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A Medicinal Extract of *Scutellaria baicalensis* and *Acacia catechu* Acts as a Dual Inhibitor of Cyclooxygenase and 5-Lipoxygenase to Reduce Inflammation

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ABSTRACT A mixed extract containing two naturally occurring flavonoids, baicalin from *Scutellaria baicalensis* and catechin from *Acacia catechu*, was tested for cyclooxygenase (COX) and 5-lipoxygenase (5-LOX) inhibition via enzyme, cellular, and *in vivo* models. The 50% inhibitory concentration for inhibition of both ovine COX-1 and COX-2 peroxidase enzyme activities was 15 μg/mL, while the mixed extract showed a value for potato 5-LOX enzyme activity of 25 μg/mL. Prostaglandin E_2 generation was inhibited by the mixed extract in human osteosarcoma cells expressing COX-2, while leukotriene production was inhibited in both human cell lines, immortalized THP-1 monocyte and HT-29 colorectal adenocarcinoma. In an arachidonic acid-induced mouse ear swelling model, the extract decreased edema in a dose-dependent manner. When arachidonic acid was injected directly into the intra-articular space of mouse ankle joints, the mixed extract abated the swelling and restored function in a rotary drum walking model. These results suggest that this natural, flavonoid mixture acts via "dual inhibition" of COX and LOX enzymes to reduce production of pro-inflammatory eicosanoids and attenuate edema in an *in vivo* model of inflammation.

KEY WORDS: • ***anti-inflammatory*** • ***arachidonic acid metabolism*** • ***baicalin*** • ***catechin*** • ***cyclooxygenase*** • ***dietary management*** • ***leukotriene*** • ***lipoxygenase*** • ***medical food*** • ***prostaglandin***

INTRODUCTION

More than 4,000 distinct, natural flavonoids exist and are commonly consumed as foods, teas, wines, traditional medicines, and pharmaceutical drugs.[1] Not only do flavonoids contribute to color and flavor in foods such as fruits, vegetables, and spices, but they also have been used as antioxidants, antimicrobials, and antivirals to fight infection and as pharmaceutical agents for chronic disease management.[2–4] Flavonoids are also both anticarcinogenic and antimutagenic.[4,5] Flavonoids from *Scutellaria baicalensis* (Chinese skullcap) and *Acacia catechu* (black catechu) have been used in many traditional medicines and pharmaceutical products, especially in Asia, for a variety of purposes, including anti-inflammatory, antiviral, antibacterial, anticancer, and cardiovascular applications.[6–13] The anti-inflammatory properties of these flavonoids are particularly interesting in the management of chronic inflammatory diseases, such as osteoarthritis (OA).

Following the trauma that initiates OA, metabolic processes play an important role in pathophysiology resulting in progressive cartilage degradation and joint pain.[16] For instance, high levels of arachidonic acid (AA) are generated from damaged cell membrane phospholipids by the action of phospholipase A_2.[14] AA is then further metabolized by the cyclooxygenase (COX) and 5-lipoxygenase (5-LOX) enzyme systems to a variety of mediator molecules, including prostaglandin (PG) E_2, thromboxanes (TXs) (TXA_2), prostacyclins (PGI_2), and highly inflammatory leukotrienes such as leukotriene (LT) B_4, LTC_4, and LTD_4 (Fig. 1).[14–16] Conventional pharmacological management of OA involves treatment with nonsteroidal anti-inflammatory drugs (NSAIDs) or selective COX-2 inhibitors that block the formation of PGs without modulating 5-LOX enzyme activity. Inhibition of one or both of the COX enzymes may "shunt" AA metabolism down the 5-LOX pathway, which can aggravate toxicity associated with the lack of PGs and excess production of LTs.[17,18] For example, NSAID-induced gastric ulcers have been shown to have high concentrations of LTB_4 in their walls, which attracts leukocytes to the stomach and may contribute to ulceration.[18,19] Recently, reports have appeared regarding so-called "dual inhibitors," agents that inhibit not only COX-1 and COX-2, but also 5-LOX.[20–23] These agents may be particularly effective for managing the metabolic processes underlying OA and reducing both gastric and cardiovascular side effects by balancing AA metabolism in the body.[16,24]

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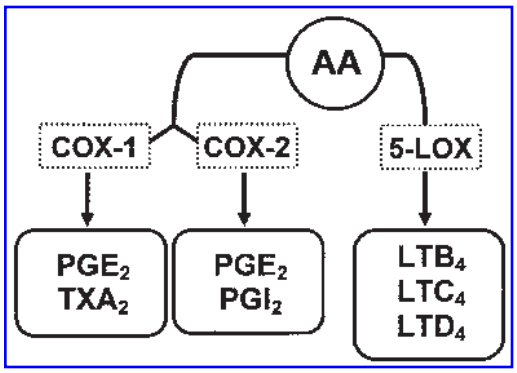


FIG. 1. Metabolic conversion of AA to inflammatory metabolites.

Using a high-throughput screening method, baicalin and catechin, from *S. baicalensis* and *A. catechu*, respectively, were previously found to inhibit COX-1, COX-2, and 5-LOX, whose AA metabolic products contribute to the development and progression of OA.[25] It is important to understand the mechanism of action of these compounds at the enzymatic and cellular levels in order to prepare for further human clinical studies. The present study demonstrates that the mixed extract containing baicalin and catechin exhibits properties of dual inhibition of both COX and LOX enzyme systems and cellular down-regulation of specific inflammatory metabolites, as well as reduction of inflammatory responses in an *in vivo* animal model. This profile, at the cellular and whole animal level, suggests the utility of a baicalin and catechin extract for the dietary management of OA.

