Research Article

In Vivo Evaluation of Genotoxic Effects of *Euphorbia Nivulia* Buch on Mice Bone Marrow Cells Using Chromosomal Aberration Test and Micro Nucleus Assay

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ABSTRACT

Euphorbia nivulia is a tall deciduous tree belonging to the family Euphorbiaceae. It is mainly found in India and is reported to have tremendous therapeutic potential. Herbal medicine may cause damage to genetic material which may lead to an increased risk of cancer and other diseases. Hence, it becomes important to assess the genotoxicity assessment of herbal medicines. The present study was conducted to evaluate the *in vivo* genotoxic effects of *Euphorbia nivulia* Buch extract on mice bone marrow cells using chromosomal aberration test and micro nucleus assay. Three different doses 250, 500 and 1000 mg/kg b.w were selected as per OECD guidelines. In micronucleus assay, single oral dose administration of *Euphorbia nivulia* extract at 250, 500 and 1000 mg/kg b.w did not increase in the mean number of micronucleated polychromatic erythrocytes or percentage of PCE in both sexes of mice indicating non clastogenicity. Similarly in chromosomal aberration test, a single oral administration of *Euphorbia nivulia* extract showed no significant changes on the mitotic indices or in the induction of chromosomal aberrations (both numerically and structurally) in proliferative cells of the bone marrow of mice in either sex. Hence it can be concluded, *Euphorbia nivulia* extract showed no significant genotoxic effect on the chromosomes of mice in either sexes. The extract was devoid of neither mutagenic nor carcinogenic and hence can be used safely.

Keywords: *Euphorbia nivulia*, genotoxicity, carcinogenic, micronucleus, mutagenic

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INTRODUCTION

Herbal medicines are the oldest remedies known to mankind. Indian has one of the well known histories for the use of medicinal herbs [1]. Accessibility and affordability of these medicines have made them popular worldwide. Currently, about 80% of the world population use herbal medicines for routine health care [2].

Assessment of the potential genotoxicity of traditional medicines is indeed an important issue as damage to the genetic material may lead to critical mutations and therefore also to an increased risk of cancer and other diseases. Genetic toxicology studies have given rise to a number of testing procedures, both *in vitro* and *in vivo*. They have been designed to assess the effects of various test substances on the genetic material and consequently, to assess the risk to living organisms including humans [3]. *In vivo* mouse chromosome aberration test and micronuclei assay has been recognized as one of the sensitive methods to test genotoxicity of plant extracts [4]. The micronucleus assay detects clastogenicity due to chromosome breakage, and aneugenicity due to chromosome lagging resulting from dysfunctioning of the mitotic apparatus. Micronuclei appear in cells due to chromosomal damage during the last mitosis [5]. Chromosomal aberrations are changes in chromosome structure resulting from a break or exchange of chromosomal material. Structural chromosome aberrations are of two types, chromosome or chromatid. Numerical aberrations are aneuploidy or polyploidy [6].

Euphorbia nivulia Buch is an open-crowned succulent-branched spiny, glabrous,

deciduous tree, belonging to family Euphorbiaceae [Fig. 1]. Potential therapeutic benefits of *Euphorbia nivulia* Buch have been reported in literature [7-10]. However, there are no scientific studies reporting on genotoxic effects of *Euphorbia nivulia* Buch. Thus, the present study undertaken to evaluate the genotoxic effects of *Euphorbia nivulia* Buch extract by *in vivo* chromosomal aberration test and micronucleus assay using mice bone marrow cells.

Figure 1: *Euphorbia nivulia* Buch

MATERIALS AND METHODS

Collection of plant material:

The leaves of *Euphorbia nivulia* Buch were collected from the Ramnagar, Karnataka, identified and authenticated by Dr. MD Rajanna, Botanical garden and Herbarium, UAS, GKVK Campus, Bangalore, Karnataka, India.

