The free radical scavenging activity of four flavonoids determined by the comet assay*

K. HORVÁTHOVÁ[1], L. NOVOTNÝ[2], A. VACHÁLKOVÁ[1]

[1]*Cancer Research Institute, Slovak Academy of Science, 833 91 Bratislava, Slovak Republic; e-mail: katarina.horvathova@savba.sk;*
[2]*Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Kuwait University, 13110 Kuwait*

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Flavonoids are reported to exhibit a wide variety of biological effects, including antioxidant and free radical scavenging activities. The aim of our study was to determine the cytotoxicity of flavonoids quercetin, rutin, apigenin and luteolin and their ability to protect DNA molecules against H_2O_2-induced damage. Cytotoxicity of studied flavonoids was tested in murine leukemia L1210 cells by the trypan blue exclusion technique. DNA strand breaks were determined using the alkaline single-cell gel electrophoresis (comet assay). Quercetin was found to possess the highest protective effect among the flavonoids studied (45%). The protective activity determined was lower for luteolin (40%). Protective effect of apigenin (600 μmol/l) was only marginal (2%). However, at the higher concentration of apigenin (1200 μmol/l), this flavonoid induced DNA single strand breaks. This indicates the ability of apigenin to serve as a pro-oxidant. Rutin had no protective effect on DNA single strand breaks induced by H_2O_2.

Key words: Quercetin, luteolin, apigenin, rutin, cytotoxicity, comet assay.

Flavonoids comprise the most common group of plant polyphenols and provide much of the flavor and color to fruits and vegetables. More than 5000 different flavonoids have been described. RUSZNYAK and SZENT-GYÖRGYI observed in 1936 that the mixture of some flavonoids decreased capillary permeability and fragility in humans [25]. Early studies of flavonoids demonstrated their significant antioxidant [1], antitumor [15], anti-inflammatory [31], anti-allergic [19], anti-osteoporotic activities [4] and hepatoprotective effect [14]. They are also able to modulate a wide range of mammalian enzyme activities of cytochrome P450, epoxide hydrolase, glutathione transferase, DNA and RNA polymerases and topoisomerases [21].

Two flavonols, quercetin (QU) and rutin (RU), and two flavones, apigenin (AP) and luteolin (LU), are attracting a lot of attention and are being studied very intesively due to their ability of preventing cardiovascular diseases and cancer in human [26].

Quercetin (5,7,3',4'-tetrahydroxyflavonol) and rutin (quercetin-3-O-glucose-rhamnose) act as powerful antioxidants preventing oxidation of LDL [3,13]. Their action may serve as the base for the explanation of beneficial epidemiologic effects related to increased consumption of fruits and vegetables and decreased cardio-vascular morbidity [30]. It was found that the dietary intake of QU estimated by the analysis of dietary records is inversely related to ischemic heart disease mortality [11]. Several more recent studies confirmed that QU is a strong antioxidant [20], and a strong inhibitor of CYP3A4 which acts as a modulator of P-glycoprotein [12]. Rutin had a slight but still obvious inhibitory effect on lipopolysacharide-induced nitric oxide production in primary peritoneal macrophages [27]. Intake of RU in proper amounts, can effectivelly improve antioxidant status as well as a level of trace elements such as iron, copper and zinc in mouse liver and brain [8]. Apigenin (5,7,4'-trihydroxyflavone) is a widely distributed plant flavonoid. It belongs among the inhibitors of growth and induces the G2/M cell cycle arrest by modulating cyclin B1 and cyclin-dependent kinase 1 (CDK1) protein levels regulators. It induces the extracellular-signal regulated kinase (ERK) mitogen-acti-

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vated protein kinase activation in breast carcinoma cells and in thyroid cancer cells [33]. Apigenin is a pleiotropic effector affecting both, protease-dependent invasiveness and associated processes as well as proliferation of tumor cells [17], by inhibition of CDK1 kinase activity [33]. Luteolin (5,7,3',4'-tetrahydroxyflavone) inhibits production of serum tumor necrosis factor-alpha *in vivo*. It also inhibits the arachidonic acid-induced ear edema formation, the 12-O-tetradecanoylphorbol-13-acetate-induced ear edema and the oxazolone-induced allergic edema. It decreases the activity of arylamine N-acetyl-transferase enzyme and formation of DNA-2-aminofluorene adducts in human and mouse leukemia cells [16].

