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Research Paper

Effects of *Eriobotrya japonica* seed extract on oxidative stress in rats with non-alcoholic steatohepatitis

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Abstract

Objectives Non-alcoholic steatohepatitis is associated with the deposition of lipid droplets in the liver, and is characterised histologically by the infiltration of inflammatory cells, hepatocellular degeneration and liver fibrosis. Oxidative stress may play an important role in the onset and deterioration of non-alcoholic steatohepatitis. We previously reported that an *Eriobotrya japonica* seed extract, extracted in 70% ethanol, exhibited antioxidant actions *in vitro* and *in vivo*. In this study, we examined the effect of this extract in a rat model of non-alcoholic steatohepatitis.

Methods The seed extract was given in the drinking water to fats being fed a methionine-choline-deficient diet for 15 weeks.

Key findings Increases in alanine aminotransferase and aspartate aminotransferase levels were significantly inhibited in rats fed the seed extract compared with the group on the diet alone. Formation of fatty droplets in the liver was also inhibited. Antioxidant enzyme activity in liver tissue was higher than in the diet-only group and lipid peroxidation was reduced compared with rats that also received the extract. Expression of 8-hydroxy-2′-deoxyguanosine and 4-hydroxy-2-nonenal was lower in the rats given the seed extract than in the diet-only group. In the former, liver tissue levels of transforming growth factor-β and collagen were also decreased.

Conclusions Thus, the *E. japonica* seed extract inhibited fatty liver, inflammation and fibrosis, suggesting its usefulness in the treatment of non-alcoholic steatohepatitis.

Keywords antioxidant enzyme activity; *Eriobotrya japonica* seed extract; non-alcoholic steatohepatitis; oxidative stress; methionine-choline-deficient diet

Introduction

In 1980, Ludwig and colleagues described a patient with no history of alcohol abuse but in whom liver biopsy showed histological features similar to alcoholic injury.[1] Since then, non-alcoholic steatohepatitis (NASH) has been investigated extensively. NASH is defined as steatosis with inflammation and fibrosis, excluding many liver disorders such as viral hepatitis. In some patients, it deteriorates to liver cirrhosis, leading to the development of hepatocellular carcinoma.[2–4] The pathogenesis of this disorder may initially involve fatty changes in hepatocytes, oxidative stress, insulin resistance, dyslipidaemia, excessive iron deposition and mitochondrial dysfunction.[5] As a factor involved in the deterioration of NASH, oxidative stress may be associated with reactive oxygen species (ROS) synthesised by the excessive amount of adipose tissue-derived free fatty acids when oxidised by mitochondria and cytochrome P450 2E1 (CYP2E1), the production of inflammatory cytokines, such as tumour necrosis factor-α and transforming growth factor-β (TGF-β), and the Fenton reaction of iron deposition in the liver.[6]

The treatment of NASH involves diet/exercise therapies,[7] insulin-sensitising agents,[8] ursodeoxycholic acid[9] and fibrate lipid-lowering agents.[10] Recent studies indicated that angiotensin II receptor blockers reduced liver fibrosis in the presence of NASH.[11,12] However, appropriate treatment has not been established.

Eriobotrya japonica seed extract (ESE) is a health food with antioxidative activity. It exhibits radical scavenging activity against ROS such as superoxide anion, hydrogen

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peroxide, hydroxyl radicals and lipid peroxide (LPO).[13] We have previously shown, using HPLC, that this extract contains various substances such as polyphenols (caffeic and chlorogenic acids), amino acids and unsaturated fatty acids.[14,15] Multiple components of ESE are considered to act additively or synergistically, showing direct antioxidant and biological regulatory actions. To date, we have reported the usefulness of ESE in the treatment of various disorders in which oxidative stress is aetiologically involved, such as nephropathy,[16] mucositis,[14] adverse reactions to anticancer agents, gastric mucosal injury related to non-steroidal anti-inflammatory drugs[15] and allergy reactions.[17,18] Furthermore, ESE decreased the level of low-density lipoprotein cholesterol in rabbits with hyperlipidaemia.[19]

According to a recent study, the histopathology of liver biopsies from rats fed a methionine-choline-deficient (MCD) diet resembles those in NASH. In this study, we administered ESE, which was extracted by 70% ethanol, to a model of NASH in rats fed an MCD diet.

