Radioprotective Effects of Hawthorn Fruit Extract Against Gamma Irradiation in Mouse Bone Marrow Cells

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Crataegus microphylla/Radioprotective/Micronucleus/Irradiation/Hawthorn.

The radioprotective effect of hawthorn (Crataegus microphylla) fruit extract against genotoxicity induced by gamma irradiation has been investigated in mouse bone marrow cells. A single intraperitoneal (ip) administration of hawthorn extract at doses of 25, 50, 100 and 200 mg/kg 1h prior to gamma irradiation (2 Gy) reduced the frequencies of micronucleated polychromatic erythrocytes (MnPCEs). All four doses of hawthorn extract significantly reduced the frequencies of MnPCEs and increased the PCE/PCE+NCE ratio (polychromatic erythrocyte/polychromatic erythrocyte + normochromatic erythrocyte) in mice bone marrow compared with the non drug-treated irradiated control (p < 0.02–0.00001). The maximum reduction in MnPCEs was observed in mice treated with extract at a dose of 200 mg/kg. Administration of amifostine at dose 100 mg/kg and hawthorn at dose 200 mg/kg reduced the frequency of MnPCE almost 4.8 and 5.7 fold; respectively, after being exposed to 2 Gy of gamma rays, compare with the irradiated control group. Crataegus extract exhibited concentration-dependent activity on 1,1-diphenyl 2-picrylhydrazyl free radical showing that Crataegus contained high amounts of phenolic compounds and the HPLC analysis determined that it contained chlorogenic acid, epicatechin and hyperoside. It appeared that hawthorn extract with antioxidant activity reduced the genotoxicity induced by gamma irradiation in bone marrow cells.

INTRODUCTION

Ionizing radiation passing through living tissues generates free radicals. Interactions of free radicals with DNA can induce DNA damage lead to mutagenesis and carcinogenesis. The damaging effects of ionizing radiation lead to cell death and are associated with an increased risk for numerous genetically determined diseases. With respect to radiation damage to human, it is important to protect humans from side effects induced by ionizing radiation. Historically, sulf-hydryl compounds have been among the first radioprotective agents. Amifostine as a thiol compound is a powerful radioprotective agent, but its application is clinically limited due to side effects and toxicity. Plant products have many compounds with medicinal value that protect biological systems from ionizing radiation with minimum side effects. We reported that citrus extract protected mouse bone marrow cells against gamma irradiation. Citrus extract contained high amounts of flavonoids. Recently we showed that hesperidin as a flavonoid had powerful protection effects on DNA damage induced by gamma irradiation.

The genus Crataegus (Hawthorn) has been widely prescribed or used in medicine. Hawthorn has many pharmacological properties, such as reducing blood pressure and total plasma cholesterol, treatment of congestive heart failure, inducing a significant decrease in mortality after ischemic reperfusion in animals and antiviral effects. Hawthorn contains phenolic and flavonoids compounds including chlorogenic acid, epicatechin, rutin, hyperoside and vitexin. In particular, antioxidant and radical scavenging activities are suggested as possible modes of action in these compounds. The aim of this study was to characterize radioprotective activity of Crataegus microphylla by using gamma rays as an oxidative DNA damaging agent, and to
investigate any reduction in the frequency of micronucleated polychromatic erythrocytes (MnPCEs) in mouse bone marrow exposed to gamma rays.

**MATERIALS AND METHODS**

**Animal**

Fifty male NMRI mice weighing 25 ± 3 g (± SD) were purchased from the Pasteur Institute (Tehran, Iran). Mice were housed in a good condition in the university animal house and were given standard mouse pellet and water ad Libitum. All animals were kept under controlled lighting condition (light: dark, 12:12h) and temperature (22 ± 1°C).

**Chemicals**

The chlorogenic acid, rutin, (–)-epicatechin, hyperoside and 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) were purchased from Fluka Chemical Co. (Buchs, Switzerland). All other reagents and acetonitrile (HPLC grade) were purchased from Merck Company.

