Protective Effect of *Pinus koraiensis* Needle Water Extract Against Oxidative Stress in HepG2 Cells and Obese Mice

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ABSTRACT Needles of pine species are rich in polyphenols, which may exert beneficial effects on human health. The present study was conducted to evaluate the *in vitro* and *in vivo* antioxidant effects of *Pinus koraiensis* needle water extracts (PKW). HepG2 cells were pretreated with various concentrations of PKW (from 10⁻³ to 1 mg/mL) and oxidative stress was induced by *tert*-butyl hydroperoxide (*t*-BOOH). In the animal model, male ICR mice were fed a high-fat diet for 6 weeks to induce obesity, and then mice were continually fed a high-fat diet with or without orally administered PKW (400 mg/kg body weight) for 5 weeks. Pretreatment with PKW prevented significant increases in cytotoxicity and catalase activity induced by *t*-BOOH in HepG2 cells. Similarly, the catalase protein expression levels elevated by *t*-BOOH were abrogated in cells pretreated with PKW. In mice fed a high-fat diet, PKW significantly increased hepatic activities of catalase and glutathione reductase and lower lipid peroxidation levels were observed in the liver and kidney of mice with PKW supplementation. The present study demonstrates that PKW protects against oxidative stress in HepG2 cells treated with *t*-BOOH and in mice fed a high-fat diet.

KEY WORDS: • functional food • HepG2 cells • obesity • oxidative stress • *Pinus koraiensis* needle

INTRODUCTION

WESTERN LIFESTYLES ARE ASSOCIATED with an increased risk of metabolic syndrome, including diabetes and fatty liver disease.^{1,2} Oxidative stress, a common feature of metabolic syndrome, is defined as an imbalance between the production and inactivation of reactive oxygen species (ROS) in biological systems.³ Overproduction of ROS results in cellular injury, including lipid peroxidation, protein oxidation, and DNA damage. Therefore, cellular antioxidant defense systems that consist of antioxidant enzymes and nonenzymatic antioxidants play important roles in counteracting these deleterious effects of ROS. Furthermore, dietary phytochemicals, including polyphenols, have antioxidant properties and prevent oxidative stress-induced development of diseases.^{4,5}

Pinus koraiensis (Korean pine) is widely distributed in Northeastern Asia, especially in Korea, China, and Russia.⁶ Its edible nut parts have been traditionally used as an important ingredient of various sauces and desserts.⁷ Recent studies have shown that a natural oil extract from Korean pine nuts may be used as an appetite suppressant through

increasing satiety hormones.^{8,9} Needle and bark of *Pinus* species contain various polyphenols, including quercetin, (+)-catechin, ferulic acid, *p*-coumaric acid, vanillic acid, and caffeic acid.^{10–12} The water extract of *Pinus densiflora* needles has been shown to alleviate oxidative stress in cells treated with hydroxyl radicals,¹³ and in rats fed a high-cholesterol diet or treated with carbon tetrachloride.^{14,15} However, it is not clear whether *Pinus koraiensis* needle water extract (PKW) has similar beneficial effects. Therefore, in the present study, we investigated the antioxidative effects of PKW in HepG2 cells treated with *tert*-butyl hydroperoxide (*t*-BOOH) and in mice fed a high-fat diet.

MATERIALS AND METHODS

Preparation of P. koraiensis needle extract

Two batches of *P. koraiensis* needles were collected from natural pine stands in Pocheon, Kyunggi-do, Korea during the summer season. The extract was prepared by water extraction of *P. koraiensis* needles. Briefly, needles were boiled in 10 volumes of distilled water at 100–125°C for 4–6 h. The extract was filtered through a 25- μ m standard sieve and was dried using a vacuum rotary evaporator under low pressure. The dried PKW was kept in an airtight container and stored at –20°C before use. PKW was analyzed for nutritional composition according to the Food Codex

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guidelines by the Korea Food Research Institute and was analyzed for minerals and heavy metals using inductively coupled plasma (ICP)–atomic emission spectrometry and ICP–mass spectrometry by the Korea Basic Science Institute.