Materials and Methods

Materials

Sufficiently sun-dried seeds of Mogi-loquant collected in Muroto and Susaki Cities in Kochi Prefecture and Shimotsu-cho in Wakayama Prefecture, Japan, were used. All other chemicals were of reagent grade.

Extraction of seeds

E. japonica seeds were extracted in 70% ethanol. Briefly, l.0 kg of seeds were crushed in a refrigerated blender at 1000 rev/min, and then stirred continuously by a mixer at 300 rev/min for 7 days after being dissolved in 70% ethanol. The supernatant was then collected and evaporated to dryness. The final yield of the extract was about 120 g. The dried extract was emulsified in 6.6 litres distilled water.

The extract was made in large quantities in this ratio and the same batch has been used for various studies since 2008.[18]

Animals

Male Wistar rats (aged 5 weeks and weighing 90–110 g) were purchased from Japan SLC, Inc. (Shizuoka, Japan) and were acclimatised for 7 days at 23 ± 2°C with free access to a normal diet (CE-2, Clea, Osaka, Japan) and water. Healthy rats were then selected and randomly divided into three groups. Rats were fed either the MCD diet (Oriental Yeast, Tokyo, Japan) or a normal diet. Three experimental groups were studied. The normal group ($n = 6$) was fed a standard diet and received water *ad libitum*. The other two groups ($n = 7$) were fed the MCD diet and water. One of these groups were also given ESE in conjunction with the MCD diet (0.27 g/day in drinking water, using a water-supply bottle at a dose of 15 ml/day). It was confirmed that the rats consumed the treated water each time. All rats were fed for 15 weeks under these conditions.

All animal experiments were performed according to the guidelines for the care and use of laboratory animals of Kochi University, and were approved by our local ethics committee for experimental animal use.

Tissue preparation

At the end of the treatment period, the rats were fasted overnight, anaesthetised with pentobarbital (50 mg/kg), and blood and liver samples taken. The liver samples were perfused *in situ* with cold phosphate-buffered saline to remove circulating blood cells. Each sample (approximately 0.4 g) was weighed and then homogenised with a cell homogeniser (Polytron PT3100, Kinematica AG, Lucerne, Germany) in an extraction buffer containing 20 mmol/l Tris-HCl, pH 7.5, 2 mol/l NaCl, 0.1% Tween 80, 1 mmol/l EDTA and 1 mmol/l phenylmethylsulfonyl-fluoride. Supernatants obtained after centrifugation at 15 000*g* for 30 min at 4°C were stored at −70°C.

Measurement of liver enzymes and markers

Plasma samples were obtained immediately by centrifuging blood samples at 3000 rev/min for 10 min. Plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were measured using a Fuji Dri-Chem analyser (Fujifilm Medical Co., Ltd, Tokyo, Japan).

Superoxide dismutase (SOD) in liver tissue was measured using a commercial kit (Dojindo Laboratories, Kumamoto, Japan). Glutathione peroxidase (GPx) and catalase activities were measured using commercial kits from Cayman Chemical Co. (Ann Arbor, MI, USA).

Levels of glutathione (GSH) in liver tissue were measured using an assay kit from Cayman Chemical Co. LPO was measured using a test kit from Wako Pure Chemical Industries (Osaka, Japan). TGF-β was measured by immunoassay using a commercial kit (Multispecies TGF-β1; BioSource Int., Camarillo, CA, USA). Collagen levels were measured using a staining kit (MCK, Tokyo, Japan).

Histopathology and immunohistochemistry

Immediately after removal, livers were fixed in 10% formalin for 48 h, and then embedded in paraffin. Sections 5 μm thick were then routinely processed for staining with haematoxylin and eosin (H&E) and Azan.

