Hepatoprotective and Antioxidative Properties of Salacia reticulata: Preventive Effects of Phenolic Constituents on CCl₄-Induced Liver Injury in Mice

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The hepatoprotective effects of the hot water (SRHW) and methanolic (SRM) extracts from the roots and stems of Salacia reticulata were examined using an oxidative stress-induced liver injury model. Both SRHW and SRM extracts (400 mg/kg, p.o.) significantly suppressed the increase in glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) activities in carbon tetrachloride (CCl₄)-treated mice. These extracts also inhibited CCl₄-induced thiobarbituric acid-reactive substance (TBA-RS) formation, which indicates increased lipid peroxidation in the liver. A good correlation (r=0.945, p<0.01) was observed between the amount of phenolic compounds in the extracts and their inhibitions of TBA-RS formation. The IC₅₀ values of the extracts on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging were less than 10 μg/ml and the antioxidative activities of six phenolic compounds from the roots of S. reticulata were examined. Mangiferin, (−)-4′-O-methylepigallocatechin, and (−)-epicatechin-(4β→8)-(−)-4′-O-methylepigallocatechin, which a principal phenolic compounds, showed potent scavenging activity on DPPH radicals and their concentrations required for 50% reduction of 40 μg DPPH radicals were 5.9, 10, and 3.2 μg, respectively. On the other hand, against the CCl₄-induced serum GOT and GPT elevations and TBA-RS formation in mice, mangiferin and (−)-4′-O-methylepigallocatechin showed potent activity at a dose of 100 mg/kg, but (−)-epicatechin-(4β→8)-(−)-4′-O-methylepigallocatechin did not. These results suggest that the antioxidative activity of the principal phenolic compounds is involved in the hepatoprotective activity of S. reticulata.

Key words Salacia reticulata; hepatoprotective activity; carbon tetrachloride; antioxidative activity; mangiferin; (−)-4′-O-methylepigallocatechin

MATERIALS AND METHODS

Animals Male ddY mice were purchased from Kiwa Laboratory Animal Co., Ltd. (Wakayama, Japan). The animals were maintained at a constant temperature of 23±2°C and were fed standard laboratory chow (MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water.

Materials Preparation of SRHW and SRM extracts, and determination of amount of phenolic compounds: Dried roots and stems of S. reticulata obtained from Sri Lanka and India were crushed and extracted with 5 times the volume of water (H₂O) at 80°C for 3 h. The solvent was evaporated under reduced pressure at 40°C to give the SRHW extract. The residue was continuously extracted with 4 times the volume of MeOH for 3 h and the SRM extract was obtained. The amount of phenolic compounds was determined by the colorimetric method using a ferrous tartrate. Briefly, each extract dissolved in H₂O (1 mg/ml) was mixed with 5 ml of ferrous tartrate reagent [FeSO₄·7H₂O (1 mg/ml) and C₆H₅KNaO₄·4H₂O (5 mg/ml) dissolved in H₂O]. This was added to 15 ml of Sörensen’s phosphate buffer (67 mM Na₂HPO₄, 67 mM KH₂PO₄, pH 7.5) and shaken. The absorbance of the mixture was measured at 540 nm. The amount of phenolic constituents was calculated using the calibration curve of ethyl gallate solution as a standard.

Phenolic constituents in S. reticulata: Mangiferin (1), (−)-epicatechin (2), (−)-epigallocatechin (3), (−)-4′-O-methylepigallocatechin (4), (−)-epiafzelechin-(4β→8)-(−)-4′-O-methylepigallocatechin (5), and (−)-epicatechin-(4β→8)-(−)-4′-O-methylepigallocatechin (6) were exam-
CCL₄-Induced Lethal Injury in Mice Mice aged 5 weeks were fasted for 20 h, and each sample suspended in 5% (w/v) acacia solution was given (10 ml/kg) orally. One hour later, were fasted for 20 h, and each sample suspended in 5% (w/v) CCl₄ diluted in olive oil was subcutaneously injected in the mice. Blood samples were collected from the infraorbital venous plexus 20 h after CCl₄ administration, and serum was obtained by centrifugation. As markers of liver injury, serum glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) activities were determined using the Reitman–Frankel method (STA test Wako, Wako Pure Chemical Industries, Ltd., Osaka, Japan). Malotilate (Daichi Pharmaceutical Industry, Tokyo, Japan) was used as a reference compound.

