

ROLE OF ROSEMARY LEAVES EXTRACT AGAINST RADIATION-INDUCED HEMATOLOGICAL AND BIOCHEMICAL ALTERATIONS IN MICE

by

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The present paper is a study of the modulatory effect of *Rosmarinus officinalis* leaves extract on radiation-induced hematological and biochemical changes in Swiss albino mice. The dose reduction factor for the Rosemary extract against gamma rays was calculated 1.53 from LD_{50/30} values. The Rosemary extract was administered orally for 5 consecutive days prior to radiation exposure. The hematological and biochemical parameters were assessed from day 1 to 30 post-irradiation intervals. The total erythrocyte count, total leucocytes count, hemoglobin, and hematocrit values in the experimental group were found to be elevated as compared to the control group of mice. Furthermore, the Rosemary extract treatment enhanced reduced glutathione content in the liver and blood against radiation-induced depletion. Treatment with the plant extract brought a significant fall in the lipid peroxidation level, suggesting rosemary's role in protection against radiation-induced membrane and cellular damage. The results from the present study suggest a radio-protective effect of the Rosemary extract against radiation-induced hematological and biochemical alterations in mice.

Key words: gamma radiation, glutathione, hematology, irradiation, lipid peroxidation
Rosmarinus officinalis, Swiss albino mice

INTRODUCTION

The problem of radiation hazard to living beings has risen due to an increasing use of nuclear energy in industrial, medical, engineering, and scientific research. The inadvertent exposure of human to radiation alters the physiological and metabolic functioning of specific body organs by causing ionization and excitation of molecules, leading to the formation of free radicals. Free radicals are believed to play a role in more than sixty different health conditions, including the ageing process, cancer, radiation damage, atherosclerosis, etc. [1, 2].

In recent years, there has been considerable interest in the possibility of modification of radiation injuries in biological systems. The development of effective radioprotectors and radio recovery drugs is of great importance in view of their potential application during both planned radiation exposure (*i. e.*, radiotherapy) and unplanned radiation exposure (*i. e.*, in the nuclear industry, natural background radiation, etc.) [3]. These drugs are also likely to be useful in nuclear warfare to provide protection to personnel [4].

Numerous drugs of both natural and synthetic origin, *e. g.*, antioxidants [5, 6], cytoprotective agents [7], metalloelements [8], immunomodulators [9, 10], sulphhydryl compounds [11, 12], vitamin A, C, and E [13, 14], have been tested in both *in vitro* and *in vivo* models, human clinical trials, as well as in animal models, to ameliorate injuries caused by exposure to radiation. Despite extensive work done in this field, not a single synthetic compound is available so far as an effective non-toxic radioprotector for practical purposes [15, 16]. Therefore, the search for alternative sources as ideal radioprotective agents, including herbs, is on going [17-19].

Various plants and plant products have been utilized successfully for the treatment of free radi-

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cal-medicated diseases in humans such as rheumatoid arthritis, cancer, Alzheimer's disease, Parkinson's disease, aging, and several other conditions including inflammatory diseases [20, 21]. It is, therefore, reasonable to expect that plants may contain certain compounds that can protect against radiation-induced reactive oxygen species (ROS) mediated damage. A number of medicinal plants evaluated for their radioprotective efficacy have shown protective effects against the damaging effects of ionizing radiation [16, 22-24]. Because of their low toxicity, naturally occurring dietary components offer opportunities for development as radioprotectors [25].

Rosmarinus officinalis L. (family *Lamiaceae*) is appreciated as a medicinal plant mainly for its antioxidant, pharmacological, as well as culinary properties. It is native to Southern Europe grows widely along the north and south coasts of the Mediterranean sea and also in the sub-Himalayan areas of India. This plant has shown to be safe in toxicity studies in animal models when added as an antioxidant to food. Well popular as "Dew of the sea", rosemary has a history of medicinal use as a tonic and stimulant, analgesic, antireumatic, carminative, diuretic, expectorant, anti-epileptic, anti-spasmodic in renal colic, dysmenorrhoea, relieving respiratory disorders effects and for effects on human fertility [26]. The present study has been undertaken to evaluate the effect of *R. officinalis*' leaves extract on radiation-induced hematological and biochemical alterations in Swiss albino mice. Rosemary leaves are used in food industry and have shown to be safe in animal tests [27, 28].

