Eur J Nutr 38:133–142 (1999)

ORIGINAL CONTRIBUTION

S. Kuntz
U. Wenzel
H. Daniel

Comparative analysis of the effects of flavonoids on proliferation, cytotoxicity, and apoptosis in human colon cancer cell lines

Abstract Flavonoids are polyphenolic compounds that occur ubiquitously in foods of plant origin. Their proposed protective role in tumor development may prevail especially in the intestinal tract due to direct exposure of intestinal epithelia to these dietary ingredients. We have screened more than 30 flavonoids for their effects on cell proliferation and potential cytotoxicity in the human colon cancer cell lines Caco-2, displaying features of small intestinal epithelial cells, and HT-29, resembling colonic crypt cells. In addition, for selected compounds we assessed whether they induce apoptosis by determining caspase-3 activation. Studies on the dose dependent effects of the flavonoids showed antiproliferative activity of all compounds with EC_{50} values ranging between 39.7 ± 2.3 µM (baicalein) and 203.6 ± 15.5 µM (diosmin). In almost all cases, growth inhibition by the flavonoids occured in the absence of cytotoxicity. There was no obvious stucture-activity relationship in the antiproliferative effects either on basis of the subclasses (i.e., isoflavones, flavones, flavonols, flavanones) or with respect to kind or position of substituents within a class.

In a subset of experiments we examined the antiproliferative activities of the most potent compound of each flavonoid subgroup in addition in LLC-PK_1, a renal tubular cell line, and the human breast cancer cell line MCF-7. Out of four flavonols tested, three displayed almost equal antiproliferative activities in all cell lines but fisetin was less potent in MCF-7 cells. The flavanones bavachinin and flavanone inhibited growth of Caco-2 and HT-29 cells with lower EC_{50} values than that obtained in LLC-PK_1 and MCF-7 cells. The lower susceptibility of LLC-PK_1 and MCF-7 cells towards growth arrest was even more pronounced in the case of the flavone baicalein. Half maximal growth-inhibition in LLC-PK_1 and MCF-7 required 2.5 and 6.6 fold higher concentrations than that needed in the intestinal cell lines. The flavonoids failed to affect apoptosis in LLC-PK_1 and MCF-7, whereas baicalein and myricetin were able to induce apoptosis in HT-29 and Caco-2 cells.

In conclusion, flavonoids of the flavone, flavonol, flavanone, and isoflavone classes possess antiproliferative effects in different cancer cell lines. The capability of flavonoids for growth inhibition and induction of apoptosis can not be predicted on the basis of their chemical composition and structure.

Key words Flavonoids – cancer-cell lines – proliferation – cytotoxicity – apoptosis

Received: 28 December 1998
Accepted: 18 March 1999

H. Daniel (✉)
Institute of Nutritional Sciences
Hochfeldweg 2
D-85350 Freising-Weihenstephan
Germany
e-mail: daniel@weihenstephan.de

S. Kuntz · U. Wenzel
Institute of Nutritional Sciences
Wilhelmstraße 20
D-35392 Giessen
Germany

Abbreviations Hepes; N-2-hydroxyethylpiperazine-N´-2-ethanesulfonic-acid; ModEM, modified Eagle medium; DMEM, Dulbecco's minimal essential medium; I.U., International Units; PMSF, phenylmethyl-sulfonyl-fluoride; DTT, dithiotreitol; FCS, fetal calf serum.

European Journal of Nutrition, Vol. 38, Number 3 (1999)

Introduction

Over 4000 flavonoids have been identified in plant sources. These low molecular weight phenylbenzopyrones are found in fruits, vegetables, nuts, seeds as well as tea and wine (1). The basic structure of flavonoids consists of a o-heterocyclic ring fused to an aromatic ring with a third ring system attached at either C3 or C4 of the heterocyclic ring. The 6 most common chemical classes are the anthocyanidins, catechines, flavones, flavonols, flavanones, and isoflavonoids (2). Their structures differ only in the position of the ring with respect to the fused ring system and by the number and positions of hydroxy- or methoxy-group substituents. In plants the flavonoids are predominantly found as glycosides (3). These glycosides appear to be resistant to the endogenous enzymes of the gastrointestinal tract but are cleaved by bacterial enzymes in the large intestine to yield the corresponding aglycons (4). The absorption of the glycosides in the small intestine is generally poor (4) but recent findings suggest that selected flavonoids may even be better absorbed in their glycosylated form (5). Flavonoids reaching the large intestine may be further metabolized subsequent to deglycosylation by the microflora (6).

On average, the daily Western diet provides 50 mg of different flavonoids but with a great variability in intake depending on the food source (7). Although there is only limited – and inconsistent – information on intestinal absorption and bioavailability of most flavonoids, the concentrations attained in the intestinal epithelial cells should by nature exceed those in circulation. Since epithelial cells of the small and large intestine are exposed to the highest concentrations of dietary flavonoids, we focused on the interaction of a variety of flavonoids with transformed human intestinal epithelial cells to explore their biological functions.

The flavonoids possess a remarkable spectrum of biochemical and pharmacological activities suggesting that they significantly affect basic cell functions such as growth, differentiation and/or programmed cell death (apoptosis). Although some epidemiological studies provided evidence that a high dietary intake of flavonoids could be associated with a low cancer prevalence in humans (8-11) others did not find such an association (12, 13, for review see 14). Animal studies or investigations employing different cellular models, however, showed selected flavonoids to inhibit tumor initiation as well as tumor progession (15-18). As possible mechanisms by which the flavonoids may affect tumorigenesis are discussed 17-β-estradiol antagonizing properties (19), antioxidant activities (20), the scavenging effect on activated mutagens and carcinogens (21, 22), interaction with proteins that control cell cycle progression (23), and altered gene expression (24).

Human colon cancer development is often characterized in an early stage by a hyperproliferation of the epithelium leading to the formation of adenomas. This is mainly a consequence of disregulated cell cycle control and/or suppressed apoptosis as usually observed in colorectal cancers (25, 26). Protective effects of flavonoids in colon cancer development should consequently be associated with inhibition of cell proliferation and/or induction of the apoptotic pathway to delete cells carrying mutations and to maintan a normal cell population. We therefore investigated whether and to what extent 36 different flavonoids belonging to the flavanone, flavone, flavonol, and isoflavone subclasses could reduce cell growth and could promote apoptosis in the two human intestinal tumor cell lines Caco-2 and HT-29. In addition, the most potent compounds of each flavonoid subgroup were also studied in two non-intestinal cell lines to determine the specificity of the effects based on the cell phenotype.

Material and methods

Materials

All flavonoids of highest purity available were purchased from Sigma (Deisenhofen, Germany), Calbiochem (Bad Soden, Germany), and Phytochem (Ichenhausen, Germany). All the materials needed for cell culture were either from Gibco (Eggenstein, Germany) or Renner (Dannstadt, Germany). The fluorophores Sytox-Green Nucleic Acid stain and fluorescein diphosphate were purchased from Bioprobes (Leiden, Netherlands). CPP-32 fluorogenic apopain substrate was obtained from Calbiochem (Bad Soden, Germany).

Cell culture

HT-29 (passage 106), Caco-2 (HTB 37, passage 31), LLC-PK_1 (CRL 1392, passage 195), and MCF-7 cells (passage 95) were provided by ATCC. Cells were cultured and passaged in RPMI-1640 (HT-29), DMEM (Caco-2), and Dulbecco‘s ModEM media (LLC-PK_1 and MCF-7), respectively. All media (Gibco) were supplemented with 10% fetal calf serum and 2 mM glutamine. Antibiotics added were 100 U/ml penicillin and 100 µg/ml streptomycin for HT-29, LLC-PK_1 and MCF-7 cells and 100 U/ml gentamycin for Caco-2 cells, respectively. With the exception of media for HT-29, all media were supplemented with 1% MEM non – essential amino acids (Gibco 01140). Media for LLC-PK_1 and MCF-7 cells contained 10 mM Hepes, 1 mM sodium pyruvate, media for MCF-7 cells in addition 10 µg/ml insulin. All cultures were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Cells were passaged at preconfluent densities with the use of a solution containing 0.05 % trypsin and 0.5 mM EDTA.

Cell proliferation and acute cytotoxicity

For determination of proliferation, cells were seeded at a density of 5×10^3 per well onto 24 well Renner cell cul-

ture plates and allowed to adhere for 24 h. Thereafter medium was replaced by fresh culture medium containing the test compounds and cells were allowed to grow for another 72 h. The flavonoids were applied in DMSO and the solvent reached a concentration not greater than 2% in all experiments. Controls were always treated with the same amount of DMSO as used in the corresponding experiments. Total cell counts were determined by SYTOX-Green that becomes fluorescent after DNA binding. Therefore, cells were lysed by 1% Triton-X 100 and cell numbers were determined based on a calibration curve. The calibration curve was generated by using cell numbers between 1 x 10^3 and 1 x 10^6, as verified by counting cells in a Neubauer chamber, and measuring fluorescence at 538 nm after excitation at 485 nm using a fluorescence multiwell-plate reader (Fluoroskan Ascent, Labsystems, Merlin Diagnostika, Bornheim-Hersel, Germany). Agents that showed an antiproliferative effect were in addition tested for their potential toxicity. Acute cytotoxicity was assessed by SYTOX-fluorescence with 5 x 10^4 adherent cells per well exposed for 3 h to 150 µM of the test compounds. The percentage of dead cells in a cell population was determined by SYTOX-fluorescence prior to cell lysis in relation to the fluorescence measured after solubilisation of the cells.

Apoptosis assay

Apopain activity was measured as described previously (27). In brief, cells were seeded at a density of 5 x 10^5 per well onto 6-well plates and allowed to adhere for 24 h. Subsequently, cells were exposed for 24 h to the test compounds and washed afterwards with PBS. Cytosolic extracts were prepared by adding 750 µl/well of a buffer containing 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, 1 mM PMSF, 10 µg/ml pepstatinA, 20 µg/ml leupeptin, 10 µg/ml aprotinin and 10 mM Hepes/KOH, pH 7.4, and the cell monolayers were homogenized by 10 strokes using a motor driven homogenizer with a teflon pestle. The homogenate was centrifugated at 100 000 x g at 4 °C and the supernatant was incubated with the fluorogenic caspase-3 tetrapeptide-substrate Ac-DEVD-amino-4-methylcoumarin at a final concentration of 20 µM. Cleavage of the apopain substrate was followed by determination of emission at 460 nm after excitation at 390 nm using the fluorescence plate reader. As a reference compound shown to induce apoptosis in a variety of human cancers and cancer cell lines, we employed the topoisomerase inhibitor camptothecin.

Calculations and statistics

To derive the EC_{50} values for growth inhibition, a nonlinear approximation model by the least square methods based on a competition curve using one component was applied (GraphPadPrism, GraphPad, USA). For each variable at least 3 independent experiments were carried out. Data are given as the mean ± SEM.

Results

The growth-inhibitory activity of various flavonoids, whose basic structures are shown in Fig. 1, was determined in preconfluent HT-29 and Caco-2 cells after 72 h of exposure to concentrations of 25 to 250 µM of the test compounds. All flavonoids inhibited the growth of both intestinal cell lines. Concentrations that caused 50% inhibition of cell proliferation when compared to controls ranged between EC_{50} values of 40 µM and 200 µM (Table 1). In Caco-2 cells, the flavones baicalein, puerarin, kaempferide, tangeretin (Table 1A), the flavonol fisetin (Table 1B), and flavanone (Table 1C) were found to be the most potent compounds with EC_{50} values of approximately 60 µM. Whereas baicalein, tangeretin, fisetin, and flavanone proved to be strong growth inhibitors also in HT-29 cells, the flavones puerarin and kaempferide required concentrations of greater than 100 µM to promote 50% growth inhibition in this cell line. Also for the flavone diosmetin EC_{50} values in HT-29 cells were twice as high as those obtained in Caco-2 cells (Table 1A). For all other flavonoids tested, the potency to inhibit cell growth was similar in both intestinal cell lines.

Assessment of plasma membrane integrity as a sensitive marker for cytotoxic effects of flavonoids was performed by measuring exclusion of the DNA-stain

Fig. 1 Structures of flavonoids; A) flavones, B) flavonols, C) flavanones, and D) isoflavones. The basic structure consists of 2 fused rings, the first ring (A) is an aromatic one, the second ring (C) is an oxygen containing heterocyclic ring which is attached by a carbon-carbon bond to a third aromatic ring (B). The flavonoids differ in their structure from each other at the C-ring.

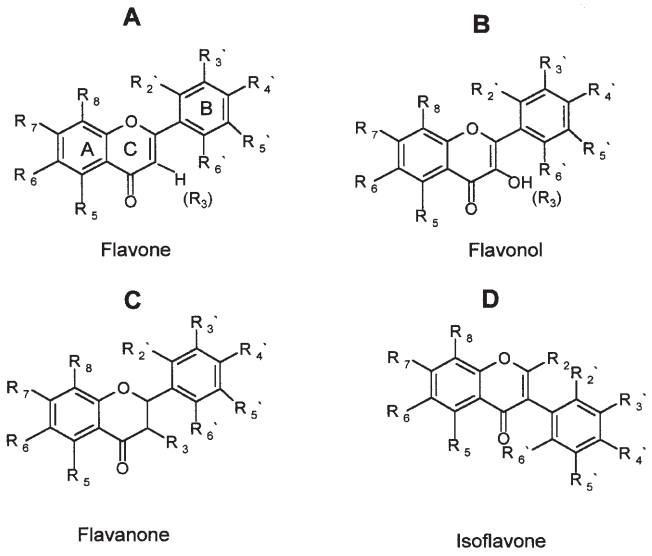

Table 1A Growth inhibitory potency and cytotoxicity of selected **flavones** in Caco-2 and HT-29 cells in culture

Substance	HT-29 EC_{50} value [µM]	Caco-2 EC_{50} value [µM]	HT-29 cytotoxicity at 150 µM (% of viable cells)	Caco-2 cytotoxicity at 150 µM (% of viable cells)
5-OH-flavone	146.3 ± 8.9	100.5 ± 10.5	97.6 ± 4.5	102.3 ± 5.5
7-OH-flavone	148.5 ± 7.4	104.6 ± 10.9	102.7 ± 6.5	100.5 ± 5.5
7.8-di-OH-flavone	155.7 ± 1.5	109.3 ± 5.7	110.0 ± 10.9	100.9 ± 14.9
apigenin	130.6 ± 6.8	115.4 ± 10.3	94.2 ± 5.0	95.6 ± 9.0
baicalein	49.4 ± 5.1	56.4 ± 4.0	107.5 ± 8.0	100.3 ± 7.0
luteolin	113.9 ± 7.8	89.7 ± 5.6	83.6 ± 0.7	82.0 ± 1.7
chrysin	126.5 ± 6.3	115.6 ± 2.1	95.0 ± 1.5	98.2 ± 2.5
acacetin	187.1 ± 9.0	102.6 ± 5.1	101.1 ± 0.9	99.1 ± 3.0
diosmin	76.5 ± 6.5	112.2 ± 6.9	101.0 ± 2.0	108.3 ± 4.0
diosmetin	203.6 ± 10.5	107.6 ± 6.8	80.1 ± 2.9	98.1 ± 4.9
apiin	136.8 ± 11.5	148.9 ± 12.7	75.0 ± 1.1	82.0 ± 2.1
kaempferide	144.7 ± 9.5	58.7 ± 5.5	80.9 ± 6.2	98.9 ± 5.2
puerarin	103.8 ± 7.1	55.9 ± 6.4	71.1 ± 2.4	87.9 ± 8.4
tangeretin	61.6 ± 4.9	56.9 ± 4.6	91.7 ± 3.3	92.1 ± 6.3
rutin	136.2 ± 15.6	136.7 ± 14.2	95.4 ± 2.1	96.5 ± 2.1

Cells were exposed for 72 hours to increasing concentrations of the test compounds and total cell numbers were determined as described in the Methods section. EC_{50} values were derived from the dose-response relationship for three experiments and are given as the mean ± SEM. Cytotoxicity as assessed by Sytox-exclusion was determined after 3 hours of exposure to 150 µM of the compounds. More than 90% viable cells were considered to be unaffected by the flavonoids, 80-90% as modestly affected, and values of less than 80% viable cells were ascribed to cytotoxic effects of the compounds.

Table 1B Growth-inhibitory potency and cytotoxicity of selected **flavonols** in Caco-2 and HT-29 cells in culture

Substance	HT-29 EC_{50} value [µM]	Caco-2 EC_{50} value [µM]	HT-29 cytotoxicity at 150 µM (% of viable cells)	Caco-2 cytotoxicity at 150 µM (% of viable cells)
3-OH-flavone	58.6 ± 2.5	65.8 ± 5.7	96.8 ± 0.4	93.1 ± 3.4
kaempferol	136.9 ± 7.4	163.2 ± 1.6	100.8 ± 4.3	101.8 ± 5.3
fisetin	54.9 ± 2.1	59.3 ± 2.9	82.9 ± 3.8	89.4 ± 1.8
quercetin	85.59 ± 8.6	96.3 ± 6.7	90.6 ± 0.5	92.5 ± 5.5
morin	117.8 ± 8.5	n.d.	89.8 ± 0.1	85.4 ± 8.1
myricetin	47.6 ± 2.3	88.4 ± 3.4	92.2 ± 2.0	98.1 ± 3.0

Table 1C Growth inhibitory potency and cytotoxicity of selected **flavanones in** Caco-2 and HT-29 cells

Substance	HT-29 EC_{50} value [µM]	Caco-2 EC_{50} value [µM]	HT-29 cytotoxicity at 150 µM (% of viable cells)	Caco-2 cytotoxicity at 150 µM (% of viable cells)
flavanone	76.5 ± 6.8	55.9 ± 4.7	78.5 ± 18.7	93.2 ± 2.5
hesperidin	55.2 ± 4.8	65.2 ± 5.5	93.9 ± 0.6	98.7 ± 4.6
hesperetin	87.3 ± 7.0	89.9 ± 5.9	88.9 ± 4.3	87.4 ± 3.5
naringin	141.3 ± 4.1	145.8 ± 8.0	78.4 ± 1.6	88.5 ± 4.6
bavachinin	39.7 ± 2.3	55.94 ± 4.9	91.5 ± 1.4	95.6 ± 3.7
didymin	108.6 ± 14.2	93.3 ± 8.7	75.0 ± 3.5	79.9 ± 2.5
neohesperidin	156.3 ± 10.0	111.0 ± 5.4	97.9 ± 2.2	92.5 ± 3.2

Table 1D Growth-inhibitory potency and cytotoxicity of selected **isoflavones** in Caco-2 and HT-29 cells

Substance	HT-29 EC_{50} value [µM]	Caco-2 EC_{50} value [µM]	HT-29 cytotoxicity at 150 µM (% of viable cells)	Caco-2 cytotoxicity at 150 µM (% of viable cells)
biochanin A	86.4 ± 5.2	92.6 ± 5.9	85.5 ± 0.4	89.4 ± 2.6
daidzein	72.7 ± 5.6	85.7 ± 5.4	96.0 ± 8.1	98.3 ± 5.1
genistein	66.6 ± 4.2	69.3 ± 4.7	90.7 ± 0.3	95.1 ± 7.3
genistin	84.3 ± 8.4	82.4 ± 5.2	104.7 ± 10.5	100.3 ± 7.2

SYTOX Green. Growth-inhibition measured, for almost all flavonoids was generally not a consequence of cytotoxic effects (Table 1). Even at concentrations of 150 µM, which caused significant growth retardation or complete inhibition of proliferation, for most compounds less than 15% of cells were found to be non-viable. Consequently, cell proliferation rates appeared to be specifically reduced by the flavonoids either by changes in cell cycle progession and/or induction of apoptosis.

