An Extract of *Phyllanthus amarus* Protects Mouse Chromosomes and Intestine from Radiation Induced Damages

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Radioprotection/ *P. amarus*/ Radiation/Micro nuclei/ Chromosomes/ Antioxidants.

We reported earlier on our preliminary study of the radioprotective effect of *Phyllanthus amarus* (*P. amarus*) in mice. *P. amarus* was found to inhibit the myelosuppression and elevated the levels of antioxidant enzymes in the blood and liver. In the present study we have evaluated the protective effect of *P. amarus* against radiation-induced changes in the intestine and mouse chromosomal damage. *P. amarus* at concentrations of 250 & 750 mg/Kg. wt were found to elevate the antioxidant enzymes in the intestine and decrease the lipid peroxidation levels. Histopathological evaluations of the intestine revealed decreased damage to intestinal cells, demonstrating that *P. amarus* protected the intestine. The genotoxic effects of radiation on mouse chromosomes were evaluated by assaying the micronuclei formation and chromosomal aberrations. *P. amarus* was found to protect the clastogenic effects of radiation as seen from decreased number of micronuclei. The administration of *P. amarus* was also found to decrease the percentage of chromosomal aberrations. Based on our present and previous reports it could be concluded that *P. amarus* extract has significant radioprotective activity.

INTRODUCTION

Radiotherapy is one of the prime choices in cancer therapy. Although the radiation is directed to tissues of malignant origin normal tissues are not often protected from radiation induced damages. The cytotoxic action of ionizing radiation is mainly mediated by the generation of oxygen free radicals. Ionizing radiation induces considerable damage to cellular macromolecules like DNA and protein resulting in alteration in gene expression, cell proliferation and apoptosis.

Protection of normal tissue during radiotherapy and during accidental radiation exposure is still a matter of active concern. Several compounds natural or synthetic origin have been evaluated for their possible radioprotective effect. However the toxicity associated with some of the synthetic compounds at the effective radio-protective concentration further demands the search for new safer and more effective compounds. Radioprotective efficacies of several plants and plant derived compounds have been reported by several workers. We had reported earlier our preliminary investigations on the radioprotective effect of *P. amarus* against gamma-radiation induced toxicity in mice. *P. amarus* was found to reduce myelosuppression, increased the bone marrow cellularity and antioxidant status of irradiated animals. In the present communication, we have evaluated the possible protective role of *P. amarus* against radiation induced damages to chromosomes and intestinal tissue of the irradiated mice.

MATERIALS AND METHODS

Animals

Inbred 4–6 week old female BALB/c mice (20–25g) were obtained from Small Animal Breeding Station, College of Veterinary and Animal Sciences, Mannuthy. Animals were maintained in ventilated polypropylene cages under the standard conditions of temperature and humidity. They were fed with normal chow (Sai Durga Feeds and Foods, Bangalore) and water ad libitum. The animal experiments were conducted after getting prior permission from Institutional Animal Ethics Committee (IAEC) and as per the instructions prescribed by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India.

Chemicals

Colchicine, 5-5’ dithiobis (2-nitrobenzoic acid) (DTNB) and glutathione reduced (GSH) were purchased from SRL, Mumbai. May-Grunwald Giemsa, giemsa powder and

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thiobarbituric acid (TBA) was obtained from Hi-Media Laboratories, Mumbai. 1, 1, 3, 3-tetramethoxy propane, 2, 2-azino-bis-(3-ethyl benzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,4,6 tripyridyl S-triazine (TPTZ) and myoglobin were obtained from Sigma-Aldrich, USA. All other chemicals used in the present study were of analytical grade.

Radiation facility

The source of radiation was a 60Co Theratron-Phoenix teletherapy unit (Atomic Energy Ltd, Canada). BALB/c mice were kept in specially designed, well-ventilated cages and exposed to whole body radiation at a dose rate of 1.44 Gy/minute in a field size of 25 × 25 cm² and at a distance of 80 cm from the source.

Preparation of the P. amarus extract

Aerial parts (stem and leaves) of P. amarus were collected from Thrissur district of Kerala State and were dried at 45°C. A voucher specimen of the plant was identified and kept in the herbarium (voucher no. Eup-9) of Amala Ayurvedic Hospital and Research Centre. Dried parts of P. amarus were powdered and extracted with twice the volume of 75% methanol by stirring overnight at the room temperature. The solution was then centrifuged at 2500 rpm to separate the supernatant and the supernatant was evaporated to dryness at 50°C using a rotary evaporator under reduced pressure. The yield of the preparation was 8.1%. Aqueous solution of this dried extract was used in the present study.

