EFFICIENCY OF RICINUS COMMUNIS EXTRACT IN MODIFYING CYCLOPHOSPHAMIDE INDUCED CLASTOGENICITY IN MICE BONE MARROW CELLS

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ABSTRACT
Genotoxicity describes a deleterious action on a cell genetic material affecting its integrity. Genotoxic substances are known to be potentially mutagenic or carcinogenic, specifically those capable of causing genetic mutation and of contributing to the development of tumors. This includes both certain chemical compounds and certain types of radiation. Typical genotoxins like aromatic amines are believed to cause mutations because they are nucleophilic and form strong covalent bonds with DNA resulting with the formation of Aromatic Amine-DNA Adducts, preventing accurate replication. Genotoxins affecting sperm and eggs can pass genetic changes down to descendants who have never been exposed to the genotoxin. All known human toxicity derives from one of three types of genotoxicity i.e. Gene mutations, stable chromosomal aberrations or changes in chromosomal number (Environmental Health Perspect, 1996). The present study will be undertaken with an objective of Evaluating the Antimutagenic activity of Extract Ricinus communis in mice using bone marrow micronucleus test and assess the genotoxicity potential of Extract Ricinus communis using Micronuclei test with Cyclophosphamide using Bone Marrow Cells in Swiss Albino Mice.

KEYWORDS: Genotoxicity, micronucleus test, Cyclophosphamide, Ricinus communis.
INTRODUCTION

Genotoxicity describes a deleterious action on a cell genetic material affecting its integrity. Genotoxic substances are known to be potentially mutagenic or carcinogenic, specifically those capable of causing genetic mutation and of contributing to the development of tumors. This includes both certain chemical compounds and certain types of radiation. Typical genotoxins like aromatic amines are believed to cause mutations because they are nucleophilic and form strong covalent bonds with DNA resulting with the formation of Aromatic Amine-DNA Adducts, preventing accurate replication. All known human toxicity derives from one of three types of genotoxicity i.e. Gene mutations, stable chromosomal aberrations or changes in chromosomal number (Environmental Health Perspect, 1996). Chemicals that exert their adverse effect through interaction with the genetic material (DNA) of cells are called genotoxic (Brusick et al., 1987, 1994). Most human carcinogens are genotoxic in nature. The science of genotoxicity mainly concerns that chemicals, which induce mutations in various experimental models, may conceivably affect the incidence of heritable mutations in man (Davidson IWF et al., 1994).

Genotoxicity tests can be defined as in vitro or in vivo tests designed to detect drugs, which can induce genetic damage directly or indirectly by various mechanisms of action. Genotoxicity tests enable hazard identification with respect to DNA damage and its fixation in the form of gene mutations, large-scale chromosomal damage, recombination and numerical chromosome changes. Drugs that are positive in these tests that detect such kind of damage have the potential to be human carcinogens and/or mutagens i.e., may induce cancer and or heritable defect (Douglass GR et al., 1988). Genotoxicity testing of new chemical entities (NCE) is generally used for hazard identification with respect to DNA damage and its fixation (Madle S et al., 1987). These damages can be manifested in the form of gene mutation, micronucleus, structural chromosomal aberration, recombination and numerical changes. These changes are responsible for heritable effects on germ cells and impose risks to future generations (Wassom J et al., 1992). In addition it has been well documented that somatic mutations can also play an important role in malignancy (Tennant R et al., 1987). These tests have been used mainly for the prediction of carcinogenicity and genotoxicity because compounds, which are positive in these tests, have the potential to be human carcinogens and/or mutagens.
1.1 MICRONUCLEUS
A micronucleus (MN) is formed during the metaphase/anaphase transition of mitosis (cell division). It may arise from a whole lagging chromosome (aneugenic event leading to chromosome loss) or an acentric chromosome fragment detaching from a chromosome after breakage (clastogenic event) which do not integrate in the daughter nuclei. Scoring of micronuclei can be performed relatively easily and on different cell types relevant for human biomonitoring: lymphocytes, fibroblasts and exfoliated epithelial cells, without extra in vitro cultivation step. MN observed in exfoliated cells are not induced when the cells are at the epithelial surface, but when they are in the basal layer. An ex vivo/ in vitro analysis of lymphocytes in the presence of cytochalasin-B (added 44 hours after the start of cultivation), an inhibitor of actins, allows to distinguish easily between mononucleated cells which did not divide and binucleated cells which completed nuclear division during in vitro culture. Indeed, in these conditions the frequencies of mononucleated cells provide an indication of the background level of chromosome/genome mutations accumulated in vivo and the frequencies of binucleated cells with MN a measure of the damage accumulated before cultivation plus mutations expressed during the first in vitro mitosis.