As a first approach to determine the mechanism by which the growth inhibition may be transmitted, we studied the most potent growth inhibitory compounds of each subclass with respect to induction of apoptosis in Caco-2 and HT-29 cells. The activation of intracellular ICE-proteases such as CPP-32 (caspase-3; apopain) is considered to represent an early event in the induction of the apoptotic pathway (28) that precedes DNA fragmentation in intestinal epithelial cells (29). Consequently, we measured apopain activity in cells exposed for 24 h to 150 µM of baicalein, genistein, bavachinin, and myricetin. The anti-cancer agent camptothecin (50 µM), known to induce apoptosis in cancer cells (30), served as a standard. As shown in Fig. 2, campothecin increased apopain activity in both cell lines four to five times, demonstrating its effectivness also in the chosen colon cancer cells. The flavonoids increased CPP-32 activity in Caco-2 and HT-29 cells comparably weaker, even when provided at three times higher concentrations than camptothecin. The most potent flavonoids were baicalein and myricetin leading to a 40% and 32% stimulation of apopain activity in Caco-2 cells, whereas both compounds induced a 2.2 fold increase in apopain activity in HT-29 cells (Fig. 2).

Although the dietary flavonoids may be presented to intestinal epithelial cells in higher concentrations than to cells in peripheral tissues, it is also important to know whether intestinal cells are more or less susceptible to the compound‘s activities. To determine the specificity of the flavonoid effects with respect to the cell type, we performed studies on growth inihibition and apoptosis employing LLC-PK_1 and MCF-7 cells. As shown in Fig. 3, the flavonols myricetin, quercetin, kaempferol, and fisetin also reduced cell proliferation in these cells without any significant cytotoxicity (> 90% viable cells in all cases). The effective concentrations required to reduce the cell counts to 50% of that in control cells were almost the same as those needed in Caco-2 and HT-29 cells. Only in case of fisetin was a 2-fold higher concentration needed for half maximal growth inhibition in MCF-7 cells when compared to the intestinal cell lines. The lower susceptibility of MCF-7 cells towards a growth inhibition was even more pronounced in case of the flavone baicalein (Fig. 4A). Here, 6.6-fold and 2.5-fold higher concentrations were needed to inhibit proliferation by 50% when compared to the intestinal and renal cell lines respectively (Fig. 4A). Although less pronounced, Caco-2 and HT-29 appeared to be more sensitive towards antiproliferative

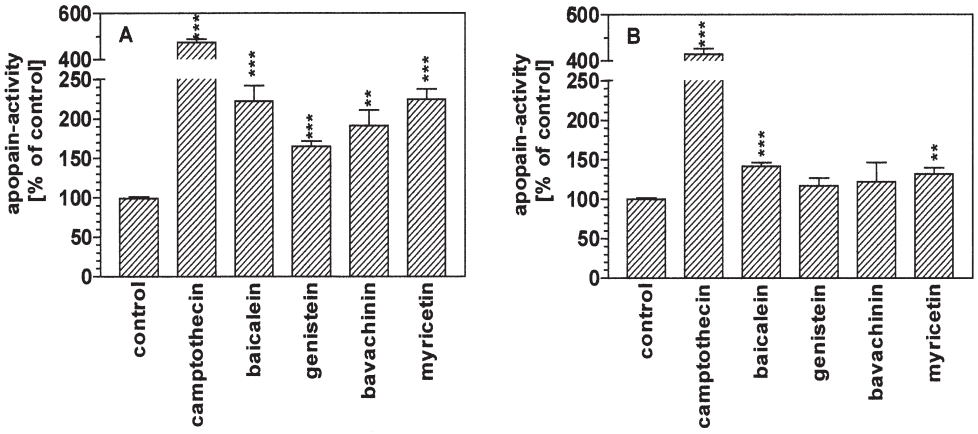

Fig. 2 Apopain-activity in HT-29 (A) and Caco-2 cells (B) after exposure (24 h) to the flavonoids (150 µM) indicated. The topoisomerase inhibitor camptothecin was used at a concentration of 50 µM as a positive control. Activity of apopain was determined based on the cleavage of the fluorogenic substrate Ac-DEVD-amino-4-methylcoumarin. Fluorescence measured in the control revealed $\Delta F = 13.5 \pm 0.6$ arbitrary units/h · 10^6 cells in HT-29 and $\Delta F = 18.3 \pm 0.4$ U/h · 10^6 Caco-2 cells. ** $P < 0.01$; *** $P < 0.001$

