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The protective effect of *Sambucus ebulus* against lung toxicity induced by gamma irradiation in mice

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Abstract

The aim of present study was to investigate the potential antioxidant and lung protective activities of *Sambucus ebulus* (SE) against toxicity induced by gamma irradiation. Hydroalcoholic extract of SE (20, 50 and 100 mg/kg) was studied for its lung protective activity. Phenol and flavonoid contents of SE were determined. Male C57 mice were divided into ten groups with five mice per group. Only the first and second groups (as negative control) received intraperitoneally normal saline fluid. Groups 3 to 5 received only SE extract at doses of 20 mg/kg, 50 mg/kg and 100 mg/kg intraperitoneally; three groups were repeatedly injected for 15 days as chronic group. Groups 6 to 8 received a single-dose of gamma irradiation just 2 hours before irradiation as acute group. The ninth and tenth groups (as positive control) received only gamma rays. Animal was exposed whole-body to 6 Gy gamma radiation. After irradiation, tissue sections of lung parenchyma were examined by light microscope for any histopathologic changes. SE at doses 50 and 100 mg/kg improved markedly histopathological changes induced by gamma irradiation in lung. Lung protective effect of SE could be due to attention of lipid peroxidation. Our study demonstrated that SE as a natural product has a protective effect against lung toxicity induced by gamma irradiation in animal.

Keywords: *Sambucus ebulusi*, gamma irradiation, histopathology changes, antioxidant, lung protective

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Introduction

Herbs, which have been used for centuries in treating various illnesses, play a major role in forming the basic platform of modern medicine (1). The therapeutic effects of many traditional herbs are due to the presence of natural antioxidants, especially phenolic compounds (2). These compounds are able to scavenge reactive oxygen species (ROS) that may cause various diseases related to oxidative stress such as cancer, hypertension, and impaiment To protect humans from oxidative stress, various herbs and plants are being utilized for their potential benefits in preventing diseases related to oxidative stress. One of these herbs is *Sambucus ebulus* (Dwarf elder). *Sambucus ebulus* (SE), family of *Caprifoliaceae*, is extracted from the roots and leaves of *Sambucus ebulus* L in traditional medicine. They are frequently used for the treatment of inflammatory diseases such as inflammatory joint diseases, rheumatic pain and sore throat (3). Several pharmacological effects have been previously reported for Sambucus species, such as anti-inflammatory (4-7), antiviral (8), antibacterial (9) and radical scavenging activities (7,10). Moreover, the significant inhibitory effects of the plant extracts reported on interleukins-1α and 1β and tumor necrosis factor-α. However, the potential of wound healing of *Sambucus* species has not been investigated so far. Therefore, the aim of present study was to investigate the potential antioxidant and Lung protective activities of SE extract induced by gamma-radiation. A number of studies have been reported on the phytochemical composition of SE leaves. Recently, six new iridoid glycosides of the Valeriana type have been isolated from the leaves of the plant (11). In addition to the

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secondary metabolites, a new family of acidic type 2 (ebulitins α, β and γ) and basic type 1 (ebulin 1) ribosome-inactivating nontoxic protein derivatives (12,13) and dimeric and mucin binding lectins and flavonoids were isolated from the matured leaves (14). However, other phytochemicals in the leaves of the plant have not been studied thoroughly. Flavonoids are used for their several therapeutic effects, such as antioxidant, anti inflammatory, antifungal, and wound healing (15, 16). Inhibition of lipid peroxidation effect by flavonoids is supposed to increase the viability of collagen fibrils by activating the DNA synthesis and preventing the cell damage (17, 18). Flavonoids are also known to promote the rapid wound healing due to their antimicrobial and astringent properties (19). Thus, wound healing effect of *SE* might belong to the phytochemicals in the leaves, accelerating the proliferation phase of wound healing. The constituents in the volatile extract and petroleum ether, ethanol, and water extract of SE exhibited clear antioxidant activities (3).

