Free Radical Scavenging and Hepatoprotective Actions of the Medicinal Herb, Crassocephalum crepidioides from the Okinawa Islands

Yoko Aniya, a,∗ Tomoyuki Koyama, a Chika Miyagi, b Manami Miyahira, a Chiho Inomata, a Shizuka Kinoshita, a and Toshio Ichiba c

a Laboratory of Molecular Pharmacology, Graduate School of Medicine, University of the Ryukyus; b Department of Laboratory Medicine, Faculty of Medicine, University of the Ryukyus; 207 Uehara, Nishihara, Okinawa 903–0215, Japan; and c Okinawa Industrial Technology Center; 12–2 Suzuki, Gushikawa, Okinawa, 904–2234, Japan.

Received July 16, 2004; accepted September 11, 2004

Free radical scavenging and protective actions against chemically induced hepatotoxicity of Crassocephalum crepidioides were investigated. A water extract of C. crepidioides strongly scavenged superoxide anion, hydroxyl radical and also stable radical 1,1-diphenyl-2-picrylhydrazyl. Galactosamine (GalN, 400 mg/kg) and lipopolysaccharide (LPS, 0.5 μg/kg) induced hepatotoxicity of rats as seen by an elevation of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and of lipid peroxidation in liver homogenates was significantly depressed when the herbal extract was given intraperitoneally 1 and 15 h before GalN and LPS treatment. Similarly, carbon tetrachloride (CCl4) induced liver injury as evidenced by an increase in AST and ALT activities in serum was also inhibited by the extract pretreatment. Isochlorogenic acids, quercetin and kaempferol glycosides were identified as active components of C. crepidioides with strong free radical scavenging action. These results demonstrate that C. crepidioides is a potent antioxidant and protective against GalN plus LPS- or CCl4-induced hepatotoxicity.

Key words natural antioxidant; galactosamine; carbon tetrachloride; free radical; medicinal herb

Reactive oxygen species (ROS) are well recognized to be the pathogenesis of various diseases such as atherosclerosis, diabetes, cancer, arthritis, etc.1−4 Thus a scavenger of ROS is expected to prevent these free radical mediated diseases. The use of antioxidants, both natural and synthetic, in the prevention and cure of various diseases is gaining wide importance in the medicinal field. Currently there is considerable interest in the antioxidant activities of dietary antioxidants, vitamins E and C, carotenoids, and plant polyphenolics. Crassocephalum crepidioides S. Moore (C. crepidioides, Japanese name; Benibanaborogiku), is wildly distributed in the Okinawa Islands and is known in folk medicine for the treatment of acute hepatitis, fever, or edema. This plant is popular as an edible wild plant in Taiwan. Recently components with antimalarial activity have been isolated from C. crepidioides and strong antimutagenicity has also been reported.5,6 However, the antioxidant and pharmacological properties of C. crepidioides have not been investigated. D-Galactosamine (GalN) and carbon tetrachloride (CCl4) were selected for an evaluation of antioxidant action of the herb in vivo. GalN is known to cause liver injury resembling drug-induced hepatitis.7,8 Early biochemical studies postulated that GalN causes liver toxicity by altering uridine pool in hepatocytes, resulting in inhibition of mRNA and protein synthesis.9,10 However, recent evidence indicates that reactive oxygen species (ROS) formed from liver macrophages or increase in the trace element (Fe) are important in liver toxicity.11−14 Furthermore, in combination with a small amount of lipopolysaccharide (LPS) with GalN more ROS is generated.15 On the other hand CCl4 is biotransformed to a trichloromethyl radical by the cytochrome P450 system in liver microsomes followed by liver injury,16−18 and thus this compound has been used for the evaluation of free radical scavenging action of antioxidants in vivo.

In the present study the antioxidant action and hepatoprotective action against this chemically induced liver toxicity of C. crepidioides were examined in vitro and in vivo and the antioxidant components were also identified.