MATERIALS AND METHODS

Animal care and handling: Animal care and handling were performed according to guidelines issued by the World Health Organization (Geneva, Switzerland) and the Indian National Science Academy (New Delhi, India). The Department's Ethical Committee has approved the present study. Adult male Swiss albino mice (6-8 weeks old) weighing 23 ± 2 g from an inbred colony were used for the present study. The animals were maintained on standard mice feed (procured from Hindustan Lever Ltd., India) and water *ad libitum*. Four animals were housed in a polypropylene cage containing paddy husk (procured locally) as bedding throughout the experiment.

Preparation of the extract: The identification of the plant *Rosmarinus officinalis* L. (Family: *Lamiaceae*) was done by Dr. Deepak Acharya (Voucher Specimen No: DDC/2001/DEPTBT/ACHARYA2430) of the Department of Botany, Danielson College, Chhindwara, Madhya Pradesh, India. The non-infected, cleaned, shade dried leaves were powdered and extracted with double distilled water (DDW) by refluxing for 36 hours at 50-60 °C. Pellets of the drug were obtained by evaporating its liquid content in the incubator. For radiation

treatment, the selected doses were prepared by dissolving the drug pellets in double distilled water (1000 mg/kg body wt.)

Determination of acute toxicity of Rosmarinus officinalis extract: The acute toxicity of the *Rosmarinus officinalis* extract (RE) was determined according to Prieur *et al.* [29] and Ghosh [30]. Briefly, the animals were fasted by withdrawing food and water for 18 hours and divided into groups of 8 each. Each group of mice was administered with various doses, *i. e.*, 100, 200, 400, 800, 1000, 1500, and 2000 mg/kg b. wt. of the plant extract orally (5 consecutive days). The animals were provided with food and water immediately after the administration of the drug. The mortality of the animals was observed up to 30 days post-RE treatment and acute LD₅₀ of RE was calculated.

Irradiation: One hour after the last administration of DDW or RE, the mice were whole body exposed to ⁶⁰Co (cobalt teletherapy unit) gamma radiation source at the Cancer Treatment Centre, Radiotherapy Department, SMS Medical College & Hospital, Jaipur, India. A batch of 12 animals was irradiated to 9 Gy radiation, each time in a specially designed well-ventilated acrylic box, at a dose rate of 0.85 Gy per minute at a source-to-animal distance (midpoint) of 80 cm.

Selection of optimum dose: The dose selection of RE was done on the basis of our previously conducted drug tolerance and animal survival study [16]. Various doses of RE (100, 200, 400, 800, 1000, 1500, and 2000 mg/kg b. wt.) were tested against gamma irradiation (9 Gy) for radiation sickness and mortality. An optimum dose (1000 mg/kg b. wt.) thus obtained was used for experimentation in details.

Protocol: The mice for this study were divided into four groups: animals in Group-I were administered orally with DDW (volume equal to RE) to serve as Normal, while animals in Group-II (RE control) were given RE orally in a dose of 1000 mg/kg body wt. per day for 5 consecutive days. Animals of Group-III (irradiation control) received an equal volume of DDW (as in Group-I) and were exposed to 9 Gy gamma rays. Animals in Group-IV (RE experimental) were given RE (as in Group-II), one hour before exposure to 9 Gy gamma irradiation.

All animals were monitored for weight change, behavioral changes, mortality, food, water consumption *etc.*, up to 30 days. The animals from the above groups were autopsied at days 1, 3, 5, 10, 20, and 30 post-treatment intervals of post-irradiation. Blood samples were collected from the orbital sinus of mice from respective groups and examined for histopathological and biochemical alterations after routine procedures.

Biochemical alterations were studied in all the groups at one hour post-exposure to 9 Gy gamma rays. Glutathione (GSH) content in the blood and liver was estimated spectrophotometrically using Ellman's reagent (DTNB) as a coloring reagent as per the method

described by Beutler *et al.* [31] and Moron *et al.* [32], respectively. The absorbance was read at 412 nm, using a UV-VIS Systronic spectrophotometer. The lipid peroxidation level in the serum and liver was measured using thiobarbituric acid reactive substance (TBARS) by the method of Ohkhawa *et al.* [33]. The absorbance was read out at 532 nm, using a UV-VIS Systronic spectrophotometer.