Extraction:

The leaves of *Euphorbia nivulia* Buch was air dried, powdered and subjected to soxhlet extraction using 70%$^{V}/_{V}$ hydro-alcohol (75-80ºC). Thereafter, the extract was concentrated using rotary flash evaporator (50ºC).

PHARMACOLOGICAL SCREENING:

Animals:

Experimental study was carried out using albino mice weighing 30-35g. The animals were procured from Biogen, Bangalore. The animals were housed in polypropylene cages. The cages were maintained clean and hygienic. Animals were acclimatized in light and temperature controlled room with a 12-12h dark-light cycle, temperature 25±2ºC and humidity 50±5%. The mice were fed with commercial pelleted feed and water *ad libitum*. The animal caring and handling were done according to the CPCSEA guidelines. The Institutional Animal Ethics Committee at Visveswarapura Institute of Pharmaceutical Sciences has approved the study.

Micronucleus test:

Albino mice were divided into 5 groups consisting of 10 mice, 5 males and 5 females in each group.

Group I : Vehicle control
Group II : Cyclophosphamide (CP) 40mg/kg b.w., i.p.
Group III : *Euphorbia nivulia* 250 mg/kg b.w., p.o
Group IV : *Euphorbia nivulia* 500 mg/kg b.w., p.o
Group V : *Euphorbia nivulia* 1000 mg/kg b.w., p.o

After 24 h of respective single dose treatment for all the above mentioned groups, animals were sacrificed and bone marrow was collected from both the femurs using fetal bovine serum. The solution was centrifuged at 1000 rpm for 10 min. Pellet was collected and smears were made. Smear glass slides was allowed to air dry, followed by fixation with methanol. Later stained using May-Grunwald's and Giemsa stain. Slides were dried and observed under oil immersion objective to determine the frequency of micronucleus (MN). The percent of

polychromatic erythrocytes (PCE) in 1000 total erythrocytes, number of micronucleus polychromatic cells in 2000 PCE and percent of micronuclei in polychromatic erythrocytes (MNPCE) were evaluated [11].

Percent of PCE = [PCE/ (PCE+NCE)] × 100,
Percent of MNPCE = (MNPCE÷PCE) × 100

Chromosoma aberration test:

Albino mice were divided into 5 groups consisting of 10 mice, 5 males and 5 females in each group.

Group I : Vehicle control
Group II : Cyclophosphamide (CP) 40 mg/kg b.w., i.p.
Group III : *Euphorbia nivulia* 250 mg/kg b.w., p.o
Group IV : *Euphorbia nivulia* 500 mg/kg b.w., p.o
Group V : *Euphorbia nivulia* 1000 mg/kg b.w., p.o

After 24h of respective single dose treatment for all the above mentioned groups, Colchicine 4mg/kg b.w i.p was given after respective treatments for all the animals 2h before sacrificing. All animals were sacrificed by cervical dislocation. Femurs and tibias were separated and adhering muscle, tissue was removed. The bone marrow was extracted from both femurs using 0.56% freshly prepared KCl solution, fixed with cold Corney's fixative and flame dried. Then slides are prepared by dropping technique and stained with 10% Giemsa solution. Slides were then air dried and observed under oil immersion objective. 100 well spread metaphase cells were screened per animal for scoring the chromosomal aberrations. Mitotic index was determined by counting 1000 cells [6, 11].

RESULTS

Micronucleus assay

Frequency of MNPCE, percent MNPCE and percent PCE were observed in both sexes of mice and results are shown in table 1. Animals treated with cyclophosphamide showed a significant increase in mean the number of MNPCE, percentage of MNPCE and percent PCE ($P<0.001$) when compared with control group. Animals when treated with 250, 500 and 1000 mg/kg b.w. of *Euphorbia nivulia* extract showed no significant increase in mean the number of MNPCE, percentage of MNPCE and percent PCE when compared with control group.

