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Effect of Flavonoids on Glutathione Level, Lipid Peroxidation and Cytochrome P450 CYP1A1 Expression in Human Laryngeal Carcinoma Cell Lines

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Summary

Flavonoids are phytochemicals exhibiting a wide range of biological activities, among which are antioxidant activity, the ability to modulate activity of several enzymes or cell receptors and possibility to interfere with essential biochemical pathways. Using human laryngeal carcinoma HEp2 cells and their drug-resistant CK2 subline, we examined the effect of five flavonoids, three structurally related flavons (quercetin, fisetin, and myricetin), one flavonol (luteolin) and one glycosilated flavanone (naringin) for: (*i*) their ability to inhibit mitochondrial dehydrogenases as an indicator of cytotoxic effect, (*ii*) their influence on glutathione level, (*iii*) antioxidant/prooxidant effects and influence on cell membrane permeability, and (*iv*) effect on expression of cytochrome CYP1A1. Cytotoxic action of the investigated flavonoids after 72 hours of treatment follows this order: luteolin>quercetin>fisetin>naringin>myricetin. Our results show that CK2 were more resistant to toxic concentrations of flavonoids as compared to parental cells. Quercetin increased the total GSH level in both cell lines. CK2 cells are less perceptible to lipid peroxidation and damage caused by free radicals. Quercetin showed prooxidant effect in both cell lines, luteolin only in HEp2 cells, whereas other tested flavonoids did not cause lipid peroxidation in the tested cell lines. These data suggest that the same compound, quercetin, can act as a prooxidant, but also, it may prevent damage in cells caused by free radicals, due to the induction of GSH, by forming less harmful complex. Quercetin treatment damaged cell membranes in both cell lines. Fisetin caused higher cell membrane permeability only in HEp2 cells. However, these two compounds did not enhance the damage caused by hydrogen peroxide. Quercetin, naringin, myricetin and fisetin increased the expression of CYP1A1 in both cell lines, while luteolin decreased basal level of CYP1A1 only in HEp2 cells. In conclusion, small differences in chemical structure of flavonoids led to drastic change of their biological effects.

Key words: flavonoids, tumor cells, cytotoxicity, glutathione, lipid peroxidation, cytochrome 1A1

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Abbreviations: CYP1A1 cytochrome 1A1, F fisetin, HEp2 human laryngeal carcinoma cells, CK2 human laryngeal carcinoma cells resistant to several cytostatics, GSH glutathione, DMSO dimethyl sulfoxide, C control, L luteolin, LDH lactate dehydrogenase, MDA malondyaldehyde, MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, M myricetin, N naringin, ROS reactive oxygen species, TBA thiobarbituric acid, Q quercetin

Introduction

Flavonoids are a group of low-molecular-mass polyphenolic substances, ubiquitous in all vascular plants (*1*). They occur naturally in a broad range of fruits, vegetables and beverages such as green tea or red wine. The daily consumption of flavonoids is difficult to estimate, because the uptake of these specific compounds may vary greatly depending on the food source. In plants, flavonoids are widely distributed in form of more polar glycosilated derivatives (mostly *O*-β-glycosides) (*1,2*) and therefore these substances are considered to be nonabsorbable (*3,4*). Nevertheless, microorganisms in the colon hydrolyze them into free flavonoids (aglycones), which are expected to be able to pass through the gut wall. Also, some glycosilated flavonoids are metabolized in liver (*3,5*).

Flavonoids from 4-oxo-flavonoid family have been reported to exert multiple biological effects, but biological outcome will depend on properties of each compound (*2*). Various experimental systems (*e.g.* membrane systems, cell cultures, animal models, and human clinical trials) are used to study the bioactivity of these plant-derived compounds. All of these systems have their advantages and limitations. Cell cultures have a number of advantages over other experimental systems: ability to conduct mechanistic studies at molecular level, easy control of experimental environment and relatively low cost, and finally, avoidance of ethical issues related to animal or human studies. However, cell cultures cannot reflect precisely the conditions found in the body. In spite of that, they are valuable tools in biomedical science.

In this work, we used two cell lines originated from the same parental cell line: human laryngeal carcinoma HEp2 cells and their drug-resistant CK2 subline. However, CK2 cells acquired (during the development of resistance to cisplatin) many alterations that can influence their response to different cytotoxic agents. Both HEp2 and CK2 cells have inducible glutathione, glutathione-*S*-transferases and cytochrome 1A1, but basal level of these enzymes is different. These properties make the selected pair of cell lines suitable for investigation of the effect of flavonoids on the expression of the mentioned proteins. We determined the ability of five flavonoids to: (*i*) inhibit mitochondrial dehydrogenases as an indicator of cytotoxic effect, (*ii*) determine the influence of flavonoids on cellular level of glutathione, (*iii*) examine their antioxidant/prooxidant effects (TBA-MDA formation) and influence on cell membrane permeability as an indicator of secondary damage of cell (lactate dehydrogenase permeability), and (*iv*) examine their influence on expression of CYP1A1.

Glutathione, a tripeptide present in the majority of cells, is responsible for hydrophilic xenobiotics conjugation. Sulphydryl group of glutathione is essential for its antioxidant activity against some forms of reactive oxygen species (ROS) in cells (*6*). There is evidence that some flavonoids can elevate intracellular basal level of GSH, allowing better tolerance of free radicals (*7*). It has been suggested that flavonoid activities depend heavily on their antioxidant and chelating properties (*1*). Therefore, numerous studies concerning biological effects of flavonoids on the *in vitro* and *in vivo* free radical-mediated processes have been carried out. Much effort has been focused on the identification of flavonoids that exert beneficial effects and mechanisms by which they inhibit cellular injury and degradation (*8,9*). Free radicals, whose origin may be molecular oxygen that has accepted an electron from variety of sources, or activated form of foreign compound that has entered into a cell, can attack unsaturated lipids in a cell, resulting in the chain reaction of the formation of free radicals. This reaction is terminated by the production of lipid breakdown products, lipid alcohols, aldehydes and malondialdehyde (MDA). Therefore, measurement of malondyaldehyde concentration is a common method for determination of primary toxic effect caused by free radicals in experiments *in vitro* (*9,10*). It has been shown that flavonoids can prevent autooxidation of linoleic acid *in vitro*, or peroxidation of phospholipid membranes, microsomal or mitochondrial peroxidation (*11–13*).

Some flavonoids can stimulate lipid peroxidation, probably depending on the number of hydroxyl substituents in the B-ring of flavonoids (hydroxyl groups are substrates for cytochrome P450) (*5*). Among the proteins that interact with flavonoids, cytochromes P450 (CYPs) play a prominent role (*5*). Flavonoid compounds influence these enzymes in several ways. They induce the expression of several CYPs and inhibit or stimulate their metabolic activity. Some CYPs participate in the metabolism. Flavonoids enhance activation of carcinogens and influence the metabolism of drugs *via* induction of specific CYPs. On the other hand, inhibition of CYPs involved in carcinogen activation and scavenging reactive species formed from carcinogens by CYP-mediated reactions can be a beneficial property of various flavonoids. Finally, cytochromes are producers of reactive oxygen species in the cells when pour coupling of the P450 catalytic cycle occurs (*5,9*).

