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Journal of Nutritional Biochemistry 17 (2006) 165–176
Journal of Nutritional Biochemistry

Chemical structure of flavonols in relation to modulation of angiogenesis and immune-endothelial cell adhesion☆

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Received 31 January 2005; received in revised form 15 June 2005; accepted 17 June 2005

Abstract

The antioxidant activity of flavonoids has been suggested to contribute to several health benefits associated with the consumption of fruits and vegetables. Four flavonols — myricetin (M), quercetin (Q), kaempferol (K) and galangin (G), all with different numbers of hydroxyl moieties (–OH) — were examined for their antioxidant activity and cytotoxicity on human umbilical vein endothelial cells (HUVECs) and for their potential antiangiogenic and cell adhesion effects. The relative antioxidant capacity of these flavonols in cell culture medium (cell-free system) and their intracellular antioxidant activity were M=Q>K=G, which correlated respectively with the presence of 3, 2, 1 and 0 moieties of –OH on their B-ring. The higher the numbers of –OH moieties on the B-ring the less toxic the flavonol was to HUVEC, and the LD50 was determined as: M (100 μM)>Q (50 μM)>K (20 μM)>G (10 μM). These flavonols at ≈0.5 LD50 doses suppressed the vascular endothelial growth factor (VEGF)-stimulated HUVEC tubular structure formation by: M (47%)>Q (37%)>K (15%)>G (14%), which was not linearly associated with their numbers of –OH moieties. However, the magnitude of flavonols' suppression of activated U937 monocytic cells adhesion to HUVEC was associated with the number of –OH moieties on the B-ring. This was prominent when U937 cells were pretreated with these flavonols. In contrast, the numbers of –OH moiety had no apparent influence on the adhesion or expression of adhesion molecules when activated HUVECs were pretreated with these flavonols. The presence of different numbers of –OH moieties on the B-ring of the flavonols may contribute to their antioxidant activity as well as their toxicity and may play an important role in their potency for biological action such as angiogenesis and immune-endothelial cell adhesion, which, respectively, are important processes in the development of cancer and atherosclerosis.

Keywords: Flavonols; Hydroxyl moiety; Antioxidant; Cytotoxicity; Angiogenesis; Cell adhesion

1. Introduction

Flavonols are polyphenol compounds possessing two benzene rings joined by a linear three carbon chain (C2, C3, C4), represented as the C6–C3–C6 system. Flavonoids have been suggested to have several potential health benefits due to their antioxidant activities, which are attributed to the presence of phenolic hydroxyl (–OH) moieties on the structure [1,2]. In molar bases, the antioxidant capacity of some of these flavonoids is much higher than those of vitamins C and E [3]. In addition to –OH moieties in the structural arrangements of flavonols, the resonance of electrons between A- and B-rings is very important for their antioxidant and biological activities. The B-ring –OH moiety is the most significant determinant factor in the scavenging of reactive oxygen species (ROS) [4,5].

Flavonoids are the important phytonutrient components present in a wide range of fruits, vegetables, nuts and beverages, including wine and tea [6]. Myricetin with three –OH moieties on the B-ring (Fig. 1A) is one of the flavonols present in a large number of plants [7], including tea, berries, fruits, vegetables and medicinal herbs [8]. It is an effective scavenger of free radicals generated by both enzymatic and nonenzymatic systems [9]. It inhibits cancer development induced by polycyclic aromatic hydrocarbons [10] in SENCAR mice. In addition, it has been shown to have antiviral activity by inhibiting the reverse transcriptase [11], antiaggregatory effects on blood platelets [12] and

☆ This manuscript is based on work supported by the US Department of Agriculture, under agreement No. 58-1950-9-001, and by the South Korea Research Foundation Grant (KRF-2003-013-F00009).
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doi:10.1016/j.jnutbio.2005.06.006

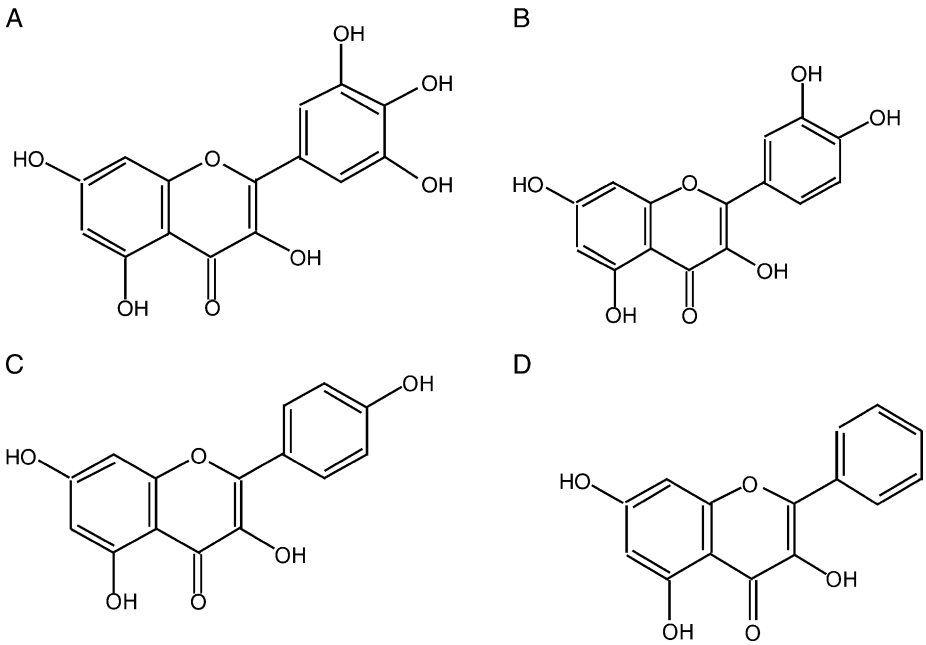


Fig. 1. Chemical structure of myricetin (A), quercetin (B), kaempferol (C) and galangin (D) bearing 3, 2, 1 and 0 hydroxyl moieties in B-ring, respectively.

