Scavenging of Reactive Oxygen Species by *Eriobotrya japonica* Seed Extract

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We have clarified that *Eriobotrya japonica* seed extract has strong antioxidative activity, and is effective for the prevention and treatment of various diseases, such as hepatopathy and nephropathy. In this study, to investigate the influences of components of *Eriobotrya japonica* seed extract on its antioxidative activity, extracts were prepared using various solvents (n-hexane (Hex), ethyl acetate (EtOAc), n-butanol (n-BuOH), methanol (MeOH) and H2O) and the antioxidative activity of the solvent fractions and components was evaluated based on the scavenging of various radicals (DPPH and O2•−) measured by the ESR method and the inhibition of Fe3+-ADP induced NADPH dependent lipid peroxidation in rat liver microsomes. The radical scavenging activities and inhibitory activities on lipid peroxidation differed among the solvent fractions and components. In the n-BuOH, MeOH and H2O fractions, radical scavenging activity and inhibitory activity on lipid peroxidation were high. In addition, these fractions contained abundant polyphenols, and the radical scavenging activity increased with the polyphenol content. In the low-polar Hex and EtOAc fractions, the radical scavenging activity was low, but the lipid peroxidation inhibition activity was high. These fractions contained β-sitosterol, and the inhibitory activity on lipid peroxidation was high. Based on these findings, the antioxidative activity of *Eriobotrya japonica* seed extract may be derived from many components involved in a complex mechanism, resulting in high activity.

**Key words** *Eriobotrya japonica*; ESR; natural antioxidant; LPO

Elucidation of the causal relationship between diseases and oxidative stress has recently progressed, and the close association of oxidation with the development and progression of not only lifestyle disorders, but also various intractable diseases and the aging process has been clarified.1,2) The ingestion of antioxidative substances is useful for the prevention and treatment of these diseases, and various investigations have been performed.3,4) *Eriobotrya japonica* has long been used as a pharmaceutical plant. Particularly, the leaves of *Eriobotrya japonica* Lindl. are used in a traditional herbal remedy for skin diseases, inflammation, cough and expectoration. Leaves of *Eriobotrya japonica* Lindl. have recently been shown to contain polyphenols, and a blood glucose-lowering action,5–7) anti-inflammatory action8,9) and anti-cancer action10) have been reported. *Eriobotrya japonica* seeds have been reported to contain nitrile aromatic compounds, such as amygdalin,11) and have been used as a substitute for An-nin in traditional herbal medicines. Nishio et al. have recently reported that, in addition to aromatic compounds, *Eriobotrya japonica* seeds contain unsaturated fatty acids, such as linoleic acid and linolenic acid, and plant sterols, such as β-sitosterol,12) and a 70% ethanol extract is effective for the prevention and treatment of disorders, such as hepatopathy12) and nephropathy.13) In this study, we estimated the antioxidative action of *Eriobotrya japonica* seed extract (ESE) with the ESR method, and the inhibitory activity on Fe3+-ADP induced NADPH dependent lipid peroxidation was measured in rat liver microsomes. In addition, we combined them and examined the influence with the components of the ESE.

**MATERIALS AND METHODS**

**Reagents** β-Sitosterol and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Nacalai Tesque Inc. Hypoxanthine (HPX), xanthine oxidase (XOD), 2-thiobarbituric acid (TBA), β-nicotinamide adenine dinucleotide phosphate reduced form (β-NADPH), sodium dodecylsulfate (SDS), Dl-xtocopherol and L-ascorbic acid were purchased from Wako Pure Chemical Industries, Ltd. 5,5-Dimethyl-1-pyrroline-N-oxide (DMPO) and diethylenetriaminepentaacetic acid (DTPA) were purchased from Sigma and Dojindo Inc., respectively. The other reagents used were commercial guaranteed-grade reagents.

