

The Effect of *Eriobotrya japonica* Seed Extract on Oxidative Stress in Adriamycin-Induced Nephropathy in Rats

Atsuhide HAMADA,* Saburo YOSHIOKA, Daisuke TAKUMA, Junko YOKOTA, Tailine CUI, Masahiko KUSUNOSE, Mitsuhiro MIYAMURA, Shojiro KYOTANI, and Yutaka NISHIOKA

Department of Pharmacy, Kochi Medical School Hospital; Kohasu, Okoh-cho, Nankoku, Kochi 783–8505, Japan.

Received May 27, 2004; accepted August 25, 2004

Eriobotrya japonica has been used as a medicinal plant for a long time, and its leaves are known to have many physiological actions such as anti-inflammatory, antitussive, and expectorant. In contrast, *Eriobotrya japonica* seeds are only known to contain amygdalin, and almost no investigations of its pharmacological action have been performed. Moreover, some anticancer agents such as adriamycin cause renal disorders as an adverse effect, and the mechanism of the adverse effect is considered to involve oxidative stress. We have reported that *Eriobotrya japonica* seed extract has an inhibitory effect on liver disorders. In this study, we prepared a 70% ethanol extract of *Eriobotrya japonica* seeds and administered the extract to rats with renal disorder induced by a single administration of 7 mg/kg body weight adriamycin, and investigated the usefulness of the extract. Increases in indices of renal function, plasma urea nitrogen, were significantly inhibited in rats treated with the *Eriobotrya japonica* extract compared to rats treated with tap water. In addition, the renal tissue level of reduced glutathione was significantly high in rats that ingested the extract, while the lipid peroxide levels in plasma and renal tissue were significantly low. However, no effect on renal tissue antioxidative enzymes was observed, suggesting that *Eriobotrya japonica* seed extract has direct antioxidative action. Based on these findings, *Eriobotrya japonica* seed extract may be effective in reducing the oxidative stress of adriamycin-induced renal disorder. Therefore, ingestion of *Eriobotrya japonica* seed extract may contribute to a reduction of the adverse effects of adriamycin.

Key words *Eriobotrya japonica*; renal disease; adriamycin; reactive oxygen species

Recent studies have shown that oxidative damage in the body induced by reactive oxygen species (ROS) and free radicals is frequently involved in the development of many diseases.^{1–3)} ROS are continuously produced physiologically, and play an important role in the expression of cell functions such as the transmission of impulse information. However, if they are produced excessively from any cause, they act cytotoxically as mediators of adverse events such as inflammation, necrosis, and apoptosis. In the kidney, ROS are important causes of acute and subacute renal failure in most cases. It has also been reported that renal failure results from decreased levels of the antioxidant vitamin E and decreased activity of the antioxidant enzyme glutathione peroxidase (GPx),^{4,5)} and can be prevented by free-radical scavengers and polyphenols.^{6,7)}

Also, certain anticancer drugs themselves produce free radicals, and act as factors promoting nephrotoxicity.⁸⁾ In particular, adriamycin (ADR), which is used to treat leukemia and lung cancer, generates superoxide anions and hydroxy radicals, thereby inducing nephrotoxicity, suggesting that the administration of antioxidant substances is effective in inhibiting nephrotoxicity. However, no clinically useful drugs are currently available.

Eriobotrya japonica is widely cultivated as a fruit crop. Among Chinese medicine preparations, *Eriobotrya japonica* folia are an ingredient in Shini-seihai-to and Biwayo-to used as antiphlogistic, analgesic, antitussive, and expectorant agents. Recently, the blood sugar-reducing^{9–11)} and antiphlogistic actions^{12,13)} of *Eriobotrya japonica* folia have also been reported. On the other hand, *Eriobotrya japonica* seeds, like those of apricots and peaches of the same genus (Rosaceae), contain amygdalin as the main constituent; therefore, they were used as a substitute medication for the latter seeds in prewar Japan.

To date, we have discovered the antioxidant action and associated hepatotoxicity-inhibiting action of an extract from *Eriobotrya japonica* seeds.¹⁴⁾ In this study, to clarify the physiological action of an extract from *Eriobotrya japonica* seeds, we administered an *Eriobotrya japonica* seed extract (ESE) with 70% ethanol to rats with ADR nephrotoxicity, and evaluated its improvement effect.