MATERIALS AND METHODS

Preparation of extracts containing baicalin and catechin

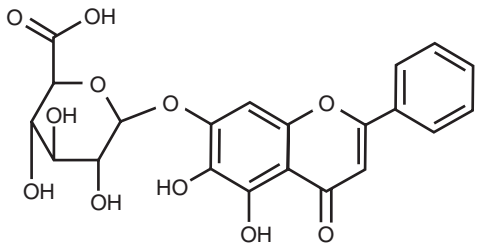
The screening process for the identification of active organic extracts containing both baicalin and catechin that inhibit COX-1, COX-2, and 5-LOX has been described.[25] In the current study, three different lots of *S. baicalensis* extracts from roots were used in the biochemical, cellular, and animal experiments. The roots were extracted with 70% ethanol and then recrystallized with an ethanol/water solvent. The *S. baicalensis* extract contained baicalin as the major component (82.2%) (Fig. 2A), as well as other minor free-B-ring flavonoids: wogonin-7-*O*-G-glucuronide, oroxylin A-7-*O*-G-glucuronide, and baicalein. Baicalein, normally a digestive product of baicalin by intestinal flora, appears in small amounts in the root of *S. baicalensis*. Three different lots of catechin extract were also obtained from repeated crystallization of an aqueous extraction of the heartwoods of a medicinal plant from India, *A. catechu*, and used in these experiments. (+)-Catechin is the major component in the *A. catechu* extract (80.4%) (Fig. 2B), plus a minor amount of its entantiomer, epicatechin, as well as minor amounts of other flavans.

Analysis of the extracts was performed separately by high performance liquid chromatography (HPLC)/photo diode array (PDA) and liquid chromatography/mass spectrometry. The results showed the major compounds to be baicalin, from the *S. baicalensis* extract, and catechin, from the *A. catechu* extract, by comparison with known standards. The structure of these compounds was then confirmed by ^{13}C and ^{1}H nuclear magnetic resonance analysis, respectively. The combined flavonoid content was analyzed by HPLC using a Phenomenex (Torrance, CA) Luna 5μ C-18 column (250 mm $\times$ 4.6 mm) with a C-18 Security Guard cartridge in a column oven at 35°C. The mobile phase had a flow rate of 1.0 mL/minute and used an isocratic 1% phosphoric acid:acetonitrile ratio of 85%:15% for the first 7 minutes, then a new gradient of 10%:90% from 7 minutes to 16.5 minutes, and then an isocratic 1% phosphoric acid:acetonitrile gradient with a ratio of 85%:15% for 7.5 minutes. The flavonoids were detected using a ultraviolet detector at 275 nm and identified based on retention time by comparison with known flavonoid standards.

Inhibition of COX enzymes

A cleavable, peroxide chromophore [*N*,*N*,*N*′,*N*′-tetramethyl-p-phenylenediamine dihydrochloride (TMPD)]

A: Baicalin



B: (+)-catechin

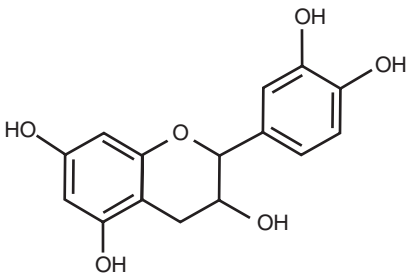


FIG. 2. Chemical structure of (**A**) baicalin and (**B**) (+)-catechin, which compose the major active compounds in UP446.

(Sigma, St. Louis, MO) was included in the assay to measure the peroxidase activity of each enzyme with AA as a cofactor.[26] Each putative inhibitor and appropriate positive controls were tested for COX-1 and COX-2 inhibition in triplicate, at room temperature over a range of concentrations from 0 to 500 μg/mL using ovine COX-1 and COX-2 enzymes (Cayman Chemical, Ann Arbor, MI), hematin (Sigma), TMPD (Sigma), and AA (Sigma). Absorbance of the cleaved TMPD substrate was read at 570 nm after a 5-minute incubation. The inhibitor concentration versus percentage inhibition was plotted, and the 50% inhibitory concentration (IC_{50}) was determined by taking the half-maximal point along the isotherm and intersecting the concentration on the *x*-axis. Different ratios of *S. baicalensis* and *A. catechu* extract were mixed to obtain equal inhibition of the peroxidase activity for both COX-1 and COX-2 enzymes *in vitro*. *S. baicalensis* to *A. catechu* ratios of 1:4, 1:1, and 4:1 were tested. The combined extract labeled UP446 (4:1 mixture *S. baicalensis* to *A. catechu*) was then compared to *S. baicalensis* and *A. catechu* extracts individually, pure baicalin (Aldrich, Milwaukee, WI), pure catechin (Sigma), ibuprofen (Sigma), aspirin (Sigma), and celecoxib (Pfizer, New York, NY).

Inhibition of 5-LOX enzyme

Other important inflammatory pathways involve nonheme, iron-containing LOXs that catalyze the addition of molecular oxygen onto fatty acids such as AA to produce unstable hyperoxides (hydroperoxyeicosatetraenoic acids) that are then converted to LTs.[27] Purified catechin from *A. catechu* was examined for its ability to inhibit 5-LOX and compared with purified baicalin and baicalein from *S. baicalensis*, UP446, and a known 5-LOX inhibitor, phenidone (Sigma), using a Lipoxygenase Inhibitor Screening Assay Kit (Cayman Chemical) in which potato 5-LOX (Cayman Chemical) was substituted for the soy 15-LOX usually present in the kit after a change of buffer from phosphate buffer, pH 6.8, to Tris-HCl, pH 7.4. The assay detects the formation of hydroperoxides through an oxygen-sensing chromagen consisting of $FeSO_4 \cdot 7H_2O$ and NH_4SCN, which changes to bright yellow and is read at 490 nm. The assay was performed in triplicate using potato 5-LOX (15.3 units), linoleic acid (Sigma) as a cofactor, and TMPD (Sigma) as a substrate with a titration of inhibitors ranging from 0 to 500 μg/mL. The inhibitor concentration versus percentage inhibition was plotted, and the IC_{50} was determined by taking the half-maximal point along the isotherm and intersecting the concentration on the *x*-axis.

