Protection against Radiation Induced Testicular Damage in Swiss Albino Mice by *Mentha piperita* (Linn)

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**Abstract.** *Mentha piperita* Linn or peppermint (Family – Labiatae) is aromatic and has stimulant and carminative properties. The protective effects of *Mentha piperita* (Linn) extract against radiation induced damage in testis of Swiss albino mice have been studied. Animals (Male Swiss albino mice) were given leaf extract of *M. piperita* orally (1 g kg⁻¹ day⁻¹) for three consecutive days prior to radiation exposure (8 Gy gamma radiation). Mice were autopsied at 1, 3, 7, 14 and 30 days of post– irradiation to evaluate the radiomodulatory effect in terms of histological alterations, lipid peroxidation, acid and alkaline phosphatases levels in testis. There was significantly less degree of damage to testis tissue architecture and various cell populations including spermatogonia, spermatids and Leydig cells. Significant decreases in the LPO and acid phosphatase level and increase in level of alkaline phosphatase were observed in testis. The methanolic extract of *M. piperita* showed high amount of phenolic content, flavonoids content and flavonols. Leaf extract of *M. piperita* has significant radioprotective effect and the amount of phenolic compounds, flavonoids and flavonols content of extract of *M. piperita* may be held responsible for its radioprotective effect.

**KEYWORDS:** Radiation protection; *Mentha piperita* Linn; lipid peroxidation; acid and alkaline phosphatases; testis.

1. Introduction

*Mentha piperita* Linn or peppermint (Family – Labiatae) is aromatic and has stimulant and carminative properties [1]. Earlier we have reported that leaf extract of *M. piperita* provide protection against radiation-induced alterations (reduction in villus height, mucosal, total cells and mitotic figures/crypt section) in intestinal mucosa of mice [2]. The radioprotective effect of leaf extract of *M. piperita* was also demonstrated. On the basis of survival percentage the LD₉₀/₅₀ values were calculated for control animals and *M. piperita* treated animals were 6.48 Gy and 11.59 Gy, therefore, 8 Gy radiation dose was selected for further investigation [3]. Pre-treatment of leaf extract of *M. piperita* protects the hematological constituents and modulates values of serum acid and alkaline phosphatases activities in Swiss albino mice against gamma irradiation [3]. Oral administration of leaf extract of *M. piperita* prior to radiation exposure was also found to be effective against chromosomal damage in bone marrow in Swiss albino mice [4]. Peppermint oil has also been found to protect the hematological constituents in peripheral blood in mice against gamma irradiation [5]. Recently, the protective effects of leaf extract of *M. piperita* against radiation induced hematopoietic damage in bone marrow of Swiss albino mice has been reported [6]. The radioprotective and antioxidant activity of leaf extract of *M. piperita* was assigned to different chemical constituents present in plant extracts [7]. Present study has been undertaken to evaluate the protective effects of leaf extract of *M. piperita* against radiation induced damage in testis of Swiss albino mice, also the leaf extract of *M. piperita* was further evaluated to determine the amount of phenolic compounds, flavonoids and flavonols.

2. Materials and Methods

2.1 Animals- Adult male Swiss albino mice (*Mus musculus*, 6-8 weeks old, weighing 25±2 g) maintained in the animal house as an inbred colony (procured from Hamdard University, Delhi) were used for the present study. These animals were maintained at a temperature of 24 ± 3°C and housed in polypropylene cages, as per norms laid down by a Departmental Ethical Committee. After weaning at three weeks of age, the animals were fed standard mouse feed (Hindustan Lever, Delhi, India), and provided tap water *ad libitum*.

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2.2 Irradiation- The Cobalt Teletherapy Unit (ATC-C9) at cancer treatment centre, Radiotherapy Department, SMS Medical College and Hospital, Jaipur was used for irradiation. Unanaesthetised mice restrained in well-ventilated Perspex boxes were whole-body exposed to gamma radiation (8 Gy) at the distance (SSD) of 77.5 cm from the source to deliver the dose-rate of 1.59 Gy/min [3].

2.3 Mentha extract (ME) - Mentha piperita Linn. (Fig. 1), Plant material was collected locally, identified and a specimen was deposited at the Herbarium, Department of Botany, University of Rajasthan, Jaipur (Voucher number-RUBL-19443, Fig. 1). Freshly collected leaves were air dried, powdered and extracted with double distilled water (DDW) by refluxing for 36 hr (12 hr x 3) at 80°C as described previously [3].

