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Radioprotective Property of the Ethanolic Extract of *Piper betel Leaf*

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Lymphoproliferation/*Piper betel*/Radioprotection/ROS scavenging/Lipids/DNA.

The radioprotective activity of *Piper betel* ethanolic extract (PE) has been studied using rat liver mitochondria and pBR 322 plasmid DNA as two model *in vitro* systems. The extract effectively prevented γ-ray induced lipid peroxidation as assessed by measuring thiobarbituric acid reactive substrates, lipid hydroperoxide and conjugated diene. Likewise, it prevented radiation-induced DNA strand breaks in a concentration dependent manner. The radioprotective activity of PE could be attributed to its hydroxyl and superoxide radicals scavenging property along with its lymphoproliferative activity. The radical scavenging capacity of PE was primarily due to its constituent phenolics, which were isolated and identified as chevibetol and allyl pyrocatechol.

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 4-8 Gy is considered fatal for humans and other mammals.[2)] Consequently, there is a 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 betel* Linn, commonly known as tambula (Sanskrit), pan (Hindi and Bengali) of *Piperaceae* family was studied.

The *Piper betel* 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 wound 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.

MATERIALS AND METHODS

Materials

Ascorbic acid, 2-thiobarbituric acid (TBA), 2-deoxyribose (DR), were obtained from Himedia Lab. Pvt. Ltd., India. Tris, ethylenediamine tetraacetic acid (EDTA), sucrose and ethanol were purchased from E. Merck (India), while $FeCl_3$ and trichloroacetic acid were from Thomas Baker, India. Superoxide dismutase (SOD), cytochrome C, xanthine oxidase (XO), methyl thiazole tetrazolium (MTT), hypoxanthine, mannitol, tetraethoxypropane and agarose were from Sigma Chemicals, U.S.A. PHA and pBR 322 DNA were obtained from Bangalore Genei Ltd, India. H_2O_2 (35%) from Lancaster, England and RPMI-1640 from Gibco, India were used. Sodium phosphate KH_2PO_4, KOH, HCl and ethidium bromide were purchased from SRL, India. Leaves of *Piper betel* were collected locally during March to May and iden-

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tified by the Botanical Survey of India as *Piper betle* Linn, of family *Piperaceae*.

Stock solutions of ascorbic acid, $FeCl_3$, EDTA and H_2O_2 were prepared in deaerated water just prior to use. The test extract was used as an aqueous solution. Stock solutions (1% w/v) of TBA were prepared in 50 mM NaOH solution and used within a week. All solutions were made with triply distilled water.

Animals

The rats were bred in the BARC Laboratory Animal House Facility and procured after obtaining clearance from the BARC Animal Ethics Committee. All the experiments were conducted with strict adherence to the ethical guidelines laid down by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted by the Animal Welfare Division of Government of India on the use of animals in scientific research.

Preparation of the plant extract

The leaves were chopped into fine pieces, soaked in 95% ethanol for seven days and the supernatant decanted. The extract was filtered through a nylon mesh. The entire process was repeated three times, the combined alcoholic extracts were evaporated in vaccuo and finally dried in a lyophilizer to obtain an amorphous yellowish brown solid in 2.2% w/w yield. This was designated as *Piper betle* ethanloic extract (PE) and was stored in a vacuum dessicator.

Isolation of chemical constituents

PE was treated with activated charcoal, filtered and concentrated in vacuum to obtain a chlorophyll-free material. This was subjected to column chromatography over silica gel. After elution with 10% methanol/chloroform, five fractions were collected which were further subjected to preparative thin layer chromatography over silica gel using 5% ethyl acetate/hexane and 5% methanol/chloroform as solvents separately. The bands obtained were scrapped from the silica gel plate, eluted with chloroform, and concentrated in vacuum to obtain two major compounds, which were identified from their infra red and ^1H nuclear magnetic resonance (NMR) spectral data.

Preparation of mitochondria

Mitochondria was isolated from male Wistar rats weighing 250 ± 20 g. Rats were killed by cervical dislocation and livers were excised and homogenized in 0.25 M sucrose containing 1 mM EDTA. The homogenate was spun at 3,000 × g for 10 min to remove cell debris and nuclei. The supernatant was centrifuged (DuPont, USA, model Sorvall RC5C) at 10,000 × g to obtain mitochondria which was washed three times with 0.05 M sodium phosphate buffer pH 7.4 to remove traces of sucrose. The final pellet was suspended in the same buffer at a concentration of 10 mg protein /ml, frozen in liquid nitrogen and stored at –70°C.