Materials and Methods

Human cell lines

Human laryngeal carcinoma HEp2 cells were grown as monolayer cultures in DMEM medium supplemented with 10 % of fetal bovine serum, 4500 mg/L glucose, pyridoxine and 1 % penicillin/streptomycin solution. Drug-resistant subline (CK2) was developed in Laboratory for genotoxic agents, at Rudjer Bošković Institute, by the treatments with stepwise increased concentration of cisplatin (*14*). CK2 cells became resistant to cisplatin and cross-resistant to several other anti-cancer drugs: vincristine, methotrexate, fluorouracil, mitomycin C and carboplatin (*15,16*). This cross-resistance was accompanied with sensitivity to amphotericin B (*17*), more hyperthermia-reduced intracellular cisplatin accumulation (*18*), increased levels of markers for invasion and metastasis, cathepsin D and urokinase plasminogen activator (*19*), and increased expression of integrins (*20*).

Chemicals

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), malondyaldehyde (MDA), NADH, sodium pyruvate, thiobarbituric acid (TBA), Ellman reagent, NADPH, glutathione and glutathione reductase were

purchased from Sigma Chemicals. Flavonoids (quercetin, fisetin, myricetin, luteolin and naringin) were also obtained from Sigma Chemicals. Primary monoclonal antibody CYP1A1 was purchased from Santa Cruz Biotechnology. Secondary antimouse antibody was purchased from Amersham Pharmacia, USA. The chemicals used in experiments were of analytical grade.

Flavonoids

Quercetin, myricetin and fisetin belong to a group of flavonols. The difference in their structure is very small (Fig. 1). Myricetin has three hydroxyl groups at positions 3′, 4′ and 5′ at B ring and one hydroxyl group at position 5 at A ring, quercetin lacks one hydroxyl group at 5′ position, and fisetin misses one hydroxyl group at 3′ position on B ring and one hydroxyl group at position 5 at A ring. Luteolin belongs to a group of flavons, lacking hydroxyl group at 3 position at C ring. Naringin, belonging to a group of flavanons, is glycosilated form of naringenin, which lacks hydroxyl groups at positions 3′ and 5′ at B ring. The structure of the examined compounds is presented in Fig. 1.

Flavonoids were dissolved in dimethyl sulphoxide (DMSO), in stock concentrations of 500 mM. Just before use, stock solutions were dissolved in growth medium. In all experiments final concentration of DMSO in the medium did not exceed 0.1 %.

Cytotoxicity assay

HEp2 and CK2 were seeded in 24-well plates in concentration of $5.4 \cdot 10^4$ cells, 1.8 mL/well. The next day, cells were treated with a range of concentrations (3.8––500 μM; see Fig. 2) of each flavonoid. Following 72 hours (period sufficient for 3 cell divisions), growth medium was removed, and cells were treated with 10 % MTT solution (*21*). Viable cells have active mitochondrial dehydrogenase enzymes which metabolize a yellow-coloured tetrazolium salt (MTT) into a blue formazan. Therefore, the number of surviving cells is directly proportional to the level of the produced formazan. Formazan was dissolved with 2-propanol, containing 0.01 M HCl. Intensity of absorbance was measured at 540 nm in Cecil spectrophotometer (Cecil Instruments Ltd, Cambridge Technical Centre, UK). Each experiment was repeated three times.

Measurement of total glutathione

A volume of 13.5 mL of cell suspension ($4.5 \cdot 10^5$ cells) was seeded in 10-centimetre Petri dishes. After attachment, the cells were treated with highest nontoxic con-

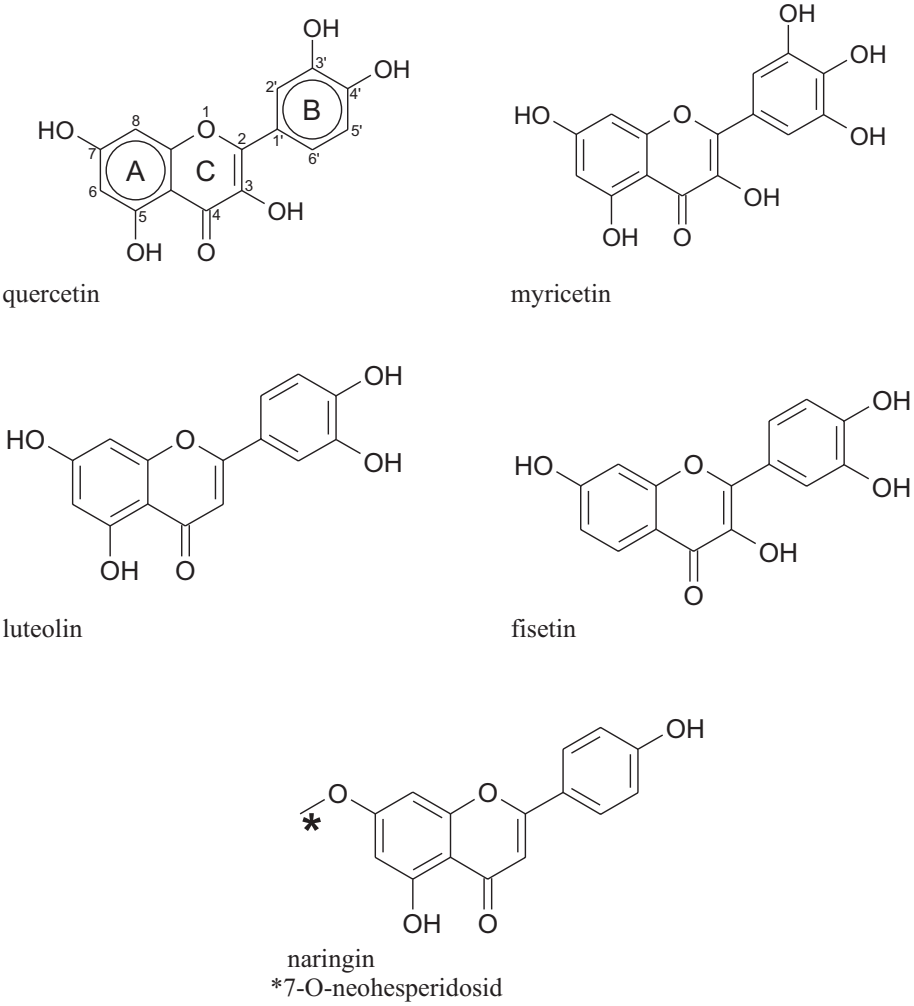

Fig. 1. Chemical structure of investigated flavonoids

centration of the examined flavonoids. After 72 hours of incubation, the cells were scraped, washed twice with cold PBS (containing Ca^{2+} and Mg^{2+} salts), lysed and centrifuged at 15 000 rpm. Supernatant was examined for the level of total glutathione by the Tietze's method (22). Briefly, cell supernatant (10 or 20 μL) was added into quivette containing 0.7 mL of 100 mM Na_2HPO_4/5 M EDTA, 200 μL of Ellman reagent (3 mM) and 100 μL of NADPH (2 mM). Prior to measurement, 10 μL of glutathione reductase (activity 12 units/mL) was added into the mixture. The absorbance was determined at 412 nm every 15 seconds for 2 min. Concentration of total gluta-

