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ANTIGENOTOXIC EFFECT OF *PIPER BETLE* TOWARDS RADIATION INDUCED CYTOGENETIC DAMAGE IN MOUSE BONE MARROW CELLS

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ABSTRACT

The chemoprotective activity of Piper betle methanolic extract has been studied using swiss albino mice bone marrow as an in vivo model. The methanolic extract (ME) effectively prevented cyclophosphamide (CP) induced chromosomal aberration. Animals were injected (i.p.) with 100mg/kg body weight of 50% methanolic extract (ME) of Piper betle as a single dose & exposed to cyclophosphamide (50mg/kg) body weight ½ hr later. Bone marrow protection was studied by scoring aberrations in metaphase chromosomes. No drug toxicity was observed at this dose (100 mg/kg) b.wt. The effectiveness of methanolic extract (ME) of Piper betle when administered gave a significant protection against CP alone group.

KEYWORDS

Piper betle, Micronuclei, Methanolic extracts

1. INTRODUCTION

Exposure of mammalian systems to radiations induces damaging effects leading to cell death and an increased risk of diseases particularly cancer. [1] A dose of 4 Gy is considered fatal for humans and other mammals.[2] Consequently; there is growing interest in developing new radioprotectants in preventive medicine as well as adjuvant therapy. Most of the effective radioprotectants such as WR-2721 developed so far are synthetic, and are reported to be toxic.[3] Thus, there is a need to develop radioprotectants from natural sources especially from edible or medicinal plants/herbs as these are regarded as non-toxic even at higher concentrations. The importance of usage of ethnomedicines is increasing nowadays as they have less or no side effects, low cost and are, often easily accessible to the common people. Almost half of the pharmaceuticals are originated from plant products. The present study was primarily aimed to this end. Wherein the radioprotecting property of *Piper betle* Linn. Commonly known as tambula (Sanskrit), pan (Hindi and Bengali) of Piperaceae family was studied.

The *Piper betle* plant is widely growing in the tropical humid climate of South East Asia and its leaves, with a strong pungent and aromatic flavour are widely consumed as a mouth freshener. The leaves are credited with would healing, digestive and pancreatic lipase stimulant activities in the traditional medicine[4, 5] Which has also been proved with experimental animals.[6] In fact, usefulness of this plant against various diseases can be traced in the ancient Vedic literature, Atharved as early as 3000-2500 BC. Its Vedic name is Saptasira.[7] Earlier, we also reported gastrocytoprotective properties of the leaf extract on experimentally induced gastric lesions and rationalized the activity in terms of its antioxidant property.[8,9] In addition, its antimicrobial.[10] antifungal and anti-inflammatory[(11)] activities are also reported. However, no study has been reported on its radioprotective effect. The present investigation

was undertaken to study the radio protective property, if any of *P.betle*

2. MATERIALS AND METHODS:

2.1 Preparation of Methanolic Extract:

2.1.1 Collection of plants – The fresh leaves of *Piper betle* were collected from the region of Madhya Pradesh (Bhopal) in the month of February and were identified by Botanist Professor Shaukat Ali, Safia College, Bhopal.

Fresh leaves were washed under tap water and shade dried and powdered. 50% methanolic extract of the powder (100gm) was prepared with the help of cold maceration. And was allowed to stand at room temperature for about 18 hrs. After shaking frequently for 6 hrs. The filterate was collected[11]. This process of extraction was repeated for three times. The combined extract was filtered and concentrated under vaccum using SC110A Speed Vac® plus at 4^0C. The extractive value of extract obtained was 14.94398% w/w.

Animals: Swiss albino mice of either sex weighing 30-40gm were obtained from Jawahar Lal Nehru Cancer Hospital and Research Centre. Animals were kept in cages and in environmentally controlled room. (23± 3^0C, 12hr light & dark cycle) with free access to water. Animals were fed with standard diet pellets *labium.*

Animals were randomly allocated to different experimental groups, three or four mice were used for each groups .Experimental protocols were approved by institutional ethical committee of JNCH & RC, Bhopal, which follow guidelines of CPCSEA (Committee for the Purpose of Control & Supervision of Experiments on Animals) that complies with international norms of INSA.

