



Contents lists available at UGC-CARE

International Journal of Pharmaceutical Sciences and Drug Research

[ISSN: 0975-248X; CODEN (USA): IJPSPP]

journal home page : <http://ijpsdr.com/index.php/ijpsdr>

Research Article

Evaluation of Mutagenic and Antimutagenic Activity of Methanol Extract of *Cousinia thomsonii* against Cyclophosphamide

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ARTICLE INFO

Article history:

Received: 23 October, 2019

Revised: 10 January, 2020

Accepted: 20 January, 2020

Published: 30 January, 2020

Keywords:

Cousinia thomsonii,
Cyclophosphamide,
Micronucleus,
Mitotic index.

DOI:

10.25004/IJPSDR.2020.120109

ABSTRACT

The present study was conducted to evaluate the mutagenic and antimutagenic potential of *Cousinia thomsonii* (CT) extract in bone marrow cells of male wistar rats using some important parameters like micronucleated polychromatic erythrocyte (MnPCE), mitotic index (MI), chromosomal aberrations (CA), and polychromatic erythrocyte (PCE) to normochromatic erythrocyte ratio (PCE/NCE). Thirty male rats of Wistar strain were divided into 6 groups with 5 rats in each group. Group 1 rats were taken as negative control having free access to distilled water and rat feed. Group 2 rats were taken as positive control treated with mutagen cyclophosphamide (CP) at a dose of 60 mg/kg b wt. for 2 days. Group 3 and 4 were treated with CT extract at dose of 100 and 200 mg/kg b wt. for 20 days. Group 5 and 6 were treated with 100 and 200 mg/kg b wt of CT extract for the first 18 days and the last 2 days with CP at dose concentration of 60 mg/kg. It was found that rats treated with CT extract alone did not produce any significant changes in MnPCE, PCE/NCE ratio, CA, and MI when compared with control-treated rats (group 1). However, in group 5 and 6 rats treated with CT extract in combination with CP, a protective effect was observed against cyclophosphamide-induced cellular mutagenicity. In concluding remark, CT was found to show antigenotoxic potential and also produce protective antimutagenic effects against CP induced chromosomal damage.

INTRODUCTION

The use of medicinal herbs is certainly as ancient as human beings themselves. Over 150,000 species of plants have been studied, many of which contain medicinal substances. During earlier times, phytotherapy was considered as one of the best forms of clinical practice to treat, heal, and prevent general disorders.^[1] Herbal and natural treatments are projected to account for about 25% in developed nations and around 80% in developing nations. The total amount of plant species with chemopreventive properties approaches one lakh, but the actual figure could be much greater because only 10–20% of plants have been analyzed till now.^[2] Much emphasis has recently been focused on the use of naturally occurring substances in

food and medicinal plants. Reports of the protection and toxicity of plants used in therapy are important because of their vast range of applications and their common use in folk medicine, which is often the only therapy of many cultures and ethnic groups.^[3] Many phytotherapy experiments have already shown that most compounds of medicinal plants have detrimental effects such as mutagenicity, carcinogenicity, and toxicity. Therefore these drawbacks limit their use as therapeutic agents.^[4,5] Despite the fact that the beneficial uses of medicinal plants, many chemicals present in plants may show mutagenic effects due to their ability to produce genomic changes, which subsequently results in severe health issues. Hence, it is necessary to check the mutagenicity of plant constituents to ensure the value of medicinal

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Relevant conflicts of interest/financial disclosures: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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compounds.^[6,7] Many assays are being used to assess the genotoxicity of test chemicals in both *in-vivo* and *in-vitro* conditions. Some parameters like micronucleus formation, chromosome abnormalities, and mitotic index, are routinely used in genotoxicity evaluation.^[8,9]