For immunohistochemical analysis, Histofine Simple Stain Rat MAX PO (Nichirei Biosciences, Tokyo, Japan) was used in combination with anti-8-hydroxy-2′-deoxyguanosine (8-OHdG), 5 μg/ml, and anti-4-hydroxy-2-nonenal

Table 1 Effect of *E. japonica* seed extract (ESE) on body weight and liver enzymes after 15 weeks

	Normal	MCD diet	MCD diet + ESE
Body weight (g)	307.3 ± 9.2	79.7 ± 0.6**	86.9 ± 1.5
ALT (U/l)	51.0 ± 4.9	160.0 ± 11.9**	103.0 ± 16.7†
AST (U/l)	91.5 ± 10.2	154.6 ± 23.3*	95.8 ± 8.1†

Values are means ± SEM (n = 5–7 experiments). $^{*}P < 0.05$; $^{**}P < 0.01$ vs normal group; $^{\dagger}P < 0.05$ vs MCD diet group (Tukey–Kramer's test). ALT, alanine aminotransferase; AST, aspartate aminotransferase; MCD, methionine-choline-deficient.

(4-HNE), 5 μg/ml (both Oxis International, Beverly Hills, CA, USA).

Statistical analysis

Data are given as means ± SEM. The level of statistical significance was determined by analysis of variance followed by Tukey–Kramer's test for multiple comparisons. P values less than 0.05 were considered significant.

Results

Effect of ESE on body weight, ALT and AST

The body weight and plasma levels of ALT and AST after 15 weeks are shown in Table 1. Body weight at week 15 was significantly lower in the diet group than in the normal group ($P < 0.01$) but was higher in the diet/ESE group than in the diet group. Plasma ALT and AST levels were significantly higher in the diet group than the normal group ($P < 0.01$ and

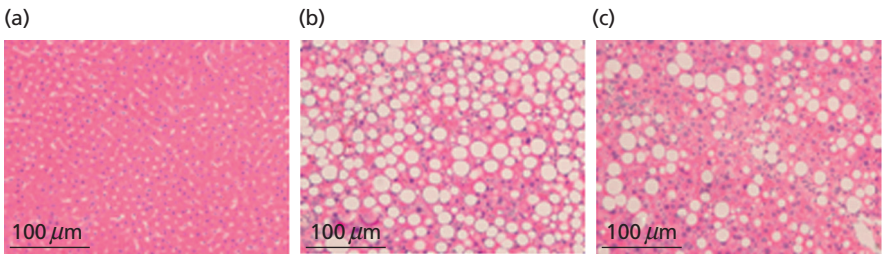

Figure 1 Effect of *E. japonica* seed extract (ESE) on diet-related fatty liver. Samples were taken from rats fed (a) a normal diet, (b) a methionine-choline-deficient (MCD) diet or (c) the MCD diet with ESE in the drinking water. Samples from rats fed the normal diet show normal hepatocytes. Samples from rats fed the MCD diet show diffuse macrosteatosis and hepatocellular ballooning. Samples from the rats fed ESE and the MCD diet show reduction in steatosis. Samples are stained with haematoxylin and eosin.

Table 2 Effect of *E. japonica* seed extract (ESE) on oxidative stress in liver tissue after 15 weeks

	Normal	MCD diet	MCD diet + ESE
SOD (U/mg protein)	332.4 ± 17.7	193.8 ± 15.1*	308.1 ± 49.0†
GPx (μmol/min per mg protein)	1315.5 ± 60.1	344.5 ± 40.1**	532.5 ± 51.5†
Catalase (μmol/min per mg protein)	2.96 ± 0.38	1.60 ± 0.14**	1.64 ± 0.06
GSH (nmol/mg protein)	186.8 ± 13.0	138.7 ± 8.4*	184.3 ± 12.0†
LPO (nmol/mg protein)	63.9 ± 4.2	126.7 ± 17.4**	73.0 ± 4.4‡

Values are means ± SEM (n = 4–6 experiments). $^{*}P < 0.05$; $^{**}P < 0.01$ vs normal group; $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.01$ vs MCD diet group (Tukey–Kramer's test). GPx, glutathione peroxidase; GSH, glutathione; LPO, lipid peroxide; MCD, methionine-choline-deficient; SOD, superoxide dismutase.

