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Metabolic transformation has a profound effect on anti-inflammatory activity of flavonoids such as quercetin: Lack of association between antioxidant and lipoxygenase inhibitory activity

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ABSTRACT

Dietary flavonoids are thought to have health benefits possibly due to antioxidant and anti-inflammatory properties. Many previous *in vitro* studies examining the bioactivity of flavonoids have failed to consider the effects of metabolic transformation on flavonoid activity. In this study we examined the effect of quercetin and its major metabolites on the production of pro-inflammatory eicosanoids by human leukocytes. Studies comparing free radical scavenging, antioxidant activity and eicosanoid production demonstrate that there are different structural requirements for antioxidant and anti-inflammatory activity. We also investigated the effect of metabolic transformation on flavonoid bioactivity by comparing the activity of quercetin and its major metabolites to inhibit inflammatory eicosanoid production from human leukocytes. Quercetin was a potent inhibitor of leukotriene B_4 formation in leukocytes ($IC_{50} \sim 2$ μM), and its activity was dependent on specific structural features, particularly the 2,3-double bond of the C-ring. Functionalisation of the 3′-OH group with either methyl or sulfate reduced inhibitory activity up to 50% while a glucuronide substituent at the 3-OH effectively removed the LTB_4 inhibitory activity. The major quercetin metabolite quercetin-3′-O-sulfate retained considerable lipoxygenase inhibitory activity ($IC_{50} \sim 7$ μM) while quercetin-3-O-glucuronide maintained antioxidant activity but had no lipoxygenase inhibitory activity at physiological concentrations. In conclusion, we have found that structural modification of quercetin due to metabolic transformation had a profound effect on bioactivity, and that the structural features required for antioxidant activity of quercetin and related flavonoids were unrelated to those required for inhibition of inflammatory eicosanoids.

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1. Introduction

There is considerable research interest in the potential health benefits of flavonoids. Results of population studies suggest that dietary flavonoids provide protection against cardiovascular disease [1–3]. There is also a growing body of evidence from controlled trials that dietary flavonoids can improve endothelial and platelet function and reduce blood pressure in humans [4], and may inhibit the development of atherosclerosis in animal models [5]. Since oxidative stress has been implicated in atherosclerosis and cardiovascular disease, one of the main properties of flavonoids thought to explain their effect is the antioxidant activity of this group of polyphenols [6]. However, there is some doubt as to whether dietary flavonoids can act as antioxidants *in vivo* and the results of intervention studies have yielded conflicting results [7,8]. This may be due to several reasons including variations in the absorption and metabolism of flavonoids which may alter antioxidant activity as well as other biological activities [9]. In particular there is doubt about the interpretation of *in vitro* studies of antioxidant activity where issues of bioavailability and metabolic transformation have not been considered [10].

Inflammation and leukocyte recruitment are considered to play key roles in atherogenesis [11]. Inflammatory processes in the vascular wall may be mediated by a range of factors, such as cytokines, eicosanoids (such as leukotriene B_4 [LTB_4]), reactive oxygen species (generated by NADPH oxidase [12] and myeloperoxidase activities [13]) and nitric oxide, which in turn modulate cellular signaling, cell growth and differentiation and a variety of other cellular processes. Arterial leukocyte recruitment is an important initiating step in atherogenesis [14]. Leukocyte–endothelial interactions and leukocyte migration to the sub-endothelium occur in response to cytokines and chemokines such as monocyte chemotactic protein-1 (MCP-1). There is evidence that potent chemotactic molecules such as MCP-1 and LTB_4 are involved in inflammatory diseases such as rheumatoid arthritis [14] and atherosclerosis [15]. Stimulated neutrophil LTB_4 synthesis has recently been suggested as a useful marker for assessing the leukotriene pathway in humans [16]. Human atherosclerotic lesions produce LTB_4 and the enzymes responsible for its production (5-lipoxygenase and leukotriene A_4 hydrolase) are associated with symptoms of plaque instability [17].

We have been particularly interested in examining the effects of dietary flavonoids on the production of pro-inflammatory eicosanoids such as LTB_4 and prostaglandin E_2 (PGE_2) by human leukocytes. Quercetin is a common dietary flavonoid which has been shown to inhibit pro-inflammatory cytokines in mononuclear cells [18] and block airway epithelial chemokine expression [19]. A recent study has demonstrated that quercetin and related flavonoids can attenuate TNF stimulated adhesion molecule expression in human aortic endothelial cells, however, exposure to cultured hepatocytes (mimicking first pass metabolism) greatly diminished this activity [20].

To address the issue of the effect of metabolic transformation on flavonoid bioactivity we have compared the ability of quercetin and its major human metabolites to inhibit inflammatory eicosanoid production from human leukocytes. We have examined quercetin, structural analogues of quercetin, and a series of quercetin phase-2 conjugates of known structure to determine structural features important for antioxidant and anti-inflammatory activity (see Fig. 1 for structures and Fig. 2 for flow diagram of experiments). We found that structural modification of quercetin due to metabolic transformation had a profound effect on bioactivity. The structural features required for antioxidant activity of quercetin and related flavonoids were unrelated to that required for inhibition of inflammatory eicosanoids.

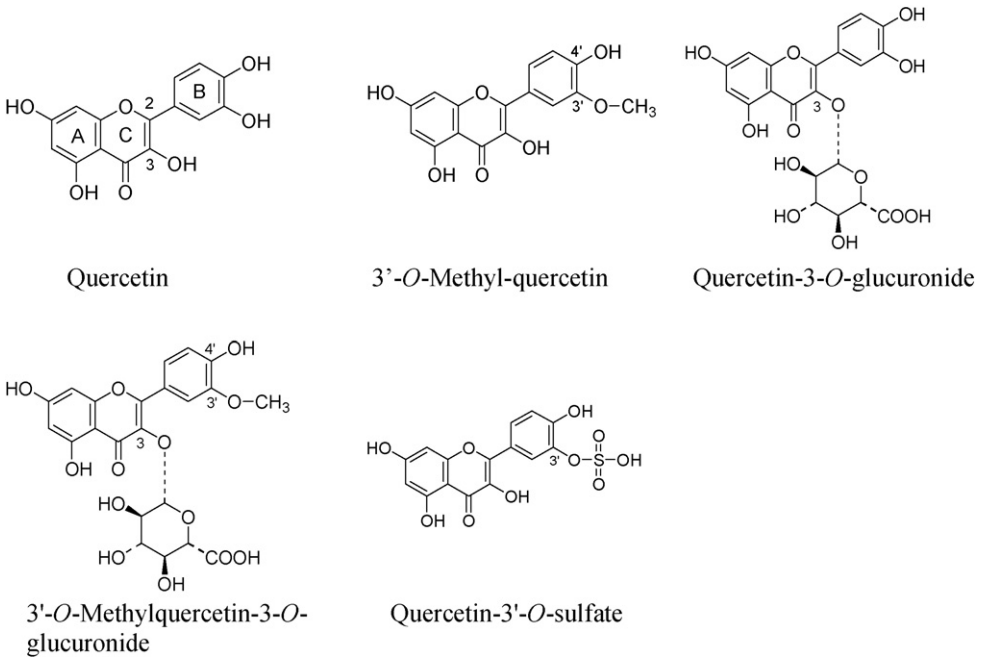


Fig. 1 – Structures of quercetin and its metabolites present in human circulation.