MATERIALS AND METHODS

Chemicals Reduced glutathione (GSH), glucose 6-phosphate, and lipopolysaccharide (Salmonella enteritidis) were obtained from Sigma Chemicals (St. Louis, MO, U.S.A.). D-Galactosamine (GalN) was obtained from Nacalai Tesque (Kyoto). 1-Chloro-2,4-dinitrobenzene (CDNB), carbon tetrachloride (CCl4), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and 2-thiobarbituric acid (TBA) were from Wako Pure Chemicals (Osaka). L-Ascorbic acid (vitamin C) from Ishizu Pharmaceuticals (Osaka) and trichloroacetic acid from Kanto Chemicals (Tokyo) were used. All other reagents were of analytical grade.

Preparation of the Herbal Extract Dried C. crepidioides was obtained from a company (Nakazen Co., Ltd.) in Okinawa, Japan. One gram of the herb was extracted with 10 ml of water at 37 °C for 1 h and filtered through filter paper. The extract thus obtained was used as an original extract and diluted with water when necessary.

Measurement of Free Radical Scavenging Action Scavenging action for DPPH by the extract was measured as described previously.19 Superoxide anion and hydroxyl radical scavenging action of the extract were also examined using an electron spin resonance spectrometer (JES-FR30, JEOL, Tokyo).19

Animals and Treatment Male Sprague–Dawley rats (7—8 weeks) and ddY male mice (9—10 weeks) from Seakku Yoshitomi (Fukuoka) were used for GalN and CCl4 experiments, respectively. Rats were randomly divided into three groups (Control, GalN plus LPS and Herb plus GalN/LPS) each with 4—6 rats. In the GalN plus LPS group rats were given intraperitoneally GalN (400 mg/kg/5 ml

∗ To whom correspondence should be addressed. e-mail: yaniya@med.u-ryukyu.ac.jp © 2005 Pharmaceutical Society of Japan
saline) and LPS (0.5 μg/kg/5 ml). In the herb treated group the herbal extract (5 ml/kg) with 50% DPPH scavenging action was injected intraperitoneally 1 and 15 h before GalN and LPS treatment. Control rats were given vehicle (5 ml/kg) in place of the herb extract or GalN and LPS. The effect of the herbal extract alone was also examined. The extract (5 ml/kg) was given to rats under the same conditions described in the hepatotoxin plus the herbal extract-treated groups except that the vehicle was injected in place of GalN and LPS. Rats were killed by decapitation 24 h after GalN plus LPS treatment after overnight starvation. In the case of CCl4-induced hepatotoxicity, CCl4 (2 ml/kg, as 50% corn oil solution) was given subcutaneously to mice and the herbal extract (a 2-fold diluted solution) (5 ml/kg) was given intraperitoneally to mice 1 and 15 h prior to CCl4 treatment. Control mice received the vehicle instead of the herbal extract or CCl4. Mice were killed 48 h after CCl4 treatment following overnight starvation.

All animals were sacrificed by decapitation and blood was taken from the stump. Serum was separated by centrifugation and stored at 4 °C until use. The liver removed after perfusion in situ with 1.15% potassium chloride solution was homogenized with 2 volumes of the same solution. Cytosol and microsomes were prepared by differential centrifugations and one-washed microsomes were used as reported previously. All the samples obtained were stored at −80 °C for 1 or 2 d except cytosol which was immediately assayed for glutathione S-transferase activity. Animal care was in compliance with Guidelines for Animal Experimentation of the University of the Ryukyus.

**Assay** Activities of glutathione (GSH) S-transferase for CDNB and GSH peroxidase for cumene hydroperoxide were measured by the methods of Habig et al. and Reddy et al., respectively. AST and ALT activities in serum were determined using an assay kit (Kainos, Tokyo). The amount of lipid peroxide in liver homogenates and in serum was estimated by the TBA method and expressed as malondialdehyde (MDA) equivalents described previously. Glutathione content in the liver homogenate was measured by high performance liquid chromatography by the method of Reed et al. Protein concentration in each fraction was measured by the method of Lowry et al.

**Isolation and Identification of Antioxidant Components** During isolation of antioxidant components the antioxidant activity was evaluated by measuring DPPH radical scavenging activity. A dried material of *C. crepidioides* (30 g) was extracted with water twice at 100 °C under 1500 psi by an Accelerated Solvent Extractor (ASE200, DIONEX). The extract solution (280 ml) was diluted to 2.8 l by distilled water and applied to a HP20 (Mitsubishi Chemical, 40×150 mm) column eluting with water and increasing the amount of methanol (MeOH) (MeOH: 10, 25, 50, 75 and 100%, 500 ml each in volume) to give six fractions. Since 25, 50, and 75% MeOH eluted fractions showed DPPH radical scavenging activity, these three were combined, and subjected to further separation by a reversed phase HPLC on C18 (Waters Symmetry, 4.6×100 mm) using a gradient solvent profile (45% MeOH to 50% MeOH containing 0.1% trifluoroacetic acid) to yield five fractions. Final purification of each fraction was carried out by HPLC on C8 using H2O/MeOH (55:45) with 0.1% trifluoroacetic acid, or HW40F (Tosoh, 15×60 mm) with 50% MeOH as an eluent. Thus the structure of six fractions obtained was identified by UV, 1H-NMR and 13C-NMR spectras by comparison with an authentic sample or the reported compound.

**RESULTS**

**Radical Scavenging Action of *C. crepidioides*** Figure 1 shows the effect of *C. crepidioides* on the DPPH radical and the 50% scavenging concentration (IC50) was 11.5 times the dilution of the original herbal extract. Superoxide anion and hydroxyl radical, as seen in Fig. 2, were also scavenged with the IC50 of 177 times and 2.3 times dilution, respectively. Thus it was clarified that *C. crepidioides* has a potent radical scavenging action.

**Hepatoprotective Action of *C. crepidioides*** As shown in Fig. 3, AST and ALT activities in serum were significantly increased in GalN plus LPS treated rats (2880% and 16933% of control, respectively) and were decreased significantly by pretreatment (i.p.) with the herbal extract (182% and 123%, respectively). Lipid peroxide content in liver homogenates increased by GalN plus LPS treatment (887%) was also significantly decreased by pretreatment with *C. crepidioides* extract.
The following six antioxidants were identified. 1) Isochlorogenic acid a: a colorless glassy solid (7 mg); UV (MeOH) λ_{max} 267, 347 nm; LR-APCI-MS, positive ion, m/z 517 [M+H]^+. 2) Isochlorogenic acid b: a colorless glassy solid (7 mg); UV (MeOH) λ_{max} 217, 245, 330 nm; LR-APCI-MS, positive ion, m/z 517 [M+H]^+. 3) Kaempferol-3-galactoside: a colorless glassy solid (3 mg); UV (MeOH) λ_{max} 267, 347 nm; LR-APCI-MS, positive ion, m/z 517 [M+H]^+. 4) Kaempferol-3-

**Fig. 3.** Effect of the Extract of *C. crepidioides* on GalN Plus LPS Induced Liver Injury

Rats received intraperitoneally (i.p.) *C. crepidioides* extract with 50% DPPH scavenging action in two doses, 15 h and 1 h before GalN (400 mg/kg, i.p.) and LPS (0.5 μg/kg, i.p.) treatments and were killed 24 h after the GalN plus LPS injection. Results are mean ± S.D. for 3—5 rats. The activities in control animals were as follows: serum AST, 152.6±12.6 Karman units, ALT, 32.7±1.6 Karman units, liver LPO, 2.3±0.4 nmol/mg, cytosolic GSH S-transferase (GSTc), 1.167±0.31 μmol/min/mg, microsomal GSH S-transferase (GSTM), 0.052±0.016 μmol/min/mg, and cytosolic GSH peroxidase (GSHpX), 0.283±0.043 μmol/min/mg. ***p<0.001, **p<0.01, *p<0.05 vs. control, ###p<0.001, ##p<0.01 vs. GalN plus LPS-treated group.

**Fig. 4.** Effect of the Oral Pretreatment of the Extract of *C. crepidioides* on GalN Plus LPS Induced Liver Injury

Rats received orally the *C. crepidioides* extract (5 ml/kg) in two doses, 15 h and 1 h before GalN (400 mg/kg, i.p.) and LPS (0.5 μg/kg, i.p.) treatments and were killed 24 h after GalN and LPS injection. Results are mean ± S.D. for 6—10 rats. The levels in control animals were serum AST, 157.3±5.6 Karman units, ALT, 39.4±2.1 Karman units, liver LPO, 8.6±1.8 nmol/mg, cytosolic GSH S-transferase (GSTc), 1.881±0.09 μmol/min/mg, microsomal GSH S-transferase (GSTM), 0.091±0.015 μmol/min/mg: ***p<0.001, **p<0.01, *p<0.05 vs. control group.

**Fig. 5.** Effect of Peritoneal Injection of *C. crepidioides* Extract on CCl4-Treated Mice

Water (control) or the 2-fold diluted extract from *C. crepidioides* (5 ml/kg) was given intraperitoneally 1 and 15 h before CCl4 (2 ml/kg, 50% corn oil solution, subcutaneously) treatment. In the case of treatment with the herbal extract alone, the extract (5 ml/kg) or water (control) was given, followed by corn oil injection in place of CCl4. Mice were killed 48 h after CCl4 injection following overnight starvation. Liver and serum parameters were measured as described in Materials and Methods. Each column shows the mean±S.D from 5 (control), 7 (CCl4-treated), 5 (herb alone) and 8 (CCl4+ the extract treated) mice. Control values are as follows: AST, 207.0±27.5 Karman units, ALT, 31.5±4.3 Karman units, liver LPO, 3.88±1.73 nmol/mg, GSH, 16.1±2.1 nmol/mg. *p<0.05, **p<0.01, ***p<0.001 vs. control, ###p<0.001 vs. CCl4-treated.

**Fig. 6.** Structure of Antioxidants Isolated from *C. crepidioides*

Isochlorogenic acid a

Isochlorogenic acid b

Quercetin glycoside

Kaempferol glycoside

**Identification of Antioxidants** The following six antioxidants were identified. 1) Isochlorogenic acid b: a colorless glassy solid (50 mg); UV (MeOH) λ_{max} 220, 241, 326 nm; LR-APCI-MS, positive ion, m/z 517 [M+H]^+. 2) Isochlorogenic acid a: a colorless glassy solid (7 mg); UV (MeOH) λ_{max} 217, 245, 330 nm; LR-APCI-MS, positive ion, m/z 517 [M+H]^+. 3) Kaempferol-3-galactoside: a colorless glassy solid (3 mg); UV (MeOH) λ_{max} 267, 347 nm; LR-APCI-MS, positive ion, m/z 491 [M+H]^+. 4) Kaempferol-3-
Glucoside: a colorless glassy solid (5 mg); UV (MeOH) \( \lambda_{\text{max}} \) 267, 347 nm; LR-APCI-MS, positive ion, \( m/z \) 491 [M+H]+. 5) Quercetin-3-galactoside: a colorless glassy solid (13 mg); UV (MeOH) \( \lambda_{\text{max}} \) 257, 352 nm; LR-APCI-MS, positive ion, \( m/z \) 507 [M+H]+. 6) Quercetin-3-glucoside: a colorless glassy solid (4 mg); UV (MeOH) \( \lambda_{\text{max}} \) 257, 354 nm; LR-APCI-MS, positive ion, \( m/z \) 507 [M+H]+. IC\(_{50}\) values of six antioxidants for DPPH radical was about 50 \( \mu \)g/ml. Isochlorogenic acids a and b showed strong scavenging action for superoxide anion with IC\(_{50}\) of 6.5 and 4.1 \( \mu \)m, respectively, which are stronger than that of ascorbic acid (Table 1).

**Discussion**

Free radical scavenging action of *C. crepidioides* was investigated in vitro and in vivo. Since DPPH is known to abstract a labile hydrogen atom,\(^9\)\(^,\)\(^10\) it has been used to screen the antioxidant action of various compounds.\(^9\)\(^,\)\(^20\) Water extract of *C. crepidioides* showed strong scavenging action for the DPPH radical with 50% inhibition (IC\(_{50}\)) at 11.5 times dilution of the original extract. The herbal extract also possessed potent scavenging action for superoxide anion and moderate action for hydroxyl radical. We thus examined the antioxidant action in vivo using animal models.

