Effects of Extract Derived from *Eriobotrya japonica* on Liver Function Improvement in Rats

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**Eriobotrya japonica** is considered a medicinal plant, and its leaves (*Eriobotrya folia*) have been used in the Kampo medicines Shini-seihai-to and Biwayo-to to treat skin diseases, as well as to relieve inflammation, pain, coughing, and sputa. In our evaluation of the pharmacological efficacy of the seed extracts, constituents of the seeds were found to contain the unsaturated fatty acids linolenic and linoleic acids and the sterol β-sitosterol in the 70% EtOH and the MeOH extracts. The seed extracts were orally administered to rats with dimethylnitrosamine-induced hepatopathy, and blood l-aspartate aminotransferase (AST) and l-alanine aminotransferase (ALT) levels, liver retinoid level, and hydroxyproline level were measured. Liver fibrosis rates calculated after Azan-Mallory staining and evaluation of the liver function-improving effects of extracts were showed that AST, ALT, and hydroxyproline levels and liver fibrosis rates were significantly lower, and retinoid levels were significantly higher in hepatopathic rats treated with 70% EtOH and MeOH extracts of the seed than in water-treated control rats. This suggests that the positive effect on liver function of the extracts varies depending on the extracting solvent used. 70% EtOH and MeOH extract of the seeds inhibited the development of liver fibrosis in hepatopathic rats, thus exhibiting potent improvement. The unsaturated linolenic and linoleic acids and the sterol β-sitosterol contained in these extracts may also contribute to the improvement of liver function.

**Key words** *Eriobotrya japonica*; liver fibrosis; retinoid; hydroxyproline; dimethylnitrosamine

\*Eriobotrya japonica\* is widely planted as a fruit tree, and its leaves (*Eriobotrya folia*) have been used in the Kampo medicines *Shini-seihai-to* and *Biwayo-to* to treat skin diseases, as well as to relieve inflammation, pain, coughing, and sputa. The hypoglycemic\(^{1-5}\) and anti-inflammatory effects\(^{6-8}\) of *Eriobotrya folia* have also recently been reported.\(^{9-16}\) As in seeds of other plants from Rosaceae such as *Persicae semen* and *Armeniacae semen*, amygdalin is a major component of *Eriobotrya japonica* seeds,\(^{17}\) and these latter seeds have therefore been used as a folk medicine substituting for these constituents of Kampo medicines. However, most *Eriobotrya japonica* seeds are currently discarded as garbage. Recently, it has been recognized that patients with liver fibrosis due to chronic hepatitis frequently develop liver cirrhosis and carcinoma; the inhibition of liver fibrosis is thus considered important in preventing liver cirrhosis and carcinoma. Although many studies have evaluated methods of inhibiting the development of liver fibrosis, no adequate means has yet been found.\(^{18-21}\)

Based on the pharmacological background of *Eriobotrya japonica*, various extracts of the seeds were orally administered to rats with dimethylnitrosamine-induced hepatopathy. Subsequently, blood l-aspartate aminotransferase (AST) and l-alanine aminotransferase (ALT) levels, liver retinoid (an index of fibroblast expression in the liver) level, and hydroxyproline (a collagen-specific amino acid) level were measured. Liver fibrosis rates were also calculated after Azan-Mallory staining of liver tissues to evaluate the effects of the extracts on liver function.

**MATERIALS AND METHODS**

**Animals** Male Wistar rats, aged seven weeks, 180—200 g, were purchased from NSC Japan. Animals were acclimatized for seven days at 23±2°C with free access to pellet food (CE-2, Clea, Osaka, Japan) and water. Healthy rats were then selected and assigned to groups.

**Materials** Sufficiently sun-dried seeds of Mogi-loquat collected at Muroto and Susaki cities in Kochi Prefecture of Japan were the *Eriobotrya japonica* seeds used. Dimethylnitrosamine, retinol palmitate, hydroxyproline, linoleic acid, linolenic acid, and β-sitosterol were purchased from Nacalai Tesque (Kyoto, Japan). All other chemicals were of reagent grade.

