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EVALUATION OF MICROTRON-ACCELERATED ELECTRON BEAM RADIATION INDUCED TISSUE DAMAGE AND THE RADIOPROTECTIVE EFFECT OF *PIPER BETLE* AND *PIPER NIGRUM* IN SWISS ALBINO MICE

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ABSTRACT

Ionising radiation has vast applications in medical diagnosis and therapy. Radiation has both stochastic and delayed type of effects and the sensitivity of different organs and tissues vary enormously, wherein the hematopoietic and rapidly proliferating cells being the most sensitive of all. In our present study we aimed at detecting the tissue damage induced by microtron-accelerated high energy electron beam radiation and the protective effects of Piper betle and Piper nigrum extracts in Swiss albino mice. To assess the histological damage induced by e-beam radiation and to detect the protective effect of Piper extracts against radiation induced tissue injury we used intestinal jejunum as target tissue. Haematological parameters were also carried out to study the toxicity of e-beam radiation and the protective effect of the selected plants. The experimental animals were divided into groups and were treated with different doses of the extracts prior to irradiation and analysis of haematological and histological parameters were carried out at different time intervals. E-beam radiation markedly decreased the TEC, TLC and Hb content which was significant compared to the sham control. There was a gradual decline in all these blood parameters which was found to be time dependent. The blood parameters were normal in CMC and extract alone treated animals. Piper extract pre-treatment improved the blood counts and Hb content in a dose dependent manner in the animals exposed to e-beam radiation. The sham control group did not show any change in the intestinal histology, as evidenced by the presence of a large density of crypts at the base of the villi. In the irradiated group, the villus height and mucosal length were found to be significantly shorter compared to the sham control, demonstrating the damaging effects of radiation on the intestinal mucosa. Irradiation reduced the number of surviving crypts significantly and mucosal thinning was also observed. The extract treated groups showed a significant increase in the number of surviving crypts compared to the radiation alone group. The villus height, crypt depth and mucosal length in the combined treatment group were found to be greater than those of the radiation alone group.

KEYWORDS: *Electron Beam Radiation, Piper Extracts, Intestinal Injury, Haematology*

Article History

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INTRODUCTION

Radiation exposure has become an indispensable part of mankind as he is exposed to natural background radiation as well as other cosmic rays. Ionising radiation has vast applications in medical diagnosis and therapy. Radiation has both stochastic and delayed type of effects and the sensitivity of different organs and tissues vary enormously, wherein the hematopoietic and rapidly proliferating cells being the most sensitive of all. Reduction in certain elements of blood is often seen following radiation exposure. This results from radiation exposure of bone marrow, and to a lesser extent, direct damage to lymphocytes in the blood stream and lymph nodes. The white cell count will be reduced, particularly the lymphocytes, and the number of platelets will be reduced. Murine hematopoietic stem cells were determined to be the most sensitive of all mammalian cells undergoing mitotic death [1]. Radiation was also shown to affect the biochemical structure of the cell membrane. It increases membrane cholesterol level, causes oxidation of membrane proteins, thiol groups and lipid peroxidation, and impairment of membrane permeability barrier [2]. Radiation injury can result in varying degrees of inflammation, thickening, collagen deposition, and fibrosis of the bowel, as well as impairment of mucosal and motor functions [3]. Submucosal fibrosis and obliteration of small blood vessels result in ischemia, which is progressive and irreversible. Ischemia initially involves the mucosa and gradually progresses to involve the submucosa and serosa [4]. In this context, the agents which have radioprotective effects may typically induce haemopoetic regeneration. It was reviewed that large number of plant extracts and plant bioactive compounds have been evaluated for their radio protective effects particularly for attenuating damage to the haemopoetic system [5]. There are several reports on the radioprotective effects of dietary antioxidants on haemopoetic cells after total body irradiation [6] as well as on radiation injury studies [7].

Search for the chemical agents that are able to protect human beings from the ill-effects of ionizing radiation is a key issue in radiation biology. The use of plants and natural products may be beneficial in protecting against the radiation induced damage, as they are less toxic or practically non-toxic compared to the synthetic compounds at their optimum protective dose levels. Therefore, interest is generated in the development of potential drug of plant origin to prevent the harmful effects of radiation [8]. In view of this, more focus is now emphasised to evaluate the radioprotective effects of natural compounds. The non-toxic protective agents will be useful in reducing the toxic side effects of various loads of radiation exposure. In this line plants and/or their bioactive compounds would be very useful in reducing the harmful effects of radiation [9].