Table 1: Frequency of MNPCE, Percent MNPCE and Percent PCE of *Euphorbia nivulia* Extract

Groups	Sex	MNPCE/2000	Percent MNPCE	Percent PCE
Vehicle control	Female	2.20 ±0.37	0.11±0.01	47.00±1.21
	Male	2.020±0.20	0.11±0.01	47.63±2.64
Cyclophosphamide	Female	14.20±1.53***	0.71±0.07***	65.84±1.60***
	Male	12.40±0.92***	0.62±0.04***	66.13±2.47***
Euphorbia nivulia extract 250 mg/kg	Female	2.00±0.31ns	0.10±0.01ns	48.94±0.87ns
	Male	1.80±0.37ns	0.09±0.01ns	49.28±1.53ns
Euphorbia nivulia extract 500 mg/kg	Female	2.40±0.24ns	0.12±0.01ns	50.02±1.54ns
	Male	2.80±0.37ns	0.14±0.01ns	51.68±1.11ns
Euphorbia nivulia extract 1000 mg/kg	Female	2.80±0.37ns	0.14±0.01ns	50.52±0.80ns
	Male	3.00±0.31ns	0.17±0.02ns	52.88±0.85ns

Data were analysed by one way ANOVA followed by Dunnett's *t* test. Number of animals in each group n = 5, All groups are compared with vehicle control group. *** $P<0.001$, ns non-significant.

Chromosomal aberrations

An *in vivo* chromosomal aberration in both the sexes of mice was performed at three different doses and results are shown in table 2. Single oral dose administration of *Euphorbia nivulia* extract at 250, 500 and 1000 mg/kg b.w. after 24 h has shown no significant induction of chromosomal aberrations in bone marrow cells of mice of either sex when compared with vehicle control group. Whereas, cyclophoshamide treated animals in both sexes has significantly (P<0.001) induced different types of chromosomal aberrations (**Table 2**). Chromosome and chromatids breaks and gaps, fragments, associations, ring formation and pulverization were seen.

Table 2: Effect of *Euphorbia nivulia* on the Chromosomal Aberrations in Bone Marrow Cells of Mice After 24 h of Treatment

Groups	No. of animals	Total no. of meta-phases observed	Total No. of aberrant metaphases /100 metaphases	Type and no. of				C	R	F	A C	Others (CAD, RT, Multiple)	P	Average aberrations /100 metaphases (without gap)
				C		C								
				G	B	G	B							
Vehicle control	5 F	500	14±0.44	15	4	7	2	1	0	1	2	1	0	13
	5 M	500	20±1.14	25	2	9	2	1	0	3	2	2	0	12
Cyclophos phamide	5 F	500	302±6.64***	60	91	35	68	13	7	68	66	20	5	449
	5 M	500	296±2.12***	58	10	37	85	18	5	97	67	16	6	560
Euphorbia nivulia	5 F	500	17±0.89ns	23	4	11	3	2	0	1	3	2	0	15
	5 M	500	22±1.00ns	25	4	12	2	2	0	2	3	2	0	15
Euphorbia nivulia	5 F	500	21±1.14ns	20	4	9	3	1	1	3	3	3	0	20
	5 M	500	22±1.58ns	28	3	7	3	1	2	2	3	3	0	19
Euphorbia nivulia	5 F	500	22±0.44ns	12	4	5	4	1	1	1	4	2	0	19

Data were analysed by one way ANOVA followed by Dunnett's *t* test. Number of animals in each group n = 5, All groups are compared with vehicle control group. *** P<0.001, ns non-significant. Cd-Chromatid; Ch-Chromosome; G-Gap; B-Break; C-Complex; R-Ring; F-Fragment; AC-Acentric; CAD-Centromeric adhesion; RT- Robertsonian translocation

Mitotic index

The percentage of mitotic index was used to determine the rate of cell division. The slides prepared for the assessment of chromosome aberrations were used for calculating the mitotic index. Cyclophoshamide treated animals in both sexes has shown a significant (P<0.001) decrease in percentage of mitotic index when compared with vehicle control group (table 3). Whereas, no significant differences in the percentages of mitotic indices were observed in both sexes of mice at three different dose levels of *Euphorbia nivulia* extract (250, 500 and 1000 mg/kg b.w.) were seen.