2.2 Mode of treatment –

2.2.1 Route of administration: The animals were administered with optimal dose of drug (100 mg/kg b.wt.) intraperitoneally before exposure to external irradiation (4 Gy) and the administration of cyclophosphamide.

2.2.2 Genotoxic agent used during study: Radiation

2.2.3 Irradiation: Gamma rays were delivered from the ^{60}Co teletherapy unit (Canada) in the dept. of Radiotherapy, JNCH Bhopal. The Gammatron unit is remote controlled. Radiation is directed downwards through a collimator. The collimator is fitted with a bulb. The field size can be adjusted according to the requirement; the dose rate was calculated every week using the decay table for ^{60}Co. The fixed dose ratio of 1.6 Gy per min. was used in all the experiments.

Mode of exposure : Unanesthetized mice were restrained in a well ventilated Perspex box (20 + 20 cm, 2 + 4cm) partitioned in to 12 chambers each chamber can accommodate a single mouse, to adjust the dose rate with the radioactive decay, the source to surface distance (SSD) of 112.0 cm-114.0 cm during the course of the study. The field was maintained at 20 + 20 cm^2.

3. MICRO NUCLEUS ASSAY

Procedure: The mice were injected i.p. with 0.025% colchicines (Sigma, USA) and left for 2 hrs. to arrest the cells in metaphase. Then the animals were sacrificed by cervical dislocation, femur were dissected out and cleaned to remove adherent muscles. The mice femur marrow was flushed out with normal saline (0.84%). Cells smear was made. and the slides stained with May-Greunwald's-Geimsa and scored under fluorescent microscope. Polychromatic and normochromatic erythrocytes bearing micronuclei were scored. The Micronucleus assay, was done by the method of Schmid[12] with some modification in staining procedure[13]

3.1 Experimental protocol

Treatment schedule – Animals were divided into following seven groups of three to four animals each.

Group-I (Control) – Double distilled water
Group-II - (Vehicle alone) – IPA
Group-III - (Methanolic extract) – 50% Methanolic extract of *Piper betle* (dose 100mg/kg)
Group-IV – Radiation alone (4 Gy)
Group-V - Radiation alone plus methanolic extract of *P.betle* (dose- 100mg/kg)

3.2 Statistical Analysis

The data were analyzed by students test. Comparison between different groups were done by One Way ANOVA (Kyplot) using Graph PAD Instant software (USA), and the histograms for chromosomal aberration assay and micronuclei assay were drawn using Microcal Origin 6.0 software.

Number of micronuclei in PCE in the bone marrow of mice treated with *Piper betle* extract (ME) Radiotherapy (Radiation)

S.No.	Treatment	Number of Micronuclei MNPCE			Total number of MNPCE
		MN1	MN2	MN3	
1.	**Normal**	11	-	-	11
2.	**Vehicle alone (IPA)**	12	-	-	12
3.	**Methanolic Extract alone (*P. betle*)**	23	-	-	23
4.	**Radiation Alone**	575	24	10	653
5.	**Radiation + ME (*P. betle*)**	329	19	12	403

MNPCE / 100 PCE in the bone marrow of mice treated with *Piper betle* extract (ME) before whole body irradiation (RT, 4Gy)

S.No.	Treatment	Percentage MNPCE (%)
1.	**Normal**	2.3 ± 0.057735
2.	**Vehicle alone (IPA)**	$2.37624 \pm 1.15\ E-005$
3.	**Methanolic Extract alone (*P. betle*)**	4.8 ± 0.011547
4.	**Radiation Alone**	$128.039 \pm 0.001155^{c, f}$
5.	**Radiation +ME (*P. betle*)**	$79.9 \pm 1.962991^{c, f, 2}$

a, 1, x, d = p < 0.05; b, 2, y, e = p < 0.01 c, 3, z, f = p < 0.001
a, b, c is compared to DDW (control); 1, 2, 3 is compared to RT alone; d, e, f is compared to ME alone.

