DNA DAMAGE PROTECTIVE AND ANTI-INFLAMMATORY ACTIVITY OF MADHUCA INDICA AGAINST ELECTRON BEAM RADIATION

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ABSTRACT
Ionizing radiation is an important genotoxic agent causes deleterious effect to living system by generating free radicals produced via radical oxygen species (ROS). Protecting against free radicals especially by a natural plant source has several potential applications. In the present study, we have examined the ability of Madhuca indica (M. indica) ethanol stem bark extract, to inhibit radiation-induced DNA damage and inflammatory mediators. Our study showed that there was protection of pBR322 DNA (in vitro) upon exposure to 4 Gy electron beam (EB) radiation at different dose rate. Whole body exposure of mouse to EB radiation cause strand breaks in the cellular DNA, as observed using the comet assay and also there was substantial increase in the levels of inflammatory mediators. When animals exposed to radiation there was an increase in the percentage of DNA in tail (%T), tail length (TL) and olive tail moment (OTM). The presence of 400 mg/kg body weight of M. indica extract prior to irradiation significantly reduced comet parameters. Simultaneously the levels of nitric oxide (NO), malondialdehyde (MDA), and sialic acid concentration were reduced significantly when compared to irradiate, contributing to the overall radioprotective and anti-inflammatory activity of M. indica extract.
KEYWORDS: Madhuca indica; MDA; NO; sialic acid; pBR322; EB radiation.

INTRODUCTION
Radiotherapy an important modality for treatment of cancer and can be used as a single modality or an adjuvant along with surgery and/or chemotherapy.[1] Radiation emitted during radiotherapy inflicts deleterious effects to living cells through the generation of ROS that damage vital macromolecules such as DNA, protein etc.[2] Radioprotection of normal tissue is also important in the radiotherapy of cancers where the tissues need to be protected when cancers are exposed to high doses of radiation. A great many compounds synthesized for this purpose showed good radioprotection in vitro and in vivo.[3] There have been various attempts to find an agent that can preferentially protect the normal tissues from radiation damage. Thus in radiation biology the search for an ideal radioprotectors is on current topics of research.

*M. indica* is a common herb belongs to the family sapotaceae is commonly known as a honey tree, found widely in Indian states and also known as Indian tropical tree that grows in deciduous forests. *M. indica* is being used from centuries for numerous purposes as a medicinal herb. The bark of *M. indica* is used for treating rheumatism, ulcer, itches, bleeding, spongy gum and tonsillitis.[4] Stem bark is also reported having, inhibitory activity on free radical release from phagocyte.[5] The methanol extract of flower, stem bark has been reported to have antibacterial activity.[6] The present investigation involves the radioprotective activity and anti-inflammatory activities of *M. indica* extract against EB radiation induce damage.

MATERIALS AND METHODS
Chemicals
pBR322 plasmid DNA was purchased from Bangalore Genei. Low melting agarose, high melting agarose, TRIS base, disodium EDTA, TritonX-100, sodium sarcosinate, DMSO and propidium iodide were used for comet assay. Thiobarbituric acid, N-1-naphthylethylenediamine dihydrochloride, sulfanilamide, sulphuric acid, sodium periodate, cyclohexanone for conducting MDA, NO and sialic acid assay were obtained from Sigma Aldrich (St.Louis, Missouri).
**In vitro DNA damage studies**

**Protection of plasmid pBR322 DNA by *M. indica* against EB radiation**

To carry out pBR322 plasmid DNA damage the experiment was divided into 6 sets. Set I: control; set II: radiated control; set III, IV, V and VI: were treated with *M. indica* extract at different concentrations of 50, 100, 150 and 200μg respectively prior to irradiation. The plasmid DNA (250ng in 0.01M sodium phosphate buffer) was added to each set except control and was exposed to 4 Gy of EB radiation. After irradiation DNA was electrophoresed on 0.8% agarose at 55V for 2 hours and the DNA damage was analyzed by UV transilluminator.

