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Radioprotective effect of *Haberlea rhodopensis* (Friv.) leaf extract on γ-radiation-induced DNA damage, lipid peroxidation and antioxidant levels in rabbit blood

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Different concentrations of *H. rhodopensis* total extract (HRE; 0.03, 0.06 and 0.12 g/kg body weight) were injected im, into rabbits 2 h before collecting the blood samples. The whole blood samples were exposed *in vitro* to 2.0 Gy ^{60}Co γ-radiation. The radiation-induced changes were estimated by using the chromosome aberration test (CA) and cytokinesis blocked micronucleus assay (CBMN) in peripheral lymphocytes, and by determining the malondialdehyde levels (MDA) in blood plasma and the superoxide dismutase (SOD) and catalase (CAT) activity in erythrocytes. Radiation significantly increased the chromosome aberration and micronuclei frequencies as well as MDA levels and decreased the antioxidant enzyme activity. On the other hand, the HRE pretreatment significantly decreased the CA, MN frequencies and MDA levels and increased the SOD and CAT activity in a concentration dependent manner. The most effective was the highest concentration of HRE (0.12 g/kg body weight). The results suggest that HRE as a natural product with an antioxidant capacity could play a modulatory role against the cellular damage induced by γ-irradiation. The possible mechanism involved in the radioprotective potential of HRE is discussed.

Keywords: Antioxidants, Catalase, Chromosome aberrations, *Haberlea rhodopensis*, Malondialdehyde, Micronuclei, Radioprotection, Superoxide dismutase

Ionizing radiation produces reactive oxygen species (ROS) or related radicals, resulting in cellular and tissue damage[1]. These reactive species can have a harmful effect on cellular macromolecules, and both the direct and indirect effects of ionizing radiation are deleterious on cellular DNA. DNA lesions induced by ionizing radiations have already been described and it is believed that chromosomal aberrations appear as a result of double strand breaks and misrepaired damage[2]. Indirect estimation of chromosome damage can be obtained through the frequency of micronuclei. Micronuclei are produced during mitosis due to various mechanisms and they can be detected in the cytoplasm beside the cell nucleus as small nucleus-like particles[3]. The frequency of micronuclei is used as an alternative for the examination of lymphocyte aberrations, as a predictive assay of radiosensitivity, and an identification of cancer-prone individuals[4,5].

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Apart from DNA damage, lipid peroxidation (LPO) is also considered a critical event during ionizing radiation-induced damage[6]. Lipid peroxidation has been found to amplify with the increase in the radiation dose in irradiated tissues[7]. To maintain the redox balance and in order to protect themselves from free radical action, living cells have evolved an endogenous antioxidant defense mechanism which includes non-enzymatic entities like glutathione, ascorbic acid, and also enzymes like superoxide dismutase, catalase, glutathione peroxidase, etc[8]. Although the endogenous cellular antioxidants act in concert to eliminate the ROS accumulation in a physiological state, under pathological conditions ROS overload might exceed the cellular antioxidant capacity, affecting critical biological macromolecules and triggering oxidative stress[9].

Therefore, to develop effective and nontoxic radioprotective and radiotherapeutic drugs, many sulphydryl compounds were synthesized and tested but none of them was found to be suitable for clinical applications due to their high toxicity. Hence, efforts have been made to develop effective,

nontoxic, inexpensive and easily available radioprotective drugs of a plant origin and several plants extracts have been tested or are in the process of being tested[10-12].

Haberlea rhodopensis (Friv.) belongs to family *Gesneriaceae* and is a Balkan endemic relict that is widely distributed mainly in the Rhodope Mountains and some regions of the Sredna gora Mountains and the Balkans. *Haberlea rhodopensis* belongs to the group of extremely desiccation-tolerant (ressurection) plants which are capable of withstanding long periods of almost full desiccation and recover quickly on water availability[13]. Carbohydrates and phenols have been found to play an important role in the survival of plants under extreme conditions[14]. Phenolic compounds, accumulated in high amounts in ressurection plants, are assumed to protect their membranes against desiccation and free radical-induced oxidation[15].

