



Original Article

Effect of an extraction solvent on the antioxidant quality of *Pinus densiflora* needle extract

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ABSTRACT

Pinus densiflora needle extract (PDNE) is widely reported to have many pharmacological activities including antioxidant potential. However, the solvent system used for extraction greatly affects its antioxidant quality. Hence, in the present study, we investigated the effect of a different ratio (vol/vol) of ethanol to water (0–100%) in the extraction of PDNE with potent antioxidant capacity. The chemical assays, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), were conducted to assess the antioxidant potential of PDNE. Subsequently, the cytoprotective effect of PDNE was determined using *tert*-butyl hydroperoxide (TBHP)-challenged HepG2 cellular model. The needle extracts from 40% ethanol (PDNE-40) showed greater radical scavenging activity followed by 60%, 20%, 80%, 0% and 100% ethanol extracts. EC₅₀ value of the most active extract, PDNE-40, was 8.56 ± 0.51 µg/mL, relative to 1.34 ± 0.28 µg/mL of the standard trolox (for ABTS radical), and 75.96 ± 11.60 µg/mL, relative to 4.83 ± 0.26 µg/mL of the standard trolox (for DPPH radical). Either PDNE-20 or PDNE-40 pretreatment remarkably decreased the levels of reactive oxygen species (ROS), lipid peroxides and protein carbonyls in TBHP-challenged HepG2 cells. In addition, both PDNE-20 and PDNE-40 significantly reversed the decreased ratio of reduced (GSH) to oxidized (GSSG) glutathione. Moreover, these two extracts showed a significant inhibitory effect on TBHP-induced nuclear damage and loss of cell viability. In summary, the inclusion of 40% ethanol in water for extraction of *Pinus densiflora* needle greatly increases the antioxidant quality of the extract.

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1. Introduction

Reactive oxygen species (ROS) are highly reactive molecules continuously produced in our body at a certain physiological level as a normal product of cellular metabolism. ROS are known to regulate many crucial cellular activities including signaling pathways, cell proliferation and differentiation [1]. However, an excessive amount of ROS can damage biomolecules such as proteins, lipids, and nucleic acids and thereby induce loss of cell function and eventually cause cell death [2–5]. While most cell types in our body are well-equipped with inherent, complex antioxidant defense mechanisms to battle any excess ROS, a prolonged increase in the production of ROS resulting from various stress conditions can collapse this defense mechanism and cause severe oxidative damage to the system [6].

Many experimental and clinical studies have strongly reported that supplementation of natural antioxidants such as pycnogenol (a standardized French maritime *Pinus pinaster* bark extract), citrus flavonoids, tea polyphenols, soy isoflavones, vitamin E, vitamin C, carotenoids, silymarin, curcumin, quercetin, and resveratrol could reduce the risk of developing oxidative stress-mediated disease complications [7–17]. *Pinus densiflora* is a common pine species found in Korea. Traditionally, the needle of this plant is considered to have numerous health-promoting properties. In East Asian traditional system of medicine, it has been used to treat various diseases including gastrointestinal, urinary, vascular and neuronal disorders. Scientific studies have shown that *Pinus densiflora* needle extract (PDNE) has anti-microbial, anti-inflammation and anti-cancer properties [18–21]. Recently, *Pinus densiflora* needle has been reported to have a protective effect against acute restraint stress-induced hippocampal memory impairment in the experimental mouse model [22]. Moreover, many reports have demonstrated the antioxidant activity of PDNE [23–25]. These reports clearly show that the solvent system used for extraction greatly

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influences the antioxidant quality of PDNE. Hence, in the present study, we determined the effect of the different ratio (v/v) of biologically-considered non-toxic solvents, ethanol to water in the extraction of antioxidant-rich PDNE. There are many chemical methods available for determining the antioxidant activity of natural products. The mechanism of these methods is either based on scavenging of free radicals, reduction of metal ions or competition with free radicals. Though limitations exist, DPPH and ABTS free radical scavenging assays have been widely used to determine the antioxidant capacity of any natural products due to their ease, speed, and sensitivity [26]. Hence, in the present study, we performed DPPH and ABTS assays to determine the antioxidant potential of PDNE.

2. Experimental

2.1. Preparation of pine needle extracts

Pinus densiflora needles were collected in July 2017, from Seoul city, Republic of Korea. A voucher specimen (PDN-017) of the authenticated plant material was deposited in the herbarium of the College of Forest Science, Kookmin University, Korea. The pine needles were washed with running tap-water and shade dried at room temperature for about three weeks. The dried needles were ground in an electronic laboratory blender into the fine powder. About 10 g of needle powder was extracted with different ratios of ethanol to distilled water at 60 °C for 15 h. The resulting extracts PDNE-0, PDNE-20, PDNE-40, PDNE-60, PDNE-80, and PDNE-100, respectively, were filtered through whatman no.1 filter paper (Cat. No. 1001 110), and the filtrates were concentrated under reduced pressure at 45 °C using a rotary evaporator. The concentrated extracts were further lyophilized and quantified. The stock solutions of extracts were prepared in dimethyl sulfoxide (DMSO; Sigma, D8418) at a concentration of 50 mg/mL and used for further experimental analysis.

2.2. High-performance liquid chromatography (HPLC) analysis of pine needle extracts

The pine needle extracts were first dissolved in methanol, filtered using 0.45 µm syringe filter, and subjected to analysis. HPLC was run in 2795 separation module (Agilent, California, USA) with PDA 996 detector (Waters, Massachusetts, USA) using HALO C₁₈ column, 2.1 mm × 150 mm, 5 µm (Advanced materials technology, USA). The mobile phases consisted of water with 0.1% trifluoroacetic acid (solvent A), and acetonitrile with 0.1% trifluoroacetic acid (solvent B). The gradient conditions were 100% A, 0–3 min; 50% A at 45 min; 100% B, 50–55 min; 100% A, 60–70 min. The flow rate was set to 0.2 mL/min. The injection volume was 10 µL. The detection wavelength was 190–400 nm.

2.3. 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical scavenging assay

ABTS assay was done according to the method of Re et al. (1999), with some modifications [27,28]. Briefly, to generate ABTS radicals, an equal volume of 7 mM ABTS and 2.45 mM potassium persulfate aqueous solutions were mixed and allowed to stand in the dark at room temperature overnight. Then, this radical solution was diluted with methanol and used for the assay. The absorbance of reaction mixtures containing 100 µL of different concentrations of extracts (3.90–500 µg/mL) or trolox (0.39–50 µg/mL) in methanol and 100 µL of ABTS radical solution was read at 734 nm, after 10 min of incubation in the dark at room temperature. The concentration of half-maximal scavenging efficiency (EC₅₀) of extracts

and trolox was calculated, and ABTS radical scavenging capacity was expressed as trolox equivalent (TE), g/100 g extract.

2.4. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The DPPH assay was performed as described previously, with slight modifications [22]. In brief, 100 µL of two-fold serial dilutions of the extracts in methanol, concentrations ranging from 3.90 to 500 µg/mL, were placed in a 96-well microplate. Then, 100 µL of 200 µM DPPH (Sigma, D9132) in methanol was added to each sample. For the blank sample, the extracts and DPPH solution were replaced with methanol. Assay control contained 100 µL methanol and 100 µL DPPH solution. Trolox at 0.39–50 µg/mL concentrations was used as a positive control. After incubation of the reaction mixtures for 15 min at room temperature, the absorbance was recorded at 515 nm. The EC₅₀ value was calculated from the non-linear regression curve. The DPPH radical scavenging potential of the extracts was expressed as trolox equivalent (TE), g/100 g extract.

2.5. Cell culture

The human liver cancer cell line HepG2 (KCLB No. 88065) was obtained from the Korea Cell Line Bank, Seoul, Korea. Cells were grown in minimum essential medium (MEM; Gibco, 51200-038) supplemented with 2 mM glutamine (Gibco, 25030081), 10% heat-inactivated fetal bovine serum (Gibco, 10082-147), 100 U/mL penicillin, 100 µg/mL streptomycin sulfate and 0.25 µg/mL amphotericin B (Gibco, 15240062). Cell cultures were maintained at 37 °C in a humidified atmosphere of 5% CO₂.

2.6. Cell viability assay

The methylthiazolyldiphenyl-tetrazolium bromide (MTT) colorimetric assay was performed to assess the cytotoxicity of extracts. Briefly, cells were seeded at a density of 2×10^4 /well into a 96-well plate and incubated overnight. Thereafter, they were exposed to different concentrations of PDNE (0, 6.25, 12.5, 25, 50 and 100 µg/mL) or vehicle (0.2% DMSO) for 24 h. After removing the drug-containing medium, cells were incubated with 0.5 mg/mL MTT (Sigma, M2128) solution, which was prepared in serum and phenol-red free culture medium, for 4 h. The resulting formazan crystals were dissolved in DMSO and absorbance was measured at 570 nm using a multi-plate reader.

2.7. ROS assay

A clear-bottom black 96-well plate was used for the ROS assay. The assay was performed as previously described in the literature with some additional experimental procedures [29,30]. After pretreatment with PDNE followed by TBHP exposure, cells were incubated with 20 µM of 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA; Cayman 85155) for 45 min. Cells were then washed twice with PBS, and DCF fluorescence intensity was recorded using a Biotek SYNERGY HTX multi-mode reader. Thereafter, an equal volume of 2% Triton X-100 in PBS was added to each well and cells were lysed by repeated pipetting. Plates were centrifuged for 15 min, at 2000 rpm and 4 °C. Then, 20 µL of supernatant from each sample was transferred to a 96-well plate and to this, 30 µL PBS and 50 µL lactate dehydrogenase (LDH) assay reagent were added. The absorbance of this reaction mixture was recorded at 450 nm with reference wavelength, 660 nm, after 15 min incubation at room temperature in the dark. DCF fluorescence was normalized to LDH absorbance value, and ROS levels were expressed as the number of fold change versus the control group.

