

Ipomoea pes-caprae Linn protects mice bone marrow cells against chemo-radiotherapy induced genotoxicity

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ABSTRACT: Chemo-radiotherapy is the gold standard for the non-surgical management of cancer disease. However, patient receiving chemo-radiotherapy may lead to the genotoxicity of bone marrow. Therefore, *Ipomoea pes-caprae* as a potential chemoprotectant was evaluated against chemo-radiotherapy induced genotoxicity in C57BL mice. The present investigation evaluated the protective effect of *Ipomoea pes-caprae* against chemo-radiotherapy induced genotoxicity in mouse bone-marrow cells during the treatment of Melanoma cancer (B16F10). Methanol extract of the plant was administered to mice (25 and 50 mg/kg b. wt.) with the treatment dose of chemotherapy (Dacarbazine 50 mg/kg b. wt.) and radiotherapy (8Gy in 4 fractions, 2Gy in every 10th day to the tumor only) over a treatment time of 40 days. Significant increase ($p < 0.001$) in the chromosomal aberrations were found for the tumor control, radiation control and dacarbazine administered groups. Significant increase in frequency of micronucleated polychromatic erythrocytes (MNPCEs) and micronucleated normochromatic erythrocytes (MNNCEs) was observed ($p < 0.01$) in the tumor control, radiation control and dacarbazine administered groups, whereas the ratio of polychromatic and normochromatic erythrocytes (PCEs/NCEs) was found to be decreased. Methanolic extract treated group has shown a statistically significant ($p < 0.01$) reduction in the aberrant metaphase and number of chromosomal aberrations as compared to tumor radiation control. Aberrant metaphase and incidence of micronuclei formation was found to be decreased in the experimental groups administered with plant extracts. The results of the present study concluded that *I. pes-caprae* has potential chemo-protection against chemo-radiotherapy induced genotoxicity during treatment of melanoma cancer in mice, which support its candidature as a potential chemo-radioprotective agent for cancer patients undergoing chemo-radiotherapy.

KEYWORDS: *Ipomoea pes-caprae*; bone-marrow cells; chemotherapy; radiotherapy; chromosomal aberrations; micronuclei formation

1. INTRODUCTION

Chemotherapeutic agents and ionizing radiation are the most effective agents in cancer therapy. It is well known that both cause side effects and toxic manifestations in patients with irreversible harmful cellular changes [1]. Cancer treatment by chemotherapy and radiotherapy stimulates cell division through oxidative stress, a critical factor in carcinogenesis that can lead to DNA damage (chromosomal aberrations and micronuclei formation) [2]. In addition, mutations in somatic cells also play an important role in the development of other chronic degenerative diseases such as atherosclerosis and heart disease, which may represent another major cause of death in the human population [3]. In this context, researchers are searching for protective compounds from natural sources. A growing body of evidence suggests that herbal medicines may favorably influence genetic responses. Herbs contain a variety of phytoconstituents that may act synergistically to scavenge free radicals and thus minimize the side effects caused by chemoradiation therapy.

Ipomoea pes-caprae (Convolvulaceae), also known as morning glory, railroad vine, and goat's foot, is common in tropical and subtropical regions. The leaves are used in the form of juice as a first aid for jellyfish stings

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[4]. In some parts of India, it is used in ritual baths to drive away evil spirits. It has antioxidant, analgesic, anti-inflammatory, antispasmodic, antinociceptive, antihistaminic and antidiabetic properties [5]. It is rich in betulinic acid, pescaproside, pescapreins, α -terpineol, naphthalinone, mellein, eugenol and 4-vinyl guaiacol, etc. Although *I. pes-caprae* possesses a broad spectrum of therapeutic activity of exceptional value, very few studies have been conducted or the antigenotoxic potential has not been reported in the literature. In the present study, the antigenotoxic potential of *I. pes-caprae* was evaluated against chemotherapy- and radiotherapy-induced genotoxicity in mouse bone marrow cells.