The total extract of *Haberlea rhodopensis* (HRE) was found to possess strong antioxidant and antibacterial activities[16,17]. Recently Berkov *et al.*[18] have revealed more than 100 compounds (amino acids, fatty acids, phenolic acids, sterols, glycerides, saccharides, etc.) and five ingredients possessing antiradical activity—syringic, vanillic, caffeic, dihydrocaffeic and p-coumaric acids in HRE. Further, a significant linear relationship was found between the antioxidant activity and the phenolic content, indicating that phenolic compounds are major contributors to the antioxidant activity of HRE[19]. The antimutagenic potential of HRE was reported in a preliminary study[20]. Therefore, the aim of present study is to investigate the protective role of the HRE against γ-radiation induced DNA damage, lipid peroxidation and antioxidant levels in rabbit blood.

Materials and Methods

Chemicals—All the chemicals were of analytical grade and purchased from Sigma Chemical Co (St. Lotus, USA) and Gibco.

Preparation of ethanolic extract of H. rhodopensis—Fresh leaves of *H. Rhodopensis* were collected from their natural habitat (the vicinity of Asenovgrad, Bulgaria) during the flowering period in May-June. They were botanically identified in Department of Pharmacology and Pharmacognosy (Medical University, Sofia, Bulgaria) by botanist-phytotherapist. Voucher specimen was deposited in the Institute of Botany, Bulgarian Academic of Science, Sofia, Bulgaria. The leaves were cut into small pieces and dried at room temperature for 1 month. After grinding the leave pieces, the dry matter was macerated for 6 h in 70% ethyl alcohol and then was percolated for 48 h. The primary extract was concentrated by evaporating ethanol in a vacuum environment in order to reach a ratio of 5% ethanol and 95% water. The obtained extract was filtered through a 0.25 μm Millipore membrane (Millipore, USA) to remove emulsified substances, chlorophyll and other particles. The extract was standardized in accordance with the method for determining the relative density (Bulgarian Pharmacopoeia Roll 2, p.19). The amount of the extracted substance(s) ranged between 0.098 and 0.142 g/cm^3 (average 0.120 g/cm^3).

Animals—Thirty male New Zeland rabbits, 5 months old, weighing 3.5–4.0 kg body weight purchased from the Animal House of the Agricultural Faculty, Trakia University were used. The experimental protocol was approved by the Department of Animal Care and adhered to the European Community Guiding Principles for the Care and Use of Animals. The animals were fed on a standard rabbit diet, had access to water *ad libitum*, and were synchronized by maintaining controlled environmental conditions (light, temperature, feeding time, etc.) for at least two weeks prior to and throughout the experiments.

Acute toxicity studies—Having determined the LD_{50} of >1250 mg/kg body weight as per Popov[21] and showing that HRE was not acutely toxic at 1250 mg/kg body weight, in this study <1/10 of LD_{50} is used.

Study design—The rabbits were divided into following 6 groups of 5 animals each. Gr. 1 (control) untreated rabbits; Gr. 2 (control 2) rabbits treated with HRE (0.12 g/kg body weight); Gr. 3 rabbits treated with double distilled water (DDW); Gr. 4 rabbits treated with HRE (0.03 g/kg body weight); Gr. 5 rabbits treated with HRE (0.06 g/kg body weight); Gr. 6 rabbits treated with HRE (0.12 g/kg body weight).

The animals from groups 2, 4, 5 and 6 were injected, im with HRE 2 h before collecting the blood. The animals from group 3 were injected, im with DDW at the same time. Blood samples from all groups were aseptically obtained from the marginal ear vein in vacutainers with heparin as an anticoagulant for the cytogenetical assays and EDTA as an anticoagulant for the biochemical assays.