It has been recognized that GalN causes hepatotoxicity by the accumulation of UDP-GalN derivatives in the liver, followed by a depletion of hepatic UTP resulting in inhibition of mRNA and protein synthesis.\(^9\)\(^,\)\(^10\) However, oxidative stress is now assumed as the primary cause of GalN-induced hepatotoxicity.\(^9\)\(^,\)\(^11\)\(^-\)\(^14\) ROS produced by activated macrophages or by eliminated metal ions might contribute to the oxidative stress. LPS is known to cause oxidative stress through cytokine (TNF-\( \alpha \)) formation.\(^15\) In addition it is reported that the combination of GalN with LPS causes massive oxidative stress.\(^15\) Thus the oxidative stress by GalN plus LPS treatment was confirmed in our experiments as seen by rising LPO levels in liver homogenate (Figs. 3, 4). GalN plus LPS induced liver injury was protected by pretreatment with the extract of *C. crepidioides* as evidenced by reduction in the increase in serum AST and ALT activities caused by GalN plus LPS. Pretreatment with the herbal extract also depressed the elevation of lipid peroxidation caused by GalN plus LPS, showing that the herbal extract scavenges ROS (such as superoxide anion and hydroxyl radical) resulting in preventing GalN plus LPS induced liver injury.

Carbon tetrachloride (CCl\(_4\)) is biotransformed by the liver microsomal cytochrome P450 enzyme system to radicals such as trichloromethyl radical (CCl\(_3\)) and peroxy radical (OOCCl\(_3\)), resulting in lipid peroxidation of membrane lipid.\(^16\)\(^-\)\(^18\) Thus it was expected that a radical scavenger may ameliorate CCl\(_4\)-induced liver toxicity. As shown in Fig. 5, the elevation of AST and ALT activities in serum and of lipid peroxidation in liver was depressed by the herbal extract treatment. It is therefore suggested that *C. crepidioides* scavenges CCl\(_4\)-derived radicals resulting in depressing the toxicity.

Since the antioxidant action of *C. crepidioides* was demonstrated in vitro and in vivo, we isolated active components of the herb and clarified that isochlorogenic acid a and b are main antioxidants in the extract and that the other flavonoids quercetin 3-galactoside, quercetin 3-glucoside, kaempferol-3-galactoside and kaempferol-3-glucoside were also involved. These components might contribute to the hepatoprotective action of *C. crepidioides*. Flavonoids are widely distributed in various plants as antioxidants but the high content of isochlorogenic acid which accounts for 67% of the antioxidants in *C. crepidioides* is quite unique.

In conclusion, the water extract of *C. crepidioides* showed strong scavenging action for DPPH, superoxide anion and moderate action for hydroxyl radical in vitro. Pretreatment with the herbal extract of GalN plus LPS-treated rats or CCl\(_4\)-treated mice prevented the chemically induced hepatotoxicities. These results suggest that ROS or radicals generated by GalN plus LPS or CCl\(_4\) treatment were scavenged by the herbal extract resulting in prevention of the hepatotoxicities. Antioxidants including isochlorogenic acid in the herb may contribute to the protective action against the liver toxicity.

**Acknowledgements** The authors thank Mr. K. Nakamoto of Nakazen Company for his kindness in supplying the medicinal herb, and Ms. N. Murayama for typing the manuscript. This study was partly supported by a Grant-in-Aid for University and Society Collaboration.

**Table 1. Antioxidant Action**

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>DPPH IC(_{50})</th>
<th>O(<em>2^•) IC(</em>{50})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract (dilution)</td>
<td>12.0</td>
<td>280</td>
</tr>
<tr>
<td>Isochlorogenic acid a (( \mu )m)</td>
<td>52.0</td>
<td>6.5</td>
</tr>
<tr>
<td>Isochlorogenic acid b (( \mu )m)</td>
<td>48.0</td>
<td>4.1</td>
</tr>
<tr>
<td>Ascorbic acid (( \mu )m)</td>
<td>12.3</td>
<td>38.5</td>
</tr>
</tbody>
</table>

**References**


