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Original Research Article

Evaluation of the Genotoxic and Antigenotoxic Potential of *Plantago major* Methanolic Extract

Sanjeev Karmakar[1], Danswrang Goyary[1], Aadesh Upadhyay[1], Dhruba Kumar Jha[2], Pronobesh Chattopadhyay[1]*

[1]Defence Research Laboratory, Post Bag No. 2, Tezpur-784 001, Assam, India.
[2]Gauhati University, Guwahati, 781 014, Assam, India

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ABSTRACT

Plantago major L. (Plantaginaceae) is a perennial herb with rosette of leaves found throughout India and widely utilized traditional medicine of Indian-Ayurvedic and Chinese-medicine system. The present study was conducted to evaluate the *in vivo* genotoxic and/or antigenotoxic potential of *P. major* methanolic extract (PmME) using Organization for Economic Co-operation and Development (OECD) guidelines. The genotoxic and antigenotoxic potential of PmME was assayed using the comet assay on lymphocyte, the micronucleus (MN) and chromosomal aberration (CA) tests in bone marrow cells of albino mice. The results exhibited that none of the tested doses of PmME demonstrated genotoxic effects by the comet test, or clastogenic effects of the MN test. On the other hand, for all cells evaluated, the three tested doses of the PmME promoted inhibition of DNA damage induced by cyclophosphamide. Based on these results, it was concluded that PmME contains no genotoxic or clastogenic effects in our experimental conditions. However, it showed a significant decrease in DNA damage induced by cyclophosphamide. These findings therefore suggest that the antigenotoxic properties of PmME may be of great pharmacological importance and safe to use as an immunomodulatory agent.

1. Introduction

From the time immemorial therapeutic plants and their parts have been utilized to cure numerous human diseases. North East part of India is one of a kind because of its rich biodiversity and profound customary learning. Home grown medicines are generally utilized and of quickly developing therapeutic framework and economic significance. As indicated by WHO report, 2002-2005, up to 80% of the number of inhabitants in Africa Continent utilizes conventional herbal medication for different diseases. Also, chemotherapeutic medications are being questioned because of the developing concern on negative/unfavorable impacts of which are pumping expanded enthusiasm for natural remedies. *Plantago major* L. is a perennial herb with a rosette of leaves ovate to elliptical with parallel venation. The flowers are little, greenish chestnut on long non-ramified spikes.

P. major is accounted for its wound healing potential in numerous countries of the world[1]. Genotoxicity of extract concentrates of *P. major* was examined by utilizing the somatic mutation and recombination test (SMART) in *Drosophila melanogaster*[2]. *Terminalia chebula, Acacia nilotica* and *Juglans regia* concentrates indicated fundamentally expanded DNA harm at different fixations[3]. *Arctium lappa* root concentrate found to incite aggregate number of chromosomal distortions and micronuclei developments[4]. The comet assay and micronucleus test were utilized to assess their harmful and clastogen impacts of

Garcinia achachairu seed concentrates[5]. The safety appraisal with respect to substances equipped for prompting mutations is of vital significance according to US FDA and OECD rules. The enthusiasm for herbal meds utilized for the aversion and helpful treatment of illness has expanded because of the increased resistance of microorganisms to synthetic antibiotics, increasing expenses and reactions of manufactured medications for the support of individual wellbeing. However, concerns have been raised over the absence of value control and experimental proof for the security of herbal pharmaceutical[6]. In addition, couple of logical examinations have investigated the security and harmfulness of natural pharmaceuticals[7,8,9]. Assessment of potential genotoxic action of *P. major* methanolic (PmME) concentrates was done utilizing a standard series of tests as per OECD rules. These tests incorporated comet test, micronucleus test and chromosomal aberration test.