The aim of the present study was to evaluate the free radical scavenging activity of QU, RU, AP and LU by measuring their DNA protective effects against H_2O_2- induced DNA strand breaks, with help of the single-cell gel electrophoresis (SCGE, comet assay). SCGE was chosen for our investigation because it is a sensitive and rapid method for the detection of DNA damage at the individual cell level [5, 18, 29]. It is suitable method for measuring and comparing the effects of the selected flavonoids on DNA.

Material and methods

Chemicals. QU, RU, AP, LU, Triton X-100, ethidium bromide and agarose electrophoresis reagent were obtained from Sigma-Aldrich Chemie, Steinheim, Germany. Agarose II, RPMI (1640) medium without L-glutamine and $NaHCO_3$ was bought from Amresco-Biotechnology Grade, USA. Na_2EDTA, NaOH, H_2O_2 and NaCl were purchased from Lachema Brno, Czech Republic. Tris(hydroxymethyl)-aminomethane was obtained from Serva Feinbiochemica, Heidelberg, Germany. Trypan blue solution (0.4%) was obtained from Fluka Chemie AG, Switzerland. Phosphate-buffered saline (PBS) was obtained from Sebak GmbH, Germany. Fetal calf serum (FCS) was purchased from Grand Island Biological Co., Grand, Island, NY, USA.

Cell culture. The murine leukemia cell line (L1210) was purchased from the American Type Culture Collection (Rockville, MD, USA). The cell line was kept in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), penicillin (100 μg/ml) and streptomycin (100 μg/ml). The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2: 95% air. All *in vitro* experiments were performed during the exponential phase of cell growth.

Cytotoxicity assay. Cells were incubated with different concentrations of flavonoids for 30 min at 37 °C in a dark incubator together with untreated control samples. Samples were then centrifuged at 1000 x g for 5 min at 25 °C. After pretreatment, the cells were washed with PBS and again centrifuged at 1000 x g for 5 min at 25 °C. Cells were stained with the trypan blue (TB – 0.4%) and the number of viable (uncoloured) and dead (colored) cells was counted. The ratio of the number of viable cells/all cells gives the percentage of viable cells. The two concentrations of a particular flavonoid that had only low effect on the viability of cells (generally less than 10–20%) were used for the investigation by comet assay.

Single cell gel electrophoresis (comet assay). The procedure of Singh et al [28] was followed with minor modifications introduced by Slameňová et al [29] and Gábelová et al [6]. Murine leukemia cells L1210 were incubated with different concentrations of flavonoids, QU (20, 70 μmol/l), RU (100, 1000 μmol/l), LU (25, 100 μmol/l), AP (600, 1200 μmol/l), for 30 min at 37 °C in a dark incubator together with untreated control samples. Samples were then centrifuged at 1000 x g for 5 min at 25 °C. After this pretreatment, cells were washed in PBS and centrifuged again at 1000 x g for 5 min at 25 °C. Assayed cells were suspended in 0.75% agarose II (low-melting point) and spread on a base layer (100 μl of 1% agarose in Ca^{2+} and Mg^{2+} free PBS buffer) on a microscope slide. The agarose was allowed to solidify. Involved slides were treated with 50 μl 100 μmol/l H_2O_2 for 5 min on ice in the dark and they were washed twice with PBS (10 mol/l H_2O_2 stock solution was kept at 4 °C and diluted immediately before use in Ca^{2+}- and Mg^{2+}-free PBS buffer at 4 °C). All slides were placed in a lysis mixture (2.5 mol/l NaCl, 100 mmol/l Na_2EDTA, 10 mmol/l Tris, 1% Triton X-100, pH 10) for 1 h at 4 °C to remove cellular proteins. The slides were then transferred to an electrophoretic solution (300 mmol/l NaOH, 1 mmol/l Na_2EDTA, pH 13) and kept in this solution for a 40 min unwinding time at 4 °C. A current of 25 V (300 mA) was then applied to them for 30 min. The slides were removed, neutralized with Tris (0.4 mmol/l, pH 7.5), and stained with 20 μl ethidium bromide (1 mg/ml). Ethidium bromide-stained nucleoids were evaluated with a Zeiss Jenalumar fluorescence microscope using the computerized image analysis (Komet 3.0, Kinetic Imaging, Ltd., Liverpool, UK). The percentage of DNA in the tail was determined as it is linearly related to the frequency of DNA breaks.