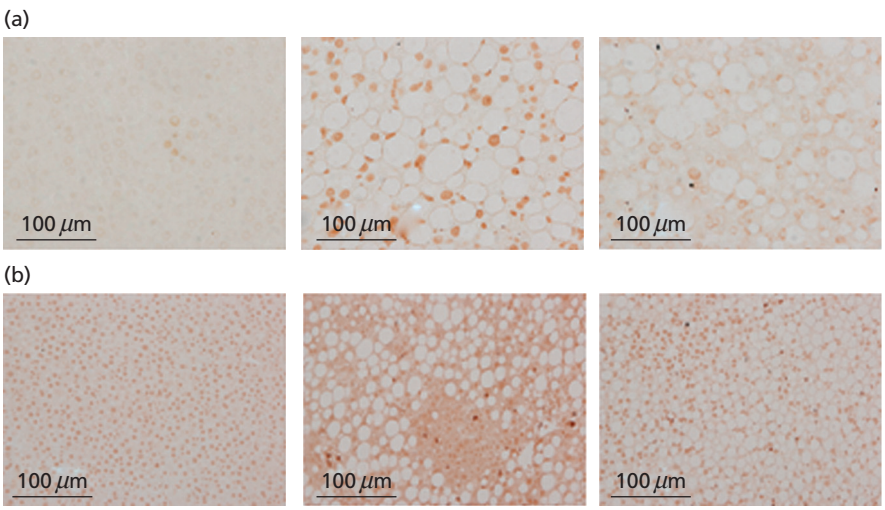

Figure 2 Effect of *E. japonica* seed extract (ESE) on expression of (a) 8-hydroxy-2′-deoxyguanosine (8-OHdG) and (b) 4-hydroxy-2-nonenal (4-HNE). Representative livers from rats fed a normal diet (left), methionine-choline-deficient (MCD) diet (centre) or MCD diet with ESE in the drinking water (right). In (a), 8-OHdG positive cells are identified by the brown nuclei in the photomicrographs. In (b) 4-HNE positive cells are identified by the brown cytoplasm in the photomicrographs.

$P < 0.05$, respectively). These increases were significantly inhibited in the diet/ESE group ($P < 0.05$ and $P < 0.05$, respectively).

Effect of ESE on diet-related fatty liver

H&E stained liver sections are shown in Figure 1. The normal group showed normal hepatic histology. The diet group showed characteristics of NASH, namely macrovesicular steatosis and hepatocellular ballooning. Slight fat deposition was seen in the diet/ESE group.

Effect of ESE on oxidative stress in liver tissue

Activities of SOD, GPx and catalase and levels of GSH and LPO in liver tissue are shown in Table 2. SOD, GPx and catalase activities were significantly lower in the diet group than in the normal group ($P < 0.05$, $P < 0.01$ and $P < 0.01$, respectively). SOD and GPx activities were significantly higher in the diet/ESE group than in the diet group ($P < 0.05$ and $P < 0.05$, respectively). There was no marked difference in catalase activity between the two groups.

The level of GSH in the liver tissue was significantly lower in the diet group than in the normal group ($P < 0.05$) but was significantly higher in the diet/ESE group than in the diet group ($P < 0.05$). The level of LPO in liver tissue was significantly higher in the diet group than in the normal group ($P < 0.01$) but was significantly lower in the diet/ESE than in the diet group ($P < 0.01$).

Effect of ESE on 8-OHdG and 4-HNE expression

Immunohistochemical staining of liver tissue is shown in Figure 2. Immunohistochemical staining of 8-OHdG (Figure 2a) is used as an index of oxidative DNA damage; 4-HNE (Figure 2b) is a secondary oxidation product of high-level unsaturated fatty acids. Neither 8-OHdG nor 4-HNE was detected in the normal group. Both 8-OHdG and 4-HNE were detected in the diet group but expression of both was inhibited in the diet/ESE group.

