Cellular & Molecular Biology
Cell. Mol. Biol. 2015; 61 (4): 46-50
Published online August 17, 2015 (http://www.cellmolbiol.com)
Received on June 17, 2015, Accepted on August 11, 2015.
doi : 10.14715/cmb/2015.61.4.8

Radioprotective effect of chicory seeds against genotoxicity induced by ionizing radiation in human normal lymphocytes

S. J. Hosseinimehr[1], V. Ghaffari-rad[1, 2], M. Rostamnezhad[1, 2], A. Ghasemi[3], T. Allahverdi Pourfallah[4] and S. Shahani[5]✉

[1] Department of Radiopharmacy, Faculty of Pharmacy, Pharmaceutical Sciences Research Center, Mazandaran University of Medical Sciences, Sari, Iran
[2] Student Research Committee, Mazandaran University of Medical Sciences, Sari, Iran
[3] Department of Radiology and Radiation Oncology, Faculty of Medicine, Mazandaran University of Medical Sciences, Sari, Iran
[4] Department of Biochemistry and Biophysics, Faculty of Medicine, Mazandaran University of Medical Sciences, Sari, Iran
[5] Department of Pharmacognosy and Biotechnology, Faculty of Pharmacy, Mazandaran University of Medical Sciences, Sari, Iran

Corresponding author: Somayeh Shahani, Department of Pharmacognosy and Biotechnology, Faculty of Pharmacy, Mazandaran University of Medical Sciences, Sari, Iran. E-mail: so.shahani@yahoo.com

Abstract

The search for less-toxic radioprotective agents has led to a growing trend towards natural products. Protective effect of the methanolic extract of chicory seeds (MCS) was investigated against genotoxicity induced by ionizing radiation in human lymphocytes. Human peripheral blood samples were collected and incubated with MCS at different concentrations (10, 50, 100, and 200 µg/mL) for two hours. The whole blood samples were exposed in vitro to X-ray at dose 2.5 Gy. Then, the lymphocytes were cultured with mitogenic stimulation to determine the micronucleus in cytokinesis blocked binucleated cell. The methanolic extract at all doses significantly reduced the frequency of micronuclei in binucleated lymphocytes, as compared with similarly irradiated lymphocytes without any extract treatment. The maximum protection was observed at 200 µg/mL of MCS, it completely protected genotoxicity induced by ionizing radiation in human lymphocytes. The extract exhibited a concentration-dependent radical scavenging activity on 1,1-diphenyl-2-picryl hydrazyl free radicals. HPLC analysis of MCS showed this extract is containing chlorogenic acid as a phenolic compound. These data suggest that the radioprotective effect of methanolic extract of chicory seeds can be attributed to the presence of phenolic compounds such as chlorogenic acid which act as antioxidant agents.

Key words: *Cichorium intybus*, micronuclei, chlorogenic acid, antioxidant.

Introduction

Ionizing radiation (IR) produces free radicals and toxic substances that are highly chemically reactive and can attack on the cellular biomolecules including proteins, lipids, and DNA. DNA breaks are caused by IR. If DNA damages can't be efficiently repaired by endogenous defense systems, it is leading to chromosome abnormalities (1). Radiation-induced genome aberration has a crucial role in the mechanisms underlying radiation-induced carcinogenesis (2). Radiation-induced DNA lesion is main reason for cell death. With respect to radiation-induced side effects on humans, it is important to protect normal cells from radiation-induced DNA damage. Amifostine as a thiol synthetic compound is a powerful radioprotective agent, but this drug has limited usage in clinical practice, due to its side effects and toxicity (3, 4). The search for less-toxic radioprotective agent has spurred interest in the development of natural products. Many natural antioxidants such as flavonoids and polyphenols can cause some level of radioprotection (5).

Chicory (*Cichorium intybus* L., Compositae) is an herbaceous plant belonging to the Asteraceae family (6) and is native to Europe and Asia (7). Different parts of this plant are used for medicinal purposes (8). It is known as Hendeba in Iranian traditional medicine and has been used as liver tonic, diuretic, laxative and appetizer (9). Previous studies on *C. intybus* have shown hepatoprotective (10), antidiabetic, anti inflammatory (11), wound healing and antioxidant (12) activities. The most secondary metabolites of this plant are sesquiterpene lactones and cinnamic acid derivatives (13). Chicory seeds are rich in polyphenols especially dicaffeoylquinic acids (14, 15). Ghamarian et al reported strong antioxidant capacity of the aqueous seed extract which exhibited short and long term effects on diabetes (16). The methanolic fraction of chicory seeds possessed hepatoprotective activity on CCl4 – induced liver damage in albino rats (17). In other research, the methanolic extract of seeds showed good antioxidant activity (18).