**Preparation of Crataegus extract**

The ripe fruits of *Crataegus microphylla* were collected from Neka area in the north of Iran. The peels of the hawthorn were dried at room temperature and powdered in a grinder. Aqueous methanol (75%) was added to the powdered peels (20g), and stirred for one hour. The mixture was kept at room temperature for 72 h. After filtration, methanol was evaporated under reduced pressure at 40°C. The remained water extract was freeze-dried. In so, 6 g of extract powder were obtained.

**Determination of total phenol**

Folin Ciocalteu reagent was used for the determination of total phenols adapted from McDonald *et al.* A dilute extract or gallic acid (standard phenolic compound) was mixed with Folins Ciocalteu reagent (5 ml, 1:10 diluted with distilled water), and aqueous Na2CO3 (4 ml, 1 M). The mixtures were remained for 15 min and the total phenols were determined by colorimeter at 765 nm. The standard curve was prepared using solutions of gallic acid in methanol: water (50: 50, V/V). Total phenol values were expressed as a gallic acid equivalent (mg/g dry mass).

**HPLC analysis**

The phenolics contained of the hawthorn fruits were analyzed by HPLC method described by Zhang *et al* with brief modification. The HPLC system consists of a model K-1001 solvent delivery system equipped with a Rheodyne injection valve (20 μl sample loop inserted) and a UV-Vis spectrophotometer detector model K-2600 set at 278 nm (all from Knauer Assoc., Germany). Chromatographic separation was performed on an ODS-C18 column (150 × 4.6 mm i.d., 5 μm particle size), (Shim-pack VP-ODS), coupled to a security guard precolumn (5 × 4.6 mm i.d., 5 μm particle size), using an isocratic mobile phase of acetonitrile and 25 mM sodium phosphate buffer (PH 2.4) (22/78 v/v), delivered at flow rate of 1 ml /min.

**Treatment**

Hawthorn extract was dissolved in distilled water and four doses of extract (25, 50, 100 and 200 mg/kg) were injected intraperitoneally to the experimental animals at 1 h before gamma irradiation. Amifostine was dissolved in distilled water and a single dose of 100 mg/kg was injected. The control group received the same volume of distilled water. Five mice were used for each treatment group.

**Irradiation**

Whole-body irradiation was performed with a cobalt-60 γ-radiation source (Teratron 780, Canada). Mice were placed in ventilated Plexiglas cages and irradiated in groups of five mice simultaneously. The source-to-skin distance was 80 cm with a dose rate of 1.03Gy/min at room temperature (23 ± 2°C). The mice were irradiated with a total dose of 2 Gy γ-rays.

**Micronucleus assay**

The mouse bone marrow micronucleus test was carried out according to the method described by Schmid. Animals were sacrificed by cervical dislocation 24 h after irradiation. The bone marrow from both femurs was flushed in the form of a fine suspension into a centrifuge tube containing fetal calf serum (FCS). The cells were dispersed by gentle pipetting and collected by centrifuge at 400 g for 5 min at 4°C. The cell pellet was resuspended in a drop of FCS, and bone marrow smears were prepared. The slides were coded to avoid observer bias. After 24 h air-drying, smear were stained with May-Grunwald/ Giemsa. With this method, polychromatic erythrocytes (PCEs) stained reddish-blue, and normochromatic erythrocytes (NCEs) stained orange, while nuclear material is dark purple. For each experimental point, five mice were used and a total of 5000 PCEs were scored per each experimental point to determine the percentage of micronucleated polychromatic erythrocytes (MnPCEs), micronucleated normochromatic erythrocytes (MnNCEs), and ratio of PCE to (PCE + NCE). The ratio of PCE to (PCE + NCE) was determined for each experimental group to assess radiation effects with or without hawthorn extract on bone marrow proliferation.

