Influence of Seed Extract of Syzygium Cumini (Jamun) on Mice Exposed to Different Doses of γ-radiation

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The radioprotective activity of the hydroalcoholic extract of jamun seeds (SCE) was studied in mice exposed to different doses of gamma radiation. The mice were injected with 0, 5, 10, 20, 40, 60, 80, 100, 120, 140 or 160 mg/kg body weight of SCE, before exposure to 10 Gy of gamma radiation, to select the optimum dose of radiation protection. The 80 mg/kg SCE was found to offer highest protection, therefore, further studies were carried out using this dose. The drug was more effective when administered through the intraperitoneal route at equimolar doses than the oral route. Since higher survival was observed for the i.p. route (50%), than the oral route (29.2%), all other studies were carried out by injecting SCE intraperitoneally. The mice treated with 80 mg/kg body weight SCE intraperitoneally before exposure to 6, 7, 8, 9, 10 and 11 Gy of gamma radiation showed reduction in the symptoms of radiation sickness and mortality at all exposure doses and caused a significant increase in the animal survival when compared with the concurrent double distilled water (DDW) + irradiation group. The SCE treatment protected mice against the gastrointestinal as well as bone marrow deaths and the DRF was found to be 1.24.

INTRODUCTION

With the realization of deleterious effects of ionizing radiation, a need was felt to protect human beings against these harmful effects by using physical and/or chemical means. The first report of use of chemicals to protect mammals against the radiation-induced damage appeared in 1949, where Patt et al.\textsuperscript{1) observed that cysteine protected mice and rats against the radiation-induced sickness and mortality. Thereafter, several chemical compounds and their analogues have been screened for their radioprotective ability. However, the practical applicability of majority of these synthetic compounds remained limited, owing to their high toxicity at their optimum protective dose.\textsuperscript{2) With increasing use of radiation for the medical diagnostic and treatment purposes it is essential to protect humans against the deleterious effects of radiation. In addition to its utility in the cancer treatment, an efficient and non-toxic radioprotector could also prove useful in occupational settings, where ionizing radiations are used (e.g., defense, airline, military and research personnel, nurses, dental assistants, radiotherapy and nuclear medicine technicians, etc.) or in accidental exposures which leave radioactivity in the environment (viz. Three Mile Island, Chernobyl and Goiania) and also during space travel to protect astronauts from the effects of high doses of radiation associated with solar flares.\textsuperscript{3) The plants have been the companion of man since time immemorial and formed the basis of several useful drugs for the treatment of various ailments. The use of plant and natural products may be beneficial in protecting against the radiation-induced damage, as they are less toxic or practically non-toxic than the synthetic compounds at their optimum protective doses.\textsuperscript{4) Therefore, screening of natural products presents a major avenue for the discovery of new radioprotective drugs. Syzgium cumini Linn. Skeels is a, medium sized to large tree, and it has been attributed to posses several medicinal properties in the folklore system of medicine.\textsuperscript{5) The bark of the plant is astringent, sweet, refrigerant, carminative, diuretic, digestive, antihelmintic, febrifuge, constipating, stomachachic and antibacterial. The fruits and seeds are used to treat diabetes, pharyngitis, splenopathy, urethrorrhea and ringworm infection. The leaves are antibacterial and used to strengthen the teeth and gums.\textsuperscript{5,6) The leaves have been extensively used to treat diabetes, constipation, leucorrhoea, stomachache, fever, gastropathy, strangury, dermopathy and to inhibit blood discharges in the faeces.\textsuperscript{5,6) Syzygium cumini has been reported to possess acetyl olean-
olic acid, triterpenoids, ellagic acid, isoquercetin, quercetin, kaempferol and myricetin\textsuperscript{5,7} in different concentrations depending on the season and climate. Most of these compounds have been reported to possess antioxidant and free radical scavenging activities.\textsuperscript{3,9}

In our earlier studies, the leaf extract of \textit{Syzygium cumini} has been reported to inhibit radiation induced micronuclei formation in the cultured human peripheral blood lymphocytes.\textsuperscript{10} However, lessons from the experience with radio-protectors world-wide are that the animal studies using death of animals as the end point are the most confirmatory. Therefore, the present study was undertaken to obtain an insight into the radioprotective effects of hydroalcoholic extracts of \textit{Syzygium cumini} in mice whole body exposed to different doses of \(\gamma\)-radiation.