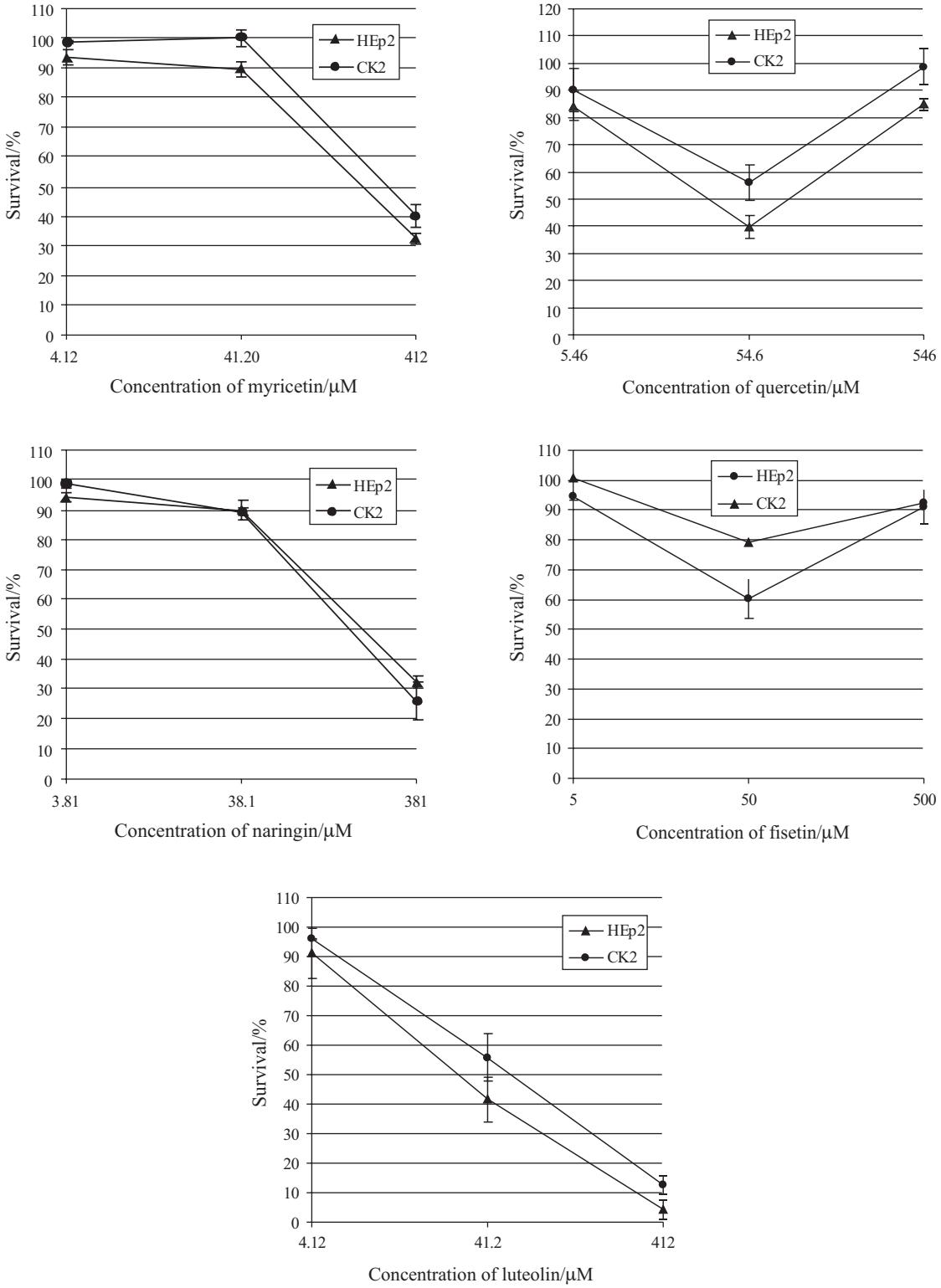

Fig. 2. Survival curves obtained after the 72-hour treatment of HEp2 (triangle) and CK2 (circle) cells with five flavonoids. Percentages of survival are expressed in comparison with negative control. Pooled data from three experiments (the mean at the point ± s.d.)

thione was calculated from standard curve. Total amount of proteins in supernatants was determined using Bradford assay (*23*). Each experiment was carried out in triplicate.

Measurements of lipid peroxidation and lactate dehydrogenase

A volume of 13.5 mL of cell suspension ($4.5 \cdot 10^5$ cells) was seeded in 10-centimetre Petri dishes. After attachment, the cells were incubated with highest nontoxic concentration of the examined flavonoids or the flavonoid and hydrogen peroxide for 72 hours. Then the cells were scraped, washed twice with PBS (containing Ca^{2+} and Mg^{2+} salts) and lysed in 520 μL of potassium chloride (1.15 %) for 30 min. The mixture was centrifuged at 5000 rpm. A volume of 500 μL of cell supernatants was incubated with 2 mL of TBA (100 g/L) for 15 min at 100 °C. The mixture was cooled with tap water, centrifuged at 1000 rpm/10 min; then 2.5 mL of supernatant were mixed with 1 mL of TBA (0.8 %) and incubated for 15 min at 100 °C. After cooling with tap water, the absorbance of the samples was examined at 532 nm and then at 600 nm (value of nonspecific absorbance). Concentration of MDA-TBA complex as an indicator of lipid peroxidation was calculated from standard curve (*24,25*). Concentration of cellular proteins was determined according to Bradford method (*23*). Each experiment was carried out in triplicate.

Changes in the membrane permeability can be detected *in vitro* as the leakage of enzymes such as lactate dehydrogenase. The measurements were done in the medium where cells were grown and treated with different flavonoids or with flavonoids and hydrogen peroxide mixture. One unit of LDH activity presents the quantity of an enzyme needed for the formation of 1 μmol of NAD^+ in one minute (*26*). Growth medium was centrifuged for 5 min at 3000 rpm to remove detached cells. In 470 μL of buffered water (5 mM) and 430 μL of MOPS buffer (500 mM), 30 μL of NADH and 30 μL of sodium pyruvate were added. Prior to measurement, 40 μL of sample were added. Absorbance was measured at 340 nm for 2 min, every 15 seconds. Activity of lactate dehydrogenase was expressed as a rate of absorbance change in unit of time. Concentration of cellular proteins was determined according to Bradford method (*23*). Each experiment was carried out in triplicate.

Western blot analysis

A volume of 13.5 mL ($4.5 \cdot 10^5$ cells) was seeded in 10-centimetre Petri dishes. After attachment, the cells were incubated with highest nontoxic concentration of the examined flavonoids for 72 hours. After that, the cells were scraped, washed twice with cold PBS (containing Ca^{2+} and Mg^{2+} salts), lysed and centrifuged at 10 000 rpm/15 min at 4 °C. The supernatant was collected and placed on ice. Protein concentration was determined according to Lowry method (*27*). An amount of 25 μg of proteins was separated by SDS polyacrilamide electrophoresis (2 h/80 mA). The proteins were transferred (1 h/400 mA) on nitro-cellulose membranes (Hybond C, Amersham Pharmacia, USA). Membranes were blocked in 5 % non-fat milk in TTBS (50 mM Tris-HCl, 200 mM NaCl and 0.05 % Tween 20) and incubated with monoclonal primary mouse antibody raised against human CYP1A1 (dilution 1:1000; Santa Cruz). Detection of CYP1A1 was enabled by incubation of membranes with anti-mouse secondary antibody (Amersham Pharmacia, USA). CYP1A1 was determined by enhanced chemiluminescence (ECL) reagent (Amersham Pharmacia, USA). Relative intensities of the bands were estimated from densitometric analysis of the blot with an Image Master VDS software (Amersham Pharmacia Biotech). Each experiment was repeated three times.