**Measurement of free radical scavenging activity**

The free radical-scavenging capacity of hawthorn extract was determined as bleaching of the stable 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH). Different concentration of hawthorn extract (0.01 to 0.2 mg/ml) was added, at an equal volume, to methanolic solution of DPPH (100 μM). After 15 min at room temperature, the absorbance was recorded at 532 nm. The free radical scavenging capacity was calculated as the percentage of the initial absorbance.
517 nm. The experiment was performed in triplicate. BHT (Butylated Hydroxytoluene) was used as an antioxidant standard.

**Statistical analysis**

Statistical analysis was performed using SPSS for windows (version 11; SPSS Inc., Chicago, IL, USA). One-way ANOVA analysis and Tukey's HSD test were used for multiple comparisons of data.

**RESULTS**

**HPLC analysis**

The separation of four standards of analytes, chlorogenic acid, epicatechin, rutin and hyperoside are shown in Fig. 1. Typical retention times are as follows: chlorogenic acid 2.54 min, epicatechin 3.12 min, rutin 3.8 min and hyperoside 4.81 min. The comparison between their retention times with that of the standards identified the peaks of the analytes in the hawthorn extract. The crateagus extract was standardized based on epicatechin by HPLC method. The calibration curve of epicatechin was linear over the range 20–80 µg/ml. The epicatechin content was 15.96 ± 1.8 mg/g in extract powder.

Total phenol was determined by the folin Ciocalteu method. It was expressed as gallic acid equivalents by reference to a standard curve. The amounts of total phenolic compounds in the Crataegus extract was 165.4 ± 10.2 mg/g of extract powder.

**Antioxidant activity**

DPPH free radical scavenging method was be used to evaluate the antioxidant activity of specific compound. Excellent scavenging effect was observed with Crataegus extract. Scavenging effects of methanolic extracts were enhanced with increasing concentration. The scavenging effects were obtained in 91% and 70% at 0.2 mg/ml for BHT and Crataegus extract, respectively.

**Effects of Crataegus on the frequency of MnPCEs induced by gamma irradiation**

The effect of gamma irradiation with or without hawthorn fruit extract on the induction of MnPCEs and the PCE/PCE+NCE ratio in bone marrow cells, 24 h after γ-irradiation, is shown in Table 1. The frequency of micronuclei was increased in all groups of mice irradiated with 2 Gy γ-irradiation in comparison with the control group treated with distilled water (p < 0.00001). The frequencies of MnPCE found in the extract treated groups were significantly lower than that of the group treated only with radiation. In comparison to the irradiation-control group, the total MnPCE values were 1.71, 2, 1.93 and 5.7 times less that those in the treatment group receiving 25, 50, 100 and 200 mg/kg Crataegus extract respectively. All four doses were effective in significantly reducing of MnPCEs (p < 0.02). The maximum reduced MnPCE was observed in mice treated with extract at a dose of 200 mg/kg. The frequency of MnPCE in the latter group was 2.02 ± 0.49% which was much lower than the control group receiving radiation alone (11.50 ± 0.87%). Administration of amifostine at dose 100 mg/kg reduced the frequency of MnPCE to 2.38 ± 0.22%. There was no significant difference between hawthorn extract at dose 200 mg/kg and amifostine treated mice at reducing the frequency of MnPCE. Determination of PCE/PCE+NCE ratio in the gamma irradiated mouse showed a pronounced cytotoxic
effect of radiation on bone marrow proliferation. Treatment of mice with hawthorn extract arrested the radiation-induced decline in the PCE/PCE+NCE ratio (Table 1), and this ratio in the extract irradiated group (at doses 25, 50, 100, 200 mg/kg) was higher than that of the irradiated-alone group (p < 0.05). The highest PCE/PCE+NCE ratio was observed in hawthorn extract treated with 100 and 200 mg/kg before γ-irradiation. Administration of amifostine increased PCE/PCE+NCE ratio to 48.4%. In this study, crataegus extract and amifostine did not indicate any genotoxic and toxic effects at extensive doses.