Lipid peroxidation assay

The radiation induced lipid peroxidation of the rat liver mitochondria was assayed by the thiobarbituric acid (TBA) method described elsewhere[12] with minor modification. In brief, the reaction mixture (final volume of 1.0 ml) containing mitochondria protein (0.25 mg) and PE (1–100 μg/ml) in KH_2PO_4-KOH buffer (pH 7.4) was irradiated with a dose of 450 Gy at a dose rate of 8 Gy/min using a ^{60}Co source. For estimating the thiobarbituric acid reactive substrates (TBARS), the reactants were heated for 15 min on a boiling water bath with the TBA reagent (0.5% TBA/ 10% trichloroacetic acid/ 6 mM EDTA/ 0.63 M hydrochloric acid). After cooling, the precipitate formed was removed by centrifugation at 1000 × g for 10 min. The absorbance of the sample was determined at 532 nm against a blank that contained all the reagents except the mitochondria. The malonaldehyde equivalents of the sample were calculated using tetraethoxypropane as the standard. The assays for the conjugated diene (CD) and lipid hydroperoxide (LOOH) were carried out as described earlier.[13]

DNA strand break assay

The assay was carried out using our own method.[14] The reaction mixtures (total volume 20 μl) containing pBR 322 DNA (10 mg/l) in a 10 mM potassium phosphate buffer, pH 7.4 in presence or absence of PE were irradiated at 25°C up to a dose of 22.5 Gy at a dose rate of 8 Gy/min using a ^{60}Co source. The test samples were added as aqueous solution to achieve the final concentration stated in the text. After γ-irradiation, the resultant supercoiled (Form I) and open circular (Form II) forms of the plasmid DNA were separated by electrophoresis and visualized under ultra violet light after staining with ethidium bromide. The relative intensities of the bands were determined with a Bio-Rad-gel documentation (Discovery series) system.

2-Deoxyribose assay

The hydroxyl radical scavenging activity of PE was determined using a reported method.[15] Briefly, the reaction mixture (total volume 1 ml) contained 28 mM 2-deoxyribose, 10 mM Tris-HCl buffer (pH 7.4), 100 μM $FeCl_3$, 100 μM EDTA, 1 mM H_2O_2 and PE. The reaction was triggered by adding ascorbic acid (300 μM). After incubating the mixture for 1 h at 37°C, it was treated with 30% ice cold HCl containing 0.75% TBA in sodium acetate. The mixture was boiled for 30 min, cooled and the absorbance of the supernatant at 532 nm was read. Mannitol was used as a positive control.

Superoxide ($O_2^{-\bullet}$) scavenging assay

The superoxide anion ($O_2^{-\bullet}$) scavenging activity of PE was determined using a reported method.[16] The amount of

$O_2^{-\bullet}$ generated in the presence and absence of PE was measured following the reduction of cytochrome C absorption at 550 nm. The assay mixtures in a total volume of 2 ml contained the following: 100 μM cytochrome C, 100 μM hypoxanthine, 10 mM Tris-HCl buffer pH 7.4 and PE (1 mg/ml). The reaction was initiated by the addition of 8 mU of XO. PE did not show any inhibition to XO under the experimental conditions. SOD (3U) was used as the positive control for the experiment. Percent inhibition of the generation of $O_2^{-\bullet}$ was calculated using the activity of SOD as 100%.

Lymphoproliferation assay

The *in vitro* cellular proliferative activity of PE was assayed using a reported method.[17] Briefly, Peripheral Blood Mononuclear Cells (PBMC) were separated from human blood. 2×10^4 Cells/well were seeded in complete RPMI-1640 medium. Different concentrations (0.25–1.0 mg/ml) of PE were added in different wells and the plates were incubated at 37°C in a 5% CO_2 environment for 72 h. PHA (5 μg/ml) was used as the standard lymphoproliferative agent. The viable cells were counted by the MTT assay.