Basically, oxygen is essential for life, but it can also be harmful to cells. This is because of reactive oxygen species (ROS), active forms of oxygen, such as hydroxyl (OH^-) and superoxide (O_2^-) radicals, hydrogen peroxide (H_2O_2), and singlet oxygen ($1O_2$) which are arisen as by-products and intermediate substances of aerobic metabolism and during oxidative stress. Similar reactions are also generated during gamma ray irradiation (20, 21). To the best of our knowledge, lung protective activity of SE has not been reported so far and nothing has been found about mechanism of SE activity. Thus, the aim of the present work was to determine the lung protective activity (or antioxidant activity) of SE against oxidant-induced gamma radiation and also to understand the usefulness of this plant as a foodstuff as well as a remedy in medicine. Moreover, gamma radiation was employed to evaluate SE lung protective activity and its correlation to histopathologic changes.

Materials and methods

Animals

Adult male C57 mice (6 to 8 weeks), weighing 25-30g were purchased from Institute of Pasteur. They were housed individually in standard mice cages in a room on a 12- hour light- dark cycle at 22 °C (22 ± 1 °C) and with 50±5% relative humidity, including food, water and *libitum*. The animals were adapted to the condition for 7 days prior to the beginning of the experiments (22). The experiments were performed during the day time (08:00-16:00 hours). A research proposal (No. 91-331) was prepared according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Plant

Dried *Sambucus ebulus* or Dwarf elder was used for this investigation (collected at km 5, Sari–Ghaemshahr Road), identified and confirmed in January 2012 and also authenticated by Dr. Bahman Eslami (Department of Biology, Islamic Azad University of Qhaemshahr, Iran). SE was dried at room temperature and an ethanol-water (1:1) extraction was made by using maceration method, soaking in the solvent mixture. The extract was collected after removing the solvent and lyophilized and then was suspend in phosphate buffer (pH 7.4) and injected to case groups (Groups 3 to 8) intraperitoneally for protective studies (23).

Determination of total phenolic and flavonoid content

Total phenolic compound content was determined by the Folin-Ciocalteau method (9, 10, 11). The extract sample (0.5 mL of different dilutions) was mixed with 2.5 mL of 0.2 N Folin-Ciocalteau reagent for 5 min, and then 2.0 ml of 75 g/l sodium carbonate were added. The absorbance of reaction was measured at 760 nm after 2 h of incubation at room temperature. Results were expressed as gallic acid equivalents. Total flavonoid was estimated according to the method of Ebrahimzadeh, MA et al (23, 24). Briefly, 0.5 mL solution of extract in methanol was mixed with 1.5 mL of methanol, 0.1 mL of 10% $AlCl_3$, 0.1 mL of 1 M potassium acetate, and 2.8 ml of distilled water and then left at room temperature for 30 minutes. The absorbance of the reaction mixture was measured at 415 nm with a double beam spectrophotometer (Perkin Elmer, USA). Total flavonoid content was calculated as quercetin from a calibration curve.

Gamma irradiation

The Cobalt Teletherapy Unite was used for irradiation at Cancer Treatment Centre, Department of Radiotherapy, Martyr Rajai

Medical College and Hospital, Babolsar (Iran) with a cobalt-60 γ- radiations source (Teratron 780, Canada). Mice were restrained in a well ventilated Perspex box and anaesthetized with ether. Then the whole-body was exposed to 6 Gy gamma radiation at a distance (SSD) of 77.5 cm from the source to deliver the dose rate of 0.85 Gy/min (25).

Experimental design

Mice were divided into ten groups with five mice per group. Only the first and second groups (as negative control) received normal saline intraperitoneally. Groups 3 to 5 received only SE extract at doses of 20 mg/kg, 50 mg/kg and 100 mg/kg intraperitoneally; three groups were repeatedly injected for 15 days as chronic group. Groups 6 to 8 received a single dose of gamma irradiation just 2 hours before irradiation as acute group. Groups 9 and 10 (as positive control) received only gamma rays. Mice were exposed whole body to 6 Gy gamma radiation (26, 27). Following the preliminary study, the dose of 50 mg/kg was selected for the remaining of the study in order to evaluate the lung protective effect of SE (27).