**DISCUSSION**

An increase in the DNA damage after γ-irradiation has been observed in different studies. In the present study, an elevation in the frequency of MnPCEs was observed after irradiation in both control and experimental groups. In a previous report we showed that the synthetic compounds containing thiol protect mice against a lethal dose of γ-irradiation. Difficulties were encountered when administering aminothiols to humans leading to adverse toxic effects such as hypotension, nausea, vomiting and allergy. Thus, the use of these agents must have limited usage due to their toxicities. Phytochemicals a antioxidant might provide a variable degree of radioprotection. Natural compounds, including phenolic acids and flavonoids, may affect scavenging free radicals, such as hydroxyl radicals, generated by γ-rays in cells. There is a possible that pretreatment with medicinal plants with antioxidant activity could protect biological system against oxidative stress. Citrus extract contains high amounts of flavonoids have strong radical scavenging properties and reduce side effects due to oxidative damage. The results of this study demonstrated the protective effects of Crataegus extract, against genotoxicity and toxicity induced by γ-irradiation in mouse bone marrow cells. The greatest protective effects were observed at dose of 200 mg/kg. Crataegus did not show any genotoxicity or toxic effects at doses up to 200 mg/kg in mice bone marrow cells. Treating 2 Gy γ-rays reduced the frequency of MnPCEs almost 5.7 fold. The administration of amifostine, as a powerful radioprotective agent, reduced the frequency of MnPCE 4.8 fold. There has not been any significant difference between extract at dose 200 mg/kg and amifostine. The percentage of PCE/PCE+NCE ratio declined in irradiated mice, since this ratio gave a direct index of cell division. Hawthorn protected mice against radiation-induced decline in cell proliferation, as supported by the increased PCE/PCE+NCE ratio. We have shown that hawthorn extract contains high amounts of phenolic compounds. Antioxidant activity of the phenolic compounds (chlorogenic acid (phenolic acid), hyperoside (flavonoid) and epicatechin (proanthocyanins)) might be the main mechanism of hawthorn for its biological effects. Chlorogenic acid scavenged directly OH free radical in a dose-dependent manner, and it eliminated ROS induced by hydrogen peroxide. The chlorogenic acid has protective properties against oxidative stress induced in neural cell line. Epicatechin exerts antioxidant activity and protects hepatocytes against oxidative stress induced by tert-butyl hydroperoxide. Epicatechin increases superoxide dismutase activity, and inhibits the lipid peroxidation and cell membrane damage. Hyperoside has many kinds of biological function such as scavenging ROS, preventing the free radical induced oxidation and increased superoxide dismu-

**Table 1. Effects of hawthorn fruits extract on the formation of radiation-induced micronuclei PCE and the ratio of PCE/PCE+NCE in mice bone marrow exposed to 2 Gy gamma irradiation**

| Group | Treatment | MnPCE/PCE (%)* | PCE/PCE+NCE (%)* |
|-------|-----------|----------------|------------------|
| 1     | Control   | 0.25 ± 0.19    | 52.6 ± 0.97      |
| 2     | Irradiation | 11.50 ± 0.87*  | 43 ± 1.73*       |
| 3     | 25 mg/kg Hawthorn + irradiation | 6.73 ± 0.46* | 47.25 ± 0.5e |
| 4     | 50 mg/kg Hawthorn + irradiation | 5.76 ± 0.96* | 48.20 ± 1.3*   |
| 5     | 100 mg/kg Hawthorn + irradiation | 5.97 ± 1.10* | 48.67 ± 2.08* |
| 6     | 200 mg/kg Hawthorn + irradiation | 2.02 ± 0.49* | 48.3 ± 0.84*  |
| 7     | 100 mg/kg Amifostine + irradiation | 2.38 ± 0.22* | 48.43 ± 1.77* |
| 8     | 100 mg/kg Hawthorn | 0.43 ± 0.24* | 52 ± 2.45      |
| 9     | 200 mg/kg Hawthorn | 0.46 ± 0.22* | 50 ± 0.73      |
| 10    | 100 mg/kg Amifostine | 0.27 ± 0.12* | 52 ± 1         |