Number of micronuclei in NCE in the bone marrow of mice treated with *Piper betle* extract (ME) before whole body irradiation (RT, 4Gy)

S.No.	Treatment	Number of Micronuclei MNNCE			Total number of MNNCE
		MN1	MN2	MN3	
1.	**Normal**	6	-	-	6
2.	**Vehicle alone (IPA)**	8	-	-	8
3.	**Methanolic Extract alone (*P. betle*)**	10	-	-	10
4.	**Radiation Alone**	54	15	6	102
5.	**Radiation + ME (*P. betle*)**	33	7	1	50

MNNCE / 1000 NCE in the bone marrow of mice treated with *Piper betle* extract (ME) before whole body irradiation (RT, 4 Gy)

S.No.	Treatment	Percentage MNNCE (%)
1.	**Normal**	1.2 ± 0.1
2.	**Vehicle alone (IPA)**	$1.21212 \pm 1.15\ E-005$
3.	**Methanolic Extract alone (*P. betle*)**	2.06 ± 0.001547
4.	**Radiation Alone**	$22.27074 \pm 1.2\ E-005^{c,\ f}$
5.	**Radiation + ME (*P. betle*)**	$14.3 \pm 0.1154701^{c,\ f,\ 2}$

a, 1, x, d = p < 0.05; b, 2, y, e = p < 0.01 c, 3, z, f = p < 0.001
a, b, c is compared to DDW (control); 1, 2, 3 is compared to RT alone; d, e, f is compared to ME alone.

Ratio of PCE to NCE (P/N ratio) in the bone marrow of mice treated with *Piper betle* extract (ME) before whole body irradiation (RT, 4 Gy)

S.No.	Treatment	No. Cells per thousand Erythrocytes		Ratio of PCE to NCE (P/N ratio)
		PCE	NCE	
1.	**Normal**	478	500	0.956 ± 0.017474
2.	**Vehicle alone (IPA)**	505	495	1.028 ± 0.011547
3.	**Methanolic Extract alone (*P. betle*)**	469.5	485	0.968 ± 0.001155
4.	**Radiation Alone**	510	458	$1.114 \pm 0.001155^{a,f}$
5.	**Radiation + ME (*P. betle*)**	505	348	$1.45 \pm 0.011547^{b,f,2}$

a, 1, x, d = p < 0.05; b, 2, y, e = p < 0.01 c, 3, z, f = p < 0.001
a, b, c is compared to DDW (control); 1, 2, 3 is compared to RT alone; d, e, f is compared to ME alone.