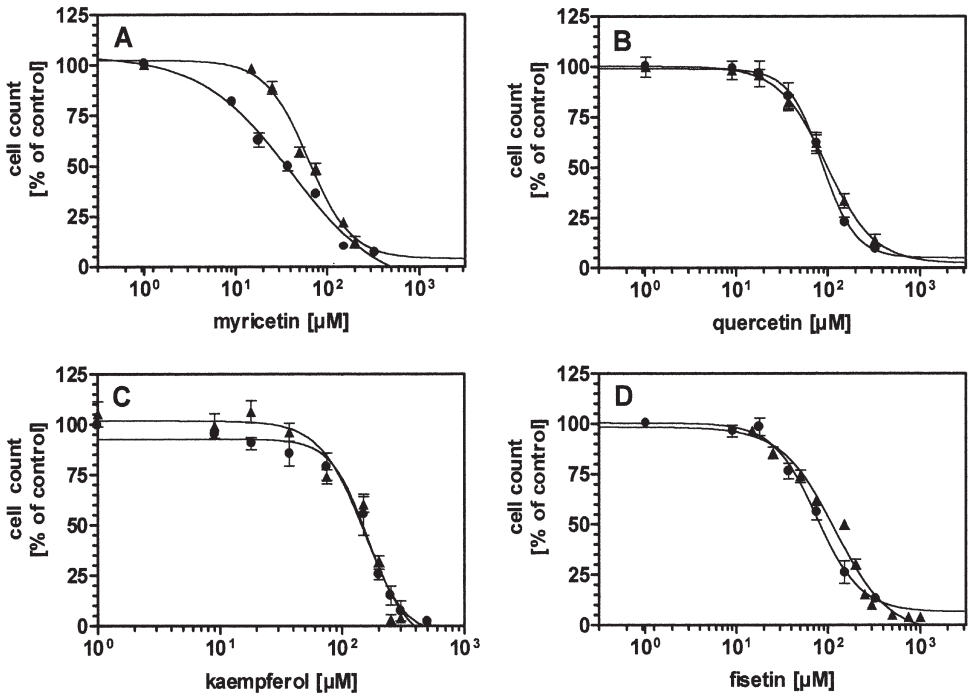

Fig. 3 Growth inhibition of LLC-PK$_1$ cells (●) and MCF-7 cells (▲) by the flavonols myricetin (A), quercetin (B), kaempferol (C), and fisetin (D). Total cell numbers were determined after a 72 h incubation period with various flavonoid concentrations using the nucleic acid stain SYTOX-Green. EC$_{50}$ values derived for growth inhibition in LLC-PK$_1$ and MCF-7 cells were 40.8 ± 2.2 µM and 62.1 ± 3.5 µM for myricetin, 86.9 ± 1.5 µM and 96.3 ± 2.3 µM for quercetin, 159.1 ± 6.1 µM and 155.4 ± 2.8 µM for kaempferol, and 75.1 ± 2.2 µM and 118.1 ± 3.5 µM in case of fisetin.

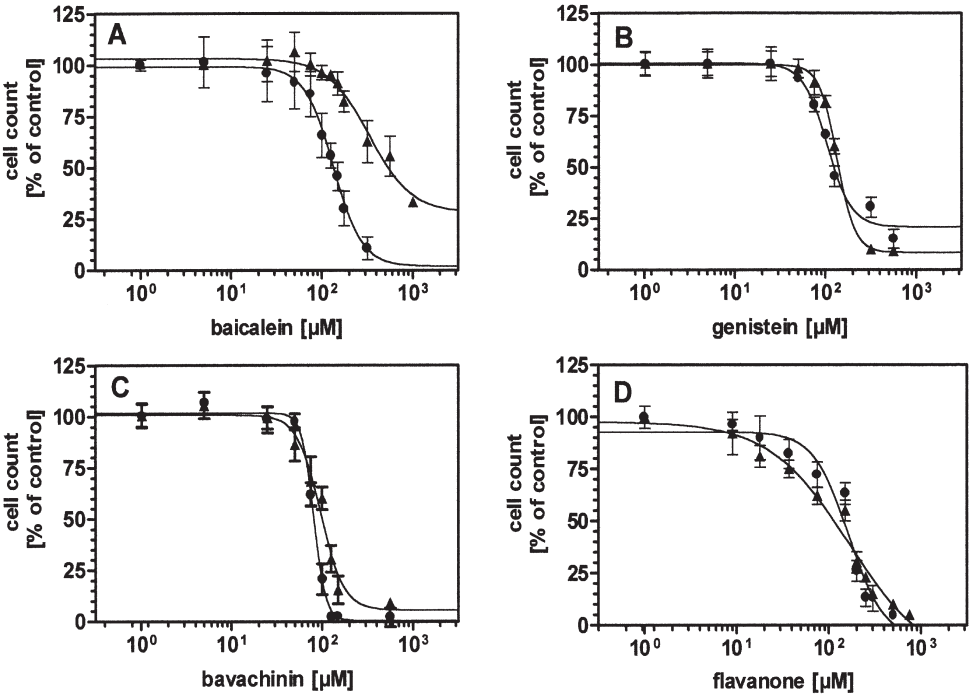

Fig. 4 Inhibition of proliferation in LLC-PK$_1$ cells (●) and MCF-7 cells (▲) by the flavone baicalein (A), the isoflavone genistein (B) and the flavanones bavachinin (C) and flavanone (D). Cell numbers were determined after a 72 h incubation period at different flavonoid concentrations. EC$_{50}$ values for growth inhibition in LLC-PK$_1$ and MCF-7 cells were 133.2 ± 2.0 µM and 331.2 ± 3.9 µM for baicalein, 103.9 ± 1.6 µM and 133.6 ± 3.3 µM for genistein, 80.1 ± 1.6 µM and 95.3 ± 2.6 µM for bavachinin, and 163.7 ± 5.5 µM and 164.3 ± 5.3 µM for flavanone.

effects of the isoflavone genistein and the flavanones bavachinin, and flavanone when compared to LLC-PK$_1$ and MCF-7 cells (Figs. 4B–4D).

When the flavonoids shown to induce apoptosis in the intestinal cell lines were applied in concentrations of 150 µM to the renal and breast cell lines, none of them increased caspase-3 activity above that in control cells (Fig. 5). In contrast, campothecin, although provided only in a concentration of 50 µM, caused an almost ten-fold increase in apopain activity in the non-transformed renal cell line LLC-PK$_1$ but only a twofold increase in MCF-7 cells (Fig. 5). MCF-7 cells, therefore, appear not only particularly resistant to induction of apoptosis by flavonoids, but also by the topoisomerase inhibitor campothecin.

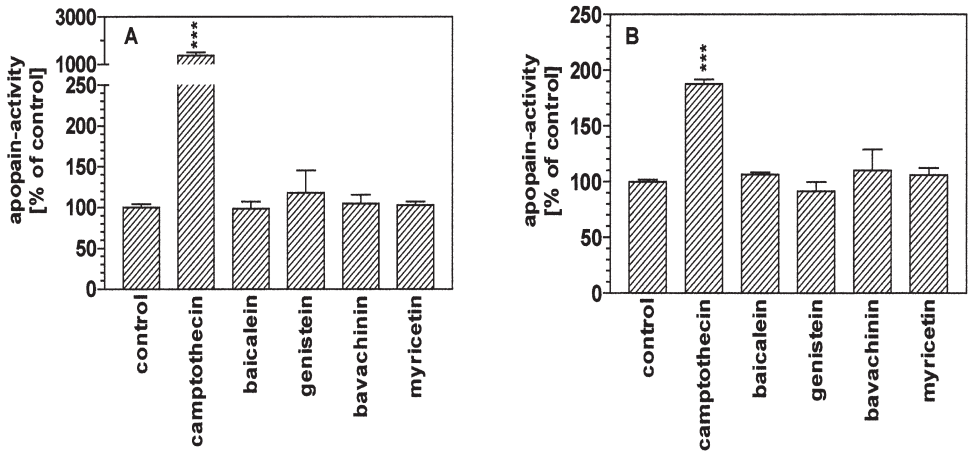

Fig. 5 Apopain activity in LLC-PK$_1$ (A) and MCF-7 cells (B). Subsequent to adherence, cells were exposed to 150 µM of the flavonoids for 24 h. Fluorescence measurements revealed a ΔF of 14.1 ± 0.8 U/h · 10^6 in LLC-PK$_1$ cells not treated with flavonoids and of 14.4 ± 0.4 U/h · 10^6 in untreated MCF-7 cells. *** $P < 0.001$