We examined the mutagenic and clastogenic impacts of PmME in a mixed bag of test frameworks utilizing *in vivo* trial model considering its proclaimed medicinal significance and the requirement for practical information on safety. Herbal drugs have been generally accepted because of the prevalent view that they display insignificant side effects[10]. However, a few herbal drugs have been accounted for to go about as mutagens and/or cancer-causing agents[11,12,13]. These genotoxic effect frequently prompt tumor[14], Alzheimer's

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Corresponding Author: Sanjeev Karmakar*, Defence Research Laboratory, Post Bag No. 2, Tezpur, Assam, 784001; E-mail: sanjeevkarmakar@gmail.com

disease[15] and other constant degenerative sicknesses, for example, atherosclerosis and heart ailments[16]. As being what is indicated, genotoxicity investigations of both actually happening and manufactured substances are of extraordinary premium on account of the broad and regularly ceaseless utilization of powerful or incapable natural cures, of advanced restorative items, of nourishment ingredients, and in addition of other family unit and ecological chemicals[17].

2. Materials and Methods
2.1 Plant Material and Preparation of Extract

Plant material: Ariel parts of *P.major* L. (Plantaginaceae) including roots were collected from Tezpur, Assam (India). A voucher specimen (BSI, ERC Accession No. 081169) was kept at the Botanical Survey of India, Shillong. About 100 g of shade dried leaf powder was successively extracted using 70% methanol by Accelerated Solvent Extractor (ASE 1.5, Dionex, USA) as described earlier[10] and concentrated in a rotary evaporator (Rotavac, Heidolph2, Germany) under reduced pressure.

2.2 Animals and dosing

Experiments were carried on 6-weeks-old Balb/c female albino mice, measuring 20-25 g. The animals were obtained from the Animal facility of Defence Research Laboratory, Tezpur, India, and kept in polyethylene boxes, in a temperature-controlled environment (25°C ± 4°C, 50-60% relative humidity and a 12 h light/dark cycle). They were fed with standard pellet diet (Ashirwad Industries, N. Delhi, India) and water *ad libitum* throughout the experiment. The experiments were performed according to the Institutional Animal Ethical Committee guidelines (1127/bc/07/CPCSEA). The Balb/c albino - mice were isolated into eight groups: (i) control (C), (ii) positive control (Cyclophosphamide, CP) and (iii-v) three experimental groups, consisting 500, 1000 and 2000 mg/kg of PmME alone by gavage, while a further (vi-viii) three groups got the same doses in addition to CP (40 mg/kg bw) for 48 hours. The dosage of PmME was determined based on the toxicity study and administered by gavage[18]. The positive control was administered at a single dose by intraperitoneal injection for 48 hours. All the mice were sacrificed after the treatments by cervical dislocation and the respective cells were prepared for further analysis.

2.3 Comet measure

The comet assay was carried out by the method described earlier[19,20]. The blood samples were collected from the retro orbital plexus after treatment and before euthanasia and treated with 1× RBC lysis buffer for 10 min at 25°C and leukocytes were isolated and suspended in 50 µl of phosphate buffered saline (pH 7.5). Cell counting was performed using an automated cell counter (Scepter™ 2.0, Millipore). Cell viability was determined by trypan blue dye exclusion. The number of trypan blue negative cells was considered to be the number of viable cells and was greater than 85%. An equal aliquot of leukocyte cell was mixed with 0.5% low melting point (LMP) agarose at 37 °C, and rapidly spread onto microscope slides, precoated with 1% normal melting point (NMP) agarose. The slides were coverslipped and allowed to polymerize at 4 °C for 20 min. The coverslips were gently removed and the slides were then immersed in cold, freshly

