Radioprotective Potential of *Prunus Domestica* against Radiation Induced Oxidative Stress in *Swiss Albino* Mice with Special Reference To Spatial Learning

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Summary

Prunus domestica fruit has been used for treatment of various disease and disorders in Ayurveda and Unani medicine. The current study has concentrated on assessment of the modulatory effect of methanolic extract of *Prunus domestica* against radiation induced oxidative stress. Optimum dose selected against radiation (10 Gy) exposure, was 400 mg/kg b.wt./d of PDE as it afforded maximum protection in terms of body weight and survivability of the mice in comparison to other doses. DRF was calculated 1.59. The activity of PDE on memory acquisition and retention was studied using Morris water maze model (MWM). Mice were divided into 5 groups (I) control – no treatment (II) PDE (III) Irradiated (IR) (IV) IR+PDE (V) PDE+IR. Administration of PDE prior/post irradiation resulted in improved spatial learning as evident by the lesser time taken to reach the platform compared to the control and increased protein concentration in brain.

Keywords- *Prunus* domestica; Dose Reduction Factor; Morris Water Maze; Oxidative Stress; Radioprotection.

Introduction

Synthetic protectors against oxidative damage to tissue have toxicity which limits their use. In Ayurveda, the traditional Indian system of medicine, several plants have been used to treat free radical-mediated ailments and, therefore, it is logical to expect that such plants may also render some protection against radiation damage. Therefore, researchers have diverted their attention towards the plant and natural products during last decades[1]. *Prunus domestica* (Family-Rosaceae) commonly known as Alu Bukhara (plum), contains immunostimulatory components that potentially may be useful in human and veterinary medicine. *Prunus domestica* (Plums) are fruits rich in phenolic compounds, characterized by relatively high antioxidant activity, higher than oranges, apples or strawberries[2,3.] The fruit contain anthocyanins (type cyanidin-3-glucoside and cyanidine-3-rutinoside which make up 42 to 62% of the total anthocyanins) and flavanols (catechin) which have recently emerged as powerful antioxidants. Catechin occurring in quantities from 1.3- to 3.9-mg/100 g was found to be a dominant compound representing biologically active monomers and dimers of flavanols. The highest quantities of hydroxycinnamic acids, especially neochlorogenic one, (46-85 mg/100 g) are found in all plum varieties. All studied plum varieties show high polyphenoloxidase activity but there are substantial differences between particular varieties ranging from 3200 to 17200 U/g[4]. 100 grams of edible portion of fruits of *Prunus domestica* also has Protein 0.7g, fat 0.5g, Carbohydrate 11.1g, Minerals and fibre 0.4g, Calcium 10mg, Phosphorus 12mg, Iron 0.6mg, Magnesium 147mg, Sodium 0.8mg, Potassium 247mg, Copper 0.13 mg, Sulphur 33 mg, Carotene (Vit A) 166µg, Thiamine (Vit. B1) 0.04mg, Riboflavin (Vit. B) 0.1mg, Niacin 0.3mg, Vitamin C 5mg and Oxalic acid 1mg according to Nutrient Database [5.] Amygdaline and prunasin, substances which break down in water to form hydrocyanic acid (cyanide or prussic acid) are also present in all members of the genus.

This exceedingly poisonous compound stimulates respiration, improves digestion and gives a sense of well being in small amounts[6]. Plums are recommended to patients suffering from arterial hypertension due to the high potassium content and advantageous sodium, potassium ratio[7,8]. Plums have useful levels of riboflavin (B2), with two (66 gram sized) plums providing about a sixteenth of an adult's recommended minimum daily intake, and fairly good amounts of vitamin C. *Prunus domestica* containing free radical scavengers like phenolics, tannins and flavonoids are known for their therapeutic activity[9.] There is a lack of scientific data regarding the effect of the authentication of traditional claims of PDE as a memory enhancer. The objectives of the present study were to investigate the toxicity, if any, induced by the fruit of *Prunus domestica* (PDE) and to find out the optimal protective dose of PDE against radiation exposure, and to investigate the possible ameliorative potential against radiation induced spatial learning deficits in *Swiss albino* mice.