Down-regulation of COX and LOX metabolites in cells

For analysis of the COX-2 inhibitory activity in cells, HOSC (ATCC #8304-CRL, American Type Culture Collection, Manassas, VA) cells, a human osteosarcoma cell line that expresses COX proteins, were cultured to 80–90% confluence, trypsinized, washed, resuspended in fresh medium, plated out in 96-well tissue culture plates, and incubated for 2 hours at 37°C in 5% CO_2 in a humidified environment. The medium was then replaced with new medium containing 1 ng/mL interleukin-1β and incubated overnight to induce COX gene expression. The medium was removed again and replaced with fresh medium. Test compounds were then added in 10 μL of medium to final concentrations of 0.75–50 μg/mL for 15 minutes at 37°C. AA, in the same medium, was added to the mixture and incubated for 10 minutes at room temperature. Supernatant was transferred to new plates containing 100 μM indomethacin in enzyme-linked immunosorbent assay (ELISA) buffer to suppress the COX-1 enzyme activity.

Immunolon-4 ELISA plates (Dynatech Laboratories, Chantilly, VA) were coated with capture antibody at 0.5–4 μg/mL against PGE_2 and TXB_2 in carbonate buffer (pH 9.2) and incubated overnight at 4°C. The plates were washed and incubated for 2 hours with blocking buffer (phosphate-buffered saline + 1% bovine serum albumin) at room temperature. The plates were washed again, and test sample was added and incubated for 1 hour at room temperature. Peroxidase-conjugated secondary antibody was added at 0.5–4 mg/mL and incubated for 1 hour at room temperature while shaking. The plates were then washed three times with phosphate-buffered saline, and 100 μL of 3,3′,5,5′-tetramethylbenzidine (Sigma) substrate was added. The plates were allowed to develop, after which the reaction was stopped by addition of 1 *M* phosphoric acid. The plates were then read at 450 nm.

To analyze the effect on 5-LOX metabolites, UP446 was titrated in triplicate at 0, 3, 10, 30, and 100 μg/mL, into fresh tissue culture medium containing confluent layers of THP-1 (ATCC #TIB 202) or HT-29 (ATCC #HTB-38™) cells, human monocyte and colorectal adenocarcinoma cell lines, which express COX-1, COX-2, and 5-LOX. A competitive ELISA for LTB_4 (Neogen, Lansing, MI) was used to assess the effect of the combined purified extracts on newly synthesized levels of LTB_4 present after 48 hours of incubation at 37°C with 5% CO_2 in a humidified environment. Each treated cell line was then harvested by centrifugation and disrupted by gentle dounce homogenization lysis in physiological buffers. The amount of newly synthesized LTB_4 was measured by ELISA.

To assess the effects of a typical NSAID on LTB_4 production, ibuprofen was added to the HT-29 cells at 3 μg/mL versus UP446 at 3 μg/mL and incubated for 48 hours. A competitive ELISA was performed as above, and 0- and 48-hour results were plotted for each.

In vivo *anti-inflammatory model for efficacy*

Outbred, pathogen-free ICR male mice (Harlan Inc., Indianapolis, IN) were used in anti-inflammatory experiments to test efficacy *in vivo*. Mice were received at 4–5 weeks of age and used after 1 week of acclimatization. Animals were housed five per cage, maintained under a light:dark schedule of 12 hours:12 hours, and provided tap water and com-

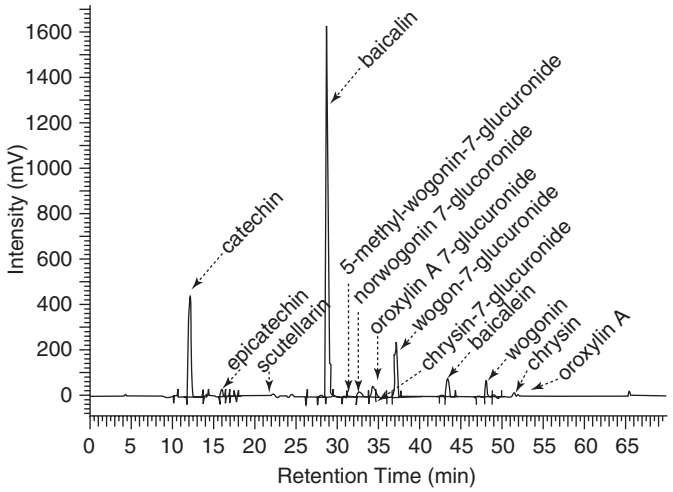


FIG. 3. HPLC profile of the combined extract, UP446.

mercial rodent chow (Teklad 2019, Harlan) *ad libitum*. Health status of the mice was monitored once daily. All animals were maintained under accepted health standards in a facility approved by the Institutional Animal Care and Use Committee and were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals*.[28]

To determine if UP446 had an effect on inflammation *in vivo*, a mouse ear swelling assay with topically applied AA was utilized.[29] Briefly, 4–5-week-old ICR mice were fed dose equivalents of 0, 50, 100, and 200 mg/kg of UP446 finely suspended in water by repeated vortex-mixing via oral gavage, while control mice were fed only water ($n = 10$ per group) 12 hours before applying 20 μL of 185 μM AA (Sigma) dissolved in 95% ethanol on one ear. The other ear served as an internal control with the application of 20 μL of 95% ethanol. Indomethacin (Sigma) administered at 50 mg/kg served a control in this experiment ($n = 10$). Ear swelling was measured twice prior to AA application and again 30–60 minutes after AA application with a spring-loaded micrometer. If measurements differed at each time point by $\geq$10% an additional measurement was taken to minimize measurement error. Inflammation was quantified as a percentage increase from baseline ear thickness to control for individual differences in basal ear thickness among mice.