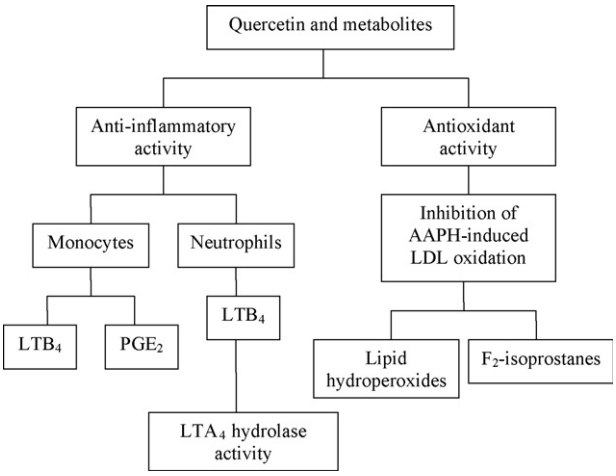


Fig. 2 – Flow diagram of the experiments.

2. Materials and methods

2.1. Chemicals and reagents

Bovine serum albumin (BSA), calcium chloride, calcium ionophore A23187, Hepes, lipopolysaccharide, MK886, quercetin, sodium phosphate dibasic, sodium bromide, sodium chloride, sodium hydrogencarbonate, trifluoroacetic acid, luteolin, kaempferol, taxifolin, xylenol orange, ammonium ferrous sulfate, butylated hydroxytoluene, hydrogen peroxide (50% by volume) and 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH) were purchased from Sigma–Aldrich (St. Louis, MO, USA); acetonitrile, magnesium sulfate and sulfuric acid from Univar (WA, Australia); Ficoll-Paque from GE Healthcare (Uppsala, Sweden); phosphate buffered saline (PBS), heat-inactivated fetal calf serum (HIFCS) and RPMI 1640 from Gibco™ Invitrogen (Calsbad, CA, USA); dextran 500 from Amersham Biosciences (Uppsala, Sweden); glucose and potassium phosphate monobasic from Merck (VIC, Australia); methanol and ethanol from Mallinckrodt (NJ, USA); and prostaglandin B_2 (PGB_2) and leukotriene A_4 (LTA_4) methyl ester from Cayman Chemical (Michigan, USA). 3′-O-Methylquercetin was purchased from Advanced Technology & Industrial Co., Ltd, Hong Kong, while quercetin-3′-O-sulfate, quercetin-3-O-glucuronide and 3′-O-methylquercetin-3-O-glucuronide were synthesized as previously described [21].

2.2. Isolation of peripheral monocytes and neutrophils

Peripheral blood mononuclear cells (PBMC) were isolated from whole human blood (containing 1 mg/mL EDTA) by centrifugation on Ficoll-Paque at $500 \times g$ for 30 min at 20 °C. The collected PBMC layer was further purified by washing with MACS buffer (0.5% bovine serum albumin, 2 mM EDTA in PBS; pH 7.2) and centrifuging at $100 \times g$ for 10 min at 4 °C to remove platelets. MACS® human CD14 Micro-beads (Miltenyl Biotec, CA, USA) (20 μL/10^7 cells final concentration) were incubated with PBMC for 15 min at 4 °C. The resulting mixture was passed through MACS® separation column (Miltenyl Biotec, CA, USA), which separated peripheral blood monocytes from other mononuclear cells. The neutrophils were isolated from the neutrophil/erythrocyte pellet from the Ficoll-Paque gradient by dextran sedimentation of red cells as previously described [22]. Cell viability was assessed using trypan blue exclusion and was typically >98%.

2.3. Stimulation and measurement of LTB_4 production

The effects of quercetin and its metabolites on the 5-lipoxygenase (5LO) pathway were examined using freshly isolated human peripheral monocytes and neutrophils. The freshly isolated monocytes and neutrophils were re-suspended in HBHS [$CaCl_2{\cdot}2H_2O$ (0.09 g), glucose (0.50 g), Hepes (0.06 g), KCl (0.20 g), KH_2PO_4 (0.03 g), $MgSO_4{\cdot}7H_2O$ (0.10 g), $NaHCO_3$ (0.18 g), NaCl (4.00 g), Na_2HPO_4 (0.02 g) and bovine serum albumin (BSA) (0.50 g) in pure water (500 mL); pH 7.4] at a concentration of 5×10^6 cells/mL. The cell suspension (1 mL) was incubated with either quercetin, 3′-O-methylquercetin, quercetin-3′-O-sulfate, quercetin-3-O-glucuronide, 3′-O-methylquercetin-3-O-glucuronide, luteolin, kaempferol or taxifolin (2–10 μM final concentration) at 37 °C for 5 min prior to 5LO stimulation. In each independent experiment, cells were incubated in duplicate for each concentration tested. Quercetin and 3′-O-methylquercetin were added using ethanol as vehicle, while the other metabolites used water as their vehicles. The cells were stimulated with calcium ionophore A23187 (2.5 μg/mL final concentration) at 37 °C for 15 min. The supernatant from the cell suspension was collected and stored at −80 °C before LTB_4 extraction and analysis. Untreated cells with ethanol and water vehicles were used as positive controls while untreated cells incubated with the leukotriene biosynthesis inhibitor MK886 (300 nM) served as negative controls [23]. In another set of experiments designed to examine specific inhibition of LTA_4 hydrolase, cells incubated with LTA_4 (final concentration 15 μM) only or LTA_4 (final concentration 15 μM) and quercetin (final concentration 10 μM) were stimulated with calcium ionophore as above. The release of LTB_4 from stimulated neutrophils was measured by HPLC. Briefly, the released eicosanoids were extracted from cell supernatant (after acidification with formic acid, and addition of a PGB_2 internal standard) with ethyl acetate, dried under nitrogen and re-suspended in mobile phase [methanol:acetonitrile:water (1:1:2)]. Components were separated by reverse phase chromatography on a C18 column (Agilent Technologies LiChrospher 100 RP-18, 5 μm) using methanol, acetonitrile, water, trifluoroacetic acid (40:40:80:0.1, v/v; pH 3) mobile phase (solvent A) with increasing gradient of methanol:acetonitrile (1:1) (solvent B) at a flow rate of 1 mL/min over 30 min (from 0% to 50% B in A) using a Hewlett Packard Series 1050 HPLC. Wavelength detection at 270 nm was used to detect conjugated trienes. Peak area was determined using Agilent Technologies Chemstation software package. The production of LTB_4 from the monocytes was measured using a specific LTB_4 enzyme immunoassay (EIA) kit (Cayman Chemical).