Based on the protective and antioxidant properties of *C. intybus*, the present study aimed to evaluate radioprotective activity of chicory seeds using in vitro radiation-induced genetic damage in volunteers' human blood lymphocytes.

Materials and methods

Plant material and extraction

Seeds of *Cichorium intybus* L. were purchased from Grand Bazzar, Tehran, Iran. A voucher specimen (102) was deposited at the herbarium of Faculty of Pharmacy, Mazandaran University of Medical Sciences, Sari, Iran. The cleaned seeds were powdered and then extracted with methanol by maceration at room temperature. The extract was concentrated by using a rotary evaporator (Heidolph, Germany) and dried by a freeze dryer (Zirbus, Germany) to remove any moisture.

Table 1. The frequency of micronuclei induced by 250 cGy X-ray radiation in cultured blood lymphocytes from human volunteers after treatment with different doses of the methanolic extract of chicory seeds (MCS).

Group	Micronuclei in binucleated lymphocyte (%)*			
	Volunteer I	Volunteer II	Volunteer III	Mean ± SD
Control	0.7	0.88	0.6	0.72 ± 0.14
IR †	4.1	5.3	5	4.80 ± 0.62
10 µg/mL MCS + IR	1.2	1.1	1.4	1.23 ± 0.15
50 µg/mL MCS + IR	1.7	1	1.5	1.40 ± 0.36
100 µg/mL MCS + IR	1.5	1.8	1.5	1.60 ± 0.17
200 µg/mL MCS + IR	0.9	0.5	0.7	0.70 ± 0.20
200 µg/mL MCS	0.4	0.2	0.2	0.27 ± 0.12

* 1000 BN cells were examined in each sample

† IR: irradiated group

X-ray irradiation and genotoxicity assay

This study was approved by research and ethical committee of Mazandaran University of Medical Sciences. Three healthy, non-smoking human volunteers, males ages between 20 to 25 years were enrolled in this study. Twelve mL whole blood were collected in heparinized tubes and divided in centrifuge tube. The extract was dissolved in ethanol and diluted in cultural medium. Ethanol concentration was same in control and extract solutions (0.5%). Blood samples were treated with 100 µl solution of MCS at the concentrations 10, 50, 100, or 200 µg/mL (final concentrations). These samples were incubated for two hours at 37°C. Tubes containing blood were irradiated at 37°C with 6 MV X-ray beams produced by a radiotherapy machine (Linear accelerator, Siemens, Primus, Germany) at a dose of 2.5 Gy with a dose rate of 190 cGy/min. Subsequently, 0.5 mL of each sample (control, irradiated and extract samples in duplicate) was added to 4.4 mL of RPMI 1640 culture medium (Gibco, USA), which contained 10% fetal calf serum. Then phytohemagglutinin (100 µl/5mL, Gibco, USA) was added to cultures as lymphocyte stimulator. All cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. After 44 h of culture, cytochalasin B (Sigma, final concentration: 6 µl/mL) was added to culture. Following 72 h of incubation, the cells were collected by centrifugation, re-suspended in 0.075 M cold potassium chloride. Cells immediately fixed in a fixative solution as methanol: acetic acid (6:1) two times. The fixed cells were dropped onto clean microscopic slides, air-dried and stained with Giemsa solution (10%). All slides were evaluated at 100 × magnification in order to determine the frequency of micronuclei in the cytokinesis-blocked binucleated cells with a well-preserved cytoplasm (19). At each concentration and for each volunteer, 1000 binucleated lymphocyte cells were examined from the control and irradiated cultures in duplicate to record the frequency of micronuclei.