Statistical analysis: The result for all the groups at various autopsy intervals was expressed as mean \pm standard error. The Student's 't' test was used for statistical comparison between the groups and significance level was set at different levels as $p < 0.05$, $p < 0.01$, and $p < 0.001$.

RESULTS

No noticeable signs of behavioral changes, sickness or mortality were observed in the sham-irradiated (Group-I) and RE-treated group (Group-II). These animals appeared quite healthy in all respects. In the sham-irradiated (Group-I) and rosemary alone treated (Group-II) mice, a consistent weight gain was observed from day 3 onwards, reaching the maximum by the last autopsy interval. The total erythrocyte counts, hemoglobin percentage, hematocrit value and total leucocyte counts did not show any significantly notable changes after sham-irradiation. The various hematological parameters as described in Group-I animals closely followed the pattern in RE alone treated mice (Group-II), depicting the fact that the plant extract alone did not bring about notable changes in blood components of Swiss albino mice (tab. 1).

Erythrocytes: In group III, erythrocytes count decreased significantly below normal ($p < 0.001$) at all autopsy intervals. The maximum decline (4.07 ± 0.12)

was recorded as early as day 1 post-irradiation, after which a gradual increase was noticed at later intervals. No animals in this group survived beyond the 10th day post-autopsy interval. In animals pre-treated with RE prior to irradiation (group IV), erythrocyte counts showed a highly significant ($p < 0.001$) increase over the control (Group-I) up to the 10th day post-treatment. However, maximum decrease (5.83 ± 0.17) was observed on day 3 post-irradiation. Thereafter, a recovery was evident, but the normal value could not be attained even after the 30th day radiation exposure (tab. 1).

Hemoglobin: In irradiated alone (Group-III) animals, a marked drop in the hemoglobin level (9.74 ± 0.13) was recorded on day 1 post-irradiation. Thereafter, a gradual increase was evident up to the 10th day, but after that no animal survived later autopsy intervals. In the RE pretreated irradiated group, the values of hemoglobin showed a significant ($p < 0.001$) increase over the control group. After the day 1 autopsy interval, a continuous recovery in the hemoglobin level was measured up to day 30 post-irradiation. However, a normal value could not be obtained even on the 30th day of post-treatment interval (tab. 1).

Hematocrit: A highly significant decrease ($p < 0.001$) from normal was observed in group III, at all autopsy intervals studied. However, the fall was maximal (28.65 ± 0.52) on the 1st day of the post-autopsy interval. Thereafter, a recovery was recorded up to day 10, but the values remained significantly below normal. In RE treated irradiated mice (Group-IV), a similar trend was noticed in both the hematocrit and erythrocytes count (tab. 1).

Leucocyte: The leucocytes count decreased significantly ($p < 0.001$) below normal with a maximum decline (2.71 ± 0.16) on day 3 post-irradiation. No animal survival was seen in this group after day 10

Table 1. Variations (mean \pm standard error) in hematological counts after exposure to 9 Gy gamma rays with (experimental) or without (control) *Rosmarinus officinalis* L. extract

| Hematological parameters | Post – irradiation intervals | | | | | | | | | | | | |
|--------------------------|------------------------------|-------|-------------------|-------|-------------------|-------|-------------------|--------|-------------------|--------|------|--------|------|
| | Groups | Day 1 | | Day 3 | | Day 5 | | Day 10 | | Day 20 | | Day 30 | |
| Erythrocytes | Control | 4.07 | 0.12 ^c | 4.13 | 0.13 ^c | 4.41 | 0.12 ^c | 5.37 | 0.21 ^c | n. s. | | n. s. | |
| | Experimental | 5.94 | 0.20 ^c | 5.83 | 0.17 ^c | 7.12 | 0.19 ^c | 7.38 | 0.14 ^c | 7.62 | 0.19 | 7.96 | 0.12 |
| Hemoglobin | Control | 9.74 | ^c | 10.18 | 0.14 ^c | 10.67 | 0.10 ^c | 11.78 | 0.22 ^c | n. s. | | n. s. | |
| | Experimental | 10.87 | 0.23 ^c | 11.33 | 0.19 ^c | 11.59 | 0.21 ^c | 11.83 | 0.16 ^c | 12.10 | 0.19 | 12.47 | 0.13 |
| Hematocrit | Control | 28.65 | ^c | 30.75 | 1.01 ^c | 30.34 | 0.48 ^c | 31.94 | 0.59 ^c | n. s. | | n. s. | |
| | Experimental | 34.83 | 0.84 ^c | 34.18 | 0.34 ^c | 35.64 | 0.60 ^c | 38.31 | 0.63 ^c | 39.59 | 0.43 | 40.45 | 0.46 |
| Leucocytes | Control | 2.86 | 0.15 ^c | 2.71 | 0.16 ^c | 3.18 | 0.17 ^c | 3.78 | 0.24 ^c | n. s. | | n. s. | |
| | Experimental | 3.96 | 0.21 ^c | 4.03 | 0.27 ^c | 4.21 | 0.12 ^c | 4.43 | 0.18 ^c | 4.84 | 0.20 | 5.06 | 0.12 |