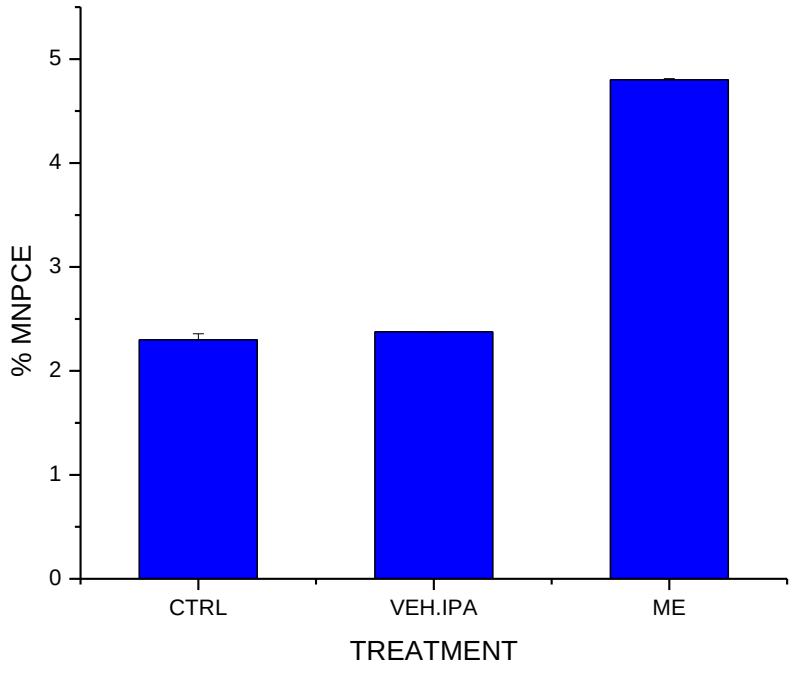


Figure.1.Effect of *P. betle* extract on the whole body γ-irradiation induced MNPCE in mouse bone marrow at 24 hrs.

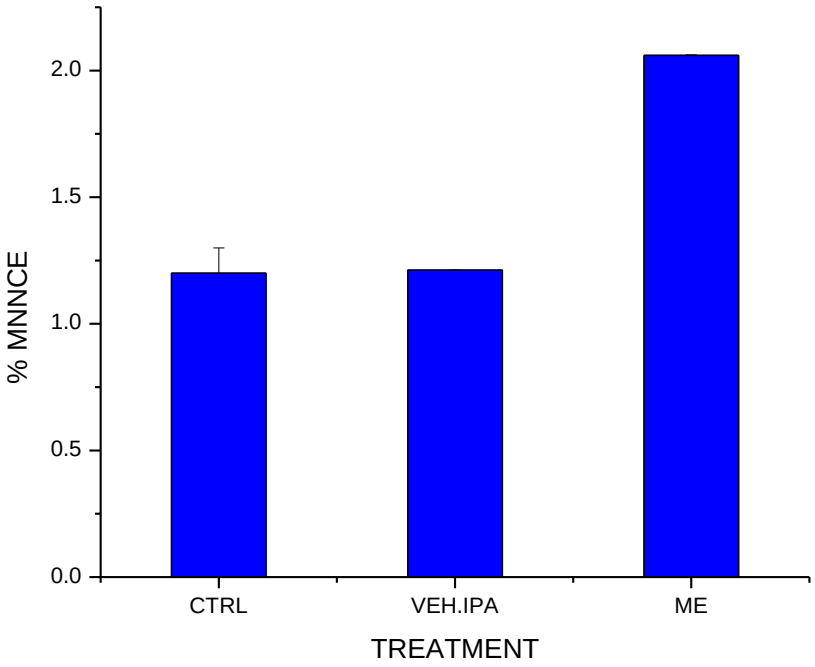


Figure.2.Effect of *P. betle* extract on the whole body γ-irradiation induced MNNCE in mouse bone marrow at 24 hrs.