After determining the anti-inflammatory activity in the ear swelling assay, 10 μL of 185 μM AA (Sigma) dissolved in 95% ethanol was injected into the intra-articular ankle space of mice ($n = 9$) that had been previously gavaged with 200 mg/kg UP446 12 hours before. Other animals, also previously gavaged with water, received injections with 10 μL of 185 μM AA ($n = 9$), 10 μL of 95% ethanol ($n = 5$), or no injection ($n = 5$) to control for the invasive procedure. Micrometer measurements of ankle swelling were taken at baseline and then 30–60 minutes after ankle injection or no treatment. Ankle swelling was quantified as the mean increase of the bilateral percent increase from baseline ankle thickness.

A "rota-rod" device, typically used for coordination studies in mice,[30] was used to assess how the mixed extract, containing baicalin and catechin, helped to maintain functional locomotor activity in control animals as well as animals injected with vehicle or AA. The animals were placed on the rota-rod (5-cm-diameter drum rotating at 20 revolutions/minute) after micrometer measurements of ankle inflammation were taken, and the latency to fall from the drum was determined.

All *in vivo* data were analyzed by analysis of variance. To probe statistically significant differences, standard polynomial contrasts and *post hoc* tests (Tukey's HSD) were employed. Significance for all statistical tests was set at $P < .05$.

TABLE 1. COMPARISON OF *S. BAICALENSIS* AND *A. CATECHU* EXTRACTS FOR INHIBITION OF COX-1 AND COX-2 WITH BAICALIN, (+)-CATECHIN, NSAIDS, AND A SELECTIVE COX-2 INHIBITOR, CELECOXIB

	IC_{50} ($\mu g/mL$)		
Compound	*COX-1*	*COX-2*	*COX-1/COX-2*[a]
S. baicalensis extract	12	6.0	2.0
A. catechu extract	4.3	10.3	0.42
Baicalin	9.8	7.3	1.34
(+)-Catechin	2.8	10.5	0.27
S. baicalensis to *A. catechu* ratio			
1:4	3.5	15	0.23
1:1	9.5	20	0.48
4:1[b]	**15**	**15**	**1.0**
Ibuprofen	10.5	90	0.12
Aspirin	2.6	40	0.065
Celecoxib	10.8	0.025	432

[a]A COX-1-selective inhibitor will have a ratio of <1, whereas a COX-2 –selective inhibitor will have a ratio of >1.

[b]This ratio of *S. baicalensis* to *A. catechu* is the final formulation of UP446.

RESULTS

Characterization of isolated and purified extracts from S. baicalensis *and* A. catechu

Eleven structures were elucidated and identified for the *S. baicalensis* standardized extract using HPLC/PDA/mass spectrometry analysis, while two were identified in the *A. catechu* extract. The mixed extract is shown in Figure 3. The major active components in the combined extract are baicalin (62.5%) from *S. baicalensis* and (+)-catechin (9.9%) from *A. catechu*, with other minor flavonoid components. Also contributing to the content from the *S. baicalensis* extract were wogonin-7-*O*-G-glucuronide (6.7%), oroxylin A-7-*O*-G-glucuronide (2%), baicalein (1.5%), wogonin (1.1%), chrysin-7-glucuronide (0.8%), 5-methyl-wogonin-7-glucuronide (0.5%), scutellarin (0.3%), norwogonin (0.3%), chrysin (<0.2%), and oroxylin A (<0.2%). In addition to the (+)-catechin, epicatechin was also present at 0.4% in the *A. catechu* extract.

Selectivity for COX-1 versus COX-2 inhibition

In order to determine the inhibitory selectivity on COX-1 and COX-2 enzymes of the combined extracts, inhibition of the recombinant ovine peroxidase enzyme, a second fatty acid-modifying activity contained within the COX-1 and COX-2 enzymes and linked to formation of PGs, was measured.[26,31] IC_{50} values are expressed in μg/mL rather than micromolar concentrations because of the impure nature of the plant extracts. Initial inhibition results for the *S. baicalensis* extract and pure baicalin showed approximately twofold more COX-2 versus COX-1 inhibition when IC_{50} values were compared (Table 1). COX-1/COX-2 ratios of <1 reflect COX-1 selectivity, whereas ratios of >1 reflect COX-2 selectivity. The *A. catechu* extract and pure (+)-catechin, however, similarly showed more COX-1 than COX-2 inhibition. Since we wished to have a balanced dual inhibitor as a therapeutic (see Discussion), different ratios of *S. baicalensis* to *A. catechu* extract were tested for COX-1 and COX-2 inhibition in an attempt to balance the activity. A 1:4 ratio of *S. baicalensis* to *A. catechu* extracts showed an IC_{50} of 3.5 μg/mL for COX-1 and 15 μg/mL for COX-2 inhibition (Table 1). A 1:1 ratio of *S. baicalensis* to *A. catechu* extracts showed an IC_{50} of 9.5 (μg/mL for COX-1 and 20 (μg/mL for COX-2 inhibition. Finally, a 4:1 ratio of *S. baicalensis* to *A. catechu* extracts showed equal IC_{50} values for both COX-1 and COX-2 inhibition for a COX-1/COX-2 ratio of 1. This formulation was coded as UP446. The traditional NSAIDs, aspirin and ibuprofen, showed more selectivity for COX-1 inhibition by IC_{50} comparison than COX-2 compared to celecoxib, a selective COX-2 inhibitor, which showed dramatic selectivity for COX-2 over COX-1 in this assay system (COX-1/COX-2 = 432) (Table 1). An example of the IC_{50} analysis by titration of increasing amounts of UP446 into both COX-1 and COX-2 assays is shown in Figure 4. For both COX-1 and COX-2, all compounds and combinations tested inhibited 100% of the enzyme activities within the 5-minute incubation period.