Normal values:

Erythrocytes = $8.67 \pm 0.14 \times 10^6/\text{mm}$, Hemoglobin = $13.18 \pm 0.13 \text{ gm/dl}$, Hematocrit = $42.22 \pm 0.38\%$, Leucocytes = $5.86 \pm 0.14 \times 10^3/\text{mm}$, Control = DDW + irradiation; Experimental = RE + irradiation; n. s. = no survival

Statistical comparison:

Control vs. Normal; Experimental vs. Control

Significance levels:

^ap ^bp ^cp 0.001

post-exposure. In Group-IV, leucocyte counts were observed as significantly ($p < 0.001$) higher than in the control group throughout the experiment. Initially, a drop in the number of such cells was noticed on day 1 post-treatment (3.96 ± 0.21). Later, it exhibited a continuous rise up to day 30th post-interval, but the values remained below normal (tab. 1)

Glutathione (GSH): Blood and liver GSH level in Group-I was estimated as 3.47 ± 0.12 and 62.87 ± 1.36 , respectively. In RE alone administered (Group-II) animals, no significant alterations in the GSH content were observed as compared to normal (Group-I). In animals exposed to 9 Gy rays alone (Group-III), a significant fall in blood and liver GSH level was recorded ($p < 0.001$) in comparison to normal. However, in RE pretreated irradiated mice (Group-IV), a significant increase ($p < 0.001$) was observed in comparison to group-III, but the difference was not significant (tab. 2).

Lipid peroxidation (LPx): Serum and liver LPx in Group-I (normal) mice was measured after 1 hour and was found to be $1.25 \mu\text{mol/ml}$ and $2.65 \mu\text{mol/mg}$, respectively. No significant alterations as compared to normal were observed in RE alone treated animals (Group-II). In the irradiated alone control (Group-III), the LPx level increased after radiation exposure of 9 Gy. RE pre-treatment (in group-IV) significantly lowered lipid peroxidation, as it was measured above normal in both blood and liver in the experimental Group-IV (tab. 2).

DISCUSSION

Several attempts have been made to inhibit radiation-induced injuries using a variety of synthetic and natural radioprotectors in mammals. But chemical compounds do not qualify radiation protection at a non-toxic, low dose in occupational or therapeutic settings. Since it is a common fact that medicinal plants and plant products are eco-friendly, harmless, and cheap, they have become a major avenue for the discovery of new radio-protective drugs [15, 34, 35].

Some earlier studies on radioprotection show that an agent tested for its radio-protective action is ef-

fective only at a particular dose [35-37]. A similar action can not be ruled out for RE, which provided an optimum protection at 1000 mg/kg, while the higher doses resulted in a steady decline in its protective properties. Hematological counts serve as sensitive parameters determining the protective efficacy of such compounds. In the present study, RE pre-treatment shows a significant increase in the total number of erythrocytes, leucocytes, hemoglobin content, and hematocrit values as compared to their respective controls at all other intervals.

To the contrary, all animals of Group-III died within 30 days when exposed to the 9 Gy radiation dose. The first death was recorded on day 7th, followed by a 100% mortality within 20 days of the 9 Gy radiation exposure. According to Casarette [38], damage to the hematopoietic system is a major factor in the mortality rate following acute radiation exposure. RE pre-treatment provided enhanced survival in mice and also, protection against hematopoietic death, probably by shielding hematopoietic stem cells responsible for the regeneration and recovery of the system. The process, possibly, stimulates cellular regeneration and, thus, causes early recovery. Similar observations have been reported earlier with some herbal preparations like Triphala, Abana, Septlin [39], *Syzygium cumini* [40], and *Brassica campestris* [41].