Analysis of total phenolic content

The total phenolic content in PKW was determined according to the modified Folin–Ciocalteu method using gallic acid as the standard.¹⁶ Briefly, 10 μ L of the PKW stock solution (6 mg/mL) was incubated with 50 μ L of deionized water and 50 μ L of the 2 normality (N) Folin–Ciocalteu reagent for 5 min. After incubation, 150 μ L of 30% Na₂CO₃ and 40 μ L of deionized water were added into the mixture, and then incubated for 2 h. The absorbance was measured at 765 nm and the total phenolic content in the extract was expressed as milligrams of gallic acid equivalents (GAE) per gram of PKW.

Analysis of total flavonoid content

Total flavonoid content of PKW was determined according to the modified spectrometric method using (+)-catechin as a standard.¹⁷ Briefly, 25 μ L of the PKW stock solution (6 mg/mL) was incubated with 100 μ L of deionized water and 10 μ L of 5% NaNO₂ for 5 min. After incubation, 15 μ L of 10% AlCl₃ was added into the mixture, and then incubated for 6 min followed by the addition of 50 μ L of 1 N NaOH and 50 μ L of deionized water. The absorbance was measured at 510 nm and total flavonoids were expressed as milligrams of (+)-catechin equivalents (CE) per gram of dried PKW.

High-performance liquid chromatography analysis of polyphenol compounds

Polyphenol compounds of PKW were analyzed by high-performance liquid chromatography (HPLC; 1090 Series II LC, Hewlett Packard) equipped with a Luna 5- μ m C18 column (250 mm $\times$ 4.6 mm; Phenomenex). The mobile phases consisted of the gradient elution using solvent A (2% acetic acid) and solvent B (methanol). The elution condition was programmed as 0% of B at 0 min, 10% of B at 6.2 min, 20% of B at 14 min, 60% of B at 40 min, and 90% of B at 50 min with a flow rate of 1.0 mL/min. The detection wavelength was 280 nm and the column temperature was 40°C. (+)-Catechin (0.005–0.1 mg/mL) and *p*-coumaric acid (0.0015–0.03 mg/mL) were used for the preparation of the calibration curves.

Cell culture and cell viability assay

HepG2 human hepatocarcinoma cells (American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium (DMEM; WelGene) supplemented with 10% heat inactivated fetal bovine serum (WelGene) in a humidified incubator in a 95% air–5% CO₂ mixture. After cells reached 70–80% confluence, the culture medium was switched to the serum-free DMEM with various concentrations of PKW (from 10^{−3} to 1 mg/mL) for 20 h. To induce

oxidative stress, cells were treated with *t*-BOOH (500 μ M) prepared in a fresh serum-free DMEM for 3 h. *t*-BOOH, which has been shown to be a consistent cellular oxidative stress inducer in liver cells for studying the protective effects of dietary antioxidants.¹⁸ Cell viability was determined by MTT assay as previously described,¹⁹ and results were expressed in relation to the control without any treatment.

Animals and diets

All treatment protocols for this study were approved by the Seoul National University Institutional Animal Care and Use Committee. Three-week-old male ICR mice were purchased from the Institute of Laboratory Animal Resources, Seoul National University. After a 1-week adaptation period, mice were randomly divided into two groups (the chow diet group and the high-fat diet group). The chow diet group (normal control, NC) was fed a standard chow diet containing 5.3% fat (Agribrands; Purina Korea, Inc.) and the high-fat diet group was fed a high-fat diet (S3282; Bio-Serv) containing 35.5% fat to induce obesity. After 6 weeks of feeding, the high-fat diet group was subdivided into three groups; the high-fat control group (HFC), the high-fat with PKW group (HFP), and the high-fat with glibenclamide group (HFG) as a positive control. For 5 weeks, PKW (400 mg/kg body weight) and glibenclamide (4 mg/kg body weight; Sigma) were orally administered to the HFP and HFG groups, respectively. Glibenclamide has been used as a positive control in type 2 diabetic animal models.²⁰ Distilled water was given to NC and HFC groups. Food intake and body weight gain were recorded twice per week. Individual food intake was calculated based on the dietary intake of two or three mice kept in the same cage. All mice were maintained under controlled conditions of temperature (23 $\pm$ 3°C), humidity (50 $\pm$ 10%), and 12-h light–12-h dark cycles (8 a.m.–8 p.m.). At the end of the study, blood samples were taken by cardiocentesis after 16 h of fasting and serum was obtained by centrifugation at 1000 *g* for 20 min at 4°C. The liver, kidney, and epididymal adipose tissues were removed, snap-frozen immediately in liquid nitrogen, and stored at −80°C until use.