2.8. Lipid peroxidation assay

The assay was performed according to the previous report with slight modifications [31]. Briefly, after pretreatment of cells with PDNE or trolox for 24 h and followed by TBHP for 2 h, the cell culture supernatant was collected and cleared by centrifugation for 5 min, at 10,000 rpm and 4 °C. In a 1.5 mL eppendorf tube, 300 µL of cell-free supernatant was added to 300 µL thiobarbituric acid, TBA (Cayman 10009199) solution (0.5% TBA in 20% trichloroacetic acid and 0.33 M hydrochloric acid solution with 0.005% butylated hydroxytoluene). The eppendorf tubes containing the reaction mixtures were placed in a boiling water bath for 60 min. Subsequently, the samples were placed on ice for 10 min. Then, 300 µL butanol was added to each sample, vortexed and centrifuged at 10,000 rpm for 5 min. From this, 100 µL of the butanol fraction was transferred to 96-well clear-bottom black plates, and fluorescence (excitation, 530 nm, and emission, 590 nm) was read using a Biotek SYNERGY HTX multi-mode reader.

2.9. Protein carbonyl assay

Here, we used a commercially available kit (Cayman, 10005020) to determine the concentration of protein carbonyls in cell lysates. The assay was performed according to the manual instructions with some modifications. Briefly, cells were lysed in phosphate buffer by sonication and cleared by centrifugation for 15 min, at 10,000 rpm and 4 °C. After removing the assay-interfering substances, nucleic acids, by the streptomycin sulfate (Amresco, 0382) precipitation method, cell lysates were mixed with 2,4-dinitrophenylhydrazine (DNPH) and incubated in the dark at room temperature for 60 min. Then, trichloroacetic acid (TCA) was added to each sample at a final concentration of 10%. After samples were placed on ice for 5 min, they were centrifuged at 10,000 rpm and 4 °C for 10 min. The resulting pellets were thoroughly washed with an ethanol and ethyl acetate mixture (1:1) three to four times and then resuspended in a guanidine hydrochloride solution. The absorbance of this solution was read at 375 nm, and protein levels in the corresponding samples were determined by the bicinchoninic acid (BCA) method (Sigma, BCA1-1KT). The amount of protein carbonyls in cell lysates was expressed as nM/mg protein.

2.10. Glutathione assay

The ratio of reduced to oxidized glutathione (GSH/GSSG) in cell lysates was determined according to the instructions from the commercial assay kit (Cayman, 703002). Cell lysates were prepared in phosphate buffer, and proteins were removed by the metaphosphoric acid (Sigma, 239275) precipitation method. The acidity of the cell lysates was neutralized with triethanolamine (Sigma, T58300) buffer. To determine the amount of oxidized glutathione alone, the samples were treated with 2-vinylpyridine (Sigma, 13229-2) for 60 min at room temperature. The standard, GSSG was serially diluted using 2-(N-Morpholino)ethanesulfonic acid (MES) buffer, as per the manual instructions. The concentrations of diluted standards were 0–8 µM. 50 µL of cell lysates or standards were mixed with 150 µL assay cocktail, which is a mixture of MES buffer, cofactor, enzyme and DTNB [5,5'-dithio-bis-(2-nitrobenzoic acid)]. The absorbance of the reaction mixtures was read at 405 nm at 5 min intervals for 30 min. The i-slope of the standard curve was used to calculate the amount of GSH or GSSG, and the ratio of GSH/GSSG was expressed as the number of fold change versus the control group.

2.11. Cytoprotective assay

HepG2 cells (4×10^4 /well) were seeded into a 96-well plate and allowed to adhere overnight. Cells were then pretreated with PDNE (12.5 µg/mL), trolox (12.5 µg/mL) or vehicle (0.1% DMSO) for 24 h. After completely washing with serum-free cell culture media, cells were incubated with 0.5 mM of *tert*-butyl hydroperoxide (TBHP; Alfa Aesar, A13926) for 4 h. Next, either the MTT colorimetric assay was performed, or cells were stained with the fluorescent dyes, Hoechst 33342 and propidium iodide, to assess the impact of extracts on TBHP-induced nuclear damage. Fluorescence images were captured using a fluorescent microscope.

2.12. Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.02 software (GraphPad Software 7825 Fay Avenue, Suite 230, La Jolla, CA 92037 USA). Data are expressed as mean \pm SD of three independent experiments. The *p* values less than 0.05, 0.01 or 0.001 were considered statistically significant.

3. Results

3.1. Extraction yield

In the present study, we used several different ratios of ethanol to distilled water as an extraction solvent for the preparation of pine needle extracts. After removing the extraction solvent using a rotary evaporator, the extracts were lyophilized and quantified. The yield was 12.4%, 15.5%, 17.0%, 20.0%, 17.9% and 18.1% for PDNE-0, PDNE-20, PDNE-40, PDNE-60, PDNE-80 and PDNE-100, respectively. A typical HPLC profile of pine needle extracts is presented in Fig. 1. The data shows there are some compositional differences between aqueous and ethanolic extracts. However, the more detailed analysis needs to be done in order to understand the exact compositional difference between the ethanolic gradient extracts.

3.2. ABTS radical scavenging activity of PDNE

ABTS is a green colored, free radical, widely used to determine the antioxidant potential of natural products. In this study, ABTS radicals were generated from oxidation of ABTS by potassium persulfate, and the assay was carried out as per the existing literature. The concentrations of extracts and trolox that reduced the absorbance of ABTS radical solution by 50% were determined, which were then used to express their antioxidant capacity in trolox equivalents (Table 1). The ABTS radical scavenging activity (TE) of extracts, PDNE-0, PDNE-20, PDNE-40, PDNE-60, PDNE-80 and PDNE-100, were 8.97 ± 1.64 , 14.78 ± 2.17 , 15.58 ± 2.31 , 14.63 ± 2.36 , 11.35 ± 2.28 , and 6.75 ± 0.95 g/100 g extract, respectively.

3.3. DPPH radical scavenging activity of PDNE

DPPH is a stable deep purple colored radical, largely used to determine the antioxidant potential of both natural and synthetic compounds. Antioxidants change the color of DPPH from purple to yellow when they are added to the DPPH radical solution and thereby decrease the absorbance of the reaction mixture at 515 nm. First, the concentrations of extracts and trolox standard that reduced the absorbance of DPPH radical solution by 50% were calculated. Then, the antioxidant potentials of extracts were expressed as TE, g/100 g of extract (Table 2). The TE of extracts, PDNE-0, PDNE-20, PDNE-40, PDNE-60, PDNE-80 and PDNE-100, were 2.76 ± 0.33 , 5.78 ± 0.11 , 6.41 ± 0.68 , 6.17 ± 0.31 , 3.82 ± 0.07 , and 1.30 ± 0.15 g/100 g extract, respectively.

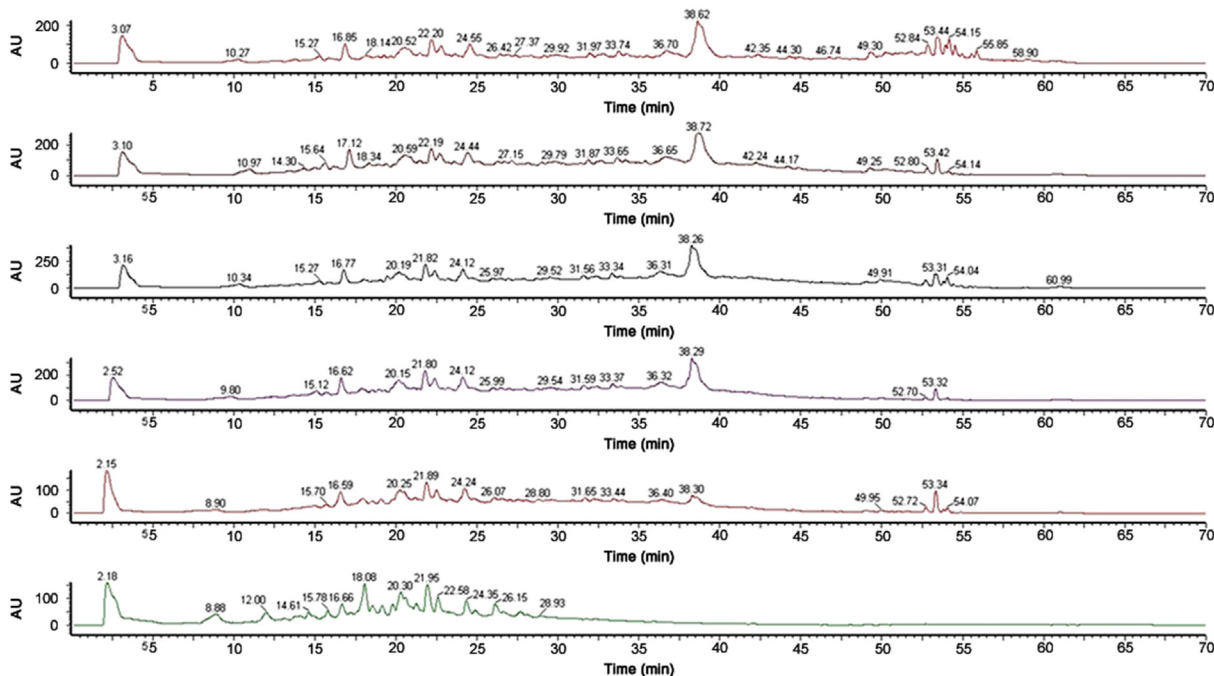


Fig. 1. Typical HPLC profile of PDNE.

Table 1
ABTS radical scavenging activity of pine needle extracts.

Pine needle extracts	ABTS (EC ₅₀ , µg/mL)	Activity ^a
PDNE-0	14.90 ± 0.37	8.97 ± 1.64
PDNE-20	9.02 ± 0.55	14.78 ± 2.17
PDNE-40	8.56 ± 0.51	15.58 ± 2.31
PDNE-60	9.12 ± 0.43	14.63 ± 2.36
PDNE-80	11.80 ± 0.08	11.35 ± 2.28
PDNE-100	19.76 ± 1.32	6.75 ± 0.95

EC₅₀ (µg/mL), the concentration of half-maximal radical scavenging efficiency.

