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Oral Administration of *Trapa taiwanensis* Nakai Fruit Skin Extracts Conferring Hepatoprotection from CCl_4-Caused Injury

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ABSTRACT: As a folk medicine, the hot-water infusion of water caltrop fruits has been used to protect the liver. In this study, the outer skins of mature water caltrop fruits (*Trapa taiwanensis* Nakai) were removed, forced-air-dried, pulverized, and subjected to extraction with hot water, and the infusion was lyophilized and pulverized to prepare a hot water extract of *T. taiwanensis* (HWETT). HWETT was subjected to assays of α,α-diphenyl-β-picrylhydrazyl scavenging activity, reducing power, Trolox equivalent antioxidant capacity, and antioxidative potency, and all determinations showed HWETT to be a potent antioxidant. As further analyzed with LC-MS, two major HPLC-detected components were elucidated as gallic acid and ellagic acid. Hepatoprotective activity of HWETT was assessed with Sprague−Dawley male rats by oral administration. Six groups of rats ($n = 8$ for each) were respectively treated, namely, control, CCl_4 (20% CCl_4/olive oil by 2.0 mL/kg bw), CCl_4 and Silymarin (200 mg/kg bw), CCl_4 and low HWETT dose (12.5 mg/kg bw), CCl_4 and medium HWETT dose (25 mg/kg bw), and CCl_4 and high HWETT dose (125 mg/kg bw). After 8 weeks, all animals were fasted for an additional day and sacrificed to collect blood, liver, and kidney for analyses. Histopathological examinations showed that oral administrations with Silymarin and HWETT were effective in protecting the liver from CCl_4-caused fatty change. Oral administration of HWETT at 125 mg/kg bw was more effective than was Silymarin at 200 mg/kg bw. On biochemical analyses, oral administrations with HWETT at medium and high doses were effective ($p < 0.05$) in lowering CCl_4-caused increases of alanine aminotransferase and aspartate aminotransferase activities. It is of merit to demonstrate HWETT as a potent source of antioxidants and hepatoprotective agents.

KEYWORDS: water caltrop, gallic acid, ellagic acid, antioxidant, CCl_4, liver protection

INTRODUCTION

Water caltrop (*Trapa* sp.), an aqueous floating plant, belongs to the Trapaceae family and grows in shallow water fields, ponds, or swamps in tropical and subtropical areas.[1] Fruits of water caltrop vary with different sizes, shapes, and colors, depending on variety and maturity. Fruits with bull-horn shape (a pair of horns on the fruit shoulders) are most commonly grown in Taiwan. The inside kernel (pulp) is generally 1−2 cm in width and 3−5 cm in length. Fruits bear pink color and change to dark brown with increase of maturity or time of postharvest storage. In the harvesting season, inside kernels are removed manually and destined for food use. Abundant fruit hulls are discarded as agricultural waste. It is of merit to extract the bioactive compounds from the hulls for value-added product development. Water caltrop has been used in traditional medicine, mostly in Asian countries, including China, India, and Bangladesh.

Carbon tetrachloride (CCl_4) is widely used to cause liver injury in animal models. Ingested CCl_4 is generally metabolized by cytochrome p450 in the endoplasmic reticulum to form reactive intermediates such as trichloromethyl radical ($^{\bullet}CCl_3$), one of most reactive free radicals.[2] The active free radicals consequently cause oxidative stress, hepatotoxicity, and tissue injury. The most remarkable CCl_4-caused injury is diagnosed from fatty liver to cirrhosis and necrosis. In prevention of hepatotoxicity, intervention of bioactive compounds from foods or dietary supplements to eliminate or decrease quantities of the active free radicals (or so-called reactive oxygen species, ROS) is critical. ROS tend to bind macromolecules, such as proteins, lipids, and DNA, resulting in physiologic dysfunction. Accordingly, blocking or retarding the ROS-involved chain reactions is important in the prevention of oxidative stress-induced hepatotoxicity. As further related, enhancement of the phase II detoxifying and antioxidant enzymes and elevation of the antioxidant substances is also important to enhance hepatoprotective activity against oxidative stress. In this study, antioxidant activities of water caltrop (*Trapa taiwanensis* Nakai) fruit skin extracts were investigated. Two of the HPLC-detected major compounds were isolated and subjected to structural elucidation. The extracts were further subjected to an in vivo experiment with CCl_4-induced Sprague−Dawley male rats by oral administration in demonstration of hepatoprotective activity. On the basis of the fact that Silymarin has long been used in clinical therapy of liver diseases[3] and has been demonstrated to be a potent antioxidant with anti-inflammatory, hepatoprotective, and growth-modulatory activities,[4] it was used in this study as a reference compound for comparison of hepatoprotective activities with rats as an animal model.[5]

MATERIALS AND METHODS

Water Caltrop and Preparation of Fruit Skin Extracts. Kernel-removed fresh fruit hulls of water caltrop (*Trapa taiwanensis* Nakai) were obtained from a local market in Chiayi, Taiwan. Outer skins

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of the hulls were removed manually and subjected to drying in a forced-air oven at 50 °C until constant weight was reached. The dry matter was then pulverized into powder by a cyclone mill, sealed in a plastic bag, and stored under −20 °C until used.