Discussion

Based on the comparative analysis of 36 flavonoids represented by the flavone, flavonol, flavanone, and isoflavone subgroups we can conclude that all compounds possess antiproliferative activity in vitro. However, this activity varies depending on the flavonoid and appears to be dependent also on the cell type employed. Flavonoids with the highest antiproliferative activity in Caco-2 and HT-29 cells displayed EC_{50} values of around 50 µM; those with the lowest activity required 150 to 200 µM for 50% inhibition of proliferation. There was no obvious structure-activity relationship either with respect to the subclasses or with respect to kind and/or positions of substituents within a subclass (Table 2). For example, isoflavonoids were equally potent in their growth-inhibitory activities irrespective of their particular composition (Table 2D) However, we did not find a flavone bearing only one or two hydroxyl groups that displayed good antiproliferative activities (Table 2A).

Table 2A **Flavones**, their substituents and their growth inhibitory activities in intestinal cell lines

number of OH-groups	compound	R3	R5	R6	R7	R8	R2'	R3'	R4'	R5'	R6'	EC_{50} <100µM	EC_{50} >100µM
1	5-OH-flavone	H	OH	H	H	H	H	H	H	H	H		•
1	7-OH-flavone	H	H	H	OH	H	H	H	H	H	H		•
2	7.8-$(OH)_2$-flavone	H	H	H	OH	OH	H	H	H	H	H		•
2	acacetin	H	OH	H	OH	H	H	H	OCH_3	H	H		•
2	chrysin	H	OH	H	OH	H	H	H	H	H	H		•
2	diosmin	H	OH	H	sugar	H	H	OH	OCH_3	H	H		•
2	apiin	H	OH	H	-apiosyl glucoside	H	H	H	OH	H	H		•
3	baicalein	H	OH	OH	OH	H	H	H	H	H	H	•	
3	apigenin	H	OH	H	OH	H	H	H	OH	H	H		•
3	diosmetin	H	OH	H	OH	H	H	OH	OCH_3	H	H		•
3	kaempferide	OH	OH	H	OH	H	H	H	OCH_3	H	H	•	•
4	tangeretin	H	OH	OH	OH	OH	H	H	OCH_3	H	H	•	
4	luteolin	H	OH	H	OH	H	H	OH	OH	H	H	•	•
4	rutin	manno/gluco-pyranosyl	OH	H	OH	H	H	H	OH	OH	H		•

EC_{50} values for growth-inhibition were taken from Table 1. Those flavonoids that displayed half-maximal inhibitory concentrations below 100 µM in one of the intestinal cell lines Caco-2 or HT-29 and above 100 µM in the other were marked in both columns. The number of OH-groups refers to those that are free and linked to the basic phenylbenzopyrone structure.

Table 2B **Flavonols**, their substituents and anti-proliferative activities in intestinal cells

number of OH-groups	compound	R3	R5	R6	R7	R8	R2'	R3'	R4'	R5'	R6'	EC_{50} <100µM	EC_{50} >100µM
1	3-OH-flavone	OH	H	H	H	H	H	H	H	H	H	•	
4	kaempferol	OH	OH	H	OH	H	H	H	OH	H	H		•
4	fisetin	OH	H	H	OH	H	H	OH	OH	H	H	•	
5	quercetin	OH	OH	H	OH	H	H	OH	OH	H	H	•	
5	morin	OH	OH	H	OH	H	OH	H	OH	H	H		•
6	myricetin	OH	OH	H	OH	H	H	OH	OH	OH	H	•	

Table 2C **Flavanones**, their substituents and anti-proliferative activities

number of OH-groups	compound	R3	R5	R6	R7	R8	R2'	R3'	R4'	R5'	R6'	EC_{50} <100µM	EC_{50} >100µM
0	flavanone	H	H	H	H	H	H	H	H	H	H	•	
1	bavachinin	H	H	methyl-butenyl	OCH_3	H	H	H	OH	H	H	•	
1	didymin	H	OH	H	-rutinosid	H	H	H	OCH_3	H	H	•	•
2	hesperidin	H	OH	H	manno/gluco-pyranosyl	H	H	H	OCH_3	OH	H	•	
3	hesperetin	H	OH	H	OH	H	H	OH	OCH_3	H	H	•	
3	naringin	H	OH	H	manno/gluco-pyranosyl	H	H	H	OH	H	H		•

Table 2D **Isoflavones** and their anti-proliferative activities

number of OH-groups	compound	R3	R5	R6	R7	R8	R2'	R3'	R4'	R5'	R6'	EC_{50} <100µM	EC_{50} >100µM
2	biochanin A	H	OH	H	OH	H	H	H	OCH_3	H	H	•	
2	daidzein	H	H	H	OH	H	H	H	OH	H	H	•	
2	genistin	H	OH	H	-glucoside	H	H	H	OH	H	H	•	
3	genistein	H	OH	H	OH	H	H	H	OH	H	H	•	

Flavonoids are considered to be antioxidants and inhibition of cell growth might therefore depend on the capacity of the compounds to serve as free radical scavengers. It has been described that flavonoids with 4-6 OH groups act as strong antioxidants in an aqueous milieu, whereas those with more or fewer OH groups show low or no antioxidant activities (31). Moreover, it was found that OH groups in the ortho-position at ring B as well as the double bond between C2 and C3 together with the carbonyl function in ring C are important structural determinants for the antioxidant effects of flavonols (32). From this point of view, one might expect that flavonoids with lower antioxidative properties possess less antiproliferative activities since flavones with 1 or 2 OH groups displayed high EC_{50} values of growth inhibition. However, there were also flavanones and isoflavones carrying only one or two OH groups that showed high antiproliferative potencies (Table 2). In addition, ortho-OH groups in ring B seemed not to promote growth inhibition since 3-OH flavone bearing no OH group in ring B proved to be a strong growth inhibitor (Tables 1 and 2).

Although flavonoids may be powerful antioxidants, they were also shown be able to generate reactive oxygen species (33). This property, however, was found to depend on the amount of dissolved oxygen in the test system (34). Consequently, it is almost impossible to predict whether pro- or antioxidative properties of flavonoids in a cell system are predominant. Previous studies employing proliferating HT-29 cells had shown the flavonols myricetin, kaempferol, and especially quercetin to be cytotoxic after 48 h of exposure and that was attributed to an autoxidation process (34). We, however, did not observe

any toxicity of these flavonols which might be due to differences in the oxidative status of the cell systems employed.