RESULTS

Prevention of γ-ray induced lipid peroxidation by PE

The protective capacity of PE against γ-ray induced peroxidation of rat liver mitochondria was studied by assaying the TBARS, LOOH and CD formed during the lipid peroxidation. Fig. 1 demonstrates radiation-induced formation of TBARS in rat liver mitochondria and its possible prevention by PE. Exposure of mitochondria to radiation at 450 Gy showed significant increase in TBARS (2.45 ± 0.38 nmol/mg protein) as against the control value (0.38 ± 0.071 nmol/mg protein) without exposure. Simultaneous exposure of mitochondria to γ-ray at the same dose with various concentrations of PE demonstrated significant protection. Even at a very low concentration (1 μg/ml), PE showed 10% decrease in TBARS, which increased in a concentration dependent manner. For example, the protections offered by 5, 10, 50 and 100 μg/ml of PE were 30, 36, 50 and 66% respectively.

Radiation exposure of the rat liver mitochondria expectedly led to the increased formations of LOOH and CD,

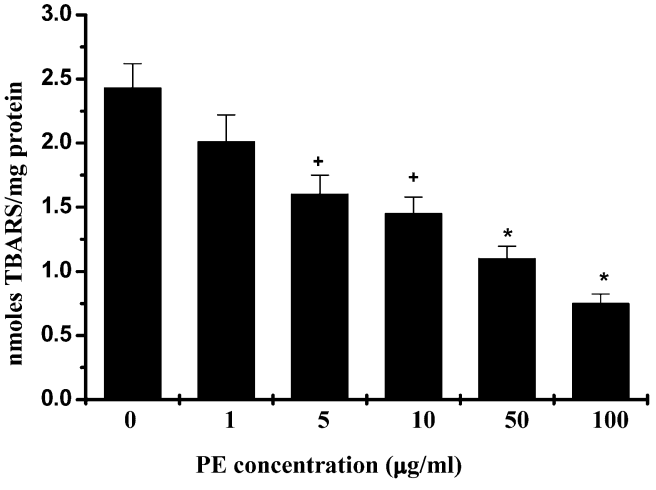

Fig. 1. Radiation-induced formation of TBARS in rat liver mitochondria and inhibition by *Piper betel* ethanolic extract (PE). Mitochondria (2 mg protein /ml) were exposed to γ-radiation at 450 Gy with and without PE (1–100 μg/ ml) and formation of TBARS was measured. The values are mean ± S. E. (n = 5). $^{8}P < 0.001$, $^{+}p < 0.01$ compared to radiation alone.

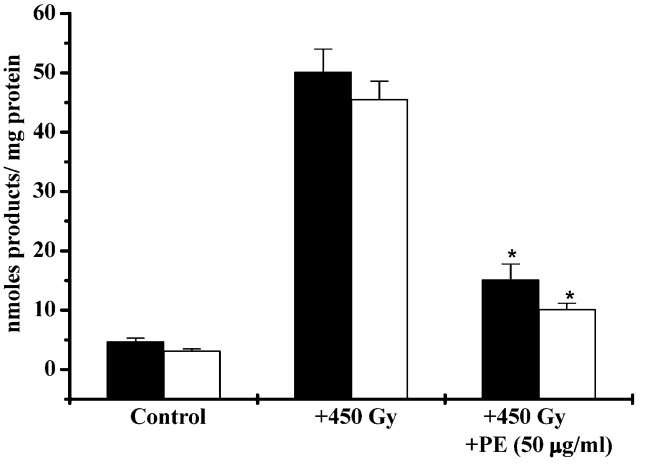

Fig. 2. Radiationñinduced formation of lipid peroxidation products (CD and LOOH) in rat liver mitochondria and prevention by *Piper betel* ethanolic extract (PE). Mitochondria (2 mg protein /ml) were exposed to gamma radiation at 450 Gy with and without PE (50 μg/ml) and the formation of conjugated diene (CD) and lipid hydroperoxide (LOOH) was measured. ■ – CD; □ – LOOH The values are mean ± S. E. (n = 5). $^{8}P < 0.001$ compared to radiation alone.

