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Research Article

EVALUATION OF MUTAGENIC EFFECT (ANTIMUTAGENIC) OF *DALBERGIA LATIFOLIA* ON SWISS ALBINO MICE

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ABSTRACT

Dalbergia latifolia Roxb (Family: Fabaceae) is a traditional herb, contain latinone and dalcriodain flavonoid having excellence medicinal value [8]. The present study was designed to evaluate the antimutagenic potential of methanolic extract of *D. latifolia*, using micronucleus (MN) and chromosomal aberration (CA) assay in mice bone marrow. The antimutagenic effect of *D. latifolia* was assessed using cyclophosphamide MN formation and CA in mice. The animals were pre-treated with the methanolic extract of *D. latifolia* orally at two doses of 100, 200 mg/kg body weight for 7 days. In MN and CA test the two doses provided protection when given 24 hrs prior to a single i.p. administration of cyclophosphamide (100 mg/kg body weight). These results demonstrate that methanolic extract of *D. latifolia* has got anti-mutagenic potential.

Keywords: *Dalbergia latifolia*, Methanolic extract, Antimutagenic activity

INTRODUCTION

All chemicals that produce DNA damage leading to mutation or cancer are described as genotoxic [1]. Genotoxicity testing is an important part of the preclinical safety assessment of any drug. It is designed to detect genetic damage such as gene mutations and chromosomal aberration (CA), which may be reflected in tumorigenic or heritable mutation potential of the drug. As the mechanisms of micronucleus (MN) formation are related to those inducing CA, both micronuclei and CA can be accepted as assay systems to screen clastogenicity induced by test compounds [2]. Toxicological studies have undergone a significant evolution during the past decade, with much greater emphasis being placed on chronic toxicity, carcinogenicity, teratogenicity, and mutagenicity. The mutations in somatic cells are not only involved in the carcinogenesis process but also play a role in the pathogenesis of other chronic degenerative diseases, such as atherosclerosis and heart diseases, which are the leading causes of death in the human population. MN test and CA test are used for studying the antimutagenic activity of a drug. One of the best ways to minimize the effect of mutagens and carcinogens is to identify the anticlastogens/anti-mutagens substances, which suppress or inhibit the process of mutagenesis by acting directly on the mechanism of cell and des-mutagens (substances which somehow destroy or inactivate, partially or fully the mutagens, thereby affecting less cell population) in our diets and increasing their use. Nature has bestowed us with medicinal plants. There is a need to explore them for use as anti-mutagenic and anti-carcinogenic food or drug additives [1].

Antimutagen is described as an agent that reduces the apparent yield of spontaneous and/or induced mutations. Mechanisms of anti-mutagenesis have been classified into two major processes one is des-mutagenesis: In which factors act directly on mutagens or inactivate them, the other is bio-anti-mutagenesis in which factors act on the processes of mutagenesis or repair DNA damages that result in a decrease in the mutation frequency. Gemcitabine used as a mutagen with anti-metabolites activity, it exerts its effect by prohibiting DNA chain elongation. Anti-mutagenesis is considered as one of the most feasible ways for inhibiting the negative effects of environmental genotoxicants including carcinogens. Nowadays, a large number of anti- mutagens of plants origins are known [3]. Evaluation of genetic toxicity is an important component of the safety assessment of chemicals, including pharmaceuticals, agricultural chemicals, food additives, and industrial chemicals. Up to the present time, genotoxicity has been regulated mainly on the basis of qualitative outcomes of hazard identification assays, i.e., decisions are often based on classification as positive or negative for genotoxic potential. Most human carcinogens are identified by epidemiological studies. These studies are necessarily long term, as no effect is expected to be observed until decades after the carcinogenic event or events [4]. However, convincing these studies are costly and exposure levels and effects are difficult to quantify. A few multiple generation mutation assays have been carried out using rodents:

- Dominant lethal
- Mouse spot test
- Heritable translocation test

These tests must be carried out on a large scale, and tend to be insensitive; in order to detect a 1% increase (which is a very strong effect) in carcinogenicity in a human population, one would need to perform an animal study to such a large scale as to cost over 25 million dollars. Genotoxicity tests can be defined as *in vitro* and *in vivo* tests designed to detect compounds that induce genetic damage by various mechanisms. These tests enable hazard identification with respect to damage to DNA and its fixation. Fixation of damage to DNA in the form of gene mutations, larger scale chromosomal damage or recombination is generally considered to be essential for heritable effects and in the multi-step process of malignancy, a complex process in which genetic changes may play only a part. Numerical chromosome changes have also been associated with tumor genesis and can indicate a potential for aneuploidy in germ cells. Compounds that are positive in tests that detect such kinds of damage have the potential to be human carcinogens and/or mutagens. Because the relationship between exposure to particular chemicals and carcinogenesis is established for humans, whilst a similar relationship has been difficult to prove for heritable diseases, genotoxicity tests have been used mainly for the prediction of carcinogenicity. Nevertheless, because germ line mutations are clearly associated with human disease, the suspicion that a compound might induce heritable effects is considered to be just as serious as the suspicion that a compound might induce cancer. In addition, the outcome of genotoxicity tests can be valuable for the interpretation of carcinogenicity studies [5].

MATERIALS AND METHODS

Animals

Eight to 10 weeks old Swiss albino mice having weight (25-30 g) were purchased from Central animal research facility NIMHANS Reg.

No.12/99 Bangalore. They were housed, five per polypropylene cage under standard laboratory conditions at room temperature (25±2°C) with 12 hrs light/dark cycle. The animals were provided with pellet chow and water ad libitum. Ethical clearance was obtained from Institutional Animal Ethical Committee of Karnataka College of Pharmacy, Bangalore.

Plant material

The fresh root of *Dalbergia latifolia* was collected from Tirupati, Andhra Pradesh, identified and authenticated by Dr. K. Madhava Chetty, Asst. Professor, Department of Botany, Sri Venkateswara University, Tirupati. All other chemicals used in the study are of AR grade.

Chemicals and drugs

1. Cyclophosphamide (Endoxan, purchased from local market.)
2. Normal saline
3. Fetal Bovine serum (Himedia)
4. E.D.T.A disodium salt LR (Merc)
5. Anesthetic Ether I.P (TKM Pharma)
6. Glacial acetic acid LR (Merc)
7. Sodium hydrogen phosphate LR (Merc)
8. Potassium Dihydrogen phosphate LR (Merc)
9. May-Grunewald stain (Himedia)
10. Giemsa stain (Himedia)
11. Sodium carbonate LR (Merc)
12. Glycerol LR (Merc)
13. Sodium hydroxide LR (Merc)
14. Methanol LR (Merc)
15. Potassium chloride LR (Merc)
16. Colchicine (Himedia)
17. Phosphate buffer saline (Himedia)

Instruments/equipments

1. Remi centrifuge
2. Digital pH meter (PHep, Hanna Instruments)
3. Coupling Jars
4. Microscopic Glass slides (Blue star)
5. Cover slips 22× 40 mm (Blue star)
6. Inverted microscope (Labomed, USA)
7. Micropipettes (Thermo Scientific)
8. RO water system (Millipore)
9. Reagent bottles
10. Pippetter tip

PREPARATION OF EXTRACTS

The leaves of *D. latifolia* was powdered (500 g) and the methanolic extract was prepared using soxhelt extraction process. The methanolic extract was evaporated under reduced pressure using rotavapor evaporator. The yield of the extract was 3.73% g. A suspension was prepared using 2% v/v tween 80 and administered orally.

Acute oral toxicity studies (oppts870.1100)

The acute oral toxicity study was performed according to the OPPTS (Office of Prevention, Pesticides, and Toxic Substances) guidelines as follows [9]. Female albino rats of Wister strain (150-200 g) were maintained under controlled standard animal house condition with access to food and water ad libitum.

Methods

Dose selection

Lethal dose 2000 mg/kg selected and two doses of 100 mg/kg & 200 mg/kg body weight of methanolic extract of *D. latifolia* leaves was selected as low dose and high dose as per the acute oral toxicity studies. The extract was subjected to the phytochemical test.

Preparation of phosphate buffer solution (pH=6.8)

Dissolved 2.366 g. of Na2HPO4 in 250 ml of distilled water = Solution A
Dissolved 2.27 g. of K2HPO4 in 250 ml of distilled water = Solution B
A volume of 50 ml of solution A and 50 ml of solution B was taken and made up the volume to 1000 ml with distilled water

Preparation of suspending medium

About 5% bovine albumin solution was prepared by dissolving the required quantity of bovine albumin power in phosphate buffer (pH=7.2). The bovine albumin power is dissolve very carefully by adding the powder little by little to the solvent and mixed thoroughly, so as to avoid any coagulum. The final 5% albumin solution should be very clear and free from any protein lumps. Two drops of 1% sodium azide were added as a preservative.