In vitro antioxidant activity

DPPH assay

In this assay, 1,1-diphenyl-2-picrylhydrazyl (DPPH), which is a stable free radical, reacts with antioxidants and gets converted into 1,1-diphenyl-2-picrylhydrazyl. The ability of a compound to scavenge the stable free radical DPPH is measured by a decrease in the absorption at 515 nm. DPPH (25 mg % in methanol) was allowed to react with antioxidants at different concentration of P. amarus (1 – 10 μg/mL) and percentage inhibition was calculated by the following formula. DPPH radical scavenging (%) = [(O. D of control – O. D of sample)/O. D of control] × 100.

ABTS assay

The assay measures the relative ability of antioxidant substances to scavenge the 2,2-azo-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS⁺) radical generated in the aqueous phase. The stock solutions of 500 μM ABTS diammonium salt, 400 μM myoglobin (Mb III), 740 μM potassium ferricyanide and 450 μM H₂O₂ were prepared in phosphate buffered saline (PBS) (pH 7.4). Methmyoglobin was prepared by mixing equal amounts of myoglobin and potassium ferricyanide solutions. The reaction mixture (2 mL) contained ABTS (150 μM), Mb III (2.25 μM), varying concentrations of P. amarus extract (1–10 μg/mL) and PBS. The reaction was initiated by adding 75 μM H₂O₂ and lag time in seconds was recorded before absorbance of ABTS⁺ at 734 nm began to increase. The concentration required for the 50% inhibition of ABTS radicals was determined.

FRAP (Ferric Reducing Ability of Plasma) assay

This method measures the ability of the antioxidants compounds to reduce ferric-tripyridyltriazine (Fe³⁺-TPTZ) to a ferrous form (Fe²⁺) with absorption at 595 nm. The reaction mixture contains 0.3 M acetate buffer (pH 3.5), 10 mM TPTZ in 40 mM HCl and 20 mM FeCl₃ in water and various concentrations of P. amarus were used and the concentration needed for 50% inhibition was calculated.

Effect of P. amarus on radiation induced changes in the intestine

Twenty-eight mice were used for the experiment and animals were randomly divided into four groups as described below.

Group I: Normal, untreated (n = 4), Group II: 6 Gy irradiated control treated with vehicle (distilled water) orally (n = 8), Group III: 6 Gy irradiated animals treated with P. amarus extract 250 mg/kg body wt. orally (n = 8), Group IV: 6 Gy irradiated animals treated with P. amarus extract 750 mg/kg body wt. orally (n = 8)

Treatment for groups II–IV began five days prior to radiation administration. All animals were exposed to single dose of radiation (6 Gy) on 6th day and the administration of P. amarus continued once daily for another 12 days. Four animals from group II–IV were sacrificed on day 3rd and 12th after irradiation and animals in the group I was also sacrificed on day 3.

Collection of intestinal mucosa

A part of the intestine was removed from the animals. The intestinal mucosa was scraped off using a clean glass slide and kept at –70°C until analysis. On the day of analysis 25% homogenate was prepared in 0.1M phosphate buffer (pH 7.4) and following parameters were evaluated. Total protein was estimated by the method of Lowry et al, the levels of GSH was estimated by the method of Moron, GPx activity was measured by Haffeman’s method and for levels of lipid peroxidation by the method of Okhawa et al was used.

Histopathological analysis

A portion of the intestine was fixed in buffered 10% formalin, were fixed in 10% neutral-buffered formalin. Sections (4 μm) were taken and stained with haematoxylin-eosin and observe under oil immersion microscope (100x). Photographs were taken.

Effect of P. amarus on radiation induced micronuclei formation in mouse chromosomes

Sixteen BALB/c mice were divided into following groups of 4 animals each as given below.

Group I: Normal, untreated (n = 4), Group II: 1.5 Gy irra-
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diated control treated with vehicle (distilled water) orally (n = 4), Group III: 1.5 Gy irradiated animals treated with *P. amarus* extract 250 mg/kg body wt. orally (n = 4), Group IV: 1.5 Gy irradiated animals treated with *P. amarus* extract 750 mg/kg body wt. orally (n = 4).