MATERIALS AND METHODS

Materials Sufficiently sun-dried seeds of Mogi-loquant collected at Muroto and Susaki cities in Kochi Prefecture of Japan were the *Eriobotrya japonica* seeds used. Adriamycin (ADR) was provided by Kyowahakko (Japan). All other chemicals were of reagent grade.

Extraction of Seed *Eriobotrya japonica* seeds were extracted by 70% ethanol. 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 being dissolved in the 70% ethanol. The supernatant was then collected and evaporated to dryness to prepare the dried extracts.

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.

ADR-Induced Renal Failure Rats were randomly divided into four groups, consisting of six animals each. They were injected intravenously *via* the tail vein with ADR at a single dose of 7 mg/kg body weight.

Administration of ESE to Renal Failure Rats ESE was administered to the renal failure rats using a water-supply bottle at a dose of 15 ml/d for 14 d.

* To whom correspondence should be addressed. e-mail: hamadaa@med.kochi-u.ac.jp

Tissue Preparation The rats were anesthetized with pentobarbital (50 mg/kg body wt), and the kidneys were perfused *in situ* with cold phosphate buffered saline (PBS) to remove circulating blood cells. Each removed tissue (approximately 0.4 g) was weighed and homogenized with a cell-homogenizer (Eilard) in an extraction buffer containing 20 mM Tris-HCl, pH 7.5, 2 M NaCl, 0.1% Tween-80, 1 mM ethylenediamine tetraacetic acid and 1 mM phenylmethylsulfonyl-fluoride. After centrifugation at $15000\times g$ for 30 min at 4 °C, the supernatant was stored at -70 °C.

Determination of Plasma Creatinine, Urea Nitrogen and Albumin Plasma creatinine, urea nitrogen and albumin were determined using DRI-CHEM 5500 (Fuji film, Tokyo, Japan).

Determination of SOD, GPx and CAT Activities Determination of SOD, GPx and CAT activities of renal tissue was carried out using the SOD Assay Kit-WST (Dojindo Molecular Technologies), glutathione peroxidase assay kit (Cayman) and Amplex[®] Red Catalase Assay Kit (Molecular Probes), respectively.

Determination of Glutathione (GSH) Determination of GSH of renal tissue was carried out using the glutathione assay kit (Cayman). This principle utilizes a carefully optimized enzymatic recycling method, using glutathione reductase for the quantification of GSH. The sulfhydryl group of GSH reacts with 5,5'-dithiobis-2-nitrobenzoic acid and produces a yellow colored 5-thio-2-nitrobenzoic acid (TNB). The mixed disulfide, between GSH and TNB, that is concomitantly produced is reduced by glutathione reductase to recycle the GSH and produce more TNB. The rate of TNB production is directly proportional to this recycling reaction, which is in turn directly proportional to the concentration of GSH in the sample. Measurement of the absorbance of TNB at 405 nm provides an accurate estimation of GSH in the sample.

Determination of Lipid Peroxide Kidney lipid peroxide levels were determined by Wasowicz's method based on the reaction of lipid peroxide with thiobarbituric acid at 95 °C. Fluorescence intensity was measured in the upper *n*-butanol phase by a fluorescence spectrophotometer adjusted to exci-

tation at 525 nm and emission at 547 nm. Arbitrary values obtained were compared with a series of standard solutions (1,1,3,3-tetramethoxypropane). Results were expressed as nmol per gram tissue protein of kidney.

Statistical Analysis Data are expressed as the mean \pm S.E.M. For comparison of two groups, the unpaired *t*-test was used. For more than two groups, analysis of variance was used. $p < 0.05$ indicated a significant difference among groups.

RESULTS

Effect of ESE on Body and Kidney Weights in Rats

Table 1 shows the daily body weight gain from the initiation of animal maintenance to completion of the experiment, as well as the ratio of kidney weight to body weight at the completion of the experiment. No significant difference was observed in daily body weight gain between the saline+water group and the saline+ESE group during the experimental period. However, administration of ADR significantly decreased the daily body weight gain, and the decrease was significantly inhibited by ESE. ADR administration significantly increased the ratio of kidney weight to body weight at the completion of the experiment, but no effect of ESE was observed, regardless of the presence or absence of ADR administration.