2. RESULTS

2.1. Chromosomal aberration

Data of chromosomal aberrations in experimental groups are presented in Table 1. The metaphase analysis of the bone-marrow cells revealed the presence of slightly higher frequencies of various types of aberrations such as chromatid breaks, fragments, ring, severe damaged cell and deletion (Figure 1) in radiation treated group as compared to normal control group (A_1). Methanolic extract treated groups (B_2 and B_3) have shown reduction in the aberrant metaphase and number of chromosomal aberrations as compared to tumor radiation control group (B_1). The reduction in the mean number of chromosomal aberrations by MeOH extract at 25 and 50 mg/kg, b. wt. was statistically significant ($p < 0.01$). Chromosomal aberrations were also found increased in the standard control dacarbazine treated group (C). Chromatid breaks, dicentric and fragments were observed in tumor bearing mice as compared to the tumor control group (A_1) but administration of MeOH extract as an adjuvant to standard control dacarbazine group (D) at 50 mg/kg, b. wt. showed significant reduction ($p < 0.01$) in the number of chromosomal aberrations.

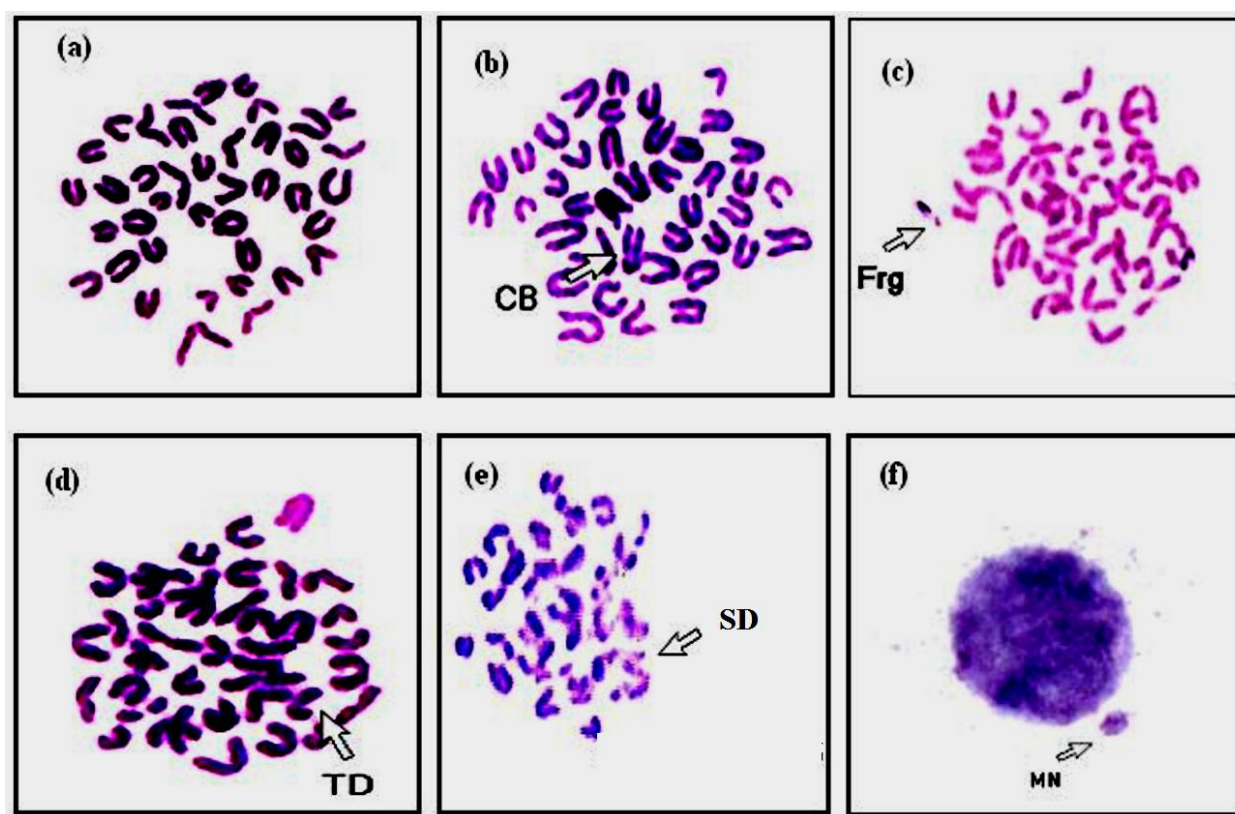


Figure 1. Photomicrograph of chromosomal aberrations in experimental groups. The metaphase analysis of the bone-marrow cells showing normal metaphase (a), and cells revealed the presence of slightly higher frequencies of various types of aberrations such as chromatid breaks (b), fragments (c), terminal deletion (d), severely damaged cells (e) and micronucleus (f) at 100 X magnification.

Table 1. Effect of Methanol extract of *I. pes-caprae* on chromosomal aberration in experimental mice.

