The Anti-gastropathic and Anti-rheumatic Effect of Niga-ichigoside F1 and 23-Hydroxytormentic Acid Isolated from the Unripe Fruits of Rubus coreanus in a Rat Model

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This study was undertaken to produce the clinical merits of two natural antinociceptive anti-inflammatory triterpenoids which synthetic anti-inflammatory drugs do not have. The triterpenoid glycoside niga-ichigoside F1 (NIF1) and its aglycone 23-hydroxytormentic acid (23-HTA), which were isolated from the unripe fruits of Rubus coreanus (Rosaceae), reduced rheumatoid arthritis (RA) factor and C-reactive protein (CRP) factor in Freund’s complete adjuvant reagent-induced rats, suggesting that these two triterpenoids had an anti-rheumatic effect. It was also shown that treatment with NIF1 or 23-HTA reduced gastric lesion extent, acidity and total gastric acid output induced by EtOH plus sodium salicylate in a gastric secretion test. Moreover, 23-HTA had a greater effect than the glycoside, NIF1. To clarify the anti-gastropathic mechanism of these two compounds, their free radical scavenging activities in the gastric mucosa were examined in a rat EtOH–sodium salicylate-induced gastropathy model. The two compounds significantly increased superoxide dismutase and glutathione peroxidase activities, indicating that the healing effects of NIF1 and 23-HTA against gastropathy are associated with free radical scavenging enzyme activities. These results support the notion that the long-term administration of NIF1 or 23-HTA should overcome the adverse effects of synthetic anti-inflammatory drugs.

Key words Rubus coreanus; Rosaceae; niga-ichigoside; 23-hydroxytormentic acid; anti-gastropathic; anti-rheumatic

The majority of synthetic steroidal or non-steroidal anti-inflammatory drugs (SAIDs or NSAIDs) are used to treat arthritis and neurotic pain. These drugs exhibit anti-inflammatory actions by reducing prostaglandin biosynthesis due to the inhibition of cyclooxygenase (COX) activity.1—3) However, it is very well known that these drugs may cause gastritis or gastric ulcer after long-term oral administration.

It has been reported that niga-ichigoside F1 (NIF1) and 23-hydroxytormentic acid (23-HTA) isolated from Rubus coreanus had anti-inflammatory effects in a carrageenan-induced acute inflammatory animal model in addition to their anti-nociceptive actions. Chemically, NIF1 is known as 23-hydroxytormentic acid 28-O-glucoside, and the aglycone 23-HTA is classified as the 19α-hydroxyursane-type triterpenoid.4,5)

23-HTA has only one more hydroxyl at the 19-position than asiatic acid, and asiaticoside, a glycoside of asiatic acid, has well-known wound-healing properties.5,6) Although the healing effect of asiaticoside in gastropathies has been previously reported,7,8) the effects of NIF1 and 23-HTA in this context have not been reported to date. This presents a research study designed to demonstrate the anti-gastropathic activity of these two multihydroxursane-type triterpenoids in terms of developing naturally occurring anti-inflammatory therapeutics that do not cause gastric damage even after long-term administration. We also performed an antirheumatoid arthritic activity test using Freund’s complete adjuvant reagent (FCA) in rats. In addition, the pharmacological effects of these two compounds were observed on gastric lesions, gastric secretion and on free radical scavenging enzyme activities in an EtOH plus sodium salicylate-treated rat gastropathy model.

MATERIALS AND METHODS

Reagents and Instruments The following reagents were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.): bovine serum albumin, NADPH, GSH (reduced and oxidized form of glutathione), SDH (sodium dodecyl sulfate), sodium salicylate, EDTA, DNTB (5,5’-dithiobis-2-nitrobenzoic acid), methylprednisolone, KH2PO4, K2HPO4, absolute alcohol. FCA was purchased from Difco Co., U.S.A. UV absorbance was measured using a Shimadzu UV-120 unit. The refrigerated centrifuge and ultracentrifuge used were a Hanil supro 22K and a Hitachi 70P-71, respectively.

Plant Material and Isolation of NIF1 and 23-HTA The roots of Rubus coreanus were purchased from a herb store in Korea and identified by Professor Sang-Cheol Lim (Department of Botanical Resources, Sangji University, Wonju, Korea). A voucher specimen (# natchem-23) has been deposited at the Laboratory of Natural Product Analysis, Department of Botanical Resources, Sangji University, Korea. The dried roots of R. coreanus (2.0 kg) were chopped and extracted three times with MeOH under reflux for 5 h. The filtered total extract was concentrated under reduced pressure and finally freeze-dried to give 10 g of a MeOH extract. Sequential solvent fractionation of the MeOH extract gave a CHCl3 extract, an EtOAc extract and a BuOH extract (23 g).
and silica gel column chromatography of the BuOH extract (20 g) yielded NIF₁ (2.3 g). Acid hydrolysis of the isolated compound (1.5 g) then afforded 23-HTA (0.8 g). A detailed description of the isolation and purification process has previously been reported.⁹

**Animals** Sprague–Dawley male rats with 150—250 g body weight as SPF (specific pathogen free) were purchased from Daehan Biolink Co. (Eumseong, Korea), adapted at 22±2 °C, 40—60% humidity under a 12 h light/dark cycle for 2 weeks and used for the experiment. These animals were cared for according to the “Guide for the Care and Use of Laboratory Animals” issued by the American Institute of Laboratory Animal Resources.

**Induction of Rheumatoid Arthritis** Freund’s complete adjuvant reagent (FCA; Difco, Detroit, Michigan, U.S.A.) was subcutaneously injected in 100 µl/kg body weight dose into the right hind paws to prepare a rheumatoid arthritis animal model. Two weeks after FCA administration the induction of rheumatoid arthritis was confirmed. Methylprednisolone (MTP), a synthetic corticosteroid, was used as the positive control. After 12 h starvation following final treatment of the samples, the animals were anesthetized and the blood was sampled from abdomen aortas. Blood samples were stored in CBC bottles and the remaining samples were coagulated at room temperature by standing for 30 min. The serum so obtained was collected by centrifuging at 600 × g for 15 min.

**RA Test and CRP Test** Rheumatoid arthritis (RA) testing was carried out utilizing the agglutination reaction between γ-globulin and rheumatoid factor. C-reactive protein (CRP) factor was measured using an antibody to purify CRP by latex slide test in serum using a commercial kit (Randox, U.K.).

**Induction of Gastropathy** The rats with gastropathy were induced by oral administration of 4 ml/kg EtOH and 200 mg/kg sodium salicylate with zonde once a day for 14 d. To prevent normal healing, saline (0.9% NaCl solution) was orally administered for 7 d. After the last administration of EtOH plus sodium salicylate, 10 and 30 mg/kg of NIF₁ or 23-HTA solutions were orally administered once a day for 7 d. Vehicle control group animals were treated with saline for 21 d.

**Measurement of Gastric Lesion** Rats used for the experiment were fasted for 24 h after the last administration, euthanized with CO₂ gas, and then stomachs were excised as described by Mizui and Dodeuchi,⁷ and fixed in 2% formalin solution for 10 min. After incising the greater curvature, extent of gastric damage in the glandular region was defined as the gastric lesion index.

**Measurement of Gastric Juice Secretion** After rats (230 g body weight) subjected to the treatment had been fasted for 24 h, they were anesthetized with ether and then the pylorus was ligated. The volume of gastric secretion was measured using the method described by Dai and Ogle.⁵ In brief, the abdomens of an anesthetized rat were opened, the pylorus was ligated, and then sealed up after the sample solutions (10 and 30 mg/kg of NIF₁ or 23-HTA) had been placed in the duodenal tract. Four hours after the sealing-up of the abdomens, the rats were anesthetized with ether, the stomachs were excised, and gastric juices were collected. These were centrifuged at 3000 rpm, and then the gastric juice volumes, pHs, and acidities were measured and total acid output was calculated. Acidity and total acid output were determined by titration versus 0.05 N NaOH using phenolphthalein as indicator.

**Preparation of Enzyme Sources** Excised stomachs were washed with saline, wiped with a filter paper, and then finely cut with scissors. Four volumes of 0.1 M KP buffer (potassium phosphate buffer pH 7.4) was poured into the stomach tissue and the tissue was then macerated in ice using a homogenizer. The homogenate was centrifuged for 10 min (600 × g), nuclei were removed, and again centrifuged for 20 min (10000 × g) to yield the mitochondrial fraction. Ultra-centrifugation of this fraction for 1 h provided the enzyme source as supernatant.

**Measurement of TBARS** The thiobarbituric acid-reactive substance (TBARS) was measured as a marker of lipid peroxidation by the method of Okawara et al.⁷ 0.4 ml of 10% liver homogenate in 0.9% NaCl was added to 1.5 ml of 8.1% sodium dodecyl sulphate, 1.5 ml of 20% acetic buffer (pH 3.5) and 1.5 ml of 0.8% TBA solution. The mixture was heated at 95 °C for 1 h. After cooling, 5.0 ml of n-butanol–pyridine (14:1) was added for extraction, and the absorbance of the n-butanol–pyridine layer at 532 nm was measured for the determination of TBA reactive substance.

**Measurement of Superoxide Dismutase (SOD) Activity** Total SOD activity was measured by determining the ability to inhibit the auto-oxidation of pyrogallol using the method described by Marklund and Marklund.⁸ The rate of auto-oxidation was determined by measuring increases in the absorbance at 420 nm. Reaction mixture containing 0.2 mM pyrogallol in 50 mM Tris–cacodylic acid buffer (pH 8.5) and 1 mM diethylenetriaminepentaacetic acid was incubated for 90 s at 25 °C. One unit of SOD activity was defined as the amount of the enzyme required to inhibit the rate of pyrogallol auto-oxidation by 50%.

**Measurement of Glutathione Peroxidase (GPx) Activity** Glutathione peroxidase activity was measured based on the method described by Paglia and Valentine.¹¹ In brief, the reagents of H₂O₂, 1 mM GSH, 0.2 mM NADPH and the enzyme source were added to 0.1 M Tris–HCl buffer solution (pH 7.2) and reacted at 25 °C for 5 min. NADPH consumed by the reduction of the oxidized form of glutathione (GSGS) was determined by measuring absorbance at 340 nm and GPx activity was calculated. Enzyme activity is quoted as the units of NADPH oxidized nmol/l mg protein/min.

**Protein Quantification and Statistics** Protein levels were quantified using bovine serum albumin as a standard using the method of Lowry et al.¹² Data are presented as means±S.D., and statistical significances were analyzed using Duncan’s multiple range test.

**RESULTS**

**Antirheumatoid Arthritic Effect** As shown in Table 1, RA- and CRP factors were considerably higher in the FCA-only treated group. The two compounds, NIF₁ or 23-HTA, and a standard drug, MTP (the positive standard), reduced these factors, suggesting that these compounds are effective against the rheumatic disease. 23-HTA exhibited a more pronounced effect than NIF₁.

**Effect on Gastric Lesions** The control group treated
with EtOH–sodium salicylate only had a lesion index of 78.9 \pm 18.5 \text{mm}. NIF1 produced no significant decrease in lesion index at 10 mg/kg dose daily, but it caused a significant decrease at a 30 mg/kg dose. However, significant decreases were observed for 23-HTA at both 10 and 30 mg/kg. These results suggest that 23-HTA has a more potent effect in anti-gastropathic effect than NIF1. In particular, 23-HTA potently decreased gastric lesion indices by 30.6 \pm 8.5 \text{mm} at 30 mg/kg. In this experiment, 23-HTA exhibited a stronger inhibitory effect than NIF1 (Table 2).

**Effect on Gastric Juice Secretion** Table 3 shows the effect of the two compounds on gastric secretion in the rat. NIF1 or 23-HTA significantly decreased gastric secretion volumes, total acid output and gastric juice acidity. The compound 23-HTA also showed a more potent effect than NIF1. However, no significant change in pH of the gastric juice was observed by the sample treatments, though cimetidine (H2-antagonist, a positive control) increased the pH.

**Effect on TBARS** Figure 2 shows the effect of NIF1 and 23-HTA on lipid peroxidation in gastric tissue. TBARS value was observed as 4.97 \pm 0.98 \text{nmol/mg protein} in the vehicle only-treated group, but as 11.3 \pm 1.49 \text{nmol/mg protein} in the control group. These results indicate that oral treatment with EtOH plus sodium salicylate caused lipid peroxidation in gastric tissue. However, treatment with NIF1 and 23-HTA (each, 10 or 30 mg/kg, p.o.) significantly inhibited lipid peroxidation caused by EtOH plus sodium salicylate, respectively, and increased dosage of these compounds exerted even more significant effects. The compound of 23-HTA had a more potent effect than NIF1.

**Effect on SOD Activity** The effect of NIF1 and 23-HTA on SOD activities is shown in Fig. 2. The SOD activity in a vehicle only-treated group was 7.98 \pm 1.01 unit/mg protein, and that of the control group was 3.92 \pm 0.86 unit/mg protein. This result indicates that the induction of gastropathy caused a considerable decrease in the free radical-scavenging activity of gastric tissue. However, treatment with NIF1 and 23-HTA significantly inhibited lipid peroxidation caused by EtOH plus sodium salicylate, respectively, and increased dosage of these compounds exerted even more significant effects. The compound of 23-HTA had a more potent effect than NIF1.

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### Table 1. Inhibitory Effect of Niga-ichigoside F1 and 23-Hydroxytormentic Acid on Freund’s Complete Adjuvant-Induced Edema of the Hind Paw in Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg, p.o.)</th>
<th>RA</th>
<th>CRP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>NIF1</td>
<td>10</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>23-HTA</td>
<td>10</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Methylprednisolone</td>
<td>10</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

### Table 2. Effect of Niga-ichigoside F1 and 23-Hydroxytormentic Acid on EtOH Plus Sodium Salicylate-Induced Gastric Lesion in Rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Lesion index (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>78.9 \pm 18.5</td>
</tr>
<tr>
<td>NIF1</td>
<td>10</td>
<td>75.2 \pm 10.6</td>
</tr>
<tr>
<td>23-HTA</td>
<td>10</td>
<td>53.7 \pm 7.4</td>
</tr>
<tr>
<td>Cimetidine</td>
<td>100</td>
<td>6.2 \pm 4.6</td>
</tr>
</tbody>
</table>

### Table 3. Effect of Niga-ichigoside F1 and 23-Hydroxytormentic Acid on Gastric Secretion in Pylorus Ligated Rats (after 4 h)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>pH</th>
<th>Vol (ml)</th>
<th>Acidity (mEq/l)</th>
<th>Total acid output</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>1.42 \pm 0.19</td>
<td>7.3 \pm 0.9</td>
<td>106.9 \pm 15.8</td>
<td>610.2 \pm 100.5</td>
</tr>
<tr>
<td>NIF1</td>
<td>10</td>
<td>1.36 \pm 0.18</td>
<td>7.0 \pm 0.8</td>
<td>94.7 \pm 10.5</td>
<td>580.6 \pm 90.3</td>
</tr>
<tr>
<td>23-HTA</td>
<td>10</td>
<td>1.40 \pm 0.19</td>
<td>6.5 \pm 0.6</td>
<td>85.2 \pm 7.9</td>
<td>429.8 \pm 79.5</td>
</tr>
<tr>
<td>Cimetidine</td>
<td>100</td>
<td>2.34 \pm 0.42</td>
<td>4.3 \pm 0.5</td>
<td>63.8 \pm 6.4</td>
<td>290.3 \pm 91.6</td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
<td>1.40 \pm 0.25</td>
<td>6.7 \pm 0.9</td>
<td>89.6 \pm 4.8</td>
<td>447.5 \pm 63.2</td>
</tr>
<tr>
<td>NIF1</td>
<td>30</td>
<td>1.42 \pm 0.30</td>
<td>4.3 \pm 0.5</td>
<td>63.8 \pm 6.4</td>
<td>290.3 \pm 91.6</td>
</tr>
<tr>
<td>23-HTA</td>
<td>30</td>
<td>1.35 \pm 0.20</td>
<td>2.0 \pm 0.3</td>
<td>31.4 \pm 5.46</td>
<td>131.8 \pm 41.8</td>
</tr>
<tr>
<td>Cimetidine</td>
<td>100</td>
<td>2.34 \pm 0.42</td>
<td>4.3 \pm 0.5</td>
<td>63.8 \pm 6.4</td>
<td>290.3 \pm 91.6</td>
</tr>
</tbody>
</table>

Value represents means \pm S.D. (n=6). Values followed by the same letter are not significantly different from control (p<0.05).
HTA significantly restored SOD activity reduced by gastropathy induction. The effect of the former compound was more potent than that of the latter.

**Effect on GPx** GPx cleaves hydrogen peroxide and lipid peroxide and is generally considered to be a free radical-scavenging enzyme. The GPx activity in the gastric mucosal tissue was considerably lowered in rats with gastropathy. The administration of NIF$_1$ or 23-HTA increased GPx activity in gastropathy-induced rats. And, once again compound 23-HTA was more potent than NIF$_1$ (Fig. 2).

**DISCUSSION**

Almost all the NSAIDs like aspirin, ibuprofen, and indomethacin and steroidal anti-inflammatory drugs are administered for long periods of time to treat chronic inflammatory diseases, *e.g.*, rheumatoid arthritis. Moreover, it is accepted that NSAIDs of the COX-2 inhibitor type have on an adverse effect like a gastropathy. NSAIDs have the ability to bind with the active sites of COX enzymes. The antinociceptive/anti-inflammatory actions of many nutraceuticals are usually associated with reduced COX-2 production, which is responsible for prostaglandin biosynthesis. Therefore, the development of anti-inflammatory agents with anti-gastropathic action or without a gastritis-inducing action makes long-term oral administration possible.

Rheumatoid arthritis is a disease whereby tissues are damaged by the overproduction of reactive oxygen species (ROS). Moreover, some triterpenoids and saponins reduce ROS level in carrageenan or Freund's complete adjuvant reagent-induced rats. FCA is a reagent frequently used to induce rheumatoid arthritis in models of this disease. RA factor is observed positively in 80% of the number of rheumatoid arthritis patients and is also increased in diffuse collagen disease. CRP factor is a diagnostic index of bacterial infection, chronic rheumatoid arthritis, supplicative arthritis, gout, malignant tumor and rheumatoid fever. As shown in Table 1, the FCA-treated rat produced significant increases in RA- and CRP factors (RA: 10, CRP: 10). Moreover, the treatment of rats with NIF$_1$ and 23-HTA potently inhibited RA- and CRP factor increases, and the reference drug, methylprednisolone (MTP) produced a potent effect. And, 23-HTA also exhibited a potent inhibitory effect on rheumatoid arthritis induced by a self-immunological reaction.

In the present study, the effects of NIF$_1$ and 23-HTA were investigated on FCA-induced rats to determine whether they have an effect on rheumatoid arthritis or gastropathy in a rat model. The present experiment demonstrated the merits of anti-inflammatory natural products in terms of avoiding the harmful effects of synthetic anti-inflammatory drugs. As shown by our experimental results, NIF$_1$ and 23-HTA reduced gastric lesion indices and gastric juice secretion in rats with gastropathy caused by EtOH plus sodium salicylate. Further, both NIF$_1$ and 23-HTA also decreased lipid peroxidation, possibly due to increased SOD and GPx activities. Histopathologic observations confirmed these anti-gastropathic effects, suggesting that NIF$_1$ and 23-HTA are potential candidates for long-term administration as anti-inflammatory drugs (Microscopic photos not shown.).

It has been reported that many natural antioxidants should inhibit the gastropathy caused by oxidative stress. In the present study, treatment with EtOH plus sodium salicylate reduced free radical-scavenging enzyme activities and increased lipid peroxidation, whereas treatment of rats with NIF$_1$ and 23-HTA attenuated the pathophysiology due to the gastropathic induction. 23-HTA, an aglycone of NIF$_1$, exhibited more potent activity than NIF$_1$, thus suggesting that the aglycone may be a more active moiety. Taken together, our findings suggest that these two species which ameliorated gastropathy have potential as therapies for the long-term treatment of rheumatoid arthritis.

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