Statistics. The results were statistically evaluated using the Student's t-test and a statistically significant increase: $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, resp. decrease of DNA strand breaks by H_2O_2 or flavonoids: $^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$ was indicated in A, B, C and D parts of Figure 2.

Results

Cytotoxicity of flavonoids on murine leukemia L1210 cells. Cytotoxicity of QU, RU, AP and LU was evaluated by TB in L1210 cells. Murine leukemia L1210 cells were treated with QU, RU, AP (50–1200 μmol/l) and LU (25–100 μmol/l) for 30 min. Results are presented in Figure 1. They show that incubation of L1210 cells in RPMI containing different

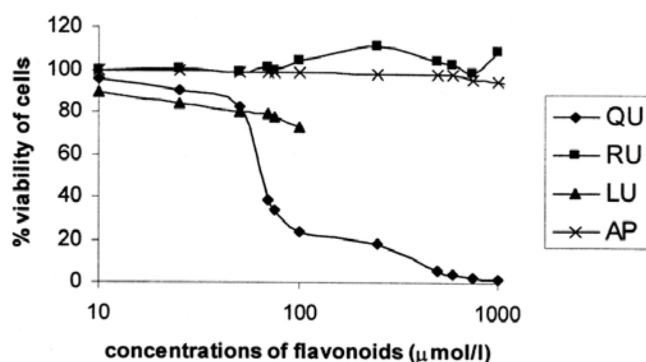

Figure 1. Percentage of viable cells after treatment of L1210 cells with flavonoids evaluated by TB exclusion technique.

concentrations QU and LU causes cytotoxicity at concentrations of >50 μmol/l. This cytotoxicity becomes remarkably high at 70 μmol/l of QU. Both, RU and AP do not have any cytotoxic effect even at substantially higher concentrations.

Influence of pre-incubation of L1210 cells with flavonoids on the level of single DNA strand breaks induced by H_2O_2. The level of DNA single strand breaks induced in L1210 cells by H_2O_2 was determined by the single cell gel electrophoresis. The optimal concentration of H_2O_2 in our experiments was 100 μmol/l. The % of tail DNA corresponding to the level of DNA strand breaks at this H_2O_2 concentration, was 60. Figure 2 represent protective effects of 30-min pretreatment of L1210 cells with QU (20, 70 μmol/l), RU (100, 1000 μmol/l), AP (600, 1200 μmol/l) or LU (25, 100 μmol/l) in combination with a short-term treatment of cells with H_2O_2 (100 μmol/l). Results presented at Figure 2A and 2B show that both QU and LU did not cause any significant increase of DNA single strand breaks but are capable to reduce the incidence of DNA strand breaks induced by H_2O_2. On the other hand, the results obtained with other two flavonoids, RU and AP, show that RU and AP at concentration of 600 μmol/l do not induce important changes in cellular DNA (Fig. 2C and 2D). On the other side AP at the concentration of 1200 μmol/l induces important increases in DNA strand breaks incidence.