**MATERIALS AND METHODS**

The animal care and handling was done according to the guidelines set by the World Health Organization, Geneva, Switzerland and the INSA (Indian National Science Academy, New Delhi, India). Eight to ten week old male Swiss albino mice weighing 30 to 36 g were selected from an inbred colony maintained under the controlled conditions of temperature (23 ± 2°C), humidity (50 ± 5%) and light (10 and 14 h of light and dark, respectively). The animals had free access to sterile food and water. Four animals were housed in a polypropylene cage containing sterile paddy husk (procured locally) as bedding throughout the experiment. The study was approved by the institutional animal ethical committee of the Kasturba Medical College, Manipal, India.

**Preparation of the extract**

The seeds of \textit{Syzygium cumini} Linn. Skeels, (or \textit{Eugenia cuminum} Linn. Druce) family Myrtaceae was collected locally during the month of June of the year, and were identified by Dr. G. K. Bhat (Department of Botany, Poorna Prajna College, Udupi, India). The plant seeds were freed of pericarp, shade dried and powdered in a mixer and the extract was prepared as described earlier.\textsuperscript{11} Briefly, one hundred grams of the seed powder was extracted with 50% ethanol at 50 to 60°C in a Soxhlet apparatus for 72 h. The liquid extract was cooled and concentrated by evaporating its liquid contents in vacuo and freeze dried. An approximate yield of 20% was obtained. The extract was stored at –70°C until further use. Henceforth, the extract of \textit{Syzygium cumini} will be called as SCE.

**Preparation of drug and mode of administration**

The required amount of SCE was dissolved in sterile double distilled water (DDW). The animals were administered with 0.01 ml/g b. wt. DDW or SCE intraperitoneally, consecutively for 5 days unless otherwise stated.\textsuperscript{4} The animals were divided into the following groups:

- **DDW + irradiation**
  - The animals of this group received DDW as described above before exposure to different doses of \(\gamma\)-irradiation.
- **SCE + irradiation**
  - This group of animals was injected with SCE intraperitoneally once daily for 5 consecutive days before exposure to radiation as described earlier.\textsuperscript{4}

**Irradiation**

One hour after the last administration of DDW or SCE the prostrate and immobilized animals (achieved by inserting cotton plugs in the restrainer) were whole-body exposed to \(\text{\textsuperscript{60}Co}\) gamma radiation (Theratron, Atomic Energy Agency, Canada) in a specially designed well-ventilated acrylic box. A batch of twelve animals was irradiated each time at a dose rate of 1.66 Gy/min at a source to animal distance (mid-point) of 70 cm. The following experiments were conducted:

**Experiment 1: Determination of acute drug toxicity of SCE**

The acute toxicity of the SCE was determined according to Prieur \textit{et al.}\textsuperscript{12} and Ghosh.\textsuperscript{13} Briefly, the animals were allowed to fast by withdrawing the food and water for 18 h. The fasted animals were divided into several groups of 10 each. Each group of animals was injected with various doses 250, 500, 750 and 1000 mg/kg body weight (b. wt.) of freshly prepared SCE intraperitoneally. The animals were provided with food and water immediately after the drug administration. Mortality of the animals was observed up to 14 days post drug treatment.

**Experiment 2: Selection of optimum SCE dose**

The animals were divided into two groups DDW + irradiation and SCE + irradiation group as described above. The former group received 0.01 ml /g b. wt. of DDW, while the SCE + irradiation group was administered with 5, 10, 20, 40, 60, 80, 100, 120, 140 and 160 mg/kg b. wt. of SCE intraperitoneally once daily for five consecutive days. One hour after the last administration on fifth day, the animals of both the groups were exposed to 10 Gy of \(\gamma\)-radiation.\textsuperscript{4} This allowed the preliminary screening and 80 mg/kg b. wt. of SCE was found to be the best radioprotective dose and therefore, further studies were carried out using this dose of SCE. Usually 24 animals were used for each drug dose in each group.

**Experiment 3: Selection of route of SCE administration**

A separate experiment was conducted to select the most effective route of administration of SCE for radioprotection, where the animals were administered orally or intraperitoneally with 80 mg/kg b. wt. of SCE once daily for five con-

secutive days before exposure to 10 Gy of $\gamma$-radiation. Twenty-four animals were used for each route of administration and a total of 72 animals were used for this experiment.