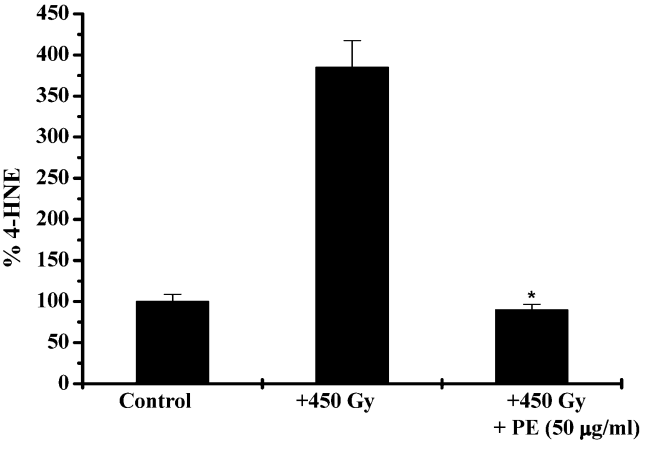

Fig. 3. Radiation-induced formation of 4-hydroxynonenal in rat liver mitochondria and prevention by *Piper betel* ethanolic extract (PE). Mitochondria (2 mg protein /ml) were exposed to gamma radiation at 450 Gy with and without PE (50 μg/ ml) and the levels of 4-hydroxynonenal were measured The values are mean ± S. E. (n = 5). $^{8}P < 0.001$ compared to radiation alone.

which, however, were prevented by PE as shown in Fig. 2. Following radiation exposure (450 Gy), a significant increase (from 4.7 ± 0.61 to 50.1 ± 3.9 nmol/mg protein) in CD was noticed. Addition of PE (50 μg/ml) to the mitochondria prior to radiation exposure could inhibit the formation of CD (15.1 ± 2.7 nmol/mg) by 77%. Similarly, the levels of LOOH that was enhanced from 3.1 ± 0.41 to 45 ± 3.1 nmol/mg protein by gamma irradiation, was reduced by 83.3% (LOOH 10.1 ± 1.01 nmol/mg protein) in the presence of PE (50 μg/ml).

Prevention of HNE formation by PE

The data on radiation-induced formation of HNE in rat liver mitochondria and its prevention by PE are shown in Fig. 3. Gamma irradiation of mitochondria to 450 Gy dose induced the formation of HNE (385% increase) in significant amount which was reduced to the basal level by addition of PE (50 μg/ml) prior to irradiation.

Prevention of γ-ray induced DNA scission by PE

Fig. 4 shows the agarose gel electrophoresis pattern of plasmid DNA irradiated at 22.5 Gy in the absence and presence of PE at three different concentrations (0.25, 0.5 and 0.75 mg/ml). Compared to the unirradiated DNA (control, lane 1), exposure of DNA to γ-radiation (22.5 Gy) led to the extensive conversion of the supercoiled form to the open circular form (lane 2). Addition of PE in increasing concentrations to the DNA, prior to irradiation, progressively reduced the intensity of the band due to the open circular form (Lanes 3–5). The quantification of the extent of radioprotection by PE is presented in Table 1.

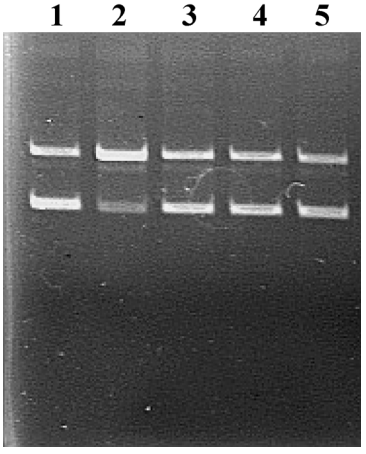

Fig. 4. Gel electrophoresis pattern of plasmid pBR 322 exposed to γ-ray in presence and absence of different concentrations of *Piper betel* ethanolic extract (PE). Plasmid DNA (200 ng) samples in 10 mM potassium phosphate buffer, pH 7.4 in a 20 μL volume were irradiated at 25°C up to a dose of 22.5 Gy using a ^{60}Co source. **1** – The DNA sample without irradiation; **2** – The DNA sample after irradiation; **3–5** – The DNA samples after irradiation in the presence of 0.25, 0.50 and 0.75 mg/ml PE respectively.

Table 1. Quantification of concentration-dependent radioprotection of *Piper betel* ethanolic extract (PE) against γ-ray induced plasmid DNA damage.