Materials and Methods

Mice

The animal care and handling was done according to the guide-lines set by INSA (Indian National Science Academy, New Delhi, India). The Departmental Animal Ethical Committee (DAEC) approved this study. Six weeks adult male *Swiss albino* mice, weighing 25 ± 2 g, from an inbred colony were used for the present study. These mice were maintained under controlled conditions of temperature and light (light: dark, 10h: 14h). Four mice were housed in a polypropylene cage containing sterile paddy husk (procured locally) as bedding throughout the experiment. They were provided standard mice feed (procured from Hindustan Levers Ltd, India) and water *ad libitum.*

Extract Preparation

Fresh fruits of *Prunus domestica* were washed, shade dried and powdered after removal of seeds. Methanolic extract was then

prepared by refluxing for 48 hours (4 × 12) at 50°C. The extract thus obtained was vacuum-evaporated so as to achieve powdered form. The extract was redissolved in doubled-distilled water (DDW) just before the oral administration.

Source of Irradiation

The cobalt teletherapy unit (ATC-C9) at Cancer Treatment Center, Radiotherapy Department, SMS Medical College and Hospital, Jaipur, Rajasthan, India was used for irradiation. Unanaesthetized Mice were restrained in well-ventilated perspex boxes and the whole body exposed to gamma radiation at a source-to-skin distance(SSD) of 77.5 cm from the source to deliver the dose rate of 1.07 Gy/min.

Experiments

Selection of optimum dose of PDE against radiation

For selection of the optimum dose of PDE against radiation, mice were given 0,100, 300, 400, 500,600, 700, 1000 and 1200 mg/kg bwt/d for 15 consecutive days. One hour after final administration, mice were exposed whole body to 10 Gy gamma-radiations. All the mice were observed for 30 days for any sign of radiation sickness, behavioural change, toxicity, and mortality if any. hanges in body weight were recorded. Usually 10 mice were used for each PDE dose and control group.

The radio-protective effect of PDE [Dose Reduction Factor (DRF)]

Mice for this study were divided into two groups (50 mice in each group). The first group received double distilled water equal to the extract, served as control, while the second group was administered PDE extract for 15 days at optimum dose level, which served as the experimental group. After 30 minutes of last treatment, on 15^{th} day mice of both the groups were divided into ten sub groups (five sub groups in each group) and exposed to different doses of gamma radiation (3, 5, 7, 10 and 12Gy). Mice of control and experimental group were checked daily for 30

days. The survival percentage of mice in each group was used to construct survival dose response curves for obtaining the $LD_{50/30}$ value for experimental as well as the control group. DRF value was calculated by the following formula-
DRF = $LD_{50/30}$ of Experimental group/ $LD_{50/30}$ of Control group.

Learning Studies Morris Water Maze task method[10]
Learning is defined as the acquisition of information and skills, while subsequent retention of that information is called memory. One of the challenging tasks for neuroscientists is to elucidate the biochemical and molecular mechanisms under lying learning and memory. To assess the learning and memory paradigms in laboratory animal, maze is used conventionally. The apparatus used was a circular water tank (100 cm diameter) filled to a depth of 30 cm with water (25^0C). Along the perimeter of the tank four points were equally distributed which served as starting locations. The tank was divided arbitrarily into four equal quadrants. A small platform (5 cm width) was located in the centre of one of the quadrants. Four different starting positions, equally spaced around the perimeter of pool, were used in a fixed order. The maximum duration of the trial was 60 second and mice not finding the platform within this 60 second were placed on it. At the end of each trial the mice was allowed to remain on the platform for 20 seconds and then returned to their home cage and left there to rest for 15 minutes, before beginning of the next trial. Mice received 2 trials for 10 consecutive days and time taken to reach the platform was noted for each trial.
For this purpose the male mice (n=10 each group) were divided into five groups *viz*.

Group I (Control): Mice of this group did not receive any treatment.

Group II (Only PDE): Mice of this group were supplemented PDE orally once every day for fifteen consecutive days at optimum dose of 400 mg/kg b. wt/d dissolved in double distilled water.

Group III (Irradiated): Mice in this group received double distilled water, which equalled to the dose of extract for fifteen days and then exposed to whole body γ-irradiation at the dose of 5 Gy.