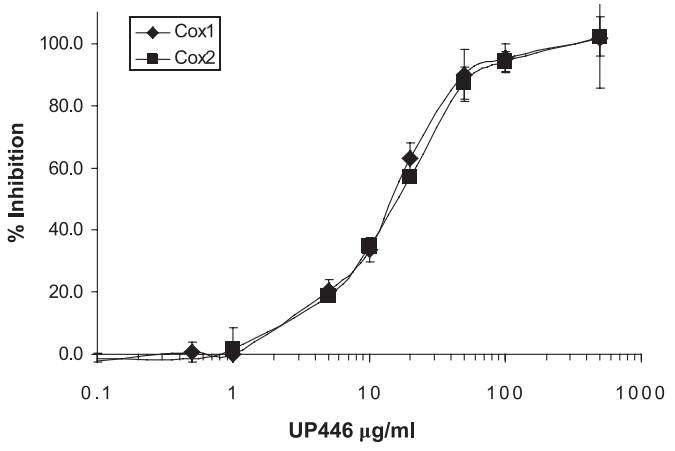


FIG. 4. Titration of UP446 for IC_{50} determination by inhibition of COX-1 (◆) and COX-2 (■) enzymes.

TABLE 2. COMPARISON OF 5-LOX ENZYME INHIBITION BY UP446, BAICALIN, (+)-CATECHIN, AND PHENIDONE

Compound rested	*5-LOX IC_{50} (μg/mL)*
UP446	29
Baicalin	ND[a]
(+)-Catechin	16.5
Phenidone	0.65

[a]ND, no inhibition detected.

Inhibition of 5-LOX enzyme

The IC_{50} for inhibition of 5-LOX enzyme activity was determined for UP446, baicalin, catechin, and phenidone, a well-characterized LOX inhibitor (Table 2).[32] The extent of inhibition for catechin was 50% at the two highest concentrations tested, and the inhibition reached a maximum of 25% without leveling off for UP446, compared to phenidone, which reached 100% (Fig. 5). Increased concentrations of UP446 were precluded from testing to establish a more accurate IC_{50} because of problems with solubility of the molecules. Thus, since 100% inhibition was not obtained at any concentration, the IC_{50} for UP446 is only an estimate based on this analysis. The absorbance for the color change in the 5-LOX enzymatic conversion of linoleic acid as measured by the oxygen-sensing system, $FeSO_4 \cdot 7H_2O$ and NH_4SCN, is measured at 490 nm. Baicalin's absorbance at the same wavelength interferes with quantitative measurement of the signal produced by the oxygen-sensing system and that of baicalin, thereby preventing an accurate determination of an IC_{50} for UP446 and baicalin. The IC_{50} was determined for catechin and UP446 after normalizing the data to 100% (Fig. 5). No inhibition

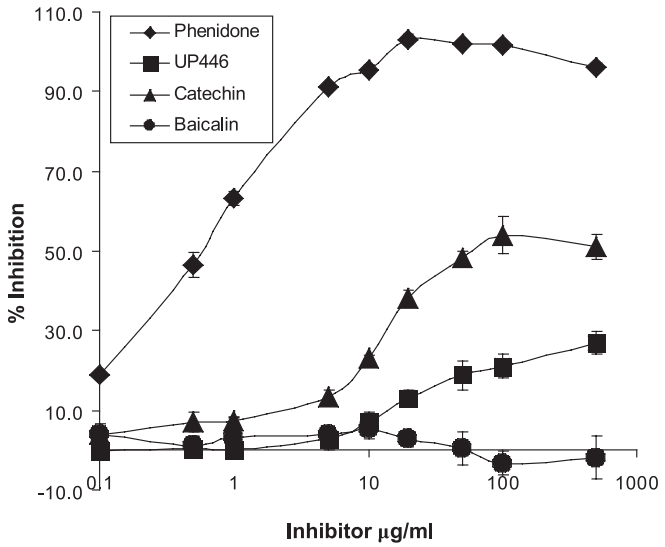


FIG. 5. Titration of UP446 (■), catechin (▲), baicalin (●), and phenidone (◆) and determination of IC_{50} for each by inhibition of the 5-LOX enzyme.

of 5-LOX was found for indomethacin, ibuprofen, naproxen, and celecoxib at the concentrations tested (data not shown).

Inhibition of cellular COX and LOX activity

Based on the *in vitro* inhibition of the COX enzymes, the ability of UP446 and celecoxib to inhibit PGE_2 production in cells was investigated. UP446 and celecoxib were titrated between 0.75 to 50 μg/mL into cultures of HOSC cells, a human osteosarcoma cell line expressing COX-2. Celecoxib was found to be a much more potent COX-2 inhibitor based on PGE_2 recovery when compared to UP446, consistent with *in vitro* enzyme inhibition results compared to UP446 (Fig. 6). UP446 at the highest concentration tested (50 μg/mL) reached approximately the same level of PGE_2 inhibition in the assay as celecoxib at the lowest concentration tested (0.75 μg/mL). Treatments of cell cultures with indomethacin inhibited any COX-1 activity and prevented production of additional PGE_2, which could interfere with the assay results (data not shown).