Biochemical determination

Homogenate samples of gamma radiation treated groups were collected and analyzed for determination of lung Glutathione reductase (GSH) level using a commercial Kit of Zist Shimie (Tehran, Iran) and Ellman's method (28).

Histological studies

Mice were sacrificed and their lungs were removed for histopathological examination. The lungs were completely excised and any extraneous tissues were also released from them. Multiple samples were then taken from each lung (mean 3 mm in diameter) and placed in 10% neutral buffered formalin. After paraffin embedding, they were cut into 4-5 μm thick sections, stained with hematoxylin-eosin and observed under a light microscope (Model N - 400ME, CEL-TECH Diagnostics, Hamburg, Germany), studied pathological factors in lung cellular damage, including necrosis and inflammatory cells infiltration.

Statistical analysis

Statistical analysis was performed using SPSS for Windows (Ver.10, SPSS, Inc., Chicago, USA). All values were analyzed by one-way analysis of variance (ANOVA) and expressed as mean ± Standard error in the mean of 4 rats (S.E.M). Student-Newman-Keuls test was used to evaluate the significance of the obtained results. $P < 0.05$ was considered to be significant.

Results

Total phenol and flavonoid contents

Total phenol compound was determined by Folin Ciocalteu method. It was used gallic acid as reference for standard curve ($y = 0.0063x$, $r^2 = 0.987$). The total phenolic content was 118.94 ± 2.78 mg gallic acid equivalent/g of SE extract. The total flavonoid content was 58.22 ± 1.34 mg quercetin equivalent/g of SE extract, by reference to standard curve ($y = 0.0067x + 0.0132$, $r2 = 0.999$).

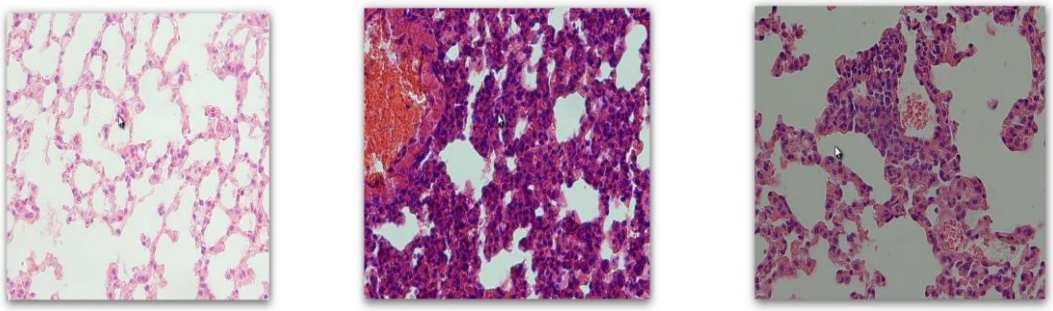
Animal behavior changes due to gamma radiation

The animals treated with gamma radiation showed signs of radiation sickness within 1-2 days after exposure to 6 Gy of gamma irradiation. Reduction in food and water intake, watering eyes, weight loss, epilation, irritability, lethargy, diarrhea, ruffled hair and slow gait in animals were the main symptoms appeased after irradiation. No toxic effects were observed in terms of sickness, body weight, urination, defection pattern and mortality in animals treated with SE alone, once a day for 6 consecutive days. The mice which were pretreated with SE (experimental group) showed delay in onset of radiation-induced mortality and symptoms of sickness as compared to the control group.

Table 1 Biochemical analyses of lung glutathione in mice treated with *Sambucus ebulus* extract and/or gamma radiation.