Figure 1: Mentha piperita Linn.

2.4 Experimental Design - Mice selected from inbred colony were divided into four groups. Animals of Group-I were administered DDW for three consecutive days to serve as normal, while Group-II received leaf extract of M. piperita orally (1 g/kg b.wt./day) for three consecutive days. Animals of Group-III received DDW (volume equal to leaf extract of M. piperita) to serve as control whereas animals of Group-IV were administered leaf extract of M. piperita orally (1 g/kg b.wt./day) for three consecutive days to serve as experimental. After 30 min of treatments on 3rd day, animals of Group-III and IV were exposed to 8.0 Gy gamma radiations. At least six animals from each group were autopsied at 1, 3, 7, 14 and 30 days of post - treatments.

2.4.1 Histopathology of Testis
Following various treatments, animals were autopsied by cervical dislocation on day 1, 3, 7, 14 and 30. Testis were surgically removed, weighed, and fixed in Bouin’s fluid. The tissue was embedded in a paraffin block after dehydrating with increasing concentrations of 70%, 90% and 100% ethanol. 5 μM sections were cut using hand microtomy, were placed on glass slide, and were stained with Harris hematoxylin and eosin. Stained tissue sections were observed under light microscope (Olympus) to determine histopathological changes.
2.4.2 Biochemical Studies

Lipid Peroxidation (LPO) Assay: Lipid peroxidation level in testis and was estimated by the method of Ohkawa et al. [8] as thiobarbituric acid reactive substances (TBARS). The testis were dissected out and chilled in ice cold 0.09% NaCl. Homogenate of desired tissues were prepared in 1.15% KCl (1 gm tissue in 9 mL of 1.15 % KCl). Sodium dodecyl sulfate (8.1%; 0.2 mL) was added 0.2 mL of sample in test tubes, and pH was adjusted to 3.5 with NaOH. To this, 1.5 mL of 0.8% aqueous solution of TBA was added. The mixture was made up to 4 mL with distilled water and heated at 95 °C for 60 min. After cooling under tap water, 1 mL of distilled water, and 5 mL of mixture of n-butanol and pyridine (15:1) were added and shaken vigorously. The solution was centrifuged at 3900g for 10 minutes. The upper organic layer was taken out and absorbance was measured at 532 nm using UV-VIS Systronic spectrophotometer.

Acid and Alkaline Phosphatase Activity: Acid and Alkaline phosphatase activities in testes were estimated using the method described by Fiske and Subbarow [9]. The tissue homogenates were mixed with trichloroacetic acid and then, centrifuged at 3900g for 10 minutes. The supernatant was then treated with molybdate solution. This resulted in the formation of phosphomolybdic acid from the phosphate present in the tissue. The phosphomolybdic acid was then reduced by 1-anilino-8 naphthalenesulfonic acid (ANSA) to produce a blue color whose intensity was proportional to the amount of phosphate liberated. The alkaline phosphatase activity is the difference between inorganic phosphate content of the incubated and control samples expressed as Bodansky units. One Bodansky unit corresponds to the liberation of 1 mg of inorganic phosphorous from the tissue in mg/gm/hr.

Determination of the amount of phenolic compounds, flavonoids and flavonols

Phenolic compounds- The content of total phenolic compounds in plant methanolic extract was determined by Folin and Ciocalteu method [10]. For the preparation of calibration curve 1 ml aliquots of 0.024, 0.075, 0.105 and 0.3 mg/ml ethanolic gallic acid solutions was mixed with 5 ml Folin-Ciocalteu reagent (diluted 10 fold) and 4 ml (75 g/L) sodium carbonate. The absorption was read after 30 min at 20 °C at 765 nm and calibration curve was plotted. One ml methanolic plant extract (10 g/L) was mixed with the same reagent as described above, and after 1 hr the absorption was measured for the determination of plant phenolics. All determinations were performed in triplicate. Total content of phenolic compounds in plant methanol extract in gallic acid equivalents (GAE) was calculated by the following formula:

\[ C = c.V/m' \]

Where, C- total content of phenolic compounds, mg/g plant extract in GAE; c- the concentration of gallic acid established from the calibration curve mg/ml; V- the volume of extract, ml; m'- the weight of pure plant methanolic extract, g.