In vitro irradiation—A cobalt teletherapy unit (Rocus M, ^{60}Co) at the Inter-District Cancer Dispensary, Stara Zagora, Bulgaria, was used for irradiation. The whole blood samples from groups 2, 3, 4, 5 and 6 were exposed to 2.0 Gy gamma rays, at a dose rate 89.18 cGy/min in a water bath (37 °C). The samples were kept at 37 °C for 1 h immediately after the irradiation and then transported to the laboratory. While conducting the experiments and the data analysis, the guidelines and recommendations of the International Atomic Energy Agency[22] were followed strictly.

Culture set up for chromosome aberrations—The method of Evans[23] with a modification for rabbits was applied. Briefly, 0.5 mL of irradiated and control whole heparinised blood was incubated in 7.0 mL (RPMI-1640) medium, 3.0 mL heat-inactivated foetal calf serum, 0.2 mL reconstituted phytohemagglutinin-M (PHA-M), 100 units/mL penicillin, and 50 µg/mL gentamicin. The cultivation flasks were placed in a thermostat in the dark at 39 °C. All cultures were incubated for 48 h. Colcemid at a final concentration of 0.2 µg/mL was added at 46 h to block the cells at a metaphase stage. At the end of the 48th h from the beginning of lymphocyte incubation, chromosomal preparations for detection of chromosomal aberrations were established. The slides were stained with 10% Giemsa (Merck), and mounted. In each group a total of 500 cells (100 cells from each donor) were scored and the frequency of aberrant cells, fragments, dicentrics and rings were registered.

Culture set up for MN—The presence of MN in a binucleated cell was assayed by blocking the cell at cytokinesis stage. Lymphocyte culture was set as described for chromosome aberrations up to the stage of addition of phytohemagglutinin-M (PHA-M). In place of colcemid, cytochalasin-B (Sigma) in concentration of 6 µg/mL was added to the culture at 44 h. The cells were harvested at 72 h. The contents of the flasks were centrifuged and the sediment was treated with 0.075 mol/L KCl. Following the fast hypotonic treatment, the cells were fixed with a freshly prepared fixative solution of methanol/acetic acid (3:1). After a treatment with the fixative twice, the cell sediment was finally re-suspended in a small amount of the fixative. The cell suspension was dispensed in drops onto microscope slides and left to air-dry at room temperature. The slides were stained with 5% Giemsa solution. In each group a total of 5000 (1000 cells from five donors) binucleated cells (BNC) were scored and the frequency of cells with one (MN1), two (MN2) and three (MN3) micronuclei was recorded. The total number of MN in each group was derived from (1×MN1) + (2×MN2) + (3×MN3).

Biochemical assays

Peripheral blood processing—Collected blood (with EDTA) was centrifuged at 3000 *g* for 15 min and plasma was separated. Then, the plasma was deproteinized with 25% trichloroacetic acid by continuous mixing for 5 min and centrifugation at 2000 *g* for 15 min. The deproteinized plasma was used for lipid peroxidation products determination.

Determination of products of lipid peroxidation—The total amount of lipid peroxidation products in plasma was assayed using the thiobarbituric acid (TBA) method, measuring spectrophotometrically malondialdehyde (MDA) reactive products at 532 nm[24].

Erythrocyte processing—The erythrocyte pellet was washed thrice with saline and lysed. The hemoglobin was separated by precipitation with ethanol/chloroformmixture. The mixture was continuously shaken for 5 min and centrifuged at 2500 *g* for 20 min. The obtained supernatants were used for determination of enzyme activity.

Determination of superoxide dismutase (SOD) activities—Erythrocyte lysates were assayed for SOD activity using the xanthine/xanthine oxidase system for superoxide anion ($O_2^{\cdot-}$) generation. This anion reduced nitroblue tetrazolium (NBT) to formazan, which was monitored at 560 nm[25].