**Experiment 4: The radioprotective effect of SCE**

To ascertain the radioprotective ability of SCE the animals were divided into two groups as described above. One hour after the last administration of DDW or 80 mg/kg b. wt. of SCE on 5th day the animals were exposed to 6, 7, 8, 9, 10 or 11 Gy of $\gamma$-radiation. Usually 24 animals were used for each dose of radiation in each group.

The animals of experiment 2–4 were monitored daily for the development of symptoms of radiation sickness, and mortality up to 30 days post-irradiation. The dose reduction factor (DRF) was calculated by the method of Miller and Tainter.\(^{14}\)

\[
DRF = \frac{LD_{50} \text{ of the SCE + irradiation group}}{LD_{50} \text{ of DDW + irradiation group}}
\]

**Statistical analysis:** The statistical significance between the treatments was determined by ‘Z’ test as described earlier by Abramowitz and Stegun\(^{15}\) using the following formula:

\[
z = \frac{\hat{p}_1 - \hat{p}_2}{\sqrt{\hat{p}(1-\hat{p})(1/n_1 + 1/n_2)}}
\]

where \(\hat{p}\) = (number of successes)/total sample size

**RESULTS**

**Experiment 1: Effect of SCE on acute toxicity**

The administration of 250 to 1000 mg/kg of SCE to mice did not induce drug-related toxicity in the animals as evident by 100% survival of the treated animals. There was no change in behavior, respiration pattern and neuromuscular co-ordination.\(^{13}\) Therefore, it was concluded that SCE as such did not induce any toxic manifestations up to a dose of 1000 mg/kg b. wt. The higher doses of SCE could not be tested owing to the problems in drug dissolution and administration.

**Experiment 2: Selection of optimum SCE dose**

The animals of DDW + irradiation group exhibited signs of radiation sickness within 2–4 days after exposure to 10 Gy of $\gamma$-radiation. The irradiated animals exhibited reduction in the food and water intake, irritability, watering of eyes, lethargy, ruffling of hair, diarrhea, weight loss, emaciation and epilation. A few animals of the DDW + irradiation group showed facial edema between one and two weeks after exposure. A few animals, also exhibited paralysis and difficulty in locomotion during the second week after exposure. The first mortality in this group was observed on day 4 and all the animals died within 14 days post-irradiation (Fig. 1).

Daily administration of different doses viz. 5, 10, 20, 40, 60, 80, 100, 120, 140 and 160 mg/kg b. wt. of SCE for five consecutive days did not cause drug-induced mortality.

![Fig. 1. Kaplan Meier’s estimate of survival of mice treated with different doses of SCE (mg/kg b. wt.) before exposure to 10 Gy of whole body $\gamma$-radiation. IR = irradiation and SIR= sham-irradiation.](image)

![Fig. 2. Effect of various doses of SCE on the survival of mice exposed to 10 Gy of $\gamma$-irradiation. Error bars (95% confidence limit). Solid: 10 day and mottled: 30 day survival.](image)

![Fig. 3. Effect of 80 mg/kg of SCE administered through oral and intraperitoneal routes in the mice exposed to 10 Gy of $\gamma$-radiation. Error bars (95% confidence limit). Solid: 10 day and mottled: 30 day survival.](image)
Treatment of mice with various doses of SCE before exposure to 10 Gy delayed or reduced the symptoms of radiation sickness and delayed the onset of radiation-induced mortality depending on the drug dose. This delay was longest for 80 and 100 mg/kg SCE, where the first mortality was reported by day 8 post-irradiation (Fig. 1). The shortest delay in

Fig. 4. Kaplan-Meier estimate of survival of mice treated with 80 mg/kg b. wt. of SCE before exposure to various doses of γ-radiation. a) 7 Gy; b) 8 Gy; c) 9 Gy, d) 10 Gy, and e) 11 Gy. (squares: DDW + irradiation; circles: SCE + irradiation).
the mortality was observed for 5 mg/kg, where the first mortality occurred on day 4 post-irradiation. The administration of 40, 60, 80 and 100 mg/kg b. wt. SCE significantly reduced 10 day mortality when compared with the DDW + irradiation group. The best activity was observed for 100 mg/kg of SCE, where 87.5% (p < 0.0001) survival was observed by day 10 post-irradiation when compared with the DDW + irradiation, where only 25% animals survived (Fig. 2).

