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Original Article

Radioprotective effects of lentil sprouts against X-ray radiation

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Abstract

The present study investigated the radioprotective efficacy of lentil (*Lens culinaris*) sprouts against X-ray radiation-induced cellular damage. Lentil seeds were dark germinated at low temperature and the sprout extract was prepared in PBS. Free radical scavenging of extract was evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and then the radioprotective potency of extract (0 to 1000 μg/mL) on the lymphocyte cells was determined by lactate dehydrogenases assay. Moreover, micronuclei assay was assessed using the cytokinesis-block technique. The irradiations were performed using 6 MV X-ray beam. The value of IC_{50} for DPPH assay was 250 μg/mL. The median lethal dose for radiation was determinate at 5.37 Gy. Pretreatment with lentil sprout extract at 1000 μg/mL reduced cytotoxicity at 6 Gy total concentration from 70% to 50%. The results of micronuclei assay indicated that cells were resistant to radiation at concentrations of 500-1000 μg/mL of exogenous lentil sprout extract. The value of median effective concentration for micronuclei assay was 500 μg/mL. The results indicated that lentil sprout extract showed actually somewhat radioprotective effect on lymphocyte cell. In addition, the obtained results suggest that extract of total lentil sprout have more antioxidant activity than radicle part.

Keywords: Radioprotective agents; Germination; X-Radiation; Legumes

INTRODUCTION

Some common components of the human diet have important effect on health. Development and use of the health-promoting foods can promote community health (1-2). Germination is an inexpensive and simple process; however, it causes important changes in the biochemical, nutritional, and sensory characteristics of plants (3-4) and provide an excellent source of dietary phenolic antioxidants (5).

Lentils are consumed as a whole food in more than 100 countries. This legume has an excellent nutrient profile and favorable levels of antioxidants (6); however, antinutritive factors such as condensed tannin and phytic acid reduce the nutritive value of lentils (7). During germination, content of low-molecular-weight phenolic compounds does not vary appreciably (8), but phytic acid and tannin decrease (9).

The total antioxidant content of lentils decrease under normal conditions, but some conditions increase the phenolic and ascorbic acid contents of lentil sprouts. Factors affecting this include temperature, light intensity, duration of germination, and elicitation agents (10,13).

Also, when the product is used in extract form, the extracting solvent can affect the properties of the extract (14). For example, lentil extract prepared with phosphate buffered saline (PBS) has a higher radical scavenging activity than ethanol extract (13). On the other hand, free radicals have a destructive influence on lives systems.

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Ionizing radiation (IR) is a main source of radical production in human bodies but there are considerable advantages for diagnostic and therapeutic applications; thus, it is impossible to neglect application of clinical rays. In these conditions, radioprotective agents may alleviate the side effects of radiation. Radioprotectives are natural or synthetic compounds that act as radical scavengers and prevent free radical activity. Generally, radioprotective compounds interact with free radicals followed by oxidization of radioprotective and conversion of free radicals to stable compounds incapable of reacting with other cellular components. Another assumption arises from hydrogen donating ability of radioprotective. A protective agent can also donate a hydrogen atom to radical and convert it to stable molecule (15). Phenolic content of lentil sprouts may play as a proton donator and scavenge free radicals.

The aim of this study was evaluation of lentil sprout as a radioprotective agent. Germination was carried out in simulated conditions that are accessible in the average home (refrigerator temperature and darkness). Radioprotective potency was evaluated using multiple chemical and cellular tests.

MATERIALS AND METHODS

Plant material and reagent

A local cultivars of lentils seed (Genotype:Kermanshah) (16) were prepared from local vegetable markets at Kermanshah province (West of Iran). The sources of cell culture requirements were as follow: RPMI 1640 (Life Technologies), fetal bovine serum (Gibco), lactate dehydrogenases (LDH) assay (Roche), penicillin/streptomycin (Sigma). Other chemicals were purchased from Merck Company.

Germination and flour preparation

The seed sterilization and soaking was carried out, similar to Swieca, *et al.* (13). Seeds were dark germinated for 6 days at 4 ºC. Sprouts were washed with distilled water and divided in two portions. Sprout radicles of the first portion were separated with scalpel blades and collected on the plate. Total sprouts and separated radicles were dried at 55 ºC overnight. Dried product were ground in a labor mill and sieved through 25 mesh. Flours were kept at -20 ºC.