S. No	Group Code	Treatment	Number and Type of aberrations / 500 cells (Mean $\pm$ SEM)						
			Chromatid Breaks	Chromosome Breaks	Dicentric	Fragments	Rings	Terminal deletions	SDC
1	A	Normal Saline, 10 ml/kg	1.16 $\pm$ 0.45 a#	0.34 $\pm$ 0.07 a#	00.00 $\pm$ 00	02.50 $\pm$ 0.20 a#	00.00 $\pm$ 00	02.14 $\pm$ 0.50 a#	00.00 $\pm$ 00
2	A ₁	Tumor + N. Saline, 10 ml/kg	28.23 $\pm$ 1.45	20.45 $\pm$ 1.78	24.12 $\pm$ 1.45	40.45 $\pm$ 3.78	19.16 $\pm$ 1.35	22.31 $\pm$ 1.58	29.23 $\pm$ 1.70
3	B	N. Saline, 10 ml/kg + Radiation (8Gy)	43.20 $\pm$ 3.63	35.30 $\pm$ 2.12 ^{b*}	30.20 $\pm$ 2.72 ^{b*}	175.50 $\pm$ 7.40 ^{b#}	24.50 $\pm$ 1.45 ^{b#}	29.20 $\pm$ 1.40 ^{b#}	38.25 $\pm$ 2.70
4	B ₁	Tumor + N. Saline, 10 ml/kg + Radiation (8Gy)	52.25 $\pm$ 3.57	47.50 $\pm$ 3.52	41.25 $\pm$ 3.35	225.46 $\pm$ 8.68	35.02 $\pm$ 2.22	41.34 $\pm$ 3.50	46.26 $\pm$ 3.90
5	B ₂	Tumor + IPCE, 25 mg/kg + Radiation (8Gy)	37.34 $\pm$ 3.45 ^{b*}	28.68 $\pm$ 2.70 ^{b#}	26.90 $\pm$ 2.65 ^{b#}	120.50 $\pm$ 6.50 ^{b#}	17.05 $\pm$ 1.40 ^{b#}	21.30 $\pm$ 1.35 ^{b#}	29.50 $\pm$ 2.48 ^{b#}
6	B ₃	Tumor + IPCE, 50 mg/kg + Radiation (8Gy)	29.68 $\pm$ 2.78 ^{b#}	25.50 $\pm$ 1.80 ^{b#}	22.28 $\pm$ 1.25 ^{b#}	89.75 $\pm$ 4.90 ^{b#}	14.35 $\pm$ 1.50 ^{b#}	17.56 $\pm$ 1.70 ^{b#}	24.34 $\pm$ 1.50 ^{b#}
7	C	Dacarbazine, 50 mg/kg	45.80 $\pm$ 3.45	28.25 $\pm$ 1.75 ^{b#}	34.50 $\pm$ 2.20	92.25 $\pm$ 4.78 ^{b#}	25.56 $\pm$ 1.56 ^{b#}	29.50 $\pm$ 1.38 ^{b#}	32.45 $\pm$ 2.95 ^{b#}
8	D	Dacarbazine, 50 mg/kg + IPCE, 50 mg/kg	32.29 $\pm$ 2.35 ^{b#}	19.41 $\pm$ 1.57 ^{b#}	20.82 $\pm$ 1.85 ^{b#}	52.43 $\pm$ 3.58 ^{b#}	18.57 $\pm$ 1.40 ^{b#}	20.38 $\pm$ 1.65 ^{b#}	23.45 $\pm$ 1.58 ^{b#}