prepared lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10.0 adjusted with NaOH and 1% sodium lauryl sarcosine plus Triton X-100 and Dimethylsulfoxide). The slides were allowed to stand at 4°C for 1 h and then placed in a high pH (>13) electrophoresis buffer (300 mM NaOH, 1 mM Na2-EDTA, pH 13.0) at 4°C for 20 min prior to electrophoresis, to allow DNA unwinding. The electrophoresis run was performed at 4°C under dim light at 300 mA and 25 V for 30 mins. The slides were then submerged in a neutralization buffer (0.4 M Tris–HCl, pH 7.5) for 15 min, and stained with 20 µg/ml 4', 6-diamidino-2-phenylindole (DAPI) for 10 min and cover slipped. The material was evaluated immediately at 400 × magnification, using a fluorescence microscope. The image analysis was performed using TriTek Comet Score™ Free version. For each treatment, 150 randomly selected non-overlapping comets per sample were scored (50 from each of three replicate slides). The cells were scored by taking the total score of three different comet classes: Class I: tail shorter than the diameter of the head (nucleus) Class II: tail length 1-2 times the diameter of the head and Class III: tail length more than twice the diameter of the head.

2.4 Micronucleus (MN) assay

The MN test was conducted in accordance with OECD guideline 474[21] and the protocols were followed as recommended[22,23]. The same group of animals used in the comet assay was also used for this protocol. The bone marrow from one femur was flushed out using 2 ml of saline (0.9% NaCl) and centrifuged for 10 min. The supernanant was discarded and smears were made on slides. The slides were coded for a blind analysis, fixed with methanol and stained with 5% Giemsa[24]. For the analysis of the micronucleated cells, 2000 polychromatic erythrocytes (PCE) per animal were scored to determine the clastogenic property of the extract. The cells were blindly scored using a light microscope at 1000 × magnification. The mean number of micronucleated polychromatic erythrocytes (MNPCE) in individual mice was used as the experimental unit, with variability (standard deviation) based on differences among within the same group[25,26]. The percentage reduction of genotoxic agent induced damage by PmME was calculated[27] using the following formula: *percentage reduction = (A-B/A-C) × 100*, where *A* corresponds to the score or MNPCE mean observed in the treatment with CP (positive control), *B* corresponds to score or MNPCE mean observed in the antigenotoxic treatment (PmME + CP) and *C* corresponds to the score or MNPCE mean in the control.

2.5 Chromosomal aberration

The chromosomal aberration test was conducted by the method as described earlier[28], according to OECD guideline[29]. The bone marrow was aspirated using 2.2% (w/v) solution of sodium citrate. The suspension was then centrifuged for 10 min at 2000 rpm. The supernatant was decanted and replaced with 0.075 M potassium chloride solution and allowed to stand for 30 min. The mixture was centrifuged again at the same speed and time. The supernatant was decanted and replaced with freshly-prepared cold fixative (methanol: glacial acetic acid, 3:1 v/v). This was allowed to stand for 10 min after which it was centrifuged for 10 min at 2000 rpm. The supernatant was decanted and replaced with

fresh fixative. The process of fixing and centrifuging was done thrice. Slides were prepared by dropping the fixed cells from a height of 30-40 cm onto clean, dry, grease-free slides. Finally, the slides were air-dried and stained with 5% Giemsa (v/v) for 10 min. All slides were analyzed using a light microscope at 1000 × magnification, blind to treatment for chromosomal aberrations. Fifty well spread metaphases were scored per albino mice for chromosomal aberration and approximately 3000 cells/concentration were analyzed scoring chromosome and chromatid breaks, ring formation, polyploidy, aneuploidy, acentric fragments, and dicentric chromosomes. The frequency of chromosomal aberration was computed as the number of aberrations per total metaphases at each concentration. The mitotic index was obtained as the number of metaphases per total cells scored at each concentration. The percentage of total chromosomal aberration (CA) was calculated as: (Total number of aberration/500) ×100%.

2.6 Statistical analysis

Data were statistically analyzed using one way analysis of variance (ANOVA) followed by Tukey's Test. A p-value <0.05 was considered statistically significant as compared to control group.