Another possible mechanism by which the antiproliferative activity of the flavonoids may be mediated could be the induction of apoptosis. We therefore determined whether selected compounds that possess a high potency for growth inhibition could induce apoptosis in Caco-2 and HT-29 cells. This was done by measuring the increase in activity of apopain, a caspase-3 protease considered to represent a specific and early marker in the apoptotic pathway in normal and transformed intestinal cells (29, 35). It became obvious that the four selected antiproliferative compounds (baicalein, genistein, bavachinin, and myricetin) increased apopain activity in the intestinal cell lines reaching levels of significance for all four flavonoids in HT-29 but only for baicalein and myricetin in Caco-2 cells. The activation of the caspase by the flavonoids was specific for the intestinal cell lines since there was no apopain stimulation detectable in LLC-PK_1 or MCF-7 cells when exposed to these flavonoids. Such a cell specific induction of apoptosis has been demonstrated for baicalein in three different hepatocellular carcinoma cell lines, leading to apoptosis only in one line (36).

Whereas, to our knowledge, this is the first report on apoptosis in intestinal carcinoma cell lines induced by baicalein, bavachinin, and myricetin, genistein has already been described to activate apoptotic pathways in HT-29 and Caco-2 cells (37). Moreover it was found to inhibit apoptosis in LLC-PK_1 (38) and to promote apoptosis in MCF-7 cells (39). The sensitivity of the breast cancer cells with regard to apoptotic stimuli is generally low (29) and this was confirmed here by only a twofold stimulation of caspase-3-like activity by camptothecin. However, the MCF-7 cells we used appeared to be even more resistant to apoptotic stimuli for example by genistein and also to growth inhibition mediated by this isoflavone. Whereas other studies reported 50% growth inhibition in MCF-7 cells by genistein at concentrations of 10 µM (40) to 40 µM (41), our studies revealed an EC_{50} value of around 130 µM. Since genistein effects are linked to growth-promoting activities of estradiol and growth factors such as EGF, the concentrations of flavonoids needed for growth suppresion are consequently dependent on the growth factors provided in the culture medium (42). We used 10% FCS as a medium supplement and therefore higher concentrations of the flavonoids may be needed for overcoming growth promoting and anti-apoptotic effects of the serum. However, by using the same FCS concentration and the same FCS batch in all studies, a direct comparison of the effects of flavonoids along the different cell lines becomes possible.

Although the mechanisms by which the flavonoids exert their cellular effects remain to be determined, it becomes evident from our studies that the intracellular events leading to apoptosis or cell cycle arrest are not strictly linked. This is supported for example by the lack of caspase-3 activation by the flavonoids in LLC-PK_1 cells. Caspase-3 activation has been shown in the renal cell line to be important for apoptosis (43) but none of the test compounds activated apopain in LLC-PK_1 cells in spite of their potent growth inhibitory activity. To which extent the induction of apoptosis by the flavonoids tested contributes to growth inhibition in the colon carcinoma cell lines remains unclear. When compared to camptothecin, the increase in apopain activitiy by the flavonoids is considerably lower, suggesting that the effects of baicalein, bavachinin, genistein, and myricetin on cell growth are most likely based on an arrest in cell cycle progression as also obserevd in MCF-7 and especially in LLC-PK_1 cells.

In conclusion, dietary flavonoids inhibit in vitro growth of a variety of cell lines mainly by cell cycle arrest. Especially in colon cancer cell lines, induction of apoptosis may contribute as well. There is no obvious structure-activity relationship with regard to the chemical composition of the flavonoids and their cell biological effects. Moreover, their proposed antioxidant activity does not relate to the inhibition of cell growth and/or induction of apoptosis. The fact that the intestinal epithelium may be confronted with much higher concentrations of the dietary flavonoids than cells in other tissues and moreover is able to accumulate certain flavonoids (44) implies that dietary polyphenols could have a significant role in the prevention of colon cancer by blocking hyperproliferation of the epithelium and by promoting apoptosis.

References

1. Middleton E, Kandaswami C (1993) The impact of plant flavonoids on mammalian biology implications for immunity, inflammation and cancer. In: Harborne JB (ed) The Flavonoids: Advances in Research since 1986. Chapmann & Hall, London, pp 619–652
2. Wiseman H (1996) Role of dietary phyto-oestrogens in the protection against cancer and heart disease. Bio Comp Food 24:795–800
3. Hempel J, Böhm H (1996) Quality and quantity of prevailing flavonoid glycosides of yellow and green french beans (Phaseolus vulgaris L.). J Agric Food Chem 44:2114–2116
4. Hollman PC, van Trijp JM, Buysman MN, van der Gaag MS, Mengelers MJ, de Vries JH, Katan MB (1997) Relative bioavailability of the antioxidant flavonoid quercetin from various foods in man. FEBS Lett 418:152–156
5. Herrmann K (1976) Flavonols and flavones in food plants: a review. J Food Technol 11:433–448
6. Winter J, Moore LH, Dowell VR, Bokkenheuser VD (1989) C-ring cleavage of flavonoids by human intestinal bacteria. Appl Environ Microbiol 55:1203–1208
7. Linseisen J, Radtke J, Wolfram G (1997) Flavonoid intake of adults in a Bavarian subgroup of the national food