Cousinia thomsonii (CT) is a perennial herb of family Asteraceae. It is commonly referred to as 'Thomsons Thistle' in English and 'Megtham' in Ladakhi language. It is an erect spinny thistle-like plant, with cottony stem, pinnately lobed spiny leaves, and with large round purple flowers. It is 1 feet 6 inch long hermaphrodite plant with flowering season in the month of July to September. Its distribution extends from Afghanistan to western Nepal, and Tibet. It is also found at high elevations 3000 to 4200 meters of Ladakh and Lahul of Jammu and Kashmir. It has been found that dried root powder of CT cure severe joint pains.^[10,11] It is also found effective in curing headache, stomach problems, measles, and boils. There is very scarce literature present regarding this plant, and little information is available about the biological effects of its constituents. No report has been found in the literature regarding the genotoxicity of CT in prokaryotic or eukaryotic mutation assay. In the present research work, the mutagenic and antimutagenic effect of CT extract was determined using micronucleus and chromosomal aberration assays. The MI and PCE/NCE ratio were also calculated to determine its toxic status.

MATERIALS AND METHODS

Chemicals

All chemicals used in this experiment were of high analytical grade.

Collection and Extraction of Plant Material

Collection of the plant was done in September 2018 from higher areas of Ladakh, J and K, India, and sent to Taxonomy center, Botany Department, University of Kashmir for identification. The identification was made based on the morphological features of the plant. A reference specimen has been stored for future reference in the herbarium of the Botany Department, Kashmir University. About 75 g of powder was extracted from the whole plant with 400 mL of methanol with the help of a Soxhlet extractor. Under reduced pressure, the methanol residue was removed from the extract by rotary evaporator, and the final extract was then stored for further analysis in the refrigerator.

Experimental Animals

In our study 30 Wistar strain male albino rats about 130–180 g in weight were used. These rats were purchased from the Indian Institute of Integrative Medicine (IIM), Canal Road, Jammu. The animals were kept in stainless steel cages in the animal house of the Zoology Department, University of Kashmir, under laboratory conditions of $24 \pm 2^\circ\text{C}$, and 12/12 hours of light and dark cycle. At

the initiation of the experiment, all animals were first acclimatized for about one week and were starved for 50 minutes. The Committee on Institutional Animal Ethics approved the research protocol, and the experiment was done under standard ethical principles.

Dosage and Design of Experimental Animals

The 30 male rats were randomly distributed into 6 groups, with 5 rats per group. The animals from each group were carefully weighed before the start of experiment and at the end of experiment. The dose was given mostly in the morning time between 10.00–11.00 a.m. The animals were treated with the CT extract and CP orally at different doses by oral gavage intubation. The animals were starved for about 12 hours before the start of the treatment protocol. The first day when the animals were treated with the plant extract was counted as experimental day one.

Group 1: (control group) treated with olive oil and distilled water for 20 days.

Group 2: (positive control) treated with (CP) at a dosage of 60 mg/kg b wt. for 2 days.

Group 3: Treated with 100 mg/kg b wt./day of CT extract orally for 20 days.

Group 4: Treated with 200 mg/kg b wt./day of CT extract for 20 days.

Group 5: Treated with 100 mg/kg b wt. of CT extract for first 18 days and for remaining 2 days with CP (60 mg/kg b wt.).

Group 6: Treated with 200 mg/kg b wt. of CT extract for first 18 days and for remaining 2 days with CP (60 mg/kg b wt.).

Chromosomal Aberration (CA) Assay

The protocol of Preston RJ^[12] was used for the analysis of chromosomal abnormalities. Briefly, rats were treated with 0.5 mL of 0.06% colchicine intraperitoneally 3 hours before cervical dislocation in order to stop cells at the metaphase stage. In a centrifuge tube, the bone marrow from both femur bones was removed in 5 mL KCl solution and incubated at 35°C for about 20 minutes. Centrifugation of the cell suspension was done at 1000 rpm for 5 minutes, and the supernatant formed was discarded. The pellets component formed was suspended in 5 mL Carnoy's fixative (glacial acetic acid and methanol in the ratio of 1:3). The centrifugation and fixation process was repeated many times. Slides were then designed, stained by Giemsa solution (5%), and microscopically displayed by using a fluorescence microscope. To determine the number of chromosome aberrations, one hundred metaphasic cells per animal were analyzed.