In our study, hematological effects of electron beam radiation were analysed by measuring blood parameters like total erythrocyte count (TEC), total leukocyte count (TLC) and haemoglobin (Hb) content and the tissue injury caused by radiation on GI tract was assessed by studying the histological details of small intestine in radiation exposed animals. The radioprotective activity of ethanolic extracts of *Piper betle* leaves and *Piper nigrum* seeds were also analysed.

MATERIALS AND METHODS

Radiation Source

High energy electron beam generated by an 8/12 MeV variable energy microtron was used. The microtron was designed, fabricated and commissioned by Mangalore University in the year 1995 in collaboration with Raja Ramanna Centre for Advanced Technology (RRCAT), Indore and Baba Atomic Research Centre (BARC), Mumbai. The microtron operates at 2998 MHz and uses a 2MW magnetron based microwave system to energize the accelerator cavity. Bremsstrahlung radiation of peak energy 8 MeV is generated by making 8 MeV electrons from the microtron to fall on a high Z material, tantalum.

Preparation of Plant Extracts

The leaves of *Piper betle* and seeds of *Piper nigrum* collected were thoroughly washed with distilled water and shade-dried. Ethanolic extract was prepared using a Soxhlet extractor for about 8-9 cycles. The extract obtained was concentrated in a rotary vacuum evaporator (Laborata 4003, Heidolph Rotovac, Germany) at 40°C. The thick concentrate obtained after the evaporation was further dried and stored in a deep freezer (-20°C) for future use.

Experimental Animals

Eight to ten weeks old Swiss albino mice, *Mus musculus*, weighing around 25±3 g. were used for the study. The animals were bred and maintained in the departmental animal house following standard ethically accepted methods. They were housed in polypropylene shoe-box type cages, bedded with rice husk and maintained in air-conditioned room at a temperature of 23±2ºC and relative humidity 65±5% with a 12 hour dark and light cycles. They were fed with standard laboratory rodent diet (Lipton India Ltd.) and water *ad libitum*. All animal experiments were conducted in accordance with the guidelines of the Committee for the Purpose of Control and Supervisions of Experiments on Animals (CPCSEA), Government of India, after prior approval from Institutional Animal Ethics Committee. The experimental animals were kept in Perspex boxes specially designed for the purpose and irradiated at a distance of 30 cm from the window of the accelerator machine. For haematological and histological studies, radiation dose of 8 Gy was chosen. For studying radioprotectivity, the animals were pre-treated with the plant extracts through oral gavage, followed by radiation exposure. Haematological and histological analyses were done at different time intervals.

Test Parameters

Haematological study was performed to assess the effect of e-beam radiation on blood parameters such as total erythrocyte count (TEC), total leukocyte counts (TLC) and the haemoglobin (Hb) content in mice. The irradiation procedure and doses selected were same as that for *in vivo* bone marrow studies. Five different time intervals were used for the study and TEC, TLC and Hb content were measured after 24hr, 48hr, 72hr, 7 days and 14 days post irradiation in different groups.

To evaluate the e-beam radiation caused tissue injury, intestinal jejunum of mice was used. Four different parameters were considered for the study, i.e., villus length, crypt depth, crypts/circumference and mucosal length. The animals were sacrificed after 3 days post irradiation and the tissues were processed for observing histological changes.

RESULTS

Haematology

The results of hematological studies for *Piper betle* are illustrated in fig 3.1 to 3.3. E-beam radiation (8 Gy) markedly decreased the TEC, TLC and Hb content which was significant compared to the sham control. There was a gradual decline in all these blood parameters which was found to be time dependent; maximum reduction was observed on 3rd day after radiation treatment (72 hr.), which was improved at later time intervals. The blood parameters were normal in CMC and extract alone treated animals. *Piper betle* extract pretreatment improved the blood counts and Hb content in a dose dependent manner in the animals exposed to e-beam radiation.

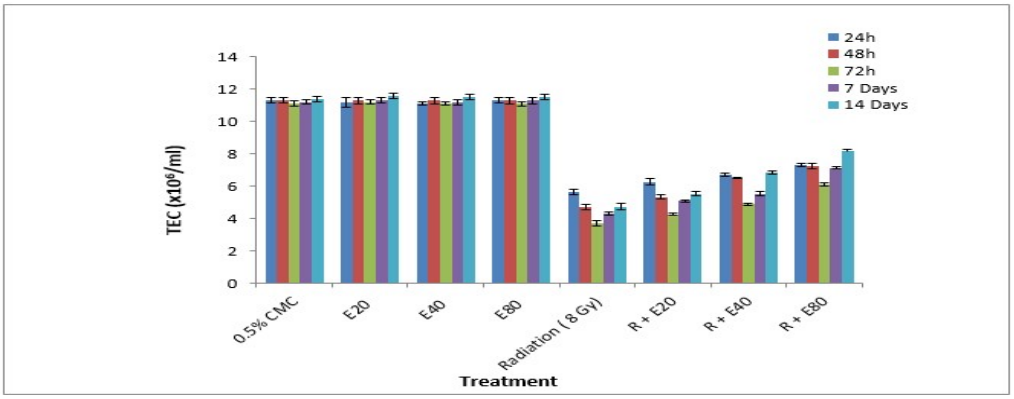

Figure 1: Effect of Piper Betle on TEC in Swiss Albino Mice Exposed to E-Beam Radiation.