consumption survey. Z Ernährungswiss 36:403–412
8. Kuo SM (1997) Dietary flavonoid and cancer prevention: evidence and potential mechanism. Crit Rev Oncog 8:47–69
9. Witte JS, Longnecker MP, Bird CL, Lee ER, Frankl HD, Haile RW (1996) Relation of vegetable, fruit, and grain consumption to colorectal adenomatous polyps. Am J Epidemiol 144:1015–1027
10. Messina M, Barnes S (1992) The role of soy products in reducing risk of cancer. J Natl Cancer Inst 83:541–546
11. Knekt P, Jarvinen R, Seppanen R, Hellovaara M, Teppo L, Pukkala E, Aromaa A (1997) Dietary flavonoids and the risk of lung cancer and other malignant neoplasms. Am J Epidemiol 146:223–230
12. Hertog MGL, Feskens EJM, Hollmann PCH, Katan MB, Kromhout B (1994) Dietary flavonoids and cancer risk in the Zutphen Elderly Study. Nutr Cancer 22:175–184
13. Goldbohm RA, van den Brand PA, Hertog MGL, Brants HAM, van Poppel G (1995) Flavonoid intake and risk of cancer: a prospective cohort study. Am J Epidemiol Suppl 141:61S.
14. Messina MJ, Parsky V, Setchell KDR, Barnes S (1994) Soy intake and cancer risk: a review of the in vitro and in vivo data. Nutr Cancer 21:333–340
15. Deschner EE, Ruperto J, Wong G, Newmark HL (1991) Quercetin and rutin as inhibitors of azoxymethanol-induced colonic neoplasia. Carcinogenesis 12:1193–1196
16. Tanaka T, Makita H, Kawabata K, Mori H, Kakumoto M, Satoh K, Hara A, Sumida T, Tanaka T, Ogawa H (1997) Chemoprevention of azoxymethane-induced rat colon carcinogenesis by the naturally occurring flavonoids, diosmin and hesperidin. Carcinogenesis 18:957–965
17. So FV, Guthrie N, Chambers AF, Moussa M, Carroll KK (1996) Inhibition of human breast cancer cell proliferation and delay of mammary tumorigenesis by flavonoids and citrus juices. Nutr Cancer 26:167–181
18. Elangovan V, Sekar N, Govindasamy S (1994) Chemopreventive potential of dietary bioflavonoids against 20-methylcholanthrene-induced tumorigenesis. Cancer Lett 87:107–113
19. Wang TTY, Sathyamoorthy N, Phang JM (1996) Molecular effects of genistein on estrogen receptor mediated pathways. Carcinogenesis 17:271–275
20. Böhm H, Boeing H, Hempel J, Raab B, Kroke A (1998) Flavonols, flavones and anthocyanins as native antioxidants of food and their possible role in the prevention of chronic diseases. Z Ernahrungswiss 37:147–163
21. Williamson G, Faulkner K, Plumb GW (1998) Glucosinolates and phenolics as antioxidants from plant foods. Eur J Cancer Prev 7:17–21
22. Calomme M, Pieters L, Vlietinck A, Vanden Berghe D (1996) Inhibition of bacterial mutagenesis by citrus flavonoids. Planta Med 62:222–226
23. Plaumann B, Fritsche M, Rimpler H, Brandner G, Hess RD (1996) Flavonoids activate wild-type p53. Oncogene 13:1605–1614
24. Gerritsen ME (1998) Flavonoids: inhibitors of cytokine induced gene expression. Adv Exp Med Biol 439:183–190
25. Potten CS (1997) Epithelial cell growth and differentiation. Am J Physiol 36:G253–G257
26. Gryfe R, Swallow C, Bapat B, Redston M, Gallinger S, Couture J (1997) Molecular biology of colorectal cancer. Curr Probl Cancer 21:233–300
27. Nicholson DW, Ali A, Thornberry NA, Vaillancourt JP, Ding CK, Gallant M, Gareau Y, Griffin PR, Labelle M, Lazebnik YA, Munday NA, Raju SM, Smulson ME, Yamin T-T, Yu VL, Miller DK (1995) Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature 376:37–43
28. Droin N, Dubrez L, Eymin B, Renvoize C, Breard J, Dimanche-Boitrel MT, Solary E (1998) Upregulation of CASP genes in human tumor cells undergoing etoposide-induced apoptosis. Oncogene 16:2885–2894
29. Grossmann J, Mohr S, Lapentina EG, Fiocchi C, Levine AD (1998) Sequential and rapid activation of select caspases during apoptosis of normal intestinal epithelial cells. Am J Physiol 274:G1117–1124.
30. Liu W, Zhang R (1998) Upregulation of p21WAF1/CIP1 in human breast cancer cell lines MCF-7 and MDA-MB-468 undergoing apoptosis induced by natural product anticancer drugs 10-hydroxycamptothecin and camptothecin through p53-dependent and independent pathways. Int J Oncol 12:793–804
31. Rice-Evans CA, Miller NJ, Bolwell PG, Bramley PM, Pridham JP, (1995) The relative antioxidant activities of of plant-derived polyphenolic flavonoids. Free Rad Res 22:375–383.
32. Bors W, Heller W, Michel C, Saran M (1990) Flavonoids as antioxidants: Determination of radical-scavenging efficiencies. Methods Enzymol 186:343– 355
33. Miura YH, Tomita I, Watanabe T, Hirayama T, Fukui S (1998) Active oxygens generation by flavonoids. Biol Pharm Bull 21:93–96
34. Agullo G, Gamet-Payrastre L, Fernandez Y, Anciaux N, Demigne C, Remesy C (1996) Comparative effects of flavonoids on the growth, viability and metabolism of a colonic adenocarcinoma cell line (HT29 cells). Cancer Lett 105:61–70
35. Shao RG, Shimizu T, Pommier Y (1998) 7-Hydroxystaurosporine (UCN-01) induces apoptosis in human colon carcinoma and leukemia cells independently of p53. Exp Cell Res 234:388–397
36. Matsuzaki Y, Kurokawa N, Terai S, Matsumura Y, Kobayashi N, Okita K (1996) Cell death induced by baicalein in human hepatocellular carcinoma cell lines. Jpn J Cancer Res 87:170–177
37. Kuo SM (1996) Antiproliferative potency of structurally distinct dietary flavonoids on human colon cancer cells. Cancer Lett 110:41–48
38. Hagar H, Ueda N, Shah SV (1997) Tyrosine phosphorylation in DNA damage and cell death in hypoxic injury to LLC–PK1 cells. Kidney Int 51:1747–1753.
39. So FV, Guthrie N, Chambers AF, Carroll KK (1997) Inhibition of proliferation of estrogen receptor-positive MCF-7 human breast cancer cells by flavonoids in the presence and absence of excess estrogen. Cancer Lett 112:127–133
40. Monti E, Sinha BK (1994) Antiproliferative effect of genistein and adriamycin against estrogen-dependent and -in dependent human breast carcinoma cell lines. Anticancer Res 14:1221–1226
41. Pagliacci MC, Smacchia M, Migliorati G, Grignani F, Riccardi C, Nicoletti I (1994) Growth inhibitory effects of the natural phyto-oestrogen genistein in MCF-7 human breast cancer cells. Eur J Cancer 30:1675–1682
42. Wang C, Kurzer MS (1998) Effects of phytoestrogens on DNA synthesis in MCF-7 cells in the presence of estradiol or growth factors. Nutr Cancer 31:90–100
43. Macen J, Takahashi A, Moon KB, Nathaniel R, Turner PC, Moyer RW (1998) Activation of caspases in pig kidney cells infected with wild-type and CrmA/SPI-2 mutants of cowpox and rabbitpox viruses. J Virol 72: 3524–3533
44. Kuo SM (1998) Transepithelial transport and accumulation of flavone in human intestinal Caco-2 cells. Life Sci 63:2323–2331