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Protective effect of *Piper longum* fruit ethanolic extract on radiation induced damages in mice: A preliminary study

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Abstract

The radioprotective property of an ethanolic extract of *Piper longum* fruits (EEPLF) was investigated in Swiss mice. The white blood cell (WBC) count in irradiated control mice was drastically reduced to 1900 cells/mm^3 on third day but in treated animals the count was 2783.3 cells/mm^3. The number of bone marrow cells and α-esterase positive cells was also enhanced by the EEPLF administration (16.7×10^6 cells/femur and 946.5/4000 cells, respectively) when compared to the radiation exposed control animals (12.2×10^6 cells/femur and 693.5/4000 cells, respectively). EEPLF reduced the elevated levels of glutathione pyruvate transaminase (GPT), alkaline phosphatase (ALP), and lipid peroxidation (LPO) in liver and serum of radiation treated animals. The extract administration also increased the reduced glutathione (GSH) production to offer the radioprotection.

Keywords: *Piper longum*; Bone marrow cells; α-Esterase; GPT; ALP; LPO

1. Introduction

A problem associated with cancer radiotherapy is the severe side effects resulting from myelosuppression and normal tissue damage [1]. In consequence, agents that protect

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normal tissues against radiation damage can increase the patient tolerance to radiotherapy. Several synthetic compounds have been found to provide good radiation protection in experimental animals, but their clinical utility is limited by the toxicity on repeated administration [2–4]. Recent studies in our laboratory shows that commonly used medicinal plants and herbal preparations [5,6] are a good source as radioprotectors in experimental models as well as in patients receiving radiotherapy [7].

Piper longum L. (Piperaceae), popularly known in India as "Pippali", is used in traditional medicine in Asia, especially in Indian medicine [8] and in Pacific islands. It is used in gonorrhea, menstrual pain, tuberculosis, sleeping problems, respiratory tract infections, gut and arthritis [9]. Moreover, analgesic and diuretic effects, relaxation of muscle tension, alleviation of anxiety [10] and immunomodulatory and antitumor activity are reported [11].

The present investigation was undertaken to study the radioprotective property of ethanolic extract of *P. longum* fruits.

2. Experimental

2.1. General

P-rosaniline hydrochloride and α-naphthyl acetate were obtained from Loba Chemie, Bombay, India. Harris hematoxylin was purchased from Glaxo India Ltd., Bombay, India. Thiobarbituric acid (TBA) was obtained from Hi-media laboratories, Mubai, India. 5,5′ Dithiobis (2-dinitro benzoic acid) (DTNB) and reduced glutathione was purchased from SRL Ltd., Mubai, India. Estimation kits for alkaline phosphatase and pyruvate transferases were purchased from Span Diagnostics Ltd., India and followed the manufacturer's protocol. All other chemicals and reagents used in this study were of analytical grade.

2.2. Plant

Authenticated *P. longum* was obtained from Amala Ayurvedic Centre.

2.3. Preparation of extract

Dried and powder fruits stirred in 70% aq. EtOH were centrifuged at 7275 rev./min for 10 min at 4 °C. The supernatant was collected and evaporated in vacuo to give a residue (yield 26%). The extract was resuspended in phosphate buffered saline (PBS) (pH 7.2). Phytochemical analysis of the extract showed the presence of alkaloids.

2.4. Animals

Swiss mice (4–6 weeks old, 20–25 g) were purchased from National Institute of Nutrition, Hyderabad, India. They were housed in ventilated cages in an air controlled room and were provided normal mouse chow (Sai feeds, India) and water ad libitum.

Experimental procedures were adopted as approved by the animal experimentation committee.