Fig:1 The combination of the micronucleus assay with fluorescence in situ hybridisation (FISH) with a probe labeling the (peri-)centromeric region of the chromosomes (FISH assay) allows discrimination between micronuclei containing a whole chromosome (centromere positive micronucleus) and an acentric chromosome fragment (centromere negative micronucleus).

The criteria for selecting binucleated cells to score are the following: Score binucleated cells with Main nuclei that are separate and of approximately equal size,
Main nuclei that touch and even overlap as long a nuclear boundaries are able to be distinguished, and Main nuclei that are linked by nucleoplasmic bridges. Do not score: Trinucleated, quadrancnucleated, or multinucleated cells or Cells where main nuclei are undergoing apoptosis.

A micronucleus is the erratic (third) nucleus that is formed during the anaphase transition of mitosis. It may arise from a whole lagging chromosome (aneugenic event leading to chromosome loss) or an acentric chromosome fragment detaching from a chromosome after breakage (clastogenic event), which do not integrate in the daughter nuclei during cell division, as illustrated by (Fenech, M. et al., 1999).

Fig:2. The illustration of MN formation from lagging whole chromosome and acentric chromosome fragments in dividing cell at anaphase.

The micronucleus assay in rodent bone marrow nucleated cells can detect a wide spectrum of changes, which result from breakage of one or more chromatids as the initial event. Breakage of chromatids or chromosomes can result into micronucleus formation if an acentric fragment is produced. Therefore, assays detecting either chromosomal aberration or micronuclei are acceptable for detecting clastogens (CSGMT 1986). The measurement of micronucleated erythrocytes in polychromatic erythrocytes is an acceptable alternative to detect clastogens/aneuploidy inducers. Male mice are more sensitive than female mice for induction of micronuclei and the differences are only quantitative, but not qualitative. (CSGMT 1992).

1.2 Mechanisms of antimitagenesis: Some of the mechanisms of antimitagenesis and antitumorogenesis are listed as under (Ferguson L.R et al., 1994). Bioantimutagens: These are equivalent to the "true" antimutagens they act on the repair and replication processes of the
mutagen-damaged dna, resulting in a decline in mutation frequency (ferguson l.r et al.,1994). Desmutagens: These must be considered only as apparent antimutagens they indirectly inactivate the mutagens.

1.3 Chemical or enzymatic inactivators: Many mutagens, directly inactivated by a range of different chemicals such as indole-3-carbinol inducers of phase II enzymes such as glutathione transferase tend to inhibit a wide range of target carcinogens e g , isothiocyanates such as benzyl isothiocyanate and antioxidants such as 2, 3-tert-butyl- 4-hydroxyarusole (bha) ( ferguson l.r et al.,1994).

1.4 Antioxidants and free radical scavengers

A wide range of chemopreventive agents has anti-oxidant or free-radical scavenging activity, eg., carotenoids, retinoids and flavonoids once formed, free radicals, having a short half-life eg., oh radical (half life =10"* sec), can be removed or inactivated ( ferguson l.r et al.,1994) .

Scavenging: A number of desmutagens are able to scavenge dietary mutagens (activated or non-activated) through binding or adsorption. In general, the mutagen remains intact during this process, but is unable to react with dna chlorophyllin and some dietary fibers appear to act in this way ( ferguson l.r et al.,1994) . Antioxidants and free radical scavengers: A wide range of chemopreventive agents has anti-oxidant or free-radical scavenging activity, eg., carotenoids, retinoids. Factors which can influence the AF/ MN relationship: For convenience, the factors that influence the observed frequencies of MN derived from a given frequency of AF are grouped under four broad headings (Streffer, 1994). Production factors,- Fragment-fate factors, Cell-kinetic factors. The time-displacement factor.