Statistical analsyes

Statistical analyses were performed with SPSS version 8.0 (SPSS Inc., Chicago, IL, USA). Each experiment is the result of three independently performed experiments. The one-way analysis of variance (ANOVA) was employed to determine whether the means of different groups were significantly different. The Dunnett post-hoc test was used to determine the significance. A probability level of $p<0.05$ was considered significant. All data are expressed as a mean ± standard error (SE).

Results and Discussion

Cytotoxicity

Cytotoxic effects of five investigated flavonoids were examined on human parental laryngeal carcinoma cell HEp2 line and their cross-resistant cell CK2 subline, which, due to alterations induced during the resistance development, acquired new properties, as shown previously (*14–20*). The results were compared and presented in Fig. 2.

Slight differences in chemical structure of flavonoids caused different toxicological effects. Myricetin was the least toxic compound. In concentrations higher than 41.2 μM, myricetin induced rapid decrease in cell survival. Therefore, for further studies, concentration of 41.2 μM of myricetin was chosen as the highest nontoxic concentration. Naringin, the only glycosilated flavonoid and the most hydrophilic compound of the five examined, was more toxic than myricetin. Survival curves of cells treated with quercetin and fisetin exhibited saddle-like forms because these flavonoids interfered with MTT dye at higher concentrations, causing false results at concentrations higher than 50 μM.

Cytotoxic action of the investigated flavonoids after 72 hours of treatment follows this order: luteolin>quercetin>fisetin>naringin>myricetin. The highest nontoxic concentrations of flavonoids were established as follows: luteolin (4.12 μM), quercetin (5.46 μM), fisetin (5 μM), naringin (38.1 μM) and myricetin (41.2 μM). These concentrations were used in further experiments. Solvent DMSO in the highest applied concentration did not cause any biological effects in control experiments. Generally, CK2 cells were more resistant to toxic effects of the examined flavonoids than parental cells.

It is known that small changes in chemical structure of flavonoids cause significant changes in their biological activities. Therefore, it is not surprising that naringin, the only glycosilated flavonoid among the tested

substances, has shown lower toxic effect on both cell lines compared to other flavonoids. This could be due to hydrophilic nature of this compound and its different bioavailability in cells.

Similar to our results, Rusak *et al.* (*28,29*) also found that quercetin is the most cytotoxic compound among the tested flavonoids after 72 hours of incubation. They also pointed out that C2–C3 double bond is an important structural requirement for the cytotoxic effects of flavonoids. All flavonoids used in our study possess C2–C3 double bond and they showed cytotoxic effects. Our data are in accordance with the data of Hodek *et al.* (*5*), who showed that 7-hydroxyl group of the ring A is an importaint factor for cytotoxic effect of flavonoids. Rusak *et al.* (*28,29*) pointed out that higher number of hydroxyl groups at ring B enhanced cytotoxic effects of flavonoids. We showed that myricetin, the flavonoid with 3 hydroxyl groups at ring B, was the least toxic compound of the tested flavonoids, suggesting that cytotoxic effects of flavonoids are cell specific. The mechanism of cytotoxic activity of quercetin and luteolin was proposed by Hirano *et al.* (*30*). They supposed that quercetin and luteolin inhibit human promyelocytic leukemia cell growth possibly *via* the cessation of DNA, RNA and protein synthesis of leukemic cells. Scambia *et al.* (*31*) estimated quercetin as a powerful antiproliferative agent against some human cancer cell lines, such as primary colorectal, ovarian, lymphoblastoid and breast cancer cells.

Glutathione levels

Probably the most important protective mechanism for free radical scavenging and inhibition of electrophilic xenobiotics attack on cellular macromolecules involves tripeptide glutathione (*6*). Due to nucleophilic thiol group, it can detoxify substances in one of three ways: (*i*) conjugation catalyzed by glutathione-*S*-transferases (GST); (*ii*) chemical reaction with a reactive metabolite to form a conjugate; and (*iii*) donation of proton or hydrogen atom to reactive metabolites or free radicals. Reactive intermediates can react with GSH either by a direct chemical reaction or by a GST-mediated reaction preventing possible cell death. In this work we have investigated the influence of flavonoids on intracellular glutathione level. These results are presented in Fig. 3.

CK2 cells have higher basal level of total GSH than parental HEp2 cells; 7.8 μM/mg of protein for HEp2 cells, 9.5 μM/mg of protein for CK2 cells, and, for comparison, 8.125 μM/mg of protein for normal human lung fibroblasts. However, according to our previous results, GSH was not involved in the resistance of CK2 cells to cisplatin (*32*). In HEp2 cell line, myricetin and quercetin elevated the level of total GSH, but this increase was significant only for quercetin. In CK2 cell line, with increased basal level of GSH, fisetin, luteolin and quercetin elevated GSH level, but again, only for quercetin this increase was statistically significant. This implicates that cells treated with quercetin can tolerate exposure to higher concentrations of different electrophilic xenobiotics, as well as compounds that cause oxidative damage and oxidative stress.

Similarly, protective *in vivo* effect of quercetin was published by Devi Priya and Shyamala Devi (*33*). They investigated the influence of quercetin on rat kidneys where rats were simultaneously treated with cisplatin and quercetin. Quercetin administration *per se* prevented lipid peroxidation and cell membrane damage. This suggests that quercetin has the same effect on GSH induction in both *in vivo* and *in vitro* systems as well as that this effect is not cell specific. Beneficial effects of quercetin were also observed by Pattipati *et al.* (*34*). They in-

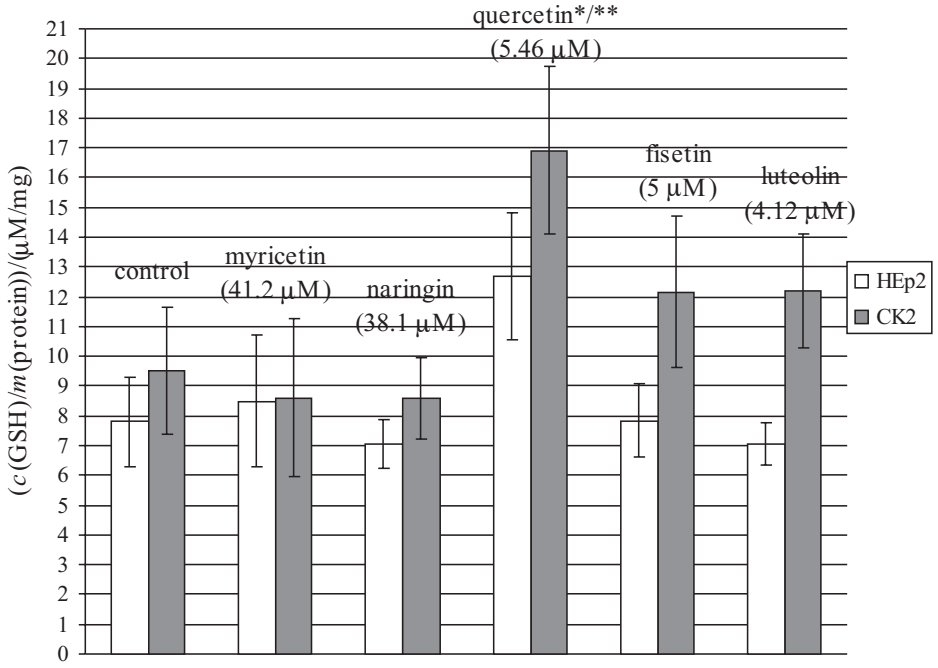

Fig. 3. Concentration of total glutathione in HEp2 (white columns) and CK2 (gray columns) cell lines after 72 hours of treatment with the highest nontoxic concentrations of flavonoids indicated as data labels. Pooled data from three experiments (the mean at the point ± s.d.). Statistically significant difference compared to control: * in HEp2 and ** in CK2 cell line