Determination of catalase (CAT) activities—CAT activity in erythrocyte lysates was assayed at 25 °C and *p*H 7 by the method of Beers and Sizer[26]. The decrease in H_2O_2 concentration was determined at 240 nm.

Haemoglobin concentrations—Haemoglobin concentrations of lysates were etermined spectrophotometrically at 546 nm by the cyanmethemoglobin method of Mahoney *et al.*[27].

Statistical analysis—The values were expressed as mean±SD. Differences between groups were assessed by one-way analysis of variance (ANOVA) using the SPSS 13.0 software package for Windows. Post hoc testing was performed for inter-group comparison using the least significance difference (LSD) test. *P* values < 0.05 was considered as significant.

Results

Effect of HRE on the frequency of chromosome aberration and MN in peripheral lymphocytes exposed to γ-radiation—The frequencies of chromosome aberrations (CA) after 2.0 Gy γ-irradiation in cultured rabbit blood lymphocytes are presented in Fig. 1. A significant increase in the frequencies of the aberrant cells – 20.6±4.66, (*P*<0.001) and CA (fragments 24.2±5.44, dicentrics 11.6±1.14 and total aberrations 37.6±5.5) was observed after irradiation, when compared with the control lymphocytes. Although the level of CA in HRE pretreated rabbits (0.03, 0.06 and 0.12 g/kg body weight) remained above that in control groups (*P*<0.05), all concentration of HRE significantly decreased the number of aberrant cells and total aberration frequencies when the lymphocytes were exposed to irradiation. Also, significant statistical differences were found between Gr. 4; Gr. 5 and Gr. 6 (Fig. 1). The highest concentration of HRE gave a yield of CA approximating the normal values.

The highest MN frequency was found in 2.0 Gy irradiated lymphocytes (120.1±13.23; *P*<0.001 compared with the control lymphocytes). Fig. 2a shows that pretreatment with HRE significantly (*P*<0.01) reduced the number of MN even at 0.03 g/kg body weight concentration. Both the 0.06 and 0.12 g/kg body weight concentrations of HRE reduced the frequency of MN to 32.1±5.1 (*P*<0.01 compared with Gr. 4) and 19.2±5.9 MN/1000 BNC (*P*<0.01 compared with Gr. 5), respectively.

Hence, the present results show that HRE decreases the MN and CA frequencies in γ-irradiated lymphocytes in a dose dependent manner.

Effect of HRE on the levels of MDA and the activities of SOD and CAT—Exposure to 2.0 Gy γ-radiation resulted in a significant increase in the plasma level of MDA – 1.58±0.19 µmol/L (P<0.001 compared with control groups), which was modified by pretreatment with HRE (Fig. 2b). HRE pretreatment (0.03, 0.06 and 0.12 g/kg body weight) showed progressively decreased concentrations of MDA when compared with the γ-irradiated group. A significantly decreased level of MDA was found only after pretreatment with 0.06 g/kg body weight HRE – 1.14±0.2 µmol/L and 0.12 g/kg body weight HRE – 1.01±0.11 µmol/L.

Reduced activities of SOD – 1051±60.9 U/gHb and CAT – 35820±675 U/gHb in the γ-irradiated erythrocytes were observed in the present study (Fig. 2c and 2d) and HRE pretreatment showed significantly increased activities of these antioxidant enzymes. Doses of 0.06 and 0.12 g/kg body weight increase SOD to 1251±54.1 U/gHb and 1290±52.2 U/gHb (*P*>0.05 compared with the control groups).