Flavonoids- The content of flavonoids was determined by a pharmacopeia method [11] using rutin as a reference compound. One ml of plant extract in methanol (10 g/L) was mixed with 1 ml aluminum trichloride in ethanol (20 g/L) and diluted with ethanol to 25 ml. The absorption at 415 nm was read after 40 min at 20 °C. Blank samples was prepared from 1 ml plant extract and 1 drop acetic acid, and diluted to 25 ml, the absorption of rutin solutions was measured under the same conditions. Standard rutin solution was prepared from 0.05 g rutin. All determinations were carried out in triplicate. The amount of flavonoids in plant extract in rutin equivalents (RE) was calculated by the following formula:

\[ X = \frac{(A . m . 10)}{(A_0 . m)} \]

Where, X- flavonoid content, mg/g plant extract in RE; A- the absorption of plant extract solution; A_0- the absorption of standard rutin solution; m- the weight of plant extract, g; m- the weight of rutin in the solution, g.
**Flavonols** - The content of flavanols was determined by Yermakov method [12]. The rutin calibration curve was constructed by mixing 2 ml of 0.5, 0.4, 0.3, 0.2, 0.166, 0.1, 0.05, 0.025, and 0.0166 mg/ml rutin ethanolic solutions with 2 ml (20 g/L) aluminum trichloride and 6 ml (50 g/L) sodium acetate. The absorption at 440 nm was read after 2.5 hr at 20 °C. The same procedure was carried out with 2 ml of plant extract (10 g/L) instead of rutin solution. All determinations were carried out in triplicate. The content of flavonols, in rutin equivalents (RE) was calculated by the following formula:

\[ X = C \times \frac{V}{m} \]

Where, \( X \) - flavonol content, mg/g plant extract in RE; \( C \) - the concentration of rutin solution, mg/ml; \( V \) - the volume of plant extract, ml; \( m \) - the weight of plant extract, g.

### 2.5 Statistical analysis

The results obtained were expressed as mean ± SE. Student’s ‘t’ test was used to make a statistical comparison between the groups. Significance levels were set at \( P < 0.05 \), \( P < 0.005 \) and \( P < 0.001 \).

### 3 Results

#### 3.1 Histopathological Changes in Testis

Animals treated with leaf extract of *M. piperita* alone, showed no significant change in weight of the testis on day 1, 3, 7 and 14 [Table 1]. Tubules showed all stages of normal germ cells with active spermatogenesis. Animals treated with radiation alone 8 Gy (Group III) showed reduction in the testis weight during all days of observation. Whereas, in leaf extract of *M. piperita*-pretreated irradiated group (Group IV: *Mentha piperita* + radiation), there was a significant increase in testis weight after day 1, 3, 7, 14, and 30 compared to irradiated animals [Table 1].

In irradiated animals, there was a drastic depletion of spermatogonial population with necrotic and pyknotic nuclei were observed [Figure 2C] when compared to animals treated with distilled water [Figure 2A]. The germinal epithelium was highly disorganized with shrinkage of tubules and cytoplasmic vacuolization [Figure 2D]. Total absence of sperm and spermatids were observed. Sertoli cells and Leydig cells showed shrinkage in their size. While in animals pretreated with leaf extract of *M. piperita*, less damage to spermatogonial population and germinal epithelium was observed with more rapid recovery [Figure 2E]. In animals pretreated with leaf extract of *M. piperita* and exposed to radiation, the quality (intact germinal epithelium, no pyknosis, necrosis, karyolysis present, less cytoplasmic vacuolization) and number of germ cells increased by day 30, the histology of testis revealed near normal histoarchitecture and lumen with full of sperms [Figure 2F].

**Table 1:** Effect of *Mentha piperita* on variation in testis weight of Swiss albino mice.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Testis Weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
</tr>
<tr>
<td>Group I</td>
<td>73.58±0.56</td>
</tr>
<tr>
<td>Group II</td>
<td>74.20±1.48</td>
</tr>
<tr>
<td>Group III</td>
<td>70.42±1.22*</td>
</tr>
<tr>
<td>Group IV</td>
<td>72.44±2.00</td>
</tr>
</tbody>
</table>

Group I, DDW alone; Group II, *Mentha* extract alone; Group III, DDW + radiation; Group IV, *Mentha* extract + radiation.