antiatherosclerotic effect via inhibition of oxidative modification of low-density lipoprotein by macrophages [13]. Quercetin (Fig. 1B) is another important flavonol with two –OH moieties on the B-ring and is found in many fruits and vegetables, as well as in olive oil, red wine and tea [14–17]. Quercetin, in addition to having antioxidant properties, has been suggested to prevent atherosclerosis and chronic inflammation [14], to modulate cell-cycle regulation, to interact with type II estrogen binding sites and to induce tumor cell apoptosis [15,16]. It may also possess antiangiogenic potential [17]. Kaempferol (Fig. 1C), with one –OH moiety on the B-ring, is widely present in broccoli [18], *Ginkgo biloba* [19], fruits and vegetables [20–22]. Several biological activities have been attributed to kaempferol, including inhibition of lipoxygenase and cyclooxygenase [23,24]. It also possesses antiaggregatory [25], antibacterial [26] and anticancer [27] activities. Galangin (Fig. 1D), with no –OH moiety on the B-ring, is another member of the flavonols and is present in high concentrations of honey and *Alpinia officinarum*, a spice plant that has also been used as an herbal medicine for a variety of ailments in Asia. Galangin is also present in propolis, which is a resinous material made by bees and is used in many Asian countries for the management of numerous diseases, including respiratory, subcutaneo-mucosal and viral infections [28,29]. Galangin has been demonstrated to possess several biological actions such as antioxidative and radical scavenging activities [30,31], antimutagenic [32,33], anticlastogenic effect [34,35], anti-inflammatory activity, [36,37] as well as an inhibitory effect on cytochrome P450 hydroxylase in human liver microsomes [38,39]. Galangin has been recently proposed as a candidate agent for cancer chemoprevention [40].

We used these flavonols containing different –OH moieties on the B-ring to examine their potential antioxidant activities in in vitro cell culture systems in relation to modulation of angiogenesis, which is necessary for solid tumor growth and immune-endothelial cell adhesion, a process important in the development of atherosclerosis.

2. Materials and methods

2.1. Flavonols

Myricetin, quercetin and kaempferol were purchased from Sigma (St. Louis, MO), and galangin was from Fluka (Buchs, Switzerland). To treat the cells with these flavonols, we first dissolved the chemicals in dimethyl sulfoxide (DMSO) and then added them into the culture medium. The concentration of DMSO in culture medium was kept below 0.1% in the flavonol-treated and control cell culture medium.

2.2. Cell culture

Human umbilical vein endothelial cells (HUVECs) were obtained from Clonetics (San Diego, CA) and cells from passages 3 to 5 were used in this study. HUVECs were cultured in EBM-2 growth medium (Clonetics) containing hydrocortisone, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF), ascorbic acid, heparin and 2% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO_2. The cells were seeded in culture flasks or on plates coated with 2% gelatin (Sigma) and allowed to grow to confluence before experimental treatment. The U937 human monocytic cell

line (American Type Culture Collection, Rockville, MD) was used for the cell adhesion assay. U937 cells were maintained in RPMI-1640 medium (Life Technologies, Grand Island, NY) supplemented with 10% FBS, 2 mM L-glutamine (Life Technologies), 1×10^5 U penicillin/L and 100 mg streptomycin/L.

2.3. Toxicity of flavonols to HUVECs and U937 cells

When HUVECs in culture reached 90% confluency, flavonols were dissolved in DMSO and added to the cell culture medium at different concentrations for 24 h. Using the Trypan blue exclusion test, the numbers of dead and alive cells were counted with a hematocytometer under the microscope.

2.4. Antioxidative capacity of flavonols

The antioxidant capacity of flavonols was measured spectrophotometrically at 593 nm by the ferric-reducing ability assay of plasma (FRAP) method [41] using 2,4,6-tri[2-pyridyl]-s-triazine (Fluka), 300 mM acetate buffer (pH 3.6), 40 mM HCl and 20 mM ferric chloride. To determine the comparative antioxidant capacity, we dissolved all flavonols in 0.1% DMSO and added them into the culture medium at a concentration of 10 μM or from all of the flavonols and at maximum nontoxic concentration of each of the flavonols: myricetin (25 μM), quercetin (15 μM), kaempfrol (10 μM) and galangin (5 μM). One unit of antioxidant capacity is designated as the equivalent antioxidant capacity of 1 mM Fe (II).

2.5. Intracellular antioxidant activity of flavonols

To determine the comparative antioxidant activity of flavonols in a cell culture system, the suppressive effects of flavonols on intracellular production of ROS in U937 cells were measured. Cells were treated with 10 μM of each flavonol at 37°C for 20 h. All treatments contained the same amount of DMSO (0.01%). After incubation, the cells were washed three times with phosphate buffered saline (PBS) and then incubated in Hank's Balanced Salt Solution (HBSS) containing 50 μmol dichlorodihydrofluorescein diacetate/L (Molecular Probes) for an additional 30 min at 37°C. Dichlorodihydrofluorescein diacetate is a nonfluorescent compound and is rapidly taken up by cells. Once inside the cell, the diacetate residues are removed by esterases, liberating dichlorodihydrofluorescein, which accumulates intracellularly as a consequence of its low membrane permeability. Dichlorodihydrofluorescein reacts predominantly with ROS, forming the fluorescent compound 2′,7′-dichlorofluorescein (DCF), which was measured by a Cytofluor (PerSeptive Biosystems) fluorescence multiwell plate reader. Then the cells were washed and maintained in HBSS. After the addition of 100 μmol/L of H_2O_2 for 45 min, fluorescence intensity was monitored at excitation and emission wavelengths of 485 and 530 nm, respectively. Data are presented as the percentage increase in DCF fluorescence compared with that in unstimulated cells [42,43].

2.6. In vitro angiogenesis

The formation of tubular structures by HUVEC in Matrigel (Becton/Dickinson, Becton, NJ) was used to assess the effect of flavonols on angiogenesis. Twenty-four-well culture plates were coated with 150 μl of Matrigel, which was allowed to solidify at 37°C for 1 h. HUVECs were seeded on Matrigel-coated wells (25 000 cells/well), then the cells were treated with flavonols at different concentrations for 24 h at 37°C in a 5% CO_2 humidified atmosphere. Five digital images in randomly selected areas of each well were obtained using a digital camera (Nikon Cool-Pix, Tokyo, Japan). The network of tube formation was quantified by determining the pixel levels of images and by using the NIH Image program.

2.7. Cell adhesion

2.7.1. Cell culture

Cultured U937 cells were concentrated by centrifugation at 1000 rpm for 5 min and suspended to a concentration of 10^6 cells/ml. U937 cells were seeded onto a 24-well plate. The U937 cells were then cultured in the presence or absence of various flavonols for the necessary experiments.

2.7.2. Fluorescent labeling of cells

U937 cells were fluorescently labeled with 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxy-fluorescein acetoxymethyl ester (BCECF-AM; Molecular Probes, Eugene, OR) for the quantitative cell adhesion assay [44]. Nonfluorescent BCECF-AM is cleaved intercellularly and becomes a highly charged fluorescent BCECF, which is retained by viable cells. Nonfluorescent BCECF-AM is lipophilic, and its methylester is cleaved intercellularly and becomes a highly charged fluorescent BCECF, which is retained by viable cells. The BCECF-AM was prepared as a 1 g/L stock in DMSO and was stored at −80°C. After labeling the U937 cells (1×10^7 cells/5 ml) with 5 μM BCECF-AM/L in RPMI-1640 medium, they were washed three times with PBS plus 1% FBS to remove the excess dye. Finally, the cells were resuspended in EBM-2 medium at a density of 5×10^5 cells/ml.

