

Effect of *Citrus medica* L Fruit Peel Extract on Genotoxicity Induced by Cyclophosphamide in Mice Bone Marrow Cells

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Abstract: Fruit waste reutilization is one of the fore most routes of employing it in many peculiar products. Here, we have evaluated the effect of *Citrus medica* L. fruit peel extract on genotoxicity induced by cyclophosphamide in mice bone marrow cells using micro nucleus assay. Mice were orally pretreated with solutions of *citrus medica* L peel extract prepared at different doses (Low dose: 5mg and High dose: 10mg) for 5 successive days. Then on the sixth day mice were injected intraperitoneally with cyclophosphamide and after 24hr the mice were killed for assessment of micronucleated polychromatic erythrocytes (MnPCEs) in bone marrow cells. Cyclophosphamide and its metabolites may be involved in the toxic reactions and cause DNA damage, inducing genotoxic effects in the cells. In our study, administration of *citrus medica* extract suspension for 5 days resulted in a dose dependent inhibition of micronuclei formation induced by cyclophosphamide in mouse bone marrow cells. *Citrus medica* extract suspension significantly reduced the frequency of MnPCEs to show a protective effect against the side effects of cyclophosphamide. The strong antioxidative activity of citrus extract contributed to reduction in the genotoxicity induced by cyclophosphamide.

Key words: Genotoxicity • Micro Nucleus • Antioxidant • DNA • Dose Dependent.

INTRODUCTION

Genotoxicity is defined as a destructive effect on a cell's genetic material (DNA, RNA) affecting its integrity by damaging them irreversibly [1-3].

Cyclophosphamide (CYP) is a cytotoxic alkylating agent used to treat sarcomas and carcinomas of the lung and mammary organs in animals. Since the CYP causes a serious side effects such as genotoxic impacts, renal and hepatic damage, thereby limiting its therapeutic use. Its cytotoxic impacts result from the responsive metabolites that alkylate DNA and form an assortment of DNA adducts that adequately modify DNA structure or capacity prompting to arrangement of chromosomal deviations and micronuclei development [4]. Subsequently, the cyclophosphamide was utilized in our review for instigating genotoxicity in mice.

Citrus medica L. (citron) belongs to family, Rutaceae is widely distributed in Himalayas and hill areas in India and also this herb is found in many parts of the tropical world, Mediterranean, south and central America [5].

Citrus medica contains citroflavonoids that have been found to possess antidiabetic activity [6]. In ayurvedic practice, the dried rind or citrus juice is used in kapha and vata diseases, as a vermifuge, for asthma and digestive disorders, as antiscorbutic and also used to counteract nausea, to increase appetite and as an antimicrobial agent [7-9]. Limited studies are available in the scientific literature on effect of *Citrus medica* peel extract on genotoxicity. Therefore, it was aimed to evaluate the possible effect of peel extract of *Citrus medica* on genotoxicity in mice bone marrow cells by micro nucleus assay.

MATERIALS AND METHODS

Cyclophosphamide injection (1g) bottle was purchased from local medical shop and 50ml of water for Injection aspirated through it to dissolve the powder and make the concentration of 20µg/ml, stored at 2°C until being used.

Preparation of Extracts: The fruits of *citrus medica* were collected from the local market and peel were dried at room temperature and powdered in a grinder. Aqueous Methanol (75%,75ml) was added to the powdered peel (50g) and then stirred for 1hr. The mixture was allowed to stand for 24h at room temperature. After Filtration Methanol was evaporated under reduced pressure at 40°C until the methanol was removed. The remaining aqueous medium was shaken with chloroform (25ml) to remove the lipid soluble substances. The chloroform phase was discharged the remaining water content followed by drying and the dried extract was kept for further use [10].

Determination of Flavonoids: 10g of the plant samples were extracted repeatedly with 100ml of 80% aqueous methanol at room temperature. The extract was filtered through Whatman filter paper No. 42 (125mm). The filtrate was later transferred into a crucible and evaporated to dryness over a water bath and weighed [11].

Experimental Design: The micronucleus assay detects chromosome damage and whole chromosome loss in polychromatic erythrocytes and eventually in normochromatic erythrocytes in peripheral blood as the red cells mature. A micronucleus is a small structure (1/5 to 1/20 the size of the nucleus) containing nuclear DNA that has arisen from chromosome fragments or whole chromosomes that were not incorporated into daughter nuclei at anaphase of mitosis. Micronuclei can be found in cells of any tissue, but only form in dividing cells.

The micronuclei in young erythrocytes arise primarily from dislocated chromosomes from disturbed mitotic spindle or chromosome fragments that are not incorporated into the daughter nuclei at the time of cell division in the erythropoietic blast cells and changes in the incidence of micronucleated polychromatic erythrocytes (MNPCE) are considered to reflect chromosomal damage [12].

For micronucleus assay, mice were maintained standard conditions of temperature and light (light: dark, 12 hrs: 12 hrs.) by providing standard mice feed and water

ad libitum. And were grouped in to five groups based on the mean body weights of 25-30g each. Grouping was done as follows Normal Control, Cyclophosphamide alone, Drug alone, High Dose Drug along with Cyclophosphamide, Low Dose Drug along with Cyclophosphamide.

Naming of the Animals: Four animals were named based on the marking with picric acid; animals were named as, Head (H), Left Femora (LF), Right Femora (RF) and Left Body (LB).

Drug Preparation: 10mg of *Citrus medica* extract was weighed and 20 ml of distilled water is added and used as high dose drug vehicle. 5mg of *Citrus medica* extract was weighed and 20 ml of distilled water is added and used as low dose drug vehicle. This suspension was gently macerated using pestle and mortar to get very fine suspension of the drug vehicle. The dose value of prepared drug is mentioned in the Table 1.

