

Modulation of ionizing radiation induced oxidative imbalance by semi-fractionated extract of *Piper betle*

An in vitro and in vivo assessment

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Key words: oxidative stress, *Piper betle*, ionizing radiation, lipid peroxidation, glutathione

Abbreviations: PBL, *Piper betle* leaf; SOD, superoxide dismutase; MN, micronuclei; DPPH, 1,1-diphenyl-2-picryl hydrazyl; BSA, bovine serum albumin; BHT, butylated hydroxy toluene; GSH, reduced glutathione; FCR, folin ciocalteu reagent; TCA, trichloroacetic acid; TBA, thiobarbituric acid; NBT, nitro blue tetrazolium chloride; DMSO, dimethyl sulfoxide; LPx, lipid peroxidation; GSSG, oxidized glutathione; TBARS, thiobarbituric acid reactive species; GAE, gallic acid equivalent

The study was planned to evaluate modulatory effect of aqueous extract of *Piper betle* leaf (PBL) on ionizing radiation mediated oxidative stress leading to normal tissues damage during radiotherapy and other radiation exposures. The total polyphenols and flavonoids known as free radical scavenger (chelators) were measured in the extract. To ascertain antioxidant potential of PBL extract, we studied free radical scavenging, metal chelation, reducing power, lipid peroxidation inhibition and ferric reducing antioxidant properties (FRAP) using in vitro assays. Mice were exposed to varied radiation doses administered with the same extract prior to irradiation to confirm its oxidative stress minimizing efficacy by evaluating ferric reducing ability of plasma, reduced glutathione, lipid peroxidation and micro-nuclei frequency. PBL extract was effective in scavenging DPPH (up to 92% at 100 µg/ml) and superoxide radicals (up to 95% at 80 µg/ml), chelated metal ions (up to 83% at 50 µg/ml) and inhibited lipid peroxidation (up to 45.65% at 500 µg/ml) in a dose dependant manner using in vitro model. Oral administration of PBL extract (225 mg/kg body weight) 1 hr before irradiation in mice significantly enhanced ($p < 0.01$) radiation abated antioxidant potential of plasma and GSH level in all the observed organs. The treatment with extract effectively lowered the radiation induced lipid peroxidation at 24 hrs in all the selected organs with maximum inhibition in thymus ($p < 0.01$). After 48 hrs, lipid peroxidation was maximally inhibited in the group treated with the extract. Frequency of radiation induced micronucleated cells declined significantly (34.78%, $p < 0.01$) at 24 hrs post-irradiation interval by PBL extract administration. The results suggest that PBL extract has high antioxidant potential and relatively non-toxic and thus could be assertively used to mitigate radiotherapy inflicted normal tissues damage and also injuries caused by moderate doses of radiation during unplanned exposures.

Introduction

Radiotherapy is an integral part of cancer treatment. However, its damaging effects on normal tissues also cannot be ignored. Extensive work, since decades, for abating normal tissue injuries inflicted during radiotherapy and other unplanned radiation exposure, has been put into. Result of this endeavor, though has not gone fruitless but encouraging output is still awaited. Synthetic drugs evaluated to meet this target, were found toxic hence, could not be put into the use.¹⁻³ Herbs rich in antioxidants and minimally toxic in nature are on extensive search to fulfill the need. *Piper betle* leaves in their unprocessed form are eaten almost daily for centuries in many parts of the world. Higher doses of this plant extract, used for many consecutive days in animals, are also found non toxic.^{4,5}

Ionizing radiation, used in radiotherapy, may induces reactive oxygen species (ROS)/reactive nitrogen species (RNS) leading to oxidative injury to the basic cellular macromolecules such as lipids, proteins and DNA, inflicting cellular dysfunction and ultimately cell death.⁶ Excessive production of oxidative stress has been implicated in many pathological disorders other than cancer, such as ageing, rheumatoid arthritis, chronic fatigue syndrome, psoriasis, asthma, cardiovascular diseases, Parkinson's disease, Alzheimer's disease etc.⁷ Low LET (linear energy transfer) radiation causes damage through ROS production mainly by the radiolysis of water present in living system.⁸ In the presence of oxygen, the condition becomes adverse by the generation of other reactive species such as superoxide radicals ($O_2^{\cdot-}$) and singlet oxygen (1O_2). To counter the deleterious effect of free radicals, cells and tissues are equipped with endogenous defense enzymatic

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Submitted: 09/22/09; Revised: 10/08/09; Accepted: 10/19/09
Previously published online: www.landesbioscience.com/journals/oximed/article/10349

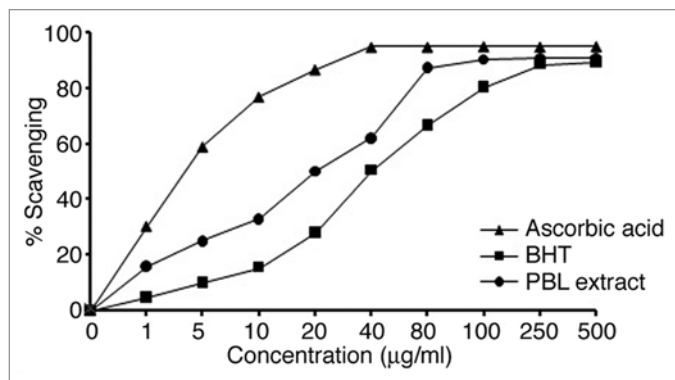


Figure 1. DPPH free radical scavenging activity of different concentrations of *Piper betle* leaf (PBL) extract. Ascorbic acid and BHT, the standard antioxidants, were used as positive controls. Methanolic solution of DPPH, mixed with PBL extract/standards, was incubated at room temperature. Decrease in absorbance was measured at 517 nm against methanol. The absorbance of DPPH was considered as 0% inhibition. PBL showed higher DPPH radical scavenging activity as compared to BHT but lower than ascorbic acid. Each value represents mean \pm SEM of data from three independent experiments.

and non-enzymatic antioxidants such as glutathione, vitamin C, α -tocopherol, superoxide dismutase (SOD), catalase, glutathione peroxidase etc. However, under extreme condition, endogenous defense system of the body gets dearranged and becomes inefficient to cope up with the free radical attack resulting to oxidative imbalance. This spurred the interest for exogenous supply of antioxidants to minimize oxidative damages. Many herbs, like *Podophyllum hexandrum*, *Hippophae rhamnoides*, *Emblica officinalis*, *Zingiber officinale*, *Osimum sanctum*, *Tinospora cordifolia*, *Phyllanthus amarus*, *Centella asiatica* etc., used in ayurvedic treatment are reported to contain plethora of secondary metabolites which could be useful in reducing ionizing radiation induced oxidative damages.