**Preparation of Samples** *Eriobotrya japonica* seeds were collected in Muroto and Suzuki, Kochi Prefecture, and dried well in the sun. *Eriobotrya japonica* seeds (1 kg) were crushed using a cooling apparatus-equipped blender (1000 rpm). After immersion in 21 of 70% ethanol, the crushed seeds were stirred continuously using a stirrer (300 rpm, 7 d), and the supernatant was collected after 7 d. The collected 70% ethanol extracts were then combined and concentrated under reduced pressure until dry. The final yield of 70% ethanol extracts (ESE) was 108 g. ESE 108 g was extracted with 500 ml of Hex, 500 ml of EtOAc, 500 ml of n-BuOH, 500 ml of MeOH and 500 ml of H2O, and the fractions were collected. The solvent of each extract was then removed under reduced pressure, and the resulting extracts were subsequently lyophilized to dryness. The final yield of Hex, EtOAc, n-BuOH, MeOH and H2O extracts was 2.3, 2.4, 1.7, 10.2 and 89.8 g, respectively. The most antioxidative active strong H2O fractions were sequentially purified using Diaion HP20 (0→100% MeOH), Sephadex LH20 and G50 (0→100% MeOH). The crude fractions were purified and the components were isolated. The structures of the isolated components were determined using JMN-NMR 400 MHz and HPLC (Instrument: HITACHI HPLC D-7000 series, column: Cosmosil 5C18-AR-II Waters 4.6×150 mm, mobile
phase: A: 10 mM phosphate buffer (pH 2.6), B: acetonitrile (gradient: 0→15 min (Bconc. 0→30%), 15→18 min (Bconc. 30→70%), 18→30 min (Bconc. 70→100%), flow rate: 1 ml/min, temperature: 40 °C, detector: Diode Array Detector (280, 320, 360 nm).

Polyphenols in ESE and the solvent fractions were measured according to the Folin–Denis method,\(^ {14}\) and the content was calculated as the (+)-catechin equivalent in the extract. Amino acid analysis was performed using model L-8500A Hitachi high-speed amino acid analyzer. Also, \(\beta\)-sitosterol, fatty acids and amigdalain were confirmed using TLC according to the method of Nishioka et al.\(^ {12}\)

**Antioxidative Activity. Measurement of Reactive Oxygen-Scavenging Action Using ESR**\(^ {15}\) The ESR spectrum was measured using a JEOL JEX-RE3X ESR spectrophotometer (Nihon Densi Inc.). The measurement conditions are shown below. Power: 5 mW (DPPH), 8 mW (\(O_2^-\)), Field: 336.0±10 mT, Sweep time: 1 min, Modulation: 100 kHz 9.45 GHz, Time constant: 0.03 s, Receiver gain: 1×1000 (DPPH), 1.6×1000 (\(O_2^-\)). To evaluate the scavenging activity of each radical, the concentration–radical scavenging rate curve was prepared for each fraction and compound using the measurement method as below, and the concentration of 50% scavenging activity (IC\(_{50}\)). We used \(L\)-ascorbic acid and \(DL\)-\(\alpha\)-tocopherol as the positive control.

**DPPH Radical** The stable free radical, DPPH, was dissolved in MeOH (100 \(\mu\)M). The DPPH solution 100 \(\mu\)l and sample solution 200 \(\mu\)l were mixed, and the DPPH radical was measured after 60 s. The sample was dissolved in mixture of acetone and 0.1 M phosphate buffer (pH 7.4) (1:1) and used. The concentration of the ESE, solvent fractions and the positive controls prepared were 1→10\(^{-4}\) mg/ml. The ESE components were prepared at 10 mg/ml (polyphenol 100 \(\mu\)g/ml). We used the spectrum that was observed when adding the solvent of the sample instead of the sample as a control. The signal intensity was investigated based on the ratio of the height of the external standard manganese signal and the height of the 3rd of 5 peaks of the DPPH radical.