Well no.	Sample	% Open circular form of DNA	% Protection by PE
1	control DNA	36.2	–
2	irradiated DNA	81.3	–
3	irradiated DNA in the presence of PE (0.25 mg/ml)	44.7	81.2
4	irradiated DNA in the presence of PE (0.5 mg/ml)	43.8	83.1
5	irradiated DNA in the presence of PE (0.75 mg/ml)	37.9	96.2

Table 2. Comparative reactive oxygen species (ROS) scavenging activities of *Piper betel* ethanolic extract (PE) and positive controls.

ROS	Sample	Scavenging capacity (%)
hydroxyl	mannitol (10 mM)	75.31 ± 5.9[a]
	PE (1 mg/ ml)	39.32 ± 1.4[a]
superoxide anion	Superoxide dismutase (SOD, 3U)	80.27 ± 3.2[a]
	PE (1 mg/ ml)	57.87 ± 2.7[a]

[a]Values are mean ± S. E. (n = 5)

•OH Radicals scavenging capacity of PE

The scavenging activity of PE against the •OH radicals was estimated by the 2-deoxyribose assay.[15] PE showed very impressive scavenging ability for the hydroxyl radicals. For instance, the •OH radicals scavenging activity of mannitol (10 mM) was 75.31 ± 5.9 (mean ± s.e., n = 5). Under similar conditions, the hydroxyl radicals scavenging activity of PE (1 mg/ml) was 52.2 ± 5.3% as that of mannitol.

O_2^{-}• Radicals scavenging capacity of PE

PE could effectively scavenge the O_2^{-}• radicals as revealed by the cytochrome C reduction assay.[16] Compared to SOD (3U), as the positive control, the scavenging capacity of PE (1 mg/ ml) was 72.1 ± 5.9% (mean ± s.e., n = 5). The results are summarized in Table 2.

Immunomodulatory activity of PE

Treatment of PBMC with PE increased the cell viability as revealed from the MTT assay. The lymphoproliferative activity of PE increased in a concentration dependent manner. For example, the absorbance (492 nm) values for the cells treated with PE at the concentrations of 0.5, 0.75 and 1.0 mg/ml were 0.14, 0.25 and 0.35 respectively. Under the same conditions, the PHA (5.0 µg/ml) treated cells showed an absorbance (492 nm) of 0.025 only.

DISCUSSION

The deleterious biological consequences of both ionizing and non-ionizing radiations especially with respect to causing mutation and carcinogenesis are well documented. The biological damages induced by the low linear energy transfer (LET) radiations are mostly indirect, and mediated by reactive oxygen species (ROS) such as •OH, $O_2^{-\bullet}$, H_2O_2 etc., generated by the radiolysis of water.[18,19] Due to the high concentration of water in metabolizing cells, radiation exposure of biological systems primarily leads to its radiolysis furnishing e^{-}_{aq}, •OH and H•. In aerobic cells, the e^{-}_{aq}, in turn, also generates the $O_2^{-\bullet}$ radicals.

These reactive species are known to cause degradation of important macromolecules including DNA and membranes.[20,21] Thus, the high level of unsaturated lipids is most susceptible to oxidative damage, resulting in disruption of cellular integrity, inactivation of cellular components etc. that lead to cytotoxicity[22] and cause several diseases and aging.[23,24] Likewise, the DNA molecules are also prone to radiation-induced lesions due to the presence of various reactive sites (base and sugar) in them.[25] For a variety of tissues, the pathophysiological importance of ROS-mediated oxidative injury caused by the exposure to radiation is widely evaluated.[26]

In the present study, the capacity of PE in preventing γ-ray induced lipid peroxidation and DNA damage were assessed. In addition, the ROS scavenging and immunomodulatory properties of PE were also evaluated to establish the mechanism of its radioprotective action.

For this, the capacity of PE to prevent lipid peroxidation in rat liver mitochondria exposed to γ-ray with a dose of 450 Gy was studied by measuring the amounts of TBARS formed in its absence and presence. It is well known that exposure of humans even to a considerably lower dose of γ-radiation causes extensive lipid peroxidation. From that perspective, the chosen radiation dose was admittedly too high. However, our *in vitro* experiments carried out by exposing rat liver mitochondria with lower doses (75, 150 and 300 Gy) of γ-irradiation did not produce significant amounts of TBARS (data not shown). Hence a high radiation dose was necessary to generate reproducible and unerroneous results. Thus, the high radiation dose was chosen primarily to demonstrate the capacity of PE in preventing the γ-ray induced lipid peroxidation. It was found that PE could inhibit the extensive lipid peroxidation caused by even a very high dose of γ-ray. Thus, it is anticipated to prevent similar lipid damage in cells.