Serum and hepatic biochemical analyses

Serum glucose, triglyceride, and total cholesterol levels and serum glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) activities were determined using commercial kits (Asan Pharmacy). Serum-free fatty acid levels were also measured with a commercially available kit (Shinyang Diagnostics). Total hepatic lipids were extracted according to the method of Folch *et al.*²¹ Briefly, liver tissue (~25 mg) was homogenized in 500 μ L of ice-cold phosphate-buffered saline and 300 μ L of the homogenate was mixed with methanol–chloroform (1:2, v/v). After incubation at 4°C for 3 h, 240 μ L of 0.88% KCl was added to homogenates. Homogenates were centrifuged at 1000 *g* for 15 min at 4°C, and the lower lipid phase was collected and concentrated by nitrogen gas. The lipid pellets were dissolved in isopropanol, and hepatic triglyceride and

total cholesterol contents were determined by an enzymatic colorimetric method using a commercial kit (Asan Pharmacy).

Measurement of antioxidant enzyme activities

After treatment, HepG2 cells were harvested in a 50 mM potassium phosphate buffer (pH 7.0) and the supernatant was collected by the centrifugation at 10,000 *g* for 15 min. Tissues (~100 mg) were homogenized in 1 mL of a homogenizing buffer containing 154 mM KCl, 50 mM Tris-HCl, and 1 mM ethylenediaminetetraacetic acid (pH 7.4). The tissue homogenate was centrifuged at 600 *g* for 10 min at 4°C and the supernatant was divided into two parts, one to measure the catalase activity and the other part was further centrifuged at 105,000 *g* for 60 min at 4°C to obtain the cytosolic fraction for measuring glutathione peroxidase (GPx) and glutathione reductase (GRd) activities. The activity of catalase was measured by the method of Abei.²² The reaction was initiated by adding H₂O₂ to tissue homogenates, and the disappearance of H₂O₂ was kinetically determined from the absorbance changes at 240 nm for 1 min. The activity was expressed as micromoles of H₂O₂ eliminated per minute per milligram of protein. The GPx activity was determined by the method of Tappel.²³ The reaction was initiated by adding cumene hydroperoxide, and the oxidation of NADPH to NADP⁺ was spectrophotometrically determined by the rate of decrease in the absorbance at 340 nm at 25°C for 1 min and expressed as nanomoles of NADPH oxidized per minute per milligram of cytosolic protein. The GRd activity was determined by the method of Carlberg and Mannervik.²⁴ The reaction is initiated by addition of the cytosolic fraction to the reaction mixture containing NADPH and GSSG in a potassium phosphate buffer, and the oxidation of NADPH to NADP⁺ was spectrophotometrically measured by the rate of decrease in the absorbance at 340 nm at 25°C for 1 min and expressed as nanomoles of NADPH oxidized per minute per milligram of cytosolic protein. Protein contents were determined using a commercial kit from Bio-Rad.

Measurement of protein expression by immunoblotting analysis

After treatment, HepG2 cells were harvested in an ice-cold protein lysis buffer. The protein concentrations of lysates were determined by a commercial kit (Bio-Rad). Equal amounts of protein were loaded into the lanes of a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel, separated, and blotted onto a polyvinylidene fluoride membrane (Millipore). After blocked with 5% nonfat milk, the membrane was probed with anti-catalase antibody (Abcam) and β -actin (Sigma), and then incubated with the horseradish peroxidase-conjugated secondary antibody for chemiluminescent detection. The band intensity was quantified using Quantity One software (Bio-Rad).

Determination of lipid peroxidation

Lipid peroxides in the liver and kidney homogenates were analyzed according to the method of Ohkawa *et al.*²⁵

Briefly, the tissue homogenate used for catalase activity measurement was mixed with SDS, acetate buffer (pH 3.5), and thiobarbituric acid. After boiling at 95°C for 1 h, the mixture was immediately placed on ice to stop the reaction. The red pigment was extracted with *n*-butanol:pyridine mixture (15:1, v/v) after centrifugation at 1500 *g* for 10 min at 4°C. Finally, the absorbance of the butanol layer was measured at 532 nm using 1,1,3,3-tetraethoxypropane as a standard. The lipid peroxide level was expressed as nanomoles of malondialdehyde (MDA) equivalents per gram tissue.

Statistical analysis

The data were analyzed using SPSS software (version 12.0). For all experiments, either the Student's *t*-test or one-way analysis of variance followed by the Duncan's multiple range test was used to assess statistical significance. Data were expressed as means $\pm$ standard error of the means and differences were considered statistically significant at *P* < .05.

RESULTS

Determination of nutritional composition and metal contents

The yield of dried PKW powders obtained from the fresh pine needles ranged from 8.8% to 9.5% for two different batches. The nutritional composition of PKW is shown in Table 1. To screen for the safety of PKW, contents of minerals (Ca, Fe, K, Mg, Na, and P) and heavy metals (Al, As, Cd, Cr, Cu, and Pb) were also assessed. In the case of minerals, the most abundant one was potassium (Table 1). The amounts of the heavy metals were under the national

TABLE 1. NUTRITIONAL COMPOSITION, MINERALS, AND HEAVY METALS OF *PINUS KORAIENSIS* NEEDLE WATER EXTRACT

Nutritional composition (%)	
Moisture	5.8
Carbohydrate	80.7
Crude protein	5.9
Crude fat	2.2
Ash	5.4
Minerals (ppm)	
Ca	2043
Fe	1.86
K	21,680
Mg	4042
Na	90.98
P	2378
Heavy metals (ppm)	
Al	7.0948
As	0.0442
Cd	0.0216
Cr	0.0769
Cu	0.0115
Pb	0.0112

FIG. 1. High-performance liquid chromatography chromatogram of *Pinus koraiensis* needle water extract (PKW). The chromatogram of a standard mixture of (+)-catechin and ρ -coumaric acid is also shown for the identification of the corresponding peaks on the chromatogram of PKW.

limits and the World Health Organization guidelines for assessing the quality of herbal materials (2007).

Characterization of bioactive compounds

The contents of total phenolics ranged from 94.0 to 105.6 mg GAE/g PKW and total flavonoids ranged from 34.6 to 40.8 mg CE/g PKW in different batches. Polyphenol compounds in PKW were analyzed by HPLC and the major peaks in the chromatogram were identified as (+)-catechin and ρ -coumaric acid (Fig. 1). Contents of (+)-catechin and ρ -coumaric acid were 2.4 mg/g PKW and 1.0 mg/g PKW, respectively.

Protective effect of PKW against oxidative stress in HepG2 cells treated with *t*-BOOH

To investigate the protective effect of PKW against *t*-BOOH-mediated oxidative stress in HepG2 cells, we treated cells with various concentrations of PKW (from 10^{-3} to 1 mg/mL). In the preliminary study, *t*-BOOH treatment induced more severe cytotoxicity compared with H_2O_2 treatment (data not shown). As determined by MTT assay, treatment with 500 μ M of *t*-BOOH significantly reduced cell viability (Fig. 2A). When cells were pre-treated with PKW at levels from 10^{-1} to 1 mg/mL, cell viability was significantly increased in the presence of *t*-BOOH. We also treated cells with PKW without *t*-BOOH to determine whether any cellular damage is caused by the extract. The viability of HepG2 cells were rarely influenced by PKW *per se*. To further investigate the protective effect exerted by PKW, we determined changes in the catalase activity and protein levels as biomarkers of antioxidant potential. Pretreatment with PKW (from 10^{-2} to 1 mg/mL) prevented a significant increase in the catalase activity induced by *t*-BOOH in a dose-dependent manner (Fig. 2B). Similarly, the induction of catalase protein levels by *t*-BOOH was abrogated in cells pretreated with PKW (Fig. 2C).

Effect of PKW on biochemical parameters in serum and liver of mice fed a high-fat diet

By the end of 6 weeks of obesity induction, there was a significant difference in body weight change in mice fed a high-fat diet compared to that of mice fed a standard chow diet (Chow diet group: 9.0 ± 1.0 g; high-fat diet group: 20.4 ± 1.0 g). After another 5 weeks of high-fat feeding, no significant differences in the body weight were observed among the high-fat groups (Table 2).

As shown in Table 2, serum glucose, triglyceride, and total cholesterol levels were not significantly different among the groups. Serum-free fatty acid levels were significantly lower in the NC group than the high-fat groups. To investigate the extent of liver damage, we also measured the activities of GOT and GPT. Although serum GOT and GPT levels tended to be lower in mice fed a high-fat diet supplemented with HFG compared to those fed a high-fat diet, the difference was not statistically significant. Hepatic triglyceride levels were significantly increased in the HFC group compared to that of the NC group. Neither PKW nor glibenclamide treatment significantly altered hepatic triglyceride levels. We did not observe any difference in hepatic cholesterol levels among the groups.

Protective effect of PKW against oxidative stress in liver and kidney of mice fed a high-fat diet

The activities of hepatic catalase and GRd were significantly increased in the HFP group compared with the HFC group (Table 3). No significant difference was observed in the activity of GPx between the HFC and HFP groups; however, PKW administration increased the GPx activity in the HFP group compared to that observed in the NC group. Glibenclamide significantly increased catalase and GPx activities in the liver of mice fed a high-fat diet. In the kidney, similar results for the GPx activity were observed (Table 3). As a marker of the lipid peroxidation, MDA levels were

FIG. 2. Effect of PKW on oxidative stress in HepG2 cells treated with *tert*-butyl hydroperoxide (t-BOOH). (A) Relative cell viability. Cell numbers were measured by MTT assay and expressed as percentage of the control value. (B) Catalase activity. (C) Representative immunoblotting for catalase with its densitometric analysis. Relative protein expression levels of catalase/beta-actin were quantitated and expressed as percentage of the control value. Each bar represents mean $\pm$ standard error of the means (SEM, $n = 3-4$) and bars with different superscripts are significantly different at $P < .05$.

TABLE 2. EFFECT OF *PINUS KORAIENSIS* NEEDLE WATER EXTRACT ON BODY WEIGHT AND BIOCHEMICAL PARAMETERS IN SERUM AND LIVER OF MICE FED A HIGH-FAT DIET

	NC	HFC	HFP	HFG
Body weight (g)	37.9 $\pm$ 1.2 ^a	50.6 $\pm$ 1.5 ^b	49.1 $\pm$ 3.3 ^b	54.4 $\pm$ 2.4 ^b
Serum				
Glucose (mg/dL)	139.3 $\pm$ 14.7	156.5 $\pm$ 9.2	149.4 $\pm$ 6.6	163.0 $\pm$ 11.5
GOT (IU/L)	65.5 $\pm$ 16.1	60.3 $\pm$ 6.0	61.9 $\pm$ 9.8	39.9 $\pm$ 2.3
GPT (IU/L)	33.8 $\pm$ 10.1	36.9 $\pm$ 8.6	31.8 $\pm$ 6.8	21.7 $\pm$ 2.6
Triglyceride (mg/dL)	108.4 $\pm$ 14.2	94.0 $\pm$ 10.7	74.2 $\pm$ 8.2	93.9 $\pm$ 7.4
Total cholesterol (mg/dL)	156.4 $\pm$ 10.7	160.3 $\pm$ 12.1	177.9 $\pm$ 18.5	161.6 $\pm$ 21.4
Free fatty acid (μ Eq/L)	1587.9 $\pm$ 115.1 ^b	1156.9 $\pm$ 86.3 ^a	1156.3 $\pm$ 79.9 ^a	1073.9 $\pm$ 100.5 ^a
Liver				
Triglyceride (mg/g)	49.6 $\pm$ 3.3 ^a	82.7 $\pm$ 7.7 ^b	78.5 $\pm$ 10.3 ^b	93.5 $\pm$ 8.5 ^b
Cholesterol (mg/g)	18.2 $\pm$ 1.9	17.5 $\pm$ 0.7	17.3 $\pm$ 1.0	20.2 $\pm$ 0.6

Values are expressed as means $\pm$ SEM ($n = 5-6$).

^{ab}Values with different superscripts within the same row are significantly different at $P < .05$.

NC, normal control; HFC, high-fat control; HFP, high fat + PKW (400 mg/kg); HFG, high fat + glibenclamide (4 mg/kg); PKW, *Pinus koraiensis* needle water extract; GOT, glutamic-oxaloacetic transaminase; GPT, glutamic-pyruvic transaminase; SEM, standard error of the means.