CCL₄-Induced Thiobarbituric Acid-Reactive Substance (TBA-RS) Formation in Mouse Liver Mice were treated with a sample and CCl₄ with the same procedure as described above and killed by cervical dislocation 4 h after CCl₄ administration. The liver was removed immediately and homogenized in a 10-fold weight of ice-cold 0.15 M KCl solution. Three milliliters of 0.1 m phosphoric acid and 1 ml of 0.04 M 2-thiobarbituric acid (TBA, Nacalai Tesque, Inc., Kyoto, Japan) aqueous solution were added to 0.5 ml of the homogenate in a 10-ml centrifuging tube. The mixture was heated for 60 min in boiling water. After cooling, 4 ml of n-butanol was added and mixed vigorously. Then the n-butanol phase was separated by centrifugation (3000 rpm, 10 min, 4 °C) and absorbance was measured at 535 and 520 nm. The difference between the absorbance at 535 nm and that at 520 nm was used as the TBA value.9 The TBA value was expressed in terms of malondialdehyde (nmol per gram of wet tissue), using 1,1,3,3-tetraethoxypropane (Sigma Chemical Co., St. Louis, MO, U.S.A.) as an external standard.

Examination of 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical-Scavenging Activity DPPH radical-scavenging activity was investigated according to the method of Uchiyama et al.8 Briefly, a solution of sample in acetate buffer (pH 5.5, 2 ml) and ethanol (EtOH, 2 ml) was treated with 2×10⁻⁴ M DPPH (Nacalai Tesque) in EtOH solution (1 ml) and the mixture was incubated at room temperature for 30 min (final concentration of DPPH, 40 μM), and the absorbance change at 517 nm was measured. The reaction solution without DPPH was used as a blank test. Measurements were performed in duplicate, and the concentration required for a 50% reduction (SC₅₀) of 40 μM DPPH radical was determined graphically.

CCL₄-Induced Cytotoxicity in Primary Cultured Mouse Hepatocytes Hepatocytes were isolated from male ddY mice (body weight, 30—35 g) by the collagenase perfusion method.9 A cell suspension of 4×10⁴ cells in 100 μl of William’s E medium (Sigma Chemical) containing 10% fetal calf serum (FCS, Life Technologies, Inc., Rockville, MD, U.S.A.), penicillin 100 units/ml, and streptomycin 100 μg/ml were inoculated onto a 96-well tissue culture plate and precultured for 4 h at 37 °C under a 5% CO₂ atmosphere. The medium was replaced with 200 μl of fresh medium, and then 10 μl of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide [MTT, 5 mg/ml in phosphate-buffered saline (PBS)] solution was added to the medium. After 4-h culture, the medium was removed, and 100 μl of isopropanol containing 0.04 m HCl was added to dissolve the formazan produced by the cells. The optical density (OD) of the formazan solution was measured using a microplate reader at 562 nm. The inhibition ratio (%) was obtained by the formula:

\[
\text{inhibition} (\%) = \frac{\text{OD(sample)} - \text{OD(control)}}{\text{OD(normal)}} - \text{OD(control)} \times 100
\]

Each test compound was dissolved in dimethyl sulfoxide (DMSO), which was added to the medium (final DMSO concentration, 0.5%).

Statistical Analysis Values are expressed as mean± S.E.M. One-way analysis of variance followed by Dunnett’s test was used for statistical analysis. p Values less than 0.05 were considered significant.

RESULTS AND DISCUSSION

Effects of SRHW and SRM Extracts on CCL₄-Induced Serum GOT and GPT Elevations and TBA-RS Formation in Mice Figure 1 shows the effects of SRHW and SRM from the roots of S. reticulata on CCL₄-induced serum GOT and GPT elevations in mice. One of the mechanisms in this model is considered to be initiated by an enzymatic reaction of CCl₄ from CCl₄ in liver microsomal cytochrome P450 (CYPs).- CCl₄ causes consecutive lipid peroxidation of the cell membrane and endoplasmic reticulum. The peroxidative products induce hypofunction of the membrane and finally cytosolic enzyme aberrates to blood. Therefore lipid peroxidation of hepatocytes has been recognized to be a major factor in the liver injury model. Oral administration of SRHW and SRM extracts significantly suppressed serum GOT and GPT elevations at a dose of 400 mg/kg. Malotilate, the reference compound, showed potent inhibitory effect at the dose of 200 mg/kg.