1.4 Ricinus communis: Plants have always been used as a common source of medicines, both in traditional remedies and in industrialized products. It is estimated that more than 80% of the world’s population use plants as their primary source of medicinal agents. The Brazilian flora has been estimated to be the largest in the world and in this country, plants have always been used for prophylactic effects and for treatment of illness and diseases. Because of this, it is extremely important that the genotoxicity tests of these preparations are made in order to assess their mutagenic potential or modulating of genotoxicity when associated with others substances.
Ricinus communis, family: Euphorbiaceae, is also known as Castor oil plant. It is a tropical plant, known as castor bean, that is distributed widely across the world. The plant is native of India and cultivated throughout the country in gardens and fields and also grows wild in waste places. Ricinus communis is a small wooden tree which grows to about 6 meters in height. Stems of Ricinus communis have Anticancer, Antidiabetic and Antiprotozoal activity. the anticlastogenic potency of the ethanolic extract of Ricinus communis and the results indicate the protective effect against cyclophosphamide and mitomycin-c induced cytogenetic damage. mast cell stabilization property of aqueous and hydrochloric extract of Ricinus communis in rat peritoneal mast cells. Ricinus communis significantly exhibit anti tumour and anti mutagenic activity.

Antitumour activity: Ricin A, a lectin isolated from R. communis possess antitumor activity, it was more toxic to tumor cells than to nontransformed cells, judged from the ED50 of the lectin towards tumor cells and non-transformed cells.

![Structure of Cyclophosphamide](image)

**STRUCTURE OF CYCLOPHOSPHAMIDE**

**Mechanism of Action**

Cyclophosphamide also known as cytophosphane, is a nitrogen mustard alkylating agent from the oxazaphosphinans group. An alkylating agent adds an alkyl group (cnh2n+1) to DNA. It attaches the alkyl group to the guanine base of DNA, at the number 7 nitrogen atom of the imidazole ring. The main effect of cyclophosphamide is due to its metabolite phosphoramide mustard. This metabolite is only formed in cells that have low levels of ALDH. Phosphoramide mustard forms DNA crosslinks both between and within DNA strands at guanine N-7 positions (known as interstrand and intrastrand crosslinkages, respectively). This is irreversible and leads to cell apoptosis. Cyclophosphamide has relatively little typical chemotherapy toxicity are present in relatively large concentrations in bone marrow stem cells, liver and intestinal epithelium.
MATERIALS AND METHODS


METHODOLOGY

processing of plant material

The leaves of R.communis were collected from Geethanjali college of pharmacy, medicinal garden, cheeryal, Andhrapradesh. The plant leaves were air dried under shed at 25°C and the dried leaves were made in to a fine powder with an auto-mix blender. The powder was kept in deep freezer until the time of use.

Preparation of Aqueous Extract: One hundred grams of dry fine powder was suspended in 250 ml of water for two hours and then boiled at 60°C to 65°C for 30 minutes (since boiled decoction of the leaf of this plant has been used as remedy for diabetes). The collected extract was pooled and passed through a fine cotton cloth. The filtrate upon evaporation at 40°C yielded 20 percent semi-solid extract. The semi solid extract was dissolved in 1% CMC (carboxy methyl cellulose). The dose of the extract 100mg/kg was calculated by the body weight basis and administered through oral route. Mitomycin C solution was administered intraperitoneally at a dose of 4mg/kg according to the body weight of the animal.

Animals: All the animals were obtained from Department of pharmacology, Geethanjali college of pharmacy, Keesara, and approved by Animal Ethics committee, Regd no : 1648/PO/a/12/CPCSEA-GCOP-IAEC-03/2013. Mice were allowed to acclimate to the
experimental room conditions for a period of five days prior to randomization and treatment. During the acclimatization period, the mice were observed for clinical signs.

Micronucleus Assay: For the micronucleus assay, the extract at the volume of 0.2 ml at different doses level such as 125, 250 and 375 mg/kg body weight was injected 24 hours before the treatment of cyclophosphamide, to six animals. The positive control group received single i. p. injection of 50 mg/kg cyclophosphamide in 0.9% saline. The animals were sacrificed by cervical dislocation and bone marrow cells were harvested.

Collection of Bone Marrow Cells: The mice were sacrificed by cervical dislocation on the third day after initial dosing. Both the femoral bones were excised and bone marrow cells were aspirated using 1.5 ml of foetal calf serum. The samples were transferred to prelabelled tubes. The cells were centrifuged at 2000rpm for 10 minutes. The collected supernatant was smeared on to a clean glass slide. The slides were stained using Giemsa and Maygrunwald stains for clear differentiation of polychromatic erythrocytes and normochromatic erythrocytes during scoring.