2.5. Irradiation

Animals were divided into two groups (6 animals each) restrained in specially designed, well-ventilated cages and exposed to whole body radiation at a rate of 1.4 Gy/min. The source of radiation was a 60Co teletherapy unit (Theratron 780, Canada). Group I and group II received single whole body radiation (6 Gy/animal). Group I served as untreated control and group II was treated with EEPLF (400 mg/kg, ip) for 5 consecutive days. Blood was collected from caudal vein, and total leukocyte count (Hemocytometer) [12], differential count, hemoglobin content and weight of the animals were recorded prior to radiation exposure and continued on every third day for 30 days.

2.6. Effect of EEPLF on bone marrow cellularity and α-esterase activity

Two groups, 18 mice each, were used for the experiment. All the animals were exposed to single dose of whole body radiation (6 Gy/animal). Group III was kept as untreated control and group IV was treated with EEPLF (400 mg/kg, ip) for 5 consecutive days. Six mice from each group were killed on days 2, 7 and 11 for analysis of bone marrow cellularity and α-esterase activity.

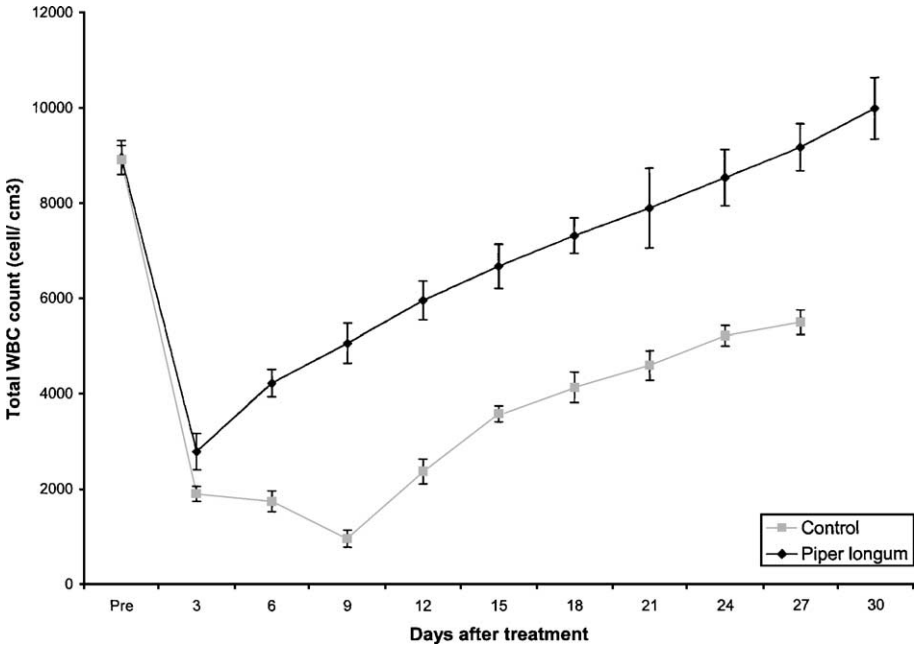

Fig. 1. Effect of ethanolic extract of *P. longum* fruits on total WBC count in irradiated mice.

Table 1
Effects of the *P. longum* fruit ethanolic extract (EEPLF) on bone marrow cellularity and α-esterase activity in irradiated mice

Treatment	Bone marrow cellularity (cells/femur) × 10^6			No. of α-esterase positive cells/4000 cells		
	48th h	7th day	11th day	48th h	7th day	11th day
Normal	14.5 ± 0.7	–	–	992 ± 71.4	–	–
Control	4.4 ± 0.5	7.4 ± 1.2	12.2 ± 1.2	200 ± 39.4	443 ± 22.9	693.5 ± 29
EEPLF + radiation	8.03 ± 0.4*	14 ± 1.1*	16.7 ± 1.20*	323.2 ± 36.2*	620 ± 56.7*	946.5 ± 94.9*

Values are the mean ± S.D. Statistically significant from untreated control.
* $P < 0.001$.