Next, we examined the effect on TBA-RS formation as a parameter of liver lipid peroxidation induced by CCl₄ administration. These extracts also inhibited CCl₄-induced TBA-
RS formation in a dose-dependent manner (Fig. 2). This result suggests that SRHW and SRM extracts have antioxidative activity. We also compared the inhibitory effects of SRHW and SRM extracts from several parts (root and stem) from *S. reticulata* in Sri Lanka and India.

Table 1 shows the effects of several SRHW and SRM extracts on CCl₄-induced serum GOT and GPT elevations and TBA-RS formation at a dose of 400 mg/kg. All extracts reduced liver injury and TBA-RS formation. On the other hand, many phenolic compounds have been reported to show antioxidative activity against CCl₄-induced lipid peroxidation. Therefore we determined the contents of phenolic compounds in these extracts by the ferrous tartrate method. As shown in Table 1, a large amount of phenolic compounds in the extracts was found to be contained in each extract, and a good correlation ($r = 0.945, p < 0.01$) was observed between the amount of phenolic compounds in the extracts and their inhibitions of TBA-RS formation (Fig. 3). This finding suggests that the hepatoprotective activity of the extracts is due to that of the phenolic compounds in them. Therefore we examined hepatoprotective and antioxidative effects of the phenolic constituents from *S. reticulata*, mangiferin (1), (-)-epicatechin (2), (-)-epigallocatechin (3), (-)-4'-O-methyllepigallocatechin (4), (-)-epicatechin-(4β→8)-(-)-4'-O-methyllepigallocatechin (5), and (-)-epicatechin-(4β→8)(-)-4'-O-methyllepigallocatechin (6) (Fig. 4).³⁵

**DPPH Radical-Scavenging Activity of Phenolic Compounds Isolated from *S. reticulata*** The DPPH radical, which is stable and shows absorption at 517 nm, has been used as a convenient tool for the radical-scavenging assay.⁸,¹² When this compound accepts an electron or a hydrogen radical to become a more stable compound, its absorption vanishes. DPPH radical-scavenging activities of the phenolic constituents were examined. As shown in Table 2, all phenolic compounds (1—6) strongly scavenged DPPH radicals, and these activities were equal to those of known antioxidants such as gallic acid, (+)-catechin, and α-tocopherol. Sato *et al.* reported that 1 showed potent antioxidative activity on a DPPH radical-scavenging system and on enzymatic lipid peroxidation by liver microsomes.¹³ The results with 1 in the present study agree with those in the previous study. This suggests that the hepatoprotective effect of *S. reticulata* on CCl₄-induced liver injury is based on the antioxidative property of phenolic compounds.

**Effects of Phenolic Compounds on CCl₄-Induced Cytotoxicity in Primary Cultured Mouse Hepatocytes** We examined the hepatoprotective effects of phenolic compounds (1—6) from *S. reticulata* on CCl₄-induced cytotoxicity in the hepatocytes. CCl₄ is metabolized to an active radical (‘CCl₃’) by CYPs and exerts oxidative activity.¹⁴ As shown in Table 3, compounds 1—6 showed significant protective effects against CCl₄-induced hepatocytotoxicity at a high concentra-
However, the effects of compounds 3, 4, and 5 were very weak.

**Effects of Principal Phenolic Compounds on CCl₄-Induced Serum GOT and GPT Elevations and TBA-RS Formation**

Finally, we examined the effects of the principal phenolic compounds from *S. reticulata*, such as 1 (0.11% from the natural medicine), 4 (0.057%), and 6 (0.014%) on CCl₄-induced serum GOT and GPT elevations in mice (Fig. 5). Oral administration of 1 and 4 significantly suppressed CCl₄-induced injury, but 6 did not. As shown in Fig. 6, 1 and 4 also inhibited CCl₄-induced TBA-RS formation in the liver in a dose-dependent manner. However, 6 lacked significant effect in this model.