Significance levels - *P < 0.05, **P < 0.01, ***P < 0.001

Statistical comparison - Group II v/s Group I; Group III v/s Group I; Group IV v/s Group III
Figure 2: Protective effect of *Mentha piperita* on radiation-induced histological changes in testis of mice

(A) Normal Testis showing compact and organized arrangement of spermatogenic cells. (B) Testis had its normal structure in *Mentha* alone treated mice. (C) Exposure to irradiation at day 1: distortion of germinal epithelium and depletion of spermatogonia were evident. (D) More shrinkage of tubules with cytoplasmic vacuolization was observed at day 7. (E) In *Mentha*-pretreated irradiated animals, less damage with increased spermatogonial population was observed at day 1. (F) In *Mentha*-pretreated irradiated animals, there was an increase in tubular diameter with reduced interstitium and testis had its normal structure at day 30.

3.2 Biochemical Changes

**Lipid Peroxidation Level in Testis:** The LPO level in testis of leaf extract of *M. piperita* treated animals was insignificantly decreased on day 30 as compared to animals treated with distilled water. In irradiated group, a noticeable elevation of LPO was observed at all intervals from day 1 [Table 2]. In
experimental group (*M. piperita* + radiation), a significant (P<0.001) reduction in LPO level from day 1 was seen as compared to irradiated group (Radiation alone).

**Acid and Alkaline Phosphatase Levels in Testis:** The acid phosphatase activity in testis was found at normal level in animals treated with leaf extract of *M. piperita* at all autopsy intervals [Table 2]. In irradiated group, a highly significant (P<0.001) elevation in the enzyme level was observed. Maximum elevation was noticed on day 1 as compared to animals treated with distilled water. In leaf extract of *M. piperita* pre-treated irradiated animals, a significant decline in acid phosphatase activity was observed at all autopsy intervals after day 1 in comparison to irradiated animals. On day 30, maximum recovery was noticed. Alkaline phosphatase activity showed no significant changes in leaf extract of *M. piperita* treated animals as compared to animals treated with distilled water [Table 2]. In irradiated group, the alkaline phosphatase activity in testis showed remarkable and significant decline (P<0.001). Maximum decline was observed on day 7 and 14. In *M. piperita* pretreated irradiated animals, a gradual recovery in alkaline phosphatase activity was observed.

**Table 2:** Variation in Alkaline phosphatase, Acid phosphatase activity (mg/gm/hr) and Lipid peroxidation activity (n mol of MDA/mg of tissue) in the testis of Swiss albino mice in different groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Alkaline Phosphatase (mg/gm/hr)</th>
<th>Acid Phosphatase (mg/gm/hr)</th>
<th>Lipid peroxidation activity (n mol of MDA/mg of tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>5.42±0.20</td>
<td>5.34±0.16</td>
<td>5.22±0.16</td>
</tr>
<tr>
<td>Group II</td>
<td>5.53±0.18</td>
<td>5.43±0.18</td>
<td>5.38±0.19</td>
</tr>
<tr>
<td>Group III</td>
<td>2.68±0.12***</td>
<td>2.81±0.14***</td>
<td>1.84±0.11***</td>
</tr>
<tr>
<td>Group IV</td>
<td>3.64±0.22***</td>
<td>3.64±0.12*</td>
<td>4.28±0.24***</td>
</tr>
</tbody>
</table>

Group I, DDW alone; Group II, *Mentha* extract alone; Group III, DDW + radiation; Group IV, *Mentha* extract + radiation.

Significance levels - *P<0.05, **P<0.01, ***P<0.001; Statistical comparison - Group II v/s Group I; Group III v/s Group I; Group IV v/s Group III

**The amount of phenolic compounds, flavonoids and flavonols**

In the present investigation, the content of phenolic compounds (mg/g) in methanolic extract of *M. piperita* was determined and expressed in gallic acid equivalents (GAE). The methanolic extract of *M. piperita* showed high amount of phenolic content (32.88±1.2). The total content of flavonoids (mg/g) in methanolic extract of *M. piperita* was also determined and expressed in rutin equivalents (RE). The methanolic extract of *M. piperita* showed significant amount of flavonoids content (4.18±0.6). The content of total flavonols (mg/g) was expressed in rutin equivalents (RE). The total concentration of flavonols was found to 0.8±0.1 in methanolic extract of *M. piperita* [Table 3]. Thus, the amount of
phenolic compounds, flavonoids and flavonols was found to be well correlated with radical scavenging activity of extract of *M. piperita*.

**Table 3**: Total amount of plant phenolic compounds, flavonoids and flavonols in leaf extract of *Mentha piperita* Linn.