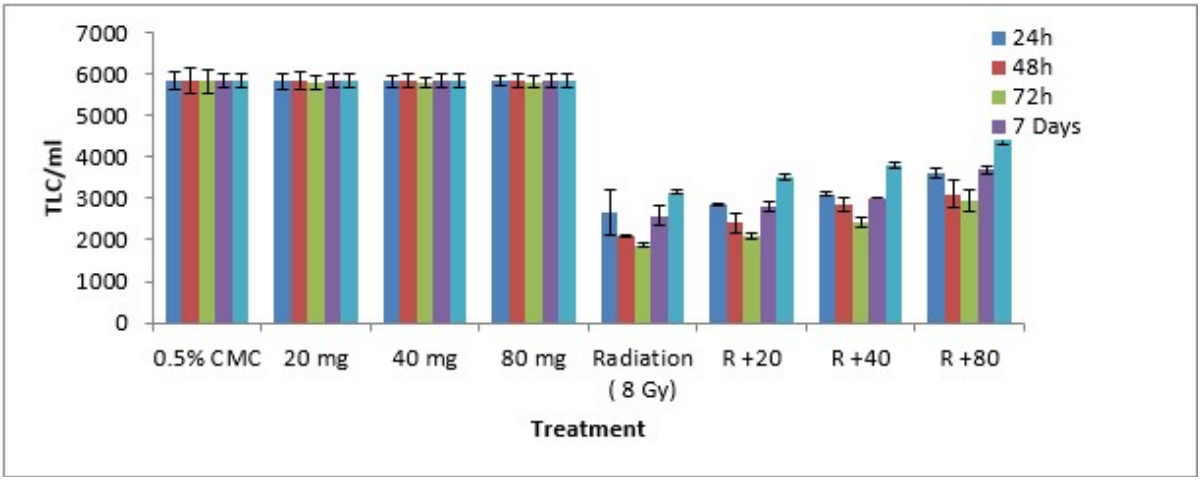

Figure 2: Effect of Piper Betle On TLC in Swiss Albino Mice Exposed to E-Beam Radiation.

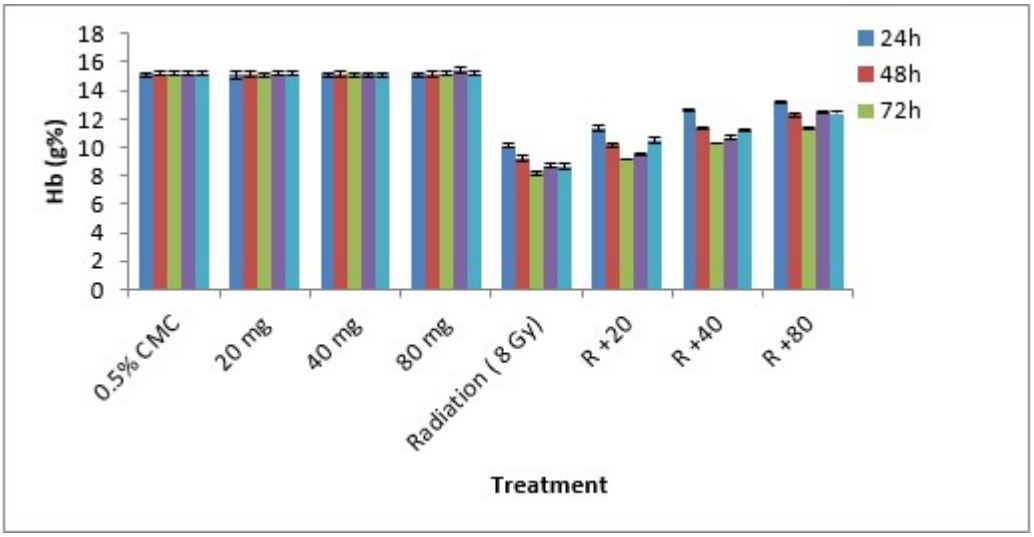

Figure 3: Effect of Piper Betle on Hb Content in Swiss Albino Mice Exposed to E-Beam Radiation.