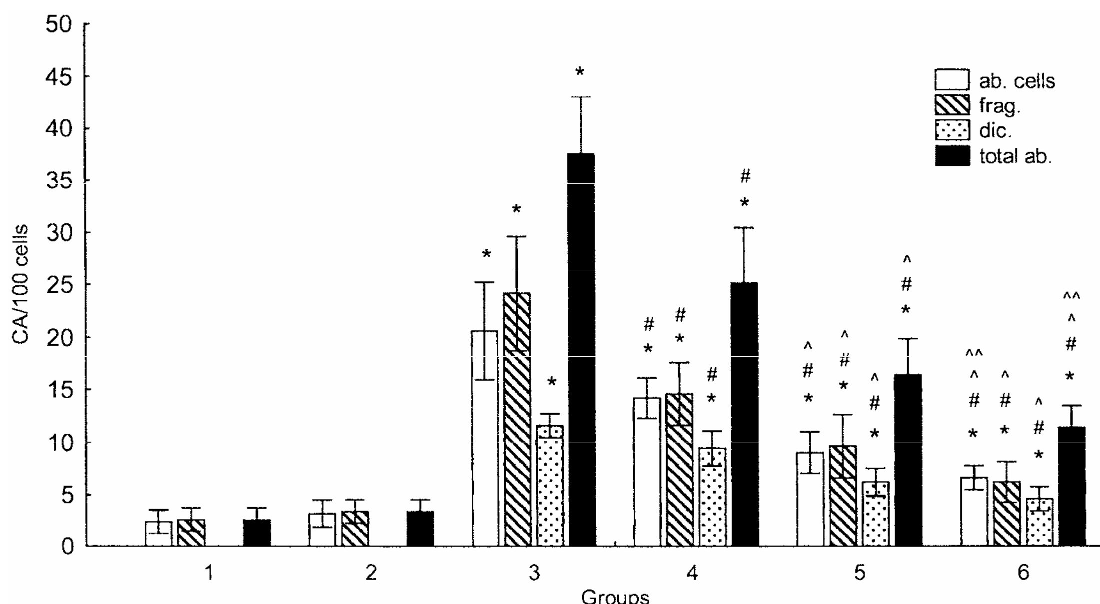

Fig.1—Effect of HRE pretreatment on the frequency of CA in γ-irradiated rabbit lymphocytes: Gr. 1 untreated control, Gr. 2 HRE (0.12 g/kg body weight) pretreated control, Gr. 3 γ-irradiated, Gr. 4 HRE (0.03 g/kg body weight) pretreated γ-irradiated, Gr. 5 HRE (0.06 g/kg body weight) pretreated γ-irradiated, Gr. 6 HRE (0.12 g/kg body weight) pretreated γ-irradiated. The values are presented as mean ±SD from 5 observations each. *statistical significance vs control groups; # statistical significance vs Gr. 3; ^ statistical significance vs Gr. 4; ^^ statistical significance vs Gr. 5