Effect of ESE on Renal Function Figure 1 shows the plasma levels of creatinine, urea nitrogen, and albumin. ESE did not affect the creatinine, urea nitrogen, or albumin level in the saline treatment group. ADR increased creatinine and urea nitrogen, and decreased albumin. The creatinine level was slightly reduced in the ADR+ESE group compared to the ADR+water group. The urea nitrogen level was significantly lower in the ADR+ESE group than in the ADR+water group. Furthermore, the albumin level tended to be higher in the ADR+ESE group than in the ADR+water group.

Effect of ESE on Antioxidative Enzyme Activity in Renal Tissue Table 2 shows SOD, GPx, and CAT activities in renal tissues. No significant differences were observed in

Table 1. Body Weight and Wet Kidney Weight

	Saline+water	Saline+ESE	ADR+water	ADR+ESE
Body weight gain (g/d)	4.071 \pm 0.690	4.286 \pm 0.202	-0.214 \pm 0.329 ^{a)}	0.843 \pm 0.121 ^{a,b)}
Wet kidney weight (mg/g body weight)	4.657 \pm 0.208	4.697 \pm 0.067	6.067 \pm 0.315 ^{a)}	6.585 \pm 0.258 ^{a)}

Data represents the mean \pm S.E. of six rats. a) $p < 0.05$ versus saline+water group. b) $p < 0.05$ versus ADR+water group.

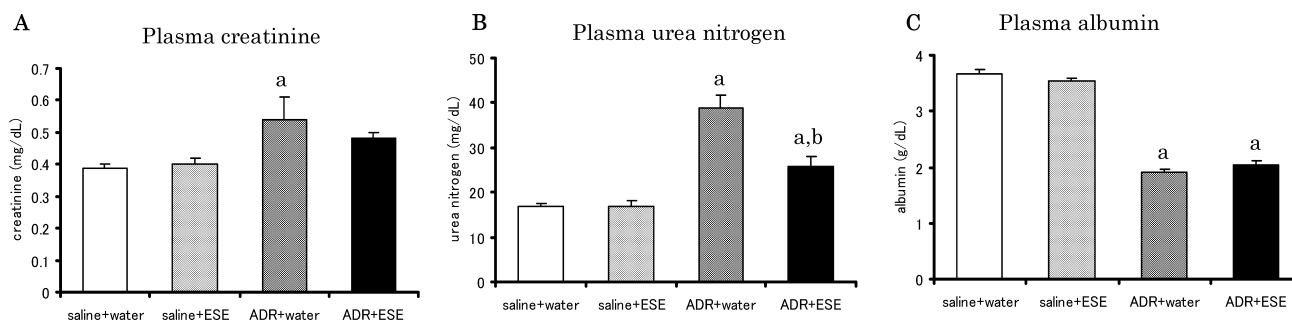


Fig. 1. Effect of ESE on Plasma Creatinine (A), Urea Nitrogen (B) and Albumin (C) Level at Day 14 after ADR Administration

Data represents the mean \pm S.E. of six rats. a) $p < 0.05$ versus saline+water group. b) $p < 0.05$ versus ADE+water group.

Table 2. Effect of ESE on SOD, GPx and CAT Activity at Day 14 after ADR Administration

	Saline+water	Saline+ESE	water+ADR	ESE+ADR
SOD (U/mg protein)	1.024±0.073	1.083±0.087	0.983±0.070	0.958±0.099
GPx (nmol/min/mg protein)	0.993±0.035	1.004±0.045	0.973±0.057	0.986±0.066
CAT (U/mg rotein)	1.362±0.076	1.402±0.078	1.179±0.087 ^{a)}	1.122±0.164

Data represents the mean±S.E. of six rats. a) $p<0.05$ versus saline+water group.

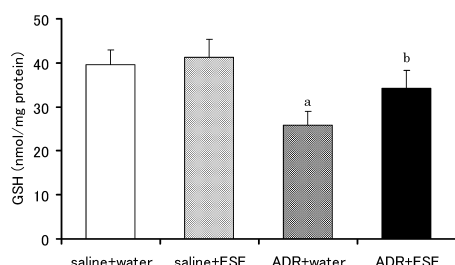


Fig. 2. Effect of ESE on GSH Level at Day 14 after ADR Administration

Data represents the mean±S.E. of six rats. a) $p<0.05$ versus saline+water group. b) $p<0.05$ versus ADR+water group.