IPCE- *Ipomoea pes-caprae* extract, Values shown are Mean $\pm$ SEM, (n=6 mice)

Significance: * p < 0.05, # p < 0.01, compared to control (one way ANOVA followed by Dunnett's multiple comparison tests)

^aA₁ vs A

^bB₁ vs B, B₂, B₃, C and D

2.2. Micronucleus assay

Micronucleus frequencies observed in polychromatic and normochromatic erythrocytes. The ratio of polychromatic to normochromatic erythrocytes of experimental mice were presented in Table 2. Incidence of MNPCEs and MNNCEs in the tumor control group was increased (Figure 2) as compared to normal control group whereas the ratio of PCEs/NCEs was decreased. Incidence of MNPCEs in the tumor radiation control was found to be increased as compared to radiation control and the ratio of PCEs/NCEs was slightly decreased. Administration of the MeOH extract as an adjuvant to radiotherapy group has shown a reduction in the percent MNPCEs as compared to tumor radiation control group with an increase of the PCEs/NCEs ratio. The reduction in the mean number of micronuclei of PCE at 25 and 50 mg/kg b.wt. with MeOH extract was statistically significant ($p < 0.01$). The standard control dacarbazine, administered alone induced a marked increase ($p < 0.01$) in the percent MNPCEs in tumor bearing mice as compared to the tumor control group. However, administration of the MeOH extract as an adjuvant to chemotherapy group, showed a significant ($p < 0.01$) reduction in the mean number of MNPCEs with an increased ratio of PCEs and NCEs. On comparison, the findings suggest that the MeOH extract found to be more effective in reducing chromosomal aberrations and incidence of micronuclei formation.

Table 2. Effect of Methanol extract of *I. pes-caprae* on frequencies of micronuclei possessing polychromatic and normochromatic erythrocytes mice bone marrow cells.

S. No	Group Code	Treatment	% MNPCs (Mean ± SEM)	% MNNCEs (Mean ± SEM)	PCEs/NCEs (Mean ± SEM)
1	A	Normal Saline (10 ml/kg)	0.46 ± 0.12 ^{a#}	0.32 ± 0.14 ^{a#}	0.32 ± 3.56
2	A ₁	Tumor + N. Saline (10 ml/kg)	13.52 ± 1.34	3.74 ± 0.15	0.27 ± 2.68
3	B	N. Saline (10 ml/kg) + Radiation (8Gy)	54.05 ± 6.45	4.95 ± 0.34 ^{b*}	0.27 ± 2.55
4	B ₁	Tumor + N. Saline (10 ml/kg) + Radiation (8Gy)	70.24 ± 6.48	8.20 ± 1.46	0.27 ± 2.57
5	B ₂	Tumor + IPCE (25 mg/kg) + Radiation (8Gy)	45.29 ± 5.45 ^{b*}	3.94 ± 0.42 ^{b#}	0.28 ± 2.27
6	B ₃	Tumor + IPCE (50 mg/kg) + Radiation (8Gy)	34.93 ± 4.47 ^{b#}	3.17 ± 0.58 ^{b#}	0.29 ± 3.56
7	C	Dacarbazine (50 mg/kg)	14.12 ± 2.57	2.08 ± 0.57 ^{a*}	0.28 ± 2.56
8	D	Dacarbazine (50 mg/kg) + IPCE (50 mg/kg)	6.78 ± 0.68 ^{a#}	1.30 ± 0.45 ^{a#}	0.29 ± 3.12

IPCE- *Ipomoea pes-caprae* extract, Values shown are Mean ± SEM, (n=6 mice)