vestigated the prevention of occurrence of dyskinesia, a serious neurological syndrome associated with long-term administration of neuroleptics to experimental animals. Repeated treatment with neuroleptics decreased the level of GSH, but treatment with quercetin significantly reduced neuroleptically induced changes in the brain of rats. Further, administration of quercetin restored intracellular GSH levels, the decrease of which was caused by chronic neuroleptic treatment.

Lipid peroxidation

Free radicals, whose origin may be molecular oxygen which has accepted an electron from a variety of sources or activated form of foreign compound that has entered into a cell, can attack unsaturated lipids in the cell, resulting in chain reaction of free radical formation. This reaction is terminated by the production of lipid breakdown products; lipid alcohols, aldehydes and malondialdehyde. Therefore, measurement of MDA concentration is a commonly used method to assess lipid peroxidation in cultures *in vitro*.

It is believed that flavonoids provide only beneficial effects on human health, which is associated with an improved antioxidant response *in vivo*. This presumption is based on their low absorption, extensive metabolism and their ability to bind to proteins. But, numerous literature data show that some flavonoids could behave as both antioxidants and prooxidants, depending on the concentration and free radical source (*8*,*35*).

In this work, we have examined the influence of five flavonoids on the formation of MDA after prolonged exposure of HEp2 and CK2 cells to nontoxic concentrations of flavonoids, as well as to the mixture of flavonoids and hydrogen peroxide. Because of low concentration of free radicals induced by highest nontoxic concentrations of flavonoids (see section *Cytotoxicity*) and hydrogen peroxide (8 μM), incubation time was prolonged to three days in order to be able to determine some biological effects.

Basal level of MDA-TBA complex in HEp2 cells was 10.64 μM/mg of protein, in CK2 cells 5.12 μM/mg of protein. For comparison, in normal lung fibroblasts basal level of MDA-TBA was 12.7 μM/mg of protein. As shown in Fig. 4, fisetin (5 μM), quercetin (5.46 μM) and luteolin (4.12 μM) induced lipid peroxidation in HEp2 cell line. Naringin (38.1 μM) reduced basal level of peroxidation but also inhibited lipid peroxidation that was induced with 8 μM H_2O_2. Luteolin and fisetin enhanced primary toxic effect of hydrogen peroxide. These two compounds behave as prooxidants showing synergistic effect with free radicals of different origin.

Contrary to our results, Shimoi *et al.* (*36*) showed that luteolin *in vivo* protected serum lipids against oxidation induced with 2,2-azobis(2-aminopropane)-dihydrochloride (AAPH). This discrepancy could be explained by the hypothesis that luteolin *in vivo* has passed through metabolic changes (glucuronidation, sulphatation, methylation) prior to entering into a blood system. We supposed that in *in vitro* system luteolin is not transformed to a more electrophilic form by enzymes involved in phase I of detoxification (this phase could be responsible for free radical formation). Kumar *et al.* (*37*) showed that

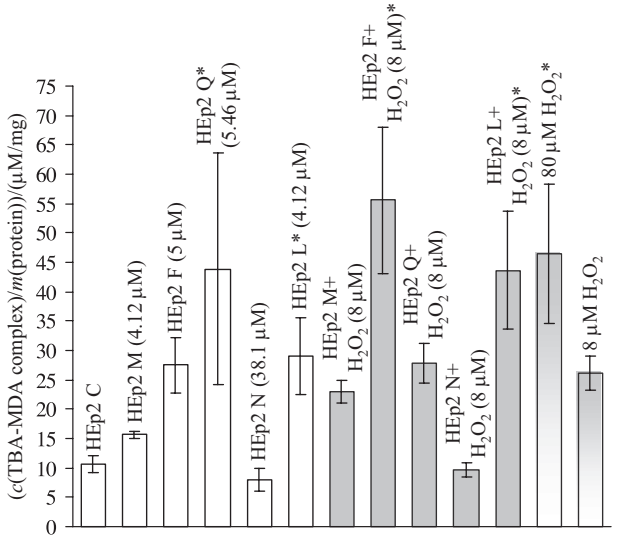

Fig. 4. Concentration of TBA-MDA complex in HEp2 cells after the treatment for 72 hours with the highest nontoxic concentrations of flavonoids (white columns) and flavonoids and hydrogen peroxide (gray columns). The highest nontoxic concentrations of flavonoids are presented in brackets. M=myricetin, F=fisetin, Q=quercetin, N=naringin; L=luteolin. *Statistically significant difference compared to nontreated control (C)

naringin protects hemoglobin from nitrite-induced oxidation to methemoglobin *in vitro.* The protection was not observed when naringin was added after autocatalytic stage of the oxidation of hemoglobin by nitrite. The ability of naringin to scavenge oxygen free radicals may be responsible for this phenomenon, because superoxide, hydroxyl and other free radicals are implicated in promoting the autocatalytic stage of oxidation of hemoglobin by nitrite.

Fig. 5 presents levels of lipid peroxidation in CK2 cells following the treatment with flavonoids. It should be pointed out that basal level of MDA in CK2 cells is

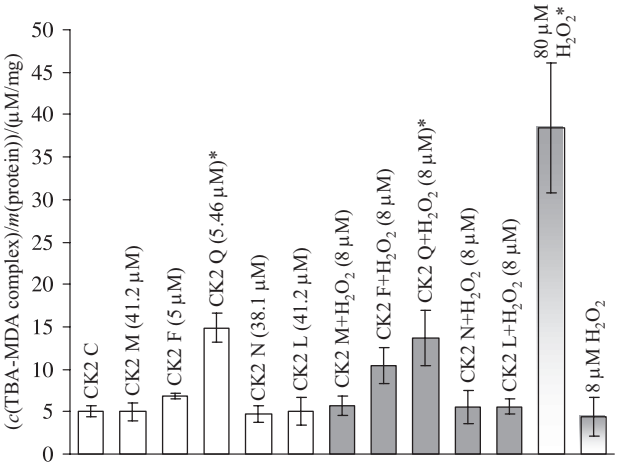

Fig. 5. Concentration of TBA-MDA complex in CK2 cells after the treatment for 72 hours with the highest nontoxic concentrations of flavonoids (white columns), and flavonoids and hydrogen peroxide (gray columns). M=myricetin, F=fisetin, Q=quercetin, N=naringin; L=luteolin. *Statistically significant difference compared to nontreated control (C)

significantly lower (5.12 μM/mg protein) than in parental cells (10.64 μM/mg of protein in HEp2 and 12.7 μM/mg of protein in normal lung fibroblasts). Furthermore, it is obvious that drug-resistant CK2 cells are less susceptible to lipid peroxidation than HEp2 cells. CK2 cells are also less sensitive to lipid peroxidation and damage caused by free radicals. Quercetin caused significant increase in MDA concentration, but not synergistic effect with 8 μM H_2O_2. Fisetin showed slight (but not statistically significant) prooxidant nature when it was added to cells treated with 8 μM H_2O_2.

