Japanese Butterbur (Petasites japonicus) Leaves Increase Hepatic Oxidative Stress in Male Rats

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We investigated the adverse effects of Japanese butterbur leaves (Petasites japonicus, Compositae) in male F344/DuCrj rats. The rats were fed a control diet or a treatment diet containing 5% butterbur leaf powder for 4 weeks. No differences were observed in body weight gain, food intake or feed efficiency between treatments, but relative liver weight in the butterbur group was significantly higher than that of the control group. In addition, thiobarbituric acid reactive substances (TBARs) and glutathione levels in the serum and liver of the butterbur group were higher than those of the control group. Furthermore, hepatic cytochrome 2E1 mRNA expression was higher than in the control group. In vitro, an acetone extract of the butterbur leaf powder showed the strongest increase in TBARs level in a hepatic homogenate through 4 d. Our findings suggest that feeding 5% butterbur leaf powder to rats can cause adverse effects by increasing oxidative stress.

Key words: Japanese butterbur (Petasites japonicus); liver; oxidative stress; rat

Japanese butterbur (Petasites japonicus, Compositae) is a perennial plant that grows wild in East Asia. It is used as a herbal remedy for asthma, and boiled and processed parts of the stalk are widely consumed as a food in Japan. Recently, butterbur species have attracted great interest because butterbur extract and certain components, including petasins, fukinolic acid, and flavonoid glycosides, have a relaxant effect, anti-inflammatory activity, an anti-migraine effect, and anti-allergic properties. In addition, Park et al. have reported that a methanol extract of butterbur had an antioxidant effect in monosodium L-glutamate (MSG)-challenged mice.

Due to the beneficial health effects of butterbur species, they are expected to be useful as herbal medicines. Indeed, an extract (Ze 339) of the roots of P. hybridus, an indigenous species of butterbur in Europe, has been used as a preventive therapy agent (Petadolex; Weber & Weber International GmbH & Co. KG, Munich, Germany) for the prophylaxis of migraines, and was also reported to improve the condition of patients with allergic rhinitis. However, butterbur species are known to contain toxic pyrrolizidine alkaloids, which are perhaps carcinogenic, hepatotoxic, and mutagenic. The metabolic formation of reactive pyrrolidyl ester metabolites, catalyzed by cytochrome P-450 monooxygenases (CYP), is primarily responsible for the hepatotoxicity and carcinogenicity of pyrrolizidine alkaloids in experimental animals. In addition to these adverse effects, long-term exposure to these alkaloids can cause cell enlargement, metabolic disturbances, and fatty degeneration. Furthermore, other pyrrolizidine alkaloid-containing plants including Senecio, Crotalaria, and Echium species, have been involved in human and animal poisoning. Hence, some researchers have suggested that unpurified butterbur or extracts can be hepatotoxic and carcinogenic in livestock.

Recently, Wildi and colleagues suggested that the leaves of P. hybridus contain a negligible level of toxicants and are clearly a good source for drug. To date, however, there have been few reports on evaluation of the safety of Japanese butterbur leaves. Hence we investigated the role of unpurified butterbur leaf powder in postprandial oxidative stress in rats.

Materials and Methods

Preparation of butterbur leaf powder and extract. Butterbur leaves were obtained locally in the Tokachi area of Hokkaido, Japan. To prepare butterbur leaf powder, leaves were gently washed with water and air dried on filter paper. Then they were weighed, cut into small pieces, and lyophilized for 48 h. Next, the freeze-dried butterbur leaves were mashed and ground to a powder. To prepare aqueous and organic solvent extracts, butterbur leaf powder (10 g) in 200 mL of distilled water (w/v) was boiled at 95 °C for 1 h with gentle stirring, and then cooled to room temperature. Next the mixture was centrifuged at 1,000 rpm for 5 min at 4 °C and filtered through Whatman no. 2 filter.
paper. The powder was re-extracted with 100 mL of distilled water twice under the same conditions. The combined supernatants were lyophylized, weighed, and then diluted with 99% methanol to a concentration of 1 mg/mL. Meanwhile, the residual filtrates on the filter papers were dried at 40 °C for 12 h and then extracted with 100 mL of acetone, as described for the aqueous extract. Finally the residual filtrates from the acetone treatment were extracted with 100 mL of ethanol. The collected organic soluble supernatants were subjected to rotary evaporation at 35 °C to remove the solvent. Finally they were lyophylized, weighed, and diluted with 99% methanol to a concentration of 1 mg/mL for each sample. The yields of the water, acetone, and ethanol extracts were approximately 23.7%, 1.46%, and 0.16% respectively, based on the dry weight of the butterbur leaves.

**Micronutrient contents.** Dietary fiber, protein, lipid, carbohydrate, moisture, and ash in the butterbur leaf powder were measured by the AOAC procedures.20) The contents were as follows (g/100 g): dietary fiber, 40.5 (soluble fiber, 12.5, insoluble fiber, 28.0); protein (calculated by multiplying the nitrogen contents by 6.25), 22.8; lipid, 6.3; carbohydrate, 15.0; moisture, 4.9; and ash, 10.5.

**Animals and diets.** The Animal Experiment Committee of Ohihiro University of Agriculture and Veterinary Medicine approved the experimental animal procedures. Male F344/DuCrj rats (7 weeks of age) were purchased from Charles River Japan (Yokohama, Japan), and randomly divided into two groups of five rats each. There were no significant differences in body weight at the start of the experiment. The control group was fed a diet based on the AIN-93G semi-purified rodent diet21) containing 200 g/kg of casein, 100 g/kg of sucrose, 3.5 g/kg of mineral mixture, 1.0 g/kg of vitamin mixture, 549.5 g/kg of a-cornstarch, 50 g/kg of cellulose, 3 g/kg of l-cystine, 50 g/kg of soybean oil, 2.5 g/kg of choline bitartrate, and 0.014 g/kg of choline bitartrate. The rats were anesthetized with Nembutal (sodium pentobarbital, 40 mg/kg of body weight; Abbott Laboratories, Irving, TX) and killed without fasting. The sera were collected quickly, and the livers were frozen (−80 °C) in screw-cap bottles until analysis. All animal procedures conformed to the principles of the “Guide for the Care and Use of Laboratory Animals.”22)

**Serum biochemical analysis.** Blood samples (1 mL) were collected weekly between 9:00 AM and 9:30 AM in tubes without an anticoagulant from the jugular veins of rats fasted for 12 h. The samples stood at room temperature for 2 h, and then serum was prepared by centrifugation at 1,500 x g for 20 min. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) activities were determined enzymatically using commercially available reagent kits (for the TDX system; Abbott Laboratories Co., Irving, TX). The glucose concentrations were also determined using commercially available reagent kits (Abbott Laboratories Co.).

**Serum and hepatic lipid peroxidation.** The degree of oxidation in the serum collected after the rats were killed was determined using a commercial assay kit (Lipid Hydperoxygenase Assay kit; Wako, Tokyo). Liver samples were homogenized in 10 volumes of phosphate-buffered saline (PBS, pH 7.4). The degree of oxidation was immediately measured by thiobarbituric acid reactive substances (TBARs) assay.25) Protein concentrations were determined by Lowry assay (Bio-Rad, Hercules, CA).24)

**Hepatic glutathione level and antioxidant enzyme activities.** The hepatic GSH level was analyzed by the method of Cohn and Lye,25) as previously described.26) Glutathione reductase (GSH-R), GST, glutathione peroxidase (GSH-Px), and catalase activities were measured by the methods of Worthington and Rosemeyer,27) Habig et al.,28) Lawrence and Burk,29) and Aebi30) respectively, as previously described.26)
Table 2. Serum Biochemical Parameters in Rats over 4 Weeks

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>AST (IU/L)</th>
<th>ALT (IU/L)</th>
<th>LDH (IU/L)</th>
<th>ALP (IU/L)</th>
<th>Glucose (mmol/L)</th>
</tr>
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<tbody>
<tr>
<td>Control Butterbur</td>
<td>Control</td>
<td>Control</td>
<td>Control</td>
<td>Control</td>
<td>Control</td>
</tr>
<tr>
<td>Week 0</td>
<td>238 ± 41</td>
<td>221 ± 37</td>
<td>43.0 ± 7.3</td>
<td>41.8 ± 3.1</td>
<td>4308 ± 1055</td>
</tr>
<tr>
<td>Week 1</td>
<td>183 ± 32</td>
<td>172 ± 29</td>
<td>37.6 ± 3.3</td>
<td>36.4 ± 1.3</td>
<td>3130 ± 773</td>
</tr>
<tr>
<td>Week 2</td>
<td>197 ± 15</td>
<td>165 ± 13*</td>
<td>37.6 ± 2.0</td>
<td>36.2 ± 2.3</td>
<td>3662 ± 338</td>
</tr>
<tr>
<td>Week 4</td>
<td>144 ± 11</td>
<td>162 ± 25</td>
<td>35.4 ± 2.2</td>
<td>35.4 ± 3.1</td>
<td>2278 ± 227</td>
</tr>
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Values are expressed as mean ± standard deviation for five rats. *Indicates significantly different from control at p < 0.05 by Student’s t-test.

Fig. 1. Hepatic TBARs and GSH Levels, and GSH-R, GST, GSH-Px, and Catalase Activities in Rats Fed the Basal (Control) and Japanese Butterbur Leaf Power (Butterbur) Diets for 4 Weeks.

