Anti-inflammatory Activity of Pectolinarigenin and Pectolinarin Isolated from Cirsium chanroenicum

Hyun Lim, Kun Ho Son, Hyeun Wook Chang, KiHwan Bae, Sam Sik Kang, and Hyun Pyo Kim

In order to identify the active anti-inflammatory ingredient(s) in Cirsium chanroenicum (Compositae), its methanol extract and several solvent fractions were prepared; the methanol extract and the ethylacetate fraction inhibited cyclooxygenase-2 (COX-2)-mediated prostaglandin E2 (PGE₂) and 5-lipoxygenase (5-LOX)-mediated leukotriene (LT) production in lipopolysaccharide-treated RAW 264.7 cells and A23187-treated rat basophilic leukemia (RBL-1) cells, respectively. Further bioactivity-guided fractionation of the ethylacetate fraction using column chromatography led to the isolation of pectolinarigenin (5,7-dihydroxy-4→6)-glucoside]. Pectolinarigenin strongly inhibited COX-2-mediated PGE₂ and 5-LOX-mediated LT production at >1 µg, indicating that it is a dual inhibitor of COX-2/5-LOX. However, pectolinarigenin did not affect COX-2 expression or nuclear transcription factor (NF-κB) activation. In addition, in vivo studies demonstrated that oral administration of these two compounds at 20—100 mg/kg resulted in similar inhibitory activities against several animal models of inflammation/allergy: arachidonic acid-induced mouse ear edema, carragenan-induced mouse paw edema and passive cutaneous anaphylaxis. All of these results suggest that pectolinarigenin and pectolinarin possess anti-inflammatory activity and that they may inhibit eicosanoid formation in inflammatory lesions. These activities certainly contribute to the anti-inflammatory mechanism of C. chanroenicum.

Key words Cirsium chanroenicum; cyclooxygenase; lipoxygenase; pectolinarigenin; pectolinarin; anti-inflammation

Among various proinflammatory chemical mediators, eicosanoids [prostaglandin (PG) and leukotriene (LT)] synthesized from arachidonic acid (AA) play an important role in many inflammatory/allergic disorders. For instance, PGs synthesized by cyclooxygenases (COX) are involved in acute and chronic inflammation, and LTs synthesized by lipoxygenases (LOX) are responsible for some allergic conditions such as bronchial asthma. Particularly, COX-2 (an inducible isoform of COX) and 5-LOX produce high amounts of PGs and LTs, respectively, in pathological lesions. Thus, an evaluation of the effects of potential anti-inflammatory agents on AA metabolism is worthwhile.

The aerial parts of Cirsium chanroenicum have been used for detoxification, to treat fever, and to enhance blood circulation in Chinese medicine. Various constituents including flavones, triterpenes, wax, polyolefins and some acetylenes were previously isolated from the genus Cirsium; several pharmacological activities of this genus were also described. The enhancing effect on hepatic alcohol-metabolizing activity was described for the methanol extract of C. japonicum. Flavonoids from the flowers of C. rivulare showed antimicrobial activity. However, phytochemical and pharmacological investigations of C. chanroenicum have not been described to date.

During a preliminary screening procedure, the methanol extract of C. chanroenicum was found to inhibit COX-2-catalyzed PGE₂ and 5-LOX-catalyzed LTB₄ production in vitro. Accordingly, the present investigation was designed to find the active principle(s) and their anti-inflammatory activities.

MATERIALS AND METHODS

Chemicals N-[2-Cyclohexyloxy-4-nitrophenyl]methane sulfonamide (NS-398) was obtained from Biomol (Plymouth Meeting, PA, U.S.A.). Nordihydroguaiaretic acid (NDGA), A23187, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), indomethacin, prednisolone, arachidonic acid (AA, 99%), λ-carrageenan and LPS (Escherichia coli 0127:B8) were purchased from Sigma (St. Louis, MO, U.S.A.). DMEM and other cell culture reagents including FBS were products from Gibco BRL (Grand Island, NY, U.S.A.). The protein assay kit was purchased from Bio-Rad (Hercules, CA, U.S.A.). All test compounds were dissolved in DMSO and diluted with serum-free DMEM to the appropriate concentrations. The final concentration of DMSO in the culture medium was adjusted to 0.1% (v/v).