At a higher dose of 9 Gy, the erythrocyte count fell more sharply up to day 7, but recovered there after. Fred and Smith [42] suggested that the radiation-induced depletion of hematopoietic stem cells may be an important factor contributing to the decline in the erythrocytic population. The acute decline in the total RBC count may be attributed to the leakage on account of hemorrhage caused by radiation induced lesions in blood vessels [43]. However, the RBC count was significantly higher in experimental Group-IV than in Group-III. Recently, Samarth et al. [44] observed a similar protective efficacy of a plant extract, *Mentha piperita*, against radiation-induced depletion in RBC and hemoglobin. A drop in the hemoglobin content (Group-III) may be attributed to the decreased erythropoiesis and, hence, a decrease in the number of red blood cells and/or the leakage of RBC into lymphatic tissue and other tissue spaces due to increased capillary permeability. The de-

Table 2. Reduced glutathione and lipid peroxidation level in blood and serum of Swiss albino mice after exposures to different doses of gamma rays with (experimental) or without (control) *Rosmarinus officinalis* L. extract

| Treatment | Glutathione | | Lipid peroxidation | |
|--------------------------|----------------------------|------------------------------|------------------------------------|------------------------------|
| | Blood [$\mu\text{g/ml}$] | Liver [$\mu\text{mol/mg}$] | Blood serum [$\mu\text{mol/ml}$] | Liver [$\mu\text{mol/mg}$] |
| Normal | 3.47 0.12 | 62.87 | 1.25 0.06 | 2.65 0.17 |
| RE treated | 3.57 0.12 | 63.32 1.13 | 1.19 0.11 | 2.54 0.12 |
| 9 Gy (control) | 2.03 0.01 ^c | 31.15 0.27 ^c | 5.63 0.41 ^c | 9.82 0.25 ^c |
| RE + 9 Gy (experimental) | 2.85 0.04 ^c | 51.20 0.43 ^c | 3.12 0.02 ^c | 5.16 0.07 ^c |

Statistical comparison:
Control vs. normal

Significance levels:
^a $p \leq 0.05$, ^b $p \leq 0.01$, ^c $p \leq 0.001$

crease in the hemoglobin content as observed in the present study may also be due to the depletion in the synthesis of hemoglobin after radiation exposure. Nunia and Goyal [45] observed the inhibition of hemoglobin synthesis in animals after radiation exposure. In RE pre-treated mice (Group-IV), the level of hemoglobin was maintained higher than their control values at all other autopsy intervals, indicating that the RE has a protective action on the hemoglobin content. The difference between hemoglobin content of the control and experimental groups at early post-irradiation intervals appears to be closely associated with the elevated RBC counts observed in RE pre-treated animals in the present study. Thus, the protection of hemoglobin is closely related with the protection of RBC and erythropoiesis by rosemary.

The decrease in the hematocrit value can be attributed to the failure of erythropoiesis, destruction of mature cells, and internal bleeding. The depression in the hematocrit value can also be attributed to the total cell depletion in peripheral blood aided by disturbances in steady-state mechanisms in blood forming organs, as well as an increase in plasma volume after irradiation. In RE treated animals, hematocrit values showed a consistent recovery over control from 24 hours onwards; however, a normal value could not be attained at a 9 Gy radiation dose.

It is a well known fact that radiation exposure reduces the number and functional activity of circulating lymphocytes and changes the distribution and ratio of their sub populations [46]. Patt [47] stated that the rapid decline in the lymphocytes number is due to the direct destruction of such cells in peripheral blood. Rosemary extract treatment to Group-IV mice may have stimulated or protected hematopoiesis and the bone marrow and brought about a subsequent rise in hematological constituents in peripheral blood.

Glutathione is a free-radical scavenger that offers protection against oxygen derived free radicals and cellular lethality following exposure to ionizing radiation [48]. Proper maintenance of GSH is critical for keeping a check on cellular homeostasis [49]. Under normal conditions, the inherent defense system, not excluding GSH and antioxidant enzymes, protects against oxidative damage [50, 51].

