

Protective Effects of Persimmon Leaf and Fruit Extracts against Acute Ethanol-Induced Hepatotoxicity

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Abstract

Persimmon is well-known as a Korean traditional medicine for alleviating coughs and enhancing blood circulation; it is also used for treatment of hypertension, cancer, diabetes and atherosclerosis. To evaluate the protective properties of persimmon leaf methanol extract (PLME) and persimmon fruit methanol extract (PFME) administration on acute ethanol-induced hepatotoxicity, C57BL/6 male mice were gavaged with or without persimmon extracts for 1 week. Hepatotoxicity was then induced by gavage of 5 g/kg BW ethanol. After 12 hr of ethanol administration, blood and liver were collected and analyzed for biochemical markers of hepatotoxicity. The results showed PLME and PFME treatments decreased the activities of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) compared with ethanol control. Both PLME and PFME reduced serum lactate dehydrogenase (LDH) activity, but elevated alcohol dehydrogenase (ADH) activity. Serum triglyceride (TG) and hepatic cholesterol levels were significantly decreased when treated with PLME and PFME. Liver malondialdehyde (MDA) levels were significantly decreased in PLME and PFME groups compared with ethanol control. Furthermore, the administration of PLME and PFME significantly increased the activities of catalase, glutathione peroxidase (GSH-Px) and glutathione reductase (GSH-red). In summary, PLME and PFME appeared to prevent hepatic injury by accelerating alcohol metabolism by increasing alcohol-metabolizing enzyme activities, by activating the antioxidative enzyme system against oxidative stress, and by decreasing fat accumulation, which is evidenced by decreased hepatotoxic indices in serum.

Key words: persimmon (*Diospyros kaki*), ethanol, hepatoprotective effect, C57BL/6 mice

INTRODUCTION

Ethanol is one of the most widely used and abused drugs. There are three major pathways for ethanol metabolism in the liver, each located in a different sub-cellular compartment: alcohol in the cytosol, microsomal ethanol oxidizing system in the endoplasmic reticulum, and aldehyde dehydrogenase in the mitochondria (1-3). All three result in the generation of reactive oxygen species (ROS), including superoxide, hydroxyl radical, and hydrogen peroxide. When the cellular antioxidant capacity is insufficient to cope with ROS accumulation, oxidative stress occurs in the liver (1,4). Alcohol-induced hepatic oxidative stress has been repeatedly demonstrated by measuring lipid peroxidation (5-7), a marker for oxidative stress in both alcoholic patients and animal models (8).

Reduced glutathione (GSH), a tripeptide consisting of glutamic acid, cysteine and glycine, in conjunction with glutathione peroxidase (GSH-Px) is involved in the protection of cells against damage caused by free radicals, peroxides and other toxic compounds (8,9). GSH-Px cat-

alyses the reduction of hydroperoxides using GSH, thereby protecting mammalian cells against oxidative damage (10). As a result, GSH is oxidized to oxidized glutathione (GSSG), which in turn is reduced back to GSH by glutathione reductase (GSH-red) at the expense of reduced nicotinamide adenine dinucleotide (NADPH) forming a redox cycle (11). GSSG, the oxidized product of GSH, has been reported to accumulate due to the inactivation of GSH-red. GSSG inactivates many enzymes containing SH groups and inhibits protein synthesis (12). Catalase is ubiquitous and is found in all oxygen-consuming organisms. This enzyme is widely distributed in cells with active oxygen metabolism and plays an important role against oxidative damage in the cells (13).

Ethanol is a powerful inducer of hyperlipidemia in humans and animals (14). It occurs when the intracellular redox potential and redox sensitive nutrient metabolisms are disturbed by alcohol. An excessive accumulation of reducing equivalents favors hepatic lipogenesis, decreases the hepatic release of lipoproteins, increases the mobilization of peripheral fat, enhances the uptake of circulating lipids and decreases fatty acid oxidation, and

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thus increases the retention of lipids in the liver (15).