<table>
<thead>
<tr>
<th>Total phenolic compounds</th>
<th>32.88±1.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total flavonoids</td>
<td>4.18±0.6</td>
</tr>
<tr>
<td>Total flavonols</td>
<td>0.8±0.1</td>
</tr>
</tbody>
</table>

Total phenolic compounds= mg/g plant extract in gallic acid equivalents (GAE)
Total flavonoids= mg/g plant extract in rutin equivalents (RE)
Total flavonols= mg/g plant extract in rutin equivalents (RE)

**4. Discussion**

The interaction of radiation with the components of living system results in the generation of free radicals. Over production of such free radicals cause oxidative damage to biomolecules leading to many chronic diseases [13]. Free radicals so formed are responsible for many detrimental effects of radiation in living system. Free radicals attack virtually all components including DNA, protein and causes lipid peroxidation. They also impair the indigenous antioxidant defense mechanism [14]. Shimoi et al. [15] concluded that plant flavonoids which show antioxidant activity *in vitro* also function as antioxidants *in vivo*, and their radioprotective effect may be attributed to their radical scavenging activity. ROS generated by ionizing radiation are scavenged by radioprotectors before they can interact with biochemical molecules, thus reducing the harmful effects of radiation. The antioxidant mechanisms of radioprotection and free radical scavenging have been attributed to flavonoids, orientin and vicenin [16]. Radioprotective/antioxidative effects of various natural products have also been reported [17]. Recently, we have reported that the chemical composition and chemical constituents in plant extracts (*Adhatoda vasica, Amaranthus paniculatus, Brassica compestris, Mentha piperita* and *Spirulina fusiformis*) have significant role in displaying variation in total antioxidant activity [7].

In the present investigation, the activity of acid phosphatase was found to increase significantly in radiation treated group as compared to control. Szeinfeld and Villiers [18] also observed a marked augmentation in acid phosphatase activity in irradiated testis. Acid phosphatase has been detected in the acrosome of spermatozoa. Its presence has also been reported in the lysosome of Sertoli cells, spermatocytes and spermatids [19]. Irradiation causes lipid peroxidation of lysosomal membrane. Thus, increased activity of acid phosphatase may be attributed due to breakdown of lysosomal membrane and liberation of the enzyme. These studies are in agreement with earlier findings [20]. A significant decline in alkaline phosphatase activity was also noticed in irradiated group as compared to control. Alkaline phosphatase is known to be associated with germinal cells [21]. It has been detected in the seminiferous tubule, basement membrane and interstitial cells. It plays vital role in transport of material from Sertoli cells to various germinal cells, differentiation and proliferation of the germinal epithelium and in the testicular metabolism. Radiation depletes germ cell population. So, decrease in alkaline phosphatase activity is correlated with the state of germ cell population. Alkaline phosphatase also plays an important role in maintaining membrane permeability. Radiation damages the cell membrane, which also might be responsible for decline in alkaline phosphatase activity [22]. The results of the present study clearly document that the extracts of *Mentha piperita* significantly restore the lipid peroxidation, acid phosphatase and alkaline phosphatase in the testis of mice exposed to irradiation. Restoration of acid phosphatase level points out the role of the leaf extract of *M. piperita* in promoting the stability of cellular, nuclear and organelle membranes.

It has been reported that *M. piperita* contains antioxidants like caffeic acid, rosmarinic acid, eugenol and α-tocopherol [23-25]. Thus, radioprotective activity of leaf extract of *M. piperita* observed may be assigned to different chemical constituents present in extract. Recently, we reported that the extract of
M. piperita showed excellent percent inhibition of DPPH* activity (93.9±1.68%) and was the most effective DPPH radical scavenger, IC_{50}=272.72 \mu g/ml. The extract of M. piperita was found to be the most active as it nearly fully scavenged ABTS**. The absorption after 6 min was 0.014±0.001. Thus, M. piperita extract showed the strong radical scavenging activity [7]. In the present investigation, the methanolic extract of M. piperita showed high amount of phenolic content (32.88±1.2), significant amount of flavonoids content (4.18±0.6) and the total concentration of flavonols was found to be 0.8±0.1. The study suggests that leaf extract of M. piperita has significant radioprotective effect and the amount of phenolic compounds, flavonoids and flavonols as well as radical scavenging activity of extract of M. piperita may be held responsible for its radioprotective effect.

Acknowledgements

Senior Research Associateship (Scientist’s Pool Scheme) to Dr. R. M. Samarth from Council of Scientific & Industrial Research [CSIR], India is gratefully acknowledged.

REFERENCES