Effect of ESE on the liver levels of TGF-β

The level of TGF-β in liver tissue is shown in Figure 3a. Levels of TGF-β were significantly higher in the diet group than in the normal group (91.5 ± 15.0 vs 32.6 ± 11.2 pg/mg protein). Levels in the diet/ESE group (34.5 ± 9.0 pg/mg protein) were significantly lower than in the diet group. There was no significant difference between the diet/ESE and normal groups.

Effect of ESE on liver fibrosis

The level of collagen in liver tissue is shown in Figure 3b. The level in the diet group was significantly higher than in the normal group (33.7 ± 4.5 vs 18.4 ± 1.7 μg collagen/mg protein). The level in the diet/ESE group (23.5 ± 1.4 μg collagen/mg protein) was significantly lower than in the diet group. There was no significant difference between the diet/ESE and normal groups.

The results of Azan staining are shown in Figure 4. The normal group showed normal hepatic histology. The diet group showed the perivenular/pericellular fibrosis that is a heterogeneous pattern of fibrosis in NASH. Slight fibrosis was seen in the diet/ESE group.

Discussion

High levels of enzymes that mop up cellular oxygen, such as SOD, GPx and catalase, are present *in vivo*. However, when the production of ROS exceeds the capacity of these enzymes, oxidative stress induces various cellular responses[20] and may also play an important role in the onset and deterioration of NASH. A study reported that, in the presence of NASH, mitochondria in hepatocytes have morphological abnormalities that are involved in the excess production of ROS.[6]