2.4. Stimulation and measurement of PGE_2 production

Freshly isolated peripheral blood monocytes (PBM) were used to evaluate the effects of quercetin and its metabolites on the cyclooxygenase-2 (COX-2) pathway. The cells were re-sus-

pended in 10% HIFCS in RPMI (3×10^6 cells/mL final concentration). The cell suspension was then incubated with quercetin or its metabolites (2–10 μM final concentrations) at 37 °C for 5 min. COX-2 stimulation was carried out by incubating cells with lipopolysaccharide (LPS, 1 μg/mL final concentration) for 20 h at 37 °C. Positive controls (LPS treated without polyphenol treatment) and negative controls (without both polyphenols and LPS treatment) were also studied. Cell supernatants were collected at the end of the incubation. The production of PGE_2 was measured by specific PGE_2 enzyme immunoassay (EIA) kit (Cayman Chemical).

2.5. Measurement of intracellular quercetin and its metabolites

Freshly isolated neutrophils suspended in HBHS were incubated with quercetin or its metabolites at concentrations ranging from 0 to 10 μM at 37 °C as described above, for a period of 20 min. The cell pellet was obtained after centrifugation at 2000 × *g* for 5 min at 4 °C, and washed once with HBBS. The cells were then lysed in buffer (30 mM NaH_2PO_4, adjusted to pH 3.0 with H_3PO_4) by sonication. The supernatant was collected and stored at −80 °C prior to HPLC analysis. An HPLC assay has previously been described to simultaneously measure the intracellular amount of quercetin and its metabolites [24,25]. Components were separated by reverse phase chromatography using a LiChrospher 100 RP-18, 5 μm column (Agilent Technologies) with sodium orthophosphate (30 mM; adjusted to pH 3 with phosphoric acid):acetonitrile (15:85, v/v) mobile phase (solvent A) with increasing gradient of acetonitrile (solvent B) at a flow rate of 0.8 mL/min over 20 min (from 0% to 50% B in A). Dual wavelength detection at 370 and 270 nm was used to detect the B-ring and C-ring contained within the 2-phenyl-γ-benzopyrone structure of quercetin, respectively.

2.6. Measurement of inhibition of lipoprotein oxidation

Low-density lipoprotein (LDL) was isolated from blood plasma by density gradient ultracentrifugation as previously described [26]. The antioxidant activity of each compound (quercetin, 3′-*O*-methylquercetin, quercetin-3′-*O*-sulfate, quercetin-3-*O*-glucuronide, 3′-*O*-methylquercetin-3-*O*-glucuronide, luteolin, kaempferol and taxifolin) to inhibit AAPH-induced LDL oxidation was analysed by measuring the formation of lipid hydroperoxides using the FOX assay [27], or F_2-isoprostanes measured by GC-MS [28]. Briefly, the test compound (final concentration 10 μM) was added to LDL (final protein concentration 0.1 mg/mL) and AAPH (final concentration 5 mM) at 37 °C. Aliquots of the mixture were analysed for lipid peroxidation products at specific time points up to 3 h and compared to control incubations without the addition of test compounds.

2.7. Statistical analysis of results

Statistical analysis of results ($n = 3$ or 5 independent experiments) was performed using SPSS version 11.5. One-way ANOVA [29] and Bonferroni post hoc analyses were performed on specific concentration points as well as the areas under the curves [30] in concentration-response results. The results analysed were considered significantly different if p value ≤ 0.05 based on 95% confidence.

3. Results

3.1. Effects of quercetin and its metabolites on LTB_4 production

The LTB_4 inhibiting actions of quercetin and its major circulating metabolites (Fig. 1) in human neutrophils and monocytes are presented in Figs. 3 and 4, respectively. Inhibitory activity is expressed as the percentage reduction in LTB_4 production compared to the untreated positive control (producing 7.0 ng/10^6 cells). None of the negative controls (MK 886 treated) showed measurable LTB_4. Quercetin exhibited a dose-dependent inhibitory effect on LTB_4 production with an IC_{50} value of 2 μM, while its metabolites showed reduced inhibitory activity at this concentration. At this low concentration (2 μM) only quercetin and 3′-*O*-methylquercetin showed any LTB_4 inhibitory activity in peripheral neutrophils (Fig. 3). Over the concentration range tested, all quercetin metabolites showed less activity than the parent compound (quercetin). Among the metabolites, 3′-*O*-methylquercetin and quercetin-3′-*O*-sulfate exhibited significant dose response effects while quercetin-3-*O*-glucuronide and 3′-*O*-methylquercetin-3-*O*-glucuronide showed virtually no activity up to a concentration of 10 μM. In peripheral monocytes, quercetin, 3′-*O*-methylquercetin and quercetin-3′-*O*-sulfate all inhibited LTB_4 production at 2 μM (approx 50% inhibition compared to controls, on average producing 2.2 ng/10^6 cells) while quercetin-3-*O*-glucuronide and 3′-*O*-methylquercetin-3-*O*-glucuronide showed minimal activity (Fig. 4). Quercetin and 3′-*O*-methylquercetin were significantly more effective in reducing LTB_4 production in monocytes ($p < 0.001$) compared to quercetin-3′-*O*-sulfate, quercetin-3-

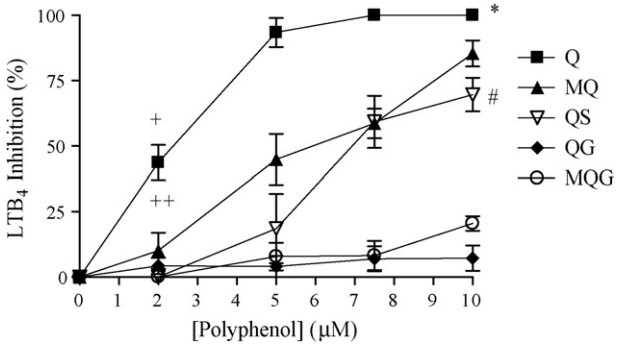


Fig. 3 – LTB_4 inhibition in peripheral neutrophils by quercetin (Q), 3′-*O*-methylquercetin (MQ), quercetin-3′-*O*-sulfate (QS), quercetin-3-*O*-glucuronide (QG) and 3′-*O*-methylquercetin-3-*O*-glucuronide (MQG) at concentrations up to 10 μM ($n = 5$). $^*p < 0.05$ vs. all quercetin metabolites using comparison of area under the curve (AUC) (ANOVA). $^\#p < 0.05$ vs. QG and MQG using AUC (ANOVA). $^+p < 0.05$ for Q compared to QS, QG and MQG at 2 μM (ANOVA). $^{++}p < 0.05$ for MQ compared to QS, QG and MQG at 2 μM (ANOVA).