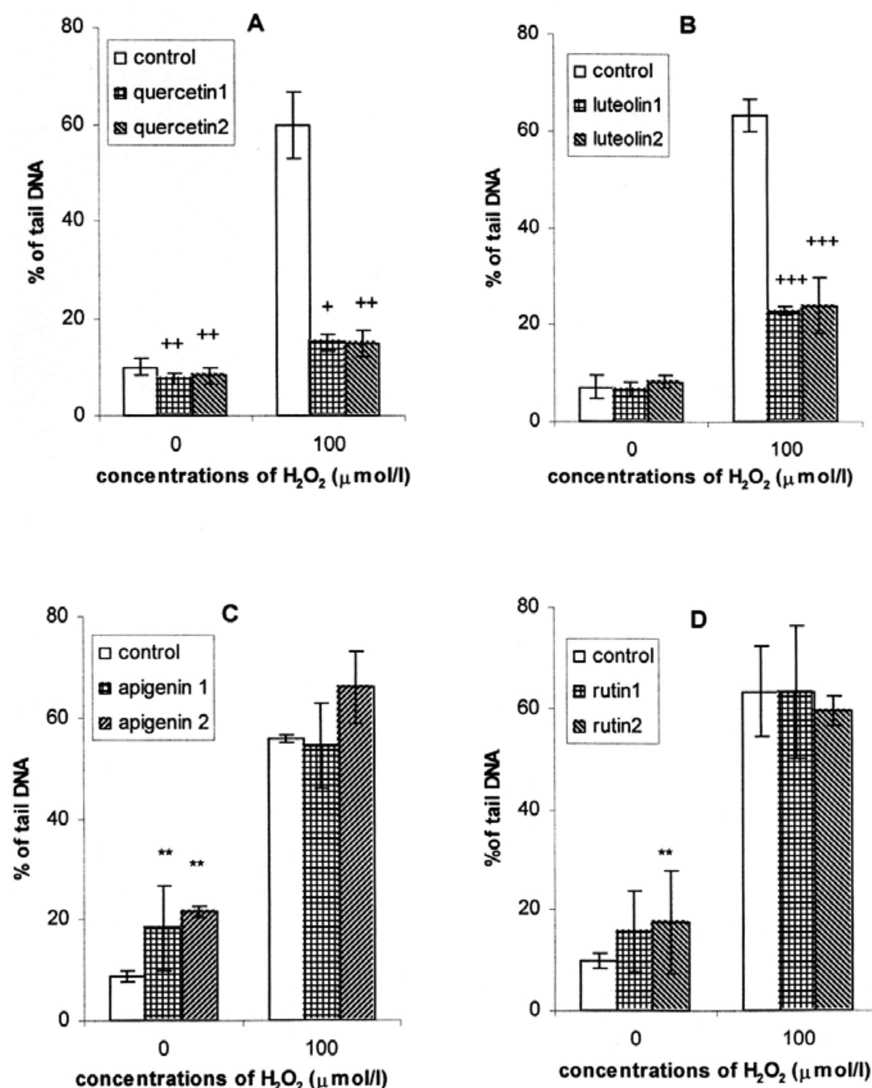

Figure 2. The incidence of DNA strand breaks in L1210 cells treated with flavonoids, H_2O_2, or by combination of H_2O_2 and flavonoids. (A) quercetin 1 (40 μmol/l) and quercetin 2 (70 μmol/l), (B) luteolin 1 (25 μmol/l) and luteolin 2 (100 μmol/l), (C) apigenin 1 (600 μmol/l) and apigenin 2 (1200 μmol/l), (D) rutin 1 (100 μmol/l) and rutin 2 (1000 μmol/l).

Discussion

Free radicals play an important role in the development of cancer, coronary heart diseases and in the aging processes through their augmentation on lipid peroxidation, oxidative damage to DNA and other processes. It is known that low-density lipoprotein (LDL) oxidation underlines atherosclerotic processes and that the inhibition of this oxidation represents an effective way in preventing or delaying progression of atherosclerosis. It was reported that the inhibitory effect of flavonoids on LDL oxidation decreases in the order of LU > QU > AP. HIRANO et al [10] suggests that 1)

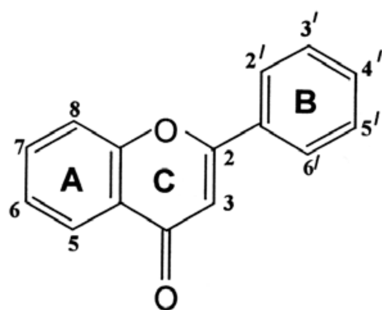

Figure 3. Universal chemical structure of flavonoids.

radical trapping effects of flavonoids is related to their structure, and that 2) flavonoids act as hydrogen donors to alpha-tocopherol radicals. Consequently, this interaction with alpha-tocopherol delays the LDL oxidation. The ability of flavonoids to scavenge reactive oxygen species, such as hydroxyl and superoxide anion radicals, can prevent oxidative damage of biomacromolecules [23].