TABLE 3. EFFECT OF *PINUS KORAIENSIS* NEEDLE WATER EXTRACT ON CATALASE, GLUTATHIONE PEROXIDASE, AND GLUTATHIONE REDUCTASE ACTIVITIES IN THE LIVER AND KIDNEY OF MICE FED A HIGH-FAT DIET

	NC	HFC	HFP	HFG
Liver				
Catalase ($\mu\text{mol}/\text{min}$ per mg of protein)	$69.8 \pm 4.5^{\text{ab}}$	$62.3 \pm 5.6^{\text{a}}$	$81.8 \pm 7.7^{\text{b}}$	$82.1 \pm 4.8^{\text{b}}$
GPx (nmol/min per mg of protein)	$747.3 \pm 26.9^{\text{ab}}$	$694.0 \pm 51.1^{\text{a}}$	$813.1 \pm 39.4^{\text{ab}}$	$830.9 \pm 44.1^{\text{b}}$
GRd (nmol/min per mg of protein)	$91.9 \pm 6.0^{\text{ab}}$	$83.6 \pm 2.4^{\text{a}}$	$104.1 \pm 8.3^{\text{b}}$	$93.4 \pm 7.4^{\text{ab}}$
Kidney				
Catalase ($\mu\text{mol}/\text{min}$ per mg of protein)	84.2 ± 6.7	75.2 ± 5.9	91.9 ± 4.1	77.9 ± 7.8
GPx (nmol/min per mg of protein)	$559.5 \pm 81.6^{\text{b}}$	$346.5 \pm 56.1^{\text{a}}$	$422.3 \pm 67.5^{\text{ab}}$	$422.6 \pm 37.3^{\text{ab}}$
GRd (nmol/min per mg of protein)	214.5 ± 12.0	218.4 ± 22.2	204.0 ± 25.3	224.7 ± 12.8

Values are expressed as means $\pm$ SEM ($n=5-6$).

^{ab}Values with different superscripts within the same row are significantly different at $P<.05$.

GPx, glutathione peroxidase; GRd, glutathione reductase.

measured (Fig. 3). MDA levels in the liver and kidney of the HFP group were significantly lower than those of the HFC group (42% and 35%, respectively). We did not observe any significant effect of glibenclamide treatment on MDA levels in the liver and kidney of mice fed a high-fat diet.

FIG. 3. Effect of PKW on lipid peroxide levels in (A) liver and (B) kidney of mice fed a high-fat diet. Each bar represents mean $\pm$ SEM ($n=5-6$) and bars with different superscripts are significantly different at $P<.05$. NC, normal control; HFC, high-fat control; HFP, high fat + PKW (400 mg/kg); HFG, high fat + glibenclamide (4 mg/kg).

DISCUSSION

Oxidative stress plays an important role in the pathogenesis of metabolic syndrome caused by obesity. It is possible that underlying mechanisms include mitochondrial dysfunction due to increased oxygen consumption and subsequent radical production, diminished antioxidant capacity, and increased toxic lipid synthesis.²⁶ Due to their ROS scavenging ability by hydroxyl groups, polyphenols are important antioxidant constituents of many plant extracts.²⁷ Previous studies have shown strong antioxidant effects of polyphenols in the liver of obese animals.^{28,29}

The present study demonstrates that PKW has protective effects against oxidative stress induced by *t*-BOOH. HepG2 cells pretreated with PKW exhibited dose-dependent resistance to oxidative stress induced by *t*-BOOH based on cell viability and catalase activity. Consistently, previous studies have demonstrated that pretreatment of HepG2 cells with quercetin or tocotrienol-rich fractions prevented the *t*-BOOH-induced increases in activities of antioxidant enzymes, including catalase, GPx, GRd, and superoxide dismutase.^{30,31} Furthermore, protein levels of catalase were significantly decreased by PKW pretreatment, suggesting that PKW prevents cellular oxidative stress probably by its potent antioxidant activity and by regulation of antioxidant enzyme expression.³² Previous studies reported that the activity of catalase is regulated at the transcriptional level in response to cellular oxidative status.^{30,33} Since there were no statistical differences in catalase protein levels among the groups treated with various doses of PKW, the present observation suggests that regulation of catalase activity by PKW pretreatment may be in part at the post-translational level. Similarly, a previous study reported that there was no difference in protein levels of GRd, but the activity of GRd was altered in the liver and brain of mice exposed to sodium arsenite.³⁴ Treatment of cells with PKW alone at doses of up to 1 mg/mL did not evoke any cellular damage. Polyphenols have been shown to be potentially cytotoxic by exhibiting pro-oxidant properties under certain experimental conditions.³⁵ Therefore, these results suggest that polyphenols contained in PKW did not lead to oxidative damage under the present cell culture system.