Treatment for groups II–IV began five days prior to radiation administration. All animals were exposed to single dose of radiation (1.5 Gy) on 6th day. 24 hr after irradiation animals were sacrificed and bone marrow cells from each femur were flushed into PBS containing 2% FCS (GIBCO). The tubes were centrifuged at 3000 rpm for 10 min and cell pellet was collected and was smeared on a clean glass slide. The slides were stained as follows. Slides were initially kept in undiluted May-Grunwald Giemsa stain (MGG) for 3 min followed by diluted MGG in water (1:1) for 2 min. Finally the slides were stained with diluted Giemsa in water (1:6) for 6 min. The slides were then rinsed in water, air-dried and mounted. Following types of cell were scored under oil immersion microscope. The slides were screened for 2000 polychromatic erythrocytes and corresponding normochromatic erythrocytes and also for the presence of micronuclei.17,18

**Effect of *P. amarus* on radiation induced chromosomal aberrations**

Sixteen BALB/c mice were divided into following groups of 4 animals per group as given below.

- Group I: Normal, untreated (n = 4), Group II: 3 Gy irradiated control treated with vehicle (distilled water) orally (n = 4), Group III: 3 Gy irradiated animals treated with *P. amarus* extract 250 mg/kg body wt. orally (n = 4), Group IV: 3 Gy irradiated animals treated with *P. amarus* extract 750 mg/kg body wt. orally (n = 4).

Treatment for groups II–IV began five days prior to radiation administration. All animals were exposed to single dose of radiation (3 Gy) on 6th day. 48 hr after irradiation animals were sacrificed. 90 min before sacrifice all the animals were given a single dose of colchicine (2 mg/kg b. wt, in saline) intraperitoneally (i.p). Bone marrow cells from each femur were flushed into PBS containing 2% FCS. Cell button was separated after centrifugation and 5 mL of 0.075 M KCl (maintained at 37°C) was added to each tube and incubated for 30 min (hypotonic treatment). Tubes were centrifuged again and 5 mL (3 mL drop wise and 2 mL at a stretch) of ice cold methanol (MeOH)-acetic acid mixture (3:1) was added (fixation step) and centrifuged again at 1000 rpm for 5 min. Five mL of MeOH-acetic acid mixture was added again, centrifuged again and cell pellet was separated. The cell pellet was dropped into a chilled clean glass slide (4 drops/slide) from a height of 60 cm and remaining droplets were blown off and slides were air dried and kept in the dark for 5 days for maturation. For staining, 2 mL of Giemsa solution was mixed with 98 mL of Sorenson buffer (4.174 g Na₂HPO₄ in 490 mL and 4.162 g KH₂PO₄ in 510 mL and these two solutions were mixed). Slides stained for 5 min and washed with water, air dried and observed under oil immersion microscope. A minimum of 100 metaphase spreads was scored for aberrations.19

**Statistical analysis**

All the values are expressed as mean ± S.D. The statistical analysis was done by one-way ANOVA followed by appropriate post hoc test using Graphpad Instat 3 Software (San Diego, USA).

**RESULTS**

**In vitro antioxidant activity**

*P. amarus* demonstrated significant antioxidant activity in vitro. The 50% inhibitory concentration (IC₅₀) was found to be 4.62 μg/mL, 1.5 μg/mL and 5.43 μg/mL respectively for DPPH, ABTS and FRAP assays. We also compared the IC₅₀ values with that of a standard compound TROLOX for DPPH and ABTS assay, which was found to be 13 μg/mL and 0.48 μg/mL respectively.

**Effect of *P. amarus* on radiation induced changes in the intestine**

The effect of *P. amarus* in GSH content of intestinal mucosa is given in Fig. 1. Radiation dose at 6 Gy caused a decrease in the GSH levels on day 3 after irradiation to 15.42 ± 2.50 nanomoles/μg protein. The administration of *P. amarus* significantly elevated the lowered levels of GSH to 23.35 ± 1.68 (p < 0.001) in case of higher dose treated group.

*Fig. 1. Effect of *P. amarus* on glutathione levels in the intestinal mucosa of radiation treated mice. Mice were exposed to a single dose radiation of 6 Gy and were sacrificed at 3rd and 12th day after irradiation. Irradiation decreased the levels of glutathione in the intestinal mucosa and administration of *P. amarus* was found to elevate the decreased levels of glutathione. Values are expressed as mean ± S.D. ***p ≤ 0.001 against radiation alone group (Dunnet’s test).***

- 3 days after irradiation
- 12 days after irradiation
Figure 2 described the activity of GPx in the intestinal mucosa. Treatment with 6 Gy radiation dose lowered the activity of GPx to $138.59 \pm 17.09$ units/μg protein on 3rd after irradiation. The administration of *P. amarus* (750 mg/Kg) increased the activity of GPx to $213.89 \pm 11.76$ (p < 0.001) indicating that *P. amarus* elevated the activity of GPx, which is one of the major enzymes in the antioxidant defense system of the body.

