In vivo radioprotective effect of Moringa oleifera leaves

Anoop V Rao\textsuperscript{a}, P Uma Devi\textsuperscript{b} & R Kamath\textsuperscript{b}

\textsuperscript{a}Kasturba Medical College, Mangalore, \textsuperscript{b}Department of Radiobiology, Kasturba Medical College, Manipal 576119, India

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Radioprotective property of Moringa oleifera leaves was investigated in healthy adult Swiss albino mice. Animals were injected (ip) with 150 mg/kg body weight of 50\% methanolic extract (ME) of \textit{M. oleifera} leaves, as a single dose, or in 5 daily fractions of 30 mg/kg each, and exposed to whole body gamma irradiation (RT, 4 Gy) 1 hr later. Five animals from each group were sacrificed at 1, 2 and 7 days after treatment. Bone marrow protection was studied by scoring aberrations in metaphase chromosomes and micronucleus induction in polychromatic erythrocytes and normochromatic erythrocytes. Pretreatment with a single dose of 150 mg/kg ME significantly reduced the percent aberrant cells to 2/3rd that of RT alone group on day 1 and brought the values to normal range by day 7 post-irradiation. A similar effect was also seen for the micronucleated cells. Fractionated administration of ME (30 mg/kg x 5) gave a higher protection than that given by the same dose administered as a single treatment. ME also inhibited the Fenton reaction-generated free radical activity \textit{in vitro} in a concentration dependent manner. These results demonstrate that pretreatment with the methanolic leaf extract of \textit{M. oleifera} confers significant radiation protection to the bone marrow chromosomes in mice and this may lead to the higher 30 day survival after lethal whole body irradiation.

A major problem associated with cancer radiotherapy is the severe side effects resulting from normal tissue damage. Consequently, agents which protect normal tissues against radiation damage can increase the patient tolerance to radiotherapy. Several chemicals have been found to provide good radiation protection in experimental animals, but their clinical utility is limited by the drug toxicity on repeated administration. The only drug approved for clinical use in cancer therapy patients is amifostine, a synthetic phosphorothioate compound, which also produces side effects of its own, like nausea, vomiting and hypotension. Moreover, amifostine is very expensive. Therefore, there is a need to find nontoxic and inexpensive drugs for clinical radiation protection. Recent studies have indicated that some of the commonly used medicinal plants may be good sources of potent but nontoxic radioprotectors. But research on the radioprotective property of plant products has not received the attention it deserves.

\textit{Moringa oleifera} (family: Moringaceae, English: Horseradish-tree, Drumstick-tree, Sanskrit: Shigru) has been an ingredient of Indian diet since several centuries. Its constituents have been shown to possess antitumour, hypotensive, antifungal, antispasmodic and anti-inflammatory activities. Its antioxidant property and possible anticarcinogenic action have also been investigated. However, no study has been reported on its radioprotective effect. The present investigation was undertaken to study the radioprotective property, if any, of \textit{M. oleifera} leaves.

Materials and Methods

\textit{Preparation of extract}—Fresh leaves were collected locally during the month of July and shade dried for 2 days. About 200 g of powder was obtained from 4 kg of leaves. Fifty percent methanolic extract of dried leaves (ME) was prepared in a Soxhlet apparatus, and concentrated under vacuum using a Speedvac System (SC110A, Savant, USA). The extract was dissolved in double distilled water (DDW, pyrogen free) freshly before injection. This solution had a pH of 8.0.

\textit{Animals}—Six to eight weeks old Swiss albino mice of both sexes, weighing 25-30 g, bred and maintained under standard conditions of light (14 hr dark; 10 hr light), temperature (23\(^\circ\)±2°C) and humidity (50-60\%) in the animal house of our department, were used for the experiments. The animals were fed on standard mouse feed (composition given by the Cancer Research Institute, Bombay) and acidified water \textit{ad libitum}.
Irradiation—The source of radiation was a $^{60}$Co Gamma teletherapy unit (Siemens, Germany) in the Department of Radiotherapy and Oncology, the S.S.B Cancer Hospital, Manipal. Animals were restrained in well-ventilated perspex boxes without anaesthesia and exposed to whole body radiation at a dose rate of 1 Gy/min, in a field size of 22 x 22 cm$^2$ and at a distance of 60 cm from the source.