**\(O_2^-\)-Scavenging Activity** \(O_2^-\) radicals were generated with a hypoxanthine–xanthine oxidase system. All solutions were dissolved in 0.1 M phosphate buffer (pH 7.4). Samples that would not dissolve in 0.1 M phosphate buffer (pH 7.4) were dissolved in acetone. The concentration of acetone was prepared to be less than 1%. The concentration of ESE, solvent fractions and the positive controls were prepared at 20—0.1 mg/ml. ESE components were prepared at 10 mg/ml (polyphenol 100 \(\mu\)g/ml). 0.1 M phosphate buffer (pH 7.4) 90 \(\mu\)l, sample solution 50 \(\mu\)l, 2 mM HPX 50 \(\mu\)l, 5.5 mM DTPA 50 \(\mu\)l, 2.7 mM DMPO 10 \(\mu\)l, and 0.27 units/ml XOD 50 \(\mu\)l were added in this order, and the DMPO-OO- spin adduct was measured using the ESR method 60 s after the addition of DMPO. The signal intensity was investigated based on the ratio of the height of the external standard manganese signal and the height of the 1st of 12 lines of DMPO-OO- spin adduct. The \(O_2^-\) scavenging activity was also used by measuring the SOD-like activity. The SOD-like activity is calculated from the relative peak height, utilizing that the signal intensity of DMPO-OO- decreases in the presence of SOD in a concentration-dependent manner. SOD is an antioxidative enzyme that scavenges \(O_2^-\) in the body. Production of the DMPO-OO adduct is inhibited by the scavenging of \(O_2^-\) by SOD in the ESR method. A calibration curve (y=0.124x+0.13, r=0.992) was prepared using standard SOD solutions (0.00—14.50 units/ml), and SOD equivalents were calculated.

**Inhibition of Fe\(^{3+}\)-ADP Induced NADPH Dependent Lipid Peroxidation in Rat Liver Microsomes.** Animals Male Wistar rats were purchased from NSC Japan. After purchase, the animals were maintained with free access to food and drinking water under constant temperature and humidity conditions for 1 week or longer before the experiment.

**Preparation of Liver Microsomes** Male Wistar rats were anesthetized with diethyl ether, and killed by exsanguination by cutting the axillary artery. A canula was inserted into the portal vein, and physiological saline was infused to flush out the blood. The liver was excised and washed with 0.25% sucrose, cut into pieces and homogenized. The homogenate was centrifuged at 8000×\(g\) for 10 min at 4 °C. The supernatant was ultracentrifuged at 105000×\(g\) for 30 min at 4 °C, and the precipitate was collected. The protein in the precipitate was measured using the Lowry method,\(^ {16}\) and a microsome suspension was prepared (protein concentration: 20 mg/ml).

**Anti-lipid Peroxidation Assay** The reaction was measured according to the method reported by Kiso et al.\(^ {17}\) To the samples at various concentrations, 10 mM ADP, 2 mM \(\beta\)-NADPH, 20 mg protein/ml liver microsomes and 167 mM KCl-74.4 mM Tris–HCl buffer (pH 7.4) were added, and the solution was preincubated at 37 °C for 5 min. Subsequently, 0.1 mM FeCl\(_3\) was added, followed by incubation at 37 °C for 20 min (final volume: 1 ml). After the reaction was stopped by cooling in ice, the peroxidized lipids were measured as purified malondialdehyde (MDA) according to the method reported by Ohkawa et al.\(^ {18}\) To the reaction solution, 8.1% SDS, 20% acetate–0.27 mM HCl–NaOH buffer (pH 3.5) and 0.8% TBA were added. The mixture was heated in boiling water for 20 min. After the reaction was stopped by cooling in ice, \(n\)-BuOH: pyridine (15:1) was added, and the solution was vigorously mixed and centrifuged at 780×\(g\) for 10 min. The absorbance of the supernatant at 532 nm was measured, and the inhibition rate relative to the control was calculated.