For hot water extraction, 5 g of the dried skin powder and 250 mL of deionized water (1:50, w/v) were deposited in a 500 mL flask and homogenized with a Polytron (Kinematica AG, Littau, Switzerland) at 12000 rpm for 1 min. Then, the suspension was heated in a boiling water bath for 30 min. After heating, the suspension was filtered through a filter cloth, and the filtrate was centrifuged at 15000*g* for 30 min. The supernatant was subjected to vacuum evaporation at 40 °C to ca. $^1/_{10}$ volume and lyophilized to prepare freeze-dried hot water extract of *T. taiwanensis* (abbreviated HWETT). Yield of HWETT powder based on five batches of the oven-dried skin powder was 139.3 ± 13.1 mg/g. All samples were protected from light exposure by covering with aluminum foil. HWETT was sealed in a plastic bag and stored under −20 °C until used.

Antioxidant Assessments. For determination of α,α-diphenyl-β-picrylhydrazyl (DPPH) scavenging activity, the procedures reported by Shimada et al.[6] and Ko et al.[7] were followed. Butylated hydroxytoluene (BHT) was determined concurrently as a reference for comparison of antioxidant potency. In determination of reducing power, the procedure of Yen and Chen[8] was followed with minor modification. Briefly, aliquots of 0.5 mL of aqueous HWETT solutions with different concentrations were mixed with 0.5 mL of phosphate buffer (0.2 M, pH 6.6) and 0.5 mL of $K_3Fe(CN)_6$. After incubation at 50 °C in a water bath for 20 min, the solution was rapidly cooled in an ice bath and replenished with 0.5 mL of 10% trichloroacetic acid (TCA). After centrifugation at 1000*g* (20 °C) for 10 min, 1 mL of the supernatant was withdrawn and mixed with 1 mL of water and 0.2 mL of 0.1% $FeCl_3$ solution and incubated at the ambient temperature (26−28 °C) for 10 min. The absorbance at 700 nm was determined (U-2001 spectrophotometer, Hitachi Co. Ltd., Tokyo, Japan) for reducing power estimation. Vitamin C was determined concurrently as a reference.

For determination of Trolox equivalent antioxidant capacity (TEAC), the improved $ABTS^{\bullet+}$ methods reported by Re et al.[9] and Li et al.[10] were followed with minor modification. Briefly, $ABTS^{\bullet+}$ radical cation was generated by a reaction of 14 mM ABTS with 4.9 mM potassium persulfate. The reaction mixture was allowed to stand in the dark at room temperature for 16 h and used within 2 days. The $ABTS^{\bullet+}$ solution was diluted with ethanol, to give an absorbance of 0.70 ± 0.05 at 734 nm. All samples were diluted appropriately to give absorbance values in the range of 20−80% to that of blank. Fifty microliters of the diluted samples was mixed with 1.9 mL of the diluted $ABTS^{\bullet+}$ solution and allowed to stand for 6 min at room temperature prior to absorbance determination at 734 nm. A series of Trolox solutions (0−15 μM) was used as reference standard. TEAC was expressed as micromoles of Trolox per gram of HWETT. For determination of antioxidative potency (AOP), the procedure of Hsu et al.[11] was followed. BHT was determined concurrently as a reference for comparison of antioxidant potency.

Compositional Analyses by HPLC and LC-MS. For analysis of the phenolic compounds, an optimized HPLC procedure in the analysis of HWETT has been developed through extensive preliminary investigations. An HPLC (L-7100 pump, L-7420 UV detector, and L-7455 diode array detector, Hitachi Co.) was equipped with an RP-18 column (Hypersil ODS, 250 × 4.6 mm, 5 μM, Thermal Hypersil Ltd., Cheshire, U.K.). The mobile phase contains A (100% methanol) and B (0.1% tetrabutylammonium hydroxide and 0.5% phosphoric acid). Each analysis was initiated with 12% A and 88% B, programmed to 25% A and 75% B in 25 min, to 100% A and 0% B in 42 min, held for 3 min, to 12% A and 88% B in 5 min, and held for 5 min. The flow rate and monitoring wavelength were 0.8 mL/min and 254 nm, respectively. As preliminarily observed, two major peaks (peaks 1 and 2) were detected (Figure 1). Gallic acid and quercetin were detected in experiments conducted for a study with water caltrop fruit pulps.[12] Gallic acid and quercetin (Sigma Chemical Co., St. Louis, MO) were respectively analyzed for comparison of HPLC retention time and spiked for analysis in detection of peak match and overlapping.

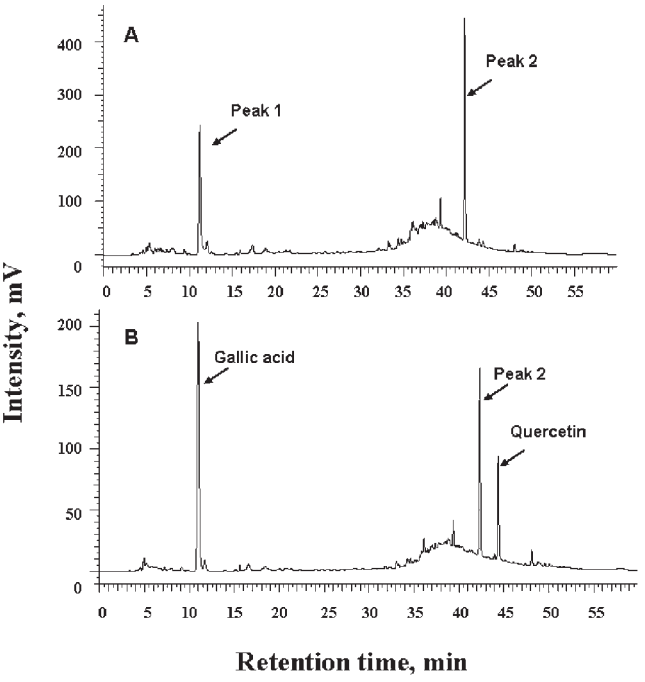

Figure 1. (A) HPLC chromatogram of the hot water extract of *Trapa taiwanensis* Nakai skins (HWETT) monitored at 254 nm, in which peaks 1 and 2 were detected as the major compounds, and (B) chromatogram of the extract (HWETT) spiked with authentic gallic acid and quercetin.

For further identification of peak 2, the peak fractions were collected, rotary evaporated, freeze-dried, and subjected to LC-MS analysis. An HPLC system (LTQ Velos, Thermo Scientific, West Palm Beach, FL) was equipped with a mass detector in series with a binary pump, an autosampler, and a controller manipulated by Xcalibur 2.0.6 software (Thermo Scientific). An HPLC column (Biobasic-18, 15 × 0.2 cm, particle size = 5 μm) (Thermo Scientific) was run with a gradient solvent system consisting of solvent A (water with 0.1% formic acid, v/v) and solvent B (acetonitrile) at a flow rate of 0.25 mL/min. A linear gradient was initiated with 100% A and linearly decreased to 95% A in B in 3 min, to 5% A in B at 3−20 min, and 5% A in B at 20−25 min. The mass detector consisted of an ion trap mass spectrometer equipped with an electrospray ionization (ESI) system. The capillary and voltage were maintained at 250 °C and 3.5 kV, respectively. Mass scan (MS) and daughter (MS-MS) spectra were measured from m/z 120 to 800. Collision-induced fragmentation experiments with collision energy set at 50% were performed in the ion trap using helium as the collision gas. Mass spectrometry data were acquired in negative ionization mode. All analyses were performed in triplicate.

In Vivo Experiment. Male Sprague−Dawley rats (200 ± 10 g) (BioLASCO Taiwan Co., Ltd., Taipei, Taiwan) were used as an animal model. The animal experiment proposal has been approved by the Institutional Animal Care and Use Committee (IACUC) of the National Chiayi University. The body weight (bw) of each rat was taken daily. After 1 week adaptation, the rats were randomly divided into six groups (8 rats/group, 4 rats in a cage), namely, control, CCl_4 (20% CCl_4/olive oil),[13,14] CCl_4 and Silymarin (200 mg/kg bw) (Sigma Chemical Co.), CCl_4 and low HWETT dose (12.5 mg/kg bw), CCl_4 and medium HWETT dose (25 mg/kg bw), and CCl_4 and high HWETT dose (125 mg/kg bw). Food and water were provided ad libitum. Gavage oral administration was conducted. On Mondays and Thursdays, each of the

rats, except those in the control group, was orally administered 20% CCl_4/olive oil (2.0 mL/kg bw). Meanwhile, each rat of the control group was orally administered olive oil (2.0 mL/kg bw). For daily treatment, the specified quantity of Silymarin or HWETT was dissolved in 0.5 mL of saline and orally administered to each of the assigned rats. For the control and CCl_4 groups, each rat was orally administered 0.5 mL of saline.

After 8 weeks of experiment, animals were fasted for an additional day and sacrificed by CO_2. Blood samples were immediately collected in blood tubes, kept at room temperature for 1 h, and centrifuged at 1000*g* for 10 min to obtain sera. The serum samples were stored at −20 °C for biochemical analyses. From each rat the liver was removed and rinsed with PBS. Then, the liver organs were excised and cut into two halves. One part was quickly frozen with liquid nitrogen and stored at −80 °C until used for the quantification of antioxidative components and determination of antioxidant enzymes. The other part from the same lobe was fixed in 10% buffered formaldehyde, processed for tissue specimen preparation, and H&E stained for histopathological examination by a veterinary pathologist with blind sample labeling. On the basis of the extent of fatty change, cytoplasmic vacuolization, and necrosis, liver tissues were examined and classified into five categories mainly based on the extent of fatty change: 0, normal and no fatty change; 1, trace fatty change; 2, slight fatty change; 3, moderate fatty change; 4, strong fatty change. Kidney, heart, lung, and spleen were also excised for visual examination and weight determination.

Serum Biochemical Analyses and Determinations of Liver Glutathione Contents and Antioxidative Enzyme Activities. Biochemical parameters of serum aspartate transaminoferase (AST) and alanine aminotransferase (ALT) and changes of blood urea nitrogen (BUN) and lactate dehydrogenase (LDH) were determined by using a Hitachi 7170 automatic biochemical analyzer (Hitachi Co. Ltd., Tokyo, Japan) according to the manufacturer's manual. Briefly, liver tissues were weighed and homogenized with a tissue homogenizer (Glas-Col, LLC, Terre Haute, IN) in ice-cooled 50 mM potassium phosphate buffer (pH 7.4) for 1 min. The homogenates were then centrifuged at 2500*g* for 30 min (4 °C) to obtain supernatants as liver extracts. Liver extracts were stored at −80 °C for further analyses. Prior to frozen storage, total protein contents of the liver extracts were determined according to the method of Bradford.[15] For each determination, 20 μL of the extract was mixed with 1 mL of Bradford reagent (Amresco Inc., Solon, OH) and incubated at the ambient temperature (26−28 °C) for 10 min. The absorbance at 595 nm was determined, and a series of bovine serum albumin (BSA) (Sigma Chemical Co.) solutions were used to construct a reference curve in estimation of protein content. For determination of liver reduced glutathione (GSH) content, the procedures of Van Dam et al.[16] and Lee et al.[17] were followed with minor modification. Briefly, 500 μL of the extract was mixed with 500 μL of 5% trichloroacetic acid (TCA) (Sigma Chemical Co.) and incubated at 4 °C for 5 min, followed by centrifuging at 8000*g* for 10 min (4 °C) to obtain supernatant. Aliquots (100 μL) of the supernatant were mixed with 900 μL of 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) (Sigma Chemical Co.) and incubated at 4 °C for 5 min prior to absorbance determination at 412 nm. A standard curve constructed by a series of authentic GSH solutions was used as a reference in estimation of GSH content and expressed as micromoles per milligram of protein.

Glutathione peroxidase (GPx) activity of the liver extract was determined following the procedure reported by Mohandas et al.[18] For each determination in a cuvette, 0.1 mL of liver extract and 0.8 mL of 100 mM potassium phosphate buffer (pH 7.0 containing 1 mM EDTA, 1 mM NaN_3, 0.2 mM NADPH, 1 unit/mL GSH reductase, and 1 mM GSH) were introduced and preincubated at ambient temperature for 5 min. Then, 0.1 mL of 2.5 mM H_2O_2 solution was introduced and mixed to initiate enzymatic reaction. The absorbance at 340 nm was determined after 5 min of incubation at ambient temperature. Enzymatic activity was estimated by the absorbance change after 5 min of incubation and expressed as nanomoles of NADPH per minute per milligram of protein.

Glutathione reductase (GRd) activity of the liver extract was determined mainly on the basis of NADPH consumption in reduction of glutathione disulfide (GSSG).[19] For each determination in a cuvette, 0.1 mL of liver extract and 0.9 mL of 0.1 M phosphate buffer (pH 7.0, containing 1 mM $MgCl_2 \cdot 6\,H_2O$, 50 mM GSSG, and 0.1 mM NADPH) were introduced, mixed, and incubated at ambient temperature for 5 min prior to absorbance determination. GRd activity was estimated by the absorbance change at 340 nm for 5 min and expressed as nanomoles of NADPH per minute per milligram of protein.

Catalase (CAT) activity of the liver extracts was determined following the procedure of Cohen et al.[20] Briefly, 2 μL of liver extract prepared as described above was mixed with 1998 μL of 50 mM (pH 7.0) potassium phosphate buffer (containing 1% Triton X-100) and 1 mL of 20 mM H_2O_2 and then incubated at ambient temperature for 2 min prior to absorbance determination. CAT activity was estimated by the absorbance change at 240 nm in 2 min of incubation and expressed as micromoles of H_2O_2 per minute per milligram of protein.

Superoxide dismutase (SOD) activity was assayed by the pyrogallol oxidation system[21] with minor modification. Briefly, each of the liver tissues was weighed and homogenized in ice-cooled 10 mM (pH 7.4) Tris-HCl buffer (1:10 w/v) for 1 min, followed by centrifugation at 13600*g* for 30 min (4 °C) to obtain supernatant, from which 25 μL was mixed with 975 μL of 50 mM (pH 7.4) Tris−cacodylic buffer, 375 μL of deionized water, and 25 μL of 10 mM pyrogallol solution (Sigma Chemical Co., prepared in deionized water) and incubated at 25 °C. SOD activity was estimated by the change of absorbance at 420 nm in 3 min of incubation. One unit of SOD activity is defined as the equivalency of 50% inhibition of pyrogallol autoxidation in 1 min at 25 °C, and the activities were expressed as units (U) of SOD activity per milligram of protein.

Statistical Analysis. All determinations are expressed as the mean ± SD. Results were statistically analyzed by one-way ANOVA and followed by Duncan's multiple-range method for significance analysis at the $p < 0.05$ level.

RESULTS AND DISCUSSION

DPPH scavenging activities of HWETT determined at various concentrations are shown in Figure 2A. As observed, the higher the concentration, the higher was the scavenging activity detected. As tested at 50, 75, and 100 μg/mL of HWETTs, their scavenging activities are 61.2, 87.0, and 90.7%, respectively. As compared to BHT, the activity of 100 μg/mL of HWETT was equivalent to 25 ppm BHT, indicating that HWETT is a potent source of free radical scavenger. When HWETTs were subjected to TEAC assay at 100, 200, 300, 400, and 500 μg/mL, their $ABTS^{\bullet+}$ scavenging activities increased linearly with increase of concentration (Figure 2B) and were equivalent to 113, 231, 308, 399, and 480 μM Trolox, correspondingly. Estimated on the basis of the linear dose dependence relationship and expressed per gram of HWETT, its $ABTS^{\bullet+}$ scavenging activity was equivalent to 1.05 ± 0.09 mmol of Trolox. As shown in Figure 2C, reducing powers of HWETT tested at 50, 75, and 100 μg/mL were correspondingly equivalent to 3.1, 6.2, and 12.3 μg/mL of vitamin C. Further subjection of HWETTs to AOP assay at 50, 75, 100, 150, and 200 μg/mL (Figure 2D) revealed AOP values of 4.3, 23.5, 68.4, 84.7, and 92.5%, correspondingly. As tested at 100 μg/mL of HWETT, the AOP was equivalent to that of BHT tested at 25 μg/mL. As generalized and compared with BHT and vitamin C, all antioxidant activity determinations showed HWETTs to be potent natural antioxidant sources. In comparison with the literature, at 10 mg/mL, lower DPPH

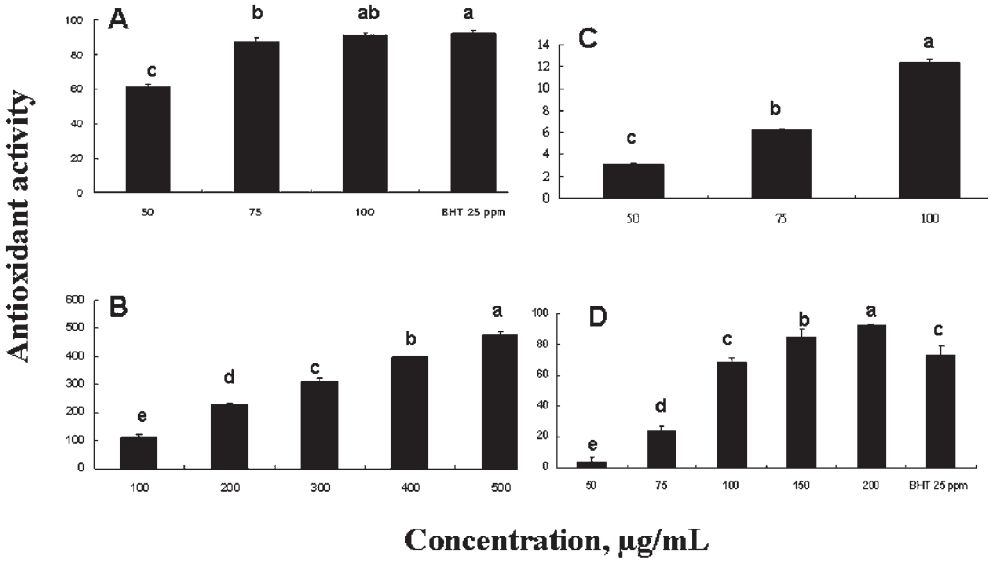

Figure 2. Antioxidant activities of the hot water extract of *Trapa taiwanensis* Nakai skins (HWETT) determined by DPPH radical scavenging activity (%) (A), Trolox equivalent antioxidant activity (μM Trolox) (B), reducing power (μg/mL of vitamin C) (C), and antioxidative potency (%) (D). Each value was expressed as the mean ± SD (n = 3). Bars with different letters are significantly different ($p < 0.05$).

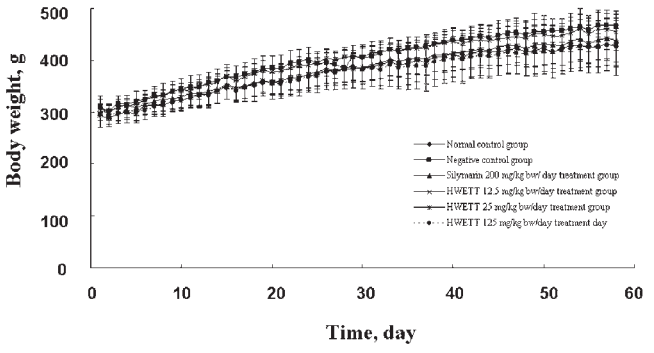

Figure 3. Body weight changes of Sprague−Dawley rats after subjection to CCl_4 treatment and oral administration with Silymarin or hot water extract of *Trapa taiwanensis* Nakai skins (HWETT) for 8 weeks. Values are the mean ± SD (n = 8).

Table 1. Liver and Kidney Weights of Sprague−Dawley Rats after Subjection to Various Treatments for 8 Weeks

	relative organ weight[a], g/100 g bw	
treatment	liver	kidney
control	2.87 ± 0.21 d	0.68 ± 0.06
CCl_4 (20% CCl_4/olive oil)	4.04 ± 0.61 a	0.72 ± 0.07
CCl_4 and Silymarin 200 mg/kg bw	3.43 ± 0.54 c	0.73 ± 0.05
CCl_4 and HWETT[b] 12.5 mg/kg bw	3.86 ± 0.43 abc	0.70 ± 0.04
CCl_4 and HWETT 25 mg/kg bw	3.93 ± 0.41 ab	0.72 ± 0.06
CCl_4 and HWETT 125 mg/kg bw	3.53 ± 0.36 bc	0.68 ± 0.09

[a] Values are the mean ± SD (n = 8); values in the same column with different letters are significantly different ($p < 0.05$). [b] HWETT, hot water extract of *Trapa taiwanensis* Nakai skins.

radical scavenging activity and reducing power were detected in methanolic extracts of the fresh and dried water caltrop pulp.[22] This reveals that hull skins may contain a more abundant quantity of antioxidants than other water caltrop matrices.

As analyzed by HPLC, two major compound peaks were detected and are shown in the chromatogram (Figure 1A), in agreement with a study conducted with water caltrop fruit pulps[12] in which gallic acid and quercetin were identified as two of the antioxidant compounds. When authentic gallic acid and quercetin were subjected and spiked for analysis, the identity of the HPLC retention time and complete peak match and overlapping of peak 1 with the spiked authentic gallic acid were observed. Accordingly, peak 1 was identified as gallic acid, and peak 2 was not quercetin. Furthermore, after repeated collection of the peak 2 fractions, the pooled peak 2 fractions were subjected to LC-MS analysis. The LC retention time was 9.91 min, and the obtained MS spectrum indicates that M − H m/z was 301. In comparison to the fragments with the reported data,[23] peak 2 was identified as ellagic acid. In the literature, gallic acid, quercetin, ferulic acid, and hydroxycinnamic acid have been detected in the fruit pulps of water caltrop (*T. taiwanensis* Nakai).[12] In this study, HPLC chromatograms (Figure 1) showed that gallic acid and ellagic acid are two major detected compounds of HWETT. Because gallic acid and ellagic acid are essential molecules of gallotannins and/or ellagitannins, the prediction that the natural fruit skins contain gallotannins and ellagitannins is very likely, and this deserves further investigations. Both gallic and ellagic acids and related tannins are well-known antioxidants. Protective effects of gallic acid and ellagic acid against hydrogen peroxide-induced oxidative stress and DNA damage in IMR-90 cells have been demonstrated.[24]

As an in vivo experiment with Sprague−Dawley rats, body weight changes of the rats in each group are shown in Figure 3. A general trend was observed for each of the groups; body weights increased gradually with an increase of feeding time. In comparison, body weights of the CCl_4-treated groups were lower than those of the non-CCl_4-treated groups. When a comparison was made among the CCl_4-treated groups, oral administration of Silymarin or various doses of HWETT did not result in significant difference. For rats orally administered with HWETT at

the highest dose of 125 mg/kg bw for 8 weeks, ceased growth or decrease of body weight was not observed. Their relative kidney and liver weights are presented in Table 1. In comparison to the control group, there was no significant kidney weight difference among the CCl_4-treated rats, HWETT-treated groups, and control group. However, by comparison to the control group, a significant increase of relative liver weight ($p < 0.05$) was observed for the CCl_4-treated rats. CCl_4 is a well-defined and studied toxic alkane causing oxidative stress and hepatotoxicity and leading to hepatocellular death or carcinogenicity.[25] When the CCl_4-treated rats were treated daily with Silymarin at 200 mg/kg bw or with HWETT at 125 mg/kg bw for 8 weeks, the CCl_4-caused increase of relative liver weights was significantly decreased ($p < 0.05$). For the other major organs including heart, lung, and spleen, there was no significant difference of relative organ/body weight ratios caused by all conducted treatments (data not shown).

Tissue photographs of rat livers subjected to histopathological examinations are shown in Figure 4. As observed, in comparison to the normal tissue of rats in the control group (scored as 0) (Figure 4A), CCl_4 treatment caused strong fatty change, cytoplasmic vacuolization, and necrosis of the liver tissues (scored as 4) (Figure 4B). CCl_4 has long been used to cause liver injury of various animal models in investigations of hepatoprotective activity.[26] In this study, oral administration of the CCl_4-treated rats with Silymarin at 200 mg/kg bw resulted in moderate fatty change (scored as 3) (Figure 4C). Oral administration of the rats with HWETT at 12.5, 25, and 125 mg/kg bw resulted in moderate, slight, and trace fatty change (scored as 3, 2, and 1), respectively (Figure 4D−F). Obviously, a dose-dependent protection of liver tissue from CCl_4-caused fatty change was observed by HWETT treatments. Oral administration of HWETT at 125 mg/kg bw performed the better effect as did Silymarin at 200 mg/kg bw on preventive protection of CCl_4-caused fatty change. On the basis of the fact that Silymarin has been commonly applied as a human and animal medicine,[27] the observed hepatoprotective activity of HWETT is of merit and deserves further investigation.

Enzyme activities of serum AST and ALT activities of rat livers as affected by various treatments are shown in Table 2. It is well-known that CCl_4 is a potent chemical agent causing hepatic injury and tremendously increasing ALT and AST activities.[25] Rats treated with CCl_4 to elevate serum AST and ALT activities provide a suitable animal model in the detection and screening of hepatoprotective compounds. Generally, after CCl_4 treatment, wide ranges of deviation of determinations among each individual rat in each group were observed. For comparison of the AST and ALT activities of the Silymarin- and HWETT-treated groups to those of the CCl_4-treated group, oral administration of Silymarin (200 mg/kg bw) was effective ($p < 0.05$) in lowering CCl_4-caused increase of serum ALT activity. Oral administrations of the rats with medium (25 mg/kg bw) and high doses (125 mg/kg bw) of HWETT were effective ($p < 0.05$) in lowering CCl_4-caused increases of both AST and ALT activities. Accordingly, hepatoprotective activity of HWETT was further supported by its potency in lowering CCl_4-caused increases of AST and ALT activities. Plasma LDH activities of the blood samples collected from the rats in each group are also shown in Table 2. As affected by treatment, medium dose of HWETT treatment at 25 mg/kg bw was effective ($p < 0.05$) in lowering CCl_4-caused increase of plasma LDH activities. However, plasma LDH activities were significantly increased ($p < 0.05$) by Silymarin treatment at 200 mg/kg bw. The reason is unclear and deserves further investigation. With regard to kidney as affected by treatments, BUN contents of the rats subjected to Silymarin and HWETT treatments were distributed in the insignificant ranges as compared with the contents of the CCl_4-treated group (Table 2). This indicates that HWETT did not alter the effects of CCl_4 on BUN, which is used as an indicator of kidney function.

Antioxidant- and detoxification-related liver enzyme activities and GSH contents of the rats after subjection to various treatments are presented in Table 3. It is well-known that GSH-related enzymes play detoxifying and antioxidant roles in metabolizing or eliminating xenobiotics through conjugation with glutathione and reducing free radicals. Phase II antioxidant-related enzymes are involved in the protection of CCl_4-induced damage of hepatitis.[28] GPx mainly converts H_2O_2 to water and

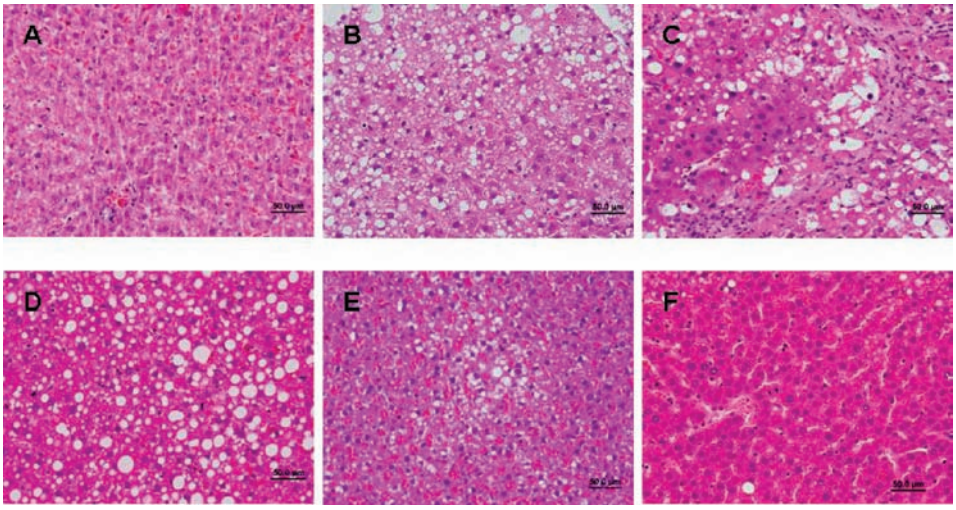

Figure 4. Histopathological examination of the liver tissues (H&E stain): (A) control (normal and absent fatty change scored as 0); (B) CCl_4 treatment (strong fatty change scored as 4); (C) CCl_4 treatment and oral administration with Silymarin at 200 mg/kg body weight (moderate fatty change scored as 3); (D) CCl_4 treatment and oral administration with hot water extract of *Trapa taiwanensis* Nakai skins (HWETT) at 12.5 mg/kg body weight (slight fatty change scored as 3); (E) CCl_4 treatment and oral administration with hot water extract of *T. taiwanensis* Nakai skins (HWETT) at 25 mg/kg body weight (slight fatty change scored as 2); (F) CCl_4 treatment and oral administration with hot water extract of *T. taiwanensis* Nakai skins (HWETT) at 125 mg/kg body weight (trace fatty change scored as 1).

Table 2. Enzymatic Activities of Serum Aspartate Transaminoferase (AST), Alanine Aminotransferase (ALT), and Lactate Dehydrogenase (LDH) and Changes of Blood Urea Nitrogen (BUN) Content of Sprague−Dawley Rats after Subjection to Various Treatments

	enzymatic activity and content[a]			
treatment	AST, U/L	ALT, U/L	LDH, U/L	BUN, mg/dL
control	104 ± 8 c	46 ± 5 c	958 ± 254 d	21 ± 2 b
CCl_4 (20% CCl4 in olive oil)	715 ± 252 a	830 ± 278 a	2355 ± 992 ab	28 ± 4 a
CCl_4 and Silymarin 200 mg/kg bw	500 ± 286 ab	453 ± 337 b	2517 ± 1361 a	26 ± 4 ab
CCl_4 and HWETT[b] 12.5 mg/kg bw	623 ± 474 ab	571 ± 356 ab	1561 ± 608 bcd	29 ± 6 a
CCl_4 and HWETT 25 mg/kg bw	367 ± 160 bc	459 ± 388 b	1380 ± 431 cd	26 ± 4 ab
CCl_4 and HWETT 125 mg/kg bw	381 ± 138 bc	427 ± 238 b	1953 ± 655 abc	31 ± 4 a

[a] Values are the mean ± SD ($n = 8$); values in the same column with different letters are significantly different ($p < 0.05$). [b] HWETT, hot water extract of *Trapa taiwanesis* Nakai skins.

Table 3. Liver Antioxidant-Related Enzyme Activities and Glutathione Contents of Sprague−Dawley Rats after Subjection to Various Treatments for 8 Weeks[a]

treatment	GPx, nmol/min/mg protein	GRd, nmol/min/mg protein	GSH, μmol/mg protein	CAT, μmol/min/mg protein	SOD, U/mg protein
control	290 ± 45 a	56.1 ± 5.4 b	328 ± 53	88 ± 9	2.3 ± 0.3 a
CCl_4 (20% CCl_4 in olive oil)	238 ± 26 b	58.5 ± 10.7 ab	374 ± 92	88 ± 14	1.9 ± 0.2 b
CCl_4 and Silymarin 200 mg/kg bw	280 ± 47 a	63.3 ± 9.1 ab	411 ± 162	96 ± 10	2.3 ± 0.6 a
CCl_4 and HWETT[b] 12.5 mg/kg bw	252 ± 42 ab	56.0 ± 8.0 b	400 ± 168	88 ± 13	2.0 ± 0.3 ab
CCl_4 and HWETT 25 mg/kg bw	255 ± 19 ab	62.1 ± 7.7 ab	398 ± 118	94 ± 10	2.2 ± 0.2 ab
CCl_4 and HWETT 125 mg/kg bw	271 ± 36 ab	67.5 ± 10.9 a	402 ± 122	97 ± 16	2.3 ± 0.3 ab

[a] GPx, glutathione peroxidase; GRd, glutathione reductase; GSH, glutathione; CAT, catalase; SOD, superoxide dismutase. Values are the mean ± SD ($n = 8$); values in the same column with different letters are significantly different ($p < 0.05$). [b] HWETT, hot water extract of *Trapa taiwanesis* Nakai skins.

also integrates with GSH in disintegrating other organic hydroperoxides. GRd is responsible for regeneration of GSH. In this study, as caused by CCl_4 treatment, GPx activities declined significantly. As compared to the CCl_4-treated group, oral administration with Silymarin at 200 mg/kg bw was effective in increasing GPx activity ($p < 0.05$). For the CCl_4-treated rats further orally administered various doses of HWETT, a dose-dependent tendency, but not statistically significant, in the increase of GPx activities was observed. As noted, the GRd activities were significantly increased by oral administration of the high HWETT dose. As further statistically analyzed, catalase activities and GSH contents were not affected by treatments with CCl_4, Silymarin, or HWETT (Table 3). For SOD determinations, oral administration of Silymarin at 200 mg/kg bw was effective ($p < 0.05$) in increasing the CCl_4-caused decrease of SOD activity. For HWETT treatments, a tendency, but not statistically significant, to increase SOD activities was observed.

In conclusion, potent antioxidant activities of HWETT were evidenced by the resultant activity determinations of scavenging α, α-diphenyl-β-picrylhydrazyl radicals, reducing power, Trolox equivalent antioxidant capacity, and antioxidative potency. By a developed HPLC procedure, two major HPLC-detected components were elucidated as gallic acid and ellagic acid, indicating that involvement of other gallotannins and ellagitannins is likely. As assessed in vivo with Sprague−Dawley male rats by gavage administration with CCl_4, Silymarin, and HWETT, histopathological examinations showed that treatments with HWETT or Silymarin at 200 mg/kg bw were effective in protecting livers from CCl_4-caused injury. In addition, CCl_4-caused increases of alanine aminotransferase and aspartate aminotransferase activities were significantly inhibited ($p < 0.05$) by HWETT treatments at 25 and 125 mg/kg bw. Generally, it is of merit to identify ellagic acid and gallic acid as the major HPLC-detected components of HWETT and, supported by an in vivo experiment, to find HWETT being effective in protecting livers from CCl_4-induced oxidative injury. Thus, future works should be focused on intensive investigations on the related active compounds in addition to gallic and ellagic acids and on mechanisms of HWETT-involved biochemical and physiological activities. Nevertheless, on the basis of the fact that HWETT is a newly emerged and scientifically investigated bioactive source, further investigations addressed to more intensive and extensive assessments on its toxicology are also necessary.

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