To investigate the antioxidant effect of PKW using an *in vivo* model, we induced obesity by feeding a high-fat diet

for 6 weeks before oral administration of PKW with a high-fat diet feeding for another 5 weeks based on the previous study.³⁶ Because obesity and metabolic disorders induced by a high-fat diet feeding to rodents resemble the human metabolic syndrome,³⁷ these animal models are popularly adopted to investigate the effect of phytochemicals against oxidative stress associated with metabolic syndrome. Although the body weight was significantly increased in the high-fat diet-fed groups compared to the normal control groups, we did not observe any significant changes in serum glucose, triglyceride, and total cholesterol levels. In the present study, there was a significant increase in hepatic triglyceride levels, suggesting a high-fat diet induced fatty liver in obese mice. However, serum GOT and GPT levels in the HFC group were not significantly increased compared with those in the NC group, suggesting that high-fat diet-induced fat accumulation may not always cause liver damage. Both PKW and glibenclamide, an antidiabetic drug, did not exert any effect on these biochemical levels. The activities of catalase, GPx, and GRd tended to be lower in mice fed a high-fat diet, but differences were not significant. Interestingly, PKW administration significantly restored the antioxidant defense system as determined by levels of lipid peroxidation and activities of catalase and GRd in mice fed a high-fat diet. These results suggest that PKW is a potentially powerful antioxidant ingredient responsible for reducing the cellular ROS. Treatment with glibenclamide also alleviated oxidative stress by increasing activities of antioxidant enzymes as observed in a previous study.³⁸

Polyphenols are commonly found in various plant species and classified into different groups, including phenolic acids, flavonoids, polyphenolic amides, and nonflavonoid polyphenols based on their chemical structures.³⁹ In the present study, we identified (+)-catechin and *p*-coumaric acid as the main polyphenol compounds in PKW. Their beneficial effects on health were well defined in the previous studies.^{40–42} Total phenolic contents (~ 100 mg GAE/g PKW) and total flavonoid contents (~ 38 mg CE/g PKW) in PKW were similar to those in water extracts of *P. densiflora* needles⁴³ and in 80% aqueous methanol extracts of *P. cembra* needles,⁴⁴ respectively. PKW exhibited a higher antioxidant activity than *P. koraiensis* ethanol extract, as determined by cell viability of HepG2 cells treated with *t*-BOOH (unpublished data). Consistently, water extraction of *P. densiflora* needles showed a higher antioxidant activity and total polyphenol contents compared with other extract methods.⁴³ These results suggest that water extraction would be a suitable method for mass production of functional foods in terms of cost and manufacturing process.

According to the Food and Drug Administration, the extrapolation of animal dose to human dose is correctly performed only through normalization to the body surface area.⁴⁵ Therefore, to convert the dose used in a mouse to a dose based on the surface area for humans, a dose of 400 mg/kg (body weight) of PKW was multiplied by the K_m factor for a mouse (3), and then divided by the K_m factor for a human (37). This calculation results in a human equivalent dose for PKW of 32.4 mg/kg, which equates to a 1945 mg

dose of PKW for a 60-kg person. According to a recent study, green tea extract, 1402 mg of tea catechins were provided to human for 12 weeks to study the effects of green tea polyphenols on skin sensitivity toward UV exposure.⁴⁶ Therefore, we believe that the consumption of ~ 2 g dose of PKW per day would be physiologically attainable.

In conclusion, our data showed that PKW significantly decreased oxidative stress in HepG2 cells treated with *t*-BOOH and in obese mice induced by a high-fat diet. The present study provides the basis for further animal and clinical studies to validate the antioxidant efficacy of PKW.

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AUTHOR DISCLOSURE STATEMENT

The authors have declared that there are no conflicts of interest.

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