Figure 3 shows the lipid peroxidation levels in the intestine as nanomoles of MDA formed/μg protein. As a result of irradiation the levels of lipid peroxides were elevated considerably. On day 3 after the whole body irradiation, the levels of MDA in radiation alone treated group were $4.90 \pm 0.50$ as compared to the normal MDA levels of $0.64 \pm 0.05$. The administration of *P. amarus* has a protective role on the
mucosal membrane against radiation induced changes. On day 12 in higher dose treated group the levels of MDA decreased to 1.34 ± 0.35 (p < 0.001).

Histopathological analysis of intestinal mucosa after irradiation

Figure 4 described the effect of *P. amarus* on the intestinal mucosa of irradiated animals. The intestinal sections taken from the radiation alone treated group showed damaged intestinal wall, the morphology of villi is damaged; the columnar lining of intestinal wall got distorted. Edema was present along with infiltration of lymphocytes (Fig. 4(b)). All these pathological changes have been reverted in *P. amarus* treated group (Fig. 4(c)). The wall of the intestine appears normal. The lining columnar cells appear normal with fewer collections of lymphocytes. The structures of villi appear normal and the damages to the mucosal gland were also reverted.

Effect of *P. amarus* on radiation induced micronuclei formation

1.5 Gy irradiation treatment induced the formation of micronuclei in the bone marrow cells. In radiation alone treated group, the percentage of micronucleated PCE and NCE was 3.18 ± 0.48 and 3.57 ± 0.69 respectively as compared to normal, which was 0.16 ± 0.08 and 0.12 ± 0.03 respectively. The treatment with *P. amarus* significantly inhibited the micronuclei formation and the percentage of micronucleated PCE and NCE was 1.58 ± 0.22 and 1.63 ± 0.46 in *P. amarus* 750 mg/Kg b.wt treated group (Table 1).

Effect of *P. amarus* on radiation induced chromosomal aberrations

The 3 Gy irradiation produced a significant chromosomal aberration in mouse chromosome as seen from the number of breaks, gaps and numerical aberrations. The percentage of aberrations in radiation alone treated group was 50.25 ± 4.50 while that of the normal unirradiated group was 3.75 ± 0.95. The treatment of *P. amarus* decreased the radiation-induced aberrations in chromosome and the percentage of aberrations in a lower dose (250 mg/kg) and a higher dose of *P. amarus* (750 mg/Kg b. wt) treated group was 34.00 ± 3.16 and 28.25 ± 1.70 respectively (Table 2).

**Table 1.** Effect of the administration of *P. amarus* on the radiation induced micronuclei formation in mouse bone marrow cells (24 h after 1.5 Gy)

<table>
<thead>
<tr>
<th>Group/Treatment</th>
<th>%Mn PCE</th>
<th>%Mn NCE</th>
<th>(%Mn PCE + %Mn NCE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.16±0.08</td>
<td>0.12±0.03</td>
<td>0.14±0.34</td>
</tr>
<tr>
<td>Radiation alone</td>
<td>3.18±0.48</td>
<td>3.57±0.69</td>
<td>3.47±0.29</td>
</tr>
<tr>
<td>Radiation + <em>P. amarus</em> 750 mg/Kg b.wt</td>
<td>1.58±0.22***</td>
<td>1.63±0.46***</td>
<td>1.63±0.23***</td>
</tr>
<tr>
<td>Radiation + <em>P. amarus</em> 250 mg/Kg b.wt</td>
<td>2.20±0.30***</td>
<td>2.18±0.29***</td>
<td>2.19±0.23***</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± S.D. Statistical significance of the treatment was done using one way ANOVA followed by Dunnet’s test. ***p ≤ 0.001 against radiation alone group (Dunnet’s test).

**Table 2.** Effect of the administration of *P. amarus* on the radiation induced chromosomal aberrations in mouse bone marrow cells (48 h after 3 Gy)

<table>
<thead>
<tr>
<th>Group/Treatment</th>
<th>Chromatid</th>
<th>Chromosome</th>
<th>Other aberrations</th>
<th>% of aberrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gap</td>
<td>Break</td>
<td>Gap</td>
<td>Break</td>
</tr>
<tr>
<td>Normal</td>
<td>1.0</td>
<td>1.25</td>
<td>0.25</td>
<td>0.75</td>
</tr>
<tr>
<td>Radiation alone</td>
<td>12.0</td>
<td>9.75</td>
<td>7.5</td>
<td>8.5</td>
</tr>
<tr>
<td>Radiation + <em>P. amarus</em> 750 mg/Kg b.wt</td>
<td>6.75</td>
<td>7.50</td>
<td>5.0</td>
<td>3.5</td>
</tr>
<tr>
<td>Radiation + <em>P. amarus</em> 250 mg/Kg b.wt</td>
<td>6.75</td>
<td>9.25</td>
<td>6.0</td>
<td>7.0</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± S.D. Statistical significance of the treatment was done using one way ANOVA followed by Dunnet’s test. ***p ≤ 0.001 against radiation alone group (Dunnet’s test).
DISCUSSION

