Original Article

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Protective effect of Phikud Navakot extract against hydrogen peroxide-induced oxidative stress in HepG2 cells

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ABSTRACT

Objective: The purpose of the study is to evaluate the hepatoprotective effects of Phikud Navakot (PN) extract against hydrogen peroxide (H_2O_2)-induced oxidative stress in HepG2 cells. **Materials and Methods:** The cells were treated with concentrations of PN up to 1 mg/ml before incubation with H_2O_2. Cell viability and reactive oxygen species (ROS) generation were assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and 2′,7′-dichlorofluorescein diacetate assays, respectively. Oxidative defense mechanisms were investigated by measuring glutathione (GSH) levels and activities of antioxidant enzymes. Expression of nuclear factor erythroid 2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1) proteins was investigated by Western blot analyses. **Results:** The results showed that PN extract at concentrations of 0.001–0.1 mg/ml significantly improved cell viability and prevented ROS generation induced by H_2O_2 in HepG2 cells. The extract also increased the activities of antioxidant enzymes (catalase, glutathione peroxidase, glutathione reductase, and superoxide dismutase), total GSH, GSH, and the GSH/glutathione disulfide (GSSG) ratio, but decreased GSSG. Pre-treatment with PN prevented the decreases in Nrf2 and HO-1 protein levels caused by H_2O_2-induced oxidative stress. **Conclusion:** This study highlights the hepatoprotective effect of PN against H_2O_2-induced oxidative stress and the associated mechanisms.

Keywords: Hepatoprotective effect, oxidative stress, Phikud Navakot

INTRODUCTION

Phikud Navakot (PN) is a Thai traditional remedy that comprises equal weights of extracts from nine species of medicinal herbs [Table 1]. These combinations were believed to produce optimal therapeutic efficacy with less associated side effects or toxicity. PN has been used for improving circulatory system symptoms such as dizziness, fainting, and syncope, which is prevalent among elderly people.[1] From a safety perspective, PN extract is relatively nontoxic in acute and subchronic administration (90 days) in Sprague-Dawley rats.[2] Nevertheless, there is a concern regarding the use

of PN at high doses for long periods since chronic exposure (12 months) to PN extract at 1000 mg/kg/day resulted in mesangiolytic glomeruli in Sprague-Dawley rats.[3] In addition, we previously found that PN extract inhibits several cytochrome P450 (CYP) isoforms *in vitro*; thus precaution should also be taken when PN is coadministered with some drugs to avoid herb-drug interactions.[4] Several studies have examined the bioactivity and underlying mechanisms of PN on amelioration of cardiovascular symptoms. Nalinratana *et al*.[5] showed that a hydroethanolic extract of PN could scavenge superoxide, hydroxyl, and nitric oxide radicals, as well as hydrogen peroxide (H_2O_2), in human endothelial ECV304 cells. PN also decreases endothelial-dependent relaxation in response to carbachol in isolated rat aorta.[6] These findings support the beneficial use of PN in circulatory disorders. Furthermore, antioxidant and cytoprotective effects of the extracts separated from the individual herbs that are constituents in PN have been reported. Hepatoprotective effects of extracts of *Angelica dahurica*,[7] *Picrorhiza kurroa*,[8] *Saussurea lappa* or *Saussurea costus*,[9] and *Atractylodes lancea*[10] have been shown *in vitro* (HepG2 cells and liver slice culture) and *in vivo* (mice and rats). Cytoprotective and antioxidant effects of extracts of other components in PN have also been examined, including *Terminalia chebula* in rat heart tissues,[11,12] *Ligusticum chuanxiong* in human umbilical vein endothelial cells,[13] and *Nardostachys jatamansi* in rat C6 glioma cells.[14] *Angelica sinensis* has antioxidant effects and enhanced immune activity in the serum of middle-aged women.[15] According to the antioxidant and cytoprotective activities of these herbs contained in PN extract, PN is likely to possess a hepatoprotective effect, which might be of advantage for elderly people who usually take multiple medications together with PN. It is known that most drugs undergo Phase I metabolism through the CYP system in the liver, in which the generation of reactive oxygen species (ROS) can occur. This process is one of the pathways that lead to liver injury.[16] Therefore, the aims of this study were to investigate the hepatoprotective effects of PN extract against H_2O_2-induced oxidative stress in a hepatocellular carcinoma cell line (HepG2) and to assess the associated mechanisms explaining this protective effects such as regulation of antioxidant enzymes and cellular defense proteins (nuclear factor erythroid 2-related factor 2 [Nrf2] and heme oxygenase-1 [HO-1]). These findings provide useful information for additional application of PN extract to protect the liver against oxidative stress besides the circulatory system application.