Persimmon grows in the countries of East Asia, such as Korea, China and Japan. The fruits of persimmon are eaten fresh and the leaves of this tree are infused with water to make a popular tea. Furthermore, persimmon extract has been known to have anti-oxidative and hypo-lipidemic effects (16,17). However, the protective effect of persimmon extract against liver damage resulting from acute alcohol-induced hepatotoxicity and its mechanism of action remain unclear. The present study was undertaken to evaluate the protective effects of persimmon extracts on ethanol-induced hepatotoxicity and to elucidate the mechanism underlying these protective effects in mice. We determined serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) to check liver damage. Serum alcohol dehydrogenase activity (ADH), GSH-red, GSH-Px and catalase activities in liver were also determined to evaluate the effects on oxidative stress. We also determined triglyceride (TG) and total cholesterol concentration to evaluate lipid profiles and malondialdehyde (MDA) to evaluate lipid peroxidation.

MATERIALS AND METHODS

Animals

C57BL/6 male mice (20~25 g, 9-week-old) were used for the study. After 1 week of adaptation, all animals were housed in polycarbonate cages in a temperature (25°C) and humidity (50%) controlled room with a 12-hr light/12-hr dark cycle. Water and a normal standard pellet diet were available ad libitum throughout the experimental period.

Preparation of persimmon leaf methanol extract (PLME) and persimmon fruit methanol extract (PFME)

PLME and PFME were prepared as follows: fresh persimmon leaves and fruits which were harvested in Gimhae on November, 2006 were washed, dried with hot air, and well ground to powder. One kilogram of persimmon leaf powder was extracted with 10 L of 90% (v/v) methanol three times and filtered using Whatman No. 4 filter (Maidstone, England). The filtrate was concentrated and dried by rotary vacuum evaporator (Büchi, Flawil, Switzerland) to get 158 g of PLME. About 0.9 kg of highly viscous PFME was obtained from 1 kg of persimmon fruit powder with the same extraction procedure above.

Acute ethanol-induced liver injury in mice

C57BL/6 mice were divided into 4 groups: control group, ethanol treatment group, PLME treatment group and PFME treatment group with 6 mice. PLME and

PFME were administered by gavage at 2 g/kg BW once daily for 7 consecutive days. Then, mice received an acute ethanol dose of 5 g/kg BW diluted in water (50%, v/v) for ethanol control, PLME and PFME groups. Control group mice received an isocaloric maltose solution (50%, v/v) of 9 g/kg BW. 12 hr after the administration of ethanol and maltose, the animals were sacrificed and blood collected from by cardiac puncture to determine biochemical parameters. Subsequently, livers were collected, weighed and frozen in liquid nitrogen and stored at -70°C for further study.

Biochemical assay in serum

Serum ALT and AST activities were measured colorimetrically using a diagnostic kit (ASAN Co., Korea) and LDH using the AceChem LDH kit, according to the instructions provided. The activity of ADH was determined spectrophotometric assay with mixture of 100 µL of 1 mM NAD, 100 µL propionaldehyde, 600 µL of 50 mM sodium pyrophosphate buffer (pH 8.5) and 200 µL of serum at 340 nm.

Lipid profile assay

Serum TG concentrations were determined using colorimetric kits (AceChem Co.). Hepatic lipids were extracted by the method of Folch et al (18). The resulting lipid pellet was dissolved in chloroform with Triton X-100 and assayed for TG and total cholesterol levels using colorimetric Kits (AmeChem Co.).

Lipid peroxidation assay

Lipid peroxidation in the liver was quantified by measuring the levels of thiobarbituric acid-reactive substance (TBARS) as described by Fraga et al. (19). Lipid peroxidation was expressed as nmol of malondialdehyde (MDA) equivalents per gram of liver, derived from a standard curve for 1,1,3,3-tetraethoxypropane.

Antioxidant enzyme activities assay

Liver sample was homogenized in 20 parts (w/v) of a 50 mM sodium phosphate buffer (pH 7.4) at 4°C. The homogenate was centrifuged at 600 g, 4°C for 10 min to remove the cell debris, and the resulting supernatant was further centrifuged at 10,000 g for 20 min to separate the mitochondrial pellet and cytosolic fractions. The protein concentration was determined by the Biuret reaction (20), using bovine albumin as the standard. The cytosolic fraction was used to determine Catalase, GSH-Px and GSH-red activities. Catalase activity was analyzed using the Abei kinetic method (21). The kinetic changes of absorbance were marked at 240 nm. Activity of catalase was defined as the quantity (in µmol) of hydrogen peroxide decomposed in 1 min per mg of liver protein (U/mg). GSH-Px activity in liver sample was as-

sayed by the Paglia and Valentine kinetic method (22). Decrease in absorbance at 340 nm was measured. Activity of GSH-Px was determined as the quantity in μmol of NADPH oxidized per min per 1 mg of liver protein (U/mg). Determination of GSH-red activity in liver sample was also assayed by the kinetic method (23). The decrease in the concentration of NADPH after reduction of oxidized glutathione back to reduced glutathione was measured. Activity of GSH-red was determined as the quantity in μmol of NADPH glutathione oxidized in 1 min per 1 mg of liver protein (U/mg).