**In vivo studies**

**Animals**

Swiss albino mice weighing 22–25 g were obtained from the institutional animal breeding Section, K. S. Hegde Medical Academy, Mangalore, India. They were maintained under standard conditions of temperature and humidity in the centre’s animal house facility. The animals were provided with a standard mouse chow (Sai Durga Feeds and Foods, Bangalore, India) and water *ad libitum*. All animal experiments in this study were carried out with the prior approval of the institutional animal ethics committee and were conducted strictly adhering to the guidelines of the committee.

**Experimental design and sample collection**

To carry *in vivo* DNA damage and anti-inflammatory studies, animals were divided into five groups of six animals each. Group I: served as control; group II: irradiated; group III, IV and V were treated with 100mg/kg, 200mg/kg and 400mg/kg body weight of extract, once daily for 15 consecutive days and on 16th the day the mice were exposed to 6Gy EB radiation. After irradiation they were observed for the next 15 days and were sacrificed by cervical dislocation on the 16th day. The blood was drawn from the mice and used to carry out comet assay and sialic acid test; the liver homogenate prepared using cold normal saline was used for conducting MDA and NO assay.

**In vivo DNA damage using comet assay**

Cellular DNA damage of mouse blood lymphocytes was measured using alkaline single cell gel electrophoresis (comet assay) as described by Tice et al. 10μl lymphocyte was mixed with 200μl of low melting point agarose in phosphate buffered saline and applied on microscopic slides pre-coated with 200μl high melting agarose. The slides were covered with
a cover slip and refrigerated for 5 min. After solidification, the slides were immersed in ice cold alkaline lysing buffer for 1 hour and they were placed on a horizontal electrophoresis tank filled with an alkaline buffer and equilibrated with the same buffer for 20 min followed by electrophoresis for 20 min at 25 V and 300 mA. Then the slide were neutralized with 0.4 M Tris buffer at pH 7.4, stained with 50 μl of ethidium bromide (20 μg/ml) and visualized with a fluorescence microscope (Olympus 40x objective) and a minimum of 50 comets per slide for a group were analyzed using CASP software[9] in which the comet parameters can be assayed easily and the results are given as mean ± SD.

**Anti-inflammatory studies**

**MDA assay**

MDA was evaluated as described by Ohkawa et al.[10] MDA level was assayed by incubating liver homogenate with 15% TCA, 0.375% TBA and 5N HCL at 95 °C for 15 min, the mixer was cooled, centrifuged and the absorbance of the supernatant was measured at 532 nm against an appropriate blank. The results were expressed as nmol/mg of protein.

**Measurement of NO**

Total NO levels were calculated by the conversion of nitrate into nitrite and it was measured by the Greiss reaction.[11] Briefly; 30 μl of each sample was incubated with an equal volume of sulfanilamide solution (1% sulfanilamide in 5% phosphoric acid) at room temperature for 5-10 min, which is protected from light. After incubation 50 μl of the NED (0.1% N-1-naphtylethlenediamine dihydrochloride in water) was added and incubated at room temperature for 10 min. Then the absorbance was measured within 30 min at 550 nm spectrophotometrically and the results were expressed as μmol/g.

**Sialic acid test**

Sialic acid was evaluated as described by Warren et al.[12] To 0.5 ml of the hydrolysate in 0.1 N H₂SO₄; 0.2 ml of sodium periodate was added, mixed thoroughly and incubated for 20 min. To this 1 ml of sodium arsenate solution and 3 ml of thiobarbituric acid was added and the mixture was heated in boiling water bath for 15 min. After cooling, 4.5 ml of cyclohexanone was added and shaken well for 15 sec till the color was taken up by the cyclohexanone supernatant. The mixture was centrifuged to get a clear pink layer of cyclohexanone. This supernatant was pipetted out and the intensity of color was measured using spectrophotometer at 550 nm. The sialic acid content was determined from the standard curve of sialic acid and has been expressed in terms of μg/ml.
Histopathological studies

Slices of skin were fixed in 5% formaldehyde, embedded in paraffin wax, sectioned at 5μ thickness and stained with haematoxylin and eosin stain. Detailed microscopic examinations of this organ were carried out.

Statistical analysis

All results were expressed as Mean ± Standard Deviation (S.D). Statistical significance was determined by one-way analysis of variance (ANOVA). P values < 0.05 were considered as significant using Dunnett’s test. All statistical analysis was carried out using Graph Pad Prism software version 5.0.