Piper betle (Family: Piperaceae), a perennial climber commonly known as paan, is mainly cultivated in most parts of Southeast Asia.⁹ The leaves of *Piper betle* are reported to contain catechin, eugenol, allylpyrocatechol and hydroxychavicol which are beneficial to human health in many ways. The leaves act as a good remedy against bad breath and also reported to be digestive, pancreatic lipase stimulant^{9,10} and hepatoprotective.¹¹ The leaf extract was found to diminish the effect of oxidative stress by elevating the level of endogenous defense enzymes,⁵ scavenging free radicals and acting as a good immunomodulatory agent.¹² Recently, *Piper betle* leaf extract was also reported to inhibit gamma radiation induced damage in plasmid DNA and lipid peroxidation in rat liver mitochondria under in-vitro conditions.¹³

The present study was aimed at evaluating potential of PBL extract in minimizing radiation induced normal tissue injuries inflicted during radiotherapy and other radiation exposures. The stress modifying properties of PBL extract were evaluated by measuring the degree of protection to plasma antioxidant potential, cellular membrane damage and non enzymatic antioxidants in mice exposed to total body irradiation. Moderate dose of gamma rays (500 cGy) was used as oxidative stress inducing agent. An

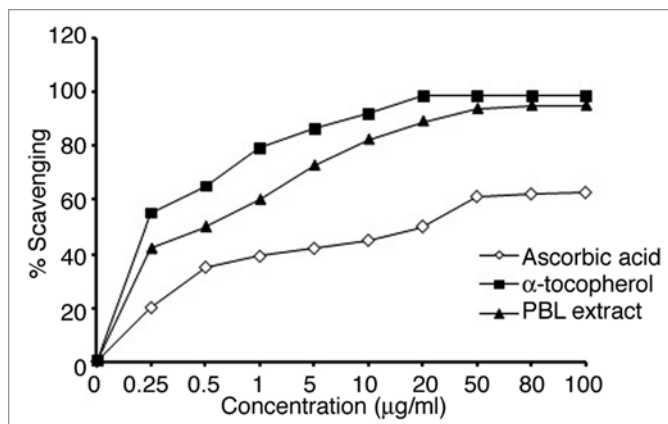


Figure 2. Superoxide radicals scavenging activity of *Piper betle* leaf (PBL) extract. BHT and α -tocopherol were used as standards for reference. Varied concentrations of PBL extract/standards were mixed with DMSO and NBT. Formation of purple colored diformazan was measured at 560 nm. The % scavenging activity of formation of colored complex was calculated with respect to control in all the samples. Values are expressed as mean \pm SEM of data from three independent assays. Superoxide scavenging activity shown by PBL was found higher to BHT but less than α -tocopherol.

array of in vitro assays was used to confirm oxidative stress mitigating potential of PBL extract by screening its properties of scavenging free radicals, chelating transition metal ions, inhibiting lipid peroxidation and reduction potential.

Results

In vitro studies. The results obtained from the present study reveal that the aqueous extract of PBL has adequate amounts of phenolic and flavonoids content. The total phenolic contents in terms of gallic acid equivalent was found to be 397.98 ± 19.26 μ g GAE/mg (the standard curve equation: $y = 0.0225x - 0.1164$, $r^2 = 0.9315$) and the total flavonoids content estimated in terms of quercetin equivalent was 265 ± 15.72 μ g QE/mg (the standard curve equation: $y = 0.0008x + 0.0621$, $r^2 = 0.9867$).

Antioxidant potential of extract. *DPPH free radical scavenging activity.* PBL extract showed maximum DPPH radicals scavenging activity up to 90.2% at 100 μ g/ml concentration while it was 94.3% at 40 μ g/ml for ascorbic acid and 89.2% for BHT at much higher concentration (500 μ g/ml) (Fig. 1). The IC_{50} values observed were 37.9 μ g/ml ($y = 0.8015 + 9.689$, $r^2 = 0.9852$) for PBL extract, 5.02 μ g/ml ($y = 0.7450 + 3.652$, $r^2 = 0.9721$) for ascorbic acid and 57.12 μ g/ml ($y = 0.7414x + 7.7285$, $r^2 = 0.9483$) for BHT. The IC_{50} value of the extract was higher than ascorbic acid but lower than BHT, revealing that the extract had higher free radical scavenging potential than BHT but lesser than ascorbic acid.

Superoxide scavenging activity. The superoxide scavenging activity as reflected in Figure 2 shows that the extract has exhibited the maximum scavenging activity (95%) at 80 μ g/ml concentration. For BHT the maximum scavenging activity was recorded 62% at 50 μ g/ml concentration whilst for α -tocopherol it was 98.7% at 20 μ g/ml concentration. The IC_{50} values observed were

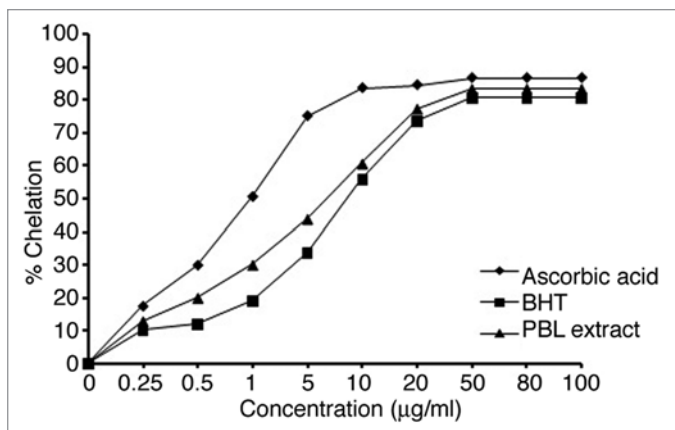


Figure 3. Effect of varying concentrations of *Piper beetle leaf* (PBL) extract on chelation of metal ions (iron). BHT and ascorbic acid were used as positive controls. Methanolic solution of o-phenanthroline and ferric chloride (200 µM), mixed with extract/standards, was incubated for 10 min at room temperature. Absorbance was recorded at 510 nm and percent chelation was calculated with respect to control. PBL extract showed marginally lower metal chelation activity as compared to ascorbic acid but higher than BHT. Results are expressed in terms of concentration vs. % chelation. Each value represents mean ± SEM of three independent experiments.