Because of the solubility issues in fully determining the enzymatic inhibition of UP446 on the 5-LOX enzyme, cell assays that showed the specific reduction of LTB_4 in a biological system were utilized. UP446 was titrated between 0 to 100 μg/mL into cultures of THP-1 cells, a monocyte cell line that expresses COX-1, COX-2, and 5-LOX. Using a competitive ELISA, it was found that the production of newly synthesized lipopolysaccharide-induced LTB_4 was almost completely inhibited by addition of UP446 to the cultures at concentrations between 3 and 10 μg/mL (Fig. 7A). The inhibition of LTB_4 production by UP446 was compared to that of ibuprofen as a negative control at 3 μg/mL. Intracellular levels of newly produced LTB_4 were measured in this assay after cellular disruption. In this system, UP446 reduced LTB_4 production by approximately 80% compared to 20% by ibuprofen (Fig. 7B).

THP-1 and HOSC cells were both tested for cytotoxicity using a lactate dehydrogenase assay.[33] UP446 showed no cytotoxicity at concentrations up to 100 μg/mL in each cell line (data not shown).[34]

In vivo *efficacy*

In vitro inhibition assays are not a true measure of efficacy for anti-inflammatory compounds. It is necessary to test the combination extract, UP446, in a biological system to determine efficacy. AA-induced ear swelling is a well-established assay for induction of COX and 5-LOX and the metabolites produced by each enzyme.[28] Edema is produced in 30–60 minutes mediated by PGE_2 and LTB_4 production and infiltration of leukocytes into the AA-treated ear.[31] In the present experiment, epicutaneous application of AA induced a robust swelling response, as evidenced by a mean 95% increase in ear thickness among animals gavaged with water (Fig. 8A). Edema was significantly reduced by acute oral UP446 pretreatment ($F_{3,31} = 6.09$, $P = .002$) in a dose-dependent manner ($P < .001$ for linear contrast). Post hoc analyses revealed that both 100 mg/kg ($P = .047$) and 200 mg/kg ($P = .001$) produced a significant attenuation of ear swelling compared to vehicle control. Indomethacin (50 mg/kg) treatment produced a trend towards reduced edema compared to vehicle that approached statistical significance ($F_{1,13} = 3.22$, $P = .096$). Importantly, UP446 did not significantly affect the thickness of the vehicle-treated ear ($P > .05$), indicating that it does not produce generalized reductions in ear thickness independent of an inflammatory stimulus.

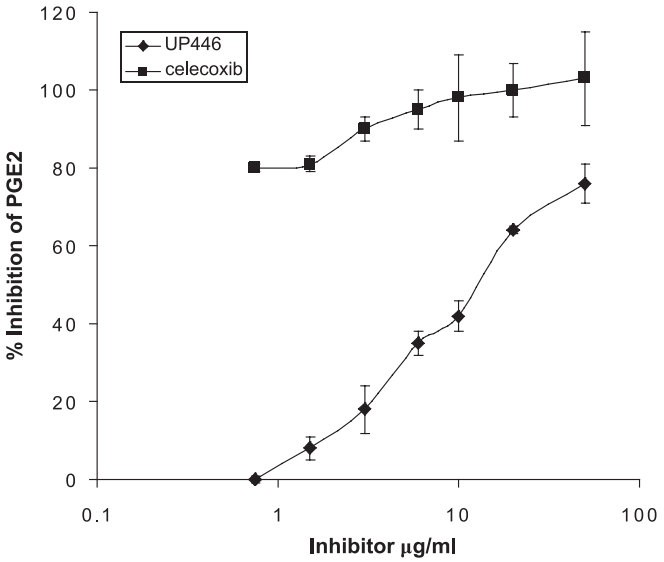


FIG. 6. Inhibition of PGE_2 production in HOSC cells by UP446 (◆) and celecoxib (■). TXB_2 activity was minimal because of indomethacin being added to supernatants.

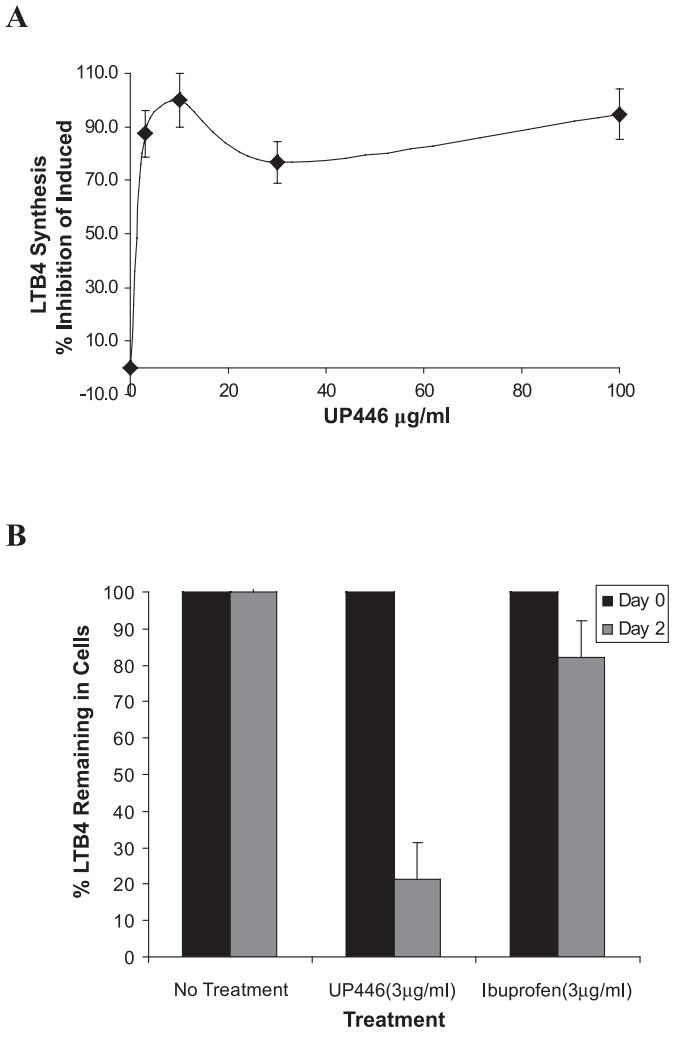


FIG. 7. (**A**) Titration of UP446 to inhibit newly synthesized LTB4 in THP-1 cells (◆). (**B**) Reduction of LTB_4 by UP446 versus ibuprofen at 3 μg/mL (0 days, black squares; 2 days, gray squares).

