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Structure of flavonoids influences the degree inhibition of Benzo(a)pyrene – induced DNA damage and micronuclei in HepG2 cells

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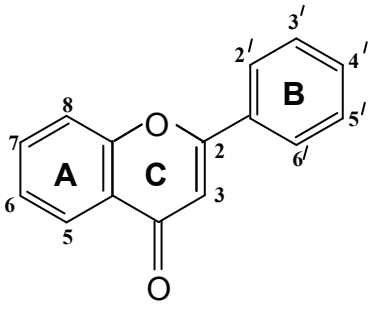
Flavonoids are plant derivatives of flavone of which chemical structure is characterized by various degrees of hydroxylation and glycosidic substitution. In the present study we investigated the protective effect of two structurally different groups of flavonoids against-benzo[a]pyrene (B(a)P)-induced genotoxic effects on human hepatocellular carcinoma (HepG2) cells. The first group of flavonoids: fisetin, kaempferol, galangin, quercetin, and luteolin, hydroxylated at the 3´,4´-position on the B ring, 3 – position of C ring and on the A ring was able to inhibit significantly B(a)P-induced genotoxic effects in a greater degree than the second group of flavonoids: chrysin, 7-hydroxyflavone, 7,8-dihydroxyflavone and baicalein (hydroxylated on the A ring) which showed a statistically significant inhibition of genotoxicity mainly at higher concentrations (10 and 25 μM). The tenth flavonoid tested rutin, which contains hydroxyl group at the position 3 of C ring, substituted by the sugar rutinose, was not able to inhibit effectively genotoxic changes induced by B(a)P. Our results, obtained with help of micronucleus test and single cell gel electrophoresis (comet assay) suggest that inhibition of B(a)P-induced DNA lesions and micronuclei correlates with the structural arrangement and organization of the hydroxyl groups in the molecular structure of the flavonoids tested.

Key words: carcinogenesis, genotoxicity, flavonoids, benzo[a]pyrene , comet assay, micronucleus assay, structural relationship

Phenolic compounds, which increasingly attract the attention of researchers, food manufacturers, as well as consumers for their antioxidant properties, are abundantly present in fruits, vegetables, and beverages such as tea and red wine. They are a large group of compounds with a similar chemical structure [1]. Flavonoids represent a group of approximately 4000 naturally occurring polyphenolic compounds that are ubiquitously present in food of plant origin [2]. They could influence several important biological functions by their free-radical scavenging ability, stimulating apoptosis, inhibiting inflammation, signal transduction pathway and inhibiting proliferation in human cancer cells [3]. Certain flavonoids are suggested to act as chemopreventive compound against lung, stomach, colorectal, epithelial, kidney, uterus and ovary cancer [4]. Flavonoids have been reported to possess a wide range of biochemical and pharmacological activities, both potentially detrimental and protective. One of the effects of flavonoids is the ability to modulate xenobiotic metabolism [5]. Various studies indicate that potential basis for protection is interference with enzymes such as specific cytochrome P450 forms that play an important role in the metabolic activation of a wide range of carcinogens [6].

It was suggested that the multifunctional effects of flavonoids were very intimately connected with their structures and functional groups. The fundamental molecular structure of a flavonoid comprises two aromatic rings (A and B) linked through a heterocyclic pyran-4-one ring C. The C2–C3 double-bond and 4-oxo functional group of the C ring are suggested to be important factors for the biological activities of flavonoids [7]. Besides, the important role in the activity of flavonoids plays hydroxyl group at C3 and the number of hydroxyl groups at B-ring which act as electron donors and are responsible for radical scavenging activity of flavonoids [8]. In this paper we investigated protective anti-genotoxic effects of 10 different flavonoids against DNA lesions and micronuclei induced in human hepatocellular carcinoma cells HepG2 by a potent mutagen and human carcinogen benzo[a]pyrene (B(a)P) [6]. B(a)P is a member of the polycyclic aromatic hydrocarbon (PAH) family, which includes more than 100 different compounds. These compounds are formed from natural and man-made