The TBA assay is fairly non-specific since TBA reacts with various chemicals including sugars[27] to give a pink red colour. Hence, the protection induced by PE at a fixed concentration of 50 µg/ml was also assayed by measuring LOOH and CD, two relatively unstable products of the process. Addition of PE to the mitochondria prior to radiation exposure could inhibit the formation of CD and LOOH by 77 and 83.3% respectively.

The LOOH produced during lipid peroxidation is known to furnish various reactive aldehydes on subsequent cleavage. Amongst these, 4-hydroxynonenal (HNE) is a toxic compound that has been associated with human diseases and experimental models.[28] HNE is known to react with glutathione and amino acids hampering the antioxidant defense and inactivating various key proteins.[29] Consequently, the preventive activity of PE against HNE formation was also assessed. It was found that while exposure of mitochondria to γ-ray of 450 Gy dose significantly increased the level of HNE, PE (50 µg/ml) could bring it down to the normalcy when administered prior to radiation exposure.

Exposure of pBR322 plasmid DNA to γ-radiation, as a function of dose, resulted in a significant increase in the single strand breaks (ssbs) of DNA. We observed a linear increase in ssbs, assessed as average strand breaks per DNA molecule, up to a dose of 30 Gy. The radiation dose of 22.5 Gy was found sufficient to convert the entire supercoiled DNA to open circular form. Hence, this dose was chosen for further studies. The present study revealed that PE could effectively protect plasmid DNA from γ-ray induced ssbs in a concentration dependent manner.

As discussed earlier, the hydroxyl and superoxide radicals are the major ROS produced during γ-ray exposure of biological systems. Of these, the hydroxyl radicals are believed to be most toxic and contribute maximum in DNA and lipid

cleavages.[30] Likewise, the superoxide radicals have been implicated to play crucial roles in ischaemia-reperfusion injury and promoting human gout.[31] Despite its involvement in many pathological processes the radical by itself is not very reactive, but can generate the hydroxyl radicals *via* a superoxide-driven Fenton process (Scheme 1). The Fenton

$$O_2^{\bullet-} \longrightarrow H_2O_2$$
$$O_2^{\bullet-} + Fe(III) \longrightarrow O_2 + Fe(II)$$
$$Fe(II) + H_2O_2 \longrightarrow Fe(III) + {}^{\bullet}OH + OH^-$$

Scheme 1 Reactions in a superoxide-driven Fenton process

process involves reaction of Fe(II) with hydrogen peroxide to generate the hydroxyl radicals. The superoxide radicals play a key role in this process. The superoxide radicals can produce the required hydrogen peroxide via disproportionation and also act as an reducing agent of Fe(III) to generate the Fe(II), propagating the process. Overall, the radicals can trigger oxidative stress directly or indirectly and lead to damages of biomacromolecules. The present study showed that PE could scavenge both hydroxyl ($^{\bullet}OH$) and superoxide ($O_2^{-\bullet}$) radicals efficiently. The scavenging activities of PE against these radicals compared well with those of the respective positive controls, mannitol and SOD (3U).

Besides the above antioxidant mechanism, many of the radioprotective compounds operate *via* their immunomodulatory activity.[32] Consequently, it was of our interest to assess the immunomodulatory property of PE. The present study revealed a significant immunomodulatory activity of PE.

The principal chemical constituents of PE were found to be the phenolics, chevibetol and allyl pyrocatechol, which were earlier isolated from the same plant.[33] In view of their phenolic nature, all these compounds might contribute to the antioxidant action of PE.

PE appears to be a potential radioprotectant, exerting the activity through its superior radical scavenging and immunomodulatory properties. Recently, the radioprotective property of *Piper betel* has been reported,[34] wherein the plant extract was found to elevate the cellular SOD level. The present study delineates other biochemical mechanism of the radioprotective action. The leaves of the plant are extensively consumed in the form of morsel by one third of the population of the Eastern World without any side effect. To the best of our knowledge, the toxicity data of PE is unavailable. However, during our own investigation on its anti-ulcerogenic activity,[9] we have found that even up to a dose of 3 g/kg body weight, it was non-toxic to rats. Consequently, further investigation with the extract appears promising in developing a new natural radioprotectant.

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