3. Results

The genotoxicity test for PmME was conducted using a set of battery test, consisting of comet assay, micronucleus and chromosomal test in female albino mice. The first battery test for genotoxicity was evaluated by comet assay in mice leukocyte. In comet assay, the alkaline version is considered most sensitive method for the detection of genomic damage induced by different genotoxic agents[30,31]. In this assay, the PmME did not affect any DNA strand breaks at the tested dose, with no significant difference in the mean scores obtained, when compared with those obtained for the control (Table 1), demonstrating the absence of genotoxic effects. On the other hand, the animals treated with CP (40 mg/kg b.w.) exhibited a higher DNA damage index (p<0.05) compared to the control group. Besides, simultaneous treatment showed a significant reduction in the extent of DNA damage for all cell types exposed to the three doses (500, 1000 and 2000 mg/kg b.w.) of the extract plus CP, compared with the CP treated group alone (Table 2). The percentages of reductions were ranged from 26 to 52% in all the cell types analysed, showing a potent antigenotoxic effect of the PmME. However, the increase in dose of the PmME did not result in a proportional reduction in genotoxicity induced by CP, indicating the lack of a response relationship.

The second battery test for genotoxicity was evaluated by micronucleus test, which is based on the variation of the frequency of polychromatic erythrocytes with micronuclei[23]. In our study, there was no statistically significant differences (p<0.05) in the frequency of micronucleated polychromatic erythrocytes (MNPCE) observed between the control and the groups treated with the three doses of PmME, indicating an absence of clastogenic / aneugenic effects of this extract (Table 3). However, animals treated with CP showed a high frequency of MNPCE in bone marrow cells as compared to the control and PmME treated groups (p<0.05). On the other hand, oral administration of different doses of PmME followed by the administration of CP led to a significant reduction in the frequency of MNPCE when compared with the group treated only with CP. This reduction ranged from 30-48% in the micronucleus test (Table 3) and indicated a potent chemoprevention of the PmME against the mutagenic effects of CP. The gradual increase in dose of PmME results in a proportional increase in the reduction in clastogenicity induced by CP.

Table No. 1: Comet assay for the assessment of genotoxicity of *P. major* methanolic extract in peripheral blood cells of albino mice *in-vivo*. Means within a column followed by same letters indicates no significant different from each other according to Tukey's Test (Significant level; *p*<0.05). Reduction percentage is calculated based on tail length as compared to CP.

Treatment	Tail Length	DNA in Tail (%)	Olive Moment	Reduction (%)
Control	0.780 ± 0.05^b	0.09	0.61	
PmME 500 mg/kg	0.723 ± 0.08^b	0.10	0.66	
PmME 1000 mg/kg	0.644 ± 0.06^b	0.08	0.60	
PmME 2000 mg/kg	0.779 ± 0.05^b	0.12	0.67	
CP 40 mg/kg	6.421 ± 0.12^a	0.96	2.12	
PmME 500 mg/kg+CP	4.622 ± 0.14^d	0.72	1.71	28.01
PmME 1000 mg/kg+CP	3.965 ± 0.18^e	0.55	1.49	38.24
PmME 2000 mg/kg+CP	$3.839 \pm 0.17^{e,f}$	0.35	1.37	40.21

Table No. 2: Number of micronucleated polychromatic erythrocytes (MNPCE) observed in the bone marrow cells of Swiss mice treated with *PmME*, and respective controls. 2000 cells per animal were analyzed.

Treatments	Number of MNPCE per animals						MNPCE (mean±SD)	Reduction (%)
	M1	M2	M3	M4	M5	M6		
Control	0	0	0	0	1	0	0.16±0.06	-
PmME 500 mg/kg	1	0	1	0	0	0	0.33±0.10	-
PmME 1000 mg/kg	0	0	0	1	1	1	0.50±0.14	-
PmME 2000 mg/kg	0	1	0	0	0	0	0.16±0.09	-
CP 40 mg/kg	15	14	13	10	09	12	12.16±1.47	-
PmME 500 mg/kg+ CP	11	13	8	7	5	7	8.32±1.16	30.96
PmME 1000 mg/kg+ CP	8	7	8	6	8	6	7.13±0.36	41.36
PmME 2000 mg/kg+ CP	5	7	8	4	7	6	6.16±2.06	49.34

