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 latinue 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, Methanol 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. 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 antigerm line substrates, 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 antimutagenesis 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. Genticide used as a mutagen with anti-metabolites activity. It exerts its effect by inhibiting 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 tumorogenesis 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.
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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. Gokhicine (Himedia)
17. Phosphate buffer saline (Himedia)

Preparation of extracts
The leaves of D. latifolia was powdered (500 g) and the methanolic extract was prepared using soxhlet 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 nts 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 KH2PO4 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, 04 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 normochromatic erythrocytes (%MNNCE) formation significantly increased (p<0.001) 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 (100mg/kg; i.p./day/single) in bone marrow cells (Fig. 3).

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 anacentric fragment

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

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Tests</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tests for steroids and triterpenes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Salkowski test</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Liebmann-Burchard test</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Kahleberg test</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Tests for alkaloids</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mayer’s reagent</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Dragendorff’s reagent</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Hager’s reagent</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Wagner’s reagent</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Tests for Saponins (Foam test)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Tests for phenolic compounds and Tannins</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ferric chloride test</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Gelatin test</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Lead acetate test</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Tests for flavonoids</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sodium hydroxide test</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Ferric chloride test</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Shindau’s test</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>ZINC-HCl reduction test</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Lead acetate test</td>
<td>+</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Plant extract</th>
<th>Percentage yield</th>
<th>Colour</th>
<th>Odour</th>
<th>Nature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>3.73</td>
<td>Dark brown</td>
<td>Characteristic</td>
<td>Non-sticky</td>
</tr>
</tbody>
</table>

Table 3: Physical properties of flavonoids

<table>
<thead>
<tr>
<th>S. no.</th>
<th>Physical properties</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>State</td>
<td>Solid</td>
</tr>
<tr>
<td>2</td>
<td>Color</td>
<td>Dark brown</td>
</tr>
<tr>
<td>3</td>
<td>Odor</td>
<td>Characteristic</td>
</tr>
<tr>
<td>4</td>
<td>Nature</td>
<td>Non-Sticky</td>
</tr>
</tbody>
</table>

Table 4: Percentage micronuclei 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; p.o./day/7 days) on micronucleus formation in bone marrow cells

<table>
<thead>
<tr>
<th>S. no.</th>
<th>Groups</th>
<th>PCE</th>
<th>MNPCE</th>
<th>%MNPEC±SEM</th>
<th>NCE</th>
<th>MNCE</th>
<th>%MNCE±SEM</th>
<th>P/N ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vehicle control</td>
<td>5500</td>
<td>28</td>
<td>0.46±0.05</td>
<td>5600</td>
<td>15</td>
<td>0.24±0.04</td>
<td>0.91±0.07</td>
</tr>
<tr>
<td>2</td>
<td>Cyclophosphamide (100 mg/kg)</td>
<td>5675</td>
<td>162</td>
<td>2.58±0.05***</td>
<td>5560</td>
<td>54</td>
<td>0.88±0.56***</td>
<td>0.63±0.01***</td>
</tr>
<tr>
<td>3</td>
<td>D. latifolia 100 mg/kg/7 days</td>
<td>5480</td>
<td>61</td>
<td>1.90±0.07***</td>
<td>5575</td>
<td>26</td>
<td>0.62±0.03**</td>
<td>0.76±0.01***</td>
</tr>
<tr>
<td>4</td>
<td>Cyclophosphamide (100 mg/kg)</td>
<td>5455</td>
<td>117</td>
<td>1.00±0.59***</td>
<td>5590</td>
<td>38</td>
<td>0.42±0.0.00***</td>
<td>0.85±0.01***</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SEM, (n=6). ***p<0.001 compared with normal control group. **p<0.01, *p<0.01 compared with cyclophosphamide groups.

PCE: Polychromatic erythrocytes, MN: Micronucleus, NCE: Norm chromatic erythrocytes, SEM: Standard error of the mean, D. latifolia: Dalbergia latifolia

Table 5: Physical properties of flavonoids

<table>
<thead>
<tr>
<th>S. no.</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Solid</td>
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<td>3</td>
<td>Characteristic</td>
</tr>
<tr>
<td>4</td>
<td>Non-Sticky</td>
</tr>
</tbody>
</table>

For the scoring of micronucl, 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.

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 compared with cyclophosphamide group
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 %MNPE and decreasing PCE:NCE ratio. Pretreatment with D. latifolia (200 mg/kg) for 7 days before CP challenge, effectively and significantly decrease the %MNPE 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

5. ICH Harmonised Tripartite Guideline.