Quercetin caused significant MDA-TBA complex formation in hydrogen peroxide-treated cells as well as in quercetin-treated cells in both cell lines, indicating its prooxidative activity. Young *et al.* (*38*) investigated the influence of quercetin present in juice on healthy volunteers. They analyzed the presence of MDA in blood samples and urine. Quercetin was excreted in urine (0.29–0.47 %), and its plasma concentration was very low. Total plasma MDA level decreased with time, indicating that reduction of lipid oxidation had occurred. 2-adipic semialdehyde, a biomarker of protein oxidation, appeared, indicating a prooxidant effect of the juice. This suggests that even within the plasma, there are several subcompartments which may respond differently to a dietary challenge. These results also contradict a general pro- or antioxidant state in blood, but also a differential protection or damage to specific structures, depending on their interaction with the dietary components that reach them. The differences between our results and those obtained by Young *et al.* (*38*) in *in vivo* system could be explained by the synergistic effects of different components present in juice. Therefore, these effects cannot be attributed solely to quercetin.

Lactate dehydrogenase activity

Changes in the membrane permeability can be detected *in vitro* as leakage of enzymes such as lactate dehydrogenase. In the present study we have examined cell membrane permeability following the treatment of cells with the highest nontoxic concentrations of flavonoids and hydrogen peroxide. One unit of LDH activity presents the quantity of an enzyme needed for the formation of 1 μmol of NAD^+ in one minute (*25*).

Basal LDH activity in HEp2 cells was 0.25 nmol/(min·mg protein), in CK2 it was 0.27 nmol/(min·mg protein). In normal lung fibroblasts, Hef cells, basal activity of LDH was 0.1 nmol/(min·mg protein). Nontoxic concentrations of fisetin and quercetin increased cell membrane permeability. LDH activity was not increased when the cells were simultaneously treated with fisetin and quercetin in the presence of 8 μM H_2O_2, suggesting that these two compounds did not enhance the toxic effect of hydrogen peroxide (Fig. 6).

CK2 cells were more sensitive to the toxic concentration of hydrogen peroxide (80 μM), possibly due to higher permeability of cell membrane. In CK2 cells, only quercetin caused significant leakage of LDH. There was no difference between cells treated with quercetin and those treated with quercetin and H_2O_2 mixture, suggesting that quercetin did not participate in LDH leakage from the cells treated with hydrogen peroxide (Fig. 7).

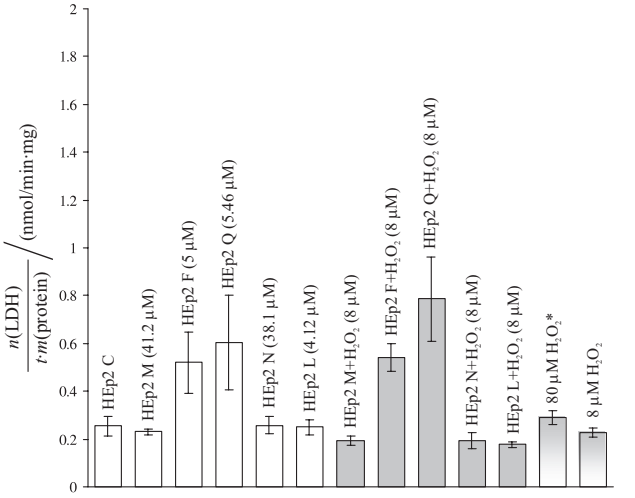

Fig. 6. Lactate dehydrogenase activity measured in growth medium as a measure of cell membrane permeability in HEp2 cells after 72 hours of treatment with the highest nontoxic concentrations of flavonoids (white columns) and flavonoids and hydrogen peroxide (gray columns). Pooled data from three experiments (the mean at the point ± s.d.). *Statistically significant difference compared to control

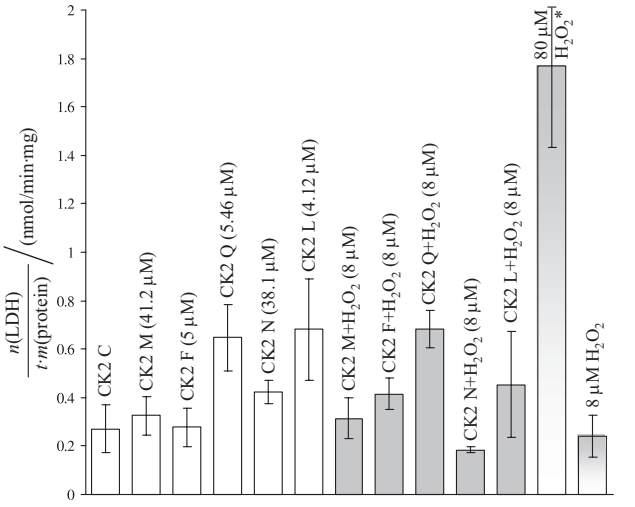

Fig. 7. Lactate dehydrogenase activity measured in growth medium as a measure of cell membrane permeability in CK2 cells after 72 hours of treatment with the highest nontoxic concentrations of flavonoids (white columns) and flavonoids and hydrogen peroxide (gray columns). Pooled data from three experiments (the mean at the point ± s.d.). *Statistically significant difference compared to control

Expression of cytochrome CYP1A1

Cytochrome P450 are heme containing mixed-function oxidases that play a key role in the metabolism of hydrophobic endogenic substances (sterols, prostaglandins, and fatty acids) and ingested foreign compounds such as drugs, pesticides and pollutants (*5*). These proteins are involved in interactions with flavonoid compounds in three ways: flavonoids can induce biosynthe-

sis of certain CYPs, they can modulate enzymatic activity of CYPs and, finally, flavonoids can be metabolized by several CYPs.

In the present study, we examined the ability of flavonoids to induce biosynthesis of cytochrome P450, CYP1A1. Induction of CYP1A1 is regulated by binding of a certain compound to aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor (*5*). Binding affinities of these xenobiotics to AhR appear to be dependent on structural constrains; planar aromatic compounds with bulky substituent groups are preferred (*5*). Depending on flavonoid concentrations, flavonoids exhibit different biological activities. At lower concentrations, flavonoids act as AhR antagonists, binding to the receptor without activation of a transcription factor, while at higher concentrations the same flavonoids can act as AhR agonists and modulate gene expression of CYPs induced by benz(a)pyrene, for example (*5*).