Significance: * p < 0.05, # p < 0.01, compared to control (one way ANOVA followed by Dunnett's multiple comparison tests)

^a A₁ vs A, C and D

^b B₁ vs B, B₂ and B₃

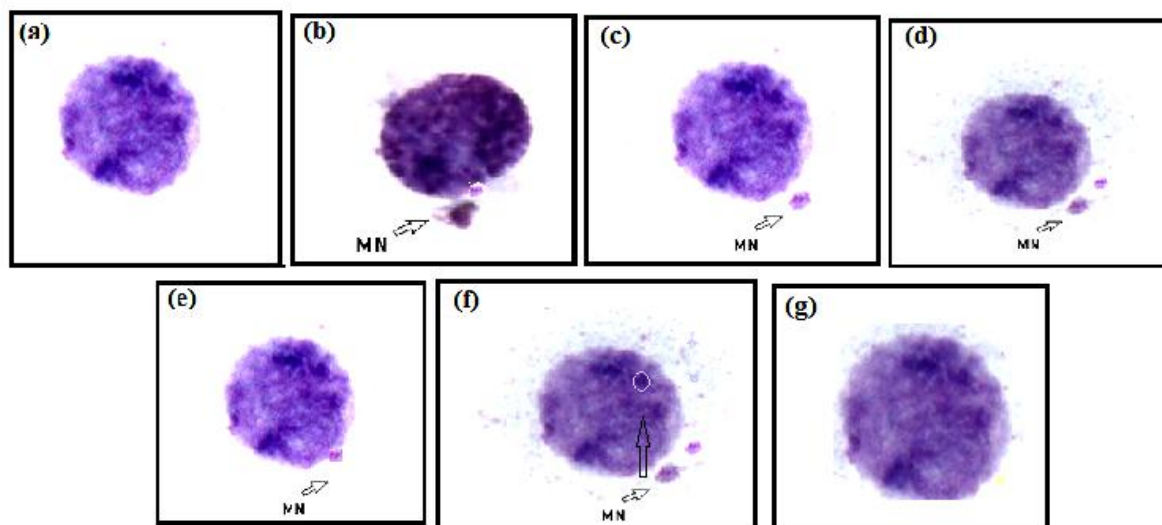


Figure 2. Comparative study showing micronucleus formation difference in various experimental groups. Normal control group showing no any micronucleus (a), while tumor control group showing micronucleus formation (b), radiation control group showing decrease in micronucleus formation (c) in comparison to tumor radiation control group (d), group treated with MeOH extract as an adjuvant to radiotherapy showing decrease in micronucleus formation (e) in comparison to standard control dacarbazine (f), likewise decrease in micronucleus formation was observed with group treated with MeOH extract as an adjuvant to chemotherapy (g) at 100X magnification.

3. DISCUSSION

In the present study, the anti-genotoxic potential of *I. pes-caprae* against chemotherapy and radiotherapy induced genotoxicity in cancer treatment was investigated. Although many pharmacological activities have been reported for this plant [6-10], no anti-genotoxic effect has been reported so far.

Chromosomal aberrations and micronuclei frequency were found more frequently in the tumor control group, radiation control group, tumor irradiation control group, and standard control group. A decrease in the PCE/NCE ratio was observed in all control groups. A significant increase in the frequency of aberrant cells and micronuclei was observed in all control groups, which may be due to the generation of free radicals that can cause genetic abnormalities [11,12]. Damage to chromosomes manifests as breaks and fragments that appear as micronuclei in rapidly proliferating cells. Chemotherapy and radiotherapy, which are most commonly used to treat cancer, can interact with non-tumor cells both in vivo and in vitro, causing genetic abnormalities such as chromosomal aberrations and micronuclei formation [13]. Bone marrow cells are highly proliferative and sensitive to radiation effects [14, 15]. Therefore, bone marrow suppression is an important side effect of radio-chemotherapy and is being investigated for such studies. Ionizing radiation can cause damage to DNA, proteins, lipids, and carbohydrates in various organs at the cellular level [16].