**Analysis of Compounds** IR spectra were measured on a JASCO FT/IR-420 spectrometer. \(^1\)H- and \(^13\)C-NMR spectra were obtained on a VARIAN UNITY INOVA 400 NMR spectrometer operating at 399.913 and 100.567 MHz, respectively. Mass spectra were recorded on a JEOL D-300 spectrometer. Analytical HPLC was carried out on a Hitachi D-6000 equipped with a Hitachi L-3000 spectrometer on a YM C Pack ODS-AQ column (6.6×150 mm); mobile phase, CH\(_3\)CN–H\(_2\)O–AcOH (80:20:1); flow rate, 0.7 ml/min; detection, 240 nm; column temperature, 30°C.

**Extraction of Seed by Various Solvents** The seeds of *Eriobotrya japonica* were extracted by various solvents. Briefly, 1.0 kg of seeds was crushed in a blender equipped with a refrigerator at 1000 rpm, and then continuously stirred by a mixer at 300 rpm for 7 d after dissolving in the respective extracting solvents (2.0 l). The supernatant was then collected and evaporated to dryness to prepare the dried extracts. Extracting solvents used in this study were 70% EtOH, MeOH, water, and hexane.

**Composition of Eriobotrya japonica Seed Extracted by Various Solvents** Using thin-layer chromatography (silica gel 60 F\(_{254}\), Merck, Darmstadt, Germany), the constituents of *Eriobotrya japonica* seed extracted by various solvents were identified with CH\(_2\)Cl\(_2–MeOH–H\(_2\)O (8:2:0.2 v/v) as the eluent. The spots were detected by heating after spraying with 10% H\(_2\)SO\(_4\) or by UV irradiation.
Preparation of Hepatopathic Rats  
After a week of acclimatization, hepatopathy was experimentally induced by a single intraperitoneal administration of 40 mg/kg of dimethylnitrosamine to the healthy 7-week-old male rats.18–20,22)

Administration of Seed Extracts to Hepatopathic Rats  
Seven days thereafter, the extract obtained by the solvents (1.0 l) (dissolved in 1.0 l distilled water) was uniformly administered to hepatopathic rats using a water-supply bottle at a dose of 15 ml/d.

The same amount of water was administered to control rats.

Evaluation of Liver Function Improvement in Hepatopathic Rats Treated with Seed Extracts  
1) Measurement of Biochemical Parameters: Blood samples were collected by sacrificing rats before (injured rats) and 7 d after administering water and seed extracts (control and extract administered group), and serum AST and ALT levels were measured using a VISION analyzer (Dainabot, Tokyo, Japan).

2) Retinoid Levels in the Liver: Retinol palmitate was measured to determine the liver retinoid level. Briefly, the liver was removed after sacrificing all rats 7 d after the administration of the Eriobotrya japonica seed extracts. Three-tenths of a gram of tissue was accurately weighed and collected from a uniform region of the liver, then homogenized on ice at 10000 rpm for 2 min using a cell homogenizer (Eilard, Germany) in the presence of 5 ml chloroform. After centrifugation at 3500 rpm for 20 min, the lower layer was recovered and used as a sample for retinoid measurement. The sample solution was filtered by a 0.5 μm membrane filter (Millipore, Bedford, U.S.A) before the measurement of retinol palmitate by the HPLC method reported previously.22)

3) Hydroxyproline Levels in the Liver: Following liver removal, another 0.3 g of the tissue was accurately weighed and collected from a uniform region of the liver, then homogenized on ice as described above (Eilard, Germany) in the presence of 5 ml chloroform. After centrifugation at 3500 rpm for 20 min, the supernatant was collected, and 1 ml was heated at 60 °C for 8 h. Subsequently, the residue was dissolved by adding 40 μl EtOH and 80 μl of 0.1 mol borate buffer solution, followed by labeling with 40 μl 100 mmol 4-fluoro-7-nitrobenzofurazan, a fluorescent labeling agent, for 15 h at room temperature in the dark. Labeling reaction was terminated by adding 840 μl of 5 mmol HCl, and the supernatant obtained by centrifugation at 3500 rpm for 20 min was used as a sample for the measurement of hydroxyproline levels by the method reported previously.23)