In this study, we determined the effects of nontoxic concentrations of investigated flavonoids on CYP1A1 expression. Basal expression of CYP1A1 in HEp2 cells was higher than in CK2 cell line (Fig. 8). Human lung fibroblasts have low basal expression of CYP1A1. As shown in Fig. 8, quercetin did not induce CYP1A1 in HEp2 cell line. Naringin, fisetin and myricetin slightly increased expression of CYP1A1 compared to control (from 22 to 27 %). Luteolin reduced basal CYP1A1 expression for 40 %. In CK2 cell line, quercetin, naringin, myricetin, fisetin and luteolin induced expression of CYP1A1 from 43 to 60 %, as compared to basal level of CYP1A1 in CK2 cell line (Fig. 8). Inhibition of CYP1A1 protein expression in HEp2 cells treated with luteolin could play an important role in cancer chemoprotection. In cells with high basal CYP1A1 expression, luteolin is probably metabolized through this system and is transformed to reactive intermediate which binds as an antagonist to AhR, inhibiting consequently polycyclic aromatic hydrocarbon adduct formation (*5,36*). Our results indicate that the same flavonoid could induce, but also suppress CYP1A1 expression in different cell lines, suggesting cell type-specific effect of flavonoids on expression of CYP1A1.

Connection between the chemical structure of the investigated flavonoids and their influence on CYP1A1 expression is difficult to establish. All examined flavonoids have 7-hydroxyl group at the ring A and 4-keto group at the ring C. They differ in number of hydroxyl groups at 3′, 4′ and 5′ positions at the ring B, absence of hydroxyl group at 5 position at A ring (fisetin) or absence of hydroxyl group at 3 position of C ring (luteolin). Flavonoids with more hydroxyl groups attached at B ring have stronger influence on CYP1A1 expression. Naringin, a flavonoid with one hydroxyl group at ring B, showed the weakest effect on CYP1A1 expression. Quercetin and luteolin, flavonoids with two hydroxyl groups at ring B, showed more potent effects on CYP1A1 expression, which depended on cellular surrounding.

Care should be taken if flavonoids are added in diet as food supplements when they are consumed with other medicaments, because their presence and chronic intake can drastically change metabolism of other drugs and their plasma level. Intensive degradation of drugs or their retention in cells due to altered expression of cytochrome P450 could lead to serious consequences and unwanted effects. It would be interesting to investigate synergistic and/or antagonistic effects of various flavonoids using the same experimental approach established in this work.

Conclusions

Small differences in chemical structure of flavonoids led to drastic change of their biological effects, and were cell specific. CK2 cell resistant to anti-cancer drugs survived the treatment with flavonoids better than parental laryngeal carcinoma HEp2 cells. CK2 cells were less perceptible to lipid peroxidation and damage caused by free radicals, as compared to HEp2 cells. Fisetin and quercetin showed prooxidant effect in both cell lines. Quercetin increased glutathione level in both cell lines, pointing out that the same compound can act as prooxidant, but also prevent severe damage of the cell by induction of GSH, which reacts with a free radical and forms harmless complex. Fisetin and quercetin damaged cell membrane, but did not enhance the damage caused by hydrogen peroxide. Quercetin, naringin, myricetin, fisetin and luteolin induced CYP1A1 protein expression in CK2 cell line, while luteolin decreased basal level of CYP1A1 in HEp2 cell line. Although the *in vitro* system provides sufficient conditions for determination of molecular mechanisms of biological effects of flavonoids, this system cannot precisely reflect conditions in the system *in vivo*. Extensive studies on structure-function relationship of flavonoids in different test systems could provide rational approach to drug and chemopreventive agent design.

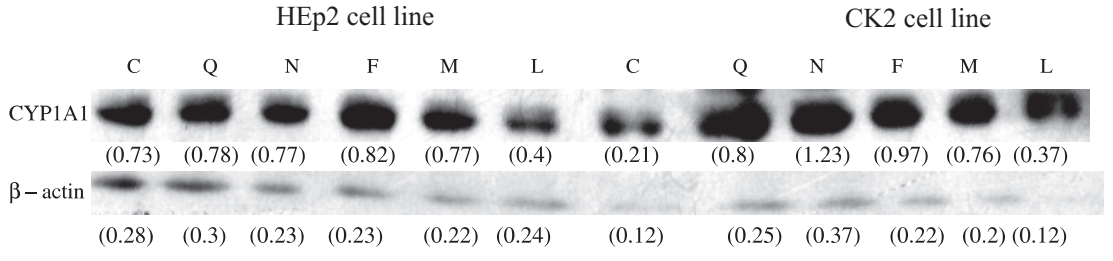

Fig. 8. Western blot analysis of cytochrome CYP1A1 expression in HEp2 and CK2 cells following 72 hours of treatment with the highest nontoxic concentrations of flavonoids in HEp2 and CK2 cell line. β-actin was used as equal loading control. Values in brackets are results of densitometry measurements. C=control, Q=quercetin, N=naringin, F=fisetin, M=myricetin, L=luteolin

