

## Luteolin and Chrysin Differentially Inhibit Cyclooxygenase-2 Expression and Scavenge Reactive Oxygen Species but Similarly Inhibit Prostaglandin-E<sub>2</sub> Formation in RAW 264.7 Cells<sup>1</sup>

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**ABSTRACT** Inflammation and oxidative stress are associated with cancer, atherosclerosis, and other chronic diseases. Dietary flavonoids have been reported to possess antiinflammatory and antioxidant properties, but their mechanisms of action and structure-activity relations have not been fully investigated. We hypothesized that differences in antioxidant activity between the structurally similar flavones, luteolin and chrysin (differing only in B-ring hydroxylation patterns), would differentially affect inflammation-associated Cox-2 expression and PGE<sub>2</sub> formation. Pretreatment of RAW 264.7 macrophage-like cells with 25, 50, or 100 μmol/L concentrations of luteolin inhibited lipopolysaccharide (LPS)-induced Cox-2 protein expression ( $P < 0.0001$ ). Chrysin pretreatment did not reduce LPS-induced Cox-2 protein expression at any level tested. Conversely, both luteolin and chrysin completely suppressed LPS-induced PGE<sub>2</sub> formation ( $P < 0.001$ ). Luteolin, but not chrysin, inhibited xanthine/xanthine oxidase-generated superoxide formation at 100 μmol/L in a cell-free system ( $P < 0.001$ ). Although both luteolin and chrysin reduced LPS-induced hydroxyl radical formation relative to the positive control ( $P < 0.001$ ), luteolin was superior to chrysin ( $P = 0.003$ ). In summary, luteolin and chrysin suppressed PGE<sub>2</sub> formation equally well, despite differential effects on Cox-2 protein expression and on superoxide and hydroxyl radical scavenging. These data indicate that flavones may display similar antiinflammatory activity via different mechanisms. *J. Nutr.* 136: 1517–1521, 2006.

**KEY WORDS:** • luteolin • chrysin • cyclooxygenase-2 • prostaglandin E<sub>2</sub> • reactive oxygen species

Numerous studies have indicated that diets high in fruits and vegetables, thus high in flavonoids, are inversely associated with cancer and heart disease (1–3). Other studies specifically examining flavonoid intake have shown similar results (4–6). The dietary flavones, luteolin and chrysin (Fig. 1), are structurally similar, differing only in that chrysin lacks hydroxyl groups at the 3' and 4' positions on the B ring. Common food sources of these flavones include broccoli, chili peppers, celery, rosemary, and honey (7,8). Anticarcinogenic and cardioprotective properties have been reported for luteolin and chrysin (9–11).

Inflammation and oxidative stress have been reported to play a role in chronic disease (12,13). Lipopolysaccharide (LPS),<sup>3</sup> a component of gram-negative bacteria cell walls, is frequently used to model inflammation because of its ability to activate macrophages. Activated macrophages produce a variety of proinflammatory mediators, including TNF-α, interleukins and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>). PGE<sub>2</sub> formation results from the release of arachidonic acid from cell-membrane phospholipids

by phospholipase enzymes and is converted to PGE<sub>2</sub> via cyclooxygenase and PGE<sub>2</sub> synthase enzymes. LPS-induced release of arachidonic acid, the resulting upregulation of cyclooxygenase-2 (Cox-2) expression, and PGE<sub>2</sub> formation, is mediated through toll-like receptor 4 in the RAW 264.7 macrophage-like cell line (14,15).

LPS-activated macrophages also produce superoxide (O<sub>2</sub><sup>-</sup>) and hydrogen peroxide. Together, these 2 compounds react to form the hydroxyl radical (HO·) (16). Recent reports have indicated that Cox-2 expression and PGE<sub>2</sub> formation are upregulated by reactive oxygen species (ROS) and their products (17,18). Flavones luteolin and chrysin have been reported to possess antiinflammatory and antioxidant activity in a variety of systems (19–22).

In this study, we examined the effects of flavones luteolin and chrysin on Cox-2 expression and PGE<sub>2</sub> formation in relation to their effects on the ROS ·O<sub>2</sub><sup>-</sup> and HO·. We hypothesized that differences in antioxidant activity between the structurally similar luteolin and chrysin would affect Cox-2 expression and PGE<sub>2</sub> formation.