Micronucleus Assay (MA)

Analysis of micronucleated cells from the bone marrow of rats was made by the method of Von Ledebur M, *et al.*^[13] Bone marrow cells from femur bones were removed with calf serum (fetal) and the mixture was centrifuged at



1000 rpm for 15 minutes. The pellets were spread on a sterilized glass slide and fixed with absolute methanol. Staining of the slides was done by undiluted May Grunwald stain for 3 minutes, then diluted May Grunwald stain for 2 minutes, and at last with 10% Giemsa stain for 10 minutes. Two thousand PCE were analyzed from each animal to check for the presence of micronucleus. The proportion of PCE to NCE from 1000 erythrocytes per rat was also evaluated to detect the toxic effects.

RESULTS

Tables 1–3 show the parameters like micronucleus assay, PCE/NCE ratio, mitotic index, and chromosomal aberrations in bone marrow Wistar rats when treated with CP, CT extract alone, and combination of CT extract and CP. It was found that CP treated group 2 rats produce a highly significant change in the formation of micronucleated erythrocytes and reduction of PCE/NCE ratio in comparison

to the control group. However, it was found that CT extract did not produce any significant micronucleated cell at a dosage of 100 and 200 mg/kg b wt. Similarly, treatment of group 5 and 6 with CT extract in combination with CP show protective effects by reducing micronucleus formation at both concentrations in a dose-dependent manner. It was also found that CT extract alone or in combination with CP improve the PCE/NCE ratio nearly above one.

The results of the micronucleus assay (MA) and PCE/NCE ratio are shown in Table 1. The mean value of MnPCE and PCE/NCE ratio in control rats (Group I) was found to be 2.20 ± 1.30 and 2.50 respectively. As expected in Group 2 (CP treated) the value of MnPCE and PCE/NCE ratio was 32.20 ± 3.49 and 0.42. However, CT extract, in combination with CP reduces the value of MnPCE from 32.20 ± 3.49 to 6.20 ± 1.78 in group 5 and to 3.60 ± 0.89 in group 6 rats.

The result of the MI is shown in Table 2. MI was calculated by considering 1000 cells/ animal ($n = 5/\text{group}$).

Table 1: Frequency of micronucleated polychromatic erythrocytes and PCE/NCE in bone marrow cells of male wistar rats exposed to varying doses of *Cousinia thomsonii* extract alone and in combination with cyclophosphamide.

Groups	Concentration	No. of MNPCE per animal					MNPCE (\pm SD)	PCE/NCE
Group 1	Olive oil (negative control)	4	2	1	1	3	2.20 ± 1.30	2.50
Group 2	Cyclophosphamide 60 mg/kg	30	38	33	30	30	$32.20 \pm 3.49^{**}$	0.42
Group 3	CT extract 100 mg/kg	3	4	0	3	3	$2.60 \pm 1.51^{\#}$	1.22
Group 4	CT extract 200 mg/kg	0	4	3	2	2	$2.20 \pm 1.48^{\#}$	1.59
Group 5	CT extract 100 mg + CP 60 mg/kg	8	8	4	5	6	$6.20 \pm 1.78^{**}$	1.00
Group 6	CT extract 200 mg + CP 60 mg	4	3	5	3	3	$3.60 \pm 0.89^{\#}$	1.04

CT = *Cousinia thomsonii*, CP = Cyclophosphamide.

Values were represented as mean \pm SD with 5 rats per group at $p < 0.05$.

*Significant ($p < 0.05$), ** highly significant ($p < 0.01$), # non-significant ($p > 0.05$).

Table 2: Frequency of mitotic index (MI) in bone marrow cells of male Wistar rats exposed to different doses of *Cousinia thomsonii* extract alone and in combination with cyclophosphamide.

Groups	Concentration	Cells counted	No. of dividing cells	Mitotic index (\pm SD)
Group 1	Olive oil (negative control)	1000	932.40	93.24 ± 2.66
Group 2	Cyclophosphamide 60 mg/kg	1000	568.00	$56.80 \pm 3.96^{**}$
Group 3	CT extract 100 mg/kg	1000	892.00	$89.20 \pm 0.83^{\#}$
Group 4	CT extract 200 mg/kg	1000	921.00	$92.10 \pm 1.59^{\#}$
Group 5	CT extract 100 mg + CP 60 mg/kg	1000	746.00	$74.60 \pm 5.54^{**}$
Group 6	CT extract 200 mg + CP 60 mg	1000	828.00	$82.80 \pm 6.76^{**}$

CT = *Cousinia thomsonii*, CP = Cyclophosphamide.

Values were represented as mean \pm SD with 5 rats per group at $p < 0.05$.

*Significant ($p < 0.05$), ** highly significant ($p < 0.01$), # non significant ($p > 0.05$).

Table 3: Frequency of chromosomal aberrations observed in bone marrow cells of male Wistar rats treated with different doses of *Cousinia thomsonii* extract alone and in combination with cyclophosphamide.

Groups	Concentration	Polyploidy	Rings	Breaks	Fragments	Stickiness	Disorientation	Total abnormality
Group 1	Olive oil (Negative control)	1.0 ± 0.0	0.4 ± 0.54	0.0 ± 0.0	0.2 ± 0.44	0.6 ± 0.54	0.4 ± 0.54	2.16 ± 1.72
Group 2	Cyclophosphamide 60 mg/kg	10.6 ± 2.40	9.0 ± 2.12	7.8 ± 1.64	7.0 ± 1.58	7.8 ± 0.83	7.8 ± 1.48	$41.66 \pm 6.40^{**}$
Group 3	CT extract 100 mg/kg	2.2 ± 0.83	1.4 ± 1.14	0.4 ± 0.54	1.4 ± 1.14	1.4 ± 0.54	1.2 ± 0.44	$6.66 \pm 2.87^*$
Group 4	CT extract 200 mg/kg	2.0 ± 0.70	1.2 ± 1.09	0.2 ± 0.44	1.2 ± 1.30	1.2 ± 0.83	1.0 ± 0.0	$5.66 \pm 2.87^*$
Group 5	CT extract 100 mg + CP 60 mg/kg	2.6 ± 0.89	2.0 ± 1.0	1.0 ± 0.0	2.0 ± 1.22	1.6 ± 0.89	1.6 ± 0.89	$9.00 \pm 2.68^{**}$
Group 6	CT extract 200mg + CP 60mg	2.4 ± 0.54	1.6 ± 0.89	0.6 ± 0.54	1.2 ± 1.30	1.8 ± 0.44	1.4 ± 0.54	$7.50 \pm 3.01^{**}$

CT = *Cousinia thomsonii*, CP = Cyclophosphamide.

Values were represented as mean \pm SD with 5 rats per group at $p < 0.05$.

*Significant ($p < 0.05$), ** highly significant ($p < 0.01$), # non-significant ($p > 0.05$).

It was found that MI in control rats (group 1) was 93.24 ± 2.66 . As expected, rats treated with CP produce a highly significant reduction in MI 56.80 ± 3.96 when compared with control rats. In groups 3 and 4 rats treated with CT extract at a dosage of 100 and 200 mg/kg, there was not found any significant change in MI when a comparison was made with control rats (group 1). However, group 5 and 6 animals, when treated with CT extract in combination with CP, a protective effect was found by increasing the MI in a dose-dependent manner.

The results of CA induced by CP, CT extract, and CT extract + CP are shown in Table 3. CAs were calculated by taking 100 cells per animal (total of 500 cells per group, $n = 5$). The different kinds of aberrations that were observed in our experiment are polyploidy, ring chromosomes, chromosome breaks, chromosome fragments, metaphase stickiness, and chromosome disorientation. It was found that group 2 rats treated with CP produce a highly significant increase in total chromosome abnormality when compared with the control group. Total abnormality found in group 2 was 41.66 ± 6.40 . CT extract treatment in groups 3 and 4 rats produce only slight changes in chromosomal aberrations at the dose concentration of 100 and 200 mg/kg when compared with the control group. However, when groups 5 and 6 rats were treated with CT extract in combination with CP, a protective effect was observed as manifested by the reduction in total chromosomal abnormality. The protective effect was found more significant at a high dose of extract.