Groups	Dose (mg/ kg, i.p.)	Lung Glutathione (μmol/g)
Control	-	22.08
Irradiation(IR)	6 Gy	6.46
20mg/kg (chronic) + IR	20	6.70
50mg/kg (chronic) + IR	50	9.50
100mg/kg(chronic) +IR	100	13.08 [a, c]
20mg/kg (acute) + IR	20	5.03
50mg/kg (acute) + IR	50	14.39 [b]
100mg/kg (acute) + IR	100	18.87 [d]

a and b represent significance at $p < 0.05$, $p < 0.01$ and $p < 0.001$ levels, respectively, when compared to control or normal saline control (10 ml/ kg, i.p.). c and d represent significance at $p < 0.05$, $p < 0.01$ and $p < 0.001$ levels, respectively when compared to irradiation or gamma radiation treated group.



(a) (b) (c)

Figure 1 Photomicrograph of alveolar space from control, gamma radiation treated group and 100 mg/kg hydroalcoholic extract of *Sambucus ebulus* treated lung. Left is a representative section of a normal Lung (a), second, an 6 Gy gamma radiation treated group (b), third is a single dose of 100 mg/kg hydroalcoholic extract of *Sambucus ebulus* treated lung (c) Hematixylin and eosin (X25).

Biochemical estimation

Gamma radiation treated animals showed a significant decreasing in glutathione level in comparison to control group ($p<0.001$) (Table 1). SE showed a significant and dose dependent increase in the lung glutathione level as compared to alone gamma radiation treated group. Dose of 100mg/kg SE, as the most potent dose of extract, increased the glutathione level from 6.46 (gamma radiation alone group) to 18.87 µmol/g ($p < 0.001$).

Light microscope observation

Histopathological examinations showed significant lung damages including necrosis and mononuclear cells infiltration in mice espoused to gamma radiation (Fig.1.b) as compared with control and extract plus irradiation groups (Fig.1.a and c).

In addition, other histopathological parameters including edematous cells and cell degeneration changed significantly with hydro alcoholic extract of SE (Table 2).

Discussion

In this study, we showed that *Sambucus ebulus* is containing high amounts of total phenol and flavonoids. Phenols and polyphenolic compounds, such as flavonoids, are widely found in food products derived from plant sources, and they have been shown to possess antioxidant activities. Studies have shown that increasing levels of flavonoids in the diet could decrease certain human diseases (29).

In the present study, irradiation of animals with gamma rays at dose 6 Gy resulted in radiation sickness within 1-2 days after exposure. The symptoms included reduction in food and water intake, irritability, weight loss lethargy, diarrhea and ruffling of hairs. The similar symptoms have been observed in mice after gamma irradiation by others studies (30-33). Whole-body irradiation primarily affected rapidly proliferating germinal epithelium, gastro-intestinal epithelium, renal, bone marrow and spleen progenitor cells. While the germinal epithelium did not have a life supporting function for the exposed individuals, the bone marrow, spleen progenitor cells and gastro-intestinal epithelium cells were crucial for the sustenance of life, and any damage to these cells would impair normal physiological host defense processes drastically, causing an adverse impact on survival.

Pretreatment of mice with SE provided protection against radiation-induced sickness and mitigated suffering. Similarly, plants such as *Ocimumsanctum*, *Moringa oleifera*, *Mentha arvensis* and *Emblica officinalis* have been reported to provide protection against sickness induced by radiation (34-36).

The increase in abnormal and binucleated pneumocytes indicated that the severity of the pathologic effect of radiation correlate by generating free-radicals. Free radicals are regularly formed in normal physiological as well as pathological processes and could be destroyed spontaneously due to their unstable nature. The rate of spontaneous destruction was determined by the action of certain enzymatic antioxidants such as catalase and glutathione peroxidase.