The analysis of thirty day survival revealed a drug dose dependent increase in the survival of irradiated animals up to a dose of 80 mg/kg in SCE + irradiation group, where a highest survival of 50% was observed (Fig. 2). A further increase in the drug dose to 100, 120 and 140 mg resulted in a 16.66, 25 and 41.67% reduction in the survival when compared with the 80 mg/kg SCE. The administration of 160 mg/kg SCE resulted in 8.33% survival up to 27 days post-irradiation, however 100% mortality was observed by day 30 post-irradiation (Fig. 1). A significant increase in the survival of irradiated animals was observed only after administration of 60 (p < 0.001), 80 (p < 0.0001), 100 (p < 0.0002) and 120 mg/kg SCE (p < 0.0002). Therefore, the optimum protective dose of SCE has been considered to be 80 mg/kg, where a maximum number of 50% survivors have been observed when compared with the DDW + irradiation group, where no survivors were observed after 14 days post-irradiation.

Experiment 3: Selection of route of SCE administration

The administration of 80 mg/kg SCE, once daily, consecutively for five days by oral and intraperitoneal routes before irradiation to 10 Gy protected the gastrointestinal tract (GI) of mice as evidenced by an increase in the 10 day survival. The oral administration of SCE increased the 10 day survival up to 54.2% as against 87.5% through i.p. route (Fig. 3). The analysis of thirty-day survival revealed that the radioprotective activity was better when the drug was administered through i.p. rather than the oral route, as 50% (p < 0.05) animals survived for the former after 30 days post-irradiation than the latter, where 29.2% survivors were observed (Fig. 3). Therefore, further studies were carried out by administering the drug through the i.p. route.

Experiment 4: The radioprotective effect of SCE

The radioprotective action of SCE was evaluated by administering 80 mg/kg SCE (the optimum dose), intraperitoneally once daily for five consecutive days before exposure to 6, 7, 8, 9, 10 or 11 Gy of γ-radiation. The irradiation of animals to different doses of radiation resulted in the development of symptoms of radiation sickness within 2–4 days after exposure, depending on the irradiation dose in the DDW + irradiation group. Facial edema was also observed in a few animals between one and two weeks after exposure to 9, 10 and 11 Gy. Some of the animals exposed to higher radiation doses exhibited paralysis and difficulty in locomotion during the second week after exposure. The severity of the symptoms increased with the increase in radiation dose.

The exposure of animals to different doses of radiation resulted in a dose dependent decline in the survival till a nadir was reached at 10 Gy, where no survivors were report-
ed beyond 17 days post-irradiation (Fig. 4). With the increase in the exposure dose the onset of mortality was also advanced (Fig. 4). The survival was plotted on to the log, while the exposure dose on the linear scale and the LD\textsubscript{30/30} was found to be 8.2 Gy for this group (Fig. 5b).

The LD\textsubscript{50/30} was found to be 9.6 Gy, resulting in an increase of 1.4 Gy and the dose reduction factor was found to be 1.24 Gy (Fig. 5b). Pretreatment of mice with 80 mg/kg SCE delayed or reduced the severity of radiation sickness and also delayed the onset of radiation-induced mortality when compared with the concurrent DDW + irradiation group (Fig. 5). This delay in the onset of mortality was almost by 4–8 days depending on the irradiation dose in the SCE + irradiation group when compared with the concurrent DDW + irradiation group. The GI deaths were fewer compared to DDW + irradiation group for all exposure doses (Fig. 5a). The number of survivors increased significantly in the SCE + irradiation group at 30 days post-irradiation (p < 0.002 for 9 and p < 0.001 for 10 Gy) when compared with the concurrent DDW + irradiation group (Fig. 4).