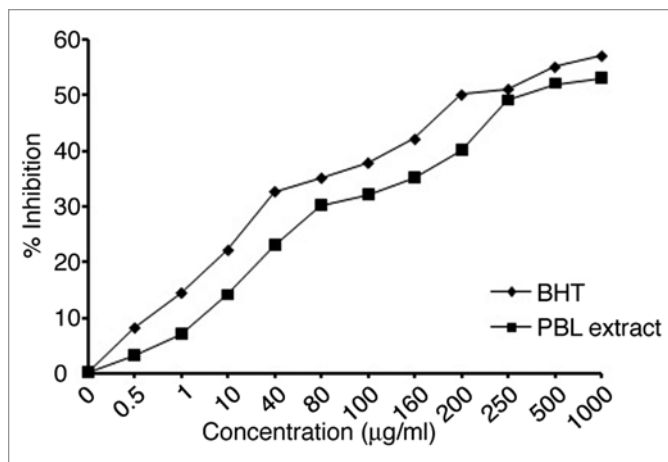


Figure 4. Anti-lipid peroxidation activity of different concentrations of *Piper beetle leaf* (PBL) extract. BHT was used as standard for comparison. Mouse brain homogenate, was added to various concentrations of PBL extract/BHT, and mixed with 2 ml of TBA-TCA solution. Thereafter, the mixtures were boiled, cooled and centrifuged. The resultant pink colored Formozan was measured at 536 nm. Percent inhibition of lipid peroxidation was calculated with respect to control. Anti-lipid peroxidation activity of PBL extract was nearly similar to standard BHT. Each value represents mean ± SEM of three independent experiments.

3.59 µg/ml ($y = 9.3465 + 16.599$, $r^2 = 0.9491$) for PBL extract, 6.86 µg/ml ($y = 6.1958 + 7.5792$, $r^2 = 0.9899$) for BHT and 2.35 µg/ml ($y = 6.1969 + 7.5780$, $r^2 = 0.9785$) for α -tocopherol. The superoxide scavenging activity of the extract was less than α -tocopherol but higher than BHT.

Metal chelation activity. The percent metal chelation activity as shown in Figure 3 indicates that the extract reflected its maximum activity (83.3%) at 50 µg/ml concentration while for ascorbic acid and BHT it was 87% and 80.3% respectively at the same concentration. IC_{50} values for extract, ascorbic acid and BHT were analyzed as 5.6 µg/ml ($y = 10.413x - 7.8282$, $r^2 = 0.9517$), 4.5 µg/ml ($y = 10.049x - 12.682$, $r^2 = 0.9756$) and 6.0 µg/ml ($y = 10.608x - 13.712$, $r^2 = 0.9381$) respectively, indicated that the metal chelation activity of the extract was higher than BHT but marginally lower than ascorbic acid.

Anti-lipid peroxidation activity. In the current study, the degree of lipid peroxidation was assayed by measuring percent inhibition of thiobarbituric acid reactive species (TBARS). The maximum inhibition activity of PBL extract was 45.65% at 500 µg/ml concentration while it was 48.69% for BHT at the same concentration (Fig. 4). The IC_{50} values observed to be 11.76 µg/ml ($y = 4.5x - 2.9286$, $r^2 = 0.9556$) for extract and 10.37 µg/ml ($y = 4.28x + 3.1234$, $r^2 = 0.9532$) for BHT. The anti-lipid peroxidation activity of the extract was found to be approximately same as that of the standard antioxidant BHT.

Reducing power assay. As reflected in Figure 5 the reducing power of PBL extract was maximum (1.64) at 1,000 µg/ml concentration which was nearly similar to standard BHT (1.11) at the same concentration.

FRAP assay. The extract has shown high potential of reducing ferric (III) form of iron to ferrous (II) (Fig. 6). In the presence of PBL extract TPTZ-Fe (III) complex was reduced to TPTZ-Fe

(II) complex (i.e., ferrous tripyridyltriazine complex). The ferric reducing antioxidant property as shown by the extract was more or less similar to the standard BHT (non-significant) but weaker than ascorbic acid (90%, $p < 0.001$) and quercetin (86%, $p < 0.001$) used as standards (Fig. 6).

In-vivo studies. Ferric reducing ability of plasma (FRAP). Figure 7 depicts ferric reducing ability of plasma in saline treated, radiation only and PBL treated irradiated groups at different time intervals. Radiation exposure to 500 cGy led to 40% ($p < 0.01$) reduction within 24 hrs in ferric reducing ability of plasma as compared to untreated animals (Fig. 7). Though, after 48 hrs of irradiation, the recovery in irradiated group also started but the FRAP values were still markedly less than the controls. Pretreatment of PBL extract significantly (34%, $p < 0.05$) countered the radiation induced decline in ferric reducing ability of plasma even after 24 hrs of irradiation and the FRAP values were almost normal after 48 hrs.

Glutathione (GSH). In radiation exposed mice, 24 hrs post irradiation, glutathione level had fallen apparently in all the observed vital organs as compared to control and the similar trend continued up to 48 hrs (Table 1). In whole blood, liver, kidney and jejunum GSH content decline within 24 hrs of irradiation was 3–5% as compared to saline treated control group. However, in thymus this fall in GSH concentration was much more drastic (74%). Pretreatment of mice with PBL extract one hour before irradiation resulted up to 5% increase in GSH concentration in all the major organs even after 24 hrs while in thymus GSH had elevated by 84% ($p < 0.001$) at the same interval of study.