The cytotoxic actions of radiations are generally mediated by the generation of free radicals. The cells of the gastrointestinal system and hematopoietic system are more prone to radiation induced damage. In the present study administration of *P. amarus* effectively prevented the intestinal damage as seen from the decreased peroxidation levels of intestinal membranes and elevated antioxidant defense system. It was concordance with our earlier observation that *P. amarus* enhanced the antioxidant system in the liver and blood of the irradiated animals. Moreover *P. amarus* also acted as an effective scavenger of free radicals *in vitro* as seen from the *in vitro* antioxidant assays. *P. amarus* inhibited the formation of lipid peroxides *in vitro*.

So it can be presumed that stimulation of the antioxidant defense system of the body is one of major mechanism behind the radioprotective effect of *P. amarus*.

Interaction of ionizing radiation with mammalian cells induces several types of molecular damage to cellular macromolecules and especially in DNA where it causes single strand breaks, double strand breaks, damage of various types of bases and cross link between DNA and protein and sometimes a combination of all of these described events. Out of these lesions DNA double strand breaks have been reported to be converted into chromosome aberrations. The micronuclei, sister chromatid exchange and chromosomal aberrations are the known cytogenetic end points generally used for evaluating genotoxicity. Micronuclei are formed in the cell due to the damage of chromosomes by external factors like radiation. They are generally found in the cytoplasm outside the main nucleus of a cell. The pretreatment of mice with *P. amarus* was effective in preventing the formation of different types of aberrations in the chromosome as well as the induction of micronuclei. The present study revealed that *P. amarus* effectively prevented the genotoxic effect of radiation on mouse chromosomes.

Several plant-derived preparations have been found to reduce the side effects of radiation. These include single plants, multiple formulations as well as isolated drugs. Plant extracts could reduce oxidant induced damage during irradiation by increasing the antioxidant potential in the body or mimics antioxidant enzymes. Some of the plants as well as formulations reduces the immuno suppression produced by irradiation, increase stem cell proliferation, and/or increase the repair of DNA induced by irradiation. Some of the plants have been useful to reduce the discomforts produced during irradiation. Use of these agents are being recommended during cancer treatment.

**Fig. 5.** The effect of reactive oxygen species on biological system. Radiation caused damage to membrane, haematopoetic system, chromosomes, gastrointestinal system increased lipid peroxidation and alters the levels of antioxidant enzymes resulting in oxidative stress. Bold single headed arrows ( ) indicate the possible site of action of *P. amarus*, resulting in the decrease of oxidative stress.
Most of these agents do not have the potential to reduce the cancer cell proliferation. P. amarus was found to be not only radioprotective but also reduces animal tumours which gives an added advantage from other plant derived radiation protectors.

P. amarus has been reported to contain several active ingredients. Some of the tannins purified from P. amarus were potent inhibitors of protein kinases. Presence of several lignans like phyllanthin and hypophyllanthin, polyphenols, flavonoids such as quercetin, astragalin and some ellagitannins like catechin and epigallocatechin has been isolated from P. amarus.

Based on our present and previous reports it can be concluded that P. amarus exerts its radioprotection by (a) suppressing the formnation of reactive oxygen species generated as a result of irradiation; (b) stimulating the antioxidant defense system of the body; (c) protection of gastrointestinal system of the body; (d) stimulating the hematopoietic system; (e) enhancing the repair and recovery process (unpublished data); (f) preventing the damage to DNA as well as to chromosome; (g) may be up regulating the activity of early response genes (Fig. 5).

Moreover P. amarus has been found to inhibit the proliferation of cancer cells during the process of carcinogenesis and in transplanted animal tumours. P. amarus extract was found to inhibit the activation of drug metabolizing enzymes both in vitro as well as in vivo. P. amarus extract was also found to produce significant protection against chemotherapy associated side effects. P. amarus did not produce any toxicity when administered at a concentration up to 1000 mg/kg body weight for fifteen days. Hence P. amarus could act both as a chemo protector, tumor growth inhibitor and as an efficient radio protector. Use of P. amarus and its isolated ingredients as a radioprotector warrants further study.

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