* Values are means ± SD for each group of five mice. a p < 0.0001 compared to control, b p < 0.001 compared to irradiation alone, c p < 0.01 compared to 100 mg/kg hawthorn + irradiation group, d no significant difference compared to control, e p < 0.01 compared to irradiation alone.
tase activity. 25) Hyperoside has protective effects on myocardial cells. 26) Phenolic acids and flavonoids had good antioxidant activity. 27) In addition, changes in the levels of endogenous antioxidative enzymes have been associated with interaction of different flavonoids with intracellular oxidative species. 28) In conclusion, we showed that Crataegus contains high amounts of phenolic compounds with antioxidant activity. These compounds could reduce the stress conditions and genotoxicity induced by gamma irradiation in bone marrow cells.

ACKNOWLEDGMENTS

This study was supported in part by a grant from Mazandaran University of Medical Sciences. The authors wish to thank Miss Mohammadifar and Miss Solimani for their assistance. We thank Dr. M. Shiran for revision of English language.

REFERENCES

1. Halliwell, B. and Aruoma, O. I. (1991) DNA damage by oxygen-derived species. Its mechanism and measurement in mammalian systems. FEBS Lett. 281: 9–19.
2. Sankaranarayanan, K. (1999) Ionizing radiation and genetic risks. X. The potential “disease phenotypes” of radiation-induced genetic damage in humans: perspectives from human molecular biology and radiation genetics. Mutat. Res. 429: 45–83.
3. Hosseinimehr, S. J., Shafiee, A., Mozdarani, H. and Akhlagpour, S. (2001) Radioprotective effects of 2-iminothiazolidine derivatives against lethal doses of gamma radiation in mice. J. Radiat. Res. 42: 401–408.
4. Hosseinimehr, S. J., Shafiee, A., Mozdarani, H., Akhlagpour, S. and Froughizadeh, M. (2002) Radioprotective effects of 2-imino-3-(chromone-2-yl) carbonyl thiazolidines against gamma irradiation in mice. J. Radiat. Res. 43: 293–300.
5. Hosseinimehr, S. J., Tavakoli, H., Pourheidari, G. R., Sobhani, A. and Shafiee, A. (2003) Radioprotective effects of citrus extract against gamma irradiation in mouse bone marrow cells. J. Radiat. Res. 44: 237–241.
6. Hosseinimehr, S. J. and Karami, M. (2005) Citrus extract modulates genotoxicity induced by cyclophosphamide in mouse bone marrow cells. J. Pharm. Pharmacol. 57: 505–510.
7. Hosseinimehr, S. J. and Nemati, A. (2006) Radioprotective effects of hesperidin against gamma irradiation in mouse bone marrow cells. Br. J. Radiol. 79: 415–418.
8. Bahorun, T., Aumjaud, E., Ramphul, H., Rycha, M., Luximon-Ramma, A., Trotin, Fm. and Aruoma, O. I. (2003) Phenolic constituents and antioxidative capacities of Crataegus monogyna (Hawthorn) callus extracts. Nahrung 47: 191–198.
9. Degening, F. H., Suter, A., Weber, M. and Saller, R. (2003) A randomised double blind placebo controlled clinical trial of a standardised extract of fresh Crataegus berries (Crataegisin) in the treatment of patients with congestive heart failure NYHA II. Phytomedicine 10: 363–369.
10. Quettier-Deleu, C., Voiselle, G., Fruchart, J. C., Duriez, P., Teissier, E., Bailleul, F., Vasseur, J. and Trotin, F. (2003) Hawthorn extracts inhibit LDL oxidation. Pharmazie 58: 577–581.
11. Zhang, D. L., Zhang, Y. T., Yin, J. J. and Zhao, B. L. (2004) Oral administration of Crataegus flavonoids protects against ischemia/reperfusion brain damage in gerbils. J. Neurochem. 90: 211–219.