Administration of *I. pes-caprae* extracts significantly reduced the percentage of MNPCE, MNNCE, and aberrant metaphases such as chromatid breaks, chromosome breaks, deletions, rings, terminal dilations, and fragmentation compared with the radiation control. A higher dose of the extracts (50 mg/kg body weight) minimized the side effects of radiation in all other control groups, which proved to be a more effective dose. Administration of dacarbazine in the standard control group resulted in a significant increase in the percentage of MNPCEs, suggesting that dacarbazine caused chromosomal damage such as fragments and chromatid breaks in bone marrow cells. These fragmented chromosomes were condensed into micronuclei that were not included in the main nucleus [17]. The extracts used as adjuvant to dacarbazine resulted in a greater reduction in MNPCE, MNNCE, and aberrant metaphases. This suggests that *I. pes-caprae* may reduce damage to genetic material and thus provide better protection. The frequency of chromosomal aberrations and MNNCEs was also lower and MNPCEs were increased compared to all other groups, similar to normal controls. It is noteworthy that the differentially treated groups with MeOH extract as adjuvant were not cytotoxic (in terms of PCE/NCE ratio) compared with the control groups. Ionizing radiation and chemotherapy targeting a cell result in radiolysis of water, producing free radicals that induce biological damage to the cell. The resulting hydroxyl radicals lead to DNA strand breaks. Natural herbs have a profound effect on the free radical generation and scavenging system operating in the body, with the net effect being to mitigate the toxic effects caused by free radicals [18]. Therefore, extracts of natural herbs are recommended as supplements in free radical-induced lesions. They have been reported to have therapeutic effects on radiation-induced normal tissue damage in experimental animals [19].

The present study revealed that the decrease in MN and the increase in the PCE/NCE ratio were dose-dependent. From the result, it can be revealed that *I. pes-caprae* has no cytotoxic effect on bone marrow cells in mice. It is an indicator of bone marrow protection against cytotoxicity or alterations in erythropoiesis.

4. CONCLUSION

The present study concludes that *I. pes-caprae* has both radioprotective and chemoprotective potential. Methanol extract (MeOH) was found to be effective in protecting against both chemotherapy-induced toxicity and the clastogenic effects of radiation (Figure 3). However, future studies are needed to explore these mechanisms in detail. In addition, this opens a research strategy for the future with the lead molecule in *I. pes-caprae* extract, which may provide valuable tools to mitigate the harmful effects of radiation and chemotherapy on normal tissues in cancer patients.

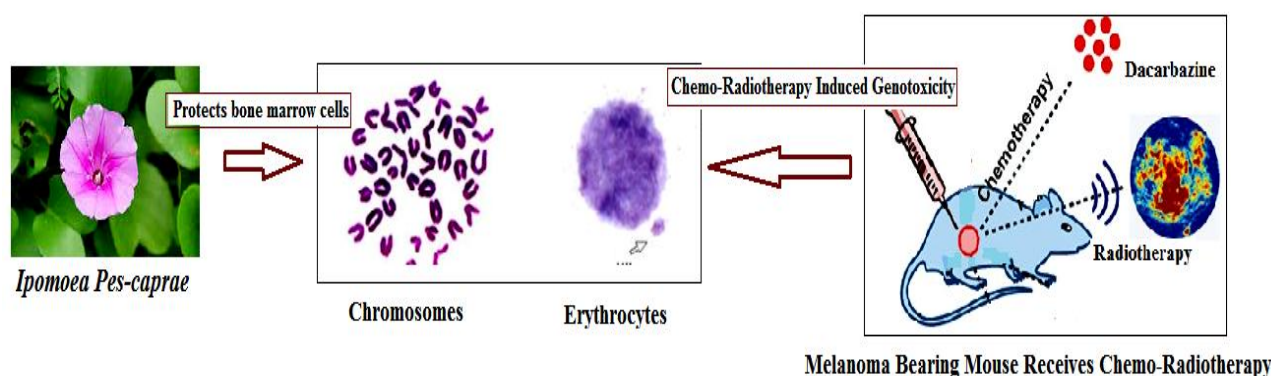


Figure 3. Illustrative flowchart to indicate the *I. Pes-caprae* protective effect against the chemo-radiotherapy induced genotoxicity.