2.7.3. U937 cell adhesion assay

HUVECs were cultured to confluency in 24-well plates (Becton Dickinson Labware, Franklin Lakes, NJ) and were treated with different concentrations of flavonols at 37°C for 20 h. Cells were then stimulated with 10 ng/ml of recombinant human IL-1β for 6 h. Then, fluorescently labeled U937 cells (2.5×10^5 cells/well) were seeded over the HUVEC monolayer and incubated for 30 min. The attached cells were lysed with 0.5 ml of 50 μM/L Tris buffer containing 0.1% sodium dodecyl sulfate. The fluorescent intensity of each well was measured with Cytofluor

(PerSeptive Biosystem, Framingham, MA), at 485 nm excitation and 530 nm emission. In addition, cell adhesion was verified by obtaining five random photomicrographs from each well under an inverted phase contrast microscope and by counting adherent cells using the NIH Image analyzer program.

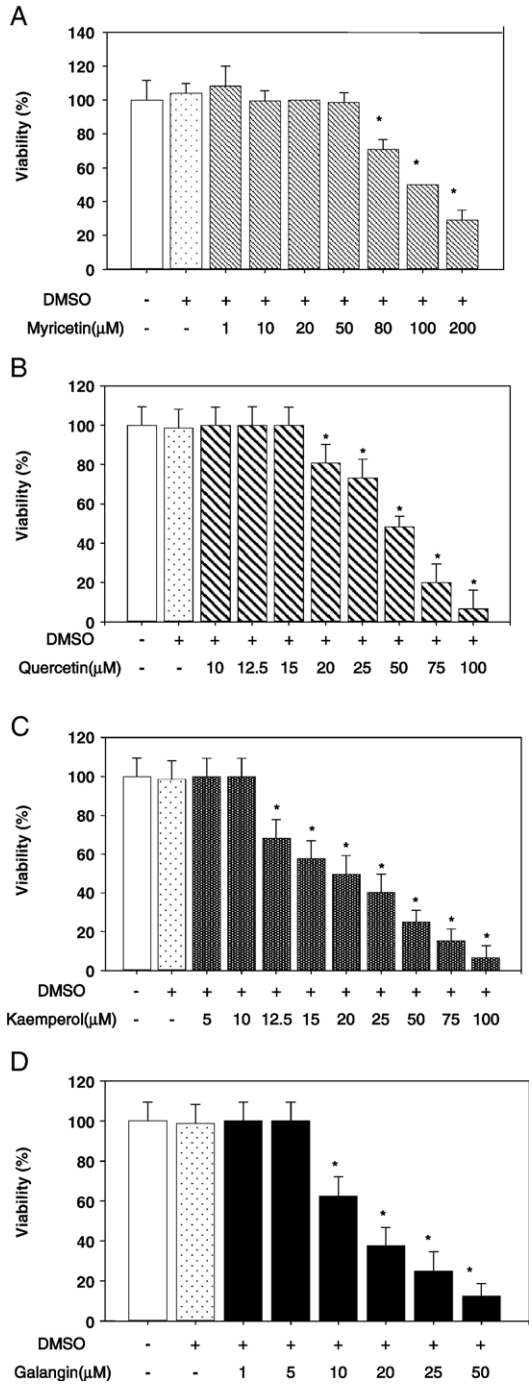


Fig. 2. Toxicity of each flavonol on HUVECs with Trypan blue exclusion test. Confluent HUVECs were incubated with different flavonoids dissolved in DMSO and added to the cell culture medium at different concentrations at 37°C. After 24 h, cells were harvested and stained with Trypan blue and counted under the microscope. Data are the mean±S.D. of three experiments, each performed in triplicate. *$P<.01$ compared with control group.

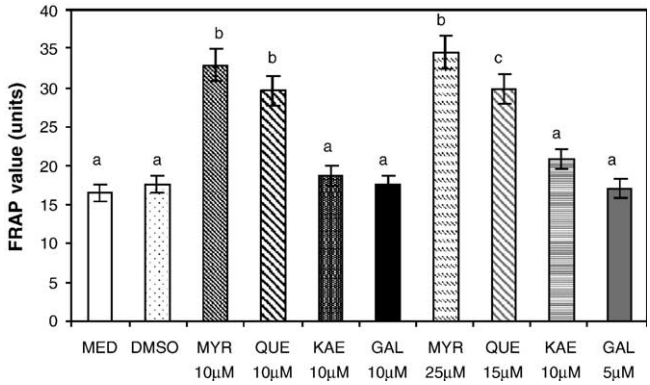


Fig. 3. Antioxidative capacities of each flavonol at 10 μM and at maximum safe doses measured by the FRAP method. Flavonols at the concentrations as indicated were dissolved in DMSO and added into the medium. DMSO alone in culture medium was used as vehicle control. Myr: myricetin; Que: quercetin; Kae: kaempferol; and Gal: galangin. Data are the mean±S.D. of three experiments, each performed in triplicate. Bars not sharing the same letter are significantly different from each other ($P<.05$).

For testing the effect of flavonols on monocyte adherence to HUVEC, U937 cells were first incubated with flavonols for 20 h, then activated with phorbol myristyl acetate [PMA, 100 (g/L)] for 2 h and seeded over HUVEC monolayer and incubated for 30 min. The adhesion of the fluorescently labeled U937 cells was measured as described above.

To test the effect of flavonols on the adhesion of monocytes to HUVECs when both cells were treated with flavonols, we treated both cell types with the same concentrations of flavonols at different doses as described above, then stimulated HUVEC with IL-1β; the number of adherent monocytes was determined as described above.

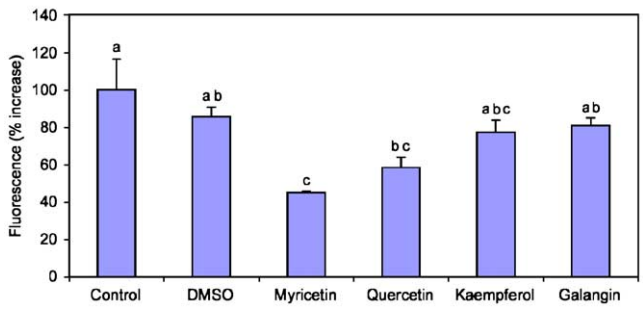


Fig. 4. The intracellular antioxidant activity of 10 μM of flavonols measured by quenching intracellular ROS as determined by the decrease in fluorescence intensity resulted from intracellular oxidation of DCHF to DCF as described in Materials and methods. The intracellular ROS was generated by the addition of 100 μM H_2O_2 into culture medium. DMSO alone was used as vehicle control. Data are the percent increase in DCF fluorescence compared with unstimulated cells. Data are the mean±S.D. of two experiments, each performed in quadruplicate. Bars not sharing the same letter are significantly different from each other ($P<.05$).

