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Moringa oleifera Lam. leaves prevent Cyclophosphamide-induced micronucleus and DNA damage in mice

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Abstract

Chemoprotective effect of ethanolic extract of *Moringa oleifera* Lam leaves was evaluated on cyclophosphamide (CP)-induced genotoxicity in the mouse. Animals were pre-treated with the extract for seven consecutive days at doses of 250, 500, 1000 and 2000 mg/kg b.w. Micronucleus in bone marrow and comet (DNA damage) in the liver were performed. Cyclophosphamide was administered intra-peritoneally on day 7 and Mice were sacrificed after 24 hours. In CP treated animals, statistically significant induction of micronuclei in polychromatic erythrocytes (PCE) was recorded. However, in the animals pre-treated with the extract, the percentage of CP-induced MN decreased with increasing concentration of the extract. Results of comet assay showed similar decrease in DNA damage in mice pre-dosed with the extract. These results point out to the presence of chemopreventive phytoconstituents in the crude extract offering protection against CP-induced genotoxicity in the mouse.

Keywords: *M. oleifera*; Anti-genotoxic; Micronucleus assay; Comet assay; Chemoprevention.

Introduction

Much focus has been laid on the role of dietary constituents as anti-mutagens and anti-carcinogens since they are non-toxic in nature [1]. *Moringa* species have long been recognized by folk medicine practitioners as having value in tumor therapy [2]. *Moringa oleifera* Lam. is the most extensively cultivated species of the Moringaceae family, found in various parts of the world. Many phytochemicals have been isolated from various parts of the plant, *viz*., phenolic compounds such as quercetin and kaempferol, flavonoids, anthocyanins, carotenoids, vitamins, minerals, amino acids, sterols, glycosides and alkaloids. It contains unique group of compounds called glucosinolates and isothiocyanates [3,4]. Recently isothiocyanates and niacimicin from this plant were shown to be potent inhibitors of cancer [5-7].Niazimicin also inhibited tumor promotion in a mouse two-stage DMBA-TPA tumor model. Seed pod extracts of *Moringa* have been demonstrated to prevent skin tumors in mice [8]. The leaves are highly nutritious, being a significant source of β-carotene, Vitamin C, protein, iron and potassium and have diverse curative properties [9 -17]. Its leaves are also used as nutritional supplement and growth promoters [18-22]. An immuno enhancing polysaccharide [23] has been reported from the

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leaves. Rich source of ascorbic acid and flavonoid pigments such as kaempferol, rhamnetin, isoquercitrin, and kaempferitrin in leaves of *M. oleifera* are known for their antioxidant properties. [24,25]. Ethanolic extract of leaves of *M.oleifera* have shown antimicrobial activity [26,27]. Radio protective effect of *M.oleifera* leaves has been established wherein radiation-induced chromosomal aberrations and micronuclei were suppressed by pre-treatment with methanolic extract [28].

Given its rich nutritional value and abundant therapeutic efficiency, we attempted to study the chemo protective effect of *Moringa oleifera* leaves on CP-induced DNA damage using micronucleus and comet assays. Ethanol was used for extraction of the phytochemicals since it is a regarded as a powerful solvent capable of isolating majority of the plant constituents.

Evaluation of micronucleus induction is the primary *in vivo* test in a battery of genotoxicity tests and is recommended by regulatory agencies around the globe as part of product safety assessment. The assay, when performed correctly, detects both clastogenic and aneugenic effects [29].Comet assay, which detects chemically induced DNA damage, has been used in a number of in vivo studies and has the advantage of investigating a wide variety of organs for different classes of DNA damage by reactive carcinogens [30]. The alkaline version of the Comet assay has been extensively employed for genotoxicity investigations as it detects double- and single-strand breaks, alkali-labile sites that are expressed as single-strand breaks and single-strand breaks associated with incomplete excision repair [4]. The present study investigates the in vivo effect of ethanolic extract of *M.oleifera* leaves on CP-induced genotoxicity in the bone marrow and liver.

Material and Methods

Preparation of the ethanolic extract

Fresh leaves of *Moringa oleifera* were collected from International institute of Biotechnology and Toxicology, India. CP (Cyclophosphamide- CAS N. 50-19-0) and Giemsa were obtained from M/S Sigma, USA and other chemicals used in the study were obtained from M/S Sigma and Merck, India. The leaves were washed with distilled water and shade dried. The dried leaves were extracted using 70% ethanol in the proportion of 1:10(w/v), leaves: solvent and mechanically stirred at room temperature for 24 hours. The crude extract thus obtained was filtered and lyophilized [31].