5. MATERIALS AND METHODS

5.1. Collection and authentication of plant material

I. pes-caprae Linn (Leaves, stems and flowers) collected in the month of December 2018 from Indian Ocean near Kuttomangalam Mandaikadu, District, Kanyakumari (T.N.) and authenticated by Botanical Survey of India, (Pune) with the reference number: BSI/WC/Tech/09/447 and voucher specimen (V. No. ASIP1).

5.2. Preparation of *Ipomoea pes-caprae* methanolic extract (IPCE)

IPCE was prepared by collecting fresh plant material (whole) followed by shade dried, powdered and sieved through 20-mesh size. 100 g powdered plant material was extracted with petroleum ether (PE) (60-80°C) followed by methanol (MeOH) in Soxhlet apparatus and filtered with Whatman filter paper.

5.3. Drugs and chemicals

Colchicine and fetal calf serum (FCS) were obtained from Hi-media Laboratories Ltd. (Mumbai, India). Giemsa stain was procured from Qualigens-Fischer scientific (Mumbai, India). All other chemicals used in the present work were of analytical grade.

5.4. Experimental animals

A total of 48 adult males 6-8 weeks C57BL mice weighing 25-30 g obtained from Animal house of Jawaharlal Nehru Cancer Hospital and Research Centre, Bhopal. The animals were maintained in air-conditioned animal house (20-25°C; relative humidity, 70-75%) in a 12 h light-dark cycle and fed on a standard diet with water ad libitum. The study was performed in accordance with CPCSEA norms, institutional regulations and national criteria for animal experiments. Institutional approval for experimental work was obtained from Animal Ethical Committee of Research, Jawaharlal Nehru Cancer Hospital and Research Centre, Bhopal, India, with the CPCSEA registration no. 500/01/a/CPCSEA/2001.

5.5. Preparation of tumor cell suspension and tumor induction

The melanoma cell line B16-F10 was purchased from National Centre for Cell Science (NCCS), Pune and then cultured in the lab at the Department of Research, Jawaharlal Nehru Cancer Hospital and Research Centre, Bhopal. The calculated amounts of viable cells were transferred to a culture flask containing 10 ml of fresh MEM media. The flask was kept aseptically in CO₂ (5%) incubator for 24h at 37°C temperature. After 24h the media from the culture flask was drained out and 2 ml of 0.5% trypsin in phosphate buffer saline was added. The disaggregated cells were collected in centrifuge tube and centrifuged at 2100 rpm for 15 min to remove trypsin. The supernatant was discarded, and the pellets were resuspended in fresh media. Resuspended pellets were again washed with fresh media and the same procedure was repeated twice. The supernatant was discarded, and the pellets were again suspended in the media. Cell viability was counted

by Trypan blue exclusion assay. For the development of B16-F10 melanoma-bearing mice, the calculated number of viable cells (Four) was injected to the hair removed dorsal surface of mice for tumor induction. After 8-10 days of injection, the tumor started growing. When the tumor was developed to a palpable level, the experiment was started for drug dosing.

5.6. Irradiation of mice

Experimental animals were subjected to radiation through $^{60}\text{Cobalt}$ Theratron Teletherapy Unit 780 C (Canada), Department of Radiotherapy, Jawaharlal Nehru Cancer Hospital and Research Center, Bhopal. Anesthetized mice were restrained in well-ventilated polypropylene box and tumor were exposed to 8 Gy radiations in four fractions (2Gy in every 10th day) at a dose rate of 1Gy/min and maintained field size at 15 x 10 cm² at a distance of 75-80 cm from the source during the whole experiment [20].