Animals Male Sprague-Dawley (SD) rats and ICR mice (4 weeks old, specific pathogen-free) were obtained from Orient-Bio Co. (Korea). Animals were fed with standard laboratory chow (Purina Korea) and water ad libitum. They were acclimatized in an animal facility (KNU) maintained at 20—22 °C, 40—60% relative humidity and 12 h/12 h (light/dark) cycle for at least 7 days prior to the experiments.

Extraction and Isolation of Active Constituents C. chanroenicum (Compositae) was collected on Mt. Dokyu (Sep., 2000) and identified by Dr. Bae, one of the authors. The voucher specimen (CNU 2151-1) was deposited at the College of Pharmacy, Chungnam National University. The aerial parts of C. chanroenicum (2.5 kg) were percolated with methanol three times and concentrated in vacuo. The dried residue (200.1 g) was suspended in H₂O and partitioned suc-
cessively with n-hexane, CH₂Cl₂, EtOAc and n-butanol, to give n-hexane (46.5 g), CH₂Cl₂ (6.5 g), EtOAc (31.3 g) and n-butanol (31.8 g) soluble fractions, respectively. A portion of the EtOAc fraction (25.3 g) was subjected to silica gel column chromatography eluted with CH₂Cl₂: methanol (100: 0→70: 10, gradient) to yield nine fractions (E1→E9). Fraction E1 was rechromatographed on a silica gel column (100:0→104.3) to give three subfractions (E1.1→E1.3). E1.2 was subjected to Sephadex LH-20 column chromatography eluted with methanol to yield pectolinarigenin (Fig. 1). Fraction E8 was recrystallized with methanol to give pectolinanin. The chemical structures of pectolinarigenin and pectolinanin were verified by comparison of the NMR data with those reported in the literature.[2,13]

Pectolinanin: ¹H-NMR (DMSO-d₆, 300 MHz) δ: 3.74, 3.84 (3H each, s, OCH₃), 6.59 (1H, s, H-8), 6.83 (1H, s, H-3), 7.08 (2H, d, J=9.0 Hz, H-9’), 7.99 (2H, d, J=9.0 Hz, H-2’, 6’), 13.01 (1H, s, 5-OH). ¹³C-NMR (DMSO-d₆, 75.5 MHz) δ: 163.5 (C-2), 103.2 (C-3), 182.3 (C-4), 152.6 (C-5), 132.7 (C-6), 156.4 (C-7), 94.5 (C-8), 152.9 (C-9), 104.3 (C-10), 123.0 (C-1’), 128.4 (C-2’, 6’), 114.7 (C-3’, 5’), 162.5 (C-4’), 60.1 (OCH₃), 55.7 (OCH₃).

Pectolinanin: ¹H-NMR (DMSO-d₆, 300 MHz) δ: 12.93 (1H, s, 5-OH), 8.04 (2H, d, J=8.9 Hz, H-2’, 6’), 7.17 (2H, d, J=8.9 Hz, H-3’), 6.94 (1H, s, H-8), 6.90 (1H, s, H-3), 5.12 (1H, d, J=6.9 Hz, anomic H of glucose), 4.58 (1H, s, anomic H of rhamnose), 3.87 (3H, s, OCH₃), 3.78 (3H, s, OCH₃). ¹³C-NMR (DMSO-d₆, 75.5 MHz) δ: 164.0 (C-2), 103.3 (C-3), 182.2 (C-4), 152.1 (C-5), 132.7 (C-6), 156.4 (C-7), 94.3 (C-8), 152.4 (C-9), 105.8 (C-10), 122.7 (C-1’), 128.3 (C-2’), 114.7 (C-3’), 162.3 (C-4’), 114.7 (C-5’), 128.3 (C-6’), 60.2 (6-OCH₃), 55.4 (4’OCH₃), 100.3 (glu-1’), 73.1 (glu-2’), 76.4 (glu-3’), 69.5 (glu-4’), 75.7 (glu-5’), 65.9 (glu-6’), 100.4 (rha-1’), 70.3 (rha-2’), 70.7 (rha-3’), 71.9 (rha-4’), 68.2 (rha-5’), 17.6 (rha-6’).