^a Trolox equivalents (TE), g/100 g extract.

Table 2
DPPH radical scavenging activity of pine needle extracts.

Pine needle extracts	DPPH (EC ₅₀ , µg/mL)	Activity ^a
PDNE-0	176.37 ± 29.84	2.76 ± 0.33
PDNE-20	83.70 ± 6.22	5.78 ± 0.11
PDNE-40	75.96 ± 11.60	6.41 ± 0.68
PDNE-60	78.46 ± 7.99	6.17 ± 0.31
PDNE-80	126.47 ± 4.38	3.82 ± 0.07
PDNE-100	373.70 ± 60.67	1.30 ± 0.15

EC₅₀ (µg/mL), the concentration of half-maximal radical scavenging efficiency.

^a Trolox equivalents (TE), g/100 g extract.

3.4. Cytotoxic effects of PDNE on HepG2 cells

Next, we determined the influence of PDNE on HepG2 cell viability according to the common MTT colorimetric assay method. The MTT assay was performed after cells were exposed to PDNE (0, 6.25, 12.5, 25, 50, and 100 µg/mL) for 24 h. PDNE affected the viability of HepG2 cells in a dose-dependent manner. Cell viability values greater than 90% were considered nontoxic. All the three examined extracts, PDNE-0, PDNE-20 and PDNE-40 showed more than 90% HepG2 cell viability for up to 12.5 µg/mL concentration (Fig. 2). Hence, the concentration of extracts was limited to 12.5 µg/mL for further experimental analysis.

3.5. Effect of PDNE on ROS generation in HepG2 cells

The influence of extracts on the cellular levels of ROS, after the extracellular addition of TBHP, was assayed using a fluorescent precursor, DCFH-DA. Following the cellular enzymatic cleavage of diacetate group from DCFH-DA, DCFH can be readily oxidized to fluorescent DCF by ROS molecules. In our study, the ROS assay results showed that the fluorescence intensity of DCF was increased by about two-fold (2.15 ± 0.24) when cells were exposed to TBHP, alone, for 30 min. However, the intensity was significantly decreased if cells were pretreated with PDNE-20 or PDNE-40 for 24 h before cells were exposed to TBHP (Fig. 3).

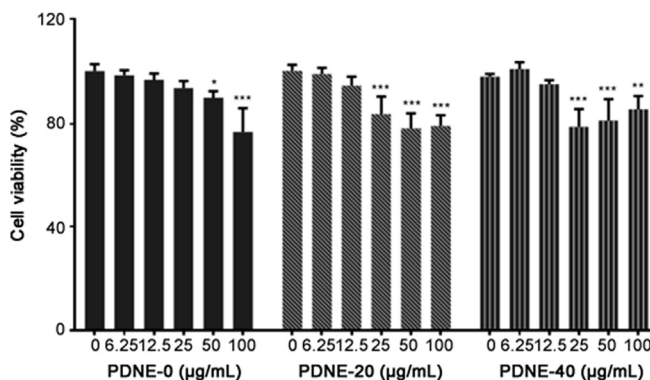


Fig. 2. Effects of pine needle extracts on HepG2 cell viability. Cells were treated with the indicated concentrations of PDNE for 24 h. MTT assay was performed, and cell viability is expressed as the percentage of the vehicle-treated control. Values are mean ± SD from three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, compared to control.

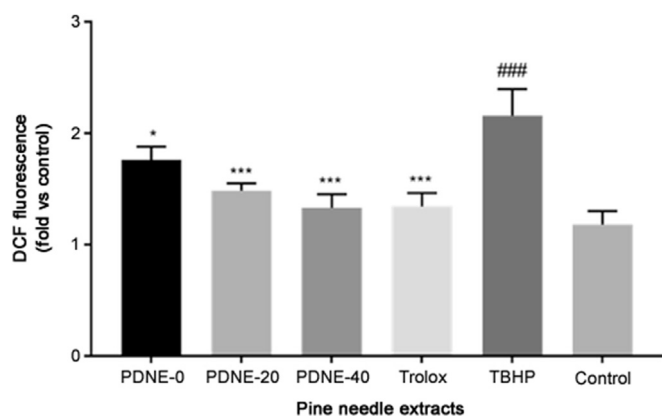


Fig. 3. Effects of pine needle extracts on *tert*-butyl hydroperoxide (TBHP)-induced enhanced levels of reactive oxygen species (ROS) in HepG2 cells. Cells were pretreated with PDNE or trolox for 24 h. Afterward, cells were exposed to TBHP (0.5 mM) for 30 min and then with the fluorescent precursor, DCF-DA for 45 min. The intensity of fluorescence was reordereed using multi-plate reader and normalized against total cellular LDH activity. Values are mean \pm SD from three independent experiments. ### $p < 0.001$, compared to control group; * $p < 0.05$, *** $p < 0.001$, compared to TBHP-challenged group.