Animals and treatment

The use of animals was approved by the Institutional Animal Ethics Committee of IIBAT, where the study was conducted. Healthy adult Swiss albino female mice, 6-8 weeks old with average body weight (b.w) of 20 to 30g were used for the study. The animals were selected randomly and numbered with ear tag and housed in autoclaved polycarbonate boxes with rice husk bedding and steel wire top. The temperature, light and humidity were maintained at 25 ± 2 ºC, 12 h dark/light and 55±7%. Gamma irradiated rodent pellet feed (M/S.Tetragon chemie pvt ltd. Bangalore, India) and reverse osmosis water were provided *ad libitum*. The animals were acclimatized for five days at 24 ± 1 ºC with alternating cycles of 12 h light/dark period.

Fifty female mice were randomly distributed into ten groups with five animals each. The negative control group, given distilled water by oral gavage; the positive control group, given a single intraperitoneal injection of the equivalent of 40 mg/kg b.w of cyclophosphamide dissolved in distilled water; the treatment group was given 250, 500, 1000 or 2000 mg/kg b.w of ethanolic extract of *M.oleifera* each day for 7 days by oral gavage; and the experimental group, given the same treatment as the treatment group except that on the seventh day the mice also received the same treatment as the positive control group. The ethanolic extract and solvent control were administered through oral gavage (10 ml/kg b.w) and CP was administered intra peritoneally. All animals were sacrificed by cervical dislocation on day eight. Femur bone and liver samples were collected.

Micronucleus Assay

Bone marrow were flushed out of the femur bone into the centrifuge tubes containing 2 mL of fetal calf serum (Gibco,USA) and centrifuged at 1000 rpm for 10 min. Smears were prepared a clean slide. The bone marrow preparations for micronucleus analysis were made according to Schmid [32] and Krishna and Hayashi [29]. The slides were fixed with methanol and stained with Giemsa. Two hundred cells per animal were counted for assessment of cytotoxicity. Two thousand poly chromatic erythrocytes (PCEs) were analyzed for presence of micronucleus using oil immersion magnification (Carl Zeiss axio star plus, Germany). To evaluate cytotoxicity 200 cells (PCE and NCE) were scored. Two thousand polychromatic erythrocytes (PCE) per mouse were scored and the number of micronucleated PCE (MnPCE) was recorded. To compare the frequencies of MnPCE and normal PCE between treated and control groups the results were expressed as mean ± standard deviation and analyzed statistically using Mann-Whitney U-test.

Comet assay

Comet assay was performed following the method of Singh *et al* [33] with necessary modifications. The liver samples were minced in ice cold phosphate buffered saline (PBS) and allowed to settle. The supernatant containing single cells was mixed with 0.7% low melting agarose dissolved in PBS and casted on to frosted microscope slides pre-coated with 1% normal melting agarose. The cells were then lysed for 1 h at 4°C in a buffer consisting of 2.5 M NaCl, 100 mM EDTA, 1% Triton X-100, 10 mM Tris, pH 10. After lysis, the slides were placed in an electrophoresis unit, allowing DNA to unwind for 20 min, in the electrophoretic buffer consisting of 300 mM NaOH, 1 mM EDTA, pH > 13. Electrophoresis was conducted at room temperature for 40 min at electric field strength 0.56 V/cm (300 mA). The slides were then neutralized with 0.4 M Tris, pH 7.5, stained with 2 µg/ml Ethidium Bromide. The nucleoids were observed at 400x magnification of a fluorescence microscope (Carl Zeiss, Germany). For DNA damage analysis, 2 slides/animal was prepared, 50 cells were scored per slide. Images were analyzed according to the method of Collins *et al* [34]. Comets were scored visually as belonging to one of five classes according to tail intensity and given a value of 0, 1, 2, 3, or 4 (0 - Undamaged, 1-mild, 2 -moderate, 3 -severe and 4 -extensive). Thus, the total score for 100 comets could range from 0 (all undamaged) to 400 (all maximally damaged). The percentage of damaged cells was calculated. The `arbitrary units' was used to express the extent of DNA damage and were calculated as follows:

$$AU = \sum_{i=0}^{4} N_i \times i$$

Where Ni=number of cells in i degree; i=degree of damage (0, 1, 2, 3, 4). Chi –square test was used to compare DNA damage profile between control and treatment groups. $P<0.05$ was considered statistically significant.

