out from each well (100 μL) was poured on previously prepared agarose slides. Later on, low melting agarose gel (0.5%) was poured (80 μL) on each slide and allowed to solidify. All of the slides were dipped in (500 mL) of lyses solution kept under refrigeration for 12 h. On second day, lyses solution was removed and slides placed in (500 mL) alkaline buffer for 25-45 min. All of the slides were placed in electrophoresis chamber having buffer [Tris base, 10.8 g, boric acid (MP Biomedicals, LLC), 5.5g and disodium ethylene diamine tetraacetic acid, 0.93 g were dissolved in 700 mL of double-distilled water and finally, volume was made upto 1 000 mL] and run at 25 v for 45 min. Slides were removed from electrophoresis chamber, rinsed two times with neutralizing buffer (Tris base, 24.25 g was mixed with 400 mL of double-distilled water and final volume was 500 mL) and finally stained with 50-80 μL of ethidium bromide solution. Slides were observed immediately under fluorescent microscope at 100× magnification with 560 nm excitation filter and 590 nm barrier filter. Sodium azide (Sigma-Aldrich) and lymphocytes alone were used as positive and negative control respectively.

Results were presented as olive tail moment (tail DNA% × tail moment) and extent tail moment (tail DNA% × length of tail) in arbitrary units[33,34]. All the values were calculated in pixels using computerized image analysis software (image j).

2.6. Mutagenecity assay

Mutagenic potential of latex (1 000, 200, 40, 8, 1.6 μg/mL) and methanolic leaves extract (10, 5, 2.5, 1.25, 0.625 μg/mL) was determined by Ames test following the protocol described by Maron and Ames[35]. Histidine dependent *Salmonella typhimurium* (TA98) (*S. typhimurium*) without (- S9) metabolic activation mixture was used to check the mutagenicity by plate incubation method. Sodium azide (1%) and normal saline were used as positive and negative control respectively.

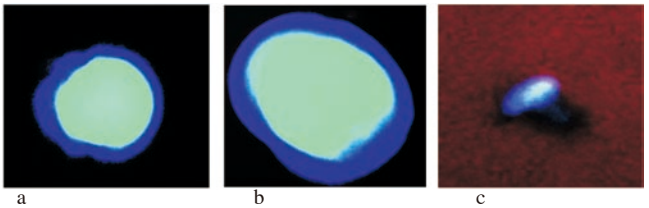
Briefly, all the selected concentrations of latex and methanolic leave extract, positive and negative control (200 μL) were incubated with bacterial culture (100 μL) and 0.2 mol/L phosphate buffer (500 μL) at 37 ℃ in sterile tubes for 20 min. Molted ager (2 mL) containing 9.6 μg/mL histidine (0.05 mmol/L) and 12.4 μg/mL biotin (0.05 mmol/L) was added to each test tube. Then each test tube was poured on separate sterile glucose minimal agar plate. Top agar layer took 2-4 min to solidify, then all the plates were inverted and incubated at 37 ℃ for 48 h and the His+ revertant colonies were manually counted. Thinning of background lawn (*i.e.* auxotrophic background) or decreased number of histidine revertant colonies (His+) was used as indicator to test the sample toxicity. Results were described in terms of mean number of revertants per plate ± SD for each concentration.

2.7. Statistical analysis

Results were presented as mean ± SD. Statistically significant difference was measured with ANOVA followed by *post hoc* Tukey Multiple comparison on SPSS version 12. Treatment groups were significantly different from negative control group at $P < 0.05$. Pearson correlation was applied to find out relationship between comet (genotoxicity) and Ames (mutagenicity) tests. Correlation was significant at 0.01 level (2-tailed).

3. Results

Latex produced no comet at selected concentration range indicating that latex did not cause DNA damage (Figure 1a). The highest concentration (10 μg/mL) of methanolic extract of leaves caused DNA damage whereas other used concentrations were safe (Figure 1b and 1c). The result was presented as olive tail moment (%tail DNA × tail moment) and extent tail moment (%tail DNA × tail length) in arbitrary units (Figure 2a). Tail and head length of comet was measured to calculate tail moment (Figure 2b). Tail and head length of comet formed at 10 μg/mL were 26.16 ± 0.07, 36.22 ± 0.02 respectively. Tail moment, cell DNA intensity and % tail DNA were 34.81 ± 0.02, 68.86 ± 0.16 and 38.13 ± 0.12 respectively. Olive tail moment was 1327.31 ± 0.05 and extent tail moment was 997.48 ± 0.08.



a b c

Figure 1. Genotoxicity potential of latex and methanolic leaves extract of *E. helioscopia* by comet assay.
a: treated with latex showing no comet; b: showing no comet when treated with methanolic leaves extract from 0.625-5 μg/mL; c: showing comet with methanolic leaves extract at 10 μg/mL.