RESULTS

Protection of plasmid pBR322 from EB radiation induced damage

The presence of extract of *M. indica* during irradiation protected plasmid pBR322 DNA from radiation induced lesions. During exposure to EB radiation, the plasmid DNA suffered strand breaks, which converted the super coiled (sc) form of plasmid DNA to open circular form (oc) (Fig. 1 Lane 2). The radiation-induced conversion of sc form to oc form was considerably reduced in the presence of the *M. indica* extract at 150μg (Fig. 1 Lane 5).

![Image of gel electrophoresis](image)

**Fig. 1: In vitro radioprotective activity of *M. indica* at different concentrations**

C: Control, R: Radiated, OC: Open Circular, SC: Super Coiled

In vivo DNA damage using comet assay

The results of alkaline comet assays performed in mice peripheral blood leukocytes of animals exposed to 6Gy radiation indicated that administration of extract of *M. indica* at different concentrations (100mg/kg, 200mg/kg and 400mg/kg body weight) prior to irradiation protected cellular DNA by inhibiting comet parameters from radiation-induced damages as represented in (Table 1).
Table 1: *In vivo* radioprotective activity of *M. indica* using comet assay

<table>
<thead>
<tr>
<th>Groups</th>
<th>Tail length</th>
<th>%T</th>
<th>OTM</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>7.3±1.61</td>
<td>0.60±0.066</td>
<td>0.52±0.181</td>
</tr>
<tr>
<td>Irradiated control</td>
<td>234.20±1.40</td>
<td>39.62±1.64</td>
<td>16.623±1.639</td>
</tr>
<tr>
<td><em>M. indica</em>100mg/kg</td>
<td>154.10±1.44</td>
<td>22.20±1.09</td>
<td>10.82±1.15</td>
</tr>
<tr>
<td><em>M. indica</em>200mg/kg</td>
<td>125.75±0.91*</td>
<td>17.81±1.40**</td>
<td>10.19±0.94**</td>
</tr>
<tr>
<td><em>M. indica</em>400mg/kg</td>
<td>85.62±1.42***</td>
<td>13.02±1.98***</td>
<td>9.16±0.55***</td>
</tr>
</tbody>
</table>

Values are reported as mean ± S.E.M. for group of six animals. The data was analyzed by Prism 5 software. Asterisks indicated statistically significant values when compared to radiation. *p<0.05, **p<0.01, ***p<0.001

**Radiation induced inflammation**

**MDA assay**

Exposure of animals to EB radiation dramatically increased MDA level in the irradiated mice group as represented in Fig 2. Pre-treatments with different dose of *M. indica* significantly decreased the MDA level and comparatively 400mg/kg body weight treated groups showed maximum activity. Rise in MDA in irradiated group could be due to increased generation of ROS due to the excessive oxidative damage.[13]

![Fig. 2: Effect of *M. indica* extract and radiation on MDA level](image)

**Nitric oxide estimation**

EB radiation caused a marked increase in the concentration of NO (Fig 3), compared with the control group. Oral intake of *M. indica* suppressed the increase in NO in a dose-dependent manner when compare to irradiated group.
Sialic acid test

Serum sialic acid concentration significantly increased in irradiated mice on EB radiation. So treatment with *M. indica* extract at 100mg, 200mg and 400mg/kg body weight concentrations showed significant reduction in the levels of sialic acid (Fig. 4).

Histopathology of Skin

Histopathology of skin (Fig. 5) in irradiated mice (Fig. 5B) showed apoptosis of epithelial cells in epidermis, condensations of cytoplasm with pyknotic nuclei and also increases in the number of keratinocytes cells, when compared to normal mice (Fig. 5A). Treatment with *M. indica* suppressed apoptosis of epithelial cells, and decreased the number of inflammatory cells.
DISCUSSION

The present study indicates that the extract of *M. indica* has significant radioprotective and anti-inflammatory activity against EB radiation. Radiation during radiotherapy produces free radicals such as hydrogen peroxide, superoxide radicals, and hydroxyl radicals; as sensitive molecules in cells are directly damaged due to interactions between radiation and water molecules in cells leading to production of ROS,[14] which damages DNA of the normal cells surrounding cancerous tissue. ROS are not only involved in damaging DNA, but also in the promotion of inflammatory processes.[15]

*In vitro* protective effect of *M. indica* was performed using pBR322 plasmid DNA. There was substantial amount of super coiling of DNA in presence of extract which was measured based on intensity of band. Among different concentrations of extract, 150μg dose showed good protection by inhibiting the formation of open circular form of DNA. Suggesting possible involvement of some endogenous cellular components involved in DNA protection.