Extraction

Flours were homogenized in PBS at specified ratio (1 g in 35 mL) by gently mixing for 1 h at room temperature (17). The mixtures were centrifuged at 10621 *g* for 5 min. The supernatants were recovered and filtrated by paper filter. The extracts were freeze-dried and stored at -20 ºC.

2,2-diphenyl-1-picrylhydrazyl (DPPH) test

The effect of total and radicles sprout extract on the DPPH radical was compared according to the method reported by Hosseinimehr, *et al.* (17). Different concentrations of extracts (100, 500 and 1000 µg/mL) in PBS was mixed to an equal volume of freshly prepared methanolic solution of DPPH (100 µM). After 15 min at room temperature, the absorbance was recorded at 517 nm. The experiment was performed in triplicate. Butylated hydroxytoluene (BHT) was used as a standard antioxidant agent. The percentage of scavenging was calculated using the following formula as percent of inhibition:

$$\text{Inhibition (\%)} = 100 \times (\text{control} - \text{test})/\text{control}$$

According to the result of DPPH test, total sprout extract was selected for the following tests.

Rats

Rats (male, Wistar strain, 6-8 weeks old) were obtained from Pasteur Institute of Iran (Tehran, Iran). All rats were housed in cages (4-5/cage) located in our center facilities maintained at 28 °C with a 50% relative humidity and a 12-h light/dark cycle. All rats had *ad libitum* access to standard rodent chow and filtered water throughout the study. The rats were housed for one week to acclimate prior to any experiments. All animal experiments were approved by the Animal Research Ethics Committee of Kermanshah University of Medical Sciences (Kermanshah, Iran) and performed in accordance with National Institute of Health Guide for the Care and Use of Laboratory Animals.

Separation of spleen lymphocyte

Lymphocytes were separated from rat spleens according to the previous report (18). In brief, native rats were euthanized (by cervical dislocation) and their spleens were disrupted in PBS (pH 7.4). The resulting suspension was passed through a 100 µm stainless steel mesh and red blood cells present were removed by incubation for 15 min on ice in lysis buffer (150 mM NH_4Cl, 1 mM $KHCO_3$ and 0.1 mM Na_2EDTA) followed by centrifugation at 3000 *g* for 5 min and 4 °C. The cells were washed twice with PBS and re-suspended in 1 mL RPMI 1640 containing 10% fetal bovine serum, concanavalin A 5 µg/mL, penicillin 100 µg/mL and streptomycin 100 µg/mL. After counting the cells, aliquots containing 8×10^5 cells were placed into each well of a 24-well plate and the plates were held at 37 °C in a 5% CO_2 incubator. The cells were used for evaluation of irradiation and extract effects at the next day.

Irradiation condition

A plaxiglas phantom was constructed with dimensions of $30 \times 30 \times 30$ cm^3. The cell plates were placed at 10 cm depth of phantom and were CT scanned. The interval between neighboring plate wells were filled with sterile water in order to minimize scattering of ray traces.

Calculation of radiation field size and number of monitor units (MU) for expose the desired radiation dose to the cells was done with ISOgray treatment planning software (Dosisoft France Co.). The irradiations were performed using 6 MV photon beam by a linac (Elekta SL75/25, placed in Imam Reza hospital, Kermanshah, Iran).

Toxicity of irradiation

The lymphocyte cells were irradiated at a dose rate of 1.6 Gy/min for total doses of 2 to 6 Gy. The cell viability was determined with LDH assay test (19). Briefly, at 72 h of irradiation, the plates were centrifuged at 200 *g* and 100 µL of the media from each well was transferred to a new 96-well plates. Thereafter, 100 µL of LDH assay mixture was added to each well and plates were incubated at 37 °C for 30 min. A group of wells was treated with 1% Triton X-100 solution for 45 min to maximum LDH release. The LDH release was estimated using a microplate reader at 495 nm according to the manufacturer's instructions. Triplicate wells were assayed for each dose. According to curve calculation formula, median lethal dose (LD_{50}) for irradiation was determinate as 5.37 Gy. According to the toxicity of different total doses, 6 Gy total dose was selected for following tests.

Treatment of the cell

The preliminary cytotoxic study of the extract was performed to check any effect of the extract. The freeze-dried extract was dissolved in PBS at a concentration of 10 mg/mL. The various doses of extract (0, 250, 500, 750 and 1000 µg/mL) was obtained with addition of concentrated extract solution (concentration: 10000 µg/mL, volumes: 0, 25, 50, 75 and 100 µL) to well of lymphocyte cell and the final volume up to 1 mL with RPMI medium. The cells were incubated for 72 h and the cell viability was determined by LDH assay (19).

After observation of non-toxic effect of extract, evaluation of radioprotective potency was carried out at similar condition. The lymphocyte cells were treated with various doses of extract (0-1000 µg/mL), 1 h before irradiation. The selected total dose (6 Gy) was used for irradiation. The cell viability was determined by LDH assay after 72 h. Triplicate wells were used for each extract dose in both cytotoxic and radioprotective assay. The experiment was performed in triplicate.

Micronuclei assay

Micronuclei (MN) assay was used as a biological endpoint for DNA damage and measured with the cytokinesis-block technique as described previously (20). Cells were reseeded (1.5×10^5 cells/dish) in the medium containing 1 µg/mL cytochalasin-B for 36 h and then fixed *in situ* with methanol:acetic acid (9:1 v/v) for 30 min. Air-dried cells were stained with Wright-Giemsa stain. MN was scored in at least 500 binucleated cells. The MN yield, Y_{MN}, was calculated as the ratio of

the number of MN to the number of binucleated cells scored. The experiment was performed in triplicate.

Statistical analysis

All data were presented as mean ± SEM. Data was analyzed by unpaired, 2-tailed t-test using Prism software to determine any significant difference.

RESULTS

DPPH test

The total and radicles sprout extract showed the different radical scavenging efficiency (Fig. 1). Appropriate scavenging was observed by the PBS extract of the total sprout. The value of IC_{50} for DPPH assay was 250 µg/mL. The extract of sprout radicles attained this percentage of inhibition at 1000 µg/mL. The radical scavenging capacity of the total sprout extract increased similarly to BHT at 500 µg/mL, but plateaued at higher concentrations. The percentage of inhibition for BHT increased to 66% at 1000 µg/mL.

Toxicity of X-ray

The toxicity of different total doses of radiation on lymphocyte cells is shown in Fig. 2. As seen, cytotoxicity increased as the dose increased.

Exposure to 2 Gy caused about 5% cell death, but this increased to 69.3% at a 6 Gy total dose.

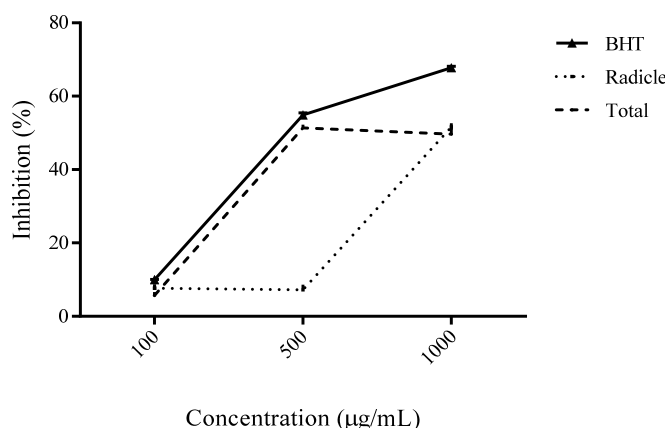

Fig. 1. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity of phosphate bufferd saline extract of total and radicle of lentil sprout and butylated hydroxytoluene (BHT) at different concentrations. Inhibition percent of total sprout extract showed more radical capturing potency toward radicle extract ($P < 0.01$).

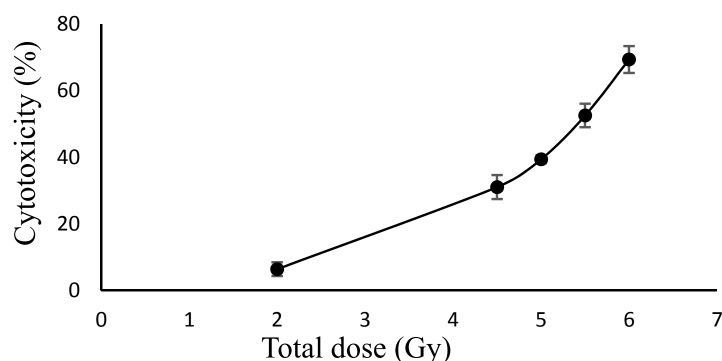

Fig. 2. The effect of different total dose of radiation on viability of lymphocyte cells. The cytotoxicity was increased with increasing total dose of irradiated X-ray (dose rate: 1.6 Gy/min, photon beam energy: 6 MV).

Protective effect of extract

Treatment of lymphocyte cells with extract showed no toxic effect at extract dose up to 1000 µg/mL. The protective effect of the extract versus the level of radiation was evaluated and the results are shown in Fig. 3. The pretreated cells showed a significant increase in the cell viability in the LDH assay. After 72 h of exposure to radiation, the cell viability of control samples (without addition of extract) was about 30%. The radioprotective activity increased with increasing the extract dose. Pretreatment with lentil sprouts at 1000 µg/mL reduced cytotoxicity to 50%.

Micronuclei assay

A MN test is applied in toxicological studies to screen for genotoxic compounds. The present study determined that exogenous lentil sprout extract protected lymphocyte cells from death and DNA double-strand break formation induced by radiation.

The result of the cytotoxicity assay indicated that 500-1000 µg/mL of exogenous lentil sprout extract increased lymphocyte resistance to radiation (Fig. 4). The values of effective concentration (EC_{50}) for MN assay was 500 µg/mL.

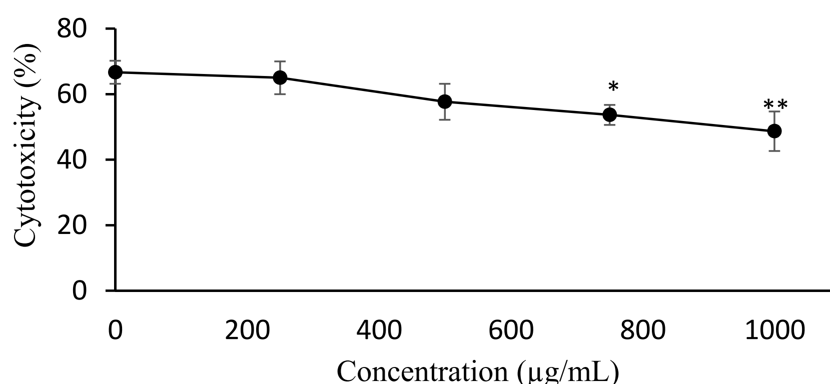

Fig. 3. The cytotoxicity of X-ray radiation (dose rate: 1.6 Gy/min; total doses: 6 Gy) on pretreated lymphocyte cell with different concentrations of lentil sprout extract (*$P \leq 0.05$, **$P \leq 0.01$).

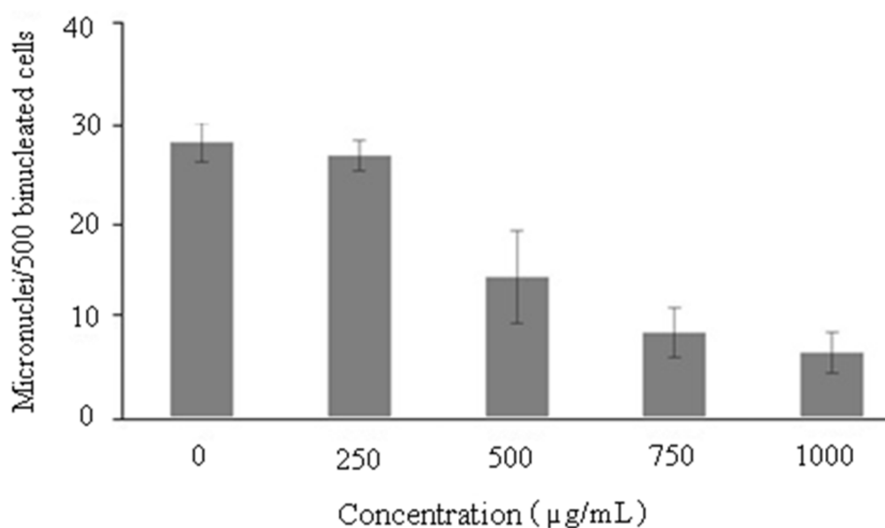

Fig. 4. The effect of different concentration of lentil sprout extract on the yield of micronuclei induced by radiation (dose rate: 1.6 Gy/min; total doses: 6 Gy).