Scoring of Slides: The slides were prepared essentially as described by Schmid (1975) and modified by Aron et al. (1980). After staining with May-Gruenwald and Giemsa, a total 1000 cells were scored at the magnification of x1000 (100 x 10x) for each group. The data are expressed as the average number of micronucleated cells/thousand polychromatied erythrocytes cells (PCE) cells/animals (±SE) for a group of six animals. The results were compared with the vehicle control group using Student’s t test with significance determined at p<0.05.
RESULTS

Fig(a): Binucleated cell without the micronucleus; Fig(b,c): Binucleated cells with the micronucleus of different size; Fig(d): Binucleated cell with two micronuclei.

Table 1: Effect of *R. communis* extract on micronucleus in mouse bone marrow cells.

<table>
<thead>
<tr>
<th>Group</th>
<th>MNPCE</th>
<th>PCE/NCE Ratio</th>
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<tbody>
<tr>
<td>Cyclophosphamide (CP)</td>
<td>3.32 ± 0.925</td>
<td>0.474 ± 0.022</td>
</tr>
<tr>
<td><em>R. communis</em> leaves ext. (125 mg/kg) + CP (50 mg/kg)</td>
<td>1.82 ± 0.484</td>
<td>0.686 ± 0.006</td>
</tr>
<tr>
<td><em>R. communis</em> leaves ext. (250 mg/kg) + CP</td>
<td>1.34 ± 0.496</td>
<td>0.745 ± 0.026</td>
</tr>
<tr>
<td><em>R. communis</em> leaves ext. (375 mg/kg) + CP (50 mg/kg)</td>
<td>0.84 ± 0.267</td>
<td>0.825 ± 0.028</td>
</tr>
<tr>
<td><em>R. communis</em> leaves ext. (125 mg/kg) + CP (50 mg/kg)</td>
<td>0.50 ± 0.23</td>
<td>0.924 ± 0.056</td>
</tr>
<tr>
<td>Solvent (DMSO)</td>
<td>0.44 ± 0.20</td>
<td>0.982 ± 0.047</td>
</tr>
</tbody>
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* Denotes Statistical Significance at P<0.05 in ‟t‟ test. When compared with respective positive control group. Each group consists of six animals.

In antimutagenicity studies, single application of *R. communis* extract at the dose of 125, 250 and 375 mg/kg body weight, 24 hours prior the i.p. administration of cyclophosphamide (at
the dose of 50 mg/kg) have significantly prevented the micronucleus formations in dose dependent manner in bone marrow cells of mice as compared to Cyclophosphamide group. However, R.communis extract alone has not induced significant micronucleus formations in bone marrow cells as compared to control group (Table 1)

DISCUSSION
This preliminary study of the clastogenic effect of R.communis using micronucleus revealed that there is no significant induction of micronucleus in formation as compared to Positive control group. The phytochemical study indicated the presence of Ricin A, a lectin isolated from R. communis extract. Flavonoids which have been shown to posse’s antimutagenic and anticarcinogenic activity and lectins reported to produce structural variation of the cell envelope. The mechanism underlying the antimutagenic action of R.communis extract and its active principles is not clear; the beneficial effect of R.communis extract may be due to either individual or combined effects of its constituents. All these data point to the possibility of developing an extract of R.communis as a novel, potential agent in the area of cancer chemotherapy. The present investigation therefore reveals that aqueous extract R.communis certainly possesses antimutagenic properties. Ricin A, a lectin isolated from R. communis possess antitumor activity, it was more toxic to tumor cells than to nontransformed cells, judged from the ED50 of the lectin towards tumor cells and non-transformed cells. However, further studies are warranted to elucidate the exact mechanism of action.

CONCLUSION
Based on study We observed A significant clastogenic effect of R.communis using micronucleus revealed that there is a significant induction of micronucleus in formation as compared to Positive control group. The present investigation therefore reveals that aqueous extract R.communis certainly possesses antimutagenic properties. All these data point to the possibility of developing an extract of R.communis as a novel, potential agent in the area of cancer chemotherapy. This study gives only preliminary evidence about the role of R.communis in clastogenic effect. Experiments involving cancer chemotherapy studies would be more confirmatory.

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