Lipid peroxidation (LPx). Exposure to 500 cGy irradiation led to considerable induction of lipid peroxidation in all the observed organs of total body irradiated mice (Table 2). After 24 hrs of

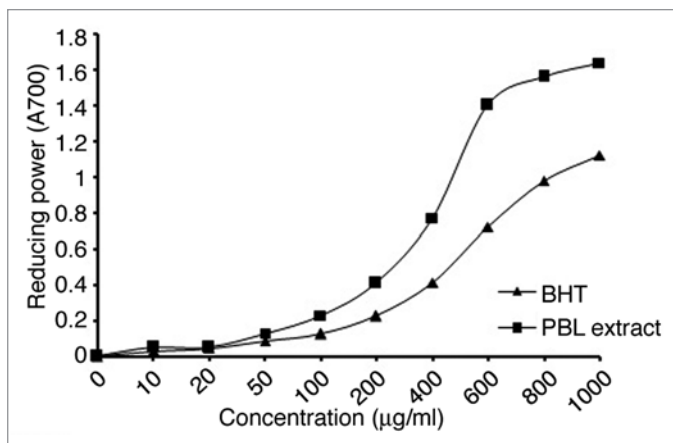


Figure 5. Reducing power of *Piper beetle leaf* (PBL) extract compared with standard antioxidant BHT by spectrophotometric detection of Fe^{3+} Fe^{2+} transformation. Different concentrations of PBL extract/BHT, after incubation with potassium ferricyanide, were mixed with 10% TCA. After administration of ferric chloride, absorbance was measured at 700 nm against blank. Reducing power of PBL was found nearly similar to BHT. Results are expressed in terms of concentration vs. absorbance. Each value represents mean \pm SEM of three independent experiments.

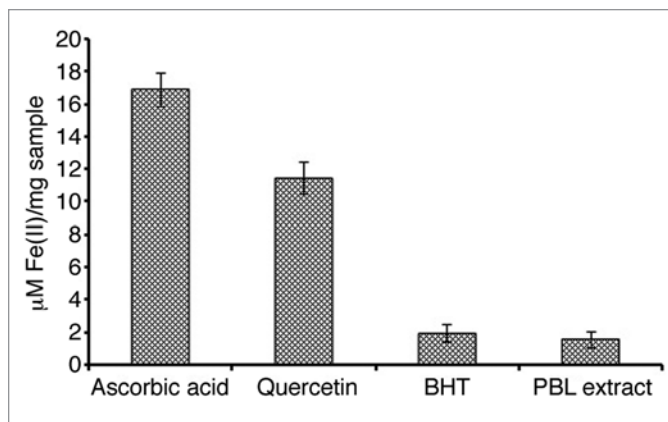


Figure 6. Ferric reducing antioxidant property of *Piper beetle leaf* (PBL) extract was evaluated by its ability to reduce TPTZ-Fe (III) complex to TPTZ-Fe (II). Ascorbic acid, quercetin and BHT were used as standards. FRAP solution (acetate buffer + TPTZ + ferric chloride) mixed with extract/standards, was incubated in dark. Formation of the colored product (ferrous tripyridyltriazine complex) was measured at 593 nm. Values are expressed as mean \pm SEM. Experiment was repeated in triplicate. Ferric reducing antioxidant property of PBL extract was found weaker as compared to ascorbic acid and quercetin but higher than BHT.

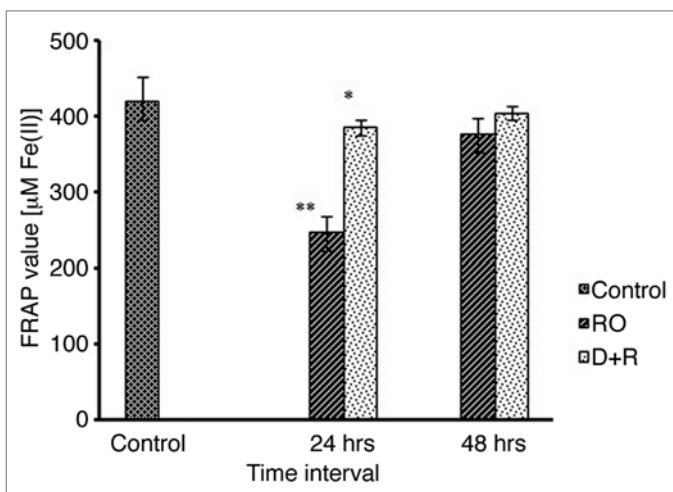


Figure 7. Effect of *Piper beetle leaf* (PBL) extract on ferric reducing ability of plasma in mice of different treated groups. Swiss albino female mice were divided into three groups: control, radiation only (RO) and PBL extract pretreated irradiated (D + R). Plasma separated from the blood of each mouse, mixed with FRAP solution and incubated in dark. Absorbance was recorded at 593 nm. Formation of $\mu\text{M FeSO}_4$ was calculated based on standard curve. Pre-irradiation treatment of animals with PBL significantly protected the antioxidant potential of plasma. Data was pooled and analyzed by three independent experiments having three animals in each group. Values are represented as mean \pm S.D. * $p < 0.05$, radiation only (RO, 24 hrs) vs. control (saline treated). ** $p < 0.01$, radiation + PBL treated (D + R, 24 hrs) vs. radiation only (RO, 24 hrs).

irradiation, most of the organs reflected significantly elevated level of malondialdehyde in radiation only group which continued up to 48 hrs. In this group, after 24 hrs, malondialdehyde concentration in thymus, brain and jejunum was enhanced by 65% ($p < 0.001$), 51% ($p < 0.001$) and 53% ($p < 0.001$) respectively.

In liver and kidney, increase in LPx activity was up to 40–43% ($p < 0.01$). Pre-treatment of PBL extract in whole body irradiated mice exhibited significant decrease (10–15%) in LPx even after 24 hrs of study in majority of the organs. But in thymus LPx inhibition was recorded up to 34% in this group. However, lipid degradation values could not be restored to normalcy up to 48 hrs of study in this group.

Micronucleus (MN) assay. Micronuclei frequency was found enhanced ($p < 0.001$) in mouse bone marrow cells in radiation only group ($23.33 \pm 1.86\%$) as compared to controls ($1.16 \pm 0.75\%$) at 24 hrs post irradiation interval. However, pre-irradiation treatment of PBL extract significantly ($p < 0.01$) lowered the micronucleated cells ($15 \pm 1.63\%$) in comparison to radiation exposed group (Fig. 8).

Discussion

Plethora of synthetic and herbal extracts is extensively screened to minimize radiation induced deterministic and stochastic effects developed after radiotherapy and other unplanned radiation exposures.^{1,2} In principle, free radicals generated during radiation exposure initiate adverse changes which, in due course, lead to the generation of immediate and late effects depending on the quantum of radicals. Though antioxidant and radioprotective potential of various extracts of *Piper beetle* based on in vitro systems have been moderately evaluated,^{5,11,13,14} but to the best of our knowledge modulation of ionizing radiation induced damage in vivo was attempted for the first time in the present study.