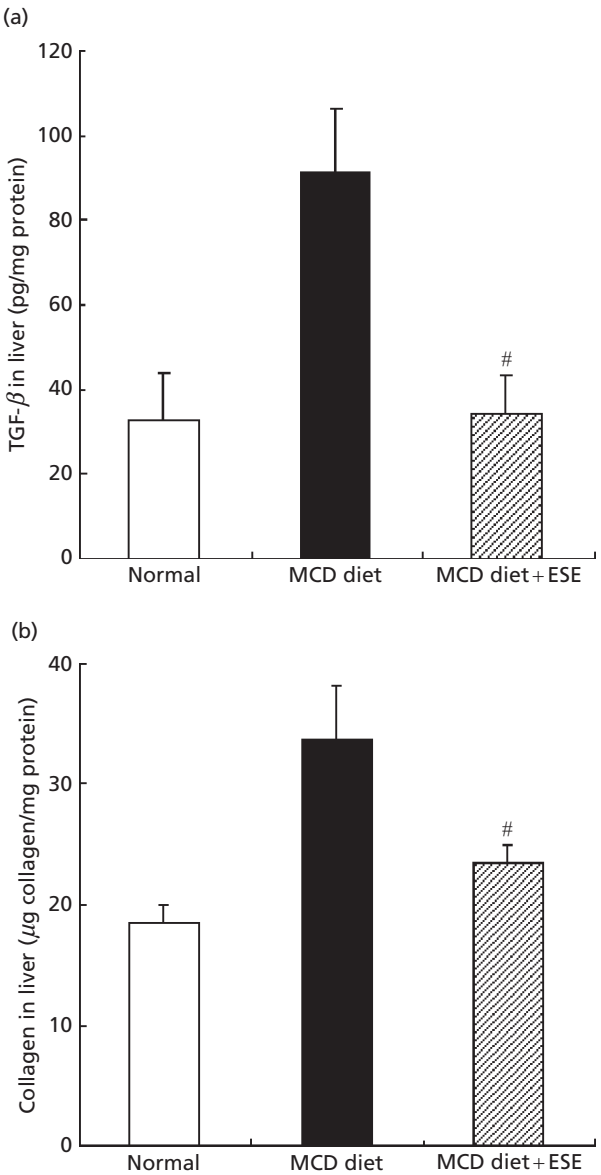

Figure 3 Effect of *E. japonica* seed extract (ESE) on liver tissue levels of (a) transforming growth factor-β (TGF-β) and (b) collagen. The significant increases in TGF-β and collagen induced by the methionine-choline-deficient (MCD) diet were reduced by concomitant treatment with ESE. Columns represent means ± SEM (n = 4–7 experiments). $^{*}P < 0.05$; $^{**}P < 0.01$ vs normal group; $^{\#}P < 0.05$ vs MCD diet group (Tukey–Kramer's test).

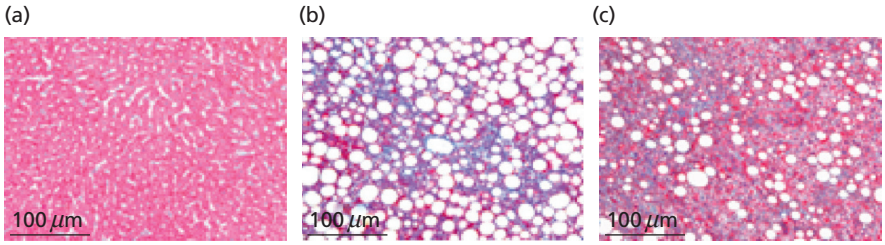

Figure 4 Effect of *E. japonica* seed extract (ESE) on liver fibrosis, demonstrated by Azan staining. Representative liver samples from rats fed (a) a normal diet, (b) a methionine-choline-deficient (MCD) diet or (c) the MCD diet with ESE in the drinking water. Collagen fibres are stained blue in the photomicrographs.

Furthermore, an excess level of fatty acids in the liver enhances mitochondrial β-oxidation, leading to oxidative stress via the excess production of CYP2E1.[21] In addition, various factors such as an excess amount of iron ions may be involved.[22,23]

In this study, we used rats fed an MCD diet as a NASH model. Decreases in the liver levels of methionine and choline promote the excess production of ROS, inducing oxidative stress and leading to hepatocellular disorder and adipose cell formation. In addition, choline deficiency affects lipid metabolism, resulting in fatty liver.[24]

We have investigated ESE because of its antioxidant actions.[14–17] In addition, the usefulness of ESE administration has also been suggested in a hyperlipidaemia model.[19]

ESE administration inhibited increases in the AST and ALT levels, preventing the onset of hepatitis. H&E staining confirmed the inhibitory effects of ESE on fat deposition in the liver. This was possibly associated with the cholesterol-reducing actions of ESE previously reported.[19] With respect to the effects of ESE on oxidative stress, ESE administration increased SOD and GPx activities and reduced oxidative stress by increasing the level of GSH (an antioxidant substance), and decreasing the LPO level. A previous study reported the hydroxyl radical and superoxide anion scavenging actions of ESE.[13]

Oxidative stress may be enhanced in the presence of a fatty liver, leading to the appearance of various types of ROS, suggesting that it can induce excessive DNA/lipid oxidation in the liver. ESE, with its antioxidant actions, inhibited the nuclear expression of 8-OHdG and intracellular expression of 4-HNE, suggesting that it reduces excessive DNA/lipid oxidation in the liver. LPO, which is generated in the presence of oxidative stress, may contribute to the progression of liver fibrosis by activating Kupffer cells, which produce collagen, and promoting the production of cytokines such as TGF-β. In our NASH model, levels of TGF-β and collagen were decreased in rats treated with ESE, suggesting that ESE has potent antioxidant actions. Azan staining confirmed the inhibitory effects of ESE administration on liver fibrosis.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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References