Group IV (PDE+ Irradiation): Mice were supplemented orally with PDE at optimum dose for fifteen consecutive days and then exposed to 5 Gy whole body irradiation.

Group V (Irradiation +PDE): Mice in this group were exposed to 5Gy whole body γ-irradiation and then supplemented orally with PDE at the optimum dose (400mg/k b.wt)
5 mice from each group were sacrificed by cervical dislocation at different autopsy intervals 1, 3,7 days post treatment. An incision was made at the sides of the jaws to separate the upper and the lower palates. The upper palate was cut in the middle and, after having cleared the surrounding tissue the brain was excised and separated from the spinal cord at the decussation of the pyramids. The intact cerebrum was then removed carefully. Cerebrum was taken out for protein estimation.

Protein assay- Estimation of protein was based on the method proposed by Bradford, 1976[11]. 10% homogenate was prepared in NaCl and 0.1 ml of the sample was taken for the assay. Three repeats of the assay from each animal were carried out. The absorbance was read at 595nm. The protein level was measured in terms of mg g-1 tissue.

Statistical analysis-
The data obtained were analyzed using one-way analysis of variance (ANOVA). The significance was observed at different levels. **Highly Significant**- *P*< 0-0.0001, **Significant**- *P*< 0.01-0.09 and **Non Significant**- *P*<.1.

Results

Selection of optimum dose of PDE against radiation-

The mice of irradiated group exhibited signs of radiation sickness within 2–3 days after exposure to 10 Gy of gamma- radiation. The irradiated mice exhibited a reduction in food and water intake, ruffling of hairs, diarrhoea, watering of eyes, and irritability. A few mice showed paralysis and difficulty in locomotion during the 2nd week after exposure. The optimal effectiveness of *Prunus domestica* extract was noted at the dose of 400mg/kg b.wt in terms of body weight changes (Fig-1). Further the following pertinent information was also gathered maximum mortality (i.e.100%) within minimum time (17 days) was observed in control group and long survivability or minimum mortality (i.e.10%) was observed with 400 mg dose within maximum observation time (30 days). Death of all mice occurred within 24 days in 100 mg dose group. In 400 mg dose group, 90% mice survived up to 30 days post irradiation. In other doses survivality ranged between 80%-60% till the last day of observation. Therefore, 400 mg/kg b. wt/d PDE has been used for the detailed investigation.

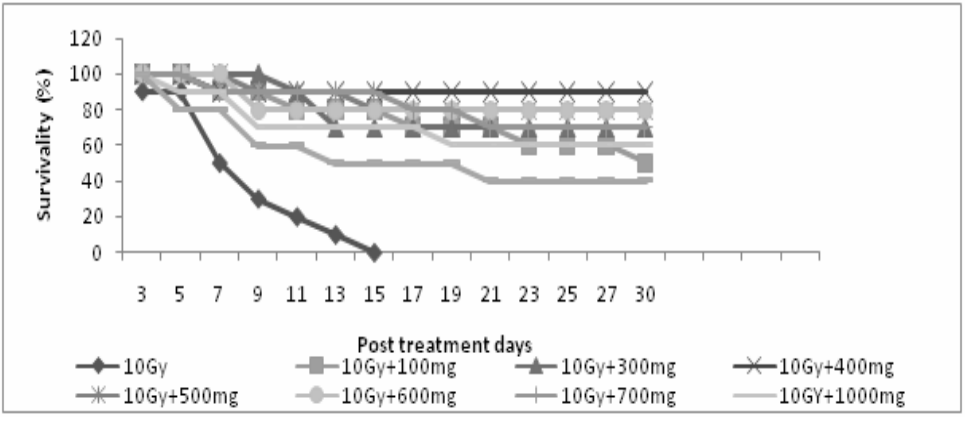

Fig-1 Curves of survival (%) after irradiation (10 Gy) (n=10) with or without different doses of PDE treatment

The radio-protective effect of PDE [Dose Reduction Factor (DRF)] – On the basis of maximum survivability a $LD_{50/30}$ values for control (irradiated alone) and experimental (PDE +irradiation) were computed as 3.77 and 2.36 Gy, respectively. DRF value was calculated as 1.59 Gy (Fig-2).