Hepatic TBARs and GSH levels and antioxidant enzymes activities

Figure 1 shows the hepatic TBARs and GSH levels and the GSH-R, GST, GSH-Px, and catalase activities in the livers of the rats fed 5% butterbur leaf powder. The hepatic TBARs and glutathione levels in the butterbur group were significantly (p < 0.05) lower than in the control group. The glucose concentration at 4 weeks was significantly (p < 0.05) higher than in the control group, and there were no significant differences between treatments at the other time points.

Table 3. Serum Glutathione and TBARs Levels in Rats at 4 Week

<table>
<thead>
<tr>
<th>Dietary group</th>
<th>Glutathione (mmol/mL)</th>
<th>TBARs (mmol/mL)</th>
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<tbody>
<tr>
<td>Control</td>
<td>75.5 ± 2.8</td>
<td>0.632 ± 0.155</td>
</tr>
<tr>
<td>Butterbur</td>
<td>147.6 ± 16.1***</td>
<td>0.886 ± 0.097*</td>
</tr>
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</table>

Values are expressed as means ± standard deviation for five rats. ***Significantly different from control at p < 0.05 and p < 0.001 respectively by Student’s t-test.

Hepatic mRNA expression

Figure 2 shows the relative quantities of mRNAs as were determined by Southern hybridization of PCR-amplified hepatic CYP 2E1, GSH-R, GST, and GSH-Px in the rats fed 5% butterbur leaf powder. The relative values of hepatic CYP 2E1, GSH-R, GST, and GSH-Px activities between treatments. However, AST and LDH activities in the butterbur leaf group at 2 weeks and ALP activity in the butterbur leaf group at 4 weeks were significantly (p < 0.05) lower than in the control group. The glucose concentration at 4 weeks was significantly (p < 0.05) higher than in the control group, and there were no significant differences between treatments at the other time points.

TBARs and GSH levels in the rats fed 5% butterbur leaf powder. These levels were also significantly (p < 0.05 and p < 0.001 respectively) higher than in the control group.

Hepatic mRNA expression

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mRNAs in the butterbur leaf group were significantly higher than in the control group.

Susceptibility to lipid peroxidation in hepatic homogenate

Figure 3 shows the susceptibility to lipid peroxidation in normal rat hepatic homogenates treated with water, acetone, and ethanol extracts from butterbur leaf powder at 37°C for 0, 1, 2, 3, and 4 days. Each of the fractions from the substrate solutions (n = 3) was obtained at 24-h intervals. The significance of differences among hepatic homogenate oxidation levels was determined by ANOVA with Duncan’s multiple-range test. Mean values were significantly different at \( p < 0.05 \). For details of procedures, see the text.

Fig. 3. Susceptibility to Lipid Peroxidation in Hepatic Homogenates from Normal Rats Treated with Water, Acetone, and Ethanol Extracts of Japanese Butterbur Leaf Powder at 37°C for 0, 1, 2, 3, and 4 Days.

Values represent mean and standard deviation, depicted by vertical bars (data obtained for five animals). \( * p < 0.05, \* \* p < 0.01 \) vs. control as determined by Student’s \( t \)-test. The values for P2E1, GST, GSH-Px, and GSH-R mRNAs are expressed relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA for all groups. Representative samples illustrating mRNA levels measured by RT-PCR and Southern blotting.

mRNAs in the butterbur leaf group were significantly \( ( p < 0.05 ) \) higher than in the control group.

**Susceptibility to lipid peroxidation in hepatic homogenate**

Figure 3 shows the susceptibility to lipid peroxidation in normal rat hepatic homogenates treated with water, acetone, and ethanol extracts from butterbur leaf powder at 37°C for 4 d. In the 0–250 \( \mu \)g/mL range of all extracts, there was no difference in lipid peroxidation below a concentration of 25 \( \mu \)g/mL, but lipid peroxidation in the liver homogenates treated with the acetone and ethanol extracts showed a slight increase at a concentration of 50 \( \mu \)g/mL (Fig. 3A). Hence, we chose to examine the effects on lipid peroxidation in liver homogenates with 50 \( \mu \)g/mL of water, acetone, and ethanol extracts over 4 d. The aqueous extract of the butterbur leaf powder did not show any difference from the control group on 4 d. The ethanol extract of the butterbur leaf powder most successfully suppressed hepatic homogenate oxidation, although it showed the highest TBARs level at the first stage. The acetone extract of the powder showed the strongest hepatic homogenate oxidation throughout the 4 d.
The susceptibility to hepatic homogenate oxidation by the equivalent of 1 mg/mL of samples was higher in the order extract of acetone > water = control > ethanol.