RAW 264.7 Cell Culture and Measurement of PGE₂ Concentrations RAW 264.7 cells obtained from the American Type Culture Collection (ATCC, Rockville, MD, U.S.A.) were cultured in DMEM supplemented with 10% FBS and 1% antibiotics under 5% CO₂ at 37°C based on previously described procedures.[14] Briefly, cells were plated in 96-well plates (2×10⁵ cells/well). After pre-incubation for 2 h, the test compounds and LPS (1 μg/ml) were added and incubated for 24 h unless otherwise specified. Cell viability was assessed by the MTT assay as described previously.[15] From the media, PGE₂ concentration was measured using an ELISA kit for PGE₂ (Cayman Chem.) according to the manufacturer's instructions. To measure direct inhibition of COX-2, the cells were incubated with LPS (1 μg/ml) for 24 h. After the cells had been completely washed with serum-free DMEM, the test compounds and AA (100 μM) were added to COX-2-preinduced RAW 264.7 cells. Fifteen minutes later, PGE₂ concentration was measured in the media.

5-LOX Assay In order to evaluate the inhibitory activity against 5-LOX, rat basophilic leukemia cells (RBL-1) purchased from ATCC were cultured in RPMI 1640 with 10% FBS, 2 mM glutamine and 1% antibiotics under 5% CO₂ at 37°C. The cells were plated in 96-well plates for 2 h. The test compounds were added and preincubated for 10 min. To activate 5-LOX, A-23187 (3 μM) was added and the cells were incubated for 15 min with a slight modification of the previously described procedure.[16] Media was collected and the concentration of the 5-LOX products, cysteinyl leukotrienes (LTC₄/D/E), was measured using an ELISA kit (Cayman Chem.) as recommended by the manufacturer.

Western Blot and Electrophoretic Mobility Shift Assay (EMSA) Western blotting was used to measure COX-2 expression.[14] RAW cells were cultured in 6-well plates (5×10⁵ cells/well) in the presence or absence of LPS (1 μg/ml) with/without test compounds for 20 h. After preparing cell homogenate, the supernatant was obtained by centrifugation at 15000 g for 30 min. Proteins were electrophoresed in Tris–glycine gels (8%) and bands were blotted to PVDF membranes. The membranes were incubated with COX-2 antibody (No-160116, Cayman Chem.) and the bands were visualized by ECL-plus reagent (Amersham, Buckinghamshire, U.K.) and subsequent exposure to X-ray film. For EMSA analysis, RAW cells were treated with LPS and various concentrations of test compounds for 3 h. To prepare nuclear fractions, the cells were washed with PBS, harvested and resuspended in 400 μl of buffer A (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, pH 7.9) for 15 min on ice. After 10% NP-40 (25 μl) was added, the tubes were vortexed vigorously for 10 s. The nuclei were collected by centrifugation at 5000 rpm for 3 min and the supernatant was saved as the cytosolic fraction. The nuclei were lysed in buffer B (20 mM HEPES, 0.4 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, pH 7.9). The nuclear transcription factor-kB (NF-kB) consensus oligonucleotide (Promega, Madison, WI, U.S.A.) was phosphorylated by T4 polynucleotide kinase (10 units) with 10 μCi of [γ-³²P] ATP (3000 Ci/mmol) at 37°C for 10 min. Unincorporated oligonucleotides were removed by Microspin G-25 column (Amersham). Nuclear extract containing 5 μg protein was incubated with ³²P-labeled NF-kB consensus oligonucleotide in gel shift binding buffer at room temperature for 20 min. The incubation mixture was subjected to electrophoresis on a 4% polyacrylamide gel in TBE buffer (0.5×) at 350 V. The gel was dried and exposed to X-ray film overnight at –70°C.