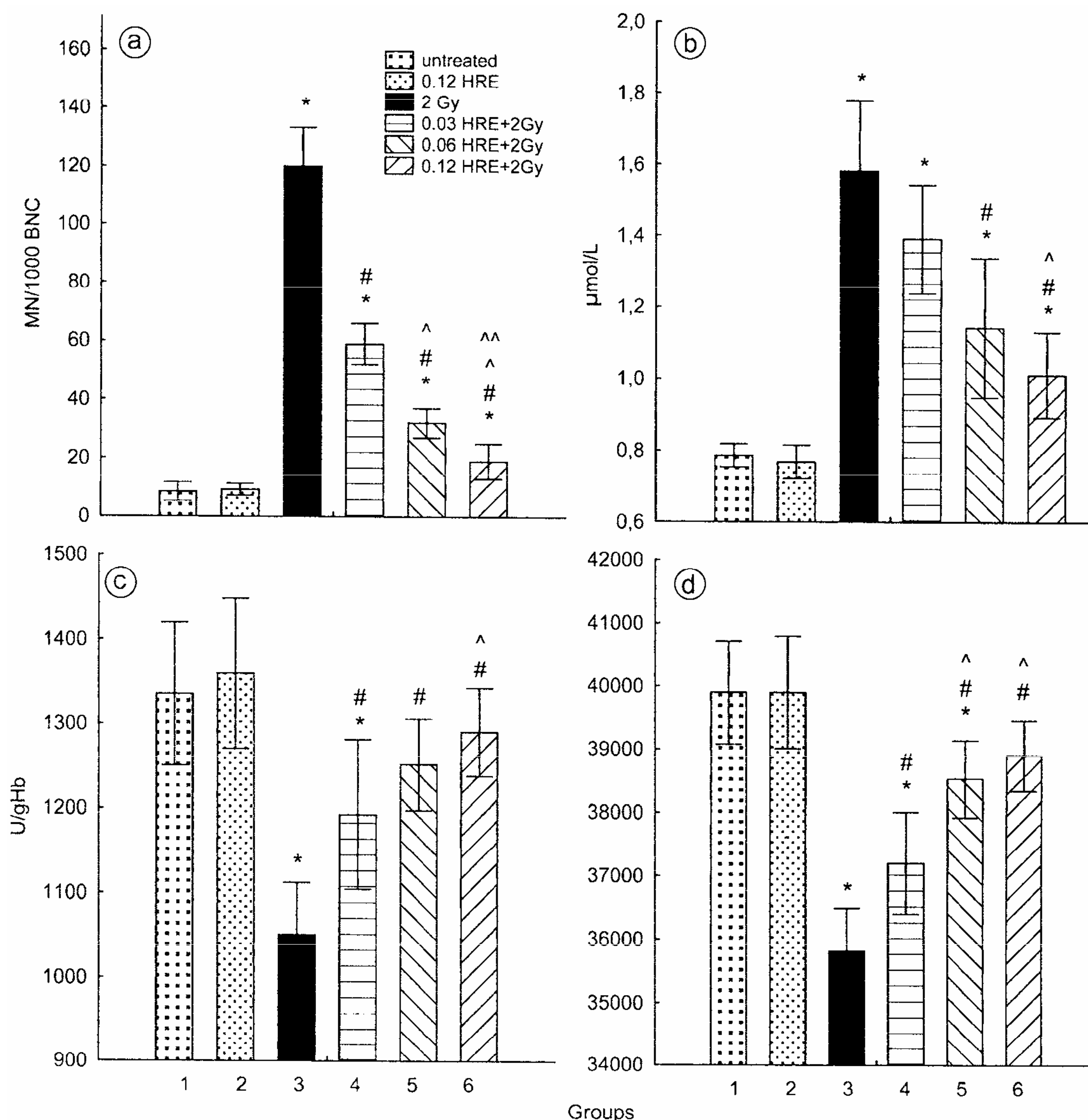

Fig. 2—Effect of HRE pretreatment on (a) the frequency of MN; (b) the level of MDA; (c) the activity of CAT; (d) the activity of SOD.* statistical significance vs control groups; # statistical significance vs Gr. 3; ^ statistical significance vs Gr. 4; ^^ statistical significance vs Gr. 5

For CAT, the most effective was 0.12 g/kg body weight HRE, which increases enzyme activity to a normal level (38901±556 U/gHb; $P>0.05$ compared with the control groups).

Hence, the present results show that HRE decreases the MDA level and increases SOD and CAT activity in γ-irradiated blood in a dose dependent manner.

Discussion

Damage to the DNA resulting from ionizing radiation is the most important factor in cell death. Such damage brings about altered cell division, depletion of stem cells, organ system dysfunction, etc. So one approach to prevent such an injury is via supplementation with or administration of natural antioxidants as natural radioprotectors[28].

In the present study, it was found that HRE renders protection against γ-radiation induced DNA damage (CA and MN frequencies) when compared with the γ-irradiated group. Also, in a previous study HRE showed antimutagenic activity against cyclophosphamide-induced mutagenicity[29]. Several investigators have demonstrated the ability of free radical scavenging compounds to protect cellular DNA against a significant proportion of the indirect effects of ionizing radiation, where the hydroxyl radicals are believed to be the primary active species responsible for the damage[30]. Free radical scavengers

like antioxidant nutrients and phytochemicals have been shown to possess a radioprotective effect against micronuclei induction and chromosome aberrations[31].