DISCUSSION

For the beneficial use of plant products as pharmacological and chemopreventive agents, it is mandatory to investigate more carefully their actual antimutagenic potential *in-vivo* in connection with other antimutagenic factors. Most of the higher plants contain a variety of compounds or phytoconstituents that can mitigate the mutagenic effects of a particular chemical.^[14] The main aim of our current work was to determine the genotoxicity of CT extract against CP induced chromosomal damage using bone marrow of wistar rats. Generally, changes in basic chromosomal architecture are considered good markers for estimating the mutagenic potential of a particular chemical. Some parameters like MnPCE, MI, PCE/NCE, and CA are being widely used to determine the cytotoxicity of chemicals. MI, which is an important parameter used to calculate the number of active dividing cells, should be neither too low nor too high when compared with the negative control. MI lower than control represents a low rate of cell proliferation, and higher than normal control represents an uncontrolled dividing rate of cells, which will ultimately lead to tumor formation.^[15]

In our study, the cytological investigation showed that a significant reduction in MI was seen in rats treated with CP. However, treatment of animals with CT extract alone and in combination with CP shows no major changes in MI when compared with control-treated rats. The restoration of MI

in CT treated rats against CP shows the protective effect of CT extract against mitotic depression. The different kinds of chromosomal aberrations induced by CP observed in our experiment were polyploidy, rings, breaks, fragments, stickiness, and disorientation. Cytotoxicity of CP was seen in the form of a reduction in PCE/NCE ratio and MI. Moreover, MnPCE and CAs were also observed at a high rate. CT extract alone at different doses did not produce any sign of mutagenicity in respect of MnPCE, CAs, MI, and PCE/NCE ratio. However, CT extract, in combination with CP shows protective antimutagenic effects against the CP damage by changing values of MnPCE, CAs, MI, and PCE/NCE ratio to negative control.

Different types of research findings were performed from time to time to investigate the mutagenic and antimutagenic potential of plant extracts, and our present experimental outcomes go in accordance with those findings. Negative genotoxicity was observed in bone marrow cells and peripheral blood of Wistar rats when treated with *Solanum melongena* extract.^[16] Later on same antimutagenic effects and protection were observed when *Solanum melongena* extract was given in combination with doxorubicin to Wistar rats.^[17]

The mushroom, *Agaricus blazei* was found to produce protection against cyclophosphamide-induced genotoxicity in rats. It was also found that three different tea extracts of *Agaricus* significantly lowers the rate of micronucleus formation in polychromatic erythrocytes and reticulocytes.^[18] The antimutagenic behavior against 2-acetylaminofluorene and aflatoxin B₁ by the aqueous extracts of rooibos tea (*Aspalathus linearis*) and honey-bush tea (*Cyclopia intermedia*) was observed.^[19] The phytochemicals derived from the bark of *Terminalia arjuna* prevented the mutagenic effects of aromatic amine, 2-aminofluorene (2-AF) and inhibited the metabolic activation of this pro-mutagen into mutagen.^[20] In a similar observation, it was found that the methanol extract of *Rhizoplaca chrysoleuca* produces antigenotoxic effects against known mutagen sodium azide.^[21] The different plant extracts also have antimutagenic effects against the mutagenicity of cyclophosphamide in mice.^[22]

In another study, it was found that the different concentrations of *Dioscorea pentaphylla* significantly inhibit the mutagenic effects induced by a potent mutagen methyl methanesulphonate (MMS).^[23] They also found that the methanolic extract was highly antimutagenic in comparison to petroleum ether and chloroform. Thus, due to non-mutagenic effects and protection against known mutagens, CT can provide a rationale for use in chemotherapeutic medicine.

CONCLUSION

Some important metabolites such as alkaloids, antibiotics, terpenoids, saponins, quinolines, and some glycoproteins are derived from plants. Lower plants play an important role in the prevention of different diseases. From the



present work, it was proved that CT did not show any mutagenic effect, but in turn, shows protection against potent mutagen cyclophosphamide.

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HOW TO CITE THIS ARTICLE: Khan IS, Bashir K, Gulzar N, Bhat YM. Evaluation of Mutagenic and Antimutagenic Activity of Methanol Extract of *Cousinia thomsonii* against Cyclophosphamide. *Int. J. Pharm. Sci. Drug Res.* 2020;12(1): 53-57. DOI: 10.25004/IJPSDR.2020.120109