Bilateral intra-articular ankle injections of AA yielded a robust increase in ankle thickness of approximately 34% among animals gavaged with water compared to baseline. Overall, there was a significant difference between treatment groups ($F_{3,24} = 10.05$, $P < .001$), with AA-treated animals that received water by gavage displaying significantly greater inflammation than all other treatment groups (all $P < .04$). Importantly, the group pretreated with 200 mg/kg UP446 12 hours before AA injection displayed virtually no change in ankle thickness and was statistically indistinguishable from animals receiving no intra-articular AA injection ($P = .986$) or ethanol injection ($P = .67$; Fig. 8B). Therefore, UP446 completely blocks AA-induced increases

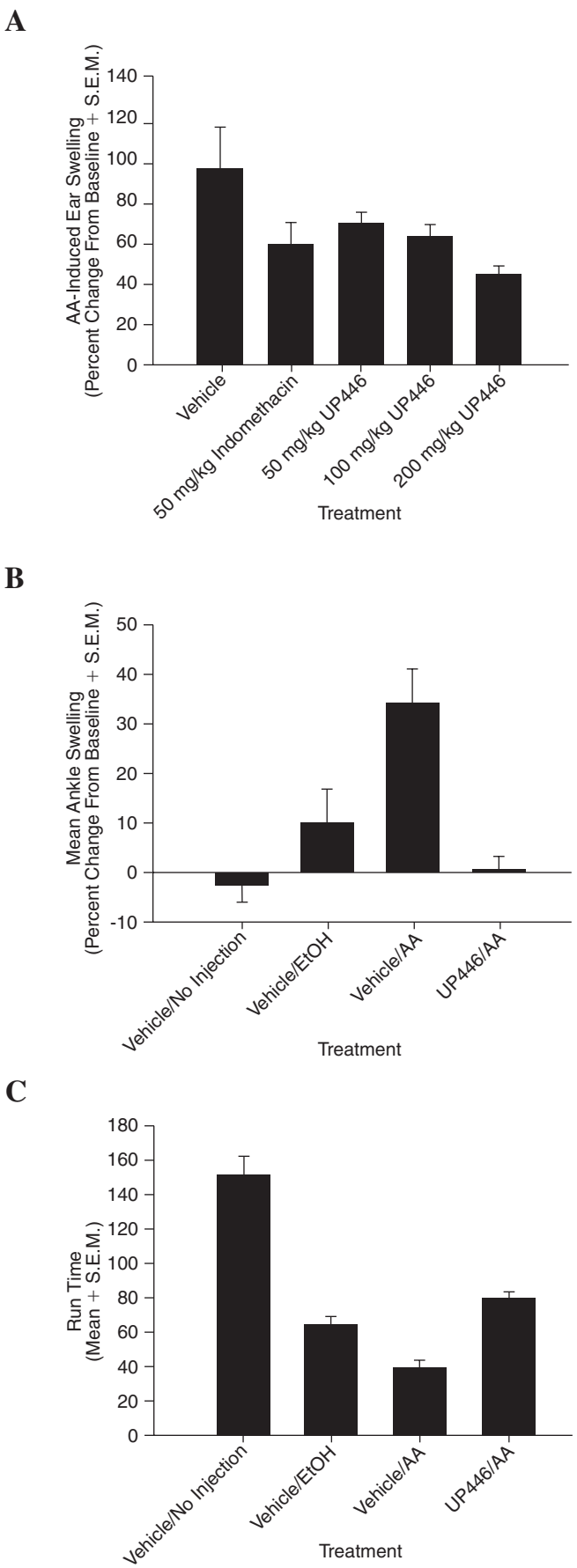


FIG. 8. (**A**) Titration of UP446 for reduction in topically applied AA-induced ear swelling. (**B**) Effect of UP446 on reduction of injected AA-induced ankle swelling. (**C**) Injected AA-induced ankle swelling timed run on a rotating drum. EtOH, ethanol.

in ankle edema. This effect was accompanied by a significant UP446-induced improvement in run time on the rotarod compared to animals gavaged with vehicle ($P < .001$; Fig. 8C). Both AA-injected animals pretreated with UP446 and animals receiving ethanol intra-articularly performed equivalently well on the rotarod task ($P = .287$); however, both of these groups performed significantly worse than uninjected controls ($P < .001$), likely because of the effects of the injection procedure. These data complement the enzyme and cellular assays and strongly support the in vivo anti-inflammatory potential of UP446 on measures of inflammation and locomotor performance.

DISCUSSION

Recently, there has been renewed interest in botanically derived products as sources of therapeutic agents because of safety concerns with synthetic drugs. Lack of characterization of agents with specific actions, especially in supplements from natural products, have made clinicians skeptical of their efficacy.[36] Only with proper studies regarding safety, mechanism of action, and efficacy can these compounds be accepted as medicinal therapeutics.