Micro Nucleus Assay: The micro nucleus test for mouse bone marrow was carried out as stated by Schmid [13] to assess chromosomal damage in experimental animals. After 24 h of cyclophosphamide treatment, the animals were sacrificed by cervical dislocation. Both the femora's were removed and bones were then freed from the muscle. The bone marrow was aspirated with 0.2ml fatal calf serum and contents were aspirated in to a disposable plastic syringe. Obtained solution was centrifuged at 1000 rev/min for 5 min. Supernatant was removed and sediment carefully mixed by aspiration.

A small drop of viscous suspension was put on the end of the slide and spread by pulling the material behind a polished cover glass held at an angle of 45° and air dried. Slide was kept in undiluted May-Greenwald (MG) stain for three minutes followed by diluted MG stain (1:1) for 2 min. Finally Giemsa stain diluted (1:6) with distilled water. Then the slides were rinsed in distilled water and blot dried with filter paper. After cleaning the back of the slide with methanol and was air dried.

Slides were analyzed for the presence of the micronucleus under 40x magnifications in fluorescent microscope. Close observation was made to determine Micronucleus. Micronucleus polychromatic erythrocytes were determined per 1000 cells [14].

Antioxidant Activity: The antioxidant activity of *Citrus medica* extract was evaluated using the stable DPPH radical according to the method of Koleva *et al.* [15] with

Table 1: Dose concentrations

Code	Group	Body weight (kg)	Drug Dose (ml)	Cyclophosphamide (ml)
H	Normal control	0.23	0.0	0.0
LF		0.28		
RF		0.19		
LB		0.28		
H	Cyclophosphamide alone	0.38	0.0	0.095
LF		0.37		0.09
RF		0.21		0.052
LB		0.27		0.067
H	<i>Citrus medica</i> extract alone	0.33	0.6	0.0
LF		0.31		
RF		0.25		
LB		0.27		
H	Cyclophosphamide with low dose drug (5 mg/kg)	0.20	0.4	0.05
LF		0.22		0.055
RF		0.25		0.062
LB		0.30		0.075
H	Cyclophosphamide with High dose drug (10 mg/kg)	0.38	1.0	0.095
LF		0.39		0.1
RF		0.21		0.052
LB		0.29		0.071

some experimental modifications. Briefly 1.0 ml of 0.1 mM solution of DPPH radical was added to sample or standard solution in methanol containing different concentrations (100 - 500 µg/ml). The reaction mixture was shaken and incubated for 30 minutes in the dark chamber. Absorbance was measured at 520 nm which showed the decrease in absorbance of the resulting solution with increase in concentration. Ascorbic acid was used as the standard antioxidant and antioxidant activity was expressed in terms of Ascorbic acid equivalents.

The capacity to scavenge the DPPH radical was calculated using the following equation:

$$(\%) \text{ scavenging} = [(Ac - As)/Ac] \times 100$$

The IC₅₀ values (extract of *Citrus medica* that causes 50% scavenging) were determined from the graph of scavenging effect percentage against the extract concentration. All determinations were carried out in triplicate.

Statistical Analysis: The data were entrusted as means ± S.D. One-way ANOVA analysis and Tukey's HSD test were used for multiple resemblances of data. A probability value of 0.05 was assumed to designated implication.

RESULTS AND DISCUSSION

The extracted *citrus medicais* further used as drug to carry out the Genotoxicity assays. *C.medica* is relevant to treatment of diabetes. The essential oil of the peel is

regarded as an antibiotic. *Citrus medica* contains citroflavonoids which have been found to possess a range of anti-inflammatory, anti-histamine, diuretic actions and cause the dilation of the coronaries [10].

The flavonoid content was 0.95g/10g of *citrus medica*. Flavonoids also have the potency to stimulate the immune system, induce protective enzymes in the liver or block damage to genetics materials. Flavonoids are very effective antioxidants and a number of flavonoids may inhibit the enzymes implicated in the oxidation of polyunsaturated fatty acids [15].

Effects of Citrus Medica Extract on the Frequency of Micronucleus Polychromatic Erythrocyte Cells (MnPCEs) Induced by Cyclophosphamide: Micronucleus cells were counted for 1000 Polychromatic Erythrocyte Cells after the collection of the bone marrow cells from the femur bone.

The micronuclei in young erythrocytes emerged predominantly from disorganized chromosomes from disconnected mitotic spindle or chromosome fragments that were are not integrated into the nuclei of the daughter at the time of cell division in the erythropoiesis blast cells and reformed in the occurrence of micronucleated polychromatic erythrocytes (MNPCE) are appraise to reflect chromosomal damage.

Antioxidant Activity: Polyphenols are a large and diverse class of compounds, many of which occur naturally in a wide range of food and plants [16]. The flavonoids are the largest and best studied group among polyphenols. Many

of them have properties including antioxidant, anti-mutagenic, anti-carcinogenic and anti-inflammatory effects that might potentially be beneficial in preventing disease and protecting the stability of genome. Antioxidant quality is a measure of the effectiveness of the antioxidant present as a pure compound or a mixture [16-18].

CONCLUSIONS

In the present study, it was concluded that the unpredictable effect of cyclophosphamide could be circumvented by utilizing the therapy of *Citrus medica* peel extracts which are being considered as a waste material of the fruit. It may be due to the presence of natural ascorbic acid, flavonoids and phenolic compounds. The results obtained showed that if *Citrus medica* is continuously consumed as a food supplement can reduce the genotoxicity induced by hazardous chemical agents in animals and human beings.

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