Preparation of staining solution

May-Grunewald's stain was prepared by dissolving 0.2 g of the strain powder in 100 ml of methanol with slight heating and stirring. After it dissolved completely, it was filtered. Giemsa's stain was prepared by dissolving 1gm of Giemsa's stain in 54 ml of glycerin. It was kept in a 60°C oven for 2 hrs. After cooling, 84 ml of methanol was added, stirred well, and filtered.

Animals

Swiss albino mice of either sex 8-10 weeks old, weighing 25-30 g were housed in plastic cages with paddy husk bedding. Animals were provided with food and water *ad libitum.*

Groups

Group 1: Animals are treated with vehicle (n-6).
Group 2: Animals are treated with cyclophosphamide (75-100 mg/kg i.p.)
Group 3: Animals are treated with 100 mg/kg with methanolic extract for 7th day followed by Cyclophosphamide as challenging dose
Group 4: Animals are treated with 200 mg/kg with methanolic extract for 7 days.

Procedure

Animals were sacrificed by cervical dislocation after 24 hrs of administration of the clastogen. 90 minutes. Prior to death, each animal was injected with 0.04% colchicine in a dose of 4 mg/kg i.p. for mitotic arrest. Colchicine solution was prepared in distilled water [6].

Animals were cut open and femur and tibia from both the legs were quickly removed and muscle mass cleaned away from the bones. For collection of bone marrow, the upper end of the femur was cut open, till a small opening was visible. A 22 gauge needle was inserted to ensure that the upper end was open. About 0.5 ml of 0.56% (or 0.075 M) hypotonic potassium chloride solution was taken in a syringe and the needle was inserted at the lower epiphyseal end. The bone marrow was flushed into a clean cavity block. If the marrow collected was solid, it was dispersed by repeated aspiration and flushing with the help of the syringe. Similarly, tibial marrow was also collected. Altogether 2 ml of hypotonic potassium chloride solution was used to collect the marrow from both femur and tibia [7].

RESULTS

Phytochemical analysis of successive extract of bark of *D. latifolia.*

Physical examination of flavonoids

The isolated flavonoids were subjected to physical examination and observation recorded in Table 3. The flavonoid was a sticky solid mass with dark green color. Its odor is characteristic.

Effect of methanolic extract *D. latifolia* (200,100, mg/kg; p.o.;/ day/7 days) on MN formation in bone marrow cells.

Percentage MN polychromatic erythrocytes (%MNPCE) formation significantly increased ($p<0.001$) after 24 hrs. Of cyclophosphamide treatment when compared to normal mice. Administration methanolic extract *D. latifolia* (200,100 mg/kg; p.o./day/7 days) to mice significantly decreased ($p<0.001$) Percentage (%MNPCE) formation levels observed after 24 hrs when compared to Cyclophosphamide control group. Percentage MN norm chromatic erythrocytes (%MNNCE) formation significantly increased ($p<0.05$) after 24 hrs. Of cyclophosphamide treatment when compared to normal mice. Administration of

methanolic extract *D. latifolia* (200,100 mg/kg: p.o./day/7 days) to mice significantly decreased (p<0.050).

%MNNCE formation levels observed after 24 hrs when compared to cyclophosphamide control group effect of methanolic extract *D. latifolia* (200,100, mg/kg; p.o.;/day/7 days) on MN formation in bone marrow cells.

Effect of methanolic extract *D. latifolia* (200,1000, mg/kg; p.o.;/day/7 days) on %MNPCE formation cyclophosphamide (100 mg/kg; i.p./day/single) in bone marrow cells (Fig. 1)

Effect of methanolic extracts *D. latifolia* (200,100, mg/kg; p.o.;/day/7 days) on p/n ratio cyclophosphamide (100 mg/kg; i.p./day/single) in bone marrow cells (Fig. 2).