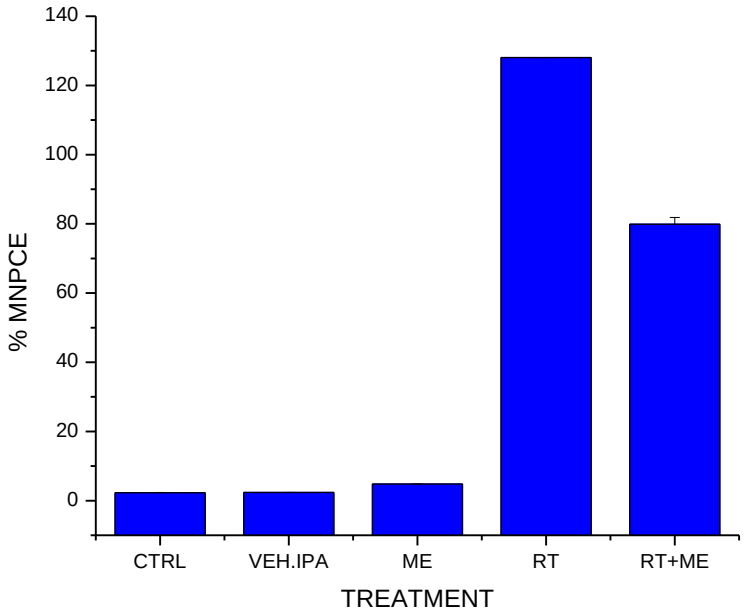


Figure.3.Effect of *P. betle* extract on the whole body γ-irradiation induced MNPCE in mouse bone marrow at 24 hrs.

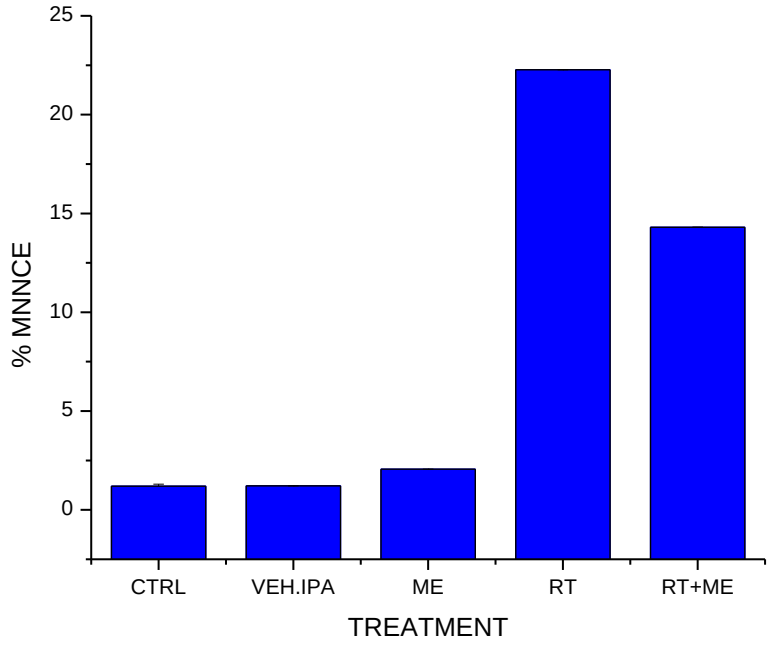
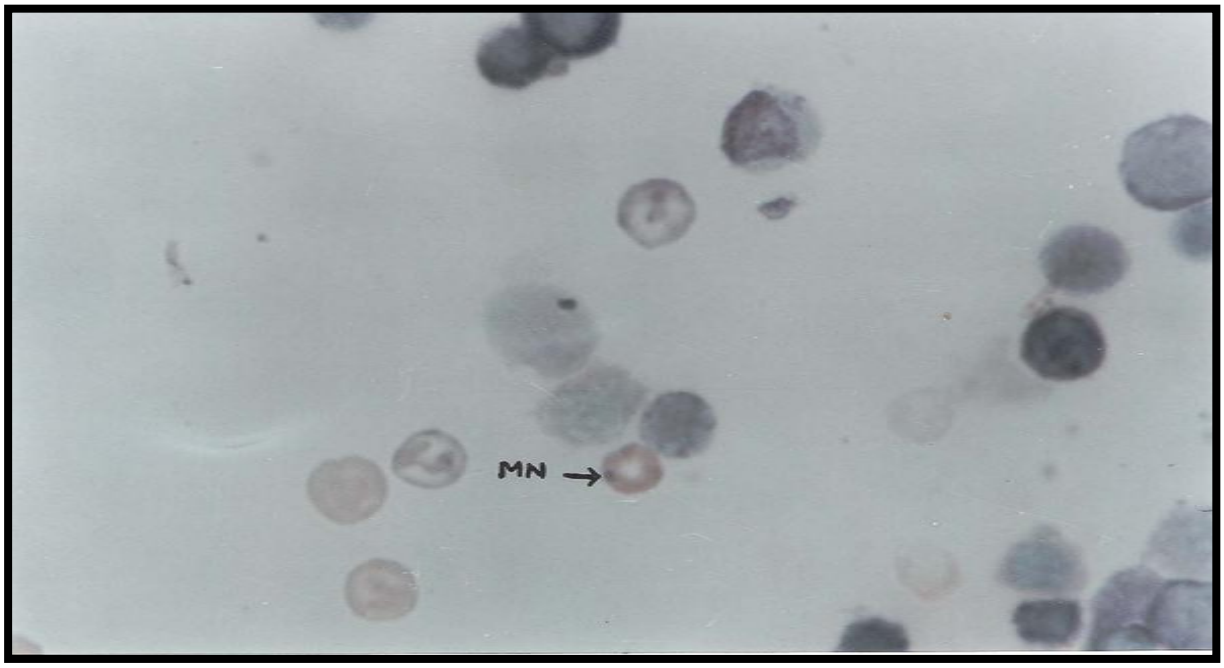