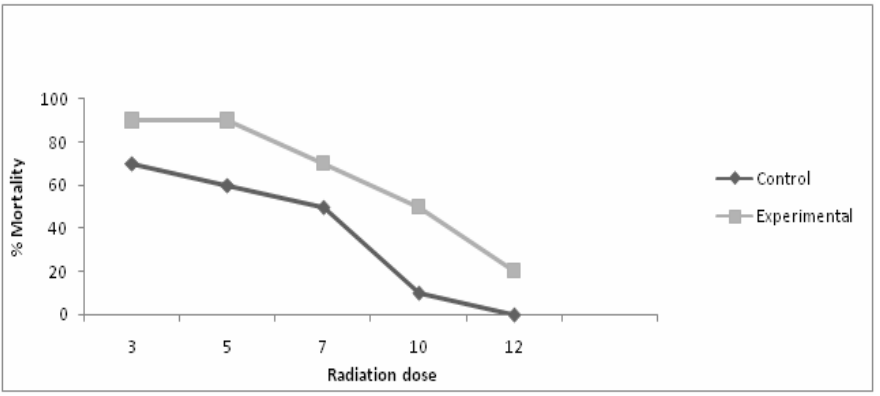

Fig-2 Mortality percentage at different doses of gamma radiation with and without optimum doses of PDE treatment

Learning Studies

In this experiment the effect on spatial learning ability was investigated after exposure to Co^{60} rays (5Gy) using water maze in which the mice were required to find a submerged platform in the circular pool. Initially upto 48 hours after irradiation the mice remained in the quadrant instead of reaching the platform. Nevertheless these mice were able to acquire the spatial information regarding the portion of the escape platform and effectively locate it in subsequent 10 day trial improving day by day as evident by decrease in time taken to reach the platform. The irradiated mice took longer time to reach the platform compared to control which was statistically significant ($F_{1,18}$ = 6.46,P<0.02). Whereas in PDE supplemented mice prior/post irradiation actively tried to acquire the spatial memory as evident by their movement in the pool all over, not just staying in the quadrant area. Supplementation of the PDE pre/post irradiation

continuously for 15 days showed decrease in escape latency as evident by the lesser time taken to reach the target compared to only irradiated mice (group III). Post irradiation supplementation of PDE extract was found to be more effective in comparison to pre irradiation supplementation of PDE. Statistical analysis by one way ANOVA also revealed significant difference between PDE+IR / IR+PDE ($F_{1,18}$=3.11,P<0.09)/ ($F_{1,18}$=5.94,P<0.02) also indicating that the post irradiation treatment is more effective compared to pre treatment.

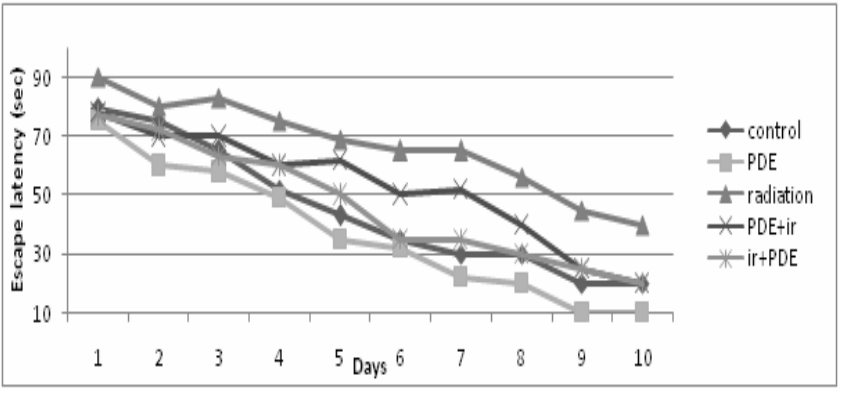

Fig-3 Average escape latency (i.e. time to reach the platform after release into the water) during the 10 days training sessions.

Protein Studies-

Only PDE supplementation (group-II) for 15 consecutive days could raise the baseline protein in cerebrum though it was statistically non significant ($F_{1,18}$ =2.32 ,P<0.14) compared to the control. In the cerebrum protein content decreased after irradiation up to 3 days thereafter it started to increase gradually. Result of one way ANOVA between control versus irradiation is as follows: day1 ($F_{1,18}$ =401.36, P < 0.0001), day3 ($F_{1,18}$=0.0001) day7 ($F_{1,18}$=352.18, P <0.0001). PDE seems to provide protection against radiation induced oxidative damage in pre and post treated irradiated group, as evident by increased level of protein at all the autopsy intervals compared to only irradiated

mice (group III). Result of one way ANOVA between irradiated versus irradiated + PDE treated is as follows: at day1 ($F_{1,18}$ =12.6, P<0.002), day3 ($F_{1,18}$ =3.64, P<0.07), day7 ($F_{1,18}$ =10.14, P<0.005) and between groups irradiated versus irradiation+ PDE treated is as follows: at day1 ($F_{1,18}$= 42.68, P<0.0001), day3 ($F_{1,18}$=86.64, P<0.0001), day7 ($F_{1,18}$ =86.64, P<0.0001). Supplementation after irradiation provided more protection in comparison to pre treatment to PDE.

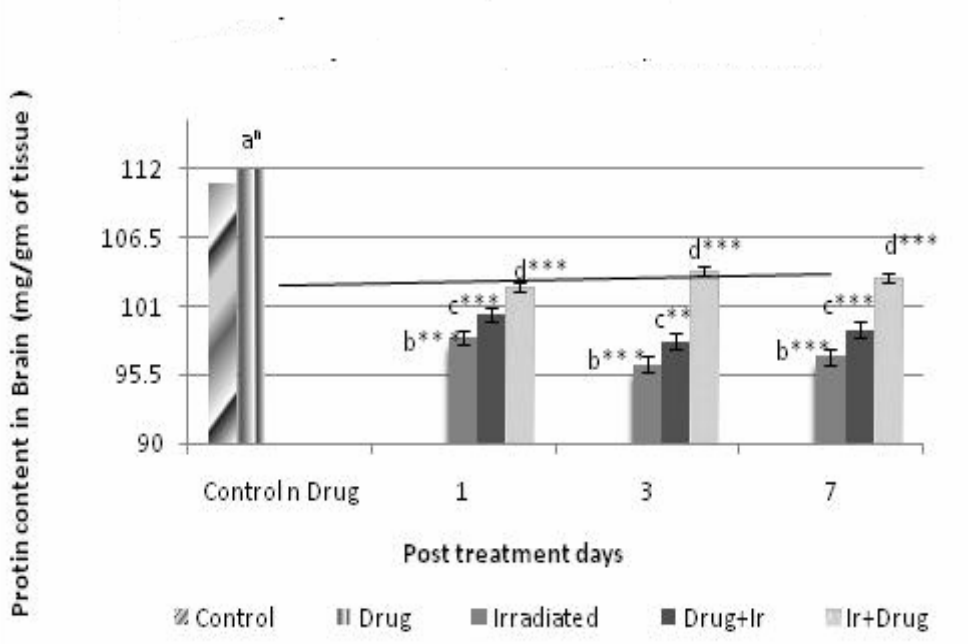

Fig-4 -The following groups were compared by ANOVA: (a) control versus PDE treated, (b) control versus irradiated, (c) irradiated versus PDE treated+ irradiation,(d) irradiated versus irradiated+ PDE treated. Symbols for P values of the ANOVA: Highly Significant- P <0-0.0001***, Significant- P <0.01-0.09** and Non significant- P<.1.

Discussion

Oral administration of a 400 mg/kg b.wt dose of PDE for 30 days, prior to radiation exposure (10 Gy), was found to be effective in terms of survivability compared to other higher and lower doses of PDE. The pattern of survival after PDE treatment was similar to that of the irradiated group except that mortality was delayed. This may be due to the effectiveness of PDE in arresting gastrointestinal (GI) death, as indicated by the increased number of survival days in all the treatment groups, compared to the irradiated mice. This reduction in GI death may also be due to the protection of intestinal epithelium, which would have allowed proper absorption of the nutrition. Anthocyanin pigments and associated flavonoids have demonstrated ability to protect against free radicals. A study using human cancer lines has demonstrated cell cycle arrest and apoptosis of mutated cells exposed to cherry anthocyanin[12,13] . So another possibility of decreased radio sensitivity may be due to cell cycle arrest in a radiosensitive phase. It is suggested that growth arrest characteristics of cyanidin are likely, at least in part, to be result of significant inhibitory effects of these cherry components on epidermal growth factor receptors[14].

