Effect of Methanol Extract of Zanthoxylum piperitum Leaves and of Its Compound, Protocatechuic Acid, on Hepatic Drug Metabolizing Enzymes and Lipid Peroxidation in Rats

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The effect of methanol extract and protocatechuic acid from the leaves of Zanthoxylum piperitum on lipid peroxidation and drug metabolizing enzymes were investigated in the liver of bromobenzene-treated rats. The methanol extract and protocatechuic acid reduced the level of lipid peroxide induced by bromobenzene. The methanol extract and protocatechuic acid reduced the activity of aniline hydroxylase that had been increased by bromobenzene, while did not affect the activities of aminopyrine N-demethylase and glutathione S-transferase. The methanol extract and compound effectively restored the activity of epoxide hydrolase which had been decreased by bromobenzene. These results may suggest that the methanol extract of Z. piperitum and of its compound, protocatechuic acid, on lipid peroxidation and the activities of hepatic enzymes involved in the formation and metabolism of epoxides were examined in bromobenzene-treated rats.

Materials and Methods

Plant material. Z. piperitum was collected by the author (J. M. Hur) in Suncheon, Jeonnam, Korea on July 25, 1998. A voucher specimen (NM0337) has been deposited at the Herbarium of Department of Oriental Medicine Resources at Sunchon National University.

Isolation of the active compound. Dried and powdered leaves (1.6 kg) of Z. piperitum were refluxed with methanol. The methanol extract (250 g) was partitioned with organic solvents of different polarity to obtain dichloromethane (80 g), ethyl acetate (29 g), n-butanol (37 g) and aqueous (70 g) fractions. The ethyl acetate fraction was subjected to silica gel chromatography, using chloroform-methanol-water (25:7:5, lower layer) and CHCl3-methanol-H2O (7:3:1, lower layer) as solvents, and to Sephadex LH-20 chromatography, using methanol as the solvent, to obtain protocatechuic acid9) (Fig. 1): 1H-NMR (DMSO-d6, 200 MHz) δ: 7.32 (1H, d, J = 2.0 Hz, H-2), 7.27 (1H, dd, J = 2.0 & 8.1 Hz, H-6), 6.77 (1H, d, J = 8.1 Hz, H-5);13C-NMR (DMSO-d6, 50.3 MHz) δ: 167.3 (C=O), 150.0 (C-4), 144.9 (C-3), 121.9 (C-6), 121.7 (C-1), 116.6 (C-2), 115.2 (C-5).

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Animals. Male Sprague-Dawley rats (Daehan Biolink, Umsung, Korea) 6 to 7 weeks old and weighing about 180–200 g, were fed ad libitum with a commercial standard basal rat diet (AIN-76) and water, and maintained at 20 ± 2°C with a 12 hr light/dark cycle. The International Chemical & Laboratory Animal Resources, United States. Laboratory Animals, established by the Institute of laboratory animals by the Institute of care and use of specific pathogen-free (SPF). The animals were cared for under the guidelines for the care and use of laboratory animals established by the Institute of Laboratory Animal Association (ICLAS) sub monitoring center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), in Korea certificated the grade of laboratory animals used for this experiment as specific pathogen-free (SPF). The animals were cared for under the guidelines for the care and use of laboratory animals established by the Institute of Laboratory Animal Resources, United States.

The animals were orally administered daily for one week with 250 or 500 mg of the methanol extract of the plant or with 5, 10 or 20 mg/kg of protocatechuic acid that had been isolated from the plant. Each sample was dissolved in 1% Tween 80. The normal group was given 0.2 ml of 1% Tween 80 per 200 g. During the final two days of the oral treatment, the rats were injected i.p. with bromobenzene (460 mg/kg) 4 times at 12 hr intervals. The animals were sacrificed by exsanguination from the aorta under anesthesia with CO2 12 hr after the final bromobenzene injection, the animals being starved for 18 hr before sacrifice in order to reduce the variation in hepatic metabolism.

Enzyme source. The liver, which had been exhaustively perfused with ice-cold 0.9% NaCl through the portal vein until uniformly pale, was immediately removed and weighed. A portion of the liver tissue was homogenized with 4 volumes of an ice-cold 0.1 M potassium phosphate buffer at pH 7.5. The homogenate was centrifuged at 600 x g for 10 min, and the resulting supernatant was recentrifuged at 10,000 x g for 20 min. The supernatant was further centrifuged at 105,000 x g for 60 min to obtain the upper fraction as cytosol. The pellet was resuspended in the same volume of the 0.1 M potassium phosphate buffer and centrifuged at 105,000 x g for 60 min to obtain the microsomal fraction. The homogenate was used to determine the contents of lipid peroxide and glutathione. The cytosolic fraction was used as the enzyme source of glutathione S-transferase, and the microsomal fraction was used to measure the activities of aniline N-demethylase, aniline hydroxylase and epoxide hydrolase.

Determination of the lipid peroxide and glutathione levels. The level of thiobarbituric acid reactive substances (TBARS) in the liver was measured as a marker of lipid peroxidation. A mixture of 0.4 ml of a 10% liver homogenate in 0.9% NaCl, 1.5 ml of 8.1% sodium dodecyl sulphate, 1.5 ml of a 20% acetic buffer (pH 3.5) and 1.5 ml of a 0.8% TBA solution was heated at 95°C for 1 hr. After cooling, 5.0 ml of n-butanol-pyridine (15:1) was added for extraction, and the absorbance of the n-butanol-pyridine layer was measured at 532 nm. The glutathione content of the liver was measured by a colorimetric method. Mixture of 0.5 ml of the liver homogenate and 0.5 ml of 4% sulfosalicylic acid was centrifuged at 2,500 rpm for 10 min. To 0.3 ml of the resulting supernatant, 2.7 ml of disulfide reagent [39.6 mg of 5,5′-dithiobis(2-nitrobenzoic acid) in 1,000 ml of 0.1 M sodium phosphate buffer (pH 8.0)] was added, and absorbance at 412 nm was measured after standing at room temperature for 20 min.

Enzymatic assays. The aminopyrine N-demethylase activity was assayed by measuring the production of formaldehyde formed by the demethylation of aminopyrine. The reaction mixture consisted of 300–400 μg of microsomal protein, a 0.1 M potassium phosphate buffer (pH 7.5) and 2.0 mM aminopyrine in a total volume of 2.0 ml. The mixture was preincubated for 3 min at 37°C, and 0.5 mM NADPH was added to initiate the reaction. The reaction was stopped after 3 min by adding 0.5 ml of 15% ZnSO4 and saturated Ba(OH)2, and the resulting mixture was cooled to room temperature. After centrifuging at 1,000 x g for 10 min, 1 ml of the supernatant was mixed with 5 ml of the Nash reagent. The tubes were then capped and heated at 60°C for 30 min. After cooling in tap water, the absorbance was read at 415 nm against a water blank. The activity is expressed as nmol of formaldehyde/mg of protein/min. The aniline hydroxylase activity was assayed by determining the p-aminophenol formation from aniline. The incubation method was similar to that just described, except that 1 mM aniline was used as the substrate. The reaction was initiated by adding 0.5 mM NADPH. After shaking for 30 min at 37°C, the reaction was terminated by adding 0.5 ml of 20% trichloroacetic acid. The mixture was centrifuged at 1,000 x g for 10 min, and 1 ml of the supernatant was added to 1 ml of 0.2 N NaOH containing 2% phenol. After mixing, 1 ml of 10% Na2CO3 was then added. The mixture was kept for 20 min at room temperature, before the absorbance was read at 640 nm against a water blank. The activity is expressed as nmol of p-aminophenol/mg protein/min. The glutathione S-transferase activity was assayed by measuring the conjugated glutathione 2,4-dinitrobenzene formation from 1-chloro-2,4-
dinitrobenzene.\(^{14}\) The reaction mixture consisted of 100 µl of the cytosol fraction, a 0.1 M potassium phosphate buffer (pH 6.5), 1 mM 1-chloro-2,4-dinitrobenzene and 1 mM glutathione in a total volume of 3.5 ml. The mixture was incubated at 25°C for 2 min. The spectrometric change per unit time at the maximal absorbance wavelength (340 nm) was calculated with a molar extinction coefficient of 9.6 mM \(^{-1}\) cm\(^{-1}\). The activity is expressed as nmol of 1,2-dinitro-4-nitrobenzene/mg of protein/min. The epoxide hydrolase activity was measured spectrophotometrically by monitoring the decrease in trans-stilbene oxide (TSO) at 229 nm. The reaction mixture consisted of 100–200 µg of microsomal protein and 3.0 mM TSO in a 0.05 M potassium phosphate buffer (pH 7.0) in a total volume of 3.0 ml. The mixture was incubated for 20 min at 37°C. The activity is defined as nmol of TSO/mg of protein/min.\(^{15}\)

**Protein assay.** The protein content was determined by the method of Lowry et al.,\(^{16}\) using bovine serum albumin as a standard.

**Statistical analysis.** The statistical differences among the experimental groups were determined by Duncan’s multiple-range test.

**Results**

The effects of the methanol extract and protocatechuic acid from the leaves of Z. piperitum on the lipid peroxide content in the liver of the bromobenzene-intoxicated rats are demonstrated in Fig. 2. The i.p. injection of bromobenzene increased the lipid peroxide content to 53.1 and 48.7 nmol of TBARS/g from the normal values of 18.4 and 19.4 nmol. However, this increased TBARS content was suppressed by 19% and 22% with pretreatment by the methanol extract (500 mg/kg) and protocatechuic acid (20 mg/kg), respectively.

The changes in the activities of hepatic aminopyrine N-demethylase and aniline hydroxylase are shown in Table 1 and Fig. 3. The treatment with bromobenzene increased the activities of aminopyrine N-demethylase and aniline hydroxylase in comparison with the normal group. The increase in aniline hydroxylase activity by bromobenzene was respectively reduced by 22% and 41% with oral pretreatment by the methanol extract (500 mg/kg) and protocatechuic acid (20 mg/kg). However, there was no such reduction in the aminopyrine N-demethylase activity from an oral treatment of either the methanol extract or protocatechuic acid.

The injection of bromobenzene increased the glutathione S-transferase activity to 278.2 nmol/mg
of protein from the normal value of 254.2 nmol/mg of protein (Table 2). No change in the glutathione S-transferase activity was apparent by treatment with protocatechuic acid. However, pretreatment with the methanol extract (250 or 500 mg/kg) for one week prior to the bromobenzene injection of 5, 10 or 20 mg/kg of body weight daily for one week, and bromobenzene was injected four times at 12-hr intervals during final two days of the oral treatment. Each value is the mean ± SD (n = 5). Values not sharing a common superscript letter(s) in a column are significantly different (p < 0.05). Unit: nmol 1,2-dinitro-4-nitrobenzene/mg of protein/min.

Table 3. Effect of Protocatechuic Acid (PA) on the Hepatic Glutathione Content in Rats Treated with Bromobenzene (BB)

<table>
<thead>
<tr>
<th>Group</th>
<th>Glutathione S-transferase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>254.2 ± 11.3^a</td>
</tr>
<tr>
<td>BB</td>
<td>278.2 ± 12.9^a</td>
</tr>
<tr>
<td>ME250 + BB</td>
<td>283.6 ± 20.6^b</td>
</tr>
<tr>
<td>ME500 + BB</td>
<td>275.4 ± 19.7^b</td>
</tr>
<tr>
<td>PA5 + BB</td>
<td>263.5 ± 19.2^b</td>
</tr>
<tr>
<td>PA10 + BB</td>
<td>251.2 ± 18.4^b</td>
</tr>
<tr>
<td>PA20 + BB</td>
<td>248.9 ± 20.3^b</td>
</tr>
</tbody>
</table>

1 The treatment has been described in Table 1. Each value is the mean ± SD (n = 5). Values not sharing a common superscript letter(s) in a column are significantly different (p < 0.05). Unit: nmol 1,2-dinitro-4-nitrobenzene/mg of protein/min.

Fig. 4. Effects of the Methanol Extract (ME) and Protocatechuic Acid (PA) from the Leaves of Zanthoxylum piperitum on the Hepatic Epoxide Hydrolase Activity in Rats Treated with Bromobenzene (BB).

Each bar represents the mean ± S.D. of 5 animals. Values not sharing a common superscript letter(s) are significantly different (p < 0.05).

Discussion

Bromobenzene is a xenobiotic liver toxin that is known to produce centrilobular hepatic necrosis through the formation of reactive epoxides as the toxic intermediates. In the metabolism of bromobenzene, the nontoxic 2,3-epoxide, which readily forms 2-bromophenol, or the toxic 3,4-epoxide are produced upon oxidation by cytochrome P-450 monooxygenases. Several pathways exist that can detoxify the reactive 3,4-epoxide; rearrangement to the 4-bromophenol, hydration to the 3,4-dihydrodiol catalyzed by epoxide hydrolase, or conjugation with glutathione. When more 3,4-epoxide is produced than can readily be detoxified, cell injury is increased.17)

Z. piperitum has traditionally been used for the treatment of vomiting and diarrhea2) and as a food treatment in Korea. The effects of Z. piperitum on the enzymes involved in the detoxification of bromobenzene in rats were investigated in the present study. The rats were orally administered daily with a methanol extract (250 or 500 mg/kg) of the leaves of Z. piperitum or with protocatechuic acid (5, 10 or 20 mg/kg) for one week prior to the bromobenzene treatment. Protocatechuic acid (3,4-dihydroxybenzoic acid) was isolated from ethyl acetate-soluble fraction of the methanol extract of the leaves of Z. piperitum by column chromatography. Bromobenzene was i.p. injected four times at 12-hr intervals during the final two days of the oral administration of the methanol extract or protocatechuic acid.

The hepatic lipid peroxide level in the normal rats was significantly increased by the i.p. injection of bromobenzene. The methanol extract of Z. piperitum and its component, protocatechuic acid, reduced the level of lipid peroxides induced by bromobenzene. Lipid peroxides are formed by oxidation of polyunsaturated fatty acids in mitochondria, microsomes, erythrocytes and platelets. The membranes of
In conclusion, the protection against bromobenzene-induced lipid peroxidation by \textit{Z. piperitum} and its component, protocatechuic acid, is thought to have been mediated via the enhanced activity of epoxide hydrolase, the enzyme responsible for removing bromobenzene epoxide, and via the reduced activity of aniline hydroxylase, the enzyme responsible for forming the epoxide.

References