Measurement of free radical scavenging activity

The free radical-scavenging capacity of the methanolic extract of chicory seeds was determined as bleaching of the stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) (20). The different concentration of the extract (0.05 to 1 mg/mL) was added, at 2 mL, to 2 mL solution of DPPH (10 mg/250 mL in ethanol). After 15 min at room temperature, the absorbance was recorded at 517 nm. The experiment was performed in triplicate and butylated hydroxytoluene (BHT) was used as a standard antioxidant agent. Percent radical scavenging was calculated using the formula [(Control-Test)/Control]*100.

HPLC analysis

Identification and quantification of chlorogenic acid in MCS was carried out by a Knauer Smartline HPLC consisting of a pump 1000 and solvent delivery system equipped with a sampler injector and a photodiode array detector model DAD 2800 and set at 270 nm (all from Knauer Assoc., Germany) with ChromGate software (Version 3.1.7). Analysis was performed by using an ODS-C18 column (250 × 4 6 mm, 5 µm particle size, Nucleodur, Duren, Germany), and the corresponding guard column. All solvent were filtered and degassed prior entering the column. The mobile phase was methanol-water-acetic acid (40:60:0.2 v/v/v). The mobile phase flow rate was 1.0 mL/min, and all the measurements were done at ambient temperature. Quantification of chlorogenic acid in MCS was done using an external standard method. Different concentrations of standard chlorogenic acid (Merck, Germany) were prepared to plot the calibration curve.

Statistical analysis

For each volunteer, at each concentration, the incidence of radiation-induced micronuclei was recorded. The data were analyzed with student *t*-test. A probability value of 0.05 was accepted to denote significance.

Results

The percentage of micronuclei in binucleated lymphocytes in three donors treated with 2.5 Gy X-ray was 4.80 ± 0.62, while the percentage in non-treated control lymphocytes was 0.73 ± 0.14. It was showed a significantly increasing of 7-fold in frequency of micronuclei in lymphocytes exposed to 2.5 Gy of X-ray ($p < 0.01$) (Table 1). The frequency of micronuclei after pre-treatment with MCS at doses of 10, 50, 100 and 200 µg/mL were 1.23 ± 0.15, 1.40±0.36, 1.60 ± 0.17 and 0.70 ± 0.20 respectively (Figure 1). The data demonstrate that the extract at all concentrations caused a significant reduction in micronuclei frequency in human lymphocytes exposed to X-ray compared to irradiated group (IR) ($p<0.01$). The total micronucleated binucleated

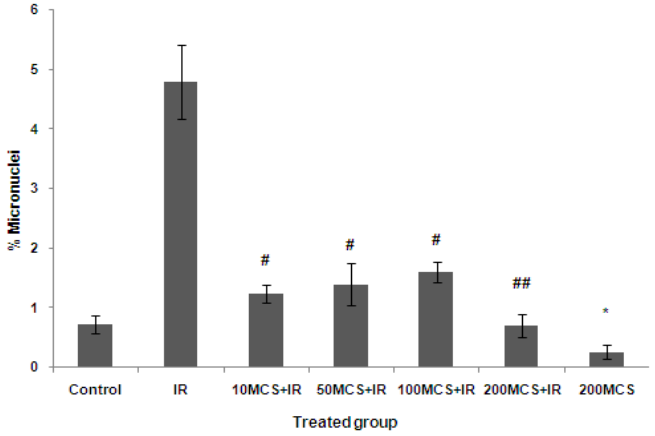

Figure 1. In vitro protection by the methanolic extract of chicory seeds (MCS) at different concentrations (10, 50, 100 and 200 µg/mL) against genetic damage induced by X-ray (2.5 Gy) in cultured whole blood lymphocytes. The data represent average ± standard deviation of three human volunteers. P<0.001: Control group compared with similarly irradiated lymphocytes (IR group). #P<0.01: IR group compared to 10MCS + IR, 50MCS + IR, 100MCS + IR and 200MCS + IR groups. ##P<0.01: between 200MCS + IR and 100MCS + IR groups. *P<0.01: between groups of 200MCS and control.

cells values were 74%, 70% , 66% and 143% fold less in 10, 50, 100 and 200 µg/mL concentrations of MCS respectively (Table 1). It was observed a concentration dependent effect for MCS on the reduction of chromosome damage in lymphocytes exposed to ionizing radiation between doses 100 and 200 µg/mL (p<0.01). The extract at concentration of 200 µg/mL exhibited no genotoxicity in cultured lymphocytes of non-irradiated sample. An interesting result is micronuclei frequency in the sample treated with 200 µg/mL of MCS was less than the control group (p<0.05).