12. Shahat, A. A., Cos, P., De Bruyne, T., Apers, S., Hammouda, F. M., Ismail, S. I., Azzam, S., Claeys, M., Goovaerts, E., Pieters, L., Vanden Bergh, D. and Vlietinck, A. J. (2002) Antiviral and antioxidant activity of flavonoids and proanthocyanidins from Crataegus sinaica. Planta Med. 68: 539–541.
13. Rohr, G. E., Meier, B. and Sticher, O. (1999) Quantitative reversed-phase high-performance liquid chromatography of procyanidins in Crataegus leaves and flowers. J. Chromatogr. A 835: 59–65.
14. Zhang, Z., Chang, Q., Zhu, M., Huang, Y., Ho, W. K. and Chen, Z. (2001) Characterization of antioxidants present in hawthorn fruits. J. Nutr. Biochem. 12: 144–152.
15. Kirakosyan, A., Seymour, E., Kaufman, P. B., Warber, S., Bolling, S. and Chang, S. C. (2003) Antioxidant capacity of polyphenolic extracts from leaves of Crataegus laevigata and Crataegus monogyna (Hawthorn) subjected to drought and cold stress. J. Agric. Food. Chem. 51: 3973–3976.
16. McDonald, S., Prenzler, P. D. and Antolovich, M. (2001) Phenolic content and antioxidant activity of olive extracts. Food Chem. 73: 73–84.
17. Schmid, W. (1975) The micronucleus test. Mutat. Res. 31: 9–15.
18. Mozdarani, H. and Gharabi, A. (1993) Radioprotective effects of cimetidine in mouse bone marrow cells exposed to γ-rays as assayed by micronucleus test. Int. J. Radiat. Biol. 64: 189–194.
19. Koleva, I., Van BEEK, T. A., Linskens, J. P. H., De Groot, A. and Evstatieva, L. N. (2002) Screening of plant extracts for antioxidant activity: a comparative study on three testing methods. Phytochem. Anal. 13: 8–17.
20. Kligerma, M. M., Glover, D. J., Thuris, A. T., Norflett, A. L., Yuhas, J. M., Coia, L. R. and Goodman, L. R. (1984) Toxicity of WR-2721 administered in single and multiple doses. Int. J. Radiat. Oncol. Biol. Phys. 10: 1773–1776.
21. Tiwari, A. K. (2001) Imbalance in antioxidant defence and human diseases: multiple approach of natural antioxidants therapy. Curr. Scienc. 81: 1179–1187.
22. Zang, L. Y., Cosma, G., Gardner, H., Castranova, V. and Vallyathan, V. (2003) Effect of chlorogenic acid on hydroxyl radical. Mol. Cell. Biochem. 247: 205–210.
23. Pavlica, S. and Gebhardt, R. (2005) Protective effects of ellagic and chlorogenic acids against oxidative stress in PC12 cells. Free Radic. Res. 39: 1377–1390.
24. Valls-Belles, V., Gonzalez, P. and Muniz, P. (2004) Epicatechin effect on oxidative damage induced by tert-BOOH in isolated hepatocytes of fasted rats. Proc. Biochem. 39: 1525–1531.
25. Breinholt, V., Lauridsen, S. T. and Drægsted, L. O. (1999) Differential effects of dietary flavonoid on drug metabolizing and antioxidant enzymes in female rat. Xenobiotica 29: 1227–
26. Luo, L., Sun, Q., Mao, Y. Y., Lu, Y. H. and Tan, R. X. (2004) Inhibitory effects of flavonoids from Hypericum perforatum on nitric oxide synthase. J. Ethnopharmacol. 93: 221–225.

27. Li, Q., Chou, G., Chen, Z. and Ma, C. (2002) Inhibitory mechanism of hyperin on the apoptosis in myocardial ischemia/reperfusion in rats. Acta. Pharm. Sin. 37: 849–852.

28. Chen, J. W., Zhu, Z. Q., Hu, T. X. and Zhu, D. Y. (2002) Structure-activity relationship of natural flavonoids in hydroxyl radical-scavenging effects. Acta Pharmacol. Sin. 23: 667–672.