Bone marrow cellularity was done according to Sredni et al. [13,14]. Bone marrow was collected from femur and made into single cell suspension. The number of cells was determined using a hemocytometer and expressed as total live cells (trypan blue exclusion) per femur.

Bone marrow cells from the above preparations were smeared on clean glass slides and stained with *p*-rosaniline and Harris hematoxylin to determine the non-specific α-esterase activity by simultaneous azo dye coupling method [15].

2.7. Effect of EEPLF on radiation induced toxicity

Two groups, 18 mice each, were exposed to single dose (6 Gy/animal) of whole body radiation. Group V was kept as untreated control and group VI was treated with EEPLF (100 mg/kg; ip) for 5 consecutive days.

Six animals from each group were killed after 2, 7 and 11 days by cervical dislocation. Blood was collected by heart puncture immediately and serum was separated. Liver and small intestine were collected to evaluate the radiation-induced toxicities. Total protein was estimated by Lowry's method [16]. Reduced glutathione (GSH) levels in liver homogenate and intestinal mucus were estimated according to Moron et al. [17] based on the reaction with DTNB.

Activities of liver homogenate and serum pathophysiological enzymes such as alkaline phosphatase [18] and glutathione pyruvate transaminases [19] were determined. The lipid peroxidation level in liver and serum was measured using thiobarbituric acid reactive substances (TBARS) according to Ohkawa et al. [20].

Table 2
Effects of the EEPLF on GSH in mice treated with radiation

Treatment	Intestine (nmol/mg protein)			Liver (nmol/mg)		
	48th h	7th day	11th day	48th h	7th day	11th day
Normal	17.2 ±	–	–	6.5 ± 0.33	–	–
Control	6.6 ± 1.34	8.1 ± 0.63	12.7 ± 0.43	2.2 ± 0.25	5.1 ± 1.8	6.1 ± 0.24
EEPLF + radiation	14.7 ± 1.5*	16.8 ± 1.8*	18.2 ± 2.2*	3.9 ± 0.50*	6.0 ± 0.22*	6.2 ± 0.22*

Values are the mean ± S.D. Statistically significant from untreated control.
* $P < 0.001$.

Table 3
Effects of the EEPLF on ALP in mice treated with radiation

Treatment	Serum (U/ml)			Liver (KA)		
	48th h	7th day	11th day	48th h	7th day	11th day
Normal	13.0 ± 0.4	–	–	14.0 ± 0.26	–	–
Control	15.8 ± 0.57	20.2 ± 0.84	14.7 ± 1.3	13.2 ± 0.15	16.1 ± 1.3	14.7 ± 0.47
EEPLF+radiation	12.4 ± 1.2*	13.5 ± 0.72*	7.5 ± 0.44*	11.7 ± 0.95*	13.7 ± 0.6*	13.8 ± 1.0

Values are the mean ± S.D. Statistically significant from untreated control.
* $P < 0.001$.

2.8. Statistical analysis

The data were subjected to Student's *t*-test to determine significant difference between the groups. The values are expressed as mean ± S.D.

3. Results and conclusion

Irradiation significantly reduced total WBC in control mice to 1900 cell/mm^3 after 3rd day of radiation exposure and gradually increased up to 5200 cells/mm^3 by day 30 (Fig. 1). EEPLF treated group had lower WBC initially 2783.3 cells/mm^3 on day 3, however the values increased significantly up to 7308.5 cells/mm^3 on the 18th day. The recovery of leucocytes in EEPLF treated animals was much better than the control animals. There was no significant effect on the differential count, hemoglobin level and body weight (data not shown).