**RESULTS**

**Various Radical-Scavenging Activity of ESE and Solvent Fractions (ESR Method)** Figure 1 shows the relationship between the concentrations of ESE and the solvent fractions and the DPPH and \(O_2^-\) radical-scavenging rates (%).

The various radical-scavenging rates varied among ESE and the solvent fractions, and the scavenging rate increased in a concentration-dependent manner for all of the radical species in ESE and the solvent fractions.

Table 1 shows the DPPH and \(O_2^-\) radical-scavenging activities (IC\(_{50}\) values) of ESE and the solvent fractions.

The IC\(_{50}\) value of ESE was 0.96×10\(^{-3}\) mg/ml to DPPH radical-scavenging activity. This showed a low value from \(DL\)-\(\alpha\)-tocopherol (0.03 mg/ml), \(L\)-ascorbic acid (8.61×10\(^{-3}\) mg/ml), which was used as the positive control. The IC\(_{50}\) value of the DPPH radical-scavenging activity was the lowest in the \(H_2O\) fraction (0.62×10\(^{-3}\) mg/ml), showing a high scavenging activity. The scavenging activity decreased in the order of the MeOH (0.79×10\(^{-3}\) mg/ml), \(n\)-BuOH (0.94×
The IC₅₀ value of ESE was 2.88 mg/ml to the O₂ radical-scavenging activity. This value was as high as L-ascorbic acid (3.90 mg/ml), which was used as the positive control. The IC₅₀ value of the O₂ radical-scavenging activity was the lowest in the H₂O fraction (1.75 mg/ml), showing high scavenging activity. As for the H₂O fraction, the O₂ radical-scavenging activity was higher than ESE. The activity decreased in the order of MeOH (2.00 mg/ml), EtOAc (2.85 mg/ml), n-BuOH (3.58 mg/ml). The IC₅₀ value of ESE was equivalent to SOD at 6.27 units/ml.

Lipid Peroxidation Inhibition Rates of ESE and the Solvent Fractions

Figure 2 shows the relationship between the concentrations of ESE and the solvent fractions and the lipid peroxidation inhibition rates (%). The lipid peroxidation inhibition rate varied among ESE and the solvent fractions, and increased in a concentration-dependent manner in ESE and the solvent fractions. The lipid peroxidation inhibition rate was not markedly different among the solvent fractions, unlike the radical scavenging activity.

Table 2 shows the lipid peroxidation inhibition activity (IC₅₀ values) of ESE and the solvent fractions. The lipid peroxidation inhibition activity (IC₅₀ value) was the lowest in the H₂O fraction (10.08 µg/ml), showing a high inhibitory activity. Unlike the radical scavenging activity, the inhibitory activity was also high in the low-polar Hex and EtOAc fractions (12.34 and 15.62 µg/ml, respectively). The value of DL-α-tocopherol, which was the positive control, was 40.21 µg/ml. For lipid peroxidation, ESE and the solvent fractions were stronger than DL-α-tocopherol.

Investigation of ESE Components

Because the antioxidative activity of ESE and the solvent fractions are different, we examined the components of ESE to clarify the different
antioxidations. Table 3 shows the polyphenol content (%) in ESE and the solvent fractions. The polyphenol content (%) varied among the solvent fractions. The content per weight of fraction was 0.56% in the H2O fraction, which is the highest and was equivalent to ESE. The content decreased in the order of MeOH, n-BuOH and EtOAc fractions. No polyphenol was contained in the Hex fraction.

We performed detailed examination about a component of the H2O fraction whose antioxidative activity was the highest. The structures of 2 polyphenols were determined. Figure 3 shows the structural formula. ESE contained 2.1 mg caffeic acid and 8.7 mg chlorogenic acid, which are phenyl propanoids, per 100 g. Table 4 shows the results of amino acid analysis of ESE. Fifteen amino acids were detected in ESE, and 7 of these were essential amino acids.