Statistical analysis

All data were expressed as mean \pm standard deviation. The statistical analyses were performed using the SPSS software. One-way ANOVA and Duncan's multiple range tests were used to examine the difference among groups, statistical significance being considered at $p < 0.05$.

RESULTS AND DISCUSSION

Serum AST, ALT, LDH and ADH levels

Serum levels of AST, ALT and LDH are cytosolic marker enzymes reflecting hepatocellular necrosis as they are released into the blood after cell membrane damage (24). Since AST and ALT were used as sensitive markers in the diagnosis of hepatic diseases (25), the extent of hepatic damage is mainly assessed by the activities of released cytoplasmic AST and ALT in the circulation (26). In the present study, acute ethanol treatment for 7 days caused significant increases in levels of AST and ALT in serum. Compared with the ethanol-treated group, treatment with PLME and PFME significantly reduced the serum ALT and AST activity (Fig. 1). Serum LDH activity, that is also a commonly used marker of tissue damage (27-29), was also significantly ameliorated by PLME and PFME treatments (Fig. 2). Silymarin (30), aqueous extract of the roots of *Decalepis hamiltonii* (31), and glycoprotein isolated from *Acatnaopanax senticosus* (25) were reported to have protective effects against ethanol-induced hepatotoxicity in mice and rats. Their effective doses were as low as 200 mg/kg, which is a much lower level than the 2 g/kg used in our study. These differences might come from the purification degree of sample or administration period of sample to animals.

Ethanol and its metabolites are primarily catalyzed by the enzyme ADH, which is located in the cytoplasm. Although ADH catalyzes the rate-limiting step in ethanol metabolism, its physiological role is uncertain. Serum ADH activity can be a specific reflection of hep-

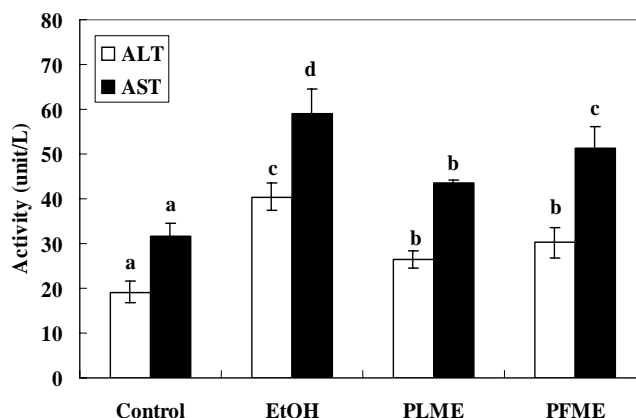


Fig. 1. Effects of persimmon leaf and fruit extracts on serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in ethanol-induced hepatotoxic mice. Each value is the mean \pm SD. Different superscripts indicate significant differences at $p < 0.05$. Control: ethanol-untreated group, EtOH: ethanol-treated group, PLME: persimmon leaf methanol extract + ethanol-treated group, PFME: persimmon fruit methanol extract + ethanol-treated group.

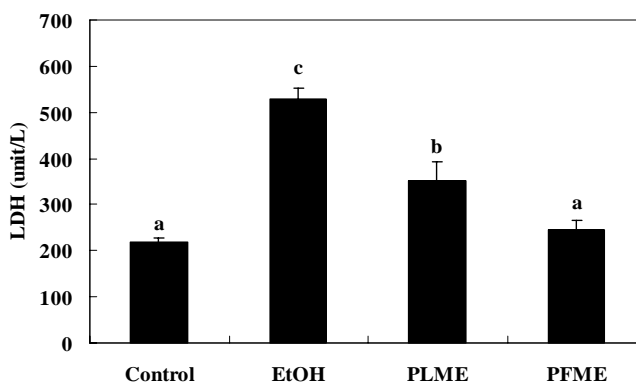


Fig. 2. Effects of persimmon leaf and fruit extracts on serum lactate dehydrogenase (LDH) in ethanol-induced hepatotoxic mice. Each value is the mean \pm SD. Different superscripts indicate significant differences at $p < 0.05$. Control: ethanol-untreated group, EtOH: ethanol treated group, PLME: persimmon leaf methanol extract + ethanol-treated group, PFME: persimmon fruit methanol extract + ethanol-treated group.