Table 1: Phytochemical analysis of successive extract of bark of *Dalbergia latifolia*

S. No.	Tests	Results
1	Tests for steroids and triterpenes	
	Salkowski test	+
	Liebermann-Burchard test	+
	Kahleberg test	+
2	Tests for alkaloids	
	Mayer's reagent	-
	Dragendroff's reagent	-
	Hager's reagent	-
	Wagner's reagent	-
3	Tests for Saponins (Foam test)	-
4	Tests for phenolic compounds and Tannins	
	Ferric chloride test	+
	Gelatin test	+
	Lead acetate test	+
5	Tests for flavonoids	
	Sodium hydroxide test	+
	Ferric chloride test	+
	Shinoda's test	+
	ZINC-HCl reduction test	+
	Lead acetate test	+

Table 2: Percentage yield and physical characters of *Dalbergia latifolia* in different solvents

Plant extract	Percentageyield	Colour	Odour	Nature
Methanol	3.73	Dark brown	Characteristic	Non-sticky

Table 3: Physical properties of flavonoids

S. no.	Physical properties	Observation
1	State	Solid
2	Color	Dark brown
3	Odor	Characteristic
4	Nature	Non-Sticky

Effect of methanolic extracts *D. latifolia* (200,100, mg/kg; p.o.;/day/7 days) on p/n ratio cyclophosphamide (100mg/kg; i.p./day/single) in bone marrow cells (Fig. 3).

For the scoring of micronuclei, the following criteria are [14]

- MN should be separated from or marginally overlap with main nucleus as long as there is clear identification of the nuclear boundary
- MN should have similar staining as the main nucleus
- The diameter of MN should be less than one-third of main nucleus.

Characteristics of the MN-test

- Biomarker of effect: Relevant for risk assessment of cancer
- Endpoint: Identification of chromosome + genome mutations
- Expression of MN requires cell division
- MN contain either a whole chromosome or an acentric fragment

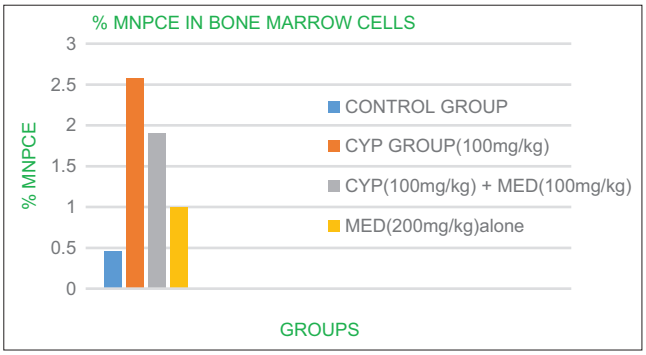

Fig. 1: Percentage of micronucleus polychromatic erythrocytes in bone marrow cells. Values are expressed as mean±standard error of the mean, (n=6) ***p<0.001 compared with normal control group. ###p<0.01 compared with cyclophosphamide group

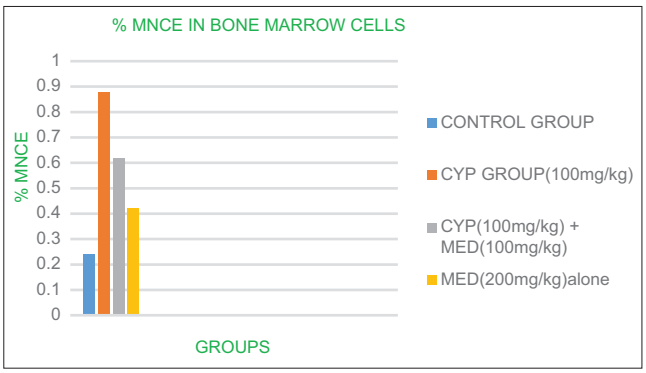

Fig. 2: Percentage of micronucleus norm chromatic erythrocytes in bone marrow cells. Values are expressed as mean±standard error of the mean, (n=6) ***p<0.001 compared with normal control group. ###p<0.01, ##p<0.01 compared with cyclophosphamide groups

Table 4: Percentage micronucleus norm chromatic erythrocytes (%MNCE) formation levels observed after 24 hrs when compared to cyclophosphamide control group effect of methanolic extract *Dalbergia latifolia* (200,100, mg/kg; po; /day/7 days) on micronucleus formation in bone marrow cells