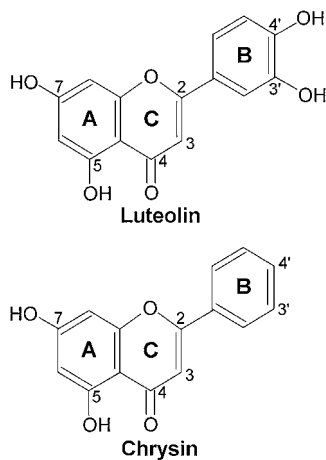
## MATERIALS AND METHODS

**Cell line, reagents, and materials.** RAW 264.7 cells, a macrophage-like cell line, were purchased from ATCC. Dulbecco's modified

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<sup>3</sup> Abbreviations used: Cox-2, cyclooxygenase-2; DMPO, 5,5 dimethylpyrrolidine-N-oxide; HO·, hydroxyl radical; LPS, lipopolysaccharide; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); O<sub>2</sub><sup>-</sup>, superoxide; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; ROS, reactive oxygen species.



**FIGURE 1** Luteolin and chrysin structures. Luteolin and chrysin possess 2 benzene rings (A,B) and a third, oxygen-containing (C) ring. Both possess a 2–3 carbon double bond, a carbonyl group at carbon 4, and lack a 3 carbon hydroxyl group. Based on these structures, they are categorized as flavones. Luteolin and chrysin also possess hydroxyl groups at carbons 5 and 7. Luteolin differs from chrysin in that it is hydroxylated at the 3' and 4' positions.

Eagle's medium (DMEM), LPS (*E. Coli* serotype 0111:B4), sodium azide, 5,5-dimethylpyrroline-N-oxide (DMPO), xanthine, and xanthine oxidase were purchased from Sigma Aldrich. Fetal bovine serum and penicillin/streptomycin were purchased from Invitrogen. Luteolin and chrysin, 99% pure, were purchased from Indofine as dry powders and stored at 25°C, as recommended by the manufacturer. All other solvents and reagents used were of analytical grade.

**Cell culture and treatment.** RAW 264.7 cells were cultured in 75 cm<sup>2</sup> flasks in DMEM supplemented with 5% fetal bovine serum and penicillin/streptomycin at 37°C in a 5% CO<sub>2</sub> atmosphere. Flavones were dissolved in DMSO immediately before experiments, then added to cell medium.

**Cell viability.** Cells were seeded into 6-well plates at a concentration of 10<sup>9</sup> cells/L. After 24 h of growth, cells were pretreated with 0, 25, 50, or 100 μmol/L luteolin for 2 h, then treated with LPS (1 mg/L) for an additional 12 h. After treatment, cells were assayed for viability using the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) method described by Mosmann (23).

**Western blotting.** Cells were grown and treated identically to those used for the MTT assay. Growth medium (used for PGE<sub>2</sub> analysis) and cell lysates were stored at -80°C. Lysates were analyzed for protein content using the BCA assay (Pierce Biotechnology). Proteins (30 μg/sample) were separated on 12% Tris-Glycine gels (Invitrogen), transferred to PVDF membranes, and blotted with the appropriate antibodies (goat polyclonal Cox-2, Actin antibodies, and anti-goat AP-linked secondary antibodies were from Santa Cruz Biotechnology; AP-linked anti-rabbit secondary antibody was from Cell Signaling) Protein bands were visualized by immunofluorescence and quantified by densitometry.

**PGE<sub>2</sub> analysis by ELISA.** Media samples were assayed for PGE<sub>2</sub> content using a 96-well plate monoclonal ELISA kits (Cayman Chemical) according to the manufacturer's instructions.

**Superoxide and hydroxyl radicals measurement.** Due to the short half-lives of ·O<sub>2</sub><sup>-</sup> and HO·, DMPO was used as a radical "trap" for electron spin resonance analyses. A quartet of peaks with hyperfine splitting is characteristic of DMPO/O<sub>2</sub><sup>-</sup> adducts, whereas a quartet of peaks in a 1:2:2:1 ratio is characteristic of DMPO/HO· adducts (24). A cell-free xanthine/xanthine oxidase (X/XO) reaction in PBS solution containing the radical spin trap DMPO (200 mmol/L final concentration) was used for the ·O<sub>2</sub><sup>-</sup> experiments. Luteolin and chrysin (0 or 100 μmol/L final concentrations) were added directly to this system. For HO· measurements, cells were pretreated for 2 h with 0 or 100 μmol/L luteolin or chrysin in 75 cm<sup>2</sup> flasks. Cells (10<sup>9</sup> cells/L) were then suspended in the same phosphate/DMPO buffer as above and treated with LPS (100 mg/L) for 1 h at 25°C. DMPO/O<sub>2</sub><sup>-</sup> and

DMPO/HO· signals were recorded using a Bruker EMX spectrometer equipped with a flat cell assembly (Bruker BioSpin).

**Statistics.** Results are representative of at least 3 independent experiments. Results were analyzed using the proc GLM (general linear models) and *P*-values were determined using the LSmeans function of SAS version 9.0 (Figs. 2–4). Differences were considered significant at *P* < 0.05.

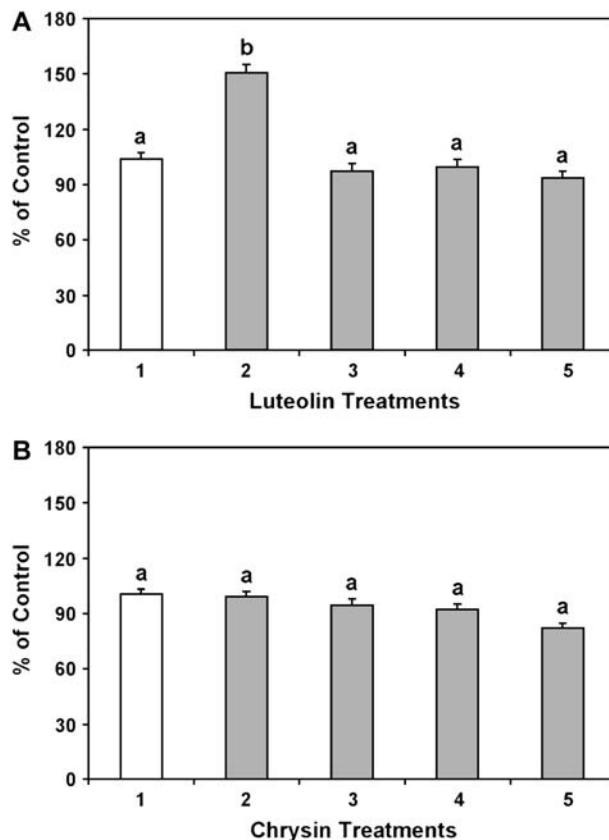
## RESULTS

**Cell viability.** The apparent viability of cells treated with 100 μmol/L luteolin alone was greater than all other treatments (*P* < 0.01), which did not differ from one another (Fig. 2).

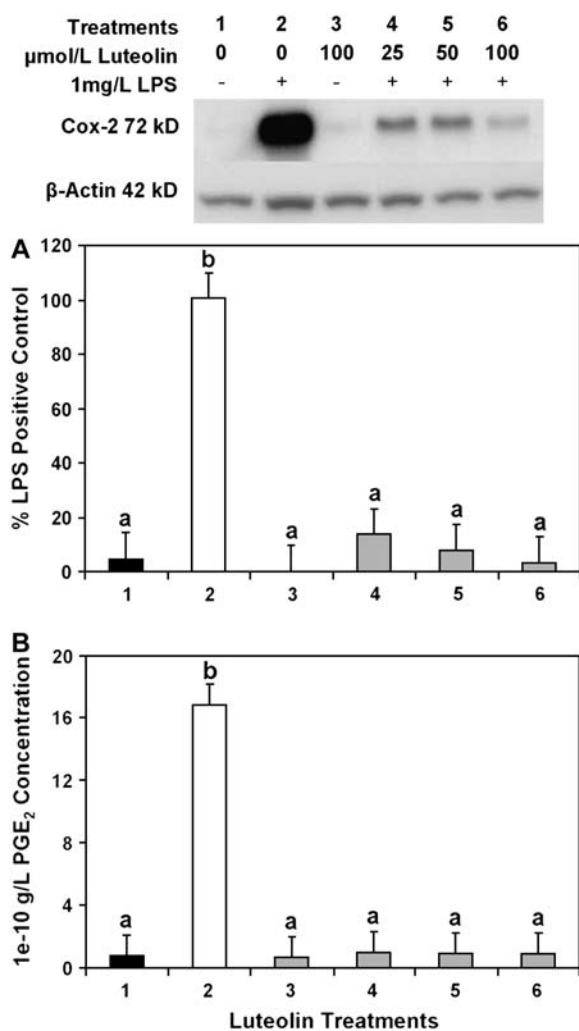
**Cox-2 protein expression.** Pretreatment with 25, 50, and 100 μmol/L luteolin reduced Cox-2 expression (*P* < 0.01) compared with the LPS positive control (Fig. 3A) at all levels. Chrysin pretreatment did not reduce LPS-induced Cox-2 protein expression at any level tested (Fig. 4A).