PmME- P. major methanolic extract; PP- Polyploidy; MNPCE- Micronucleated polychromatic erythrocytes CP- Cyclophosphamide

Table No. 3: Chromosomal aberration test in mice bone marrow cells

Treatment	Br/Fr	Ring	Aneu	Poly	Del	Total aberration	Reduction (%)
NC	4	1	1	0	1	1.4	
PmME 500 mg/kg	3	5	1	0	2	2.2	
PmME 1000 mg/kg	5	6	2	1	3	3.4	
PmME 2000 mg/kg	5	4	1	1	4	3	
CP 40 mg/kg	55	6	3	2	15	16.2	
PmME 500 mg/kg+ CP	42	8	4	2	11	13.4	17.28
PmME 1000 mg/kg+ CP	41	6	6	0	9	12.4	23.45
PmME 2000 mg/kg+ CP	40	5	2	2	7	11.2	30.86

Total CA%=(Total Number Of Aberration/500)×100%

NC: Negative control (water); Vehicle: DMSO (0.5 ml); CP: Cyclophosphamide (40 mg/kg bw); *PmME- P. major* methanolic extracts (500, 1000 and 2000 mg/kg b.w.); Br/Fr: Breakage/Fragments; Aneu: Aneuploidy; Poly: Polyploidy; Del: Deletion

Data are represented by mean of triplicates, each containing 25 nos. of cells having different comet classes (Class I: tail shorter than the diameter of the head (nucleus) Class II: tail length 1-2 times the diameter of the head and Class III: tail length more than twice the diameter of the head). MA: Mean average; Smd: Significative mean difference; PA: Punarnavine; CP: Cyclophosphamide

Chromosome analysis of bone marrow cells *in vivo* has turn into a standard system for testing the potential mutagenic effects of infections, radiation, drugs, and chemical pollutants[32,33]. Examination of mitotically active cells that have been arrested at metaphase for structural changes and re-arrangement of their chromosomes is a paramount importance *in-vivo* genetics.

Chromosomal mutations cause many human genetic diseases and there is substantial evidence that chromosomal mutation causes alterations in oncogenes and tumor suppressor genes in somatic cells, which are involved in cancer induction in humans and experimental animals. Therefore, a third battery test for genotoxicity was studied by chromosomal aberration tests to screen for possible mammalian mutagens and carcinogens. In this study, the results showed that the different concentration of PmME did not show any chromosomal aberration. As expected, the positive controls (CP- treated animals) showed significant increases in the frequency of metaphases with aberrant chromosomes when compared with the control and CP-treated groups.

4. Discussion

Herbal medicine has become one of the most widely used primary therapeutics or supplements for improving health related problems[34]. However, scientific evidence for the safety of the herbal products also requires regulatory clearance for wide acceptance. Although many studies have reported extensive investigations of PmME properties, its genotoxicity and/or antigenotoxicity has not been investigated properly so far. Therefore, we evaluated the genotoxicity and/or antigenotoxicity of PmME using standard battery tests recommended as per Organization for Economic Co-operation and development (OECD) guidelines.

In the present study, our result indicated the absence of genotoxicity of the PmME. Interestingly, our result indicated that this extract acted as a protective effect against CP induced DNA damage. Under the present experimental conditions, the PmME, administered by gavage, showed no genotoxicity in leukocytes and bone marrow cells of albino mice by the comet assay, micronucleus test and chromosomal aberration test. In contrast, PmME showed a preventive effect, inhibiting the genotoxicity induced by the CP, in all the evaluated cells. Although further investigations are needed to clarify the protective mechanism of the PmME, the antigenotoxic properties of this extract is of great pharmacological importance, and may be helpful for disease counteractive action.

5. Acknowledgements

6. Conflict of Interest

All authors declare that they have no conflicts of interest.

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