sources, but the man-made B(a)P is quantitatively the most significant [9]. B(a)P has been implicated in the evolution of various cancers of lung and colon; people are exposed to B(a)P through cigarette smoking [10], occupational exposure [11], and intake of broiled or smoked food [12]. B(a)P has been shown to induce gene mutations, chromosomal aberrations and other types of genotoxic effects both *in vitro* and *in vivo* [13]. The aim of our study was to evaluate the protective effect of selected 10 flavonoids varying in molecular structure (fisetin (FI), kaempferol (KA), galangin (GA), quercetin (QU), luteolin (LU), chrysin (CH), 7-hydroxyflavone (7FL), 7,8-dihydroxyflavone (7,8FL), baicalein (BA), and rutin (RU)), against B(a)P-induced DNA strand breaks and micronuclei in human hepatoma cells HepG2. The flavonoids studied differ in the number of hydroxyl groups in the skeleton (Fig. 1). Two standard methods, micronucleus assay and the single cell gel electrophoresis (SCGE, the comet assay) were used to evaluate the genotoxicity of B(a)P and its reduction by flavonoids.



Flavonids/ OH group position	3	5	6	7	8	3′	4′
Quercetin (QU)	OH	OH		OH		OH	OH
Fisetin (FI)	OH			OH		OH	OH
Kaempferol (KA)	OH	OH		OH			OH
Galangin (GA)	OH	OH		OH			
Luteolin (LU)		OH		OH		OH	OH
Chrysin (CH)		OH		OH			
7-hydroxyflavone (7FL)				OH			
7,8-dihydroxyflavone (7,8FL)				OH	OH		
Baicalein (BA)			OH	OH			
Rutin (RU)	Rham*	OH		OH		OH	OH

* Rham – rhamnosyl-glucosyl

Figure 1. The chemical structures of flavonoids tested.

Materials and methods

Cell culture. Malignant cell line HepG2 (human hepatocellular carcinoma cells) was obtained from A.R. Collins (University of Oslo, Oslo, Norway). The cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics (penicillin 200 U/ml, streptomycin 100 μg/ml, kanamycin 100 μg/ml) on glass Petri dishes (Ø = 10 cm) at 37 °C in humidified atmosphere of 5% CO_2.

Chemicals. Na_2EDTA, NaOH, KCl, and NaCl were purchased from Lachema Brno, Czech Republic. FI, KA, GA, QU, LU, CH, 7FL, 7,8FL, BA, RU, ethidium bromide, Triton X-100, B(a)P (CAS No. 50-32-8), dimethyl sulphoxide (DMSO), 4′,6-diamidino-2-phenylindole (DAPI, CAS No. 28718-90-30) and agarose electrophoresis reagent were obtained from Sigma-Aldrich, St. Louis, USA. 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. Agarose II, RPMI (1640) medium without L-glutamine and $NaHCO_3$ was bought from Amresco-Biotechnology Grade, USA. Fetal calf serum (FCS) was purchased from Grand Island Biological Co., Grand Island, NY, USA. The flavonoids tested and B(a)P were dissolved in DMSO (final concentration <1%) in both assays. All chemicals were of analytical grade. Stock solution of flavonoids was prepared in DMSO at concentration of 5×10^{-3}M freshly before use. Appropriate dilutions of flavonoids were added to medium RPMI 1640 to reach the final concentrations (25, 10, 5, 2.5 μM). The stock solution of B(a)P in DMSO (4×10^{-3} M) was added to medium to reach the final concentration (10 μM).

Treatment of cells for cytotoxicity, SCGE and micronucleus assays. HepG2 cells were seeded into the series of Petri dishes ($4{,}5\times10^5$, Ø=60 mm) and cultured in the RPMI 1640 medium. Exponentially growing cells were then pre-incubated in the presence of flavonoids (100, 50, 25, 10, 5, 2.5, 1 μM) or without flavonoids (control) for 24 h. Then the samples of cells were washed, exposed to B(a)P (2 h, complete culture medium, 37°C) and divided into two groups. One half of samples was used for evaluation of the number of micronuclei (micronucleus assay) and the second half of samples was trypsinized, re-suspended in a fresh culture medium and used for testing of both cytotoxicity by the trypan blue exclusion technique and the level of DNA lesions by the comet assay.

Micronucleus assay. The cells treated as described in the chapter above, were processed after 24-h post-B(a)P-treatment as follows: the cells adhered to Petri dishes were washed with PBS, incubated in mild hypotonic solution (0.075 M KCl/0.9% NaCl, 1:19) for 10 min at 37°C, and fixed with methanol – glacial acetic acid (3:1) for 15 min at 37°C, rinsed with distilled water and air dried. Fixed cells were stained with DAPI (2 μg/ml) for 30 min in the dark at room temperature, rinsed with McIlvaine's buffer and distilled water, dried, and mounted with glycerol. Micronuclei were identified based on the criteria specified by Miller et al. [14]. One thousand cells per dish were analyzed using the fluorescence microscope Olympus BX51. Data are mean ±S.D. of at least two parallel dishes per one experiment from three independent experiments.

Single cell gel electrophoresis (SCGE, the comet assay). The procedure of Singh et al. [15], modified by Collins et al. [16] and Gabelova et al. [17] was followed. In brief, treated and control HepG2 cells embedded in 0.75% LMP agarose and spread on a base layer of 1% NMP agarose in PBS buffer (Ca_2^+ and Mg_2^+ free) were placed in a lysis solution (2.5 M NaCl,

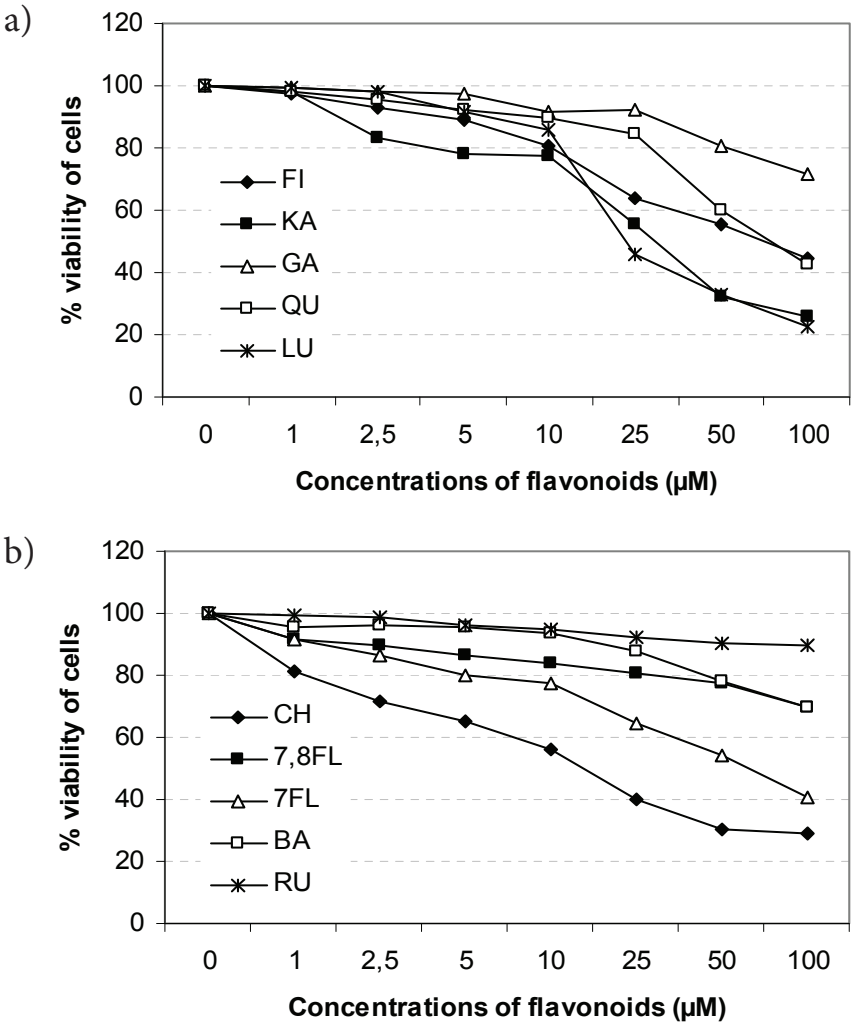


Figure 2. Cytotoxicity of different concentrations of (a) FI, KA, GA, QU, LU and (b) CH, 7,8FL, 7FL, BA, RU evaluated after 24h incubation of HepG2 cells with the flavonoids.