**PGE<sub>2</sub> formation.** LPS treatment at 1 mg/L induced PGE<sub>2</sub> formation (*P* < 0.0001) compared with untreated controls (Fig. 3B). Pretreatment with 25, 50, and 100 μmol/L luteolin reduced LPS-induced PGE<sub>2</sub> formation at all levels tested (*P* < 0.001). Like luteolin, chrysin inhibited PGE<sub>2</sub> formation at all levels tested (*P* < 0.001) (Fig. 4B).

**Superoxide and hydroxyl radical formation.** In a cell-free system, 100 μmol/L luteolin, but not chrysin, scavenged X/XO-produced ·O<sub>2</sub><sup>-</sup> (Fig. 5A) compared with the X/XO only positive control (*P* < 0.001). Both luteolin and chrysin



**FIGURE 2** Viability in RAW 264.7 cells treated with 1 mg/L LPS (1, positive control), pretreated with 100 μmol/L of either flavone only (2), or pretreated with 25 (3), 50 (4), or 100 (5) μmol/L of either flavone, and treated with 1 mg/L LPS as assessed by the MTT assay. Values are means ± SEM, *n* = 3. Means without a common letter differ, *P* < 0.05.

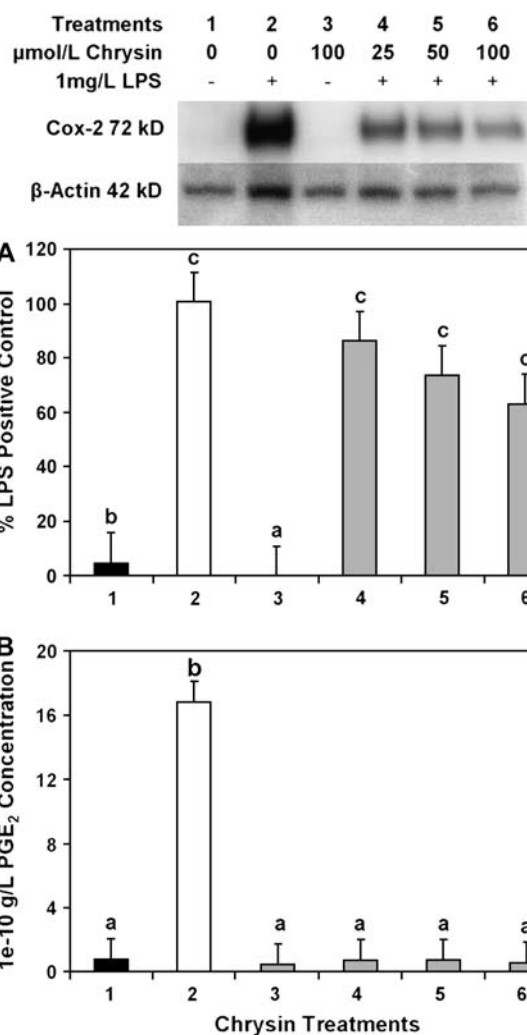


**FIGURE 3** LPS-induced Cox-2 protein expression and PGE<sub>2</sub> formation in RAW 264.7 cells that are untreated (1), treated with 1 mg/L LPS (2, positive control), pretreated with 100 μmol/L luteolin only (3), or pretreated with 25 (4), 50 (5), or 100 (6) μmol/L luteolin, and treated with 1 mg/L LPS as assessed by Western blots (A) and ELISA (B). Values are means ± SEM, *n* = 3. Means without a common letter differ, *P* < 0.05.

pretreatment inhibited (*P* < 0.001) LPS-induced HO· formation in RAW 264.7 cells (Fig. 5B). Luteolin pretreatment reduced HO· formation to a greater degree than did chrysin (*P* = 0.003).