To study the neurobiological mechanisms that underlie spatial learning and memory, age-associated changes in spatial navigation and the ability of nootropic agents to influence specific cognitive process Morris Water Maze task has been used extensively[15]. Stress affects learning and memory[16] . In fact Radiation is considered as an environmental stress factor. In our study we have observed that radiation results in the impairment of learning spatial memory which can be modulated by supplementation of PDE extract prior/post irradiation. Since it is well known that performance in the Morris Water Maze is dependent on the hippocampus, it is plausible to assume that irradiation in our experiments affected the brain area. Furthermore, the function of the hippocampus might be affected by radiation exposure possibly due to disturbance of the blood-

brain barrier, which has been reported to occur. This behavioural phenotype is reminiscent of that observed during normal aging since spatial learning impairment in aged rats is associated with change in hippocampal connectivity and plasticity in mice and rats[17,18]. Considering that memory functions similarly in mice and human with respect to the involvement of the hippocampus[19]. . Irradiation is associated with a decline in motor coordination which leads to deficit in the ability to learn new motor skills. This loss of function might be correlated with decline in cerebella beta-adrenergic receptor function that resembles to what is observed in normal aged rats. This effect of hyperoxia is blocked by antioxidants[20]. Diets supplemented with spinach, strawberries, blueberries, nutritional antioxidants, and reverse age induced declines in beta-adrenergic receptor function in cerebeller purkinje neurons were measured using electrophysiological techniques. The spinach diet improved learning on a run way motor task, previously shown be modulated by cerebella nor epinephrine[21].

Dietary supplementation with fruit or vegetable extracts high in antioxidant (eg. Bluebarries, strawberries, walnuts and concord grape juice) can decrease the enhanced vulnerability to oxidative stress that occurs in aging and these reduction are expressed as improvement in behaviour[22]. Learning ability is always associated with the formation of new protein molecules. In our study we have observed that protein content estimated in cerebrum increases after supplementation of PDE prior/post irradiation. It was also noticed that the loss of spatial memory after radiation improved after supplementation of PDE extract. The reduction in the protein biosynthesis could be attributed to any of the following factors:(1) activation of RNAase; (2) depletion of mRNA; (3) effect on the formation and/or maturation of RNAase. Radiation may also include local deficits in the microstructure of protein molecules, which becomes centre of thermal denaturation and cross linkage, thus causing tissue damage[23]. Increase in protein concentration after PDE supplementation is assigned as the compensatory beneficial

effect. It is highly possible that these changes cause alternation in congnitive function related proteins, such as androgen receptors and apolipoprotein A [24] . Several factors that elevate production of new neurons are also associated with enhanced learning both running and living in an enriched environment double the number of surviving newborn cells and improve water maze performance[25]. The question whether the memory impairment is reversible is open for exploration by further experiments.

The mechanism of action of herbal extract preparations differ in many respects from those of synthetic or single substances[26]. The radioprotective activity of plant and herbs may be mediated through several mechanisms, since they are complex mixtures of many chemicals. Scavenging of radiation- induced free radicals and elevation of cellular antioxidants by plants and herbs in irradiated system could be leading mechanism for radioprotection. The plants and herbs may also inhibit activation of protein kinase (PKC), mitogen activated protein kinase (MAPK), cytochrome P- 450,nitric oxide and several other genes that may be responsible for inducing damage after irradiation[27]. The exact mechanism of action of PDE is not known. However, it is possible that scavenging of free radicals by PDE may play an important role in providing protection against radiation-induced damage. Prophylactic action of *Prunus domestica* against radiation-induced metabolic disorders may be due to the presence of antioxidants such as anthocyanin, ascorbic acid, vitamins, minerals etc.

Conclusion-

From the present study it can be concluded that prophylactic action of *Prunus domestica* against radiation-induced metabolic disorders may be due to the protection afforded by PDE due to synergistic action of various antioxidant constituents present in it. It may act as memory enhancer and may be also useful as a supportive adjuvant in the treatment of impaired memory function.

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