DISCUSSION

People are exposed to IR originates from natural, man-made, medical, and occupational sources (21). The development of effective radiomodifiers have great medical importance considering the application of IR in medical practices (e.g., radiotherapy and nuclear medicine) and accidental exposure to radiation (e.g., industrial nuclear accident). Radioprotective agents are synthetic compounds or natural products that are administered immediately before irradiation to reduce injury caused by IR (22). The lentil sprout is a candidate possessing this potential. The present study used a variety of tests to evaluate the protective capacity of lentil sprouts.

The free radical scavenging activity of lentil sprout extract was evaluated by DPPH assay and compared with results using BHT as the standard. The free radical scavenging potential of the total sprout extract reached a plateau at 500 µg/mL, but total sprout extract showed an increasing protective effect on cells. The high concentration of catechin gallate, saponin, quercetin diglycoside and kaempferol glycoside acylated may be responsible for significant antioxidant properties of lentil sprout extract (11,23).

It is commonly believed that radicles have benefits that are superior to the total sprout. This possibility was tested using DPPH assay and the results showed that radicle extract had a weaker free radical scavenging ability, which is contrary to popular opinion. Although, profile of antioxidant agent for radicle and other part of sprout was not studied, but similar results have been obtained for other sprout as maize. Concentration of peroxidized lipids (as malonylaldehyde) in desiccated radicles of maize sprouts increase much more than total sprouts after 72 h germination (24).

It is known that the radiobiological effects of radiation doses depends on factors such as cell type and dose rate (25-26). The radioresistance of lymphocyte cells was examined with a dose rate 1.6 Gy/min and for total doses of 2 to 6 Gy. The pattern of change in the results of the cytotoxicity assay was similar to the first half of a sigmoid curve. At total doses of 4 to 6 Gy, cytotoxicity increased about 15% as the total dose increased 0.5 Gy. The curve calculation formula indicated that LD_{50} radiation was determinate at 5.37 Gy. In a similar study, Nada, *et al.* indicated that LD_{50} in rat hepatocyte irradiation was determinate at 3.2 Gy at a dose rate of 0.61 Gy/min (27). Pulmonary endothelial cells (EA.hy926) show more resistance to γ-irradiation and that cell proliferation decreased to 20% for a total dose of 10 Gy at a dose rate of 1.35 Gy/min (28).

A total dose of 6 Gy was selected for survival and MN assays. The higher cytotoxic potency of this total dose provided better evaluation of the radioprotective potential of the extracts. Under these conditions, the lentil extract had a moderately protective effect. The protective potency of the lentil extract depended on the concentration. An extract dose of 1000 µg/mL protected 20% of cells from death. Radioprotective activity increased as the extract dose increased. Pretreatment with lentil sprout extract at 1000 µg/mL reduced cytotoxicity to 50%. Considering the results of DPPH test, these findings suggest that the radioprotective activity of lentil extract is not only based on radical scavenging activity.

MN assay has been applied in the toxicological study to screen for genotoxic compounds. The study determined that exogenous lentil sprout extract protected lymphocyte cells from death and DNA double-strand break formation induced by radiation. These findings confirm the hypothesis that lentil sprout extract offers a protective effect against radiation by inhibition of ROS production. Although some plant such as *Zataria multiflora* (17) and black tea (29) show more radioprotective effect, comparison to our result with studied cereal like wheat (30) and legume like peanut (31), lentil sprout demonstrated appropriate radioprotective efficiency. This study gives new insights into the role of lentil sprout extract in attenuating lymphocyte damage induced by radiation.

CONCLUSIONS

Our data showed that extract of total lentil sprout have more antioxidant activity than

radicle part. The LDH assay showed that extract concentration at 1000 μg/mL protected 20% of cells against X-ray irradiation at 6 Gy total dose. A similar trend was observed in protection of different concentration using MN assay. In conclusion, these results suggest that lentil sprout extract has a moderate protective effect against irradiation.

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