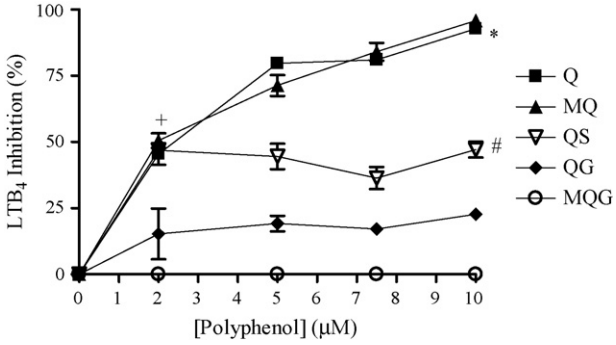


Fig. 4 – LTB_4 inhibition in peripheral monocytes by quercetin (Q), 3′-O-methylquercetin (MQ), quercetin-3′-O-sulfate (QS), quercetin-3-O-glucuronide (QG) and 3′-O-methylquercetin-3-O-glucuronide (MQG) at concentrations up to 10 μM ($n = 3$). $^{*}p < 0.001$ for Q and MQ compared to QS, QG and MQG using AUC (ANOVA). $^{\#}p < 0.001$ for QS compared to Q, MQ, QG and MQG using AUC (ANOVA). $^{+}p < 0.05$ for Q, MQ and QS compared to QG and MQG at 2 μM (ANOVA).

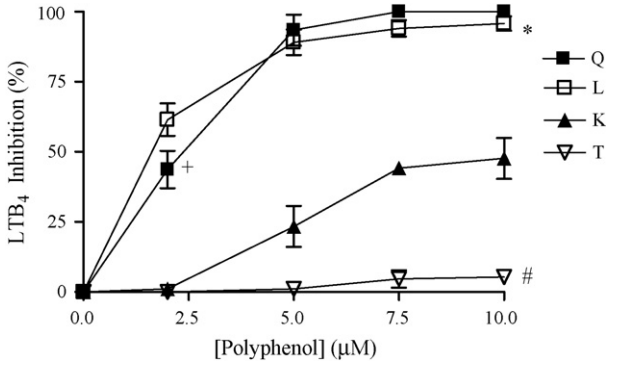


Fig. 6 – LTB_4 inhibition in peripheral neutrophils by quercetin (Q), luteolin (L), kaempferol (K) and taxifolin (T) at concentrations up to 10 μM ($n = 5$). $^{*}p < 0.001$ for Q and L compared to K and T using AUC (ANOVA). $^{\#}p < 0.001$ T compared to Q, L and K by AUC. $^{+}$ $p < 0.001$ for Q and L compared to K and T at 2 μM (ANOVA).

O-glucuronide and 3′-O-methylquercetin-3-O-glucuronide (Fig. 4). In neutrophils the LTB_4 inhibiting activity observed follows the same declining order: quercetin > 3′-O-methylquercetin > quercetin-3′-O-sulfate ≫ quercetin-3-O-glucuronide > 3′-O-methylquercetin-3-O-glucuronide. LTB_4 inhibiting activity was similar in monocytes, except that quercetin and 3′-O-methylquercetin were equipotent in monocytes.

3.2. *Effects of quercetin and its metabolites on PGE_2 production*

The effects of quercetin and its metabolites on PGE_2 production were examined in LPS treated peripheral monocytes. Without LPS stimulation (negative controls) no PGE_2 was measurable. Quercetin and 3′-O-methylquercetin exhibited similar dose-dependent reduction of PGE_2 production in peripheral monocytes ($IC_{50} = 4$ μM) compared to the untreated positive controls (producing 3.2 ng/10^6 cells), while quercetin-3′-O-sulfate, quercetin-3-O-glucuronide and 3′-O-methylquercetin-3-O-glucuronide showed minimal activity (Fig. 5). At 2 μM, both quercetin and 3′-O-methylquercetin showed significantly greater PGE_2 inhibition (25%) than the other three metabolites ($p < 0.001$).

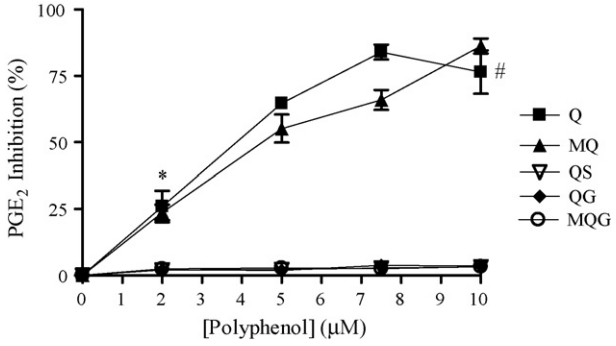


Fig. 5 – PGE_2 inhibition in LPS stimulated peripheral monocytes by quercetin (Q), 3′-O-methylquercetin (MQ), quercetin-3′-O-sulfate (QS), quercetin-3-O-glucuronide (QG) and 3′-O-methylquercetin-3-O-glucuronide (MQG) at concentrations up to 10 μM ($n = 3$). $^{*}p < 0.001$ for Q and MQ compared to QS, QG and MQG at 2 μM (ANOVA). $^{\#}p < 0.001$ for Q and MQ compared to QS, QG and MQG using AUC (ANOVA).