There was a drastic reduction in bone marrow cellularity in radiation treated animals after 48 h (4.4×10^6cells/femur). EEPLF treatment significantly increased the bone marrow cellularity to 8.03×10^6 cells/femur after 48 h and increased gradually to 16.7×10^6 cells/femur on day 11, which was comparable to normal animal (Table 1). The effect of EEPLF on α-esterase positive cells is also given in Table 1. The number of α-esterase positive cells in bone marrow of radiation treated animals was low (200/4000 cells) on the 48th h and did not reach the normal level even after 11 days. In the EPLF treated irradiated animals, there was a significant increase in α-esterase positive cells

Table 4
Effects of the EEPLF on GPT in mice treated with radiation

Treatment	Serum (U/ml)			Liver (U/ml)		
	48th h	7th day	11th day	48th h	7th day	11th day
Normal	61 ± 2.0	–	–	50.3 ± 4.0	–	–
Control	120 ± 9	98.5 ± 4.7	76.6 ± 1.9	89.8 ± 3.3	72.1 ± 2.3	56.9 ± 2.3
EEPLF+radiation	80.5 ± 6.1*	74.4 ± 5.9*	64.0 ± 5.5*	84.8 ± 1.7*	63.2 ± 2.3*	50.6 ± 1.6*

Values are the mean ± S.D. Statistically significant from untreated control.
* $P < 0.001$.

Table 5
Effects of the EEPLF on LPO in mice treated with radiation

Treatment	Serum (nmol/ml)			Liver (nmol/mg protein/min/mg protein)		
	48th h	7th day	11th day	48th h	7th day	11th day
Normal	1.42 ± 0.24	–	–	1.26 ± 0.11	–	–
Control	2.9 ± 0.15	3.5 ± 0.53	1.6 ± 0.28	4.5 ± 0.4	3.04 ± 0.7	1.9 ± 0.03
EEPLF + radiation	1.37 ± 0.16*	2.58 ± 0.82*	1.55 ± 0.27*	3.2 ± 0.35*	1.87 ± 0.62*	0.96 ± 0.05*

Values are the mean ± S.D. Statistically significant from untreated control.
* $P < 0.05$.

(323.2/4000 cells) on 48th h compared to the control and after day 11 it was similar to normal level (946.5/4000 cells).

The animals exposed to radiation had a lowered level of GSH in intestinal mucosa (6.6 nmol/ml) and liver homogenate (2.2 nmol/ml) after 48 h. Administration of EEPLF showed a significant increase in GSH content (intestinal mucosa 14.7 nmol/ml as well as in liver 3.9 nmol/mg) with respect to control (Table 2).

Whole body irradiation elevated the levels of the pathophysiological enzyme ALP in serum (15.8 U/ml) and in liver (13.2 U/mg tissue). Administration of EEPLF protected the damages by decreasing the activities of enzyme ALP in serum (12.4 U/ml) and in liver (11.7 U/mg) after 48 h, which gradually reached the normal level by day 11 (Table 3).

Serum and liver GPT (120 U/ml and 89.8 U/ml, respectively) increase after radiation were significantly lowered in treated group (serum 80.5 U/ml, liver 84.8 U/ml) after 48 h (Table 4).

An increase in thiobarbituric acid reactive substances level in serum (2.9 nmol/ml) and liver (4.5 nmol/mg protein formed/min/mg protein) was evident in control animals 48 h after the radiation exposure. In EEPLF treated irradiated animals at the same time point the level of lipid peroxidation products was reduced to1.37 nmol/mg protein formed/min/mg protein in serum and 3.2 nmol/ml in liver. After 11 days of radiation exposure the LPO level reached normal level (serum 1.55 nmol/ml; liver 0.96 nmol/mg protein formed/min/mg protein) in EEPLF treated animals (Table 5).

This study was carried out mainly to determine the effect of ethanolic extract of *P. longum* fruits on toxicity induced by radiation in mice. These results indicate that administration of EEPLF protected mice from lethal effects of radiation and promotes the recovery of bone marrow cells and leukocytes. The mechanism by which the EEPLF protects animals from radiation is not known. Further studies are needed to better evaluate the radioprotective activity of the ethanolic extract of *P. longum* fruits and its usefulness as a radioprotector agent.

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