Experimental design

The following experiments were conducted:

Determination of acute drug toxicity—Fifty mice were deprived of food for 18 hr and 10 mice each were injected intraperitoneally (ip) with 5, 6, 7 or 9 g/kg body weight of ME. The animals were observed for acute mortality and the dose to kill 50% of animals within 72 hr (LD$_{50}$) was calculated for acute mortality and the dose to kill g/kg body weight of ME. The animals were observed 200 maximum at 4 hr after an ip injection of 150 mg/kg body wt of ME.

Radioprotection by ME—In a dose-response study, where mice were injected with 10 to 200 mg/kg body wt of ME ip before whole body gamma irradiation with a lethal dose of 11 Gy, it was found that 30 day mouse survival increased with ME dose to a maximum at 150 mg/kg, without further increase at 200 mg/kg (Table 1). Based on this experiment, 150 mg/kg body wt of ME was selected to study protection of bone marrow chromosomes.

Mice were divided into groups of 15 each and treated as follows:

1. Single dose ME treatment—Control: Mice received vehicle, DDW, ip, in volume equal to that injected with ME and were sham-irradiated after 1 hr.

2. Fractionated dose ME treatment—Control: Mice were injected ip with DDW, daily for 5 consecutive days and sham-irradiated 1 hr after the last injection.

3. ME alone: Mice were injected ip with 30 mg/kg body wt of ME for 5 consecutive days and sham-irradiated as above.

4. RT: Mice were exposed to 4 Gy, as described earlier.

5. ME+RT: Mice were injected ip with 30 mg/kg body wt of ME for 5 consecutive days and exposed to 4 Gy 1 hr after the last ME dose.

On days 1, 2 and 7 after irradiation/sham irradiation, 5 animals from each group were taken to study the cytogenetic damage in the bone marrow cells, using one femur for chromosome analysis and the other femur for micronucleus count from each animal.

Chromosomal aberrations—The mice were injected ip with 0.025 % colchicine (Sigma, USA) and left for 2 hr to arrest the cells in metaphase. Then the animals were sacrificed by cervical dislocation, femurs were dissected out and cleaned to remove adherent muscles. Metaphase spreads were prepared by the air drying method, as described earlier.

Briefly, the bone marrow cells were flushed out, treated with hypotonic saline, fixed in methanol: acetic acid, stained with 4% Giemsa (Sigma Chemical Co., USA) and observed under a light microscope (Reichert, USA). A total of 500 metaphase spreads were scored per animal and the number of aberrant cells were counted and expressed as percentage of total metaphases scored. The individual aberrations, namely chromosome and chromatid breaks, fragments, rings and dicentrics as well as cells with polyploidy, pulverization and severely damaged cells (SDC, i.e. cells containing 10 or more aberrations) were also scored.

Micronucleus induction—The bone marrow was flushed out using Eagle’s minimum essential medium (MEM) and the slides were prepared by the method of Schmid. Bone marrow was centrifuged and the