3.3. *Effects of luteolin, kaempferol and taxifolin on LTB_4 production*

The LTB_4 inhibiting activity of quercetin was compared with luteolin, kaempferol and taxifolin (see structures in Fig. 11). These compounds bear close structural resemblance to quercetin but have specific OH functional group substitutions that enable a structure activity relationship (SAR) to be made. Luteolin showed similar LTB_4 inhibiting activity as quercetin, while kaempferol exhibited a much reduced activity level compared to quercetin ($p < 0.001$, Fig. 6). Taxifolin showed minimal activity compared to all the compounds tested ($p < 0.001$). At 2 μM concentration, there were no significant differences in activity between quercetin and luteolin and between kaempferol and taxifolin, but significant differences were observed between these two sets of flavonoids ($p < 0.001$).

3.4. *Effects of quercetin on LTA_4 hydrolase*

To examine the effect of quercetin on LTA_4 hydrolase we measured the amount of LTB_4 produced by peripheral neutrophils in the presence or absence of added LTA_4 (15 μM) with or without quercetin treatment (Fig. 7). Peripheral neutrophils produced significantly higher amounts of LTB_4 after LTA_4 supplementation and this was not affected by the presence or absence of quercetin ($p < 0.001$) compared to the control (without LTA_4 and quercetin). This result shows that quercetin does not exert its inhibitory effect on the enzymatic hydrolysis of LTA_4 to LTB_4.

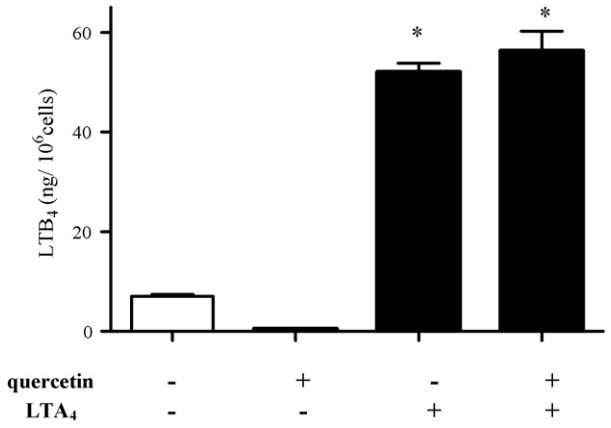


Fig. 7 – The effect of quercetin (10 μM) on LTA_4 hydrolase activity in peripheral neutrophils ($n = 3$). LTA_4 (15 μM) was added to calcium ionophore A23187 stimulated cells in the presence or absence of quercetin (filled bars) and compared to controls (without added LTA_4 ± quercetin, open bars). $^*p < 0.001$ compared to control (ANOVA).

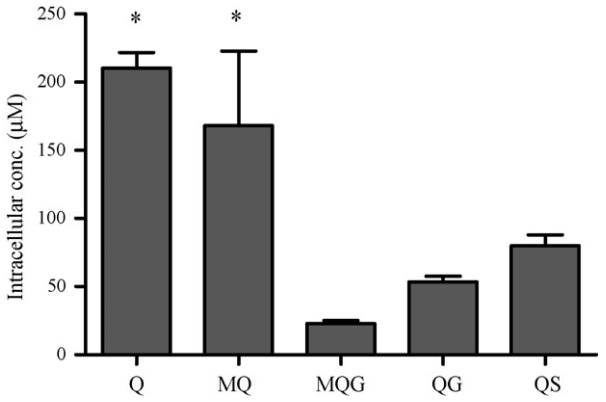


Fig. 8 – Intracellular concentrations of quercetin and its metabolites in peripheral neutrophils after incubation with each compound at a concentration of 10 μM. Concentrations are based on the cellular volume of neutrophils. $n = 3$. $^*p < 0.05$ for Q, MQ and QS compared to QG and MQG (ANOVA).

3.5. *Cellular uptake of quercetin and its metabolites*

The intracellular amounts of quercetin and its metabolites are expressed as their concentrations based on the published average cell volume of human peripheral neutrophils (~330 fL/cell) [31]. Neutrophils were able to accumulate quercetin and its metabolites within their cellular matrices to up to a concentration of 25 μM when they were incubated with a 2 μM concentration and there was no significant difference in cellular uptake between any of the compounds (data not shown). However, when the treatment concentrations were increased to 10 μM, significantly lower uptakes of quercetin-3′-*O*-sulfate, quercetin-3-*O*-glucuronide and 3′-*O*-methylquercetin-3-*O*-glucuronide were observed ($p < 0.05$) compared to that of quercetin, while cellular uptake of quercetin and 3′-*O*-methylquercetin did not differ significantly (Fig. 8).

3.6. *Antioxidant activity*

To determine the antioxidant activity of these compounds in a more physiological setting, we investigated the formation of lipid hydroperoxides and F_2-isoprostanes in AAPH-induced LDL oxidation. Fig. 9A shows the time course for LDL lipid hydroperoxide formation in the presence of quercetin and its metabolites, while Fig. 9B shows the comparison of the effect of quercetin with luteolin, kaempferol and taxifolin. Fig. 10 shows the formation of F_2-isoprostanes (a stable biomarker of lipid peroxidation) at 120 min after exposure of LDL to AAPH in the presence or absence of quercetin or its metabolites or structural analogues. These data show that quercetin, and to a slightly lesser extent 3′-*O*-methylquercetin and quercetin-3-*O*-glucuronide, are very effective at inhibiting LDL oxidation at 10 μM ($p < 0.001$ compared to LDL control). Quercetin-3′-*O*-sulfate and 3′-*O*-methylquercetin-3-*O*-glucuronide were only partially capable of inhibiting LDL oxidation at this concentration. The quantitation of F_2-isoprostanes at 120 min (the time at which F_2-isoprostanes reach maximal concentration in AAPH-induced LDL oxidation) confirms the same order of antioxidant activity for quercetin and its metabolites as seen for lipid hydroperoxide formation. Similarly the inhibition of LDL oxidation by luteolin, kaempferol and taxifolin (Fig. 9B) is mirrored by the quantitation of F_2-isoprostanes at 120 min (Fig. 10). Using this data we can construct an order of antioxidant potency; quercetin > 3′-*O*-methylquercetin = taxifolin > quercetin-3-*O*-glucuronide > kaempferol > luteolin > quercetin-3′-*O*-sulfate > 3′-*O*-methylquercetin-3-*O*-glucuronide.

4. Discussion

In this study, we have shown that quercetin is a very potent inhibitor of LTB_4 production in human peripheral monocytes and neutrophils at a realistic physiological concentration (~2 μM). However, some of its major metabolites show significantly diminished activity. Conjugation at 3′-OH of quercetin's phenylbenzopyrone structure (3′-*O*-methylquercetin and quercetin-3′-*O*-sulfate, Fig. 1) decreased LTB_4 inhibitory activity by up to 50% while metabolism at the 3-OH (quercetin-3-*O*-glucuronide and 3′-*O*-methylquercetin-3-*O*-glucuronide, Fig. 1) greatly diminished LTB_4 inhibiting activity within the physiological concentration range tested. When quercetin was compared with structural analogues (luteolin, kaempferol and taxifolin, Fig. 11), it became apparent that the 3′-OH of the B-ring played a more critical LTB_4 inhibiting role than the 3-OH of the C-ring. The absence of the 3-hydroxyl group of the C-ring in luteolin had minimal effect on its action, while the absence of 3′-hydroxyl group of the B-ring in kaempferol reduced its effect by up to 60%. These results also highlighted that the 2,3-double bond within the C-ring in quercetin is an essential structural requirement for inhibition of LTB_4 production in neutrophils, as its absence (as in taxifolin) totally diminished the inhibiting action (Fig. 11). In addition, we have observed a remarkable dissociation

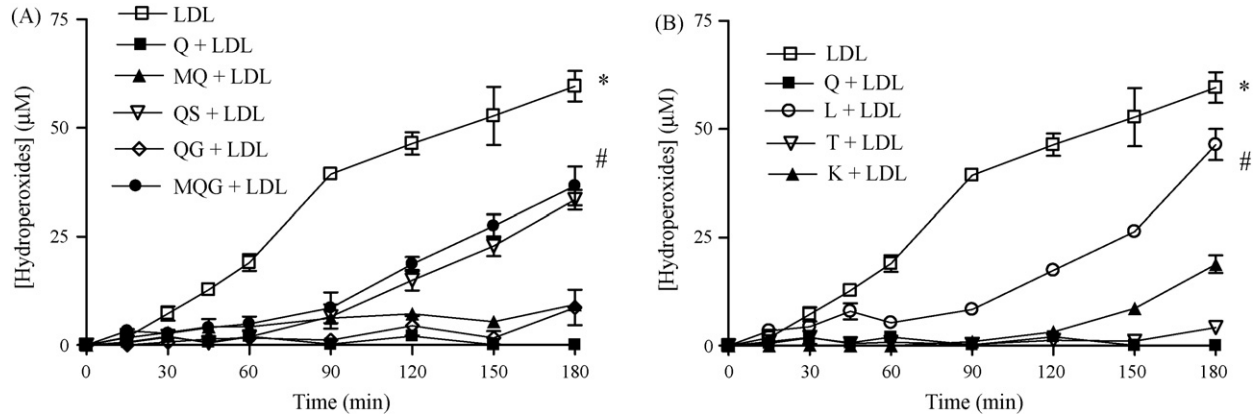


Fig. 9 – Time course for the production of lipid hydroperoxides in LDL after exposure to peroxyl radicals produced by AAPH. (A) quercetin and its metabolites were incubated with LDL (0.1 mg/mL) at a concentration of 10 μM and lipid hydroperoxides determined by FOX assay at time points up to 180 min ($n = 3$). $^{*}p < 0.001$ for control compared to all other treatments, $^{\#}p < 0.05$ for MQG and QS compared to Q (using AUC). (B) quercetin is compared to luteolin (L), kaempferol (K) and taxifolin (T) at a concentration of 10 μM ($n = 3$).$^{*}p < 0.001$ for control compared to all other treatments, $^{\#}p < 0.05$ for L compared to Q (using AUC).

between structural features that determine anti-inflammatory activity and antioxidant activity, which are illustrated in Fig. 11. The profound effect that structural modification can have on bioactivity as reported in this work further highlights the need for *in vitro* studies to use actual metabolic forms of flavonoids rather than the free aglycone or glycosides occurring in the diet.

Daily intake of flavonols such as quercetin has been estimated at between 20 and 35 mg/day, in the form of various glycosides, although intact glycosides are not found in plasma. Following supplementation with flavonol rich foods (such as onions or apples), or various quercetin-glycosides, at doses of 50–200 mg equivalents, the plasma concentration of quercetin can reach between 2 and 7 μM ([32], and references therein). In plasma, quercetin is not present as the aglycone but only in conjugated forms, with 20–40% methylated at the 3′ position and other identified metabolites being the 3-*O*-glucuronide, 3′-*O*-sulfate or the 3′-*O*-methylquercetin-3-*O*-glucuronide [32]. A recent human intervention study had identified quercetin-3′-*O*-sulfate, quercetin-3-*O*-glucuronide and 3′-*O*-methylquercetin-3-*O*-glucuronide as major quercetin metabolites in the plasma after ingestion of onions [33]. The synthesis of each of these metabolites has enabled us to study the activity of each individual metabolite and evaluate the likely consequences of metabolic conversion of flavonoids such as quercetin. It has been previously demonstrated that exposure of flavonols such as kaempferol to cultured hepatocytes (mimicking first pass metabolism) greatly diminished inhibitory activity towards endothelial cell adhesion molecule expression, although the nature of the metabolic conversion was not identified [20].

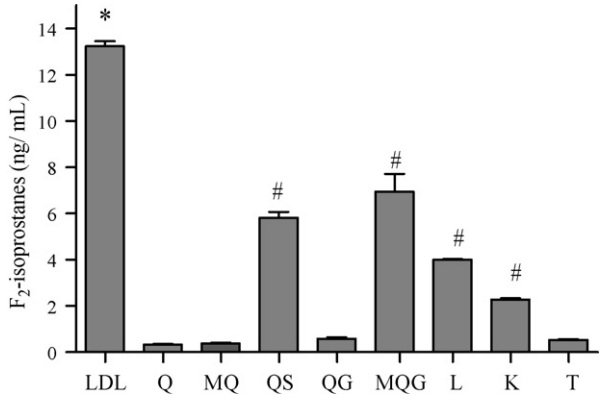
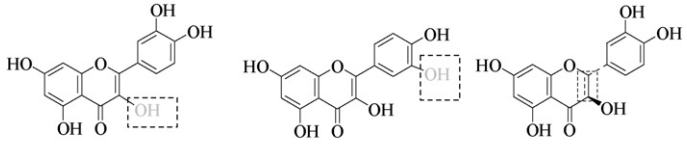


Fig. 10 – The effect of flavonoid compounds on production of F_2-isoprostanes during AAPH-induced LDL oxidation. The compounds (10 μM) were added to LDL (0.1 mg/mL) prior to incubation with AAPH and samples removed for F_2-isoprostane analysis at 120 min ($n = 3$). Compounds tested were Quercetin (Q), 3′-*O*-methylquercetin (MQ), quercetin-3′-*O*-sulfate (QS), quercetin-3-*O*-glucuronide (QG) and 3′-*O*-methylquercetin-3-*O*-glucuronide (MQG), luteolin (L), kaempferol (K) and taxifolin (T). $^{*}p < 0.001$ for control compared to all other treatments, $^{\#}p < 0.05$ for MQG, QS, L and K compared to Q.

The production of LTB_4 by leukocytes is dependent on the translocation of 5-lipoxygenase from the cytosol to the nuclear membrane in response to increased intracellular calcium [34]. It requires substrate, arachidonic acid, which must be generated from membrane phospholipids via phospholipase A_2. Five-lipoxygenase activating protein (FLAP) acts as a docking protein on the nuclear membrane and together with leukotriene A_4 hydrolase and phospholipase A_2 completes the complex that is required for LTB_4 synthesis [34]. Certain indole derivatives such as MK-886 selectively bind to FLAP and prevent the activation of 5-lipoxygenase and subsequent synthesis of leukotrienes [34]. Other compounds such as certain redox active flavonoids and phenols can directly inhibit 5-lipoxygenase, presumably by reducing the iron at the active site. We have been able to establish that quercetin does not suppress the hydrolysis of LTA_4 to LTB_4 (Fig. 7) indicating that quercetin acts directly on 5-lipoxygenase or possibly FLAP. There is evidence that quercetin and other flavonols



	Luteolin (L)	Kaempferol (K)	Taxifolin (T)
Antiinflammatory activity	Strong activity, similar to Q	Some activity	No activity
Antioxidant activity	Much less activity than Q	Modest activity	Strong activity

Fig. 11 – Structures of luteolin (L), kaempferol (K) and taxifolin (T). The structural features shown in grey represent the difference in structure between these compounds and quercetin (Q). A comparison of the antioxidant and anti-inflammatory (inhibition of LTB_4) activity with that of quercetin (Q) is summarized.

such as epicatechin can directly inhibit human 5-lipoxygenase. Using a recombinant enzyme, Schewe et al. [35] showed direct inhibition in a cell-free system with quercetin having IC_{50} = 0.6 μM for 5-lipoxygenase and 4 μM for inhibition of 15-lipoxygenase. Since these were cell membrane-free systems the involvement of FLAP appears to be ruled out. Moreover the flavonols appear to be non-specific lipoxygenase inhibitors [35].

Whatever the mechanism of action for the inhibition of 5-lipoxygenase by flavonoids, it appears to be distinct from the antioxidant properties of these studied compounds. Our results comparing antioxidant activity with leukotriene inhibitory activity clearly demonstrate this distinction. For example, the 2,3-double bond is critical for leukotriene inhibitory activity but has little effect on antioxidant activity as observed with taxifolin. The C3-hydroxyl group is not critical for leukotriene inhibitory activity but its absence significantly reduces antioxidant properties as seen with luteolin. The results are consistent with previous reports that the 2,3-double bond of the C-ring in flavonols is essential for inhibition of adhesion molecule expression in endothelial cells [20] and inflammatory cytokine production in mouse macrophages [36].

Similarly metabolism of quercetin which introduces a glucuronide at the C3-oxygen position (quercetin-3-O-glucuronide and 3′-O-methylquercetin-3-O-glucuronide) almost completely abolishes leukotriene inhibitory activity. This result is in agreement with a previous observation that formation of the 3-O-glucuronide by incubation of quercetin with liver cell-free extracts substantially reduces lipoxygenase inhibitory activity [37]. Interestingly, glucuronide formation at other sites (3′, 4′ and 7) had little effect on soybean lipoxygenase inhibition in a cell-free preparation [37]. Metabolites with groups attached to the 3′-O position of the B-ring (3′-O-methylquercetin and quercetin-3′-O-sulfate) maintain some inhibitory activity towards both leukotriene production and PGE_2 formation. Another factor which may influence the activity of quercetin metabolites is change in polarity and reduced cellular uptake. This is particularly noticeable with the polar moieties such as glucuronide and sulfate, while methylation has little effect on cellular uptake (Fig. 8). However, the actual mechanisms by which neutrophils sequester quercetin remain unknown at this time.

Overall our study suggests that at least two of the major *in vivo* metabolites of quercetin retain significant activity for the inhibition of pro-inflammatory eicosanoids such as LTB_4 and PGE_2. While *in vivo* studies of the anti-inflammatory action of flavonols is limited flavonols have been shown to reduce leukotriene production in humans [38] and direct injection of quercetin into joints of the rat significantly reduced inflammation [39]. The availability of synthetic standards of specific flavonoid metabolites will enable their bioactivity to be more clearly defined. It is now becoming clear that structural modification of flavonoids by metabolic transformation is likely to have a profound effect on biological activity.

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Conflict of interest

None of the authors have a conflict of interest.

REFERENCES

[1] Fisher NDL, Hollenberg NK. Flavanols for cardiovascular health: the science behind the sweetness. J Hypertens 2005;23:1453–9.
[2] Arts IC, Hollman PC. Polyphenols and disease risk in epidemiologic studies. Am J Clin Nutr 2005;81:317S–25S.
[3] Mink PJ, Scrafford CG, Barraj LM, Harnack L, Hong CP, Nettleton JA, et al. Flavonoid intake and cardiovascular disease mortality: a prospective study in postmenopausal women. Am J Clin Nutr 2007;85:895–909.
[4] Vita JA. Polyphenols and cardiovascular disease: effects on endothelial and platelet function. Am J Clin Nutr 2005;81:292S–7S.