Baicalin is a free-B-ring flavonoid as it lacks any side chains in the B-ring of its structure (Fig. 2A). Baicalin has specifically been shown to down-regulate the expression of cytokines and PGE_2, nitric oxide formation, and neutrophil invasion in a carrageenan-induced paw edema model.[37] Similarly, baicalein has been shown to inhibit the production of LTC_4 in the same inflammatory model, suggesting an inhibitory effect on 5-LOX as well, though whether it was at the gene expression level or via enzyme inhibition was not determined.[38] Baicalein was also shown to reduce LTC_4 and LTD_4 in sensitized lungs of guinea pigs.[39] Extracts of *S. baicalensis* as well as isolated baicalin and baicalein seemed to inhibit COX-2 gene expression[40] but not COX-2 enzyme as assessed by PGE_2 production directly compared to celecoxib in head and neck squamous cell carcinoma cells *in vitro*.[41]

Catechin derivatives purified from green tea and black tea, such as epigallocatechin-3-gallate, epigallocatechin, and epicatechin-3-gallate showed inhibition of COX and LOX-dependent metabolism of AA in human colon mucosa and colon tumor tissues.[42] Green tea catechins, when added to the diets of Sprague-Dawley male rats, lowered the activity level of platelet phospholipase A_2 and significantly reduced platelet COX-1 levels.[43] Catechin and epicatechin reportedly weakly suppressed COX-2 gene transcription in human colon cancer DLD-1 cells.[44] Green tea extract has been formulated with seven other plant extracts for reducing inflammation by inhibiting the COX-2 enzyme, without identification of any of the specific active components.[45] It was reported that five flavan-3-ol derivatives, including (+)-catechin and gallocatechin, isolated from four plant species (*Atuna racemosa*, *Syzygium carynocarpum*, *Syzygium malaccense*, and *Vantanea peruviana*) exhibit equal to weaker inhibitory activity against COX-2, relative to COX-1, with IC_{50} values ranging from 3.3 μM to 138 μM.[46] (+)-Catechin, isolated from the bark of *Ceiba pentandra*, inhibited COX-1 with an IC_{50} value of 80 μM.[47] Even with this evidence, the exact mechanism for control of inflammation by free-B-ring flavonoids, such as baicalin, and flavan-3-ols, like (+)-catechin, has not fully been elucidated, and the combination of the two compounds has never been tested. Therefore, the present study represents an important extension of this work by determining the relative selectivity of these flavonoids as COX and 5-LOX inhibitors, as well as demonstrating their *in vivo* anti-inflammatory efficacy.

There is considerable evidence that selective COX-2 over COX-1 inhibition is associated with an increased risk for cardiovascular events, including edema, hypertension, stroke, and myocardial infarction.[48–50] This suggests that an excess of TXA_2, which acts as a vasoconstrictive molecule in the cardiovascular system over reduced vasodilatory PGI_2, as a consequence of selective COX-2 inhibition, is the primary reason for increased cardiovascular toxicity.[50] Evidence is also available that COX inhibition shunts AA metabolism toward the 5-LOX pathway generating LTB_4, which attracts neutrophils producing toxicity in various organ systems.[17,18] Therefore, dual inhibition of the COX and 5-LOX enzymes may provide therapeutic benefit with a greater degree of safety than currently available modalities.[24]

To this end, standardized extracts of *S. baicalensis* enriched for baicalin and *A. catechu* containing a high level of (+)-catechin were isolated and tested for their COX-1, COX-2, and 5-LOX enzyme inhibition capabilities. The anti-inflammatory capacity of these compounds was measured by their ability to inhibit the peroxidase activity of the COX enzymes. In both enzymes, the COX activity oxygenates AA to produce PGG_2, which then is converted to PGH_2 by the peroxidase activity followed by cell-specific synthetase conversion to individual PGs such as PGE_2 or the TX, TXA_2.[51] One could argue that inhibition of the peroxidase activity is not a direct measure of COX inhibition. Since the two enzyme activities are coupled and required in the enzyme to produce the end-product PGs, however, inhibition of one or both activities reflects down-regulation of overall COX enzyme activity.[31] Using this activity as a measure for COX enzyme inhibition, the defined extracts of *S. baicalensis* and *A. catechu* exhibited inhibition similar to that of pure compounds, baicalin and (+)-catechin, respectively, when tested on COX-1 and COX-2. This suggests that the inhibition activity, within the extracts, was due to each individual flavonoid component (Table 1). When the extracts were mixed at selected ratios, the combined extract exhibited the desired dual inhibition properties on COX and 5-LOX with balanced COX-1 and COX-2 inhibitory activity. One drawback of the current study is that we were unable to establish a true IC_{50} for the UP446 mixed extract on 5-LOX because of enzyme assay interference. Further work must be done to characterize the true inhibition of the mixed extract on 5-LOX enzyme.

Tests in cell models expressing PGE_2 and LTB_4 demonstrated that the observed enzyme inhibition activity was reflective of the mixed extract's COX and LOX inhibition ac-

tivity in a biological system at the cellular level. Coupled with the dose-dependent reduction in edema in the AA-induced ear swelling model, as well as in ankles injected intra-articularly with AA, which allowed animals to maintain a higher level of locomotor function over non-treated animals, these observations suggest that the extract containing both baicalin and (+)-catechin directly inhibits the production of inflammatory fatty acids by acting on the COX and LOX enzymes. Since elevated AA metabolism is part of the etiology of arthritis,[14–16] inhibition of the production of these inflammatory mediators via dual inhibition of the COX and LOX pathways may provide a way to manage OA safely while providing acceptable efficacy. Toxicological safety studies in animals and humans have shown the combination of *S. baicalensis* and *A. catechu* extracts to be safe.[34] Additional studies are under way to further clarify the exact mechanism of action of baicalin and (+)-catechin at the protein and gene expression levels, and clinical trials are in process to evaluate the therapeutic potential of this extract as a prescription medical food for the dietary management of OA.

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Primus Pharmaceuticals, Inc. markets a Food and Drug Administration-regulated medical food composed of a purified mixed extract of *S. baicalensis* and *A. catechu*. Unigen Pharmaceuticals, Inc. markets supplement ingredients composed of a less pure mixed extract of *S. baicalensis* and *A. catechu*. As such, all authors have an interest in their respective companies and the success of their products.

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