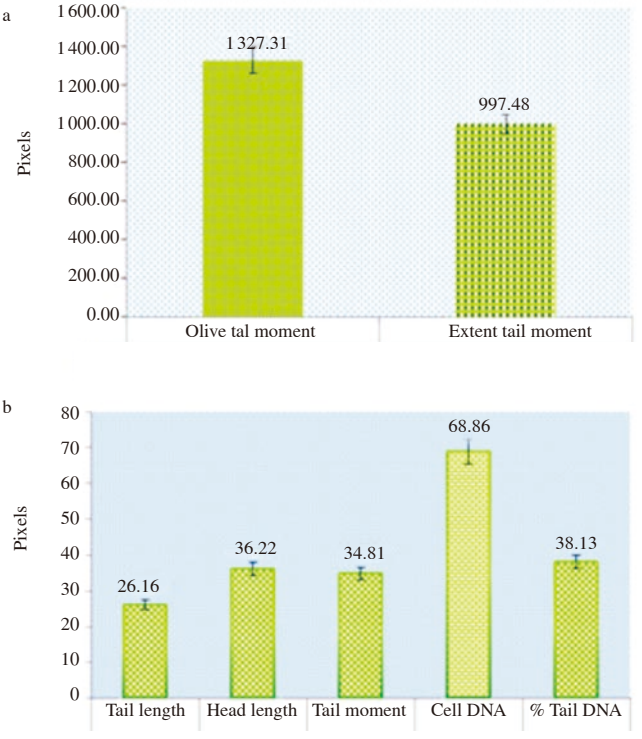
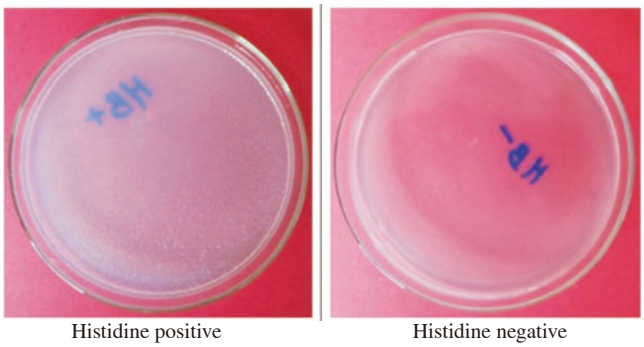


Figure 2. Quantification of COMET appeared at concentration of 10 μg/mL methanolic leaves extract of *E. helioscopia*.
a: Comet quantifying parameters; b: Supportive parameters to quantify comet.

Prior to perform the mutagenicity test, *S. typhimurium* strain TA98 was confirmed and standardized on *Salmonella Shigella* agar medium. Pink colonies were developed within 24 hours which turned yellow with time and after 48-72 hours a black spot appeared in the center of each colony.

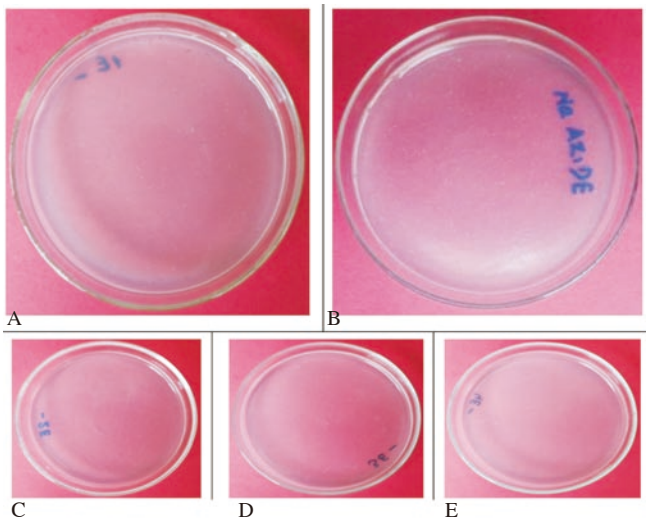
Ames test was standardizes on histidine positive and negative media to check natural revertants (Figure 3). No revertants of *S. typhimurium* were found in latex treated plates.