## DISCUSSION

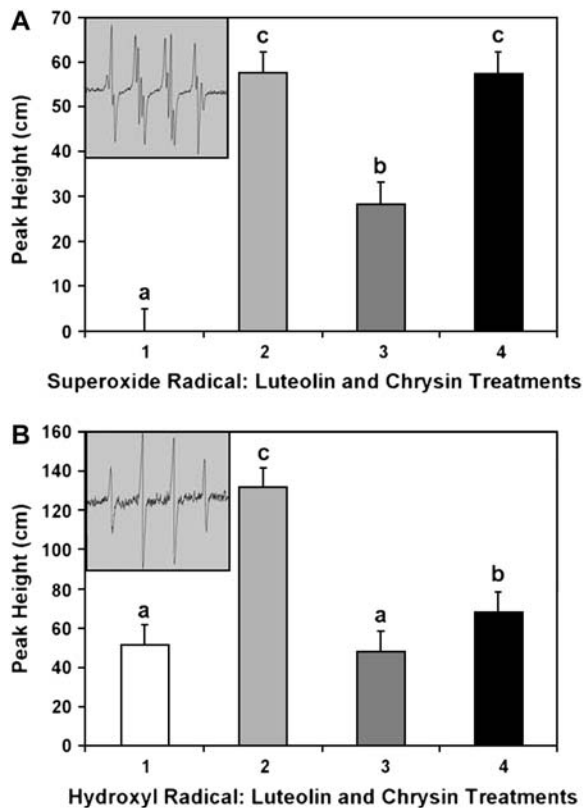
In this study, we observed that pretreatment with luteolin or chrysin suppressed PGE<sub>2</sub> formation equally well, despite luteolin's greater ability to inhibit Cox-2 expression, to scavenge HO· in LPS-induced RAW 264.7 cells, and to scavenge ·O<sub>2</sub><sup>-</sup> in a cell-free system. Thus, our original hypothesis, that differences in flavone antioxidant activity would result in differences of Cox-2 expression and PGE<sub>2</sub> formation, was partially disproven. One explanation for the observed differences in Cox-2 inhibition is chrysin's lack of 3', 4' hydroxylation on the "B" ring. Two authors have reported on the effects of flavonoid B ring hydroxylation on Cox-2 expression. Ko et al. (25) reported that 2' or 4' B ring hydroxylation mediated the inhibition of phorbol ester-induced Cox-2 expression by flavanones. Hou et al.



**FIGURE 4** LPS-induced Cox-2 protein expression and PGE<sub>2</sub> formation in RAW 264.7 cells that are untreated (1), treated with 1 mg/L LPS (2, positive control), pretreated with 100 μmol/L chrysin only (3), or pretreated with 25 (4), 50 (5), or 100 (6) μmol/L chrysin, and treated with 1 mg/L LPS as assessed by Western blots (A) and ELISA (B). Values are means ± SEM, *n* = 3. Means without a common letter differ, *P* < 0.05.

(26) reported that the inhibition of LPS-induced Cox-2 expression in RAW 264.7 cells by anthocyanins requires an ortho-hydroxyl group. Accordingly, we observed that luteolin, which possesses 3', 4' hydroxylation, significantly inhibited Cox-2 expression at all levels tested, whereas chrysin did not. A second explanation for chrysin's lack of effect on Cox-2 expression is luteolin's greater antioxidant activity. The structures reported to be essential to flavonoid antioxidant activity include 3', 4' hydroxylation, the presence of a double bond between carbons 2 and 3, and the presence of a carbonyl group on carbon 4 (27). Luteolin possesses these structures and effectively scavenged HO· and ·O<sub>2</sub><sup>-</sup>, whereas chrysin did not scavenge ·O<sub>2</sub><sup>-</sup>, and scavenged HO· less effectively than luteolin.

We show here, to our knowledge for the first time, that luteolin and chrysin inhibit LPS-induced HO· production in RAW 264.7 cells. It is unclear whether the effects of luteolin and chrysin on HO· formation are due to antioxidant activity alone, to effects on LPS-induced cell signaling, or to a combination of the two. Other workers have reported that luteolin inhibits HO· formation in cell-free systems, indicating that it possesses the capacity to scavenge HO· (21,28). Based