Table 3: Effect of *Euphorbia nivulia* on the Mitotic index (MI) in Bone Marrow Cells of Mice After 24 h of Treatment

Groups	Sex	Percent Mitotic index
Vehicle control	Female	3.8±0.09

	Male	3.52±0.19
Cyclophosphamide	Female	2.02±0.08***
	Male	2.22±0.14***
***Euphorbia nivulia* extract 250 mg/kg**	Female	3.80±0.19ns
	Male	3.32±0.11 ns
***Euphorbia nivulia* extract 500 mg/kg**	Female	3.96±0.13 ns
	Male	3.56±0.18 ns
***Euphorbia nivulia* extract 1000 mg/kg**	Female	4.00±0.07 ns
	Male	3.68±0.15 ns

Data were analysed by one way ANOVA followed by Dunnett's t test. Number of animals in each group n =5, All groups are compared with vehicle control group. *** P<0.001, ns non-significant.

DISCUSSION

Mice and rats are the most widely used animals for general toxicologic, carcinogenic, pharmacokinetic and toxicokinetic studies. Genotoxic studies are useful to identify the level of DNA damage induced by plant extracts, as well as to give a clue about the possible clinical consequences of human exposure [12]. Genotoxic agents may cause genetic damage in any cell of the body. If the damage occurs, in somatic cells, it may lead to cancer or perhaps some other degenerative diseases. Mutagenicity testing has now been recognized as an essential part in the evaluation of the genetic toxicity of plant extracts. This exciting idea of utilizing nuclear anomalies in bone marrow smear as a rapid method to analyse the mutagenic potential of plant extracts provide impetus to others working in the field of mutagenesis [11]. The rodents (mouse and rat) bone marrow micronucleus test and chromosomal aberration test are commonly used to evaluate the genetic toxicity of test substance *in vivo* [13].

In the micronucleus formation, when a bone marrow erythroblast develops into a PCE, the main nucleus is extruded; any micronucleus that has been formed may remain behind in the otherwise anucleated cytoplasm. Visualization of micronuclei is facilitated in these cells because they lack a main nucleus. Micronuclei are cytoplasmic chromatin masses with the appearance of small nuclei that arise from chromosome lagging at anaphase or from acentric chromosomal fragments. They provide a quantifiable measure of recent DNA injury that result from when acentric fragments or whole chromosomes are left behind the main nucleus at telophase. An increase in the frequency of micronucleated polychromatic erythrocytes (MNPCEs) in treated animals is an indication of induced chromosome damage [14-17].

In chromosomal aberrations test, it is necessary to arrest the dividing cells at metaphase stage. To facilitate this, treatment with colchicine and hypotonic solution is very essential. Colchicine is a mitotic arrestant and disrupts the spindle fibers there by preventing the anaphase segregation. The action of hypotonic solution causes swelling of cells by osmosis, which allows the proper spreading of the chromosomes. Mitotic index, which represent fraction of cells in a given population that undergo mitosis at a given time, indicates the cell proliferation activity.

In the present study, the *Euphorbia nivulia* extract was evaluated for genotoxicity potential by means of *in vivo* micronucleus and chromosomal aberration test in mice bone marrow. At three different doses of *Euphorbia nivulia* extract there was no increase in the mean number of MNPCEs neither in percentage of PCE in both sexes of mice indicating non clastogenicity. Similarly in chromosomal aberration test a single oral administration of 250, 500 and 1000 mg/kg b.w. of *Euphorbia nivulia* showed

no significant changes in the mitotic indices or on the induction of chromosomal aberrations (both numerically and structurally) in proliferative cells of the bone marrow of mice in either sex. These results indicate that *Euphorbia nivulia* did not interact with the spindle apparatus nor with the gene function.

CONCLUSION

Hence it can be concluded that *Euphorbia nivulia* extract did not exhibit any genotoxicity. The extract was devoid of neither mutagenic nor carcinogenic and hence can be used safely.

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