atocellular necrosis, since the reduction of ADH activity is proportional to the severity of liver disease (32). Thus, the level of this enzyme in serum can indicate liver disease with comparatively good selectivity (33). In our acute ethanol treatment model, PLME and PFME administration significantly increased ADH activity, implying hepatic function was improved by the administration of PLME and PFME (Fig. 3).

TG & cholesterol levels

Fig. 4 showed that ethanol treatment significantly elevated serum TG compared to ethanol-untreated control, while PLME and PFME treatments significantly decreased serum TG level compared to ethanol control,

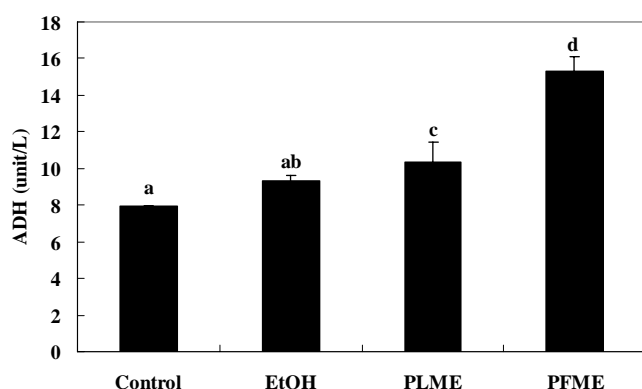


Fig. 3. Effects of persimmon leaf and fruit extracts on serum alcohol dehydrogenase (ADH) in ethanol-induced hepatotoxic mice. Each value is the mean \pm SD. Different superscripts indicate significant differences at $p < 0.05$. Control: ethanol-untreated group, EtOH: ethanol treated group, PLME: persimmon leaf methanol extract + ethanol-treated group, PFME: persimmon fruit methanol extract + ethanol-treated group.

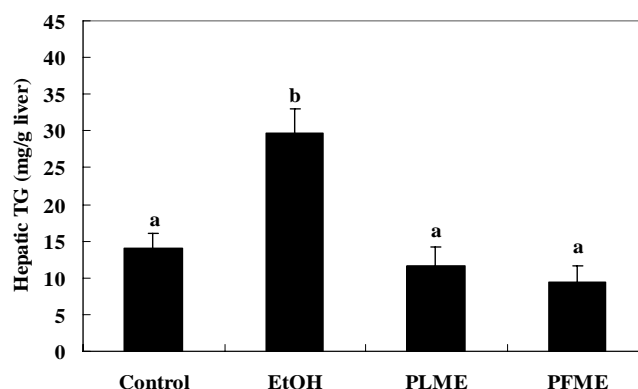


Fig. 5. Effects of persimmon leaf and fruit extracts on hepatic triglyceride (TG) concentration in ethanol-induced hepatotoxic mice. Each value is the mean \pm SD. Different superscripts indicate significant differences at $p < 0.05$. Control: ethanol-untreated group, EtOH: ethanol treated group, PLME: persimmon leaf methanol extract + ethanol-treated group, PFME: persimmon fruit methanol extract + ethanol-treated group.

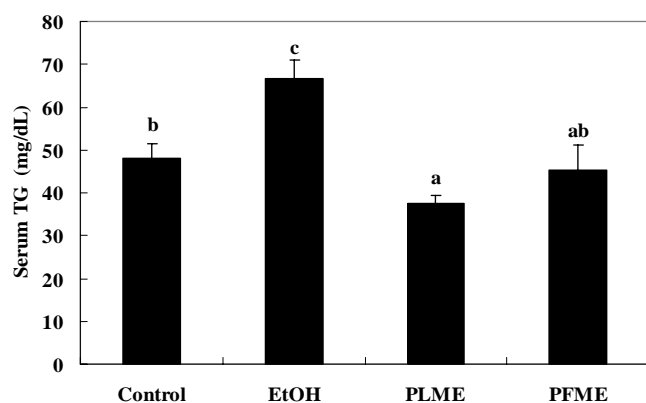


Fig. 4. Effects of persimmon leaf and fruit extracts on serum triglyceride (TG) concentration in ethanol-induced hepatotoxic mice. Each value is the mean \pm SD. Different superscripts indicate significant differences at $p < 0.05$. Control: ethanol-untreated group, EtOH: ethanol treated group, PLME: persimmon leaf methanol extract + ethanol-treated group, PFME: persimmon fruit methanol extract + ethanol-treated group.