In antioxidant assay, a considerable scavenging effect was observed with the extract. Scavenging effects of MCS on DPPH radicals increased with the increasing of concentrations, it was 93% at 0.4 mg/mL that was more than BHT (65%) (Figure 2).

In this study, an isocratic elution of methanol-water-acetic acid (40:60:0.2 v/v/v) was used to achieve complete separation of chlorogenic acid in the extract. This phenolic acid had a typical retention time of 5-6 min. Purity of chlorogenic acid peak in HPLC chromatogram was confirmed with photodiode array detector. Chlorogenic acid was identified in the chromatogram of MCS by comparing the retention time and UV spectra with those of the standard (Figure 3). The extract was standardized based on chlorogenic acid by HPLC method. The calibration curve of chlorogenic acid was linear over the range 0.05 - 0.75 mg/mL. The chlorogenic acid content of MCS was 0.016 ± 0.001 mg/ mg of extract powder.

Discussion

In this study we demonstrated that the methanolic extract of chicory seeds has potent radioprotective effect against DNA damage induced by X-ray in human lymphocytes. MCS reduced the chromosome damages in human lymphocytes that caused by IR. This protective effect was observed for MCS at all tested concentrations of 10, 50, 100 and 200 µg/mL. Furthermore, considerable antioxidant activity with free radical scavenging property was observed from the extract. HPLC analysis showed this extract is containing chlorogenic acid as a phenolic compound. IR causes many side effects in patients undergo radiotherapy or in personnel who are working with sources of ionizing radiation. The side effects of IR are related to increased intracellular level of reactive oxygen species (ROS). ROS interact with macromolecules to induce DNA damage (21). The micronucleus assay in peripheral blood cells is one of the widely used in vitro cytogenetic assays in the field of genotoxic biomonitoring in human (23). Micronuclei are scored in binucleated cells only, which enables reliable determining of chromosome damage in their cell division (19). Recently, we showed that atorvastatin as a cholesterol-lowering drug markedly protected human lymphocyte against genotoxicity induced by ionizing radiation. Antioxidant and anti-inflammatory effects were proposed for mechanisms of action of this drug (22) Natural compounds such as flavonoids and phenolic compounds may play a crucial role in scavenging free radicals. In this study, MCS exhibited radioprotective effect on reducing micronuclei induced by X-ray. Treatment of whole blood with MCS for two hour prior irradiation reduced the frequency of micronuclei.

Despite the widespread traditional use of *C. intybus*, little research has been done on its pharmacological effects (8). Chicory seeds have been used by traditional healers as a ingredient of several recipes for treatment of hepatobiliary disorders (24). Hepatoprotective (25), antidiabetic (16), antimicrobial (26) and antioxidant (15, 18) activities have been reported from the extracts of chicory seeds. Milala et al. evaluated the antioxidant activity of different parts of chicory i.e. roots, leaves and seeds. The extract obtained from chicory seeds, which was the richest in dicaffeoylquinic acids, exhibited the highest antioxidant activity (27). The literature review showed that the extract of chicory seeds is a rich source of chlorogenic acid and its derivatives (15) . In a research, chlorogenic acid showed radioprotective effects and decreased the DNA damage induced by X-ray

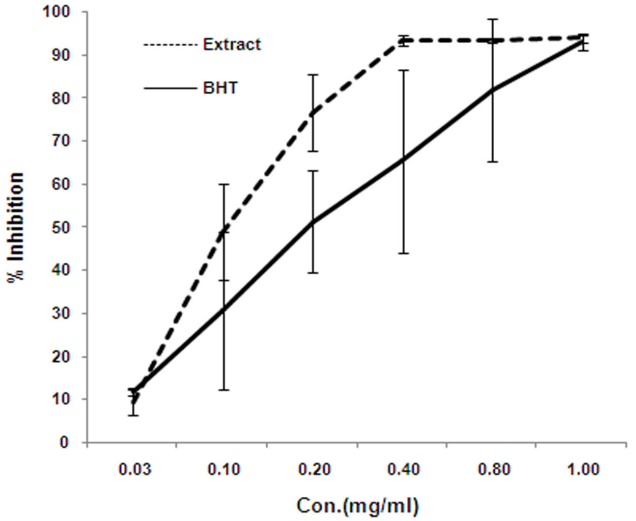

Figure 2. Scavenging effect of different concentrations of the methanolic extract of chicory seeds (extract) and Butylated hydroxytoluene (BHT) on the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical at 517 nm.

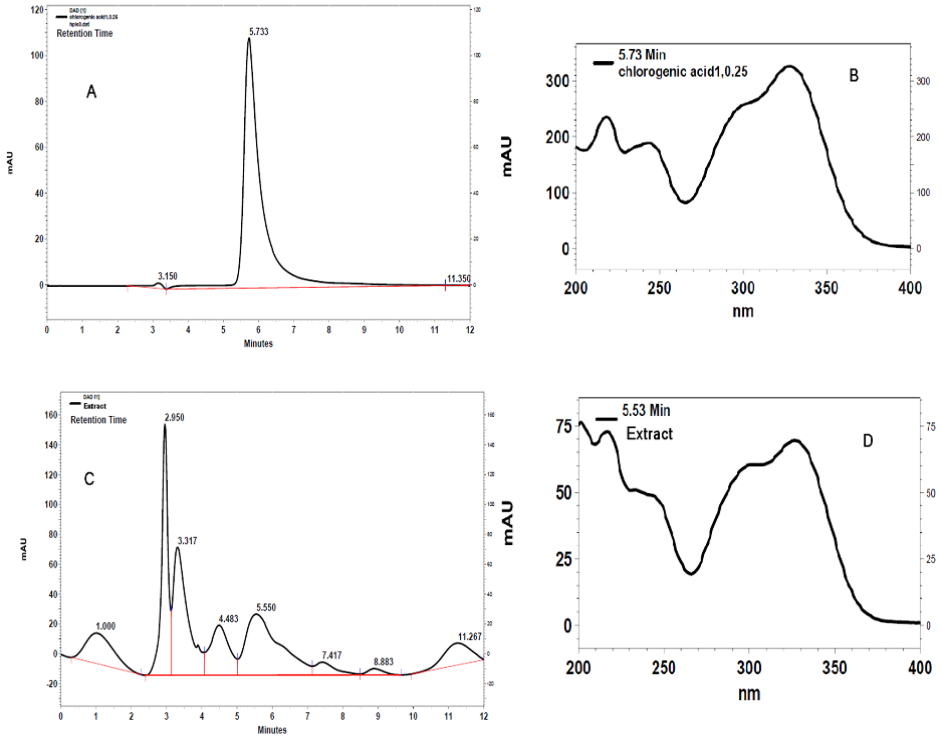

Figure 3. HPLC profiles of chlorogenic acid and the methanolic extract of chicory seeds (MCS). a: HPLC chromatogram of standard chlorogenic acid (retention time: 5.7 min), b: UV spectrum of chlorogenic acid, c: HPLC chromatogram of MCS (retention time: 5.5 min), d: UV spectrum of peak with retention time at 5.5 min).

irradiation in human blood lymphocytes in vitro (28). Based on these findings, the chlorogenic acid content of the extract was determined.

Previously we showed that the extracts of *Zataria multiflora* and hawthorn as medicinal plants protected human lymphocytes against genotoxicity induced by gamma irradiation. These extracts with strong antioxidant activities may affect scavenging free radicals such as hydroxyl radicals generated by γ-rays in cells (29, 30). Based on our findings, MCS at high concentration 200 µg/mL completely protected DNA damage induced by IR on human lymphocyte. This result is very promising that a natural product showed an excellent radioprotective effect that we previously did not observed the same results with other herbal extracts.

As a result, the presence of phenolic compounds such as chlorogenic acid in MCS with antioxidant properties may contribute to reduce genotoxicity induced by IR in human lymphocytes. It seems that chicory seeds can help body's defense system against side effects induced by irradiation in patients during radiotherapy.

Acknowledgments

This study was supported by a grant from Mazandaran University of Medical Sciences, Sari, Iran. This research was the subject of a Pharm.D thesis of Vahid Ghaffari-rad as a student of Mazandaran University of Medical Sciences.

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