The alkaline comet assay is an elegant and effective technique to monitor the extent of DNA damage and its protection. When mice blood lymphocytes were exposed to EB radiation, the cellular DNA undergoes damage, as reflected in the increase of the comet parameters. The presence of extracts of *M. indica* during irradiation of cells decreases the comet parameters, indicating its significant role in protection. One of the deleterious consequences of DNA damage from exposure to ionizing radiation is the induction of cancer. Protecting cellular DNA from radiation damage might result in the prevention of the cancer induced by radiation.
Radiation exposure of mice results in over production of inflammatory mediators such as prostaglandins, bradykinin, NO, cytokines, sialic acid, MDA and CRP, leading to cytotoxicity and tissue damage.[16] MDA a reactive aldehyde caused by toxic stress in cells and form covalent protein, the inflammatory effect would result in the accumulation of MDA.[17] In this study the concentration of MDA increased drastically in liver homogenate upon radiation. Rise in MDA could be due to increased generation of ROS and due to the excessive oxidative damage generated in liver homogenate of mice. Pretreatment of M. indica significantly inhibited production of MDA at different concentrations, but when compared to other lower dose 400mg/kg body weight showed significant inhibition as plotted in Fig 2. This observation suggests that EB radiation at 6Gy induce inflammation as indicated by increase in MDA level. Similar observations of elevated MDA levels have been reported in rats with gamma radiation.[18] In contrast to our study, Kajanachumpol et al. reported no significant change in MDA levels in rats expose to microwave radiation compared to controls.[19]

NO another important mediator for host defense and inflammatory response which on inflammation by radiation produces excessive amount of NO formation by generating peroxy nitrite which has strong injurious effect during inflammation process.[20] These results suggest that increase in NO is mainly due to increase in nitrate concentrations generated by nitric oxide synthase by reacting with radical oxygen.[21] Hence inhibition of NO by M. indica may prove extracts anti-inflammatory activity.

Significant increase in the serum sialic acid levels was observed in mice irradiated parallel to inflammation. The increase in levels of sialic acid might be considered as a defense molecule against the increased oxidative stress. Antioxidant property of sialic acid as a H2O2 scavenger has been reported by Tanaka et al.[22] It is well documented that serum sialic acid concentrations rapidly increase following the onset of inflammatory disease or injury. This result suggests that increase in sialic acid during irradiation is due to elevated release of globulins in the first inflammatory reaction.[23] There by suppression of activity of sialic acid by M. indica extracts at 400mg/kg body weight inhibited the release of globulins thus exhibiting anti-inflammatory activity. Therefore, measure of increase in serum sialic acid concentrations may be a good indicator of inflammatory process.

Inflammation to skin due to radiation can result in epithelial cell death via apoptosis.[24, 25] Death of these cells is ubiquitous in inflammatory diseases.[26] On irradiation there was over
production of keratin due to hyperkeratosis and death of epithelial cells with pyknotic nuclear formation when compared to control. Pretreatment with *M. indica* protected epidermis by reducing number of apoptotic cell formation and by decreasing number of inflammatory cells. Based on such experimental evidence, it may be deduced that *M. indica* might render significant relief from the side effects of both chemotherapy and radiotherapy.

**CONCLUSION**

In conclusion, these results indicated that *M. indica* extract exerted radioprotective and anti-inflammatory activity dose dependently against EB radiation. The radioprotective activity of *M. indica* has proven by protecting DNA both *in vivo* and *in vitro*. The anti-inflammatory mechanism of *M. indica* might be related to the superior protective effect against the free radicals generated in the liver. Therefore, it may be suggested that the anti-inflammatory effect of *M. indica* might occur via inhibition of MDA NO and sialic acid production which may be due to increase in the activities of antioxidant enzymes.

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