MATERIALS AND METHODS

Chemicals

Avidin, biotin-horseradish peroxidase (HRP), Dulbecco's modified Eagle's medium, fetal bovine serum, neutravidin biotin-binding protein, and trypsin-ethylenediaminetetraacetic acid (EDTA) were purchased from Gibco (Grand Island, NY, USA). Assay kits for catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), total glutathione (GSH), and superoxide dismutase (SOD) were purchased from Cayman Chemical (Ann Arbor, MI, USA). EDTA, H_2O_2, sodium chloride, and sodium fluoride were purchased from Merck Millipore (Darmstadt, Germany). N-acetylcysteine (NAC), 2′,7′-dichlorofluorescein diacetate (DCFH-DA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 3,3′-diaminobenzidine, ethylene glycol tetraacetic acid (EGTA), phenylmethanesulfonyl fluoride, protease inhibitor, sodium deoxycholate, sodium orthovanadate, and Tergitol® type NP-40 were purchased from Sigma (St. Louis, MO, USA). Sodium dodecyl sulfate (SDS) and Tris base were purchased from Bio-Rad (Hercules, CA, USA). All other reagents and solvents used were of the highest grade available.

Preparation of PN Extract

PN extract was prepared and provided by Dr. Narueporn Sutanthavibul (Department of Pharmaceutics and Manufacturing Pharmacy, Faculty of Pharmaceutical Sciences, Chulalongkorn University). Briefly, crude nine herbs were purchased from a Thai traditional pharmacy in Bangkok, Thailand and identified by Dr. Uthai Sotanaphun (Faculty of Pharmacy, Silpakorn University, Nakhon Pathom, Thailand). Voucher specimens (MUS1122-1130) have been deposited at the Museum of Natural Medicines, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. Equal weights of nine herbs as coarse powder were soaked in 10 times by weight of 80% ethanol overnight and extracted at 100°C for 3 h. This process was repeated twice, and then the collected extracts were mixed and concentrated. The resulting semisolid extract yield was kept at room temperature until analysis. High-performance liquid chromatography coupled with a photodiode array detector was used to quantify the analytical markers in the PN extract. The amounts of these markers were 3.4, 2.9, and 0.8% w/w for gallic acid, vanillic acid, and ferulic acid, respectively.[17]

Table 1: Herbal constituents of Phikud Navakot

Scientific name	Family	Common name	Used part
Angelica dahurica Benth. and Hook. f.	Umbelliferae	Kot Soa	Root
Atractylodes lancea (Thunb.) DC.	Compositae	Kot Khamao	Rhizome
Artemisia vulgaris L.	Compositae	Kot Chula-lumpa	Aerial part
Angelica sinensis (Oliv.) Diels.	Umbelliferae	Kot Chiang or Dang Gui	Root
Ligusticum chuanxiong Hort.	Umbelliferae	Kot Hua Bua	Rhizome
Nardostachys jatamansi (D. Don) DC.	Valerianaceae	Kot Jatamansi	Root, rhizome
Picrorhiza kurrooa Royle ex Benth.	Scrophulariaceae	Kot Kan Prao	Rhizome
Saussurea costus (Falc.) Lipsch.	Compositae	Kot Kradook	Rhizome
Terminalia chebula Retz.	Combretaceae	Kot Phung Pla	Gall

2,2-Diphenylpicrylhydrazyl (DPPH) Radical Scavenging Activity of PN Extract

The antioxidant activity of PN extract was evaluated by DPPH radical scavenging assay according to the method of Sokmen *et al*. with slight modification.[18] PN extract was dissolved in absolute ethanol and performing serial dilution for seven concentrations, and 20 μl of each concentration were added to 96-well plate. The 0.5 mg/ml of DPPH solution of 180 μl was added into each well. The plates were covered with plate covers and shacked at room temperature for 30 min. The optical density (OD) was measured at 517 nm using PowerWave XS2 microplate reader (BioTek, Winooski, VT, USA). Linear regression analysis was carried out for calculating half-maximal inhibitory concentration of sample required to scavenge DPPH radical by 50% (IC_{50}).