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References

1. L.G. Korkina, I.B. Afanas'ev, Antioxidant and chelating properties of flavonoids, *Adv. Pharmacol. 38* (1997) 151–163.
2. S.A. Aherne, N.M. O'Brien, Dietary flavonols: Chemistry, food content and metabolism, *Nutrition, 18* (2002) 75–81.
3. A.J. Day, M.R.A. Morgan, M.J.C. Rhodes, G. Williamson: The First Step in the Metabolism of Flavonoid Glycosides. In: *Dietary Anticarcinogens and Antimutagens: Chemical and Biological Aspects*, I.T. Johnson, G.R. Fenwick (Eds.), Royal Chemistry Society, Cambridge, UK (2000) p. 55.
4. J.B. Harborne: Nature, Distribution, and Function of Plant Flavonoids. In: *Plant Flavonoids in Biology and Medicine: Biochemical, Pharmacological and Structure-Activity Relationships*, V. Cody, E. Middleton, J.B. Harborne (Eds.), Alan R. Liss, New York, USA (1986) p. 15.
5. P. Hodek, P. Trefil, M. Stiborova, Flavonoids – Potent and versatile biologically active compounds interacting with cytochromes P450, *Chem. Biol. Interact. 139* (2002) 1–21.
6. N.H.P. Cnubben, I.M.C.M. Rietjens, H. Wortelboer, J. van Zanden, P.J. van Bladeren, The interplay of glutathione-related processes in antioxidant defense, *Environ. Toxicol. Pharmacol. 10* (2001) 141–152.
7. L. Cipak, E. Berceliova, H. Paulikova, Effects of flavonoids on glutathione and glutathione-related enzymes in cysplatin-treated L1210 leukemia cells, *Neoplasma, 50* (2003) 443–446.
8. A.W. Boots, J.M. Balik, A. Bast, G.R.M.M. Haenen, The reversibility of the glutathionyl-quercetin adduct spreads oxydised quercetin-induced toxicity, *Biochem. Biophys. Res. Commun. 338* (2005) 923–929.
9. N.M. O'Brien, J.A. Woods, S.A. Aherne, Y.C. O'Callaghan, Cytotoxicity, genotoxicity and oxidative reactions in cell culture models: Modulatory effects of phytochemicals, *Biochem. Soc. Trans. 28* (2000) 22–26.
10. J. Timbrell: Primary Events: Lipid Peroxidation. In: *Principles of Biochemical Toxicology*, J. Timbrell (Ed.), Taylor and Francis, London, UK (2000) pp. 204–213.
11. J. Torel, J. Cillard, Antioxidant activity of flavonoids and reactivity with peroxy radical, *Phytochemistry, 25* (1986) 383–387.
12. J. Terao, M. Piskula, Q. Yao, Protective effect of epicatechin, epicatechin gallate and quercetin on lipid peroxidation in phospholipid bilayers, *Arch. Biochem. Biophys. 308* (1994) 278–284.
13. M.R. Cholbi, M. Paya, M.J. Alcaraz, Inhibitory effects of phenolic compounds on CCl_4 induced microsomal lipid peroxidation, *Experientia, 47* (1991) 195–199.
14. M. Osmak, Collateral resistance or sensitivity of human larynx carcinoma HEp2 cells resistant to *cis*-dichlorodiammineplatinum(II) or vincristine sulfate, *Neoplasma, 39* (1992) 197–202.
15. M. Osmak, L. Beketić-Orešković, M. Matulić, J. Sorić, Resistance of human larynx carcinoma cells to cisplatin, gamma irradiation and methotrexate do not involve overexpression of c-*myc* or c-Ki-*ras* oncogenes, *Mutat. Res. 303* (1993) 113–120.
16. L. Beketić-Orešković, M. Osmak, Human larynx carcinoma cells resistant to *cis*-diamminedichloroplatinum(II): Cross-resistance pattern, *Neoplasma, 41* (1994) 171–176.
17. L. Beketić-Orešković, M. Osmak, Modulation of resistance to *cis*-diamminedichloroplatinum(II) by amphotericin B and aphidicolin in human larynx carcinoma cells, *Cancer Chemother. Pharm. 35* (1995) 327–333.
18. L. Beketić-Orešković, M. Jakšić, S. Orešković, M. Osmak, Hyperthermic modulation of resistance to *cis*-diamminedichloroplatinum(II) in human larynx carcinoma cells, *Int. J. Hyperth. 13* (1997) 205–214.
19. M. Osmak, D. Nikšić, A. Brozović, A. Ambriović-Ristov, I. Vrhovec, J. Škrk, Drug resistant tumor cells have increased levels of tumor markers for invasion and metastasis, *Anticancer Res. 19* (1999) 3193–3198.
20. A. Ambriović-Ristov, J. Gabrilovac, T. Čimbora-Zovko, M. Osmak, Increased adenoviral transduction efficacy in human laryngeal carcioma cells resistant to cisplatin is associated with increased expression of integrin $\alpha_v\beta_3$ and coxsackie adenovirus receptor, *Int. J. Cancer, 110* (2004) 660–667.
21. G. Mickish, S. Fajta, G. Keilhauer, E. Schlick, R. Tschada, P. Alken, Chemosensitivity testing of primary human renal cell carcinoma by tetrazolium based microculture assay (MTT), *Urol. Res. 18* (1990) 131–136.
22. F. Tietze, Enzymatic method for quantitative determination of nanogram amounts of total and oxidized glutathione: Applications to mammalian blood and other tissues, *Anal. Biochem. 27* (1969) 502–522.
23. M.M. Bradford, A rapid and sensitive method for the quantification of microgram quantities of protein utilising the principle of protein-dye binding, *Anal. Biochem. 72* (1976) 248–254.
24. C. Cereser, S. Boget, P. Parvaz, A. Revol, Thiram-induced cytotoxicity is accompanied by a rapid and drastic oxidation of reduced glutathione with consecutive lipid peroxidation and cell death, *Toxicology, 163* (2001) 153–162.
25. S. McCarthy, M. Somayajulu, M. Sikorska, H. Borowy-Borowski, S. Pandey, Paraquat induces oxidative stress and neuronal cell death; Neuroprotection by water soluble coenzyme Q-10, *Toxicol. Appl. Pharm. 201* (2004) 21–31.
26. E. Hurna, S. Hurna, Influence of zinc on cadmium induced cytotoxicity in cultured rat liver cells, *J. Trace Microprobe Tech. 16* (1998) 437–444.
27. O.H. Lowry, N.J. Rosenbrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, *J. Biol. Chem. 193* (1951) 265–275.
28. G. Rusak, H.O. Gutzeit, J. Ludwig Müller, Structurally related flavonoids with antioxidative properties differentially affect cell cycle progression and apoptosis of human acute leukaemia cells, *Nutr. Res. 25* (2005) 141–153.
29. G. Rusak, H.O. Gutzeit, J. Ludwig-Müller, Effects of structurally related flavonoids on hsp gene expression in human promyeloid leukaemia cells, *Food Technol. Biotechnol. 40* (2002) 267–273.
30. T. Hirano, M. Gotoh, K. Oka, Natural flavonoids and lignans are potent cytostatic agents against human leukemic HL-60 cells, *Life. Sci. 55* (1994) 1061–1069.
31. G. Scambia, F. Ranelletti, P.B. Panici, M. Piantelli, G. Bonanno, R. De Vincenzo, G. Ferrandina, N. Maggiano, N. Capelli, S. Mancuso, Inhibitory effect of quercetin on primary ovarian and endometrial cancers and synergistic antiproliferative activity with *cis*-diamminedichloroplatinum (II), *Gynecol. Oncol. 45* (1992) 13–19.
32. L. Beketić-Orešković, M. Osmak, M. Jakšić, Human larynx carcinoma cells resistant to *cis*-diamminedichloroplatinum(II): Mechanisms involved in resistance, *Neoplasma, 41* (1994) 163–169.
33. S. Devi Priya, C.S. Shyamala Devi, Protective effect of quercetin in cisplatin-induced cell injury in the rat kidney, *Indian J. Pharmacol. 31* (1999) 422–426.

34. S.N. Pattipati, A. Singh, S.K. Kulkarni, Reversal of reserpine-induced orofacial dyskinesia and cognitive disfunction by quercetin, *Pharmacology, 70* (2004) 59–67.

35. N. Cotelle, Role of flavonoids in oxidative stress, *Curr. Top. Med. Chem. 1* (2001) 569–590.

36. K. Shimoi, H. Okada, J. Kaneko, M. Furugori, T. Goda, S. Takase, M. Suzuki, Y. Hara, N. Kinae: Bioavailability and Antioxidant Properties of Luteolin. In: *Natural Antioxidants and Anticarcinogens in Nutrition, Health and Disease*, J.T. Kumpulainen, J.T. Salonen (Eds.), Royal Society of Chemistry, Finland (1998) pp. 166–174.

37. M.S. Kumar, M.K. Unnikrishnan, S. Patra, K. Murthy, K.K. Srinivasan, Naringin and naringenin inhibit nitrite-inuced methemoglobin formation, *Pharmazie, 58* (2003) 564–566.

38. J.F. Young, S.E. Nielsen, J. Haraldsdotir, B. Daneshvar, S.T. Lauridsen, P. Knuthsen, A. Crozier, B. Sandstörm, L.O. Dragsted: Effect of Fruit Intake on Urinary Quercetin Excretion and Biomarkers of Antioxidative Status. In: *Natural Antioxidants and Anticarcinogens in Nutrition, Health and Disease*, J.T. Kumpulainen, J.T. Salonen (Eds.), Royal Society of Chemistry, Finland (1998) pp. 193–203.