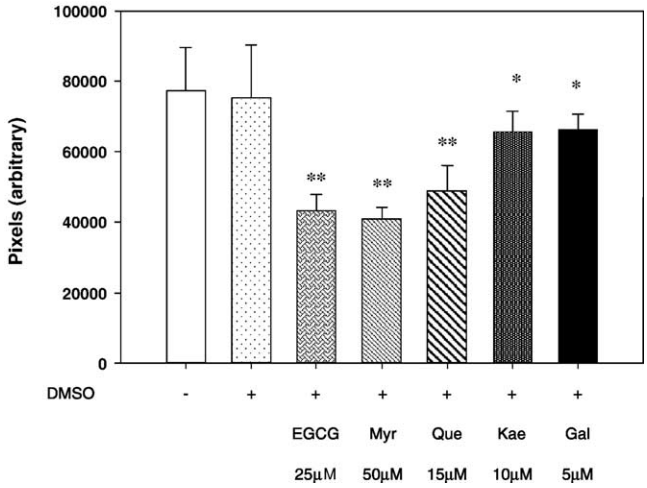


Fig. 5. Suppression of VEGF-induced in vitro HUVEC angiogenesis by flavonols at their maximum safe doses. HUVECs were seeded on Matrigel-coated wells (25 000 cells/well), then the flavonols at different concentrations dissolved in DMSO were added into the cell culture medium, and cells were incubated for 24 h at 37°C. Dimethyl sulfoxide was used as vehicle control. The network of tubular structure formation was quantified by measuring pixel levels in five photomicrographs randomly obtained from each well. Epigallocatechin gallate was used as a positive control. Myr: myricetin; Que: quercetin; Kae: kaempferol; and Gal: galangin. Data are the mean±S.D. of three experiments, each performed in triplicate. $^{**}P<.01$, $^{*}P<.05$ compared with control group.

2.7.4. Determination of cell adhesion molecules by ELISA

Measurements of cell surface adhesion molecules were carried out by enzyme-linked immunosorbent assay (ELISA). Confluent HUVECs in 96-well plates were cultured in the absence or presence of myricetin, quercetin, kaempferol and galangin for 20 h at 37°C. After washing, 5 ng/ml recombinant human IL-1β (Endogen, Woburn, MA) was added to stimulate the cells at 37°C for 6 h. Then the medium was removed, and the cells were fixed with 1% paraformaldehyde at room temperature for 30 min. The plates were then washed two times with PBS+0.5% Tween and blocked with 10% FBS in PBS for 1 h and washed again two times with PBS+0.5% Tween. Monoclonal antibodies to human CD106 (ICAM-1), CD54 (VCAM-1) and 62E (E-selectin) (BD Biosciences, Pharmigen) in 10% FBS in PBS were added at 2, 5 and 5 μg/ml, respectively, and incubated at 37°C for 2 h. A secondary antibody (horseradish-peroxidase-conjugated antimouse IgG) (Bio-Rad, Hercules, CA) was added at 1:1000 dilution and incubated at room temperature for 1 h. The visualization agent, horseradish peroxidase substrate (Bio-Rad), was then added for 1 h. The plates were read at 405 nm OD in a plate reader

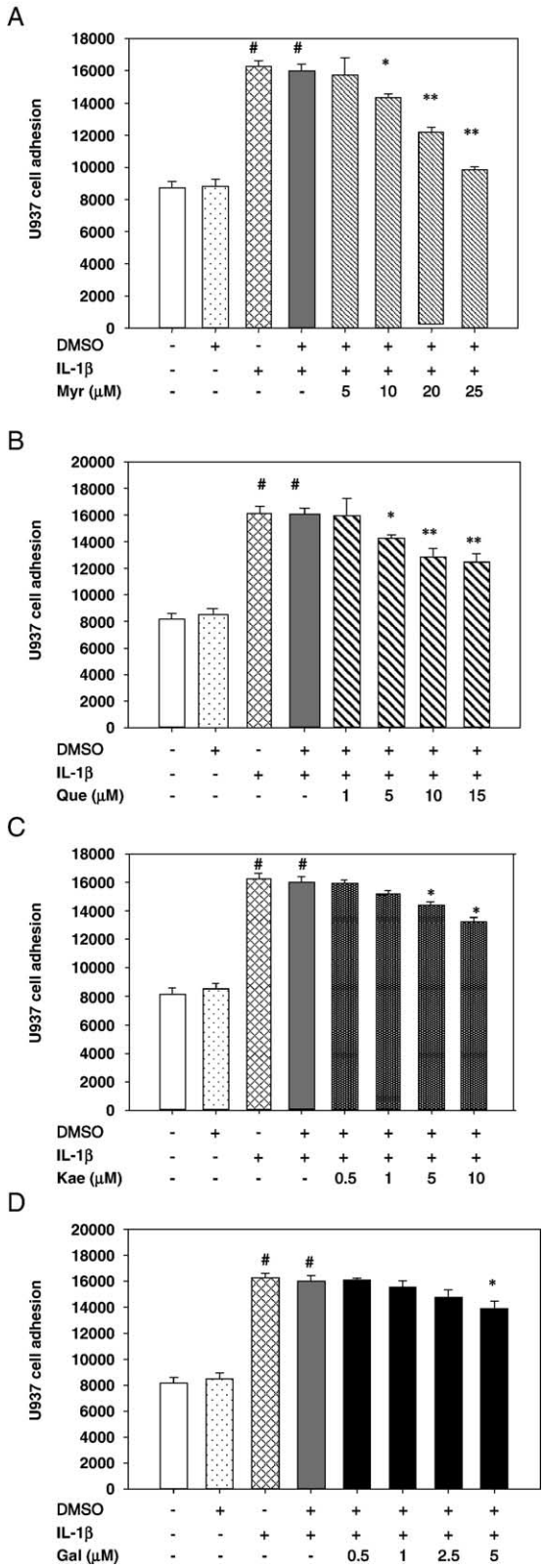
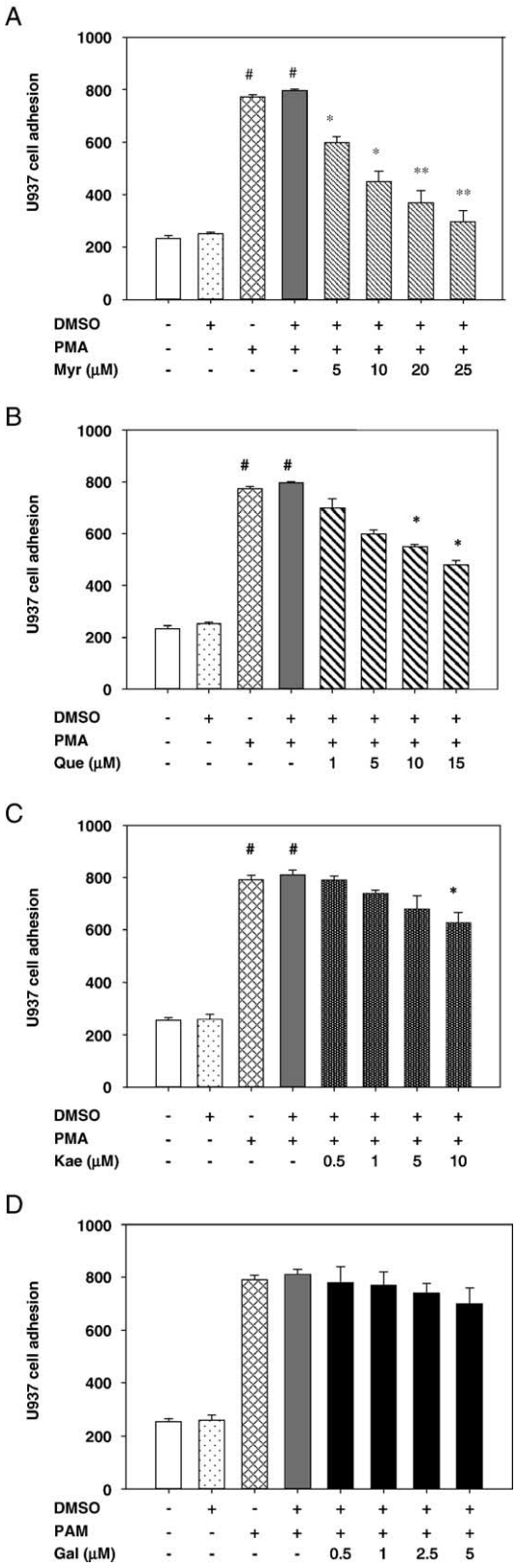


Fig. 6. The effect of maximum safe doses of flavonols on the inhibition of U937 cells adhesion to HUVEC monolayer with or without IL-1β stimulation: (A) myricetin, (B) quercetin, (C) kaempferol, (D) galangin. The pretreated HUVECs were stimulated with IL-1β (10 ng/ml) at 37°C for 6 h. Fluorescently labeled U937 cells were added onto HUVECs and incubated at 37°C for 30 min. The numbers of adherent cells were measured with a fluorescent plate reader. Data are the mean±S.D. of three experiments, each performed in triplicate. $^{**}P<.01$, $^{*}P<.05$ compared with activated control group; $\#P<.01$ compared with unstimulated control group.

(Bio-Tek Instruments, Winooski, VT). Background (OD reading of wells without adding antibodies) was subtracted from all the other wells.



2.8. Cell proliferation

HUVECs were seeded into 24-well plates and treated with nontoxic concentrations of each flavonol. Cell growth was then determined every 24 h for 5 days by counting viable cell numbers with a hematocytometer.

2.9. Statistical analysis

Data are expressed as mean±S.D. Statistical analysis was carried out using Student's *t* test following an ANOVA. $P<.05$ was considered significant.

3. Results

3.1. Toxicity of flavonols to HUVEC and U937 cells

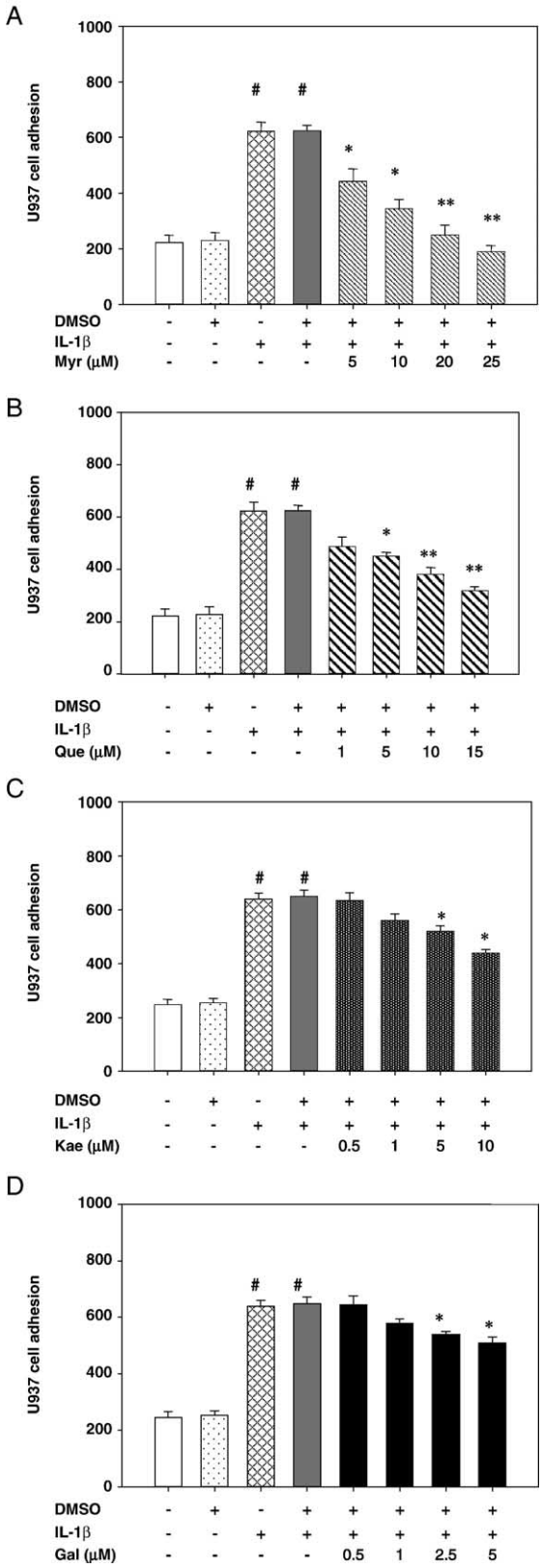
The Trypan blue exclusion test showed that the higher the numbers of –OH moieties on the B-ring the less toxic the flavonol was to HUVECs and U937 cells. The approximate LD50 to HUVEC and ranking order of the four flavonols — myricetin, quercetin, kaempferol and galangin, respectively, having three, two, one, and no –OH moieties — were 100>50>20>10 μM. The nontoxic level of these flavonols to HUVEC was 50, 15, 10 and 5 μM, respectively (Fig. 2) [45]. The concentrations of these flavonols at which 95% of U937 cells remained viable were 50 μM for myricetin, 20 μM for quercetin, 10 μM for kaempferol and 5 μM for galangin (data not shown). The toxicity of these flavonols correlates with their lipophilicity [46].