Measurement on total polyphenols and flavonoids in PBL extract demonstrated their presence in rich quantities. Flavonoids are reported to bear wide spectrum of antioxidant properties like they act as free radical scavenger by rapid donation of hydrogen

Table 1. Effect of pre-irradiation administration of aqueous extract *Piper betle* leaf (PBL) (225 mg/kg b.w., -1 hr) on radiation altered GSH level in different tissues of Swiss albino female mice at different time intervals

Group	Thymus	Spleen	Whole blood	Brain	Liver	Kidney	Jejunum
Control	50.6 ± 3.84	18.3 ± 1.57	12.2 ± 1.31	3.3 ± 0.77	6.9 ± 0.95	9.0 ± 1.97	8.6 ± 1.39
24 hrs	Radiation only	13.4 ± 0.71 ^{a***}	13.4 ± 1.53 ^{a**}	9.9 ± 0.33 ^{a*}	3.3 ± 0.89	5.3 ± 0.32	6.4 ± 0.57 ^{a*}
	Radiation + PBL treated	56.5 ± 3.93 ^{b***}	16.0 ± 1.21	10.8 ± 0.92	4.1 ± 1.00	8.9 ± 0.63 ^{b*}	9.8 ± 0.60 ^{b*}
48 hrs	Radiation only	14.9 ± 0.71	14.0 ± 1.79	9.4 ± 1.05	3.2 ± 0.20	6.2 ± 1.12	7.4 ± 1.86
	Radiation + PBL treated	57.1 ± 3.38 ^{c***}	15.1 ± 1.62	11.6 ± 1.33 ^{c*}	4.9 ± 0.85	7.7 ± 1.52	9.8 ± 0.59 ^{c*}

The supernatants of individual tissue homogenate were mixed with DTNB; Appearance of yellow color was measured spectrophotometrically at 412 nm; Values are expressed as µg GSH/mg protein and level of significance of the difference is indicated in terms of p-values; Data represents mean ± SD of three independent experiments containing three animals in each group; ^aradiation only (24-hrs) vs. control (saline treated); ^bradiation + PBL treated (24 hrs) vs. radiation only (24 hrs); ^cradiation + PBL treated (48 hrs) vs. radiation only (48 hrs); *p < 0.05, **p < 0.01, ***p < 0.001.

Table 2. Effect of pre-irradiation administration of PBL extract (225 mg/kg b.w., -1 hr) on inhibition of lipid peroxidation in different tissues of Swiss albino female mice

Group	Thymus	Spleen	Brain	Liver	Kidney	Jejunum
Control	0.14 ± 0.001	0.20 ± 0.034	0.18 ± 0.002	0.04 ± 0.003	0.06 ± 0.005	0.09 ± 0.009
24 hrs	Radiation only	0.41 ± 0.015 ^{a***}	0.26 ± 0.024 ^{a**}	0.37 ± 0.021 ^{a***}	0.06 ± 0.008 ^{a*}	0.10 ± 0.003 ^{a**}
	Radiation + PBL treated	0.27 ± 0.032 ^{b**}	0.22 ± 0.047 ^{b*}	0.33 ± 0.029 ^{b*}	0.06 ± 0.006	0.08 ± 0.001 ^{b*}
48 hrs	Radiation only	0.34 ± 0.012	0.24 ± 0.025	0.25 ± 0.036	0.06 ± 0.004	0.09 ± 0.003
	Radiation + PBL treated	0.27 ± 0.015 ^{c*}	0.21 ± 0.018 ^{c*}	0.21 ± 0.027 ^{c*}	0.04 ± 0.005 ^{c*}	0.07 ± 0.002

Animals were divided into three groups: control, radiation only (RO) and PBL extract pretreated irradiated (D + R); Tissue homogenates, mixed with TBA-TCA solution were boiled, cooled and centrifuged; Absorbance of the supernatants was measured at 535 nm; The experiment was performed in triplicate with three animals in each group; Lipid peroxidation was expressed as nanomoles of MDA formed x10⁶/gram of protein; Values represent mean ± SD of three independent experiments and level of significance of the difference is expressed in terms of p-values; ^aradiation only (24 hrs) vs. control (saline treated); ^bradiation + PBL treated (24 hrs) vs. radiation only (24 hrs); ^cradiation + PBL treated (48 hrs) vs. radiation only (48 hrs); *p < 0.05, **p < 0.01, ***p < 0.001.

atom from their hydroxyl group to radicals and results in the formation of less reactive flavonoid phenoxyl radicals.¹⁵ Most of the plant preparations reflect high antioxidant potential due to possession of larger quantity of polyphenols. During current study, PBL extract has been reported to have significant potential to chelate metal ions and to scavenge free radicals which was elucidated by its property to scavenge DPPH and superoxide radicals under in vitro systems. These findings are in corroboration to the earlier reports.^{13,14,16} Flora et al.^{17,18} recently suggested significant delineation in metal induced biochemical alterations following administration of synthetic and natural antioxidants. PBL has also revealed the properties of good electron donor, thus showing its reducing ability by converting Fe³⁺ to Fe²⁺. These properties are attributed to the presence of polyphenols in the extract. Bioactive phenolic components of *Piper betle*, like hydroxychavicol, chevitbetol and pyrocatechol are reported repeatedly^{5,13,19} for reflecting free radical scavenging activity. Our preparation of *Piper betle* (PBL extract) in aqueous form also appears to be working on the similar principle due to presence of these phenolic components.

It is amply explained that gamma radiation induces damage mainly by the generation of free radicals by indirect mode of action.⁶ Initially generated free radicals get multiplied and converted into more powerful hydroxyl radicals (OH^{*}) in the presence of trace metal anion. These hydroxyl radicals induce oxidative deterioration of lipids by the process lipid peroxidation which

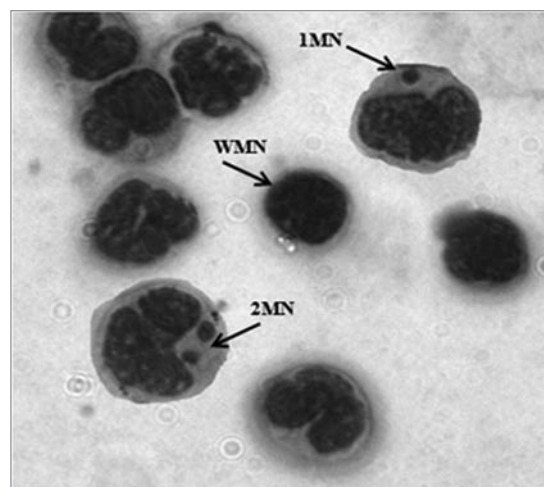


Figure 8. Mouse bone marrow cells with micronuclei (MN) viewed at 100X under trinocular microscope. Cells with micronuclei were counted in bone marrow of Swiss albino mice at 24 h after whole body gamma irradiation (200 cGy). Pre-irradiation treatment of animals with PBL effectively protected bone marrow cells and frequency of micronucleated cell formation was significantly inhibited. WMN, Bone marrow cell without micronuclei; IMN, Bone marrow cell with one micronucleus; 2MN, Bone marrow cell with two micronuclei.