Histidine positive Histidine negative

Figure 3. Mutagenicity assay (Ames test) standardization by growing histidine dependent *S. typhimurium* on histidine positive and negative media plates.

Revertant *S. typhimurium* colonies were revealed on Petri plate on which bacteria treated with the highest concentration (10 μg/mL) of methanolic leaves extract while rest of the concentrations showed no mutagenic potential (Figure 4). There was statistically significant difference, $P < 0.05$, at 10 μg/mL/plate concentration with reference to negative control (Table 1). High consistency was found between results of comet and Ames tests by Pearson correlation ($r = 0.948$).



A B C D E

Figure 4. Mutagenic potential of methanolic leaves extract of *E. helioscopia* by Ames test.
A: 10 μg/mL (plate showing revertants); B: Positive control; C: 5 μg/mL; D: 2.5 μg/mL; E: 1.25 μg/mL; C-E: Plates showing no revertants.

Table 1

S. typhimurium revertants appeared at various concentrations of latex and methanolic leaves extract of *E. helioscopia* in Ames test.

Methanolic leaves extract		Latex	
Concentration (μg/mL/plate)	No. of revertants	Concentration (μg/mL/plate)	No. of revertants
Negative control	0	Negative control	0
Positive control	1350 ± 0.21*	Positive control	1350 ± 0.21*
0.625	0	1.6	0
1.25	0	8	0
2.5	0	40	0
5	0	200	0
10	190 ± 0.51*	1 000	0

*: $P < 0.05$ when compared with negative control; Values were expressed as mean ± SD; Positive control: Sodium azide.

4. Discussion

There are number of biomarkers to detect the DNA damage in cells as a consequence of chemical exposure, but DNA damage measured by single cell gel electrophoresis (comet) is quite rapid and sensitive method. Researchers focused on this technique because of its low cost and sensitivity of method. Any nucleated cell can be employed in this assay[33,34,36-39]. The sensitivity of method allows detecting the DNA damage on individual cell level and it has capability to measure genotoxic potential, quantitatively in prokaryotic and eukaryotic cells[40-47]. Methanol crude extracts of *Euphorbia hirta* (*E. hirta*) showed genotoxicity on *Allium cepa* assay at 1 000 μg/mL as reported Bajpayee *et al*., and Lah *et al*., used comet assay to quantify DNA damage[44,48]. Scoring of comet, on the basis of nucleoids with and without tail diameter, method can also be used to quantify the comet[32,47,49]. *E. hirta* extracts showed DNA damage in dose-dependent manner with comet assay[50]. *Calendula officinalis* (Asteraceae) is herbal medicine, traditionally used in the treatment of several disease showed no DNA damage with comet assay[51].

Ames test is more quick and reliable method to evaluate mutagenic potential of plants and chemicals[32,49,52-54]. *E. hirta* extracts (aqueous and methanol) did not show any mutagenic activity on *S. typhimurium* strains TA98 and TA100[55].

Folklore use of herbal products is due to zero people interest in drugs of natural origin and their faith on safety of natural drugs but safety is strictly linked with therapeutic dose for particular indication[56]. Each drug possessed toxic effects but a valuable pharmacologically active compound should have balance between toxic or untoward and therapeutic effects[57]. To ensure the safety and efficacy of natural products, a battery of genotoxic and/or mutagenicity assays are required to be performed to screen the toxicity mechanism[58]. No single test can gather enough data to forecast the chemical hazards to human health.

Mutagenicity and genotoxicity studies were conducted on variety of plants *e.g. Acacia nilotica*, *Juglans regia, Terminalia chebula, Pothomorphe umbellate* and *Physalis angulate*[59-61].

Latex showed no mutagenic and genotoxic potentials and is safe to use for various pharmacological activities. Less than 10 μg/mL concentration of methanolic leaves extract was found safe.

Conflict of interest statement

We declare that we have no conflict of interest.

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