S. no.	Groups	PCE	MNPCE	%MNPCE±SEM	NCE	MNNCE	%MNNCE±SEM	P/N ratio
1	Vehicle control	5500	28	0.46±0.05	5600	15	0.24±0.04	0.91±0.07
2	Cyclophosphamide (100 mg/kg)	5675	162	2.58±0.05***	5560	54	0.88±0.56***	0.63±0.01***
3	*D. latifolia* 100 mg/kg/7 day+ Cyclophosphamide (100 mg/kg)	5480	61	1.90±0.07###	5575	26	0.62±0.03##	0.76±0.01###
4	*D. latifolia* 200 mg/kg/7 days	5455	117	1.00±0.59###	5590	38	0.42±0.0###	0.85±0.01###

Values are expressed as mean±SEM, (n=6), ***p<0.001 compared with normal control group, ###p<0.001, ##p<0.01 compared with cyclophosphamide group.
PCE: Polychromatic erythrocytes, MN: Micronucleus, NCE: Norm chromatic erythrocytes, SEM: Standard error of the mean, *D. latifolia: Dalbergia latifolia*

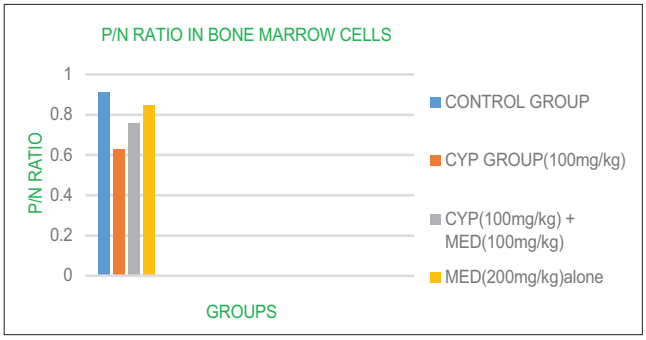

Fig. 3: P/N ratio in bone marrow cells. Values are expressed as mean±standard error of the mean, (n=6) ***p<0.001 compared with normal control group. ###p<0.01 compared with cyclophosphamide group

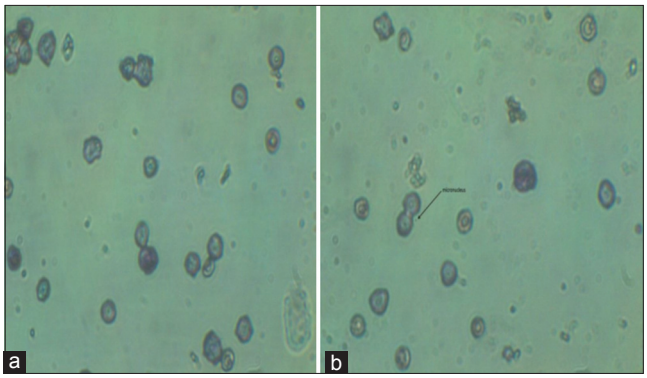

Fig. 4: (a) Normal control (b) Micronucleus

- Discrimination between mononucleated cells and binucleated cells in the cyto-B assay.

DISCUSSION

Mutagenicity is a broader term that refers to the ability to interact with DNA and/or the cellular apparatus that regulates the fidelity of the genome. It can be due to the exposure to various environmental, pharmaceutical pollutants, xenobiotic, and some category of drugs produces unexpected and unidirectional changes in the genome. A chemical is considered to be mutagenic if it is capable of inducing heritable changes in the genotype of a cell as a consequence of alteration to or loss of genes, chromosome or parts or chromosome. Many anticancer drugs, immunosuppressants, etc., are the important categories used in the treatment of various human cancers invariably have cell toxicity and can also induce genotoxicity. One such example is cyclophosphamide. The toxicity of cyclophosphamide is attributed to the generation of free radicals during its metabolism CYP is an effective anticancer drug that belongs to the class of nitrogen mustards. The cytotoxic effect of CYP is directly connected with free radical mediated metabolism. It is rapidly metabolized in the liver by cytochrome P-450 enzymes and generates active alkylating metabolites such as 4-hydroxycyclophosphamide, aldophosphamide, and acrolein, which interfere with cellular DNA synthesis in dividing cells and induce DNA single strand breaks [11] that may result in MN formation and cell death [12,13].

Cyclophosphamide induced MN formation and protection by pretreatment with *D. latifolia.*

Experimental evidences have strengthened the concept that DNA damage plays a crucial role in the initiation of genotoxicity. Error in the DNA molecules causes CA and MN.