These results suggest that the hepatoprotective effects of *S. reticulata* on CCl₄-induced liver injury depend on the antioxidative activity exerted by phenolic compounds. Compounds 1—6 scavenge free radicals and thus prevent oxidative injury. In particular, oral administration of the principal phenolic constituents 1 and 4 potently inhibited this liver injury in vivo. On the other hand, 4 was not effective against CCl₄-induced cytotoxicity in an *in vitro* assay, but showed potent activity in an *in vivo* assay. These results suggest that 4 may be metabolized to active compounds in the animal body. Conversely, 6, which showed potent inhibition against CCl₄-in-

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**Table 2. DPPH Radical-Scavenging Activities of SRHW, SRM, and Phenolic Compounds (1—6) in S. reticulata**

<table>
<thead>
<tr>
<th>Compound</th>
<th>SC₅₀ (μg/ml)</th>
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<tbody>
<tr>
<td>SRHW (root, Sri Lanka)</td>
<td>5.1</td>
</tr>
<tr>
<td>SRM (root, Sri Lanka)</td>
<td>3.1</td>
</tr>
<tr>
<td>Mangiferin (1)</td>
<td>5.9</td>
</tr>
<tr>
<td>(−)-Epicatechin (2)</td>
<td>4.1</td>
</tr>
<tr>
<td>(−)-Epigallocatechin (3)</td>
<td>4.3</td>
</tr>
<tr>
<td>(−)-4′-O-Methylepicatechin (4)</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>3.2</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>4.1</td>
</tr>
<tr>
<td>(−)-Catechin</td>
<td>5.9</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>11</td>
</tr>
</tbody>
</table>

Each experiment was done in duplicate. a) Concentration required for 50% reduction of 40 μM of DPPH radical.

**Table 3. Effects of Phenolic Compounds (1—6) from S. reticulata on CCl₄-Induced Cytotoxicity in Primary Cultured Mouse Hepatocytes**

<table>
<thead>
<tr>
<th>Conc. (μM)</th>
<th>Inhibition (%)</th>
</tr>
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<tbody>
<tr>
<td>10</td>
<td>2±4 20±2** 38±3**</td>
</tr>
<tr>
<td>30</td>
<td>(−)-Epicatechin (2) 9±3* 18±1** 59±2**</td>
</tr>
<tr>
<td>100</td>
<td>(−)-Epigallocatechin (3) 13±8 15±3* 17±2**</td>
</tr>
<tr>
<td>10</td>
<td>(−)-4′-O-Methylepicatechin (4) 3±3 6±1 18±1**</td>
</tr>
<tr>
<td>30</td>
<td>5 0±1 0±0 14±1**</td>
</tr>
<tr>
<td>100</td>
<td>6 10±3* 34±3** 66±2**</td>
</tr>
</tbody>
</table>

Hepatocytes (4×10⁶ cells) in 100 μl of William’s E medium containing 10% FCS, penicillin 100 units/ml, and streptomycin 100 μg/ml were inoculated onto a 96-well tissue culture plate and cultured for 4 h. The medium was replaced with 200 μl of fresh medium containing a test sample and 0.5 μM CCl₄, and the cells were cultured for 40 h. The viability of the cells was assessed by the MTT assay. Each value represents the mean±S.E.M. of 4 experiments. Significantly different from control, *p<0.05, **p<0.01.
duced cytotoxicity in vitro, lacked the activity against in vivo injury. Compound 6 may not be easily absorbed from the intestinal tract or may be metabolized to inactive compounds in the body.

Previous studies demonstrated that CYPs metabolize CCl₄ to CCl₃ and inhibitors of CYPs (malotilate, rifampicin) inhibit liver injury induced by CCl₄. Additionally, many phenolic compounds inhibit CYPs activity. Therefore we consider that inhibition of CYPs may be one of the mechanisms of action of 1 and 4.

In conclusion, we found hepatoprotective and antioxidative activities in extracts from S. reticulata, and six phenolic constituents (1—6) from the roots of S. reticulata in Sri Lanka were examined. Among these constituents, principal phenolic constituents 1 and 4 showed potent hepatoprotective effects in vivo.

REFERENCES AND NOTES

1) Although the genus Salacia is classified as a Celastraceae or Hippocrateaceae, the latter has recently been used in many reports.