A significant decrease in levels of GSH was registered in Group-III mice in comparison to the normal (Group-I) animals. Furthermore, in animals pre-treated with RE prior to gamma radiation (Group-IV), an increase (as compared to group III) in hepatic and blood GSH level was recorded. The present study demonstrates that oral administration of RE to Swiss albino mice did not significantly influence the endogenous GSH level. The lower depletion of liver and blood GSH in Group-IV could be due to the higher availability of GSH, which increases the ability to cope with the free radicals produced by radiation.

In the present study, an increase in LPx level (hepatic as well as serum) was observed following radiation exposure. It was observed that, although RE treatment did not significantly alter the LPx level in non-irradiated animals, it considerably diminished the generation of radiation-induced lipid peroxidation in terms of malondialdehyde production. The increase of lipid peroxidation byproduct (malondialdehyde) following radiation exposure as revealed in the present study is a clear indication of increased oxidative stress. Kilic *et al.* [52] observed that lipid peroxidation starts as soon as the endogenous GSH gets exhausted, and the addition of GSH stops further peroxidation promptly. In Group-IV, an increase in the GSH concentration, towards normal, could have resulted in the reduced levels of LPx, thereby protecting against damage caused by radiation. Shimoi *et al.* [53] proposed that plant flavonoids, which show antioxidant activity *in vitro*, function as antioxidants *in vivo*, and their radioprotective effect may be attributed to their radical scavenging activity.

The radio-modulatory effect observed after the *R. officinalis* leaves extract treatment may be due to the significant elevation in GSH level and a decreased MDA formation in the blood and liver of Swiss albino mice. There are several pathways of radioprotection that have been suggested against the damaging effects of ionizing radiation in mammalian cells. The mechanisms that radio-protectors implicate include free radical scavenging that protects against ionizing radiation-generated ROS or chemotherapeutic agents and hydrogen atom donation that facilitates direct chemical repair at sites of DNA damage.

Rosemary contains active constituents like carnosol, carnosic acid, caffeic acid, rosmarinic acid, ursolic acid, different phenols, diterpenes, and flavanoids [26, 54, 55] that have been subjected to various pharmacological investigations. Findings of Offord *et al.* [56] confirm that natural polyphenols found in rosemary have not only potent antioxidant activities but also anticarcinogenic properties.

The RE might have scavenged ROS generated by ionizing radiation before they could interact with biochemical molecules, thus reducing the harmful effects of radiation. To investigate the exact mechanism of action and clinical applicability of *R. officinalis* as a radioprotector, further work is in progress. The results of the present study suggest that *R. officinalis* leaves extract modulates radiation-induced hematological and biochemical alterations, probably by providing protection from free radicals.

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УПОТРЕБА ЕКСТРАКТА РУЗМАРИНОВОГ ЛИСТА ПРОТИВ ЗРАЧЕЊЕМ ИЗАЗВАНИХ ХЕМАТОЛОШКИХ И БИОХЕМИЈСКИХ ПРОМЕНА У МИШЕВА

Рад представља проучавање модулаторних ефеката на зрачењем изазване хематолошке и биохемијске промене код швајцарских белих мишева услед примене екстракта листа *Rosmarinus officinalis*. Фактор редукције дозе до 1,52 за рузмаринов екстракт као средство против гама зрачења израчунат је из LD_{50/30} вредности. Рузмаринов екстракт уношен је орално пет узастопних дана пре излагања зрачењу. Хематолошки и биохемијски параметри процењивани су у интервалу од првог до тридесетог дана по озрачивању. Укупан број еритроцита и леукоцита, вредности хемоглобина и хематокрита у експерименталној групи биле су повишене у поређењу са контролном групом мишева. Поступак рузмариновим екстрактом повисио је редуковани садржај глутатиона у јетри и крви на супрот зрачењем изазваног слабљења. Лечење биљним екстрактом довело је до значајног пада нивоа липидне пероксидазе, указујући на улогу рузмарина у заштити од зрачењем изазваног оштећења мембране и ћелије. Резултати проучавања упућује на ефекте заштите рузмариновим екстрактом од зрачењем изазваних хематолошких и биохемијских промена у мишева.

Кључне речи: *зама зрачење, глутатион, хематологија, озрачивање, липидна пероксидаза, Rosmarinus officinalis, швајцарски бели миш*