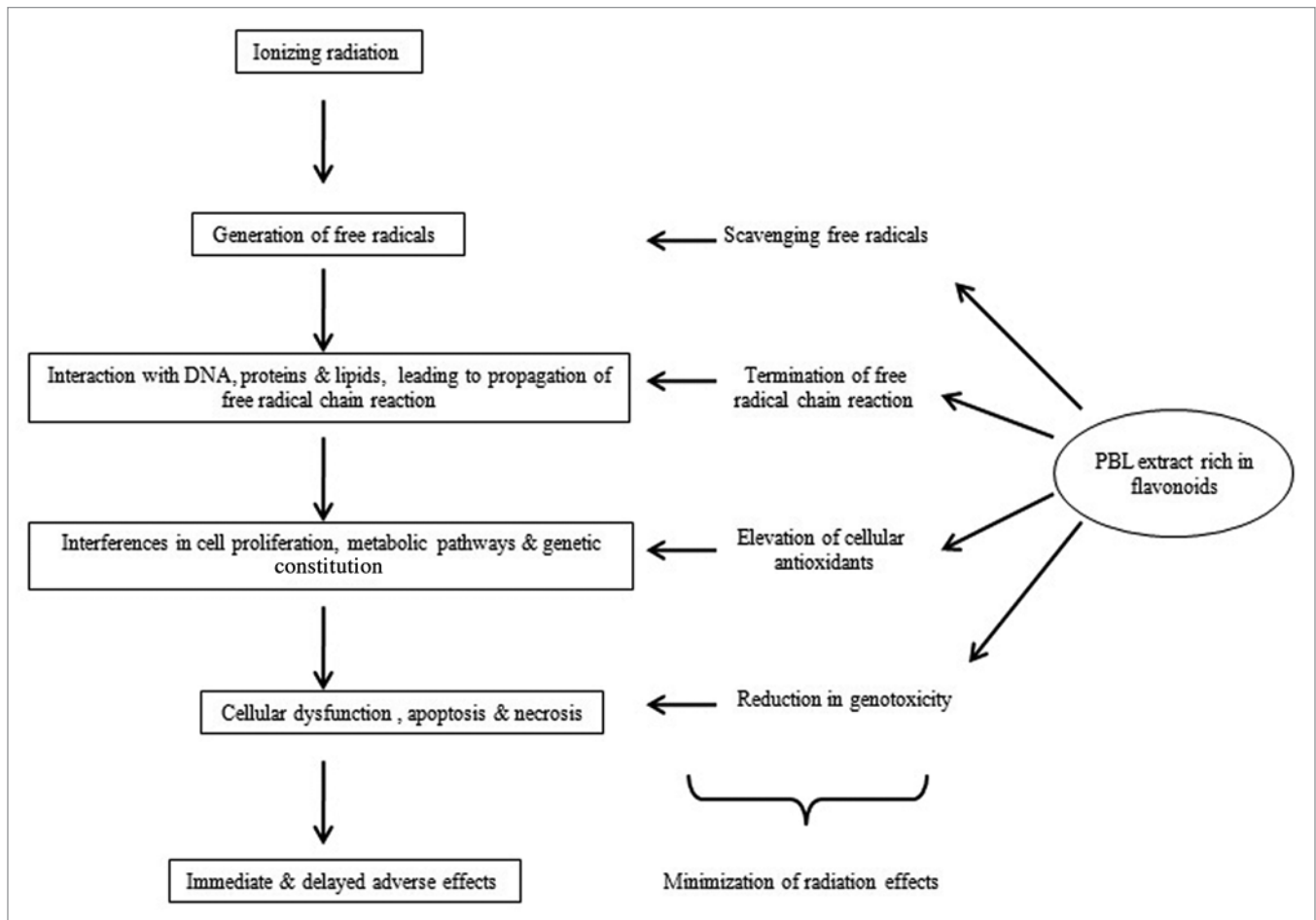


Figure 9. Diagrammatic representation showing potential pathways of injury by ionizing radiation and possible mechanism of radioprotection rendered by *Piper betle* leaf extract.

results into the formation of fatty acid radicals. These radicals later react with molecular oxygen and produce other generation of free radicals which disrupts cellular integrity by attacking on cellular membranes.²⁰ The cycle continues until an antioxidant acts as chain terminator. Pre-irradiation treatment of PBL extract resulted into significant reduction of lipid peroxidation (Table 1) clearly expressed its ability to scavenge chain propagating free radicals and minimizing metal ions involved in fenton reaction. Under in vitro investigation also, decrease in the concentration of TBARS with increased concentration of extract was reported which further strengthen the anti-lipid peroxidation activity of PBL extract.

The total antioxidant capacity of plasma which was measured in terms of its ferric reducing ability declined sharply as a result of γ -radiation induced damage in plasma.^{21,22} Herbal preparations are well documented in exerting their antioxidant potential multidirectionally. They have been also reported to enhance reducing ability of plasma.^{21,23} In consonance, our findings on PBL extract led to significant increase in plasma reducing ability (Fig. 7) and thus helping in restoring its antioxidant potential. Glutathione, non enzymatic antioxidants produced in body, a linear tripeptide, plays a pivotal role in maintenance of the balance of cellular

redox status. It directly quenches reactive hydroxyl free radicals and protects the cells and tissues from lipid peroxidation. Whole body irradiation of animals resulted in depletion of GSH content in different tissues due to the oxidation of GSH with the free radicals resulting in the formation of thiyl radicals that react to produce oxidized glutathione (GSSG) which is toxic to the cells. Depletion in GSH causes elevation in hydroxyl radicals which in turn attack on lipid membranes and results in lipid peroxidation.²⁴ In our observation also a similar correlation was observed between depletion of GSH and increase in LPx.

DNA breaks and fragments resulted from chromosomal damage appear as micronuclei in rapidly proliferating cells. Various plants flavonoids have been reported to provide protection against induction of micronuclei by radiation and this property of flavonoids might be attributed to their direct hydroxyl radical scavenging potency.^{1,25} Being rich in flavonoids PBL extract has also indicated reduced percentage of micronucleated cells reflecting protection to DNA against ionizing radiation. Finally, oral administration of the PBL extract before irradiation mitigate the deleterious effect of radiation predominantly by scavenging the free radicals, acting as chain breaking antioxidant, inhibiting the lipid peroxidation, elevating the level of endogenous antioxidants

and reducing the frequency of micro nucleated cells. Pathways reflecting radiation injury and possible mechanism of action for their mitigation are summarized in **Figure 9**.

Studies on *Piper betle* leaf extracts, to ameliorate the oxidative stress is still in the stage of infancy but the preliminary data obtained from the current study suggests the possible role of this plant extract in treatment of the free radicals mediated diseases. Supported with significant number of reports on almost non toxic status of this extract even after large, multiple and consecutive doses of this extract, make it safe in abating radiotherapy inflicted normal tissue injuries. In addition, this extract might be of immense use in mitigating damaging effects infused during whole body exposure to moderate doses of ionizing radiation. Though an elaborative work in this direction is warranted before making any further statement.

Materials and Methods

Chemicals. 1,1-Diphenyl-2-picryl hydrazyl (DPPH), bovine serum albumin (BSA) ascorbic acid, α -tocopherol, quercetin, 5,5'-dithio-bis(2-Nitrobenzoic Acid) (DTNB) and aluminium chloride (AlCl_3) were procured from Sigma Chemical Co., (St. Louis, USA). Folin Ciocalteu reagent (FCR), trichloroacetic acid (TCA), thiobarbituric acid (TBA), nitro blue tetrazolium chloride (NBT), potassium ferricyanide, 2,4,6-Tri-(2-Pyridyl)-1,3,5-triazine (TPTZ), m-phosphoric acid, sodium carbonate (Na_2CO_3), sodium hydroxide (NaOH) and ferric chloride (FeCl_3) were from SRL and gallic acid (GA), butylated hydroxy toluene (BHT) and sodium nitrite (NaNO_2) were purchased from Merck Co., (Germany). All the other chemicals used were of commercially high purity and analytical grade.

Plant material. The fresh leaves of *Piper betle*, identified by institute's botanist, were collected from Central market of Delhi, India. The plant material was made free from dust and insects by repeated washing in free flowing water. The leaves were chopped, mixed with de-ionized water and grounded. The resultant mixture was filtered through Whatman no.1 filter paper. The filtrate was dried under vacuum evaporation and stored in air tight containers in refrigerator. The preparation was analytically examined to rule the possibilities of any metal contamination and finally prepared super fine powder was assigned the name PBL extract as code.

Animals and γ -ray irradiation. Swiss albino Strain 'A' female mice (26 ± 2 g), 8–10 weeks old, fed with standard food pellet and water ad libitum were used for study. They were housed in polypropylene cages bedded with sterilized rice husk under the control environmental conditions ($24 \pm 2^\circ\text{C}$; 12 hrs alternating dark and light cycle). The experiments were conducted strictly adhering to the guidelines of the Institutional Animal Ethics Committee (IAEC). Mice were exposed in ^{60}Co ., gamma chamber (Model-220, Atomic Energy of Canada Ltd.,) at the dose rate of 0.33 cGy/sec. Fresh air was circulated continuously in the irradiation chamber to avoid hypoxic conditions. Dosimetry was carried out using Baldwin Farmer's secondary dosimeter and Fricke's chemical dosimetry method.

In-vitro studies. Total phenolic content determination. Total phenols in the aqueous PBL extract were evaluated by using Folin ciocalteu reagent.²⁶ The aliquots containing different concentration of the extract/Gallic acid (standard phenolic compound) were mixed with 5 ml of 1N FCR and 4 ml of 1 M Na_2CO_3 . The mixtures were allowed to stand for 15 min and the total phenols were determined by colorimetry at 765 nm. The standard curve was prepared by using different concentrations of gallic acid (10–100 $\mu\text{g}/\text{ml}$). Total phenolic content was expressed as μM gallic acid equivalent (GAE) using the equation based on the calibration curve: $y = 0.0225x - 0.1164$, $r^2 = 0.9315$, where y was the absorbance and x was GAE ($\mu\text{M}/\text{mg}$).

Total flavonoids determination. Total flavonoids in the PBL extract were measured using the method of Zhishen et al. 1999.²⁷ Briefly, various concentrations of the extract/quercetin were mixed with 0.03 ml of 5% NaNO_2 and 10% AlCl_3 and incubated at room temperature for 5 min. After adding 0.2 ml NaOH and 0.24 ml double distilled water, absorbance was measured at 510 nm. The standard curve was plotted using different concentrations of quercetin (50–1,000 $\mu\text{g}/\text{ml}$). Total flavonoids content was calculated by calibration curve based equation: $y = 0.0008x + 0.0621$, $r^2 = 0.9867$ where y and x was the absorbance and quercetin equivalent (μg QE/mg) respectively.

Antioxidant activity. DPPH radical scavenging assay. The stable 1,1-diphenyl-2-picryl hydrazyl (DPPH) was used for determination of free radical scavenging activity of PBL extract.²⁸ In brief, different concentrations of test samples with 1 ml methanolic DPPH (0.1 mM) were incubated at room temperature for 30 min in dark and absorbance was recorded at 517 nm. Ascorbic acid and BHT were used as reference while methanol and DPPH mixed in equal amounts served as control. The percent DPPH free radical scavenging for all the test samples was calculated.

Superoxide scavenging assay. Superoxide scavenging was assayed by the method of Govindrarajan et al. 2003.²⁹ Alkaline dimethyl sulfoxide (DMSO) was used as a superoxide generating system. Briefly, to 0.5 ml of different concentrations of test sample, 1 ml of alkaline DMSO and 20 mM NBT in phosphate buffer (pH 7.4) were added. Absorbance of the test mixtures was recorded at 560 nm. BHT and α -tocopherol were used as standard antioxidants for comparison. The percent superoxide radicals scavenging was calculated for each sample individually.