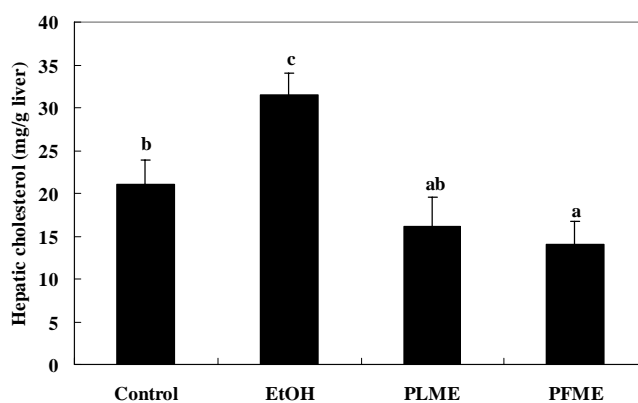


Fig. 6. Effects of persimmon leaf and fruit extracts on hepatic total cholesterol concentration in ethanol-induced hepatotoxic mice. Each value is the mean \pm SD. Different superscripts indicate significant differences at $p < 0.05$. Control: ethanol-untreated group, EtOH: ethanol treated group, PLME: persimmon leaf methanol extract + ethanol-treated group, PFME: persimmon fruit methanol extract + ethanol-treated group.

suggesting that PLME and PFME prevent hyperlipidemia induced by ethanol. Furthermore, hepatic TG (Fig. 5) and hepatic cholesterol (Fig. 6) levels were decreased both in PLME and PFME groups compared to the ethanol control group. Levels of TG and cholesterol in serum or liver tissue increased from ethanol consumption might be due to several processes, such as increased availability of free fatty acids and L-glycerophosphate, decreased secretion of very low density lipoprotein (VLDL) into the serum, and decreased removal of TG and cholesterol from serum due to diminished lipoprotein activity (34). Therefore, decreased TG and cholesterol levels by PLME and PFME treatments may suggest that PLME and PFME treatments prevent hep-

atic damage and abnormal liver function induced by ethanol.

Liver is the most common site of damage in laboratory animals administered drugs and other chemicals (35). Ethanol can cause alterations in lipid metabolism and induces fatty liver, but its exact mechanism is complex (36,37). Hepatic oxidation of ethanol to acetaldehyde, reduces NAD^+ to NADH , and produces a striking redox change associated with metabolic disorders; a decrease in the NAD^+/NADH ratio inhibits the tricarboxylic acid (TCA) cycle and fatty acid oxidation, resulting in hepatic lipogenesis. Fat accumulation in hepatocytes leads to the development of fatty liver (steatosis), which is a reversible condition (38). Day and James (39) reported that

steatosis increases the sensitivity of the liver to a variety of injurious mechanisms, or plays a role in alcohol-induced liver injury. In support, studies in animal models have shown that steatosis increases endotoxin-mediated necro-inflammation (40) and the degree of lipid peroxidation (41). In our results, levels of TG and cholesterol in the liver and serum are increased in ethanol-treated mice compared with ethanol-untreated mice. However, PLME and PFME treatments diminished TG and cholesterol elevations in the liver and serum induced by ethanol administration. These results demonstrated that PLME and PFME can prevent hyperlipidemia and fatty liver induced by ethanol consumption.