100 mM Na_2EDTA, 10 mM Tris–HCl, pH 10 and 1% Triton X-100) at 4°C for 1 h. Slides were transferred to an electrophoretic box and immersed in an alkaline solution (300 mM NaOH, 1 mM Na_2EDTA, pH> 13). After 40min unwinding time, a voltage of 25 V (0.3 A) was applied for 30 min at 4 °C. Slides were neutralized with 3×5min washes with Tris–HCl (0.4 M, pH 7.4), and stained with ethidium bromide (EtBr, 10 μg/ml). EtBr-stained nucleoides were examined with Zeiss Imager. Z2 fluorescence microscope using the computerized image analysis (Metafer 3.6, Meta Systems GmbH., Altlussheim, Germany). The percentage of DNA in the tail (% of tail DNA) was used as a parameter for measurement of DNA damage (DNA strand breaks). One hundred comets were scored per each sample in one electrophoretic run. The differences between defined groups were tested for significance using Student's *t*-test. For the purpose of comparing dose-response effects linear regression analysis was used.

Results

Cytotoxicity of flavonoids and benzo(a)pyrene. Cytotoxic effects of 24 h influence of different concentrations of flavonoids and 2 h influence of B(a)P (10 μM) were evaluated in HepG2 cells by the trypan blue exclusion technique, at which the adherent cells were loosen by trypsin, stained by trypan blue (0.4%) and the number of viable and dead cells was scored. HepG2 cells treated during 2 h with B(a)P did not show any decrease of viability. Cytotoxicity of 24 h long treatment of cells with flavonoids is summarized in Figs. 2a and 2b IC_{50} values (median inhibitory concentrations that cause approximately 50% cell death) represent : ~60 μM (FI), ~30 μM (KA), >100 μM (GA), ~60 μM (QU) and ~20 μM (LU). This group of flavonoids is in this paper indicated as the group one (Fig. 2a). Fig. 2b represents IC_{50} values of the further five flavonoids which are in this paper indicated as the group two: ~17 μM (CH), ~60 μM (7FL), >100 μM (7,8FL),

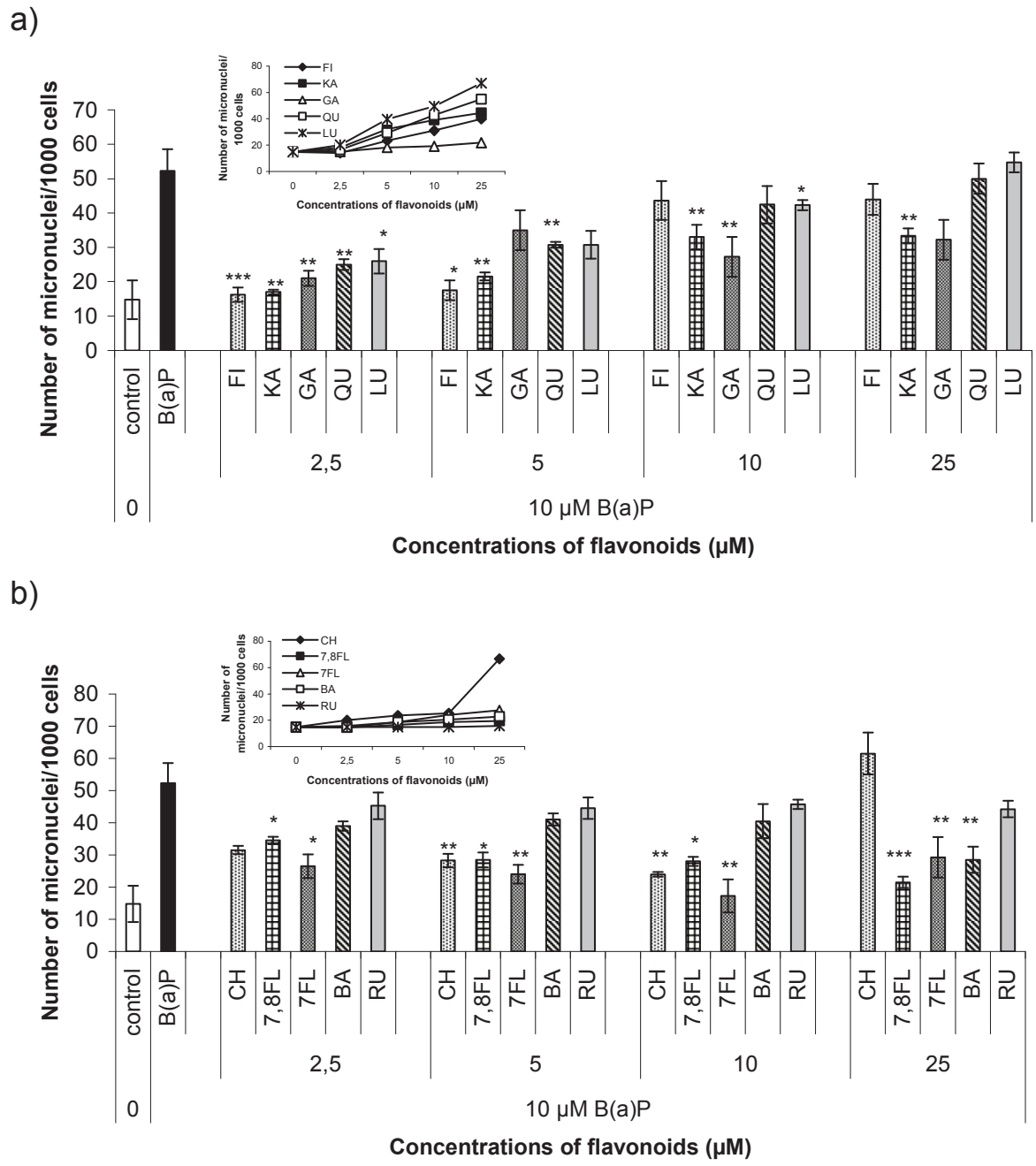


Figure 3. The frequency of micronuclei in control (white column) and B(a)P-treated HepG2 cells (black column) and in B(a)P-treated HepG2 cells after pre-incubation with (a) FI, KA, GA, QU, LU and (b) CH, 7,8FL, 7FL, BA, RU (grey hatched columns).
Inserted panels represent the frequency of MN in HepG2 cells treated with flavonoids tested alone.
Significantly different from the B(a)P value, *p< 0.05; **p< 0.01; ***p<0.001.

>75 μM (BA) and >100 μM (RU). Degree of cytotoxicity did not correlate with the molecular structure of flavonoids. Cytotoxicity was increased in the order CH>LU>KA>FI, QU, 7FL>BA>GA, 7,8FL, RU. The studies aimed at genotoxic and protective effects of flavonoids were evaluated at $IC_{\sim 10\text{-}40}$.

Induction of micronuclei. Micronuclei (MN) formation has become an important endpoint in genotoxicity studies because there exists a positive correlation between carcinogenicity of chemical agents and induction of MN [18]. The frequencies of B(a)P- induced MN in HepG2 cells treated with B(a)P (1-10 μM) are represented in Table 1. The ability of flavonoids (2.5, 5, 10 and 25 μM) to reduce the level of B(a)P (10 μM) induced MN is shown in Figs. 3a and 3b. Fig. 3a shows inhibiting effect of 24 h pre-B(a)P-incubation of cells with FI,

Table 1. The frequency of micronuclei and the level of DNA strand-breaks in HepG2 cells treated with different concentrations of B(a)P. The mean ± S.D. from at least two independent experiments on each triplicate of sheets.

Compound	Conc.(μM)	Number of micronuclei /1000 cells	% of tail DNA
	0	19,25 ± 2,39	7,46 ± 0,18
	2,5	36,50 ± 5,59	13,30 ± 0,60
B(a)P	5	46,32 ± 3,87	15,80 ± 0,87
	10	56,21 ± 5,54	17,07 ± 1,62
	15	-	20,25 ± 1,25
	20	-	22,53 ± 2,20

KA, GA, QU and LU and Fig. 3b with CH, 7,8FL, 7FL, BA and RU. Inserted panels represent the percentage of MN induced by the individual flavonoids alone. Regardless of the fact if the flavonoids tested induced (at higher concentrations) MN or not, most of them, except of RU, decreased effectively the levels of B(a)P-induced MN. While flavonoids indicated in this paper as the first group (FI, KA, GA, QU, and LU) were more effective at the concentration 2.5 μM, the flavonoids indicated as the second group (CH, 7FL, 7,8FL, and BA) were effective at higher concentrations, 10 and 25 μM, respectively. Only one of the flavonoids tested, RU, was not able to reduce the level of MN induced by B(a)P.

Induction of DNA lesions. The level of DNA strand breaks induced in HepG2 cells by different concentrations of B(a)P measured by the comet assay is presented in Table 1. We chose concentration of 10 μM as the optimal genotoxic concentration of B(a)P at which the level of DNA-strand breaks increased from ~ 7% (control) to ~17%. Figs. 4a and 4b represent inhibiting effects of 24 h pre-B(a)P-treatment of HepG2 cells with the flavonoids (2.5-25 μM) of the first or second group on the induction of DNA lesions by B(a)P. Inserted panels show the percentage of DNA strand breaks induced by the individual flavonoids alone. Similarly as in the case of MN, regardless of the fact if the they induced DNA strand breaks or not, flavonoids of the first group (FI, KA, GA, QU, LU) inhibited significantly (at concentration 2.5 μM) DNA damage induced by 10 μM B(a)P (Fig. 4a), however, flavonoids of the second group CH, 7FL, 7,8FL, BA, (hydroxylated on the A ring), showed inhibition at higher concentrations only (10 and 25μM) (Fig. 4b). Only one of the flavonoids tested, RU, was not able to reduce the DNA damage induced by B(a)P.

Discussion

The aim of our work was to investigate anti-genotoxic effects of flavonoids against B(a)P-induced genotoxicity in human hepatoma cells HepG2 and to judge the relationship between obtained results and the chemical structure of flavonoids. B(a)P exerts its genotoxic and carcinogenic effects completely after activation to a variety of mutagenic and carcinogenic electrophiles which can be covalently bound to the nucleophiles (DNA, RNA or proteins) [19]. Generally, B(a)P is catalyzed by cytochromes P450 to form B(a)P-7,8-diol which is activated to catechol through aldo-keto-reductase (AKR) pathway. This pathway produces PAH metabolites that form DNA adducts or reactive oxygen species (ROS) leading to oxidative DNA damage, such as 8-hydroxy-2`-deoxyguanosine (8-OHdG). Auto-oxidation of the intermediate catechols can generate ROS that cause oxidative DNA damage [20]. Although it is not possible to characterize exactly the earliest genotoxic effects induced in mammalian cells by B(a)P, it is known [21] that human lung tumor cells NCI-H322 metabolize B(a)P at a rate of 160 pmol/10^6 cells/h for at least 8 h. On the basis of these data we suppose that while 2 h treatment of HepG2 cells with B(a)P followed by 24 h post-B(a)P-treatment in complete growth medium enabled creation of MN as a biomarker of chromosomal breakage or whole chromosome loss [22], 2 h long treatment of HepG2 cells with B(a)P (without any post-B(a)P-treatment) induced in cellular DNA predominantly oxidative DNA lesions which we detected by the comet assay as DNA strand breaks (Table 1).

To the structure-dependent biological and pharmacological activities of flavonoids were over the past years paid a great attention [23,24]. The hydroxylation at the A and C ring moieties is proven to be important structural requirement for a significant *in vitro* antioxidant [25] and myeloperoxidase inhibitory activity [26]. The presence of a dihydroxyl group (catechol-type) or three adjacent hydroxyl groups (pyrogallol-type) on the B ring are also recognized to be very important for some of their biological activities such as antioxidant and antiradical activity [27]. The B ring dihydroxyl is even proposed to be an indicator of antioxidant activity [28]. It has been reported that 3-hydroxylation of the C ring, 7-hydroxylation of the A ring and a catechol or pyrogallol group on the B ring favour genotoxicity of flavonols *via* auto-oxidation [29]. Furthemore, the 3-hydroxyl group and 2,3 double bond also appear important in the co-mutagenicity of flavonoids with 2-acetylaminofluorene (2-AAF) [30].

The first group of flavonoids tested, i.e. FI, KA, GA, QU and LU are hydroxylated at the 3´,4´-position on the B ring, 3-position of C ring and on the A ring (Fig 1). Treatment of HepG2 cells with the above mentioned flavonoids reduced the number of B(a)P-induced MN by 69% (FI), 67% (KA), 60% (GA), 52% (QU) and by 50% (LU) at concentration 2.5 μM (Fig. 3a). The results correspond with results of the comet assay (Fig. 4a). The most effective DNA-protective effect showed FI, less but significant effect had also other flavonoids of our interest. Their DNA-protective/inhibitive effect decreased at a following sequence: FI>QU>GA>KA>LU. Both methods demonstrably showed that flavonoids at the concentration 2.5 μM had significantly protective/inhibitive effect towards B(a)P. Our results indicate that the absence of OH group in the 3-position of C ring of (LU) could play a significant role regarding to its DNA-protective activity. The flavonoids hydroxylated on the C ring at the position 3-(FI, KA, GA and QU) also inhibited the level of B(a)P-induced