**FIGURE 5** Superoxide production in a cell-free system (A) containing no X/XO (1), X/XO only (2, positive control), X/XO and 100  $\mu\text{mol/L}$  luteolin (3), X/XO and 100  $\mu\text{mol/L}$  chrysin (4) as assessed by electron spin resonance. Sample  $\cdot\text{O}_2^-$  spectra, upper left hand corner. LPS-induced HO $\cdot$  formation from RAW 264.7 cells (B) that are untreated (1), treated with 1 mg/L LPS (2, positive control), pretreated with 100  $\mu\text{mol/L}$  chrysin (3), or 100  $\mu\text{mol/L}$  luteolin (4), and treated with 1 mg/L LPS as assessed by electron spin resonance. Sample HO $\cdot$  spectra, upper left hand corner. Values are means  $\pm$  SEM,  $n = 3$ . Means without a common letter differ,  $P < 0.05$ .

on our observation that chrysin inhibited HO $\cdot$  formation, although not as well as luteolin, B-ring hydroxylation (absent in chrysin) does not appear to be essential for HO $\cdot$  scavenging. Nagao et al. (29) reported that luteolin and chrysin inhibited  $\cdot\text{O}_2^-$  formation by inhibiting xanthine oxidase activity rather than by scavenging  $\cdot\text{O}_2^-$  directly. In contrast, we observed that only luteolin inhibited  $\cdot\text{O}_2^-$  formation. It is unclear whether this was due solely to  $\cdot\text{O}_2^-$  scavenging or to inhibition of xanthine oxidase as well. Chrysin did not inhibit  $\cdot\text{O}_2^-$  generation and, therefore, did not appear to have inhibited xanthine oxidase. Martinez et al. (30) reported that  $\cdot\text{O}_2^-$  upregulates Cox-2 expression and PGE $_2$  formation in LPS-induced mouse peritoneal macrophages. Accordingly, we observed that luteolin, an effective inhibitor of  $\cdot\text{O}_2^-$  in a cell-free system, inhibited Cox-2 and PGE $_2$  formation in cell culture.

The differential effects of luteolin and chrysin on Cox-2 expression and on HO $\cdot$  and  $\cdot\text{O}_2^-$  scavenging do not explain their similar effects on PGE $_2$  formation. Based on luteolin's highly significant reduction of Cox-2 expression, this alone may have been sufficient to inhibit PGE $_2$ , whereas chrysin may have inhibited PGE $_2$  formation via other mechanisms or combinations of mechanisms. Luteolin and chrysin may also have reduced PGE $_2$  formation independently of their effects on ROS and Cox-2. This idea is supported by the observations that neither complete HO $\cdot$  quenching (neither flavone did this) nor

the inhibition of Cox-2 expression (luteolin only) was essential for the inhibition of PGE $_2$  formation. There continues to be debate as to the importance of Cox-2 expression on PGE $_2$  formation. Some reports have indicated that PGE $_2$  formation is regulated via Cox-2 at transcriptional, post-transcriptional, or enzyme activity levels (31,32). Other workers have reported that PGE $_2$  formation is controlled not by Cox-2 but by phospholipase-A2 (33). We observed that both flavones uniformly inhibited PGE $_2$  formation despite chrysin's lack of effect on Cox-2 expression, which suggests that factors other than COX-2 protein expression may have affected PGE $_2$  formation in our study.

The ultimate physiological effects of luteolin and chrysin (with the exception of direct affects on the digestive tract) depend on their bioavailability and achievable concentration in vivo. This, in turn, depends on the solubility of the compounds. Walle et al. (34) estimated the oral bioavailability of chrysin to be between 0.003 and 0.02% in healthy human volunteers. Free luteolin has also been detected (but not quantified) in human serum (35). Ng et al. (36) reported solubilities of 4.34 and 59.95  $\mu\text{mol/L}$  in PBS at 37°C, respectively. Oral administration of luteolin, but not chrysin, was reported to inhibit two inflammatory end points (LPS-induced TNF- $\alpha$  formation and phorbol ester-induced ear edema) in mice (37). Chrysin's low bioavailability and lack of reported biological activity relative to luteolin may thus be a function of its low solubility. Although serum concentrations have not been reported for luteolin and chrysin, the estimated maximum serum concentration for flavonoid aglycones in general is 1  $\mu\text{mol/L}$  (38). For this reason, we chose to use treatments in the micromolar range.

Future studies will examine the mechanisms by which these flavones, with different Cox-2-inhibitory and antioxidant properties, similarly inhibit PGE $_2$  formation. The observations that luteolin, but not chrysin, inhibited Cox-2 expression, and quenched  $\cdot\text{O}_2^-$  in a cell-free system, coupled with luteolin's more effective scavenging of HO $\cdot$  suggest that these flavones inhibit PGE $_2$  formation via different mechanisms.

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