3.6. Effect of PDNE on TBHP-induced oxidation of lipids and proteins in HepG2 cells

Due to their special chemical characteristics, ROS can extensively oxidize various biomolecules including lipids and proteins. Hence, determination of the most general, oxidized forms of lipids and proteins such as lipid peroxides and protein carbonyls, respectively, are used as an indicator of oxidative stress in many cell types. As shown in Fig. 4, HepG2 cells induced with TBHP showed more than two-fold increased levels of these oxidative markers. However, pretreatment of cells with PDNE-20 or PDNE-40 significantly reduced the TBHP-induced increased levels of both markers, lipid peroxides, and protein carbonyls.

3.7. Effect of PDNE on TBHP-induced GSH depletion in HepG2 cells

Glutathione (GSH) is a tripeptide made of three amino acids: glutamate, cysteine, and glycine. It is considered one of the major molecules of the cellular antioxidant defense mechanism. GSH is readily oxidized to the disulfide dimer GSSG when it donates an electron to the enzyme glutathione peroxidase in the process of

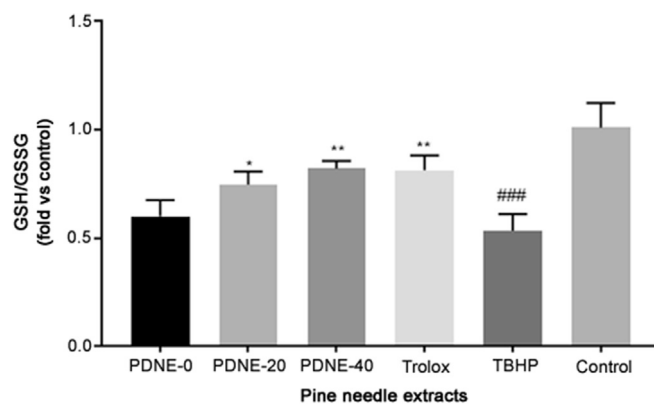


Fig. 5. Effects of pine needle extracts on *tert*-butyl hydroperoxide (TBHP)-induced altered levels of reduced (GSH) and oxidized glutathione (GSSG) in HepG2 cells. Cells were pretreated with PDNE or trolox for 24 h. Cells were then exposed to TBHP (0.5 mM) for 2 h. The levels of GSH and GSSG were determined using a commercial assay kit, according to their manual instructions. The ratio of GSH to GSSG was calculated and presented as the number of fold change versus a control group. Values are mean \pm SD from three independent experiments. ### $p < 0.001$, compared to control group; * $p < 0.05$, ** $p < 0.01$, compared to TBHP-challenged group.

reduction of hydroperoxides. Hence, the ratio of GSH to GSSG depends mainly on the amount of hydroperoxides produced in cells under stress conditions. In this study, we observed an extracellular addition of TBHP greatly reduced the GSH/GSSG ratio when compared to untreated cells. However, pretreatment of cells with PDNE-20 or PDNE-40 significantly rescued the TBHP-induced GSH depletion (Fig. 5).

3.8. Cytoprotective effect of PDNE

The protective effect of PDNE-0 and PDNE-40 on TBHP-induced oxidative damage was determined by the MTT cell viability assay method. As shown in Fig. 6, TBHP treatment led to a dramatic decrease in cell viability when compared to the vehicle-treated group. However, pretreatment of cells with PDNE-20 or PDNE-40 significantly decreased the TBHP-induced loss of cell viability. These results depict the cytoprotective nature of PDNE.

3.9. Effect of PDNE on TBHP-induced nuclear damage

TBHP is known to induce nuclear condensation in various cell types. In this study, pretreatment of HepG2 cells with PDNE-20 or

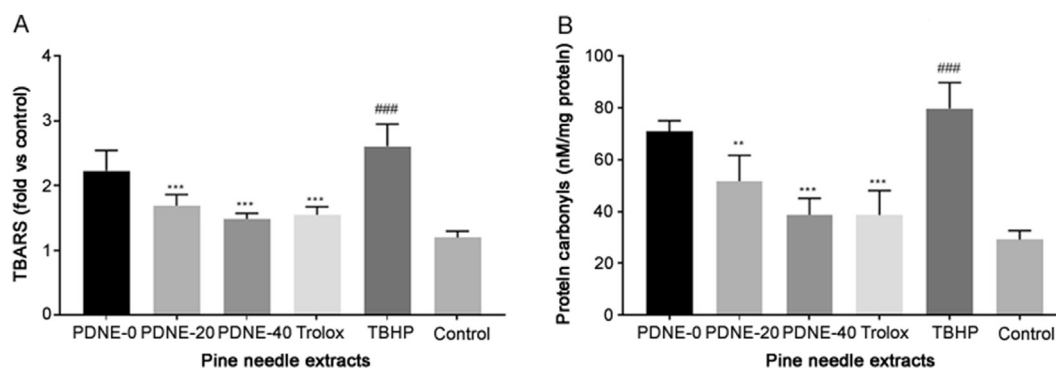


Fig. 4. Effects of pine needle extracts on *tert*-butyl hydroperoxide (TBHP)-induced enhanced levels of (A) lipid peroxides (TBARS) and (B) protein carbonyls in HepG2 cells. Cells were pretreated with PDNE or trolox for 24 h. Then, cells were exposed to TBHP (0.5 mM) for 2 h. The cell-free culture supernatant was used to determine TBARS levels, according to previous literature with some modifications. TBARS is expressed as the number of fold changes versus a control group. The whole-cell lysate was used to quantify protein carbonyls using a commercial assay kit. Protein carbonyl levels are presented as nM/mg of protein. Values are mean \pm SD from three independent experiments. ### $p < 0.001$, compared to control group; ** $p < 0.01$, *** $p < 0.001$, compared to TBHP-challenged group.