H_2O_2, a well-defined and investigated oxidative agent, does not react with DNA. It is envisaged that biological membrane-crossing H_2O_2 penetrates to a cell nucleus and reacts with ions of iron or copper to form ·OH radicals. Attack of ·OH radicals on DNA results in breaks of nucleotide sugar moieties and a terminal sugar residue fragment [9, 22]. A direct rejoining repairs these single breaks of DNA. In addition to this, ·OH radicals attack DNA bases and produce thymine glycol, 8-hydroxyguanine or 2,6-diamino-4-hydroxy-5-formamidopyrimidine. Most of these oxidative DNA lesions are repaired by base excision repair. However, some forms of oxidative damage of bases are repaired by systems that apparently recognize elements of DNA helix distortion rather than specific base damage [2].

Many authors studied antioxidant potential of dietary flavonoids in relation to oxidative DNA damage caused by H_2O_2 using vitamin C as positive control. It was reported that the prooxidant properties of flavonoids and other polyphenolic substances could participate in induction of tumor cell apoptosis thus playing an important role in chemoprevention of cancer [7]. The mechanisms participating in this chemoprevention may be very different but all of them result in reduced oxidative DNA damage. Romanová et al [24] proved the strong protective effect of AP, LU and QU in experimental system using pBR322 DNA plasmid interacting with free radicals generated by H_2O_2 or Fe^{2+} cations. Their free radical scavenging capacity and capability to form complexes with transition metals are responsible for their DNA-protective potential.

The aim of our work was to investigate the relationship between the chemical structure of flavonoids and their protective effects against H_2O_2-induced DNA damage in L1210 cells. We demonstrated that statistically significant differences exist among studied flavonoids and that these differences correlate with their chemical structure.

Quercetin as a tetrahydroxyflavone has two hydroxyl groups in rings A and B. It also has a hydroxyl at the position 3 of the ring C (Fig. 3). The importance of this hydroxyl for biological activity of flavonoids is well recognized. The mutagenic activity of QU has been investigated by Ames test [32] and it was determined to be significant. It is now know that flavonoids with the hydroxyl at the position 3 and with a double bond between the positions 2 and 3 are indeed mutagenic. At the same time, they are effective scavengers of free radicals. We determined the protective action of low concentrations of QU towards DNA damage induced by H_2O_2. Quercetin manifested the highest (45%) DNA-protective effect among the flavonoids studied (Fig. 2A).

Luteolin is also a tetrahydroxyflavone. The hydroxylation of the ring B is identical to QU. LU as a flavone does not possess a hydroxyl group in the position 3 of the ring C. This difference in chemical structure represents a reason for its non-mutagenic characteristics. On the other hand, its free radical-scavenging activity is also lower compared to QU. The extent of DNA protection determined for LU was 40% (Fig. 2B).

Apigenin belongs to trihydroxyflavones. In comparison to LU, it has no hydroxyl in the position 3' of the ring B. Its protective effect towards H_2O_2-induced damage of L1210 cells at concentration 600 μmol/l is low (only 2%). However, at the higher concentration (1200 μmol/l), it increases frequency of DNA damage to 10%. These results suggest that at higher concentrations, AP acts as a pro-oxidant (Fig. 2C).

Rutin is a glycoside of QU and the hydroxyl at the position 3 is substituted by the sugar rutinose. This flavonoid does not protect cellular DNA against the formation of single DNA strand breaks induced by H_2O_2 (Fig. 2D). This is in good agreement with the hypothesis that a free hydroxyl at the position 3 of flavonoid structure is required for the significant antioxidative activity. This may be simply because the glycosylation of any hydroxyl in the structure of flavonoid may prevent or decreases its transport toward DNA molecules inside a cell.

In conclusion, the results obtained using the comet assay may be summarized as follows: the protective effect of flavonoids studied against the H_2O_2-induced DNA damage in L1210 cells is the highest in the case of QU and lower for LU. AP and RU had no protective potential even at very high concentrations. The obtained results show the importance of the free hydroxyl at the position 3 of the ring C. Hydroxylation of a hydroxyl prevents the activation of protection against DNA damage.

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