5.7. Experimental design

Animals were divided into 8 groups, each included 6 animals. Animals of Group A, receiving normal saline (10 ml/kg b.wt.) served as Normal Control, Group A1 (tumor bearing mice) receiving normal saline (10 ml/kg) served as Tumor Control. Animals of Group B receiving normal saline (10 ml/kg) along with radiation dose served as Radiation Control. Group B1 (tumor bearing mice) receiving normal saline (10 ml/kg) along with radiation dose served as Tumor Radiation Control. Group B2 and B3 (tumor bearing mice) receiving IPCE (25 & 50 mg/kg b.wt.) along with radiation dose respectively, served as Sample Drug Adjuvant to Radiotherapy. Group C (tumor bearing mice) receiving Dacarbazine (50 mg/kg, alternate days) served as Standard Control. Group D (tumor bearing mice) receiving Dacarbazine (50 mg/kg, alternate days) along with IPCE (50 mg/kg b.wt.) served as Sample Drug Adjuvant to Chemotherapy. Dacarbazine and IPCE were administered intra-peritoneally over a treatment time of 40 days.

5.8. Genotoxicity studies

5.8.1. Chromosomal aberration assay

The chromosomal aberration assay was performed according to reported method [21, 22]. All the animals were injected *i.p.* with 0.025% colchicine (0.01 ml/gm b.wt.) after 24 h of last dose of treatment. Mice were sacrificed by cervical dislocation post 2 h of colchicine injection and femur and humerus bones were excised. All the attached muscle fibers from the bone were removed and cleaned with gauge pieces. The proximal and distal ends of bone were cut down and bone marrow was aspirated by flushing with normal saline (0.9% NaCl) in the centrifuge tube. The suspension was flushed and centrifuged for 10 min at 1000 rpm. Supernatant was discarded and pellets were treated with pre warmed (37°C) 0.57 % KCl solution. The cell suspension was left in water-bath at 37°C for 30 min, then centrifuged and the supernatant discarded. The pellets were treated with freshly prepared Carnoy's fixative. Slides were made with airdrop method by dropping the cell suspension on pre-cleaned and pre-coded slides from the sufficient height. The suspension film on the slide surface was instantly blown to remove the excess of solvent. Slides were dried using the slide warmer and then stained with 4% Giemsa stain and observed under microscope initially under 40X followed by 100X magnification. A total of 500 metaphases per animal were scored for aberrations like chromatid breaks, chromosome breaks, fragments, rings and dicentrics, as well as scored for severe damage cells (SDC) [23].

5.8.2. Micronucleus assay

Micronucleus assay was performed according to reported method [24, 25]. Animals were sacrificed and dissected as mentioned above. The proximal and distal ends of bones have been reduced down, bone marrow turned into flushed via way of means of FCS (1ml) and centrifuged for 10 min at 1000 rpm, and the supernatant discarded then resuspended the pellet with FCS. One drop of sample as fine smear was placed on pre-cleaned and pre-coded dry slide. Then the slide turned into air dried and stuck through losing absolute methanol. The slide was stained with May-Gruenwald-Giemsa solutions for conventional assessment of the micronucleus frequencies. The slides were observed under microscope in 40X and then in 100X magnification. From each animal, 1000 erythrocytes were observed and the total number of polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE) with micronuclei (MNPCE and MNNCE) was recorded.

A criterion for scoring the frequencies of micronucleus become executed as prescribed through reported literature [26]. Micronuclei were scored by their presence in young erythrocytes. PCE (immature red blood cell, containing RNA) seize bluish stain of their internal rim, whilst NCE (mature red blood cell, lacking in RNA) are stained pinkish while stained with May-Gruenwald-Giemsa.

5.9. Statistical analysis

The experimental data were expressed as mean $\pm$ SEM. The significance of difference among the treated groups and control group was analyzed by using one-way ANOVA followed by Dunnett's multiple comparison tests using GraphPad InStat Software (San Diego, CA, USA). p values less than $p < 0.01$ and $p < 0.05$ were considered statistically significant.

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Conflict of interest statement: The authors declared no conflict of interest.

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