Results

The frequency of MnPCE ± the standard deviation (SD), for female Swiss albino mice in the distilled water negative control group was 1.52 ± 0.25. For CP -treated groups the frequency of MnPCE was 23.6 ± 4.3, which was significantly higher (U-test, $p < 0.05$) when compared with the solvent control (Table 1). In the treatment group, the MnPCE frequency at all concentrations was comparable to the negative control group suggesting that the extract is not genotoxic to the bone marrow cells of mice. For the experimental groups pre-treated with the extract at doses 250,500, 1000 and 2000 mg/kg b.w, followed by CP administration the MN frequencies appeared to gradually decrease with increase in concentration of the extract suggesting a protective effect. At doses 250, 500 and 1000, there was statistically significant micronuclei frequency, however at the highest dose employed (2000mg/kg b.w) , frequencies of MnPCE was comparable to the negative control

group, suggesting a protective effect of the extract. This indicates that upon oral administration, a higher dose of *M.oleifera* extract is required to ameliorate the DNA damage induced by genotoxins such as cyclophosphamide. The treatment group had no statistically significant MnPCE values, reiterating the fact that the extract has no pro-mutagenic components.

Table 1. Results of the micronucleus assay.

Group	Description	Treatment	PCE:NCE (200 Cells) Mean ± SD	%MnPCE Mean ± SD (2000 cells)
I	Treatment group	250	0.91± 0.10	0.82 ± 0.36
II		500	0.90 ± 0.10	0.53 ± 0.16
III		1000	0.85 ± 1.16	0.68 ± 0.15
IV		2000	0.71 ± 0.18	0.51 ± 0.28
V	Experimental group	250 +CP	0.82 ± 0.16	11.04 ± 3.42**
VI		500+CP	0.86 ± 0.10	6.71 ± 1.81**
VII		1000+CP	0.67± 0.16	4.35 ± 0.94**
VIII		2000+CP	0.83 ± 0.14	2.27 ± 0.87
IX	Controls	Solvent	0.73 ± 0.10	1.52 ± 0.25
X		Positive	0.70 ± 0.10	23.6 ± 4.3**

** P<0.05 Mann –Whitney 'U' test. CP – Cyclophosphamide, SD – Standard Deviation, PCE – Poly Chromatic Erythrocyte, NCE – Normo Chromatic Erythrocyte, MnPCE – Micronucleated Poly Chromatic Erythrocytes.

Comet assay was performed in the liver cells. In the negative control group, the percentage of DNA damage was 15.25 ± 3.10, which is the baseline DNA damage possibly due to endogenous and environmental factors. Cyclophosphamide induced DNA damage mean percentage was 87.26 ± 2.23. In the experimental group, similar to the results of the micronucleus assay, the mean percentage of DNA damage was on par with the negative control (Table 2). In the treatment group, the mean percentage of DNA decreased with increase in the concentration of the extract. The study implies that oral administration of *M.oleifera* ethanol extract has potential in inhibiting cytotoxic and clastogenic damage produced by CP. Figure 1(a) represents the effect of the ameliorating effects of the extract on CP-induced DNA damage. Figure 1(b) represents the lack of genotoxic effect of the extract.

Discussion

The results of the present study clearly show that ethanolic extract of *M.oleifera* leaves have a dose-dependent modulatory effect on CP-induced micronuclei. Cyclophosphamide metabolizes into its mutagenic intermediate phosphoramide mustard through enzymatic and nonenzymatic metabolic activation. It is initially acted upon by the mixed function oxygenases to form 4-hydroxy-CP which exists as aldophosphamide. Aldophosphamide further metabolizes to form

cytotoxic (acrolein and phosphoramide mustard) and non-cytotoxic (4-ketocyclophosphamide, carboxyphosphamide and aldophosphamide) intermediates [35].

Table 2. Results of comet assay.

Group	Treatment	Damage category					% DNA damage	AU
		0	1	2	3	4		
I	250	85.00 ± 2.12	13.25 ± 1.06	2.00 ± 2.83	0.00 ± 0.00	0.000 ± 0.00	15.25 ± 1.00	17.50
II	500	82 .00± 1.41	13.00 ± 5.66	5.00 ± 7.07	0.00 ± 0.00	0.00 ± 0.00	18.00 ± 1.41	23.00
III	1000	84 .00± 1.00	8.00 ± 1.53	5.00 ± 1.15	5.00 ± 4.36	1.00 ± 1.00	19.00 ± 5.29	36.67
IV	2000	93.00 ± 2.60	6.07 ± 2.88	0.75 ± 1.50	0.25 ± 0.50	0.00 ± 0.00	7.07 ± 2.60	8.50
V	250 +CP	22.27 ± 7.71	11.16 ± 5.34	11.44 ± 2.14	51.44 ± 11.20	3.70 ± 6.42	77.73 ± 7.71**	203.33
VI	500+CP	31.50 ± 6.36	23.61 ± 15.01	40.29 ± 3.83	4.50 ± 4.95	0.00 ± 0.00	68.41 ± 6.23**	117.70
VII	1000+CP	40.99± 19.68	22.61 ± 6.20	21.77 ± 3.65	13.08 ±8.85	1.55 ± 1.98	59.01 ± 19.68**	111.60
VIII	2000+CP	69.00 ± 4.24	8.5 ± 3.54	10.00 ± 1.41	5.5 ± 0.71	7.00 ± 1.41	31.00 ± 4.24**	73
IX	Solvent	84.75 ± 3.10	4.75 ± 0.96	3.25 ± 1.89	3.5 ± 1.29	3.75 ± 1.26	15.25 ± 3.10	36.75
X	CP	12.75 ± 2.22	2.51 ± 1.01	1.75 ± 1.71	2.25 ± 2.06	80.75 ± 2.06	87.26 ± 2.23	334.5