Status of oxidative stress and antioxidative enzyme activities

Numerous studies have demonstrated that oxidative stress plays an important etiological role in the development of alcoholic liver disease (42-44). That is, the generation of lipid peroxidation by free radicals has been proposed as a mechanism for ethanol-induced hepatotoxicity (42). Alcohol administration causes accumulation of ROS, including superoxide, hydroxyl radical, and hydrogen peroxide (45). Reactive oxygen species, in turn, cause lipid peroxidation of cellular membrane lipids, and protein and DNA oxidation, which result in hepatocyte injury (46-48). Hepatic lipid peroxidation associated with acute ethanol administration has often been assessed in both animal models and in human clinical trials as an indicator of oxidative stress (6,49). In our study, a slight increase in lipid peroxidation was noted after acute ethanol administration, and this increase was significantly attenuated by persimmon extract pretreatments (Fig. 7). Furthermore, ethanol and its metabolites can alter the redox balance towards a more oxidized state in the liver, which acts in a pro-oxidant manner and/or reduces antioxidant cellular defenses. Our study demonstrated that lipid peroxidation was also increased by ethanol treatment, whereas the activities of catalase, GSH-Px, and GSH-red were decreased when compared with ethanol untreated control. However, treatments with

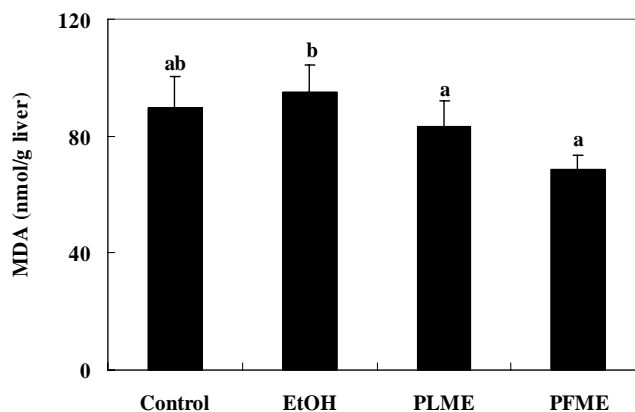


Fig. 7. Effects of Persimmon leaf and fruit extracts on hepatic malondialdehyde (MDA) concentration in ethanol-induced hepatotoxic mice. Each value is the mean \pm SD. Different superscripts indicate significant differences at $p < 0.05$. Control: ethanol-untreated group, EtOH: ethanol treated group, PLME: persimmon leaf methanol extract + ethanol-treated group, PFME: persimmon fruit methanol extract + ethanol-treated group.

PLME and PFME before ethanol treatment caused increases in catalase, GSH-Px and GSH-red activities (Table 1), as well as a decrease in MDA level, which alleviates deleterious effects induced by ethanol.

A few researchers have reported that persimmon leaves and fruits contain various polyphenols and flavonoids in addition to vitamin C and have antioxidative activity (16,50,51). Furthermore, Choi et al. (51) found that persimmon peel, with higher phenolic compounds and flavonoids, had stronger nitrite scavenging activity and inhibitory effects on xanthine oxidase and electron donating ability compared to persimmon flesh with lower concentrations of phenolic compounds and flavonoids. Therefore, PLME and PFME appear to overcome hepatotoxicity caused by acute ethanol consumption by direct antioxidant function and by inducing the activities of antioxidant defense systems in liver impaired by ethanol. According to the above results, pretreatment of PLME and PFME significantly attenuated liver injury against acute ethanol induced hepatotoxicity as evidenced by lowered activities of AST and LDH, hepatic

Table 1. Effects of persimmon leaf and fruit extracts on hepatic anti-oxidation enzyme activities in ethanol-induced hepatotoxic mice (unit/mg protein)

	Group			
	Control	EtOH	PLME	PFME
Catalase	7.28 \pm 0.28 ^b	5.98 \pm 0.86 ^a	7.01 \pm 0.41 ^b	6.64 \pm 0.18 ^{ab}
GSH-px	43.1 \pm 1.21 ^{bc}	37.8 \pm 1.21 ^a	39.0 \pm 1.23 ^{ab}	43.7 \pm 3.21 ^c
GSH-red	647.4 \pm 37.7 ^b	546.6 \pm 45.6 ^a	670.2 \pm 31.0 ^{bc}	716.3 \pm 57.6 ^c

Each value is the mean \pm SD. Different superscripts indicate significant differences at $p < 0.05$. Control: ethanol-untreated group, EtOH: ethanol-treated group, PLME: persimmon leaf methanol extract + ethanol-treated group, PFME: persimmon fruit methanol extract + ethanol-treated group. GSH-px: glutathione peroxidase, GSH-red: glutathione reductase.

MDA, TG and cholesterol levels. Hepatoprotective effects of PLME and PFME might come from the anti-oxidant effects of persimmon extracts themselves or indirect action through the induction of hepatic GSH enzymes in the ethanol-induced hepatotoxic animal model.

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