SOD or GPx activity in renal tissues among any of the groups. CAT activity was significantly lower in the ADR+water group than in the other groups, but no significant difference was observed in the ADR+ESE group. ESE administration to saline-treated rats slightly increased SOD, GPx, and CAT activities.

Effect of ESE on GSH Level in Renal Tissue Figure 2 shows the GSH levels in renal tissues. ESE did not affect the renal tissue GSH level in saline-treated rats. However, ADR significantly decreased the GSH level, and the decrease was inhibited by ESE, showing a significantly higher level in the ADR+ESE group, compared to that in the ADR+water group.

Effects of ESE on Lipid Peroxide Levels in Renal Tissue Figure 3 shows the lipid peroxide levels in renal tissues. No effect of ESE was observed in rats treated with saline. The renal tissue lipid peroxide level was significantly higher in the ADR+water group than in the other groups. However, the increase was inhibited by ESE, showing a significantly lower value in the ADR+ESE group than in the ADR+water group. Furthermore, the level was higher in the ADR+ESE group than in the saline+water and saline+ESE groups, but the differences were not significant.

DISCUSSION

Oxidant stress has been reported to be involved in the development of various diseases, and treatment methods aimed at eliminating oxidant stress have been attracting attention.^{1–3)} In the kidney, as in other organs, oxidant stress is considered to cause the progression of nephrotoxicity. Indeed, adriamycin-, puromycin-, or cisplatin-induced nephrotic syndrome has been reported to be exacerbated by reactive oxygen species.^{15–20)} In addition, studies have reported that the antioxidant vitamin E and the antihypercholesterolemic agent probucol reduce nephrotoxicity.^{21,22)} However, few drugs with antioxidant activity are clinically available, and such drugs are not used for antioxidant purposes. In this study, we investigated the physiological action and mechanism of action of ESE on ADR nephropathy, which

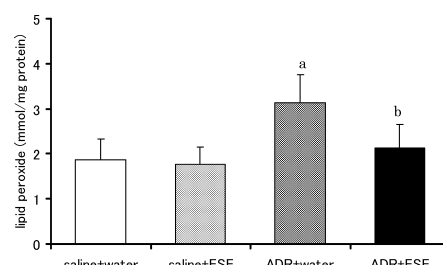


Fig. 3. Effect of ESE on Lipid Peroxide Level of Kidney at Day 14 after ADR Administration

Data represents the mean±S.E. of six rats. a) $p<0.05$ versus saline+water group. b) $p<0.05$ versus ADR+water group.

was considered to be strongly influenced by oxidant stress. The results showed the improvement of plasma urea nitrogen levels, suggesting the usefulness of ESE in the treatment of nephrotoxicity. In addition, the administration of ESE increased GSH levels and reduced lipid peroxide levels in renal tissue. It is thought that ADR is reduced by cytochrome P-450 to a semiquinone free radical, which donates electrons to oxygen to generate active oxygen species such as superoxide anions and hydroxyl radicals, leading to tissue injury. Therefore, it has been reported that, in ADR-administered rats, tissues are exposed to oxidant stress, which induces decreased GSH levels and increased lipid peroxide levels. These observations suggest that decreased creatinine and BUN levels, increased GSH levels, and decreased lipid peroxide levels result from the antioxidant action of ESE on ADR-induced renal tissue oxidant stress. Furthermore, it has been reported that, in a model of ADR administration, the levels of antioxidant enzymes such as SOD, GPX, and CAT do not significantly change. Also, in this study, the administration of ADR as well as ESE did not significantly change the renal tissue levels of antioxidant enzymes. These results suggest that the antioxidant action of ESE is not mediated by antioxidant enzymes, but that the ESE constituents absorbed in the body directly exhibit antioxidant activity. The antioxidant action and non-toxicity of ESE examined in this study have been demonstrated in studies to date. We believe that ESE can be used safely for long periods, and can be ingested routinely as a prophylactic drug for various diseases. Further studies are needed to identify the active constituents in ESE, and to elucidate their antioxidant actions in models of other disease states, as well as in ADR nephrotoxicity. Most *Eriobotrya japonica* seeds are currently discarded as garbage without being utilized. Therefore, the utilization of *Eriobotrya japonica* seeds in the prevention and treatment of diseases contributes greatly to society from the standpoint of utilization of natural resources, such as the extended use of farm products and the effective use of waste.

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