****** P< 0.05 (Chi square test: with control), 0 -Undamaged, 1-Mild, 2 –Moderate, 3 -Severe, 4 –Extensive, AU – Arbitrary Unit

The protective effect may be attributed to the potential involvement of the phytomolecules of the extract to interfere with the enzymes participating in the biotransformation of CP to cytotoxic metabolites. Free radical scavenging represents one of the important strategies in antimutagenesis and anticarcinogenesis. Leaves of *M. oleifera* contain rich amount of antioxidants [36, 37]. It is reported that *M.oleifera* has 46 antioxidants and 36 anti-inflammatory compounds naturally occurring in it [38-41]. A possible explanation for the protective effect recorded in the present investigation could be the involvement of its antioxidant and scavenging properties. Antioxidants provide protection by scavenging reactive oxidative species (ROS) that damage DNA and initiate diseases such as cancer. Ethanolic extract of *M.olerifera* leaves have been reported to contain tannins, saponins, flavonoids, glycosides and terpenoids [27]. Antioxidant vitamins, flavonoids, glucosinolates and organo-sulfur compounds have been proven to have antimutagenic or anticarcinogenic potential [42, 43].

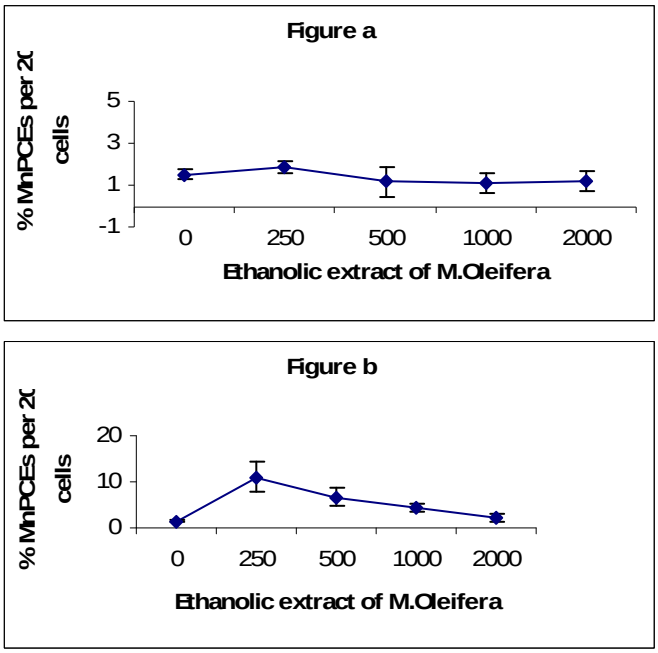

Figure 1. (a) represents the lack of genotoxic effect of ethanolic extract of *M.oleifera*. (b) represents the effect of the ameliorating effects of the extract on CP-induced DNA damage.

Therefore protection against the clastogenic effects of CP could arise from the scavenging ability of *M.oleifera* leaves to trap hydroxyl radicals originating from metabolites of CP with an OH functional group. A similar decrease in MnPCEs induced by CP has been described for other antioxidants like stobadine, eugenol which possess the potential to protect DNA from reactive oxygen species and metabolism-dependent mutagens [44].

Aqueous and methanolic extract of *M. oleifera* leaves have been reported only to limited extent for their antioxidant properties [25]. In that regard, the anti-genotoxic effect delivered by the ethanolic extract of *M.oleifera* leaves could probably be attributed to the appreciable amount of antioxidant constituents. However, such comparative studies need to be conducted.

Our study shows that in the mouse micronucleus test *M. oleifera* ethanolic extract prevents the genotoxic effects of CP when administered for a period of one week. Furthermore, the extract was non-clastogenic because it did not induce chromosome breakage in the bone marrow cells. Similar results were recorded in the comet assay. It reduces the percentage of DNA damage induced by CP in the liver cells. The results show that the extract has anti-genotoxic effects on CP-induced lesions in mice. The present results eventually lead us to conclude that ethanolic extract of *M.oleifera* leaves possess anti-genotoxic phytoconstituents.

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