[5] Waddington E, Puddey IB, Croft KD. Red wine polyphenolic compounds inhibit atherosclerosis in apolipoprotein E-deficient mice independently of effects on lipid peroxidation. Am J Clin Nutr 2004;79:54–61.
[6] Kris-Etherton PM, Keen CL. Evidence that the antioxidant flavonoids in tea and cocoa are beneficial for cardiovascular health. Curr Opin Lipidol 2002;13:41–9.
[7] Lotito SB, Frei B. Consumption of flavonoid-rich foods and increased plasma antioxidant capacity in humans: cause, consequence, or epiphenomenon? Free Rad Biol Med 2006;41:1727–46.
[8] Hodgson JM, Croft KD. Dietary flavonoids: effects on endothelial function and blood pressure. J Sci Food Agric 2006;86:2492–8.
[9] Manach C, Scalbert A, Morand C, Rémésy C, Jiménez L. Polyphenols: food sources and bioavailability. Am J Clin Nutr 2004;79:727–47.
[10] Kroon PA, Clifford MN, Crozier A, Day AJ, Donovan JL, Manach C, et al. How should we assess the effects of exposure to dietary polyphenols in vitro? Am J Clin Nutr 2004;80:15–21.
[11] Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. Circulation 2002;105:1135–43.
[12] Cathcart MK. Regulation of superoxide anion production by NADPH oxidase in monocytes/macrophages: contributions to atherosclerosis. Arterioscler Thromb Vasc Biol 2004;24:23–8.
[13] Klebanoff SJ. Myeloperoxidases: friend or foe. J Leukoc Biol 2005;77:598–625.
[14] Libby P. Inflammation in atherosclerosis. Nature 2002;420:868–74.
[15] Chen M, Lam BK, Kanaoka Y, Nigrovic PA, Audoly LP, Austen KF, et al. Neutrophil-derived leukotriene B_4 is required for inflammatory arthritis. J Exp Med 2006;203:837–42.
[16] Jala VR, Haribabu B. Leukotrienes and atherosclerosis: new roles for old mediators. Trends Immunol 2004;25:315–22.
[17] Qiu H, Gabrielsen A, Agardh HE, Wan M, Wetterholm A, Wong C-H. Expression of 5-lipoxygenase and leukotriene A4 hydrolase in human atherosclerotic lesions correlates with symptoms of plaque instability. Proc Nat Acad Sci USA 2006;103:8161–6.
[18] Nair MP, Mahajan S, Reynolds JL, Aalinkeel R, Nair H, Schwartz SA, et al. The flavonoid quercetin inhibits proinflammatory cytokine (tumor necrosis factor alpha) gene expression in normal peripheral blood mononuclear cells via modulation of the NF-{kappa}{beta} system. Clin Vaccine Immunol 2006;13:319–28.
[19] Nanua S, Zick SM, Andrade JE, Sajjan US, Burgess JR, Lukacs NW, et al. Quercetin blocks airway epithelial cell chemokine expression. Am J Respir Cell Mol Biol 2006;35:602–10.
[20] Lotito SB, Frei B. Dietary flavonoids attenuate tumor necrosis factor α-induced adhesion molecule expression in human aortic endothelial cells: structure-function relationships and activity after first pass metabolism. J Biol Chem 2006;281:37102–10.
[21] Needs PW, Kroon PA. Convenient syntheses of metabolically important quercetin glucuronides and sulfates. Tetrahedron 2006;62:6862–8.
[22] Wu JHY, Ward NC, Indrawan AP, Almeida C, Hodgson JM, Proudfoot JM, et al. Effects of α-Tocopherol and mixed tocopherol supplementation on markers of oxidative stress and inflammation in type 2 diabetes. Clin Chem 2007;53:511–9.
[23] Rouzer CA, Ford-Hutchinson AW, Morton HE, Gillard JW. MK886, a potent and specific leukotriene biosynthesis inhibitor blocks and reverses the membrane association of 5-lipoxygenase in ionophore- challenged leukocytes. J Biol Chem 1990;265:1436–42.
[24] Manach C. The use of HPLC with coulometric detection in the analysis of flavonoids in complex matrices. In: Santos-Buelga C, Williamson G, editors. Methods in polyphenol analysis. Cambridge, UK: The Royal Society of Chemistry; 2003. p. 63–91.
[25] Santos-Buelga C, Garcia-Viguera C, Tomas-Barberan FA. On-line identification of flavonoids by HPLC coupled to diode array detection. In: Santos-Buelga C, Williamson G, editors. Methods in polyphenol analysis. Cambridge, UK: The Royal Society of Chemistry; 2003. p. 92–127.
[26] Dwyer KP, Barrett PHR, Chan D, Foo JI, Watts GF, Croft KD. Oxazolinone derivative of leucine for GC-MS: a sensitive and robust method for stable isotope kinetic studies of lipoproteins. J Lipid Res 2002;43:344–9.
[27] Nourooz-Zadeh J, Tajaddini-Sarmadi J, Wolff SP. Measurement of plasma hydroperoxide concentrations by the ferrous oxidation-xylenol orange assay in conjunction with triphenylphosphine. Anal Biochem 1994;220:403–9.
[28] Mori TA, Croft KD, Puddey IB, Beilin LJ. An improved method for the measurement of urinary and plasma F_2-isoprostanes using gas chromatography-mass spectrometry. Anal Biochem 1999;268:117–25.
[29] Townend J. Practical statistics for environmental and biological scientists. England: John Wiley & Sons Ltd.; 2002.
[30] Matthews JNS, Altman DG, Campbell MJ, Royston P. Analysis of serial measurements in medical research. Br Med J 1990;300:230–5.
[31] Rizoli SB, Rotstein OD, Kapus A. Cell volume-dependent regulation of L-selectin shedding in neutrophils. A role for p38 mitogen-activated protein kinase. J Biol Chem 1999;274:22072–80.
[32] Manach C, Williamson G, Morand C, Scalbert A, Rémésy C. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. Am J Clin Nutr 2005;81(Suppl):230S–42S.
[33] Mullen W, Edwards CA, Crozier A. Absorption, excretion and metabolite profiling of methyl-, glucuronyl-, glucosyl- and sulpho- conjugates of quercetin in human plasma and urine after ingestion of onions. Br J Nutr 2006;96:107–16.
[34] Ford-Hutchinson AW. FLAP: a novel drug target for inhibiting the synthesis of leukotrienes. Trends Pharmacol Sci 1991;12:68–70.
[35] Schewe T, Kuhn H, Sies H. Flavonoids of cocoa inhibit recombinant human 5-lipoxygenase. J Nutr 2002;132:1825–9.
[36] Comalada M, Ballester I, Bailon E, Sierra S, Xaus J, Galvez J, et al. Inhibition of pro-inflammatory markers in primary bone marrow-derived mouse macrophages by naturally occurring flavonoids: Analysis of the structure-activity relationship. Biochem Pharmacol 2006;72:1010–21.
[37] Day AJ, Bao Y, Morgan MRA, Williamson G. Conjugation position of quercetin glucuronides and effect on biological activity. Free Rad Biol Med 2000;29:1234–43.
[38] Schramm DD, Wang JF, Holt RR, Ensunsa JL, Gonsalves JL, Lazarus SA, et al. Chocolate procyanidins decrease the leukotriene-prostacyclin ratio in humans and human aortic endothelial cells. Am J Clin Nutr 2001;73:36–40.
[39] Mamani-Matsuda M, Kauss T, Al-Kharrat A, Rambert J, Fawaz F, Thiolat D, et al. Therapeutic and preventive properties of quercetin in experimental arthritis correlate with decreased macrophage inflammatory mediators. Biochem Pharmacol 2006;72:1304–10.