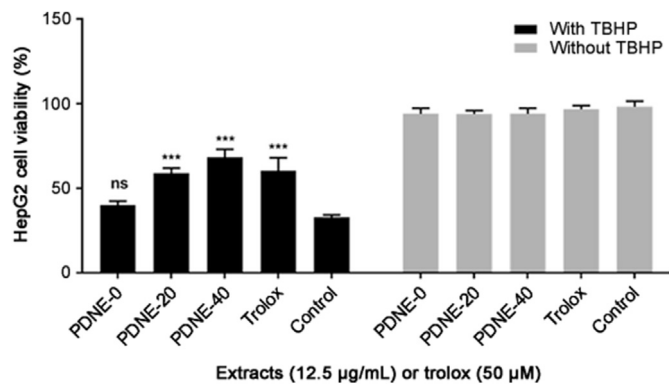


Fig. 6. Cytoprotective effects of pine needle extracts. HepG2 cells were pretreated with PDNE or trolox for 24 h. Thereafter, cells were incubated with or without TBHP (0.5 mM) for 4 h. Then, the MTT cell viability assay was performed. The results are expressed as the percentage cell viability versus a control group. Values are mean \pm SD from three independent experiments. *** $p < 0.01$, compared to TBHP-challenged group. ns: not significant versus TBHP-challenged group.

PDNE-40 significantly inhibited the TBHP-induced nuclear damage (Fig. 7). Moreover, we observed both PDNE-20 and PDNE-40 showed a greater protective effect than PDNE-0.

4. Discussion

Existing evidence clearly shows that chronic oxidative stress is one of the major risk factors associated with the initiation and progression of many pathological complications [32]. Oxidative stress is developed either due to overproduction of ROS or failure of the cellular, endogenous, antioxidant defense mechanism to counteract the oxidative damage of biological molecules [33]. In this perspective, it has been suggested that supplementation of exogenous antioxidants, such as plant-derived secondary metabolites, through diet, is highly essential for maintaining a balanced cellular antioxidant status. Moreover, many studies have reported that the dietary intake of plant-derived products is inversely

associated with the risk of progression of oxidative stress-mediated disease complications, as they are able to interrupt the propagation of free-radical reactions through their potent antioxidant ability [34–38]. In the present study, we determined the antioxidant potential of pine needle extracts by using the widely-considered, chemical radical scavenging assays such as DPPH and ABTS. In addition, these cell-free antioxidant assays can also be useful for the determination of the best extraction method [39]. In our study, the solvents used for the extraction of pine needle greatly influenced the antioxidant potential of the extracts. We observed that the inclusion of 20%–60% ethanol in distilled water, for extraction, significantly improved the antioxidant capacity of pine needle extracts. The antioxidant ability of natural products is generally expressed as equivalent of trolox, a well-established standard antioxidant. The PDNE-40, from 40% ethanol in distilled water, showed greater antioxidant potential than the aqueous and other ethanolic extracts, in both ABTS and DPPH radical scavenging assays.

Since the same antioxidant exhibits different effects in chemical and cell-based assays, we further investigated the antioxidant potential of pine needle extracts using a cell-based model. As HepG2 cells, a transformed human hepatoma cell line, has a relatively high and steady-state antioxidant defense system than that in normal hepatocytes and other non-transformed cells, it is largely utilized for screening the beneficial effects of various antioxidants [40]. Further, it is evident that the initiation and progression of many liver diseases are generally associated with prolonged, increased, oxidative stress [41]. Hydrogen peroxide (H_2O_2), a non-radical derivative of oxygen, is widely used as an exogenous oxidizing agent to induce oxidative stress in cell-based antioxidant assays [42–44]. H_2O_2 is a byproduct of normal cellular aerobic metabolism and mitochondrial respiration. Though it is relatively weak when compared to other oxidizing agents, it can induce toxicity in cells when in excess. Further, it has many unique biochemical properties, such as its presence in all biological systems and long half-life. As it is soluble in both aqueous- and lipid-containing media, it can easily diffuse through the cell membrane [45]. Hence, for many decades, H_2O_2 has been used for the induction of oxidative stress in cellular assay systems. But in the recent past, most of such screening studies