In Vivo Studies The experimental designs using animals were approved by the Committee for Animal Experimentation of the College of Pharmacy, Kangwon National University (CN-2008/19). The ethical guidelines described in the KFDA Guide for Care and Use of Laboratory Animals were followed throughout the experiments.

Arachidonic Acid-Induced Ear Edema in Mice: In order to
evaluate the inhibitory activity of pectolinarigenin and pectolinarin in an animal model of acute inflammation, mouse arachidonic acid-induced ear edema was employed as previously described. Briefly, 2% arachidonic acid in acetone was topically applied to ears of mice (20 μl/ear). One hour later, ear thickness was measured using a dial thickness gauge (Mitutoyo, Japan). Test compounds dissolved in DMSO (0.05 ml/mouse) were administered orally 1 h prior to arachidonic acid treatment.

λ-Carrageenan (CGN)-Induced Paw Edema in Mice: To further establish the anti-inflammatory activity of the test compounds against acute inflammation, the mouse CGN-induced paw edema assay was also used with slight modification from the original procedures. Test compounds were administered orally to mice. One hour later, 1% CGN (w/v) dissolved in pyrogen-free sterile saline solution (0.05 ml/paw) was injected in the right hind paw; after 5 h, paw volume was measured using a plethysmometer (Ugo Basile, Italy). The increase in paw volume from the initial non-treated paw volume was regarded as edema.

Passive Cutaneous Anaphylaxis (PCA) in Rats: To examine anti-allergic activity, the backs of the rats were shaved and injected intradermally with monoclonal anti-dinitrophenyl (DNP) mouse IgE (100 μl/site, 1:1000 dilution). After 48 h, PCA was induced by intravenous injection of the antigen (1 mg of DNP-bovine serum albumin) in PBS containing 1% Evans blue. Test compounds were orally administered twice 1 h prior to each IgE and antigen injection. Thirty minutes after antigen challenge, the skin was removed. Dye that leaked into the skin was extracted and quantified as previously described.

Statistical Analysis Experimental values were represented as arithmetic mean ± S.D. One-way ANOVA followed by Dunnett’s test was used to determine the statistical significance.

RESULTS

Inhibition of PGE₂ and LT Production Treatment of the mouse macrophage-like cell line, RAW 264.7, with LPS (1 μg/ml) for 24 h induces COX-2, producing large amounts of PGE₂. Also, upon activation with A23187 (calcium ionophore), RBL-1 cells produce great amounts of cysteinyl-LTs via 5-LOX. For example, in our experiments, the concentrations of PGE₂ and LTCl₂/D₂/E₁ increased to 18.8 ± 2.3 nM and 1872.2 ± 50.3 pg/ml from the control levels of 1.3 ± 0.2 nM and 51.2 ± 17.7 pg/ml, respectively. Under these conditions, we examined the methanol extract of C. chamaeactinum and its solvent fractions for their inhibitory activities. Among the samples tested, the methanol extract and the ethylacetate fraction considerably inhibited PGE₂ and LT production (Table 1). Based on these results, the ethylacetate fraction was selected for further isolation of the active principle(s).

Through activity-guided isolation from the ethylacetate fraction using column purification, pectolinarigenin was successfully isolated, along with pectolinarin. Table 2 shows that pectolinarigenin significantly inhibited COX-2-mediated PGE₂ and 5-LOX-mediated LT synthesis at >1 μM, whereas pectolinarin exhibited no inhibitory activity at concentrations up to 50 μM. As expected, the reference compounds, NS-398 (selective COX-2 inhibitor) and NDGA (LOX inhibitor), potentely inhibited COX-2 and 5-LOX, respectively. No significant cytotoxicity was observed in any groups tested in these experiments as measured by MTT assay (data not shown). To elucidate the mechanism of inhibition of PGE₂ production by pectolinarigenin, COX-2 expression was examined by Western blotting and the effect on NF-κB activation was measured by EMSA analysis. As shown in Fig. 2, pectolinarigenin did not affect COX-2 expression or NF-κB activation, clearly indicating that the inhibition of COX-2-mediated PGE₂ production was not due to COX-2 down-regulation. On the other hand, pectolinarigenin did significantly inhibit PGE₂ production from COX-2-preinduced RAW cells (44.8% inhibition at 50 μM), while NS-398 showed 73.8% inhibition at 0.1 μM. These results suggest that pectolinarigenin may directly inhibit COX-2 enzymatic activity. Therefore, pectolinarigenin is likely a dual inhibitor of COX-2/5-LOX.

In Vivo Anti-inflammatory Activity To establish the in vivo anti-inflammatory activities of these compounds, pectolinarigenin and pectolinarin were tested in COX-2/5-LOX inhibitor-sensitive animal models of inflammation/allergy: arachidonic acid-induced ear edema, CGN-induced paw edema, and passive cutaneous anaphylaxis. Table 3 lists the in vivo anti-inflammatory activities of pectolinarigenin and pectolinarin. By oral administration, both significantly inhib-
without allergen treatment showed absorbance, 0.291.

**Table 4.** Inhibition of Passive Cutaneous Anaphylaxis in Rats

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose (mg/kg)</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allergen-treated</td>
<td>—</td>
<td>0.807±0.139 (—)</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>20×2</td>
<td>0.439±0.256 (71.3)</td>
</tr>
<tr>
<td>Pectolinarigenin</td>
<td>20×2</td>
<td>0.648±0.228 (30.8)</td>
</tr>
<tr>
<td>Pectolinarin</td>
<td>20×2</td>
<td>0.662±0.085* (28.2)</td>
</tr>
</tbody>
</table>

All compounds dissolved in DMSO were administered orally. Negative control group without AA and CGN treatment showed 0.239±0.133. a) n=8 (arithmetic mean±S.D.). b) % inhibition compared to the allergen-treated control group. * p<0.05, significantly different from the allergen-treated control group.

**DISCUSSION**

The present investigation clearly demonstrates that pectolinarigenin and pectolinarin exhibit anti-inflammatory activity with similar potencies in animal models of inflammation and allergy, but are less potent than the reference drugs, indomethacin and prednisolone. The cellular mechanism of anti-inflammatory action of pectolinarigenin was, at least in part, due to COX-2/5-LOX inhibition that did not affect COX-2 expression. However, pectolinarin, a glycoside derivative of pectolinarigenin, did not inhibit COX-2/5-LOX in cell culture experiments, although it did show in vivo anti-inflammatory activity. Similar findings were previously reported. For example, flavonoid aglycones strongly inhibited lymphocyte proliferation and iNOS-catalyzed NO production in cell cultures, while their glycosides generally showed much less or no inhibition. However, oral administration of flavonoid glycosides sometimes gave similar or even higher in vivo activity compared to the corresponding flavonoid aglycones. This phenomenon may be caused by the inaccessibility of flavonoid glycosides to the inside of the cell due to their hydrophilicity. On the other hand, orally administered flavonoid glycosides may enter the circulation through the intestinal wall, probably after hydrolysis of their glycosidic portion. The corresponding flavonoid aglycones and their methylated derivatives were found in the bloodstream, exerting in vivo pharmacological activity. Therefore, orally administered pectolinarigenin and pectolinarin may enter the bloodstream in the aglycone form (pectolinarin) and/or its related derivatives, thus showing similar potencies for anti-inflammatory activity in vivo.

Many flavonoid derivatives have previously been shown to possess anti-inflammatory activity in vitro as well as in vivo. Their anti-inflammatory cellular mechanisms include inhibition of eicosanoid metabolizing enzymes such as phospholipase A2, COXs and LOXs. In particular, flavonoids with a 5,7-dihydroxy substitution and C-6 hydroxyl/methoxyl group possess 5-LOX inhibitory activity. For instance, baicalein (5,6,7-trihydroxyflavone) was previously reported to inhibit 5-LOX activity. Some C-5,6,7-substituted flavones that have an alkoxy group at C-5 or C-6 also inhibit 5-LOX. These synthetic flavones with an alkoxy group with 5—10 carbons had higher 5-LOX inhibitory activity. The structurally similar flavone, nepetin (3’,4’,5,7-tetrahydroxy-6-methoxyflavone), inhibited COX and LOX. In accordance with these reports, pectolinarigenin, which has a C-6 methoxyl group, inhibits 5-LOX. All of these findings suggest the importance of C-6 substitution with a hydroxyl/methoxyl group for anti-inflammatory action. While pectolinarin did not affect COX-2 expression, the structurally-related flavonoids, oroxylin A (5,7-dihydroxy-6-methoxyflavone) and wogonin (5,7-dihydroxy-8-methoxyflavone), were previously reported to down-regulate COX-2 and iNOS from the activated macrophages. Thus, it is suggested that B-ring substitution in pectolinarin may abolish the down-regulating capacity.
It is well known that COX/LOX inhibitors inhibit AA-induced ear edema and CGN-induced paw edema, while 5-LOX inhibitors and steroidal anti-inflammatory drugs such as prednisolone are active against PCA.\textsuperscript{31,32} Pectolinarigenin and pectolinarin exhibited considerable inhibitory activity against these animal models, suggesting that they may also behave as COX/LOX inhibitors \textit{in vivo}.

In this study, pectolinarigenin and pectolinarin were successfully isolated as the active anti-inflammatory principles from the aerial parts of \textit{C. chanroenicum}. Pectolinarigenin and pectolinarin were also found to be the constituents of other Compositae family members\textsuperscript{33)}, isolated from \textit{C. japonicum} DC they inhibited tumor formation in mice.\textsuperscript{35} Pectolinarin was previously found to possess \textit{in vivo} anti-inflammatory and analgesic activities.\textsuperscript{35} Recently, pectolinarigenin and pectolinarin isolated from \textit{C. setidens} were demonstrated to reduce galactosamine-induced hepatic injury in rats.\textsuperscript{36} To date, however, no study concerning the anti-inflammatory action of \textit{C. chanroenicum} and pectolinarigenin has been described. This is the first report to describe phytochemical and pharmacological activity of \textit{C. chanroenicum}, and to demonstrate anti-inflammatory activity and possible cellular mechanisms of pectolinarin.

In conclusion, pectolinarigenin and pectolinarin were successfully isolated from the aerial parts of \textit{C. chanroenicum}. Pectolinarigenin strongly inhibited COX-2-mediated PGE\textsubscript{2} synthesis and 5-LOX-catalyzed LT production from LPS-treated RAW 264.7 cells, leading to reduced eicosanoid production. In addition, oral administration of pectolinarigenin and pectolinarin inhibited animal models of inflammation and allergy. Therefore, these constituents may contribute to the anti-inflammatory action of \textit{C. chanroenicum} at least in part by inhibiting eicosanoid synthesis. They also have potential as new anti-inflammatory/anti-allergic agents since COX inhibitors are used as anti-inflammatory agents and 5-LOX inhibitors show anti-allergic activity.

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\textbf{REFERENCES}