Metal chelating activity. Metal chelating activity was estimated by using method reported by Benzie and Strain, 1996.³⁰ Varying concentrations of test samples were dissolved in 1 ml of 0.05% o-phenanthroline in methanol and 2 ml ferric chloride (200 μM). After 10 min incubation at room temperature, absorbance was measured at 510 nm. Ascorbic acid and BHT were used as reference. The metal chelating activity was calculated in terms of percent scavenging.

Reducing power assay. Oyaizu, 1986 method³¹ was followed for this assay. In brief, different concentrations of the test samples (50 μl), mixed with 200 μl phosphate buffer (0.2 M, pH 6.5) and 0.1% potassium ferricyanide, were incubated at 50°C for 20 min. Addition of 10% TCA (250 μl) to the mixtures was followed by centrifugation at 6,000 rpm for 10 min.

The resulting supernatants mixed with 500 μ l double distilled water and 100 μ l of 0.1% ferric chloride were further incubated at 30°C for 10 min and absorbance was measured at 700 nm. The color development was recorded against blank containing phosphate buffer, potassium ferricyanide and TCA. The control antioxidant BHT was utilized to compare reducing power.

Lipid peroxidation assay (LPx). Anti-lipid peroxidation property of PBL extract was measured by using John et al. 1984 method.³² Brain tissues of anesthetized mice, excised carefully after decapitation, were homogenized with cold 0.15 M KCL to make 10% homogenate. Brain homogenate (0.5 ml) was added to 1 ml of various concentrations of the extract and incubated at 37°C for 30 min. Lipid peroxidation was induced in the homogenate by exposure to ionizing radiation (25,000 cGy). Various concentrations of extract/BHT with 2 ml of TCA-TBA solution (15% w/v TCA-0.37% w/v TBA) were boiled, cooled and centrifuged at 1,000 rpm for 10 min. The absorbance of the supernatants was recorded at 536 nm and percent inhibition of lipid peroxidation was calculated.

Total antioxidant activity (FRAP). Ferric reducing antioxidant property of the extract was analyzed by using the modified method of Benzie and Strain, 1996.³⁰ The fresh working FRAP solution prepared by mixing 50 ml acetate buffer (300 mM), 5 ml TPTZ (10 mM) and 5 ml $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (20 mM), was incubated at 37°C before use. Thereafter, to the different concentrations of the test samples, 3 ml working FRAP solution was added and allowed to stand in dark for 30 min. Absorbance of the colored mixtures was recorded at 593 nm. Results were calculated by using equation based on the calibration curve: $y = 0.0007x - 0.0068$, $r^2 = 0.9992$ where y was the absorbance and x was $\mu\text{M Fe(II)}$ /mg sample. Ascorbic acid, quercetin and BHT were used as reference.

In vivo studies. Experimental protocol. The animals (Swiss albino Strain 'A' female mice) were divided into three groups with three animals in each group: control, radiation only and radiation + PBL extract treated. The animals in control group were orally administered 250 μ l normal saline only. Radiation only group animals were irradiated with a dose of 500 cGy. In Radiation + PBL treated group, mice were orally administered with aqueous extract of PBL (225 mg/kg b.w.) one hour prior to 500 cGy exposure. For micronucleus assay 200 cGy dose was used. The animals were dissected at 24 hrs and 48 hrs after exposure to gamma radiation. Each experiment was repeated in triplicate.

Preparation of mice tissue homogenate. The animals were dissected after cervical dislocation. Different organs such as brain, liver, kidney, spleen, thymus and jejunum were excised and homogenized individually in cold buffered saline (pH 7.4) to make 10% homogenate. Blood was drawn from the heart puncture of the animals.

Ferric reducing activity of plasma (FRAP). The antioxidant potential of plasma was evaluated by its ferric ions reducing

ability.³⁰ In brief, plasma was separated from the blood drawn by heart puncture of mice. The plasma, diluted with water in 1:4 ratio, was mixed with freshly prepared FRAP solution. After incubation for 10 min. in dark, OD was taken at 593 nm. Formation of $\mu\text{M FeSO}_4$ was calculated based on the standard curve (100 μ l–1,000 μ l).

Lipid peroxidation determination. Lipid peroxidation (LPx) level in different tissues was estimated by the method of Beuge and Aust, 1978.³³ Tissues homogenate (100 μ l), prepared in phosphate buffer saline (pH 7.4), was mixed with 15% TCA followed by addition of 0.37% TBA. The samples boiled at 95°C (30 min) were centrifuged and absorbance of the supernatants was recorded at 535 nm. The concentration of TBARS was determined by using an extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$. Result was expressed as nanomoles of MDA formed per gram of protein.

Antioxidant glutathione (GSH) determination. Beutler et al. 1975 method³⁴ was followed to determine GSH concentration in different tissues excised from experimental mice. Briefly, the proteins of the samples were precipitated by adding precipitating solution (1.67% metaphosphoric acid + 0.2% EDTA + 30% NaCl). The samples were centrifuged and to the 2 ml of filtrate, 8 ml phosphate solution (0.3 M) and DTNB (40 mg in 1% 100 ml sodium citrate) was added. The absorbance was recorded at 412 nm. Glutathione concentration was expressed as $\mu\text{g GSH}/\text{mg}$ protein.

Micronucleus (MN) assay. Micronuclei were scored in mouse bone marrow cells by the method described by Chaubey et al. 1993.³⁵ The animals were sacrificed 24 hrs after irradiation. Both the femurs were taken out from each animal and bone marrow cells were aspirated in PBS with the help of syringe. The suspension was centrifuged, mixed with few drops of fetal bovine serum and smear was drawn on clean slides. After fixing in methanol, the slides were stained for 10 min with May-Grunwald Giemsa stain diluted in Sorensen's buffer (pH 6.8). The slides were mounted in DPX and observed under microscope. For each animal 1,000 cells were scored and percentage of micronucleated cells was calculated.

Protein determination. Protein contents were measured by Bradford, 1976 method.³⁶ Standard curve was plotted by using different concentrations of bovine serum albumin (BSA) as a standard.

Statistical analysis. For in vitro studies, the experiment results were expressed as mean \pm SEM of three replicates. IC_{50} values were calculated by using linear regression analysis. Student's t-test was used for analyzing the data of in-vivo studies. A value of $p < 0.05$ was considered as statistically significant.

Acknowledgements

The authors thank Dr. R.P. Tripathi, Director INMAS for his support and encouragement. Financial assistance from Defense Research and Development Organization (DRDO) is gratefully acknowledged.

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