Similar results were obtained for *Piper nigrum* also. The ethanolic extract of the seeds protected the animals from radiation induced haematopoietic syndrome. It helped in restoration of blood cell counts and Hb content in a dose dependent manner. The TLC, TEC and the Hb content showed a sharp decline on the 3rd day post irradiation and recovery was observed after 7th day. The results are given in fig 3.4 to 3.6.

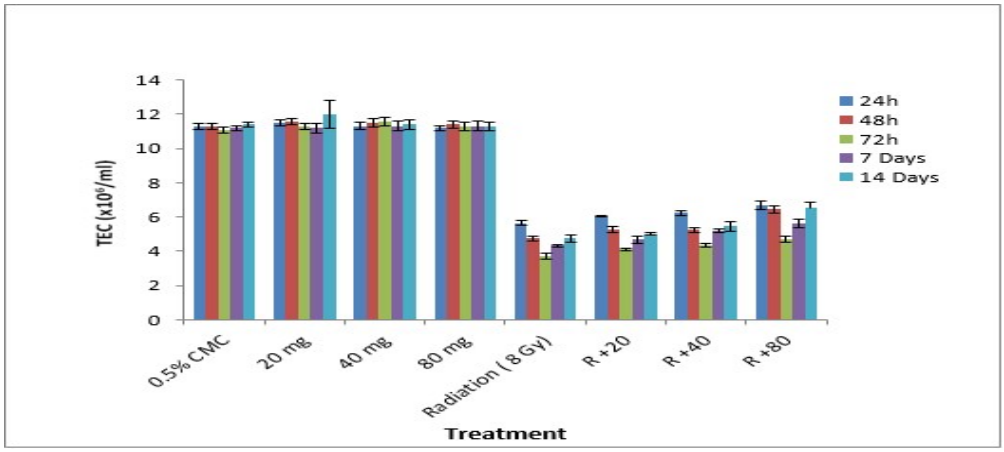

Figure 4: Effect of Piper Nigrum on TEC in Swiss Albino Mice Exposed to E-Beam Radiation.

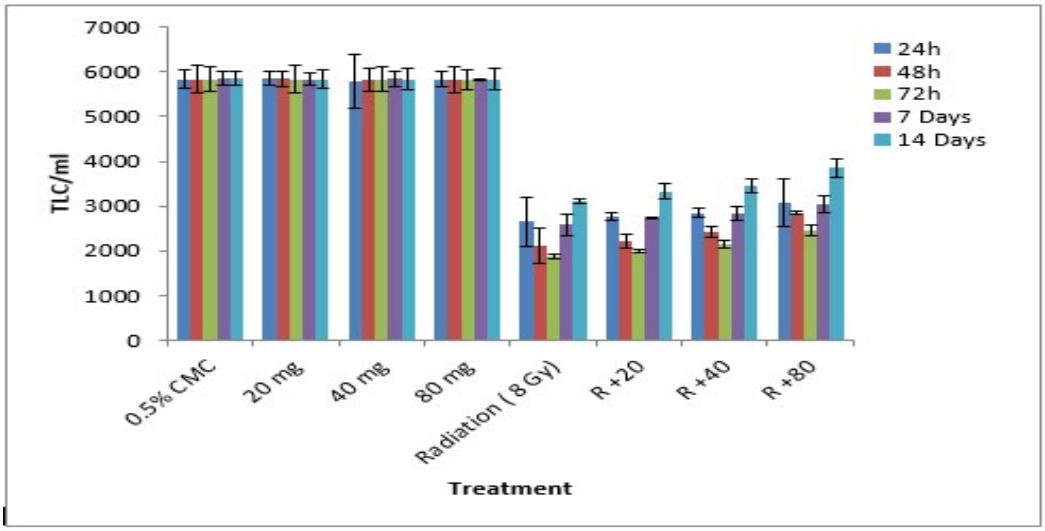

Figure 5: Effect of Piper Nigrum on TLC in Swiss Albino Mice Exposed to E-Beam Radiation.