3.2. Antioxidant capacity of flavonols

The antioxidant capacity of flavonols in cell culture media was measured via the FRAP method. The results shown in Fig. 3 support the concept that the more –OH moieties present in the B-ring the higher the antioxidant capacity of the flavonol [47]. However, the relationship between –OH moiety on the B-ring with antioxidant activity is not linear. One –OH moiety difference between myricetin and quercetin for antioxidant activity, as shown in Fig. 3, is not the same as one –OH moiety difference between quercetin and kaempferol. Therefore, the relative ranking order of these flavonols for their antioxidant activity can be as follows: myricetin=quercetin>kaempferol=galangin. It is noteworthy that it is

Fig. 7. The effect of maximum safe doses of flavonols on the inhibition of U937 cell adhesion to HUVEC monolayer stimulated with PMA: (A) myricetin, (B) quercetin, (C) kaempferol, (D) galangin. U937 cells were pretreated with flavonols at their different concentration for 20 h and stimulated with PMA (100 μg/L) at 37°C for 2 h. Fluorescently labeled U937 cells were added onto HUVECs and incubated at 37°C for 30 min. The numbers of adherent cells were measured with a fluorescent plate reader. Data are the mean±S.D. of three experiments, each performed in triplicate. $^{**}P<.01$, $^{*}P<.05$ compared with activated control group; $^{\#}P<.01$ compared with unstimulated control group.

possible to achieve a higher antioxidant activity with the maximum safe dose of myricetin compared to other tested flavonols in this study.



3.3. Intracellular antioxidant activity of flavonols

The relative intracellular antioxidant activity of 10 μM of each flavonoid as measured by quenching intracellular ROS is shown in Fig. 4. The intracellular antioxidant activity of these flavonols was somewhat parallel to that of antioxidant capacity as observed above in cell-free system (cell culture medium). However, myricetin and quercetin relative to control were significantly more effective to suppress the intracellular ROS levels in U937 cells.

3.4. In vitro angiogenesis

The quantitative analysis of tube formation by endothelial cells on Matrigel was performed by determining the pixel levels of five photomicrographs obtained from the random field of cell cultures in each well. We have found that the measured pixel levels strongly and significantly correlate with the total length of the tubular structure measured on the photomicrographs (r^2=.95) (data not shown).

We tested the maximum safe, nontoxic doses (≈ .5 LD50) of flavonols for their antiangiogenic effect. Fig. 5 shows the differential suppression of flavonols on tubular structure formations in VEGF-stimulated HUVECs on Matrigel. Since we have shown earlier that epigallocatechin gallate (EGCG) inhibits angiogenesis [48], we used 25 μM EGCG as a positive control, which inhibited angiogenesis by 44.0% (P=.006). Our results show that at a maximum safe dose of 50 μM myricetin (with three –OH moieties on the B-ring) and 15 μM quercetin (with two –OH moieties on the B-ring), both of these flavonols inhibited angiogenesis by 47% and 37%, respectively, and both were as effective as 25 μM of EGCG at inhibiting in vitro angiogenesis, whereas maximum safe doses of 10 μM kaempferol (with one –OH moiety on the B-ring) and 5 μM galangin (with no –OH moiety on the B-ring) suppressed the HUVEC angiogenesis by 15% and 14%, respectively.

3.5. Cell adhesion

3.5.1. U937 cell adhesion to IL-1β-stimulated HUVEC

The monocyte-like U937 cells did not adhere significantly to unstimulated HUVECs (Fig. 6). However, when HUVEC was stimulated with IL-1β, adhesion increased significantly (Fig. 6A). This stimulated adhesion was clearly inhibited by myricetin in a dose-dependent manner. At 10, 20 and 25 μM, myricetin significantly suppressed U937 cell

Fig. 8. The effect of maximum safe dose of flavonols on the inhibition of U937 cell adhesion to HUVEC monolayer stimulated with IL-1β: (A) myricetin, (B) quercetin, (C) kaempferol, (D) galangin. The HUVEC and U937 cells were incubated with safe doses of flavonols for 24 h. HUVECs were stimulated with IL-1β (10 ng/ml) at 37°C for 6 h. Fluorescently labeled and pretreated U937 cells were added onto HUVECs and incubated at 37°C for 30 min. The numbers of adherent cells were measured with a fluorescent plate reader. Data are the mean±S.D. of three experiments, each performed in triplicate. **P<.01, *P<.05 compared with activated control group; $^{\#}P$<.01 compared with unstimulated control group.

adhesion by 27%, 56% and 87%, respectively. Quercetin, at nontoxic doses of 5, 10 and 15 μM, significantly suppressed U937 cell adhesion by 39%, 47% and 51%, respectively (Fig. 6B). Myricetin and quercetin appear to be more powerful in suppressing the adhesion interaction compared to kaempferol and galangin. Kaempferol, at doses of 5 and 10 μM, significantly reduced adhesion by 24% and 42%, respectively (Fig. 6C), while galangin was only effective at 5 μM, which significantly reduced U937 adhesion to HUVECs by 31% (Fig. 6D). While the maximum safe doses of flavonols can be used to achieve a differential inhibition of adhesion, nevertheless, when all four flavonols were compared at the same dose level of 5 μM, there was little difference in their potency to inhibit monocyte adhesion. In fact, myricetin with three –OH moieties on the B-ring at 5 μM concentrations was less effective at suppressing adhesion than other flavonols.

3.5.2. Phorbol myristyl acetate-stimulated U937 cell adhesion to HUVECs

U937 cells were treated with each of the flavonols at different doses and stimulated with PMA (100 μg/L) for 2 h and tested for their adherence to untreated and unstimulated HUVECs (Fig. 7). The dose-dependent inhibitory effect of flavonols on the reduction of U937 cell adhesion to HUVECs was more pronounced when U937 cells were treated with flavonols compared to when HUVECs were treated with these flavonols (Fig. 6 vs. Fig. 7). A dose-dependent decrease in adhesion (by 36%, 64%, 78% and 92%) was observed in U937 cells treated with 5, 10, 20 and 25 μM myricetin, respectively (Fig. 7A). Quercetin significantly inhibited U937 cell adhesion by 45% and 58% at their safe doses of 10 and 15 μM (Fig. 7B). While kaempferol, at the safe dose of 10 μM, was only effective in reducing adhesion by 33% (Fig. 7C), galangin at 5 μM of a nontoxic dose had no significant effect on inhibiting adhesion (Fig. 7D). It is worth noting that treatment of U937 cells with 10 μM of myricetin was more effective at inhibiting their adhesion to HUVEC (by 64%) compared to when U937 cells were treated with 10 μM of quercetin or kaempferol (45% and 33%, respectively).

3.5.3. U937 cell adhesion to HUVECs when both cells were treated with flavonols

The adhesion of U937 cells to IL-1β-stimulated HUVECs was prominently decreased when both cell types were pretreated with flavonols (Fig. 8). Myricetin dose dependently ($P<.05$) inhibited the adhesion of U937 cells to HUVECs by 46%, 71%, 95% and 100% when both cell types were pretreated with 5, 10, 20 and 25 μM, respectively (Fig. 8A). Significant decrease in adhesion was also observed with quercetin (44%, 62% and 77% with 5, 10 and 15 μM, respectively) (Fig. 8B), kaempferol (33% and 53% with 5 and 10 μM, respectively) (Fig. 8C) and galangin pretreatment (28% and 35% with 2.5 and 5 μM, respectively) (Fig. 8D). In a dose–response manner, myricetin was the most effective and galangin was the least effective flavonoid in inhibiting cell–cell adhesion. However, when the effect of flavonols was examined at equal dose of 5 μM, myricetin and quercetin had an equally higher inhibitory effect (46% and 44%, respectively) on cell–cell adhesion compared to kaempferol and galangin showing 33% and 35% inhibition, respectively.

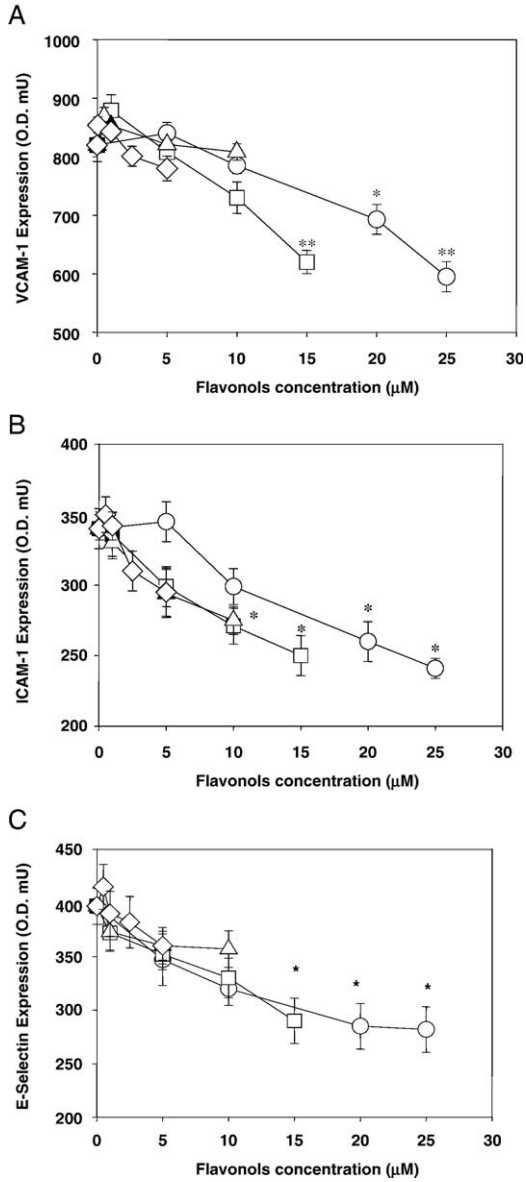


Fig. 9. Inhibition of endothelial cell adhesion molecule expression by different flavonols at their safe doses. Confluent HUVECs in 96-well plates were cultured in the absence or presence of myricetin, quercetin, kaempferol and galangin for 20 h at 37°C. Five nanograms per milliliter of recombinant human IL-1β was added to stimulate the cells at 37°C for 6 h. The expression of VCAM-1, ICAM-1 and E-selectin on the cell surface was measured using ELISA. ○, Myricetin; □, quercetin; △, kaempferol;◇, galangin: Data are the mean±S.D. of three experiments, each performed in triplicate. $^{**}P<.01$, $^{*}P<.05$ compared with activated control group.

3.5.4. Expression of adhesion molecules by HUVEC

Myricetin and quercetin reduced IL-1β-stimulated HUVEC expression of VCAM-1, ICAM-1 and E-selectin dose dependently. Myricetin, at the safe dose of 25 μM, significantly inhibited expression of all three adhesion molecules by 29% (Fig. 9A–B). Quercetin, at the maximum safe dose of 15 μM, significantly suppressed the expression of these adhesion molecules by 24% to 27%, whereas kaempferol and galangin, up to the safe, nontoxic doses of 15 and 5 mM, respectively, had no significant effect on the inhibition of the expression of these adhesion molecules by HUVEC. Thus, it appears that within "safe doses," myricetin and quercetin bearing three and two –OH moieties on the B-ring compared to kaempferol and galangin containing one and no –OH moiety on the B-ring strongly inhibited expression of adhesion molecules, which mediate cell–cell interaction. However, at the same molar concentration of 5 μM, while there was some inhibition on the expression of adhesion molecules, there were no differential effects of flavonols according to their numbers of –OH moiety.

3.6. Cell proliferation

Myricetin was only significantly ($P<.05$) effective at reducing cell growth by 16% and 29% at doses of 20 and 25 μM, respectively, and quercetin was only effective at reducing it by 10% at 15 μM ($P<.05$). However, kaempferol and galangin at their safe doses were not effective at reducing HUVEC proliferation (data are not shown). Thus, flavonols containing more –OH moieties on the B-ring were more effective at the inhibition of cell proliferation without causing cell toxicity.

4. Discussion

Our results show that the numbers of –OH moieties in this group of flavonols with similar chemical structure are important for their toxicity, in their antioxidant activity and, to some extent, in their effect on modulating endothelial cell angiogenesis, expression of adhesion molecules and cell–cell adhesion.