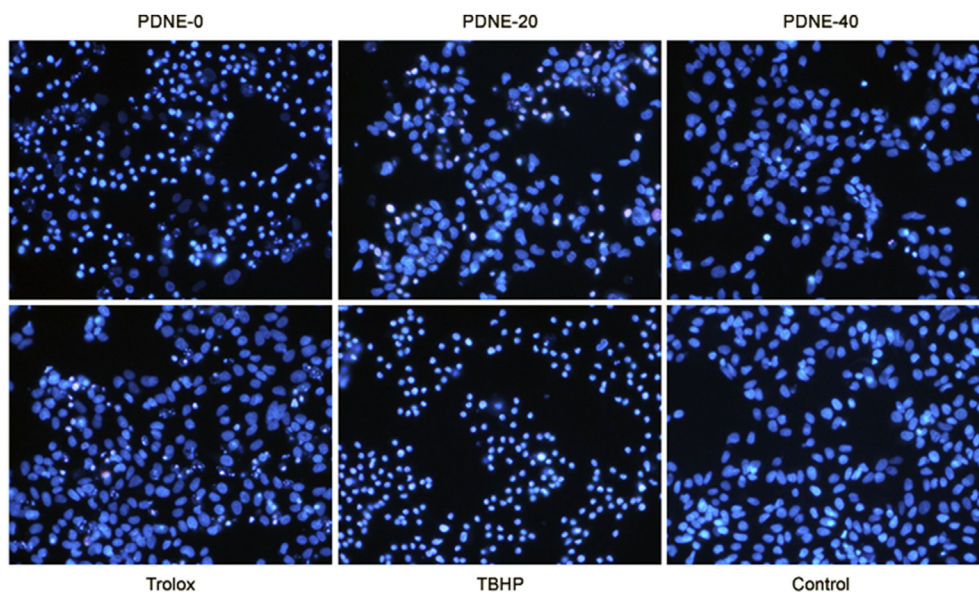


Fig. 7. Effects of pine needle extracts on *tert*-butyl hydroperoxide (TBHP)-induced nuclear chromatin damage in HepG2 cells. After cells were pretreated with PDNE or trolox for 24 h, followed by TBHP (0.5 mM) exposure for 4 h, cells were stained with the nuclear dye Hoechst 33342 for 15 min, at room temperature. Images were captured under a fluorescent microscope (Magnification, 400 \times).

have replaced H₂O₂ with *tert*-butyl hydroperoxide (TBHP), a synthetic derivative of H₂O₂, which is considered to be more stable than the corresponding H₂O₂ [46–49]. Based on this background, in the present study, we used TBHP to induce oxidative stress in HepG2 cells to determine the antioxidant efficiency of pine needle extracts.

The antioxidant potential of natural compounds against TBHP-induced oxidative injury in cells can be tested by determining the levels of various cellular oxidative stress markers, such as ROS, TBARS, a marker of lipid peroxidation and protein carbonyls, a marker of protein oxidation, as well as a reduced glutathione concentration [29,50,51]. A cell-permeable, fluorescent precursor, DCFDA is widely used to determine the levels of intracellular ROS. At the intracellular space, DCFDA is converted to DCFH by the action of cellular deacetylase enzymes. Then, the cellular pro-oxidants like H₂O₂ oxidize DCFH to fluorescent DCF. The amount of DCF fluorescent intensity directly indicates the cellular oxidative status. In the present study, HepG2 cells exposed to TBHP showed around two-fold increased DCF fluorescent intensity. However, this was significantly reduced by pretreatment of pine needle ethanolic extracts; the aqueous extract was less effective. Further, TBHP-induced, increased levels of lipid peroxides and protein carbonyls in HepG2 cells were significantly reduced by pretreatment with pine needle ethanolic extract. The data clearly demonstrate that the greater antioxidant potential of ethanolic extracts of pine needle strongly inhibits the intracellular generation of ROS, lipid peroxidation and protein oxidation in HepG2 cells challenged with TBHP. Glutathione is an important, non-enzymatic antioxidant that plays a major role in the inherent, antioxidant defense mechanism of cells. It exists in two different forms: reduced glutathione (GSH) and oxidized glutathione (GSSG). Normally, cells maintain a higher level of GSH than GSSG. However, under oxidative stress condition, GSH is oxidized to GSSG [52]. Hence, the ratio of GSH to GSSG is considered a good indicator of cellular antioxidant status. In this study, exogenous addition of TBHP to HepG2 cells induced a dramatic decrease in the concentration of GSH where GSSG level was increased. This was significantly reversed by pretreatment with pine needle ethanolic extracts. Moreover, we observed the pretreatment of PDNE-20 and PDNE-40 significantly inhibited the TBHP-induced nuclear damage and loss of HepG2 cell viability.

5. Conclusions

The results of the cell-free chemical antioxidant assays such as DPPH and ABTS clearly showed that *Pinus densiflora* needle extracted with 20%–80% ethanol in distilled water has increased antioxidant potential. Further, the cell-based assays provide useful information about the protective effects of *Pinus densiflora* needle ethanolic extract against oxidative stress-induced cell death. Our study provides evidence that *Pinus densiflora* needle extracted with 40% ethanol in water has a greater antioxidant capacity.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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