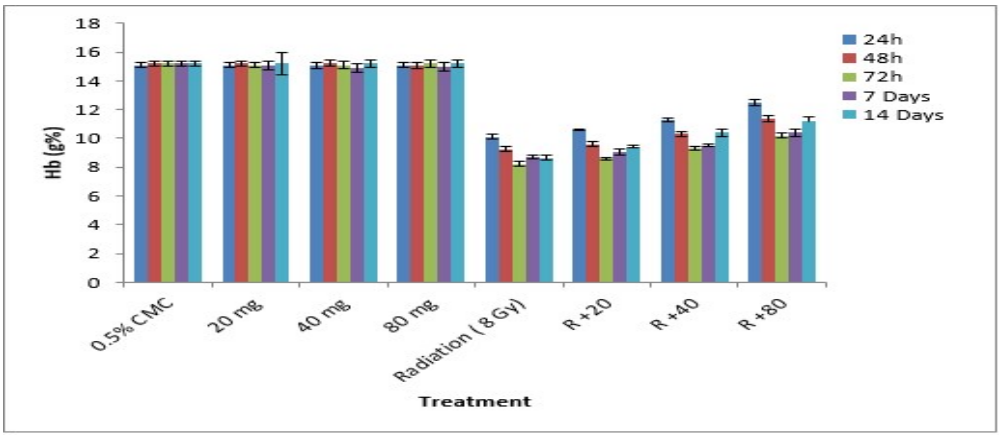

Figure 6: Effect of Piper Nigrum on Hb Content in Swiss Albino Mice Exposed to E-Beam Radiation.

Intestinal Histology

The result of histoprotective studies is given in fig. 3.7 to 3.10. The sham control group did not show any change in the intestinal histological structures, as evidenced by the presence of a large density of crypts at the base of the villi. In the irradiated group, the villus height and mucosal length were found to be significantly shorter compared to the sham control, demonstrating the damaging effects of irradiation on the intestinal mucosa. Irradiation also reduced the number of

surviving crypts significantly. Mucosal thinning was also observed. Both *P.betle* and *P.nigrum* extracts showed histoprotective effects; the former being more potent than the latter. The extract treated groups showed a significant increase in the number of surviving crypts compared to the radiation alone group. The villus height, crypt depth and mucosal length in the combined treatment group were found to be greater than those of the radiation alone group.

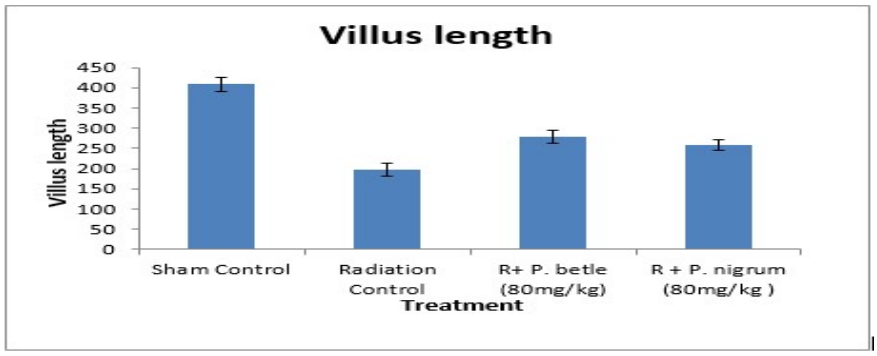

Figure 7: Effect of Piper Betle and Piper Nigrum on E-Beam Radiation Induced Changes in Villus Length.

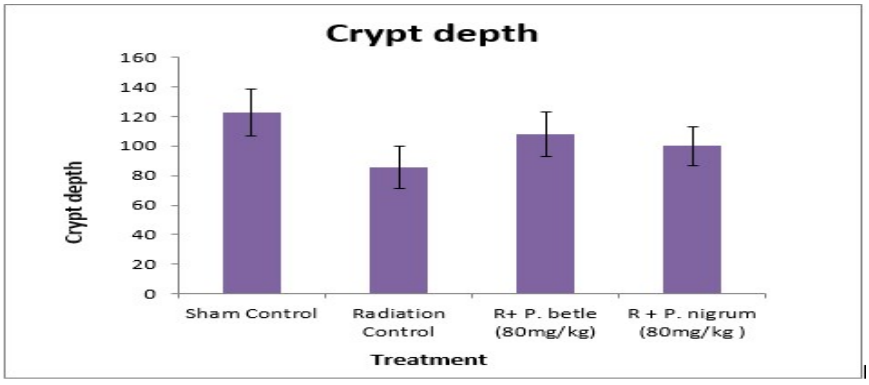

Figure 8: Effect of Piper Betle and Piper Nigrum on E-Beam Radiation Induced Changes in Crypt Depth.

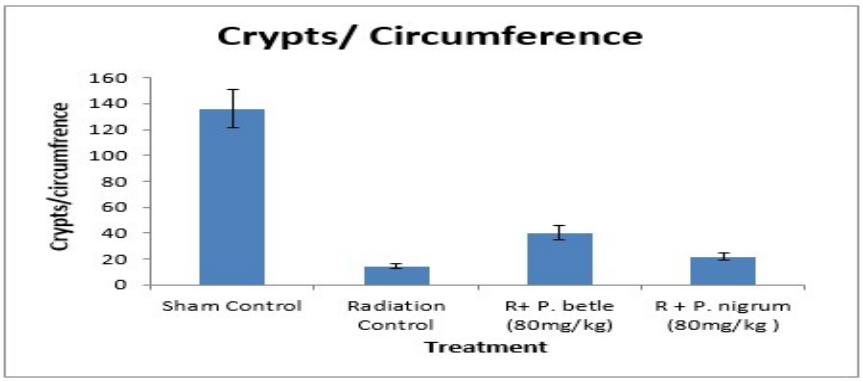

Figure 9: Effect of Piper Betle and Piper Nigrum on E-Beam Radiation Induced Changes in the Crypt Number.

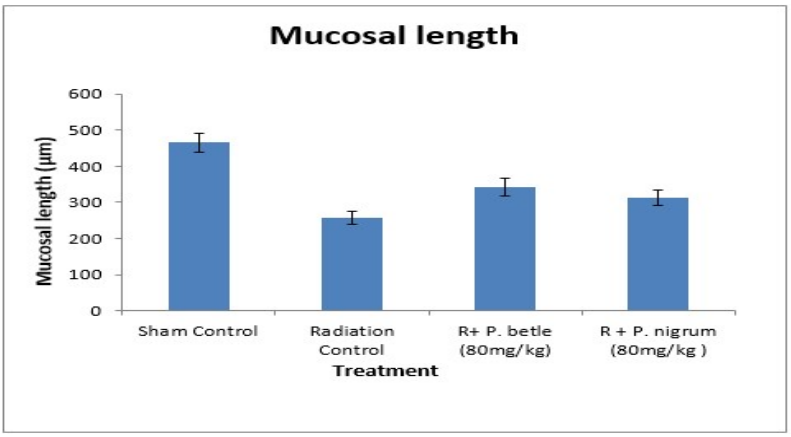

Figure 10: Effect of Piper Betle and Piper Nigrum On E-Beam Radiation Induced Changes in Mucosal Length of Intestine.

DISCUSSION

Currently the use of radiation in medical diagnosis in high technology societies is expanding [10, 11]. The number of radiological and nuclear medicine examinations performed has been increasing rapidly in recent years [12]. The effects of radiation exposure that become apparent to the clinician or the patient during the weeks, months and years after radiotherapy are seen both in tumor tissues and in the normal tissues that surround a tumor and which are unavoidably exposed to radiation [13]. The responses of normal tissues to radiation exposure range from those that cause mild discomfort to other severe effects that are life threatening. Radiobiology plays a crucial role in understanding these effects. The primary task of radiation biology is to explain observed phenomena and to suggest improvements to existing therapies. Understanding the processes that occur at the cellular level during a radiotherapy treatment presents major challenges [14].

Our study is in agreement with the findings of others [15]. In their experiment on the gamma radiation induced hematological and biochemical alterations in Swiss albino mice, they found that there was a significant decrease in the hematological constituents of peripheral blood in animals of the irradiation alone group. They stated that the decline in hematological constituents may be attributed to a direct damage by radiation. It was observed that there was a considerable decrease in the haematological values like erythrocytes, leukocytes, haematocrit and haemoglobin, post-irradiation with gamma radiation as compared to non-irradiated counterparts in Swiss albino mice [16]. Maximum decline in pro- and normoblasts, erythrocytes, leukocytes, haematocrit, haemoglobin and non-neutrophilic granules was observed on day 3 following irradiation. It has been reported that a significant decrease of human neutrophilic granulocyte function at 3.5 Gy and 4.0 Gy sublethal radiation dose [17]. Decrease in the haemoglobin content is a measure of anaemia and is attributed to the decline in the number of red blood cells [18]. In the present investigation, it was observed that haemoglobin levels declined significantly following radiation exposure. These observations are in agreement with the findings of others [19].

There are reports indicating a reduction in nucleated marrow cells, erythrocytes, leucocytes, platelets, and reticulocytes noticeably on day 1 post irradiation [20]. Our observations are in corroboration with these findings. However, there was a slight variation in our results, wherein there was a steep decline in the values of all the three blood parameters on the 3rd day post-irradiation for all the doses chosen. The blood cell count and Hb content decreased significantly from 24 to 72 hr post-irradiation, but showed an increase in their counts on the 7th day and the same condition was maintained on the 14th day as well. However, the recovery rate was slower for higher radiation doses when compared to that of lower doses.