In Vitro Cytotoxicity Assay

Cytotoxicity was evaluated using a mitochondrial-based MTT cell viability assay. HepG2 cells (ATCC, Manassa, VA, USA) were seeded in a 96-well culture plate (1×10^4 cells/well) and incubated overnight at 37°C in a CO_2 incubator. The cells were then treated with 0, 0.0001, 0.001, 0.01, 0.1, or 1 mg/ml PN extract or 0, 5, 10, 50, 100, 200, 500, or 1000 μM H_2O_2 for 24 h. Control cells were treated with media containing 0.1% dimethyl sulfoxide (DMSO) since DMSO was used to dissolve the PN extract. The cytoprotective effects of PN were examined by pretreating the cells with 0, 0.0001, 0.001, 0.01, and 0.1 mg/ml of the extract or 5 mM NAC as a positive control.[19] After 3 h, the culture medium was discarded and the cells were treated with 500 μM H_2O_2 for 24 h to induce oxidative stress. After incubation, 25 μl of 5 mg/ml MTT reagent in culture medium was added to each well (equal to 1 mg/ml MTT in a total volume of 125 μl), and the cells were further incubated for 3 h. The resulting formazan was dissolved in 100 μl of DMSO. OD was measured at 570 nm using a PowerWave XS2 microplate reader (BioTek, Winooski, VT, USA).

Detection of Intracellular ROS

Intracellular ROS levels were determined using the DCFH-DA fluorescent probe substrate. HepG2 cells were seeded in 96-well black plates (1×10^4 cells/well) and treated with 0, 0.0001, 0.001, 0.01, 0.1, or 1 mg/ml PN extract for 3 h at 37°C in a CO_2 incubator. At the end of the incubation period, 50 μM DCFH-DA was added to each well and incubated for 40 min. The fluorescent intensity of DCF was detected using a PowerWave XS2 microplate reader at excitation and emission wavelengths of 485 and 528 nm, respectively. To study the inhibition of intracellular ROS by PN extract, cells were pre-treated with 0, 0.0001, 0.001, 0.01, or 0.1 mg/ml PN extract for 3 h or 5 mM NAC (positive control) for 1 h. The media were then discarded and cells were incubated with 500 μM H_2O_2 for 3 h, after which intracellular ROS was detected as above.

Effects of PN Extract on GSH Levels and Activities of Antioxidant Enzymes (CAT, GPx, GR, and SOD)

HepG2 cells were seeded in Petri dishes (1×10^6 cells/dish) and incubated for 24 h at 37°C in a CO_2 incubator. Then, the cells were treated with 0, 0.01, or 0.1 mg/ml PN extract for 3 h before incubation with 500 μM H_2O_2 for 24 h. After this treatment, the cells were harvested and dispersed in cold homogenizing buffers: 50 mM potassium phosphate buffer containing 1 mM EDTA, pH 7.0, for CAT, GR, and GSH; 50 mM Tris-HCl containing 5 mM EDTA and 1 mM DTT, pH 7.5, for GPx; and HEPES buffer containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose, pH 7.2, for SOD. The cells were then homogenized on ice using an ultrasonic probe sonicator (Sonics Vibra Cell®, Newtown, CT, USA). CAT, GPx, GR, and SOD activities and GSH levels in HepG2 cells were determined using commercial assay kits.

Effect of PN Extract on Nrf2 and HO-1 mRNA Expression

HepG2 cells were seeded on a 12-well plate (1×10^5 cells/well), incubated overnight, and then treated with 0.1 mg/ml PN extract. Total RNA was extracted from the cells after 0, 3, 6, and 12 h of PN exposure using an RNAiso plus (Takara Bio Inc., Shiga, Japan). RNA concentration was determined by measuring the absorbance at 260 nm with a NanoDrop 2000C and using Beer's law with an assumed molar extinction coefficient of 40 for RNA. cDNA was then generated with a random hexamer primer using a ReverTra Ace® quantitative polymerase chain reaction real-time (qPCR RT) kit (Toyobo Co., Osaka, Japan). The expression levels of Nrf2 and HO-1 mRNA were determined by quantitative qRT-PCR on a MX3000 RT PCR system with Power SYBR green and primer pairs specific for each gene [Table 2]. Each PCR reaction mixture had a final volume of 10 μl and contained ×1 Power SYBR green master mix, 0.4 μM of each forward and reverse primer and 1 ng cDNA template. PCR reactions were performed at 95°C

Table 2: Sequences of polymerase chain reaction primers used in gene expression analysis

Gene name	Primer (5′-3′)	Accession number
NFE2L2 (Nrf2)	F: ACACGGTCCACAGