Pectolinarin and Pectolinarigenin of *Cirsium setidens* Prevent the Hepatic Injury in Rats Caused by d-Galactosamine via an Antioxidant Mechanism

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To identify the hepatoprotective component from the leaves of *Cirsium setidens* (Compositae), the methanolic extract was divided into two fractions, chloroform and butanol fractions, and their hepatoprotective efficacy was evaluated in a rat model of hepatic injury caused by d-galactosamine (GalN). Hepatoprotective activity was measured by the activity of serum aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH). Glutathione metabolism was measured via biochemical parameters such as glutathione (GSH), glutathione reductase (GR), γ-glutamylcysteine synthetase (GCS), glutathione S-transferase (GST), and superoxide dismutase (SOD) levels. We subjected the butanol fraction, which had higher activity, to column chromatography to yield pectolinarin, which was further hydrolyzed to yield pectolinarigenin. Administration (10, 20 mg/kg, p.o.) of the main flavonoid glycoside component, pectolinarin, and its aglycone, pectolinarigenin, for 2 weeks significantly decreased the activity levels of AST, ALT, ALP and LDH, indicating that the two compounds have hepatoprotective activity. Pectolinarin and pectolinarigenin also increased activity levels of GSH, GR, GCS, and GST, as well as SOD. The significant effect was only seen in SOD activity. This suggests that the two components exhibit hepatoprotective activity mainly via SOD antioxidant mechanism.

Key words *Cirsium setidens*; hepatoprotective; antioxidant; pectolinarin; pectolinarigenin; glutathione

\( \text{d-Galactosamine (GalN)} \) is a hepatotoxicant, an inducer in hepatic injury models, both in vivo and in vitro. In vivo, GalN causes hepatic damage resembling viral hepatitis and drug-induced hepatitis.\(^1,2\) This hepatotoxicity causes the inhibition of RNA and protein synthesis via loss of uridine nucleotides and accumulation of UDP hexosamines in hepatocytes.\(^3,4\) In the rat, activation of Kupffer cells by a low dose of endotoxin is related to GalN toxicity.\(^4—6\) GalN itself increases hepatic sensitivity to tumor necrosis factor (TNF-α), which contributes to the injury.\(^7\)

*Cirsium setidens* (Compositae) is a wild, perennial plant species found only in Korea. The young leaves and stems are edible and rich in protein, calcium, and vitamin A. *C. setidens* has been used in the treatment of hemostasis, hematemesis, hematuria and hypertension in Korean medicine.\(^9\) Activity-guided fractionation led to isolation of pectolinarin as a primary component, with pectolinarigenin obtained by acid hydrolysis of pectolinarin and used for hepatoprotective activity. Martínez-Vázquez et al.\(^9\) reported on the analgesic and anti-inflammatory activities of pectolinarin isolated from *C. subcoriaceum*. Tundis et al.\(^10\) showed that pectolinarin from *Linaria reflexa* Desf had an antitumor effect in COR-L23, Caco-2, and C32 cell lines, and Liu et al.\(^11,12\) showed that pectolinarin from *C. japonicum* had anticancer activity in S180 and H22 mice. Do et al.\(^13\) isolated pectolinarin from the aerial part of *C. nopponicum* and Roh et al.\(^14\) did from *Melampyrum roseum* var. *hirsutum*.

Here, we evaluated the hepatoprotective effects of two components of the *C. setidens* extract, the primary component pectolinarin and pectolinarigenin, an aglycone, in GalN-induced liver injury.

**MATERIALS AND METHODS**

**Plant Material** The leaves of *C. setidens* (Compositae) were collected in Jinbu-myon, Pyongchang-gun, Gangwon-do, Korea. This plant was identified by Dr. Won-Bae Kim and a voucher specimen was deposited in the laboratory of Natural Product Chemistry, Division of Environmental Botany, Sangji University, Korea. The collected plant was dried without using sunshine and pulverized for extraction.

**Extraction and Fractionation** Plant material (628 g) was extracted for 5 h with methanol (MeOH) three times under reflux. The extracted solution was filtered and concentrated under reduced pressure to give a viscous MeOH extract (128 g). A part of the MeOH extract (108 g) was suspended in 800 ml distilled water and partitioned three times with chloroform (CHCl3). The CHCl3-soluble portion was dried in vacuo to yield the CHCl3 fraction (44 g). The H2O layer was successively fractionated with butanol (BuOH) and dried in vacuo to yield the BuOH fraction (6 g).

**Isolation** Five grams of the BuOH fraction was subjected to silica gel column (SiO2, Art No. 7734, Merck, Germany, 280 g, 5×55 cm) chromatography and eluted with CHCl3–MeOH–H2O (7 : 3 : 1, lower layer). The eluting solution was collected in 200 ml, checked by TLC, and combined into three fractions (Fr. 2, retention volume 1400—2200 ml). The Fr. 2 fraction was dried in vacuo and crystallized from MeOH to yield a white amorphous powder (compound 1, Fig. 1, C13H22O15, MW 622.58).

**Compound 1:** White amorphous powder from MeOH, mp 250—253 °C; \(^1\)H-NMR (500 MHz, pyridine-d$_3$) δ: 6.86 (1H, s, H-1), no peak (H-8), 8.04 (2H, d, J=9.0 Hz, H-2,6'), 7.25 (2H, d, J=9.0 Hz, H-3,5'), 4.05 (3H, s, H-6), 3.71 (3H, s, H-4'); Sugar moieties, Glc-5.73 (1H, d, J=7.5 Hz, H-1'); 4.34
Preparation of Pectolinarigenin Pectolinarigenin was obtained by acid hydrolysis of compound 1. In brief, compound 1 was hydrolyzed in 5% \( \text{H}_2\text{SO}_4 \) in 50% MeOH solution for 3 h under reflux. After cooling, the solution was fractionated with CHCl3 three times. The CHCl3 fraction was obtained by acid hydrolysis of compound 1a. which was identified as pectolinarigenin by spectroscopy (Fig. 1, C\textsubscript{17}H\textsubscript{14}O\textsubscript{6}, MW 314.29).

Preparation of Pectolinarigenin Pectolinarigenin was obtained by acid hydrolysis of compound 1. In brief, compound 1 was hydrolyzed in 5% \( \text{H}_2\text{SO}_4 \) in 50% MeOH solution for 3 h under reflux. After cooling, the solution was fractionated with CHCl3 three times. The CHCl3 fraction was obtained by acid hydrolysis of compound 1a, which was identified as pectolinarigenin by spectroscopy (Fig. 1, C\textsubscript{17}H\textsubscript{14}O\textsubscript{6}, MW 314.29).

Compound 1a: Orange yellow powder, mp 200—205 °C \( ^{1} \text{H}-\text{NMR} \) (500 MHz, DMSO-\( d_6 \)) \( \delta \): Genin 164.5 (C-2), 104.1 (C-3), 153.0 (C-5), 133.9 (C-6), 157.6 (C-7), 95.0 (C-8), 122.8 (C-1'), 128.6 (C-2', 6'), 115.0 (C-3', 5'), 162.9 (C-4'), 60.6 (6-OCH3), 55.2 (4'-OCH3); Glc-102.3 (C-1'), 74.5 (C-2'), 78.3 (C-3'), 71.1 (C-4'), 77.5 (C-5'), 67.4 (C-6'); Rha-102.2 (C-1'), 71.9 (C-2'), 72.7 (C-3'), 73.8 (C-4'), 69.6 (C-5'), 18.3 (C-6').

Experimental Animals Male Sprague-Dawley rats were purchased from Daehan Bio Link Co., allowed to adapt to laboratory conditions (temperature: 20 °C, dampness: 40—60%, light/dark cycle: 12 h) for a week, and rats weighing 200±10 g were used for animal experiments. Animals were fasted for 24 h before the experiment. To avoid variations in enzyme activity during the day, the animals were sacrificed at a fixed time (10:00 a.m.—12:00 p.m.). These experiments were approved by the University of Kyungsung Animal Care and Use Committee. All procedures were conducted in accordance with the "Guide for Care and Use of Laboratory Animals" published by the National Institutes of Health.

Galactosamine-Induced Liver Injury in Rats Liver injury was induced by intraperitoneal injection with \( \text{d}-\text{galactosamine} \) (GalN; 400 mg/kg body weight) (Sigma, St. Louis, MO, U.S.A.) dissolved in saline. Blood was collected from abdominal aortas 24 h after the final GalN injection.

Experimental Design There were 4 groups of animals: untreated (normal, received distilled water), GalN (control), and GalN plus \( C. \text{setidens} \) extracts (methanol/butanol/chloroform fractions; 100, 200 mg/kg, respectively) or pectolinarin (10, 20 mg/kg; 16, 32 \( \mu \text{mol/kg} \))/pectolinarigenin (10, 20 mg/kg; 32, 64 \( \mu \text{mol/kg} \)) rats (n=9) for 2 consecutive weeks after GalN treatment. The rats were treated orally. Rats were fasted 7 h after the final sample treatment, anesthetized with \( \text{CO}_2 \), and blood was collected from the abdominal aorta. Serum was separated by centrifugation at 2500×\( g \) for 15 min and tested for aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) enzyme activity. Extracted liver was washed with saline for removing blood and contaminants and homogenized with 0.1 m potassium phosphate buffer (pH 7.4). Homogenates were centrifuged at 10000×\( g \) for 20 min and 105000×\( g \) for 60 min at 4 °C. Cytosolic fractions from the supernatant were used for measurement of Glutathione (GSH), glutathione-S-transferase (GST), glutathione reductase (GR), \( \gamma \)-glutamylcysteine synthetase (GCS), and superoxide dismutase (SOD) activities.

Measurement of Serum AST, ALT, ALP, LDH, and TBARS AST and ALT were measured using a kit (Asan Pharm. Co., Korea) prepared by Reitman and Frankel.15 ALP activity was measured using a kit (Asan Pharm. Co.) based on the method of Roos.16 LDH activity was measured with the method of Kim et al.17 Lipid peroxidation was measured with thiobarbituric acid reactive substances (TBARS) as a marker of lipid peroxidation using a modification of the method described by Ohkawa et al.18

Measurement of Hepatic GSH, GST, GR, GCS, and SOD Measurement of hepatic GSH was determined by a modified method of Gaitonde et al.19 GST activity was estimated by the method of Habig and Jakoby.20 GR activity was determined by a modified method of Mize and Langdon.21 GCS activity was determined by a modified method of Richman and Meister.22 SOD activity was measured according to the method of Marklund and Marklund.23

Determination of Protein Concentration and Statistical Analysis Protein concentrations were measured by the Lowery method with bovine serum albumin (Sigma Chemical Co., St. Louis, MO, U.S.A.) as the standard. Results are expressed as means±S.D. (n=9). Statistical analysis was performed with Duncan’s multiple range tests. Differences were considered significant at \( p<0.05 \).

RESULTS AND DISCUSSION

The methanol (MeOH) extract was divided into chloroform (CHCl3) and butanol (BuOH) fractions to search for the active fraction. Since the hepatoprotective effect of the BuOH fraction was more potent than the CHCl3 fraction, we separated the BuOH fraction by column chromatography to yield pectolinarin (Fig. 1 (1)), which was identified by spectroscopic evidence. This compound has not been reported in C. setidens. To obtain the aglycone, pectolinarin was hydrolyzed in an acid solution to yield pectolinarigenin (Fig. 1 (1a)). The structure was identified by spectroscopic evidence. Compound 1 and 1a were used to hepatoprotective assays.

Aminotransferase mediates the catalysis of amino transfer reactions and is a marker for clinical diagnosis of liver injury.24 During the low cellular energy, intracellular K+ ions leak out and Na+/\( \text{Ca}^{2+} \)/\( \text{H}_2\text{O} \) enter the cell. Cell membranes can swell and release hepatic intracellular AST and ALT. Serum AST and ALT in the blood are prognostic markers in liver disease.25 To determine the hepatoprotective effect of C. setidens extract fractions (MeOH extract and CHCl3- and

Fig. 1. Structure of Pectolinarin (1, C\textsubscript{29}H\textsubscript{34}O\textsubscript{15}, MW 622.58) and Pectolinarigenin (1a, C\textsubscript{17}H\textsubscript{14}O\textsubscript{6}, MW 314.29) Obtained from C. setidens

(1H, m, H-2’), 4.34 (1H, m, H-3’), 4.08 (1H, m, H-4’), 4.27 (1H, m, H-5’), 4.72 (1H, dd-like, Ha-6’), 4.14 (1H, dd-like, Hb-6’), Rha-5.47 (1H, br s, H-1”), 4.62 (1H, dd, J=1.5, 9.0 Hz, H-2”), 4.51 (1H, dd, J=3.0, 9.0 Hz, H-3”), 4.16 (1H, dd-like, H-4”), 4.27 (1H, d, J=6.5 Hz, H-5”), 1.55 (3H, d, J=6.5 Hz, H-6’); \( ^{13} \text{C}-\text{NMR} \) (125.5 MHz, pyridine-\( d_5 \)) δ: Genin 164.5 (C-2), 104.1 (C-3), 153.0 (C-5), 133.9 (C-6), 157.6 (C-7), 95.0 (C-8), 122.8 (C-1’), 128.6 (C-2’, 6’), 115.0 (C-3’, 5’), 162.9 (C-4’), 60.6 (6-OCH3), 55.2 (4’-OCH3); Glc-102.3 (C-1’), 74.5 (C-2’), 78.3 (C-3’), 71.1 (C-4’), 77.5 (C-5’), 67.4 (C-6’); Rha-102.2 (C-1’), 71.9 (C-2’), 72.7 (C-3’), 73.8 (C-4’), 69.6 (C-5’), 18.3 (C-6’).
BuOH fractions), rats were treated with GalN and the extract fractions. GalN injection increased serum AST and ALT activities significantly compared with the untreated group (Table 1), and extract treatment ameliorated this increase in a dose-dependent manner. Treatment with MeOH extract and BuOH extract suppressed AST activity by 13.8% and 19.6%, respectively, at 200 mg/kg; and ALT activity by 21.4% and 25.2%, respectively. Treatment with pectolinarin (10, 20 mg/kg), the primary component of the BuOH fraction, decreased GalN-induced increases in aminotransferase activity by 16.5% and 31.7% (AST), respectively, and 23.3% and 31.5%, respectively, and LDH activity by 39.6% and 53.2%, respectively (Table 2). Pectolinarigenin had weaker activity than pectolinarin. Thus, pectolinarin is the main hepatoprotective component of *C. setidens*.

GalN treatment increased serum activities of ALT, AST, ALP and LDH. ALP is a hydrolase enzyme responsible for removing phosphate groups from nucleotides and proteins, is produced primarily in the liver and brain, and is a marker of hepatic function. LDH is a general indicator of acute or chronic hepatic damage, as well as determining organ, cell, and tissue condition. GalN treatment increased serum ALP and LDH activities compared with the untreated group, whereas treatment with the MeOH and BuOH extracts reduced this increase (Table 1). The MeOH extract and BuOH fractions (both 200 mg/kg) suppressed ALP activity by 14.4% and 16.9%, respectively, and LDH activity by 28.1% and 45.7%, respectively (Table 1). Oral administration of 10 and 20 mg/kg of pectolinarin reduced ALP activity by 14.5% and 31.5%, respectively, and LDH activity by 39.6% and 53.2%, respectively (Table 2). Pectolinarigenin activity was again weaker than pectolinarin. Thus, pectolinarin is the main hepatoprotective component of *C. setidens*.

GalN treatment significantly increased TBARS levels compared with the untreated group, whereas treatment with the BuOH fraction significantly reduced these levels at 100 and 200 mg/kg (Fig. 2A). Pectolinarin and pectolinarigenin
enzymes. Here, GalN treatment decreased GR and GCS activities or by increased activity of glutathione breakdown through decreased activity of glutathione synthesizing mechanisms. Therefore, these two components could be potential hepatoprotective agents, as could the parent herb, C. setidens, pectolinarin, and its aglycone, pectolinarigenin, prevented GalN-induced hepatotoxicity via an antioxidant mechanism. Therefore, these two components could be potential hepatoprotective agents, as could the parent herb, C. setidens. In conclusion, a main component of the MeOH extract of C. setidens, pectolinarin, and its aglycone, pectolinarigenin, were effective as well (Fig. 2B), indicating that the samples inhibited hepatic lipid peroxidation caused by GalN. GalN treatment significantly reduced GSH levels in the control group compared with the untreated group, but these changes were reduced by administration of the MeOH extract and BuOH fraction (Table 3). Pectolinarin was again more effective than pectolinarigenin in this assay (Table 4).

The assay procedures are described in the Materials and Methods. Data represent means±S.D. (n=9). Values followed by the same superscript letter are not significantly different from each other (p<0.05) by Duncan’s multiple range test. GST, glutathione S-transferase; SOD, superoxide dismutase; GR, glutathione reductase; GCS, γ-glutamylcysteine synthetase.

Table 3. Antioxidant Effect of Active C. setidens Extracts on Liver Injury Induced by α-Galactosamine

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>GSH (μmol/mg protein)</th>
<th>GR (μmol/mg protein)</th>
<th>GCS (μmol/mg protein)</th>
<th>GST (μmol/mg protein)</th>
<th>SOD (μmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
<td>7.17±0.83a</td>
<td>45.4±3.17a</td>
<td>16.42±3.96a</td>
<td>238.4±39.2a</td>
<td>10.6±0.85a</td>
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<td>Control</td>
<td></td>
<td>4.13±0.91a</td>
<td>29.4±6.11bc</td>
<td>8.17±2.93b</td>
<td>117.7±40.5b</td>
<td>5.4±0.43b</td>
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<tr>
<td>MeOH</td>
<td>100</td>
<td>5.19±0.51b</td>
<td>28.5±3.16bc</td>
<td>8.64±3.13c</td>
<td>136.8±43.9b</td>
<td>6.1±0.49b</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>5.29±0.48c</td>
<td>30.6±4.13bc</td>
<td>8.59±4.62c</td>
<td>160.4±38.7b</td>
<td>6.5±0.53b</td>
</tr>
<tr>
<td>BuOH</td>
<td>100</td>
<td>5.25±0.47c</td>
<td>31.8±2.65bc</td>
<td>8.02±3.95c</td>
<td>154.9±41.5b</td>
<td>6.8±0.39c</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>5.49±0.56b</td>
<td>33.7±2.43b</td>
<td>8.83±2.97b</td>
<td>163.9±49.6b</td>
<td>7.6±0.42c</td>
</tr>
<tr>
<td>CHCl3</td>
<td>100</td>
<td>5.01±0.77b</td>
<td>28.4±3.19bc</td>
<td>8.25±3.45c</td>
<td>125.3±39.5b</td>
<td>5.5±0.38b</td>
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<tr>
<td></td>
<td>200</td>
<td>5.59±0.42c</td>
<td>29.7±2.47bc</td>
<td>8.31±4.06b</td>
<td>132.8±40.7b</td>
<td>5.8±0.48b</td>
</tr>
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</table>

Table 4. Antioxidant Effect of Active C. setidens Compounds on Liver Injury Induced by α-Galactosamine

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>GSH (μmol/mg protein)</th>
<th>GR (μmol/mg protein)</th>
<th>GCS (μmol/mg protein)</th>
<th>GST (μmol/mg protein)</th>
<th>SOD (μmol/mg protein)</th>
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<tbody>
<tr>
<td>Normal</td>
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<td>7.32±0.68a</td>
<td>46.7±4.07a</td>
<td>15.47±2.87a</td>
<td>257.6±34.5a</td>
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<td>Control</td>
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<td>4.36±0.98a</td>
<td>28.7±5.21b</td>
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<td>5.35±0.48b</td>
<td>33.8±4.28bc</td>
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<td>20</td>
<td>5.43±0.39b</td>
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<td>5.28±0.25b</td>
<td>29.3±2.58bc</td>
<td>8.83±3.47b</td>
<td>148.7±45.2b</td>
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<td>5.30±0.37b</td>
<td>30.1±3.34c</td>
<td>8.52±2.55b</td>
<td>162.2±43.9b</td>
<td>7.8±0.39b</td>
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</tbody>
</table>

The assay procedures are described in the Materials and Methods. Data represent means±S.D. (n=9). Values followed by the same superscript letter are not significantly different from each other (p<0.05) by Duncan’s multiple range test. GST, glutathione S-transferase; SOD, superoxide dismutase; GR, glutathione reductase; GCS, γ-glutamylcysteine synthetase.

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