The decrease in the values of haematological parameters following radiation exposure may be assigned to direct damage caused by a lethal dose of radiation [21]. Although, 3 Gy total body dose is required to produce detectable depletion in total erythrocyte cells, the whole body irradiation of the moderate dose range (5-10 Gy) leads to a decreased concentration of all the cellular elements in the blood. This may be due to a direct destruction of mature circulating cells, loss of cells from the circulation by haemorrhage, or leakage through capillary walls and loss of production of cells [22]. The acute radiation syndrome occurs after whole or significant partial body irradiation of 1 Gy delivered at a relatively high dose rate [23].

Haematological studies revealed that the TEC decreased with an increase in radiation dose and at 72 hr there was a sharp decline in their numbers. There was a recovery after 7 days. However, the recovery was not significant for 10 Gy. Similar results were observed with reference to TLC as well. There was a significant decrease in their count with an increase in radiation dose, with a maximum decrease on the 3rd day. After the seventh day there was a rapid increase in their numbers at lower doses and at higher doses recovery was slow. The haemoglobin content also showed a steady decline with increase in radiation dose with least value obtained on the 3rd day after irradiation. After the 7th day there was an increase in the haemoglobin content in the radiation exposed animals.

Exposure of animals to ionizing radiation causes a series of physiological changes known as acute radiation syndrome, which is dependent on the exposure dose and may lead to death. The damage to the haematopoietic system is a major factor in the mortality following an acute radiation exposure [16]. The bone marrow stem cells are more sensitive to radiation damage than the intestinal crypt; however, the peripheral blood cells have a longer transit time than the intestinal cells and hence the hemopoietic syndrome appears later than the gastrointestinal syndrome. In mice, death due to irradiation from 11 to 30 days is due to the hemopoietic damage inflicted by radiation to the hemopoietic organs like the bone marrow [24].

We found that e- beam radiation induced sufficient damage in the intestinal mucosa. The sham control group showed the presence of a large density of crypts at the base of the villi. There was a significant reduction in the number of surviving crypts in the irradiation group. In the irradiation control group, the villus height and mucosal length of mice 3 days after irradiation with the dose of 8 Gy was found to be significantly shorter compared to the sham control mice, demonstrating the injurious effects of irradiation on the intestinal mucosa.

Our findings are in concert with the observations of other scientific groups [25]. They reported the changes in epithelial and non-epithelial cells in small intestine after irradiation using gamma radiation and found a drop in number of visible enterocytes and endocrine cells at 1 and 3 days post irradiation. They also found that by 7 days after irradiation, the number of enterocytes and other epithelial cells returned to control levels. Katanyutanon et.al. (2008) The mechanisms of radiation-induced GI tract injury are complicated and there exists a relationship between alterations of plasma GI peptides and symptoms and signs of the radiation sickness. The levels of GI peptides measured in their study returned to normal at 8 days post irradiation. This is probably due to the recovery of GI peptide-producing cells and mucosa from radiation-induced injury [26]. It is found that after 12 Gy γ-irradiation, the villus height and mucosal depth significantly decreased in mice and the recovery from irradiation-induced intestinal injury is dependent on colonogenic stem cell survival. At least one stem cell in the crypt must survive in order for the crypt to survive. If too many crypts are sterilized, ulceration of the intestine will ensue, and if extensive ulceration occurs the animal will die within 3– 10 days. Such sterilized crypts disappear over the first 2 days post-irradiation. Crypts that contain one or more surviving clonogenic stem cells regenerate to form a crypt structure at 3–4 days post-irradiation [27].

Various studies in animals and humans have shown that both small and large intestinal permeability is increased following ionising radiation exposure [28, 29, 30]. These alterations are most marked in the acute response period and are associated with epithelial cell loss and modifications in junctional complexes. Renewal of the intestinal epithelial barrier depends upon an active stem cell compartment similar to the haematopoietic system and it is this compartment that has been shown to be particularly sensitive to ionising radiation exposure. With increasing radiation dose, the stem cells cannot produce enough cells to repopulate the villi, which results in blunting and diminution in villus height and eventual functional incapacity [31].

After whole body exposure to very large doses of radiation, sensitive cells die, resulting in serious damage to select organs and systems; while a sufficient genetic alteration may lead to cancer, a sufficient cell killing contributes to radiation sickness. Radiation sickness is caused by damage to organs or systems after exposure to very high doses of radiation. There is general scientific consensus that no matter how small, radiation exposure always increases the risk of tissue injury [32]. High doses of radiation can also produce effects long after the exposure (late effects) resulting in adverse health effects within a short time of minutes (CNS Syndrome), days (GI Syndrome) to weeks (Hematopoetic Syndrome) or years (Cancer) after exposure [33].