**DISCUSSION**

The animals of the DDW + irradiation group exhibited signs of radiation sickness within 2–4 days after exposure to 10 Gy which is in good agreement with earlier findings where a similar observation has been made.\textsuperscript{4} The death of remaining 25% animals between 11 to 30 days is due to hematopoetic syndrome characterized by symptoms like, irritability, epilation, weight loss, lacremation, emaciation, lethargy and ruffling of hairs.\textsuperscript{4}

Pretreatment of mice with different doses of SCE resulted in a dose dependent reduction in the radiation-induced mortality up to 80 mg/kg, while a further increase in the drug dose resulted in the decline in the animal survival when compared with the 80 mg/kg SCE. This is in agreement with our earlier study on human peripheral blood lymphocytes, where an optimum protection was observed at 12.5 µg/ml leaf extract of *Syzygium cumini* against the radiation-induced DNA damage, while a further increase in the drug concentration reduced the radioprotective effect of SCE.\textsuperscript{10} Similarly, MPG a synthetic thiol has been reported to provide maximum protection against the 30 day mortality in the whole body irradiated mice at 20 mg/kg b.wt. (optimum dose) while a further increase in the drug dose did not increase the survival significantly.\textsuperscript{10} The earlier studies on radioprotection have shown that an agent in test (for radioprotective action) acts only at a particular dose and radioprotective effect may decline thereafter.\textsuperscript{4,10,13} A similar action cannot be ruled out for SCE, which gave an optimum protection at 80 mg/kg, while the higher doses resulted in a steady decline in its protective action.

The pattern of survival after SCE treatment was similar to that of the irradiated control group except that the mortality was delayed. This clearly indicates the effectiveness of SCE in arresting GI death, where the number of survivors for all the treatment groups was higher than that of the DDW + irradiation group. The administration of 40, 60, 80 and 100 mg/kg SCE was better than the other doses of SCE in reducing the GI deaths. A similar effect was observed earlier for mint extract.\textsuperscript{4} This reduction in GI death may also be due to the protection of intestinal epithelium, which would have allowed proper absorption of the nutrients.

The same schedule when followed for the oral administration at equimolar dose did not afford significant protection when compared to the intraperitoneal route, where 29.2% animals survived 30 days post-irradiation as against 50% for i. p. route. This may be due to the faster absorption of the SCE through the i. p. route and oral administration may require higher dose of SCE to maintain required blood levels for radioprotection. In spite of the lower survival, oral administration of SCE delayed the radiation-induced sickness and mortality when compared with the concurrent DDW + irradiation group. A similar observation has also been reported for Ocimum sanctum, Tinospora cordifolia Phyllanthus amarus and abana, a polyherbal formulation.\textsuperscript{17–20} The treatment of mice with SCE was effective in reducing the radiation-induced sickness and mortality and the highest effect was observed for 80 mg/kg. This increase in 30 day survival may be owing to the protection afforded by SCE to the stem cell compartment of the bone marrow, which continued to supply the requisite number of cells in the survivors.

The mechanism of action of herbal drugs and their extract preparations differ in many respects from that of the synthetic drugs or single substances.\textsuperscript{21} It can be characterized as a polyvalent action and interpreted as additive, in some cases, potentiating. The exact mechanism of action of SCE is not known. However, it is plausible that scavenging of free radicals by SCE may have played an important role in providing the protection against the radiation-induced damage. The flavonoids like quercetin, kaempferol and myricetin, which are present in the SCE have been reported to scavenge free radicals like OH and superoxide and inhibit lipid peroxidation.\textsuperscript{7,9,22,23} Kaempferol and quercetin have also been reported to suppress the cytotoxicity of superoxide ion and hydrogen peroxide in V79 cells.\textsuperscript{24,25}

Myricetin, an important flavonoid present in SCE has been reported to increase the expression of DNA polymerase beta gene in a dose dependent manner, an enzyme responsible for the error-free DNA repair\textsuperscript{22} and thus increasing the survival of mice. The polyphenol ellagic acid, which is also present in *Syzygium cumini* has been reported to be antimutagenic, chemopreventive,\textsuperscript{9} antioxidant and has also been found to inhibit the radiation-induced lipid peroxidation in the liver of mice.\textsuperscript{26} The presence of flavonoids and ellagic acid in SCE extract might have been responsible for its radioprotective activity.
CONCLUSIONS

From our study it is clear that SCE, provided protection against the radiation-induced sickness and mortality and the optimum protective dose of 80 mg/kg is safe from the point of drug induced toxicity, as the animals did not show any signs of adverse effects up to 1 g/kg b. wt. The intraperitoneal administration of SCE prior to irradiation resulted in enhancement of the radiotolerance by 1.4 Gy. The scavenging of free radicals by the SCE seems to be an important mechanism of radioprotection.

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REFERENCES