The antioxidant activity of flavonoids has been considered to be one of the important factors in their biological potency. In this regard, the association of the number of –OH moieties with the antioxidant activity of a flavonoid has been investigated [9,47,49–53]. Cao et al. [47] showed that different forms of kaempferol with one, two, three and four –OH moieties in the 5-position had oxygen reactive absorbance capacity values of 0, 1, 1.6 and 2.7, respectively [2,47,54,55]. The presence of more –OH moieties on the B-ring plays an important role in electron resonance and in the donation of electrons to the oxidizing agent. Oxidation potential is affected by the number and pattern of –OH substitution on the B-ring and by the presence of structural groups required for extended conjugation between the B- and C-rings. It has been suggested that myricetin bearing pyrogallol moiety has a lower oxidation potential than quercetin containing cathecol moiety [9]. Our results show that among this group of flavonols, myricetin and quercetin, with three and two –OH moieties on the B-ring, respectively, have the highest antioxidant capacity and intracellular antioxidant activity in this group of flavonols, whereas kaempferol and galangin, with one and no –OH moiety on the B-ring, respectively, have the lowest, which is in part in agreement with published data [9,47]

The toxicity of a compound occurs via the uptake of the compound into the cell or through interaction with the cell membrane and associated molecules. Our results on the cytotoxicity of flavonols tested in this in vitro cell culture system indicate that myricetin, with three –OH moieties in the B-ring structure, is the least toxic flavonoid, and galangin, with no –OH moiety, possesses the highest toxicity to both HUVEC and U937 cells.

While previous studies have also shown the relationship between structure–activity relationships and antioxidant capacities [2,47,54,55], the association of the numbers of –OH moieties to antioxidant capacities in relation to a biological function has not been well established. In the present study, we have examined the effect of these flavonols on two biological functions in vitro: angiogenesis and immune-endothelial cell adhesion. Since we first determined the dose–response relationship of these flavonols on HUVEC viability, we chose to test the maximum safe and nontoxic dose of these flavonols on angiogenesis. We found that the maximum safe and nontoxic dose ($\approx$0.5 LD50) of both myricetin at 50 μM and quercetin at 15 μM in a cell culture system equally suppressed the VEGF-induced angiogenesis, which was comparable to the effect of 25 μM of EGCG (was used as a positive control). However, the maximum safe and nontoxic dose of kaempferol and galangin was less effective than that of myricetin and quercetin. Although we did not test the lower doses of myricetin to compare to that of quercetin, one can infer that its inhibitory effect at the 15-μM dose would have been less than that of 15 μM quercetin. Similarly, a lower dose of kaempferol at 5 μM would have been less effective on the suppression of VEGF-induced angiogenesis than that of 5 μM galangin. Thus, it appears that the inhibition of in vitro angiogenesis of HUVECs on Matrigel with these flavonols is associated with their antioxidant activity and, noticeably, is not linearly associated with the numbers of –OH moieties on the B-ring. Rather, the structural polarity, the membrane binding, and their affinity and ability in docking on a specific receptor such as the VEGF receptor, in interacting with intracellular proteins and in altering the redox status of the cell may govern their potency for this biological effect. It is also important to note that during angiogenesis, in addition to cell migration and tube formation, the proliferation of endothelial cells is a necessary step. We found that endothelial cell proliferation in our in vitro cell culture system is differentially affected by these flavonols, which might have contributed to their efficacy on the inhibition of angiogenesis. Our data suggest

that while flavonols containing more –OH moieties on the B-ring were more effective in the inhibition of HUVEC proliferation, the association was again not found to be linearly related to the number of their free –OH moieties.

Antioxidants have been shown to inhibit immune-endothelial cells' interaction [56–58], which is mediated through endothelial expression of several cell surface adhesion molecules and ligands from monocytes during inflammation and atherosclerosis [59]. To test the efficacy of these flavonols with different antioxidant activity on immune-endothelial cell adhesion, we conducted the dose–response study. Our data suggest that the dose-dependent suppression of U937 cell adhesion to the HUVEC monolayer by a flavonol in relation to the numbers of –OH moieties on the B-ring was more apparent when U937 cells were pretreated with a flavonol as opposed to when the HUVEC monolayer was pretreated with the same flavonol. This inference was drawn from our data showing that when HUVECs were treated with the "maximum safe dose" of flavonols, the inhibitory effect was in the magnitude order of myricetin>quercetin>kaempferol>galangin, whereas when all four flavonols are compared at the same dose of 5 μM, there was no difference between them. This was also, to some extent, apparent when the expression of adhesion molecules was examined as the HUVECs were treated with the equal molar concentration of flavonols.

In contrast to HUVECs, when U937 cells were treated with the same dose of flavonols, the presence of higher numbers of –OH moiety on the B-ring of flavonols appeared to be important in the inhibition of PMA-stimulated U937 cell adhesion to the HUVEC monolayer. For instance, pretreatment of U937 cells with 5 μM of myricetin significantly suppressed their PMA-stimulated adhesion by 32%, whereas pretreatment of U937 cells with the same concentration of quercetin, kaempferol and galangin was not effective to suppress adhesion. Furthermore, at a higher dose of 10 μM, myricetin suppressed the adhesion of U937 cells more strongly than quercetin or kaempferol. Interestingly, when both cell types were pretreated with flavonols, the effect of –OH moiety observed with U937 cells was blunted.

In conclusion, our data demonstrate that the presence of different numbers of the –OH moiety on the B-ring of flavonols may contribute to their toxicity and the proliferation of endothelial cells, in part, through providing differential antioxidant activity. However, the numbers of –OH moiety on the B-ring of the flavonol are not directly related to their potency for inhibiting in vitro angiogenesis or cell–cell adhesion when HUVECs were pretreated with these groups of flavonols. In contrast, pretreatment of monocytic U937 cells with myricetin, the flavonol with the highest numbers of –OH moiety on the B-ring, was more effective at suppressing their adhesion to HUVECs as compared to flavonols with the lower numbers of –OH moiety on the B-ring. It is important to note that in a dose–response manner up to the "maximum safe dose" in our cell culture system, there was a direct relationship between the biological activity of flavonols and –OH moiety on the B-ring in the following magnitudes of order: myricetin>quercetin>kaempferol>galangin. It is also important to bear in mind that in the present study, we used in vitro cell culture systems to gain insights into the role of –OH moieties in these flavonols as one of the potential determinant factors in defining their comparative biological activity. However, the actual in vivo effect of these flavonols on the prevention of angiogenesis and atherosclerosis in relation to –OH moieties on their structure and their bioavailability remains yet to be demonstrated. It is important to note that the "maximum safe dose" of the flavonols used in this study was based on the viability of cells in culture and should not be directly extrapolated to in vivo. Intestinal degradation, absorption and metabolism of these flavonols occur before reaching the targeted tissues. Nevertheless, the information from this study provides a basis to explore the molecular mechanism of action and the potential application of these compounds through different routes of administration to target specific tissue where the inhibition of angiogenesis and atherosclerosis is desired.

Acknowledgment

Any opinions, findings, conclusions or recommendations expressed in this publication are those of the authors and do not necessarily reflect the view of the US Department of Agriculture. We would also like to thank Ms. Stephanie Marco for her assistance in the preparation of this manuscript.

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