The gastrointestinal (GI) tract is among the most radiosensitive organ systems in the body. In addition to the intestinal epithelium crypt, radiation exposure damages supporting structures such as endocrine glands of the GI tract [34]. Ionizing radiation damages human tissue in many ways [35]. It can interact directly with cellular macromolecules such as DNA, mRNA and proteins to break their covalent bonds, and irreversibly destroy their structure. Ionizing radiation can also indirectly interact with cells by causing hydrolysis reaction of cellular water resulting in hydrogen molecules and hydroxyl (free radical) molecules that disrupt adjacent cellular architecture and genomic information. These interactions may eventually lead to either mutation of the cell or cell death [26].

In our current study, we demonstrated that exposure of mouse to e-beam radiation significantly decreases the blood counts which were ameliorated by *Piper betle* and *Piper nigrum* extracts rendering protection against radiation-induced damage as evidenced in all haematological parameters studied. Both *Piper betle* and *Piper nigrum* elicited protection against radiation injury on the blood cells. There was an increase in the TEC, TLC and Hb content in radiation exposed animals when treated with the plant extracts in a dose- dependent manner. Both *Piper betle* and *Piper nigrum* elicited protection against the effect of radiation. The recovery rate was slow in the earlier days after radiation exposure and there was a steady decline in the TEC, TLC and Hb content on the 3rd day. Restoration was seen after the 7th day and rate of restoration of Hb content was at a slower pace when compared to RBC and WBC counts.

Histological preparations of the sham control group did not show any change in the intestinal structures, as evidenced by the presence of a large density of crypts at the base of the villi. In the irradiated group, the villus height and mucosal length were found to be significantly shorter compared to the sham control, demonstrating the damaging effects of irradiation on the intestinal mucosa. Irradiation also reduced the number of surviving crypts significantly. Mucosal thinning was also observed. Both *P.betle* and *P.nigrum* extracts showed histoprotective effects; the former being more potent than the latter. The extract treated groups showed a significant increase in the number of surviving crypts compared to the radiation alone group. The villus height, crypt depth and mucosal length in the combined treatment group were found to be greater than those of the radiation alone group.

These observations are in agreement with the findings of others. In one of the studies, pre-treatment with MGN-3 (Biobran) protected blood cells from radiation-induced damage and improved the blood count [36]. A significant decrease in WBC and RBC counts in animals irradiated with 7.5 Gy of gamma radiation was observed. Pre-treatment of mice with thymol showed a marginal increase in WBC and RBC counts compared to the radiation alone group [37]. Treatment with *Prunus avium* showed a rise in haematological parameters in radiation exposed animals [38].

There are several reports on the gastrointestinal protectivity of plant extracts/ compounds against radiation. *Hippophae rhamnoides* has been shown to provide protection to the gastrointestinal system against lethal whole-body γ-radiation. Administration of a hydroethanolic extract before irradiation increased the number of surviving crypts in the jejunum and villi cellularity in comparison with the irradiated control [39]. It was reported that *Emblica officinalis treatment rendered protection against* Radiation sickness, tissue injury and survival studies in mice exposed to γ- radiation [40]. The protective effects of *Isatis indigotica on* Radiation injury and Antiinflammatory activity was demonstrated by using γ- radiation [41].. *Rosemarinus officinalis* rendered radioprotection by restoration of small intestine architecture and showed antioxidant activities [42, 43]. Whole body irradiation of mice resulted in distorted villus morphologies, with depopulated and degenerating crypts and reductions in villus height and crypt cell numbers. Mice that received thymol before irradiation had decreased amounts of damage compared to the irradiated controls [37].

In our investigation, we evaluated the histoprotective effects of *Piper* extracts against e-beam radiation. The villus height and mucosal length in the plant extract treated groups were observed to be higher than those of the irradiation + CMC treatment. The crypt depths were increased in the extract-treated groups compared to that of the irradiation control group. These observations indicate the protection rendered by the plant extracts of our study against radiation induced tissue injury.

CONCLUSION

Radiotherapy is one of the important treatment strategies in combating various dreaded diseases including cancer. The need of the hour is to find a potent radiosensitizer or radioprotector derived from natural source which offers protection to the normal cells and tissues against the side effects of radiation exposure. The plants of our study, *Piper betle* and *Piper nigrum* have tremendous medicinal properties and have been used extensively in traditional medicine. Our approach was to explore the radioprotective property of these plants and we found that both the plants alleviated the electron-beam induced tissue-damage as evidenced by the haematological parameters and histological observations. There is scope for isolating phytochemicals rendering radioprotectivity and the use of purified compounds isolated from these plant sources for combination therapy cannot be ruled out.

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