1. Ludwig J *et al*. Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease. *Mayo Clin Proc* 1980; 55: 434–438.
2. Younossi ZM *et al*. Nonalcoholic fatty liver disease: an agenda for clinical research. *Hepatology* 2002; 35: 746–752.
3. Kojima S *et al*. Increase in the prevalence of fatty liver in Japan over the past 12 years: analysis of clinical background. *J Gastroenterol* 2003; 38: 954–961.
4. Farrell GC. Non-alcoholic steatohepatitis: what is it, and why is it important in the Asia-Pacific region? *J Gastroenterol Hepatol* 2003; 18: 124–138.
5. Day CP, James OFW. Steatohepatitis: a tale of two "hits"? *Gastroenterology* 1998; 114: 842–845.
6. Le TH *et al*. The zonal distribution of megamitochondria with crystalline inclusions in nonalcoholic steatohepatitis. *Hepatology* 2004; 39: 1423–1429.
7. Suzuki D *et al*. Liver failure caused by non-alcoholic steatohepatitis in an obese young male. *J Gastroenterol Hepatol* 2005; 20: 327–329.
8. Marchesini G *et al*. Metformin in non-alcoholic steatohepatitis. *Lancet* 2001; 358: 893–894.
9. Laurin J *et al*. Ursodeoxycholic acid or clofibrate in the treatment of non-alcohol-induced steatohepatitis: a pilot study. *Hepatology* 1996; 23: 1464–1467.
10. Saibara T *et al*. Bezafibrate for tamoxifen-induced non-alcoholic steatohepatitis. *Lancet* 1999; 353: 1802.
11. Yokohama S *et al*. Therapeutic efficacy of an angiotensin II receptor antagonist in patients with nonalcoholic steatohepatitis. *Hepatology* 2004; 40: 1222–1225.
12. Hirose A *et al*. Angiotensin II type 1 receptor blocker inhibits fibrosis in rat nonalcoholic steatohepatitis. *Hepatology* 2007; 45: 1375–1381.
13. Yokota J *et al*. Scavenging of reactive oxygen species by *Eriobotrya japonica* seed extract. *Biol Pharm Bull* 2006; 29: 467–471.
14. Takuma D *et al*. Effect of *Eriobotrya japonica* seed extract on 5-fluorouracil-induced mucositis in hamsters. *Biol Pharm Bull* 2008; 31: 250–254.
15. Yokota J *et al*. Gastroprotective activity of *Eriobotrya japonica* seed extract on experimentally induced gastric lesions in rats. *J Nat Med* 2008; 62: 96–100.

16. Hamada A *et al*. The effect of *Eriobotrya japonica* seed extract on oxidative stress in adriamycin-induced nephropathy in rats. *Biol Pharm Bull* 2004; 27: 1961–1964.
17. Sun G *et al*. Effect of orally administered *Eriobotrya japonica* seed extract on allergic contact dermatitis in rats. *J Pharm Pharmacol* 2007; 59: 1405–1412.
18. Onogawa M *et al*. Animal studies supporting the inhibition of mast cell activation by *Eriobotrya japonica* seed extract. *J Pharm Pharmacol* 2009; 61: 237–241.
19. Nishioka Y. Medicine composition that contains *Eriobotrya japonica* seed extract to adjust amount of lipid in body fluids. 2003; JP 3438029.
20. Sies H. Oxidative stress: from basic research to clinical application. *Am J Med* 1991; 91: 31S–38S.
21. Chalasani N *et al*. Hepatic cytochrome P450 2E1 activity in nondiabetic patients with nonalcoholic steatohepatitis. *Hepatology* 2003; 37: 544–550.
22. Valenti L *et al*. Alpha 1-antitrypsin mutations in NAFLD: high prevalence and association with altered iron metabolism but not with liver damage. *Hepatology* 2006; 44: 857–864.
23. Nelson JE *et al*. HFE C282Y mutations are associated with advanced hepatic fibrosis in Caucasians with nonalcoholic steatohepatitis. *Hepatology* 2007; 46: 723–729.
24. Takei Y *et al*. Choline deficiency and NAFLD. In: Saibara T, ed. *Nonalcoholic Fatty Liver Disease: NAFLD – from the base to clinic*. Tokyo: Ishiyaku Publishers Inc, 2006: 159–162.