Liver Fibrosis Areas and Rates in Rats  
Following liver removal, the tissues were embedded in paraffin and stained with Azan-Mallory. Pathomicrographs of the tissues were input to a computer, and liver fibrosis areas were measured using MacScope, an image analysis software (Mitani Shoji, Tokyo, Japan). After establishing a color threshold in each pathomicrograph, the blue color of the stained collagen fibers was extracted, and the percentage of these fibers in the liver was calculated by dividing the total blue-colored area by the total area of the liver, excluding margins in all visual fields.23)

The liver fibrosis rate was subsequently calculated by dividing the percentage of the blue collagen fibers in hepatopathic rats by that in the control rats.

Statistical Analysis  
Statistical analysis was performed using one-way analysis of variance (ANOVA), and the significance of differences between the two groups was tested by the Bonferroni method.

RESULTS

Recovery Rates of the Seed Extracts  
The following amounts of extracts were recovered per kg Eriobotrya japonica seed after extraction by various solvents: 10 g (1.0%) by 70% EtOH, 9 g (0.9%) by MeOH, 15 g (1.5%) by water, and 4 g (0.4%) by hexane.

Investigation of Constituents  
Figure 1 shows a representative thin-layer chromatogram of Eriobotrya japonica seed extracted by various solvents.

The thin-layer chromatogram of the extracts varied depending on the extracting solvent. The thin-layer chromatogram pattern of water-extracted seed consisted of a single spot at the starting point, and spots at the starting point were smaller for Eriobotrya japonica seed extracted by 70% EtOH and MeOH than for the water-extracted seed. Constituents of the 70% EtOH-extracted seed showed Rf values of 0.53, 0.56 and 0.63, and those showing Rf values of 0.33, 0.41, and 0.71 were respectively obtained from the seed extracted by 70% EtOH and MeOH. Components with Rf values of 0.33, 0.41, and 0.71 were isolated and identified as linolenic acid, β-sitosterol, and linoleic acid, respectively after comparison with reference standards of various chemical substances, for example, IR, Mass, 1H- and 13C-NMR spectra. All constituents of hexane-extracted Eriobotrya japonica seed showed Rf values above 0.71.

Effects of the Administration of Eriobotrya japonica Seed Extracted by Various Solvents on the Improvement of Liver Functions in Hepatopathic Rats  
Table 1 shows the effects of the administration of Eriobotrya japonica seed extracted by various solvents on AST, ALT, liver retinoid, and hydroxyproline levels in hepatopathic rats after 7 d of treatment.

Before administration of the extracts, both AST and ALT levels were markedly elevated in these rats compared to those in normal rats. AST levels in hepatopathic rats treated with
70% EtOH or MeOH extracts decreased to approximately 0.51—0.62 times the levels in water-treated control rats. However, AST levels in the hepatopathic rats treated with water and hexane extracts did not significantly differ from those in control rats. ALT levels in hepatopathic rats treated with the seed extracts decreased to approximately 0.55—0.66 times the levels in water-treated control rats, regardless of the extracting solvent.

Moreover, liver retinoid levels were markedly lower in the hepatopathic rats than in control rats before the administration of any seed extract. The levels in hepatopathic rats treated with 70% EtOH, MeOH, and water extracts of the seed increased to approximately 1.15—1.40 times the levels in control rats, although, these levels did not differ between the two groups when hepatopathic rats were treated with hexane extract of the seeds.