The evaluation of MN frequencies *in vivo* is one of the primary genotoxicity test recommended internationally by the regulatory agencies for product safety association.MN are well-characterized biomarker of structural and numerical chromosomal damage. The MN in young erythrocytes arises mainly from chromosomal fragments that are not incorporated into the daughter nuclei at the time of cell division in the erythropoietic blast cells. Immature cells are called as polychromatic erythrocyte stains bluish due to the high content of RNA and mature cells called as normochromatic erythrocytes stains pink. Decrease in the PCE:NCE ratio is responsible for the induction of bone marrow cytotoxicity.

Results of our study reveal that cyclophosphamide treatment shows cytotoxicity by increasing %MNPCE and decreasing PCE:NCE ratio. Pre-treatment with *D. latifolia* (200 mg/kg) for 7 days before CP challenge, effectively and significantly decrease the %MNPCE thereby brought the PCE:NCE ratio to the normal levels.

CONCLUSION

From the present study, it was found that a significant decrease in mitotic index of cyclophosphamide treated animals, which can be due to the affected cell division in the bone marrow (Gonzalves *et al*., 2008). Methanolic extract of *D. latifolia* significantly inhibited the disturbances in the cell division of mouse bone marrow and therefore it showed anti-mutagenicity in MN tests in bone marrow cells of mice. Mutation is one of the principle pathways that lead to cancer. The anti-mutagenic effects may be an important contributor in the use this compound as a potential anti-carcinogenic drug. Methanolic extract *D. latifolia* significantly inhibit the disturbances in the cell division by increasing mitotic index *in vivo*.

Hence, we concluded that methanolic extract *D. latifolia* doesn't possess genotoxicity.

In conclusion, methanolic extract *D. latifolia* showed significant anti-mutagenicity in MN in bone marrow cells of mice and also showed potent antimutagenic activity.

REFERENCES

1. Sumanth M, Chowdary GN. Antimutagenic activity of aqueous extract of *Momordica charantia*. Int J Biotechnol Mol Biol Res 2010;1(4):42-6.
2. Genotoxicity: Specific aspects of regulatory genotoxicity tests for pharmaceuticals. Available from: http://www.tga. gov.au/docs/pdf/euguide /vol3b/3bs6aen.pdf. [Last accessed on 2010 Apr 26].
3. Mohammed BM, Karim KJ, Yaseen NY. Antimutagenic effects of *Thymus syriacus* extract. J Duhok Univ 2009;12(1):216-26.
4. Gonzalez Borroto JI, Creus A, Marcos R, Molla R, Zapatero J. The mutagenic potential of the furylethylene derivative 2-furyl-1-nitroethene in the mouse bone marrow micronucleus test. Toxicol Sci 2003;72:359-62.
5. ICH Harmonised Tripartite Guideline.
6. Seetharama Rao KP, Narayana K. *In vivo* chromosome damaging effects of an inosine monophosphate dehydrogenase inhibitor: Ribavirin in mice. Indian J Pharmacol 2005;37(2):90-5.
7. Adler ID, Ramarao G, Epstein SS. *In vivo* cytogenetic effects of trim ethyl phosphate and of TEPA on bone marrow cells of male rats. Mutat Res 1973;13(3):263-73.
8. Parrotta JA. Healing Plants of Peninsular India. USA: CABI Publishing; 2001. p. 387.
9. Acute Oral Toxicity: Organization for Economic Cooperation and Development. OECD Guidelines for the Testing of Chemicals. Guideline 425: Acute Oral Toxicity-Up-and-Down Procedure. Adopted: December, 2001.
10. Organization for Economic Cooperation and Development. OECD Guidelines for the Testing of Chemicals. Guideline 420: Acute Oral Tox-icity-Fixed Dose Method. Adopted: December, 2001.
11. Matter B, Schmid W. Trenimon-induced chromosomal damage in bone-marrow cells of six mammalian species, evaluated by the micronucleus test. Mutat Res 1971;12(4):417-25.
12. Schmid W. The micronucleus test. Mutat Res 1975;31(1):9-15.
13. Schmid W. The micronucleus test for cytogenetic analysis. Chemical

Mutagens, Principles and Methods for their Detection. New York: Plenum Press; 1976. p. 31-53.
14. Fenech M, Chang WP, Kirsch-Volders M, Holland N, Bonassi S, Zeiger E, *et al*. HUMN project: Detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. Mutat Res 2003;534(1-2):65-75.