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Post treatment time (days)</th>
<th>1</th>
<th>2</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: ME, single dose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DDW</td>
<td>0.6 ± 0.4</td>
<td>0.7 ± 0.1</td>
<td>0.6 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>ME</td>
<td>4.1 ± 0.6</td>
<td>1.5 ± 0.3</td>
<td>1.1 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>RT</td>
<td>55.7 ± 2.7$^{a}$</td>
<td>6.4 ± 0.7$^{a}$</td>
<td>3.1 ± 0.3$^{a}$</td>
<td></td>
</tr>
<tr>
<td>ME + RT</td>
<td>37.1 ± 1.8$^{a,b}$</td>
<td>4.4 ± 0.6$^{a}$</td>
<td>2.1 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>B: ME, fractionated dose (30 mg/kg x 5 days)</td>
<td>0.6 ± 0.3</td>
<td>0.6 ± 0.2</td>
<td>0.6 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>DDW</td>
<td>2.9 ± 0.6</td>
<td>1.4 ± 0.3</td>
<td>1.2 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>ME</td>
<td>53.9 ± 2.8$^{a}$</td>
<td>6.0 ± 0.7$^{a}$</td>
<td>2.6 ± 0.5$^{a}$</td>
<td></td>
</tr>
<tr>
<td>ME + RT</td>
<td>32.8 ± 1.2$^{a,b}$</td>
<td>3.4 ± 0.6$^{a}$</td>
<td>1.9 ± 0.5</td>
<td></td>
</tr>
</tbody>
</table>

P values: $^{a,b}$<0.01; $^{a}$<0.05 compared to DDW
$^{a,b}$<0.01; $^{a}$<0.05 compared to ME
$^{a,b}$<0.001 compared to RT
pellet was resuspended in 1 ml of fetal calf serum. Smears were prepared on clean glass slides, stained with acridine orange and observed under a fluorescent microscope (PHM III, Zeiss, Germany) for micronuclei in polychromatic erythrocytes (PCE) and normochromic erythrocytes (NCE). A total of 2000 erythrocytes were scored in each animal. The number of PCE and NCE and the frequency of micronucleated PCE (MPCE) and micronucleated NCE (MNCE) were recorded. The data are expressed as the number of MPCE or MNCE per 1000 PCE or NCE, respectively. The ratio of PCE to NCE (P/N ratio) was also calculated.

Antioxidant activity—*M. oleifera* is reported to contain antioxidants such as vitamin C\(^1\). We, therefore, examined the free radical scavenging activity of ME at concentrations of 10, 50, 100 and 150 mg/dl, using the Fenton reaction induced degradation of deoxyribose in vitro. Fenton reaction in the Fe\(^{3+}\)-EDTA-ascorbate-\(H_2O_2\) reaction mixture produces hydroxyl radicals, which attack deoxyribose and set off a series of reactions that eventually result in the formation of thiobarbituric acid reactive substances (TBARS). When a molecule scavenges the OH radicals, it decreases the TBARS formation. TBARS activity was measured using a spectrophotometer (VIS-260, Shimadzu, Japan)\(^{19,20}\).

**Results**

**Acute toxicity**—No mortality was seen up to 6 g/kg of ME. But 20% animals died at 7 g/kg and 100% mortality was recorded at 9 g/kg. LD\(_{50}\) was calculated to be 7.42 g/kg body wt.

**Chromosomal Aberrations**

**Single dose ME treatment**—Sham-treated control showed <1% aberrant metaphases. ME alone did not

**Table 2**—Different types of aberration in the bone marrow of mice on day 1 after treatment with ME and radiation (RT, 4 Gy)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Chromatid breaks</th>
<th>Chromosome breaks</th>
<th>Fragments</th>
<th>Rings</th>
<th>Dicentrics</th>
<th>Polyploids</th>
<th>SDC</th>
<th>Pulverization</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDW</td>
<td>0.16 ± 0.01</td>
<td>0.04 ± 0.01</td>
<td>0.52 ± 0.01</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>ME</td>
<td>1.24 ± 0.08</td>
<td>0.76 ± 0.43</td>
<td>2.84 ± 0.29</td>
<td>0.16 ± 0.29</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>RT</td>
<td>10.08 ± 3.05(a)</td>
<td>9.90 ± 2.95(a)</td>
<td>34.56 ± 4.83(a)</td>
<td>2.24 ± 0.48(a)</td>
<td>1.68 ± 0.80</td>
<td>2.48 ± 0.80</td>
<td>4.80 ± 1.90</td>
<td>5.36 ± 1.88</td>
</tr>
<tr>
<td>ME + RT</td>
<td>5.88 ± 1.60</td>
<td>5.68 ± 1.50</td>
<td>21.80 ± 4.36(a)</td>
<td>1.64 ± 0.68</td>
<td>1.72 ± 0.05</td>
<td>1.76 ± 0.60</td>
<td>2.32 ± 1.02</td>
<td>1.80 ± 1.12(a)</td>
</tr>
</tbody>
</table>