Discussion

Information on adverse effects of unpurified Japanese butterbur is limited, but some researchers have suggested that butterbur species cause hepatotoxicity, carcinogenesis, and genotoxicity in humans due to pyrrolizidine alkaloids. The most toxic pyrrolizidine alkaloids are classified into three types the retronecine type, the heliotridine type, and the otonecine type. Among these, the retronecine type, including senecionine and integerrmine, are reported to be major pyrrolizidine alkaloids in butterbur species, although at low levels. Pyrrolizidine alkaloids usually do not have toxic effects per se, but the formation of reactive pyrrolic ester metabolites catalyzed by cytochrome P-450 monoxygenase is primarily responsible for hepatotoxicity and carcinogenicity in experimental animals. Despite similar structures, it has been observed that differing CYP modulation is dependent on the type of pyrrolizidine alkaloid present. Gordon et al. reported that retrorsine of the retronecine type increased hepatic CYP1A1/2, CYP2B1/2, and CYP2E1 expression in rats, while Savin et al. reported that heliotrine of the heliotridine type decreased hepatic cytochrome P-450 enzymes. In the present study, hepatic CYP2E1 mRNA expression in the butterbur group was higher than that of the control group, which suggests that butterbur species contain the retronecine type as a major pyrrolizidine alkaloid. This is consistent with a report of Gordon et al. but there are no data on the components of Japanese butter leaf powder in this study.

GSH plays an important role in the detoxification of reactive pyrrolic ester metabolites, and of produces detoxified products such as 7-glutathionyl-6,7-dihydro-1-hydroxymethyl-5H-pyrrolizine and 7,9-diglutathionyl-6,7-dihydro-1-hydroxymethyl-5H-pyrrolizine, which are excreted in the bile. Phase II metabolizing enzyme GST is also thought to be involved in the detoxification step by catalyzing such conjugation. Lin et al. found a higher hepatic GSH level following increased hepatic GSH synthetase and GST activities in rats treated with a single dose (65 mg/kg, i.p.) of monocrotaline of the retronecine type as compared to controls. In the present study, our results indicate elevated hepatic GSH level as well as GST activity and mRNA expression in the butterbur group. Fu et al. suggested that elevation of the hepatic GSH level coupled with increased GST activity is a phenomenon of the early stage of non-severe pyrrolizidine alkaloid poisoning, which may be a self-defense mechanism preventing pyrrolizidine alkaloid intoxication in the body, some researchers have observed that long-term exposure to pyrrolizidine alkaloids causes cell enlargement, metabolic disturbances, and fatty degeneration without any clinical signs or changes in serum biochemistry in animals. In the present study, there was no evidence of hepatic damage as estimated by the serum transferase activity in the rats.

Beyond those effects, 5% butterbur leaf powder in the diet increased lipid peroxidation in the liver and serum. There is limited information on the oxidative stress induced by unpurified Japanese butterbur, but Segall et al. suggested that trans-4-hydroxy-2-hexenal (t-4HH) of reactive metabolites from senecionine of the retronecine type can cause lipid peroxidation in hepatic microsomal lipids. Their follow-up study revealed that senecionine and t-4HH increased the formation of thiobarbituric acid-reactive products in isolated rat hepatocytes. Additionally, Guarnieri and Mascari found elevation of superoxide production and the malondialdehyde concentration in the cardiac muscle of monocrotaline-treated rats. In the present study, oxidative stress was also observed in an in vitro system using hepatic homogenates exposed to acetone extract of butterbur leaf powder. But aqueous extract of butterbur leaf powder showed no difference as compared to the control group treated with methanol alone. Ethanol extract suppressed increases in hepatic homogenate oxidative stress, although it showed the highest TBARS value at the beginning of the study. It is clear that butterbur species contain toxic pyrrolizidine alkaloids, but also beneficial components such as petasin, fukinolic acid, and flavonoid glycosides, exhibiting several health effects. Indeed, Lee et al. reported that an ethanol extract of butterbur reduced reactive oxygen species production in cells of bronchoalveolar lavage fluid from mice. Therefore, it is possible that the ethanol extract has a double-edged effect due to pyrrolizidine alkaloids and the beneficial components.

In conclusion, the present study indicates that Japanese butterbur leaves might cause adverse effects in rats judging by the enhanced postprandial oxidative stress. Hence unpurified Japanese butterbur leaves may not be acceptable for consumption as an herbal medicine or a food source without further evaluation, it is necessary to investigate further the composition of unpurified Japanese butterbur leaf powder to reduce or eliminate the toxic effects associated with it.

Acknowledgment

We wish to thank Ms. Setsuko Iijima of the Department of Food Science of Obihiro University of Agriculture and Veterinary Medicine for technical support, and Dr. Yeonhwa Park of the Department of Food Science of University of Massachusetts Amherst for a very helpful critical reading of the manuscript.

References

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