**Free Radical Scavenging Activities and the Anti-lipid Peroxidation of ESE Components** The anti-lipid peroxidation and free radical scavenging activities of ESE components were tested. The activity was lower in all of the compounds, compared to the above polyphenols, and comparison by calculation of the IC50 value was difficult. Thus, the scavenging rates (%) at a concentration of 10 mg/ml were presented. The polyphenol showed a value in 100 µg/ml. As for the polyphenols, the highest antioxidative action was suggested in the components. The antioxidative activity of fatty acids was not shown. However, β-sitosterol exhibited a higher lipid peroxidation inhibition rate.

**DISCUSSION**

Various radicals are produced in oxidative reactions in the body, and exert useful actions for the body in the immune system. However, various excessively produced radicals react with biological components, such as DNA, proteins and phospholipids, and induce oxidative disorders, leading to heart disease, diabetes and cancer. Antioxidative substances are expected to be effective for such disorders.

In the previous report, we clarified that ESE was useful for the improvement of nephropathy in rats with adriamycin-induced nephropathy, and the action was not indirectly exerted through antioxidative enzymes, but was due to the direct antioxidative action of ESE components absorbed in the body.

In this study, we clarified the components of ESE and investigated their antioxidative action. Regarding the various radical scavenging activities and lipid peroxidation inhibition activity of ESE, a high antioxidative action was detected in the high-polar solvent fractions when ESE was fractionated with solvents with different polarities. This finding clarified the involvement of the high-polar solvent fractions in the antioxidative action of ESE.

The components were investigated in each fraction, and the high-polar solvent fractions contained abundant polyphenols. These polyphenols were isolated, and the structures were determined. As a result, caffeic acid and chlorogenic acid were identified. These components exhibited high antioxidative action, clarifying that these polyphenols are closely involved in the exertion of the antioxidative action of ESE.

<table>
<thead>
<tr>
<th>Pure compounds</th>
<th>DPPH</th>
<th>Superoxide</th>
<th>Anti-lipid peroxidation</th>
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<tbody>
<tr>
<td>Caffeic acid</td>
<td>85.9</td>
<td>80.1</td>
<td>76.5</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>88.5</td>
<td>79.3</td>
<td>72.8</td>
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<tr>
<td>β-Sitosterol</td>
<td>10.7</td>
<td>&lt;1</td>
<td>76.3</td>
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<tr>
<td>Linoleic acid</td>
<td>25.3</td>
<td>12.6</td>
<td>15.8</td>
</tr>
<tr>
<td>Lysine</td>
<td>&lt;1</td>
<td>4.5</td>
<td>10.2</td>
</tr>
<tr>
<td>Amigdaline</td>
<td>&lt;1</td>
<td>10.3</td>
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<tr>
<td>Benzaldehyde</td>
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<td>&lt;1</td>
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<tr>
<td>Benzoic acid</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Mandelonitril</td>
<td>7.0</td>
<td>23.7</td>
<td>6.8</td>
</tr>
</tbody>
</table>

Each value represents the mean of six tests. Concentration of each compound was 10 mg/ml. a) Each value represents the mean of six tests. Concentration of each compound was 100 µg/ml.
amino acids themselves, and various physiological functions, such as antioxidative action, are exhibited through absorption into the body.\textsuperscript{23,24)}

Based on the above information, caffeic acid derivatives and polyphenols that capture ·OOH, ·OH and ·O$_2^-$ as radical scavengers, and β-sitosterol, which exerts a preventive action by inhibiting the excess production of active oxygen by various cells, may be involved in the antioxidative action of ESE in a complex manner.

Multiple components of ESE may act additively or synergistically, and have a direct antioxidative action and biological regulatory function, which is useful for the prevention and treatment of various disorders.

Further investigation of components, and the effects on other active oxygen species, such as NO and related disorders, is necessary.

REFERENCES