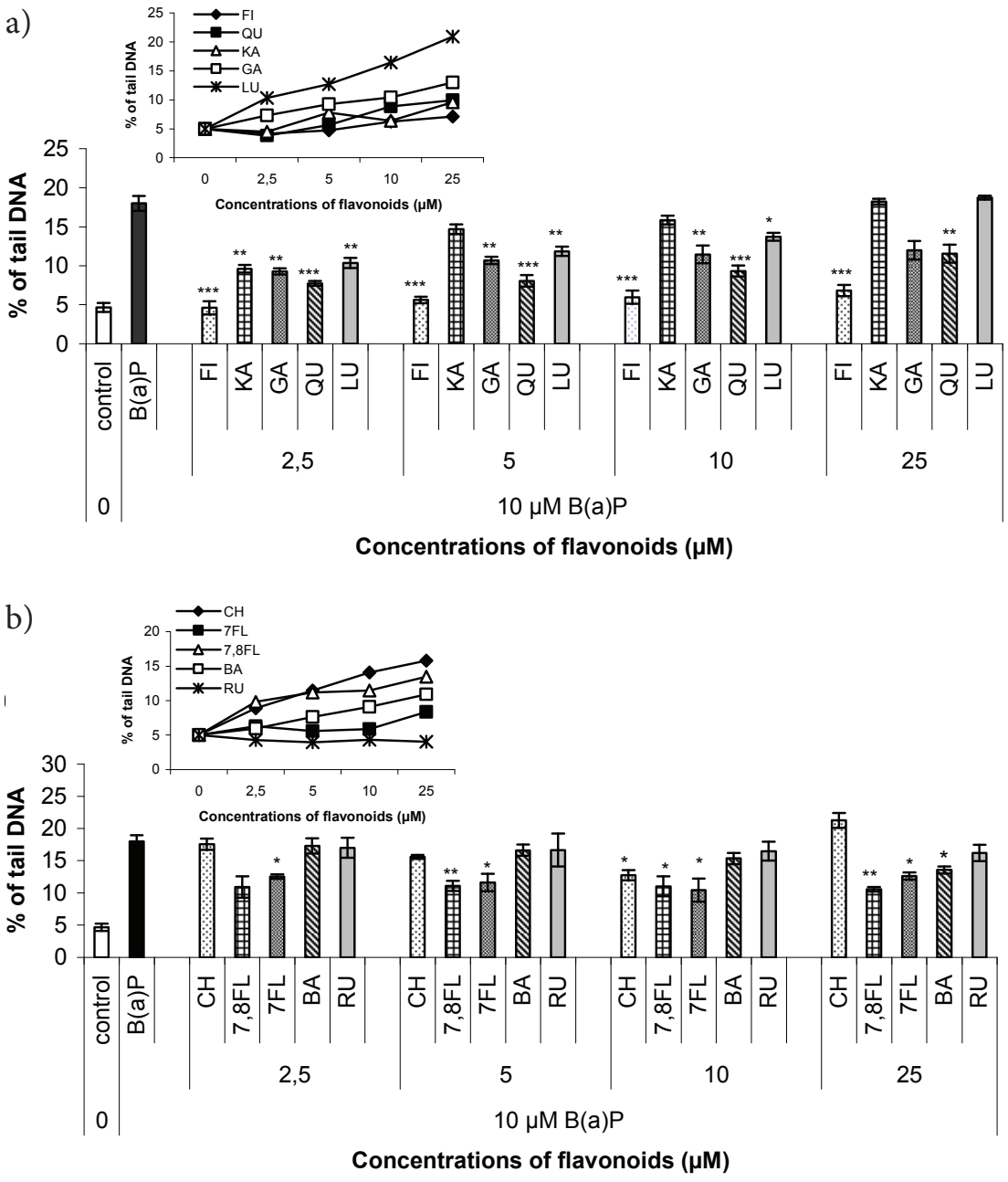


Figure 4. The incidence of DNA strand breaks (SCGE) in control (white column) and B(a)P-treated HepG2 cells (black column) and in B(a)P-treated HepG2 cells after pre-incubation with (a) FI, KA, GA, QU, LU and (b) CH, 7,8FL, 7FL, BA, RU (grey hatched colum).
Inserted panels represent the incidence of DNA strand breaks in HepG2 cells treated with flavonoids tested alone.
Significantly different from the B(a)P value, *p< 0.05; **p< 0.01; ***p<0.001.