Figure.4.Effect of *P. betle* extract on the whole body γ-irradiation induced MNNCE in mouse bone marrow at 24 hrs.



Erythrocytes with Micronuclei (MN)

4. RESULT

The DDW control group showed a P/N ratio without a significant difference with the vehicle alone treated group as well with ME alone treated group. Radiotherapy brought about a significant increase in the frequency of MNPCE and MNNCE with a significance of $P < 0.001$ when compared to control, vehicle alone, ME

alone group and a significant decrease in the P/N ratio.

Treatment with methanolic extract of *P.betle* before radiation treatment produced a significant reduction in the number of MNPCE and MNNCE when compared to radiation alone with significant increase in the P/N ratio.

RT (4 Gy) increased the Percent polychromatic and normochromatic erythrocytes bearing micronuclei when compared to control, vehicle alone and ME alone groups. RT produced 128.039 ± 0.0011 and 22.27074 ± 1.2 resp.but methanolic extract of *P.betle* before radiation treatment produced a significant reduction in the percentage of MNPCE and MNNCE.

5. DISCUSSION

This study demonstrates a radioprotective property of the *P.betle* leaf extract. The data clearly show that a single dose of 100 mg/kg of ME (*P.betle*) before whole body irradiation can significantly decrease the radiation induced chromosomal damage. Administration of the ME further enhanced the bone marrow protection, as indicated by the significant reduction in the micronucleated erythrocytes and chromosomal aberrations at 24 hr. after irradiation compared to ME treatment.

The number of PCE's in relation to NCE's is an index of the rate of proliferation.[14]The decrease in P/N ratio at 24hr. after irradiation may reflect the early effects of radiation on cell cycle leading to mitotic inhibition, as also observed by other investigators.The significant increase in the 24hr. P/N ratio by pretreatment with ME indicates that the extract reduces the radiation induced cell cycle effect.The radioprotective effect of several natural products has been associated with their antioxidant property[15,16] Earlier studies from other laboratories have shown that *P.betle* possesses antioxidant activities.[17]

This may have a role in the protective effect of ME against radiation clastogenecity, evident in the reduced chromosomal aberrations and micronuclei frequency in the bone marrow cells.

6. CONCLUSION

Thus, the present study demonstrates that nontoxic doses of an extract of the leaves of *P.betle* protect bone marrow chromosomes exposed to whole body gamma irradiation. B*etle* leaves have been reported to contain the antioxidants like Vit.C, flavonoid[17] and other constituents, which may be responsible for the radioprotective properties of the extract. As *P.betle* leaves are used as a masticatory, it is credited with many properties; it is aromatic digestive, stimulant & carminative and is freely available in India, it is worthwhile to conduct detailed studies in order to explorer the full potential of this plant in human radiation protection.

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