Table 2 Histo-pathological effects of *Sambucus ebulus* extract at different concentrations of 20, 50, 100 mg/kg on lung mice exposed to gamma irradiation

Sample	Pneumocystis (hyperplasia)	Edematous cells	Hemorrhage	Necrosis
20 mg/kg (chronic)	4+	3+	3+	4+
50 mg/kg (chronic)	4+	4+	3+	4+
100 mg/kg (chronic)	1+	1+	1+	1+
20 mg/kg (acute)	4+	4+	3+	4+
50 mg/kg (acute)	4+	4+	3+	4+
100 mg/kg (acute)	1+**	1+	1+	2+*
Control (chronic)	1+	2+	1+	1+
Control (acute)	1+	2+	1+	1+
Irradiation	4+**	4+**	3+*	4+*

No (-), Minor (+1), Medium (+2), Major (+3), high (+4) , super (+5) effects, irradiation (6Gy of gamma irradiation) , control (10ml/kg of normal saline) *P<0.05, **P<0.01, significantly different from control using Fisher' exact test. Data are means of three replicates.

Therefore, the net effect of free- radical injury depended upon the rate of their elimination.

In this study, we sought to determine how we could prevent or decrease the lung toxic effect of gamma irradiation. The decrease in reduced glutathione by irradiation could be due to the oxidation of the sulphydryl group of GSH due to the decrease in glutathione reductase and the enzyme which reduces the oxidized glutathione (GSSG) into a reduced form using NADPH as a source of reducing equivalent (36). Glutathione depletion has been shown to correlate with lipid peroxidation in lung parenchyma. Accordingly, when SE extract was used as antioxidant, lung toxicity effect of gamma irradiation was reduced almost about 40-50% of control.

Antioxidants, such as vitamin E and selenium have been proposed to prevent membrane damage of lipid peroxidation not only by glutathione peroxidase but also by allowing hydrogen to be abstracted from their own structure rather than from the allylic hydrogen of unsaturated lipid. Thus, they interrupted the free radical chain reaction (37). Treatment with SE extract has been shown to significantly decrease the toxicity of gamma irradiation. This might be through the mechanism mentioned above as well as extracts which had good reductive capability for reducing Fe^{+3} to Fe^{+2} by donating an electron Fe^{+2} chelating activity and anti-lipid peroxidation activity (38). Further investigations of individual compounds to elucidate the antioxidant activities and mechanisms are needed.

Our data showed that administration of SE extract caused edema which can be assessed by histopathological examination. These findings were in agreement with the fact that an irradiation could cause increase in Oncotic pressure, or colloid osmotic pressure (39). In addition, isolated organs had a time-dependent tendency to absorb water, as with relatively protein-free medium water which gradually escaped from the vascular space and so interstitial edema developed (39). Histopathological examination revealed significant hemolysis as assessed by the hemolytic index (Fig.1.a).

This could be due to altered calcium homeostasis concomitant with a significant increase in cytosolic calcium, which has been previously reported for *Phytolacca americana* in liver (40). Moreover, the disturbances of intracellular calcium homeostasis have been shown to be associated with a variety of toxicological and pathological processes. Accumulation of SE extract in the lung tissue, as the target organ, has been shown to cause protection (41). In a similar

manner, the result of this study also showed lung protection and SE extract decreased formation of reactive oxygen species and an oxidative stress resulting in lipid peroxidation and resulting in protection on lung toxicity induced by ionizing radiation (42, 43).

Conclusion

In this study we showed *Sambucus ebulus* as a natural product to have radioprotective effects against lung toxicity induced by gamma radiation. SE increased gluthathione level in irradiated mice. Since, SE contains high amounts of phenol and flavonoids, which have antioxidant activity, protective effect of SE is probably due to mitigation of oxidative stress caused by gamma radiation. Future studies are needed to find the exact mechanisms related to radioprotective effect of SE.

Conflict of interest statement

The authors declared no potential conflict of interest with respect to the authorship, and/or publication of this study.

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