Antioxidant enzymes such as SOD, CAT and GPx are important in providing protection against radiation exposure[32], the proper balance of the enzymes in specific cells and in the whole organism required for maximum radioprotection. Therefore, a reduction in the activity of these enzymes can result in a number of deleterious effects due to the accumulation of superoxide radicals and H_2O_2. Membrane lipids are the major targets of ROS and the free radical chain reactions thus initiated cause extensive membrane lipid peroxidation. The increase in the levels of lipid peroxidation products such as malondialdehyde, hydroperoxides, TBARS and conjugated dienes are the indices of membrane lipid damage. Moreover, the enhanced levels of lipid peroxidation induced by radiation are accompanied by a decrease in the activities of SOD, CAT and GPx[33].

In the present study, a significant decrease in the level of enzymatic antioxidant (SOD, CAT) and an increase in the level of lipid peroxidation (MDA) in the γ-irradiated group were observed. However, the pretreatment of rabbits with HRE prior to the radiation exposure of blood samples increased the antioxidant status at enzymic levels and decreased the level of MDA. This effect is supposed to be due to the antioxidant property of HRE and shows that it acts as a good scavenger against free radical generation and thereby inhibits lipid peroxidation.

HRE contains high level of flavanoid antioxidants. Several phenolic compounds were identified, mainly phenolic acids (ferulic acid, sinaric acid, caffeic acid, p-coumaric acid, ect); flavanoid-aglycones and glycosides (luteolin, myricetin, hesperidin, rutin, etc.)[19]. Michailova *et al.*[19] suggested that the major antioxidant activity of HR was due to the phenolic compounds, while the remaining activity may be attributed to the other compounds found in its leaves. The free radical scavenging and antioxidant properties of HRE have been also reported by Berkov *et al.*[18].

High phenolic content is an important factor in determining antioxidant activity[34]. Birošová *et al.*[35] reported that the ferulic and caffeic acids decreased the mutagenic effect of 5NFAA (Salmonella mutagenicity assay) by about 50%. The antimutagenic and radical scavenging activity of phenolic acids was reported by other authors[36,37] as well.

Several studies also suggest that flavonoids may act as antioxidants, free radical scavengers, or radioprotectors[38,39]. A number of flavanoids (genistein, quercetin, luteolin) have been found to reduce the frequency of micronucleated reticulocytes in the peripheral blood of irradiated mice[40]. Protection of DNA from oxidative stress by luteolin, quercetin and rutin was demonstrated *in vitro* in the Comet assay[41]. Luteolin also induces the expression of antoxidant enzymes, as shown by Sharma *et al.*[42] in normal astrocytes. The results obtained by Kalpana *et al.*[43] indicate that hesperidin shows an optimum protection against radiation by effectively decreasing the MN frequencies, dicentric aberrations and comet attributes in human lymphocytes. Lodovici *et al.*[44] showed that 4-coumaric acid, stabilizes the SOD activity in rabbit corneal-derived cells when exposed to UVB radiation through its free radical scavenging activity.

The present results corroborate with all these results and data by other authors and show that the radioprotective effect of HRE can be explained by its scavenging capability of free radicals before they have caused damage to the cellular macromolecules since HRE is a potent antioxidant with a capacity for free radical trapping.

Conclusion

The results show that γ-irradiation induces increased frequencies of micronuclei and chromosome aberrations, elevated levels of MDA, and decreased activities of the antioxidant enzymes SOD and CAT due to oxidative damage in rabbit blood. Nevertheless HRE, probably through its free radical scavenging properties, protects from γ-irradiation in a concentration-dependent manner.

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