DNA-damage. The high DNA-protective potential of FI, KA, GA and QU, which contain OH group in the 3-position of C ring demonstrated also Melidou et al. [31] using Jurkat cells. In our previous studies we proved the protective effect of quercetin and luteolin against DNA strand breaks induced by H_2O_2 in murine leukemia cells L1200 [32], and human myelogenous leukemia cells K562 [33] as well as reduction of chromosomal aberrations induced by antineoplastic drug melphalan in human melanoma HMB-2 cells [34]. The high scavenging activity of the *o*-dihydroxy structure on the B ring confers a higher degree of stability to the flavonoid phenoxyl radicals by participating in electron delocalization. It is therefore an important determinant of antioxidative potential [35]. Huang et al. found that FI, LU, QU, KA, CH

and RU inhibited the mutagenic activity resulting from the metabolic activation of B(a)P by rat liver microsomes [36]. QU represents the most frequently studied flavonoid. The protective effects of QU against the formation of oxidative DNA-damage (H_2O_2) and bulky DNA adducts (B(a)P) in human lymphocytes were investigated by Wilms et al. both *in vitro* and *ex vivo* [37]. The anticarcinogenic effect of QU might be interpreted reduction of B(a)P-derived DNA adducts in lymphocytes upon exposure to B(a)P. An indication of this protective mechanism was suggested by Kang et al. [38], who proved inhibition of *CYP1A1* gene expression by QU, and a subsequent reduction of B(a)P-derived adducts in B(a)P-exposed human HepG2 cells. In additon, QU strongly reduced of B(a)P-induced DNA-strand breaks and oxidized pyrimidines in HepG2 cells [39].

The second group of flavonoids tested was represented by CH, 7FL,7,8FL and BA. They belong to the group of flavonoids hydroxylated on A ring only (Fig,1). The number and position of OH groups on the A ring is important but seems not to be crucial in determination of DNA-protective potential in these cases. According to our results, these flavonoids had no protective effects in lower concentration (2.5 μM) but they are effective at the higher concentrations (10-25 μM). We found that 7FL and CH effectively decreased the frequency of MN (Fig. 3b), as well as DNA-strand breaks (Fig. 4b) at the concentration 10 μM. The level of B(a)P-induced MN was decreased by 67% and 54% and the level of DNA-strand breaks was reduced by 37% and 25%. 7,8 FL and BA reduced the levels of MN by 57% and 44% and the level of strand breaks by 38% and 20%. The results of our study correlate with the studies performed in various experimental systems [40,41].

To the second group of flavonoids we added also rutin (RU; 3-rhamnosyl-glucosyl-quercetin). RU is glycoside of QU, the hydroxyl group at the position 3 is substituted by the sugar rutinose. Its protective effect towards B(a)P-induced genotoxicity was not in HepG2 cells significant at any of concentrations testes (Fig. 3b and Fig. 4b). This result is in a good agreement with the hypothesis that the free hydroxyl at the position 3 of flavonoid structure is required for the significant DNA-protective activity. The consequence of the glycosylation of any hydroxyl in the structure of flavonoid may prevent or decrease its transport toward DNA molecules inside a cell. These results support also our previous findings, showing that RU has only marginal protective effect towards H_2O_2-induced DNA-damage in two types of leukemia cells [32,33] and data obtained by Ramos and coworkers [42], who did not find any DNA-protective effect of RU in HepG2 cells treated with *tert*-butyl hydroperoxide (*t*-BHP).

These results obtained using both the micronucleus assay and the comet assay suggest that several structural characteristcs of flavonoids, namely : dihydroxy groups in the 3′,4′-*ortho* position, the presence of the hydroxyl group at position 3 of ring C in combination with the oxo group at position 4, and the presence of the C_2, C_3 double bond (but not the number and position of OH groups on the A ring) support in all probability their antioxidant activity and are the crucial determinants of anti-genotoxic potential of flavonoids.

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