**Table 3**—MPCE/1000 PCE in the bone marrow of mice treated with *M. oleifera* extract (ME) before whole body irradiation (RT, 4 Gy)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Post-treatment time (days)</th>
<th>1</th>
<th>2</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDW</td>
<td></td>
<td>2.3±0.1</td>
<td>2.1±0.1</td>
<td>2.4±0.2</td>
</tr>
<tr>
<td>ME</td>
<td></td>
<td>5.0±0.2(a)</td>
<td>4.1±0.2</td>
<td>2.7±0.2</td>
</tr>
<tr>
<td>RT</td>
<td></td>
<td>127.9±1.4(a)</td>
<td>14.2±0.7(a)</td>
<td>5.6±2.0(a)</td>
</tr>
<tr>
<td>ME + RT</td>
<td></td>
<td>83.9±1.5(a)</td>
<td>5.7±0.2(a)</td>
<td>3.1±0.2(a)</td>
</tr>
</tbody>
</table>

**P values:** \(\leq 0.05\), \(\leq 0.01\), \(\leq 0.001\) compared to DDW

On day 1, the difference between single and fractionated ME + RT is highly significant, \(P<0.001\).
induce any significant changes. On day 1, RT alone produced a very significant (P<0.001) increase in the percent aberrant cells to 55% (Table 2). A corresponding increase was observed in all the individual aberrations, including chromosome and chromatid breaks, fragments, rings, dicentrics, and in the SDC and cells with pulverization (Table 3). When treated with ME before RT, the percent aberrant metaphases dropped to 37%, which was very significant (P<0.001) compared to the RT alone group (Table 2). A significant decrease was also seen in the individual aberration types (Table 3).

On day 2 and 7 the percent aberrant metaphases in the DDW control remained similar to that on day 1. In the ME alone group, the values were not significantly different from those of control. In the RT alone group, the percent aberrant cells showed a steep decline from day 2, but remained significantly (P<0.01) above the control value on day 7. The mice treated with ME before irradiation also showed a marked reduction in the percent aberrant metaphases on day 2, which further decreased with time and were non-significant from control on day 7 (Table 2).

Fractionated dose ME treatment—Treatment with 30 mg/kg for 5 days before irradiation produced a higher reduction in the percent aberrant metaphases than the single dose treatment, but the difference was only marginally significant (P=0.08) on day 1 (Table 2). Only the fragments showed a higher reduction in the fractionated ME dose group when compared to the single dose ME group on day 1 (Table 3). The aberrant metaphases further declined sharply on day 2 (3%) and reached the control range on day 7 (Table 2).

Micronucleus induction

Single dose ME treatment—MPCE: Changes in the micronucleus frequency corresponded to that of chromosomal aberration data. DDW control showed between 2 and 2.5 MPCE per 1000 cells on days 1, 2 and 7. ME alone significantly increased the MPCE on day 1 (P<0.05), which dropped to the control value by day 7. RT resulted in a highly significant (P<0.001) increase in the frequency of MPCE compared to control on day 1. The value declined with time after irradiation, but remained significantly above control level on day 7 (P<0.001). Administration of 150 mg/kg ME before RT decreased the frequency of MPCE significantly (P<0.001) on day 1, which further decreased with time and was nonsignificant from control on day 7 (Table 4).

MNCE: ME treatment alone did not have any significant effect on the MNCE. Radiation produced a highly significant (P<0.001) increase in their value from control on day 1 post irradiation, which decreased with time, but remained significantly above those in the DDW- and ME-alone treated groups on
day 7 \((P<0.001)\). ME treatment before irradiation resulted in a highly significant decrease in the MNCE from that of the RT group on day 1 and the normal value was restored by day 7 (Table 5).

P/N ratio: The DDW control group showed a P/N ratio of $$>1$$ on all the days of observation. ME alone did not have any effect on this parameter. RT significantly \((P<0.001)\) reduced the P/N ratio on day 1. It increased from day 2 onwards, but remained significantly below the control value on day 7 \((P<0.01)\). Administration of ME before RT resulted in a significant increase in the P/N ratio on day 1 \((P<0.001)\) compared to RT alone group and the control value was achieved by day 7 (Table 5).

**Fractionated dose ME treatment**—Pretreatment with 30 mg/kg ME for 5 days before RT produced a higher reduction in the radiation induced MPCE and MNCE than in the mice treated with single a dose of ME on all days of observation (Tables 4 and 5); this difference was statistically significant on day 1 post irradiation \((P<0.001)\). Similarly, there was a higher increase in the P/N ratio \((P<0.001)\) in this group when compared to single dose ME treated animals (Table 5).

**Antioxidant property**—The TBARS inhibition by ME was concentration-dependent and increased from 8% at 10 mg/dl to 28% at 150 mg/dl (Fig. 1).

**Discussion**

This is the first study that demonstrates a radio-protective property of the *M. oleifera* leaf extract. The data clearly show that a single dose of ME before whole body irradiation can significantly decrease the radiation induced chromosomal damage. Protection was obtained at an optimum dose of 150 mg/kg body wt, which is about 1/50th its LD$_{50}$ (7.42 g/kg). Administration of the same dose of ME in 5 equal fractions further enhanced the bone marrow protection, as indicated by the significant reduction in the micronucleated erythrocytes and chromosome fragments at 24 hr after irradiation compared to single dose ME treatment. This is similar to the earlier findings with the leaf extract of the medicinal plant *Ocimum sanctum*, which also gave a higher protection against radiation lethality in mice when the optimum protective dose was given in 5 fractions rather than as a single dose$^5$. If this similarity reflects similar mechanisms of action needs to be studied.

The number of PCEs in relation to NCEs is an index of the rate of proliferation. The decrease in P/N ratio at 24 hr after irradiation may reflect the early effects of radiation on cell cycle leading to mitotic inhibition, as also observed by other investigators$^{21}$. The significant increase in the 24hr P/N ratio by pretreatment with ME indicates that the extract reduces the radiation induced cell cycle effect. The restoration of normal P/N ratio by 7 days post-irradiation in the extract pretreated animals suggests an acceleration of the regenerative process by ME.

The radioprotective effect of several natural products has been associated with their antioxidant property$^{20,22}$. Our study has also shown that ME possesses antioxidant activity against hydroxyl radicals generated by Fenton reaction in *vitro*. This may have a role in the protective effect of ME against radiation clastogenicity, evident in the reduced chromosomal aberrations and micronuclei frequency in the bone marrow cells. Chromosome protection of the hemopoietic cells will result in higher stem cell survival and regeneration, leading to the increased 30 day survival of mice receiving ME treatment before whole body lethal irradiation.

Thus, the present study demonstrates that low nontoxic doses of an extract of the leaves of *M. oleifera* protect bone marrow chromosomes and increase survival of mice exposed to whole body gamma irradiation and also possess antioxidant activity in *vitro*. *Moringa* leaves and ponds have been
reported to contain the antioxidant vitamin C\textsuperscript{18}, which may be responsible for the antioxidant and radioprotective properties of the extract. As \textit{Moringa} leaves are used as a dietary vegetable and is freely available in our country, it is worthwhile to conduct detailed studies in order to explore the full potential of this plant in human radiation protection.

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References