Before administration of the extracts, hydroxyproline levels were markedly higher in hepatopathic than in normal rats; rats treated with 70% EtOH, MeOH, and water extracts of the seed decreased to approximately 0.52—0.58 times the levels in control rats. Levels did not differ between the two groups, however when epatopathic rats were treated with hexane extract.

Effects of the Administration of Eriobotrya japonica Seed Extracted by Various Solvents on the Liver Fibrosis Rate in Hepatopathic Rats Figure 2 shows pathomicro-

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AST (U/l)</th>
<th>ALT (U/l)</th>
<th>Retinoid (%)</th>
<th>Hydroxyproline (nmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal rats</td>
<td>53.7 ± 20.0</td>
<td>44.1 ± 8.1</td>
<td>92.3 ± 8.9</td>
<td>170.2 ± 33.5</td>
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<tr>
<td>Injured rats</td>
<td>420.0 ± 46.0</td>
<td>300.1 ± 38.3</td>
<td>23.2 ± 4.3</td>
<td>962.0 ± 235.0</td>
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<tr>
<td>After administration of water</td>
<td>419.7 ± 95.0</td>
<td>352.5 ± 92.3</td>
<td>59.1 ± 12.8</td>
<td>998.7 ± 274.8</td>
</tr>
<tr>
<td>(control)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>After administration of water</td>
<td>395.6 ± 77.8</td>
<td>194.5 ± 76.1</td>
<td>73.6 ± 16.8</td>
<td>581.8 ± 151.5</td>
</tr>
<tr>
<td>Eriobotrya japonica seed by</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>214.0 ± 38.5</td>
<td>210.8 ± 41.2</td>
<td>67.9 ± 7.5</td>
<td>581.8 ± 37.5</td>
</tr>
<tr>
<td>70% EtOH</td>
<td>260.1 ± 31.7</td>
<td>231.7 ± 30.8</td>
<td>82.9 ± 26.9</td>
<td>520.1 ± 28.2</td>
</tr>
<tr>
<td>MeOH</td>
<td>393.6 ± 68.1</td>
<td>215.4 ± 67.4</td>
<td>52.0 ± 12.4</td>
<td>950.4 ± 166.2</td>
</tr>
<tr>
<td>Hexane</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Each value is the mean ± S.D. of 10 experiments.  

Table 1. Effects of Administration of Eriobotrya japonica Seed Extracted by Several Solvents on AST, ALT, Retinoid and Hydroxyproline Levels in Hepatopathic Rats for 7 d

Fig. 2. Photomicrographs of Liver Tissue from Liver-Injury Rats (Stain Azan-Mallory; ×25)  

graphs of rat liver tissues.

Azan-Mallory staining of the tissues demonstrated that there were fewer blue-stained collagen fibers in hepaticopathic rats treated with 70% EtOH and MeOH extracts of *Eriobotrya japonica* seed (Figs. 2D, E) compared to those in control rats (Fig. 2B). The number of blue-stained collagen fibers in hepaticopathic rats treated with water and hexane extracts of the seed (Figs. 2C, F) exceeded those in control rats.

Table 2 shows the effects of the administration of seed extracts on the liver fibrosis rate in hepaticopathic rats after 7 d of treatment.

The rate was lower in treated hepaticopathic rats than in control rats. Liver fibrosis rates varied depending on the extracting solvent, and those in hepaticopathic rats treated with 70% EtOH and MeOH extracts were lower than those in hepaticopathic rats treated with water and hexane extracts of the seed.

**DISCUSSION**

In recent years, chemical investigations on the leaves of *Eriobotrya japonica* which are used medicinally have been reported, and many constituents were isolated: megastigmene glycosides, triterpenes, methylchlorogenic acid and flavonoid glycosides. Amygdalin, however, has long been known to be a major component of the seeds of this plant.

When constituents of *Eriobotrya japonica* seed were investigated by thin-layer chromatogram after extraction with various solvents, thin-layer chromatogram patterns varied depending on the solvent used. The thin-layer chromatogram pattern of water extract of the seed consisted of a single spot at the starting point, while the thin-layer chromatogram pattern of hexane extract was composed of spots showing *Rf* values above 0.71. However, thin-layer chromatogram patterns of 70% EtOH and MeOH extracts consisted of many spots distributed from the origin to a distant point with *Rf* values of 0.87, probably because the extraction efficiency varied with the extracting solvent. These findings suggest that 70% EtOH and MeOH are useful for extracting unsaturated fatty acids such as linoleic acid and sterols like β-sitosterol. When various of these seed extracts were administered to hepaticopathic rats, neither the AST nor the ALT level, both of which are related to the improvement of inflammation, improved with water or hexane extracts of the seed. These levels were improved, however, in hepaticopathic rats treated with 70% EtOH and MeOH extracts of the seed. These findings together with the results of thin-layer chromatography suggest that constituents showing *Rf* values between 0.06 and 0.63 may contribute to improving the levels in this rat group.

When liver retinoid and hydroxyproline levels (indices of improved hepatopathy) were evaluated in hepaticopathic rats after administration of extracts, liver retinoid and hydroxyproline levels were increased and liver hydroxyproline levels were decreased in the animals treated with 70% EtOH and MeOH extracts of *Eriobotrya japonica* seed, thus resulting in improved liver function in these rats. These findings together with the results of thin-layer chromatography suggest that linoleic acid and β-sitosterol contained in the above two extracts may contribute to the improved liver function in the rats, although, further detailed evaluations are needed in the future.

We reported previously that Sho-saiko-to extract (Kampo medicine) was useful in suppressing Ito cell activation, the cause of liver fibrosis, in rats with dimethylnitrosamine-induced liver injury. Compared to those in hepaticopathic rats after administration of 0.75% Sho-saiko-to extract (390 mg/kg), AST, ALT, liver retinoid and hydroxyproline levels were almost the same in hepaticopathic rats treated with 70% EtOH extracts of *Eriobotrya japonica* seed (375 mg/kg; this study).

The results of this study confirmed that 70% EtOH and MeOH extracts of *Eriobotrya japonica* seed inhibit the development of liver fibrosis, as well as improving liver functions in rats with dimethylnitrosamine-induced hepatopathy. The results also suggest that the unsaturated fatty acid linolenic and linoleic acids and the sterol β-sitosterol greatly contribute to the improvement of liver functions.

Although most *Eriobotrya japonica* seeds are currently discarded as garbage, the extracts of these seeds may greatly contribute to medicine by inhibiting the development of liver fibrosis. Thus, *Eriobotrya japonica* seed extracts may also contribute to society through their practical use as a bioresource such as the extended use of agricultural products and efficient use of waste.

**Acknowledgements** We are deeply indebted to Associate Professor Dr. K. Kohiro and Professor Dr. T. Hosokawa for measurement of the IR, NMR and mass spectra and to the members of the Department of Environmental Systems Engineering of Kochi University of Technology.

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**Table 2. Effects of Administration of *Eriobotrya japonica* Seed Extracted by Several Solvents on Liver Fibrosis Area in Hepaticopathic Rats**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Liver fibrosis area (%)&lt;sup&gt;6&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>After administration of</td>
<td>100.0±30.7</td>
</tr>
<tr>
<td>water (control)</td>
<td></td>
</tr>
<tr>
<td>After administration of</td>
<td></td>
</tr>
<tr>
<td>extract of <em>Eriobotrya japonica</em> seed by</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>66.8±17.4</td>
</tr>
<tr>
<td>70% EtOH</td>
<td>46.4±20.9&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>MeOH</td>
<td>58.1±11.6&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hexane</td>
<td>63.8±15.7</td>
</tr>
</tbody>
</table>

Each value is the mean±S.D. of 10 experiments. a) Liver fibrosis area was expressed as a comparison of fibrosis degree in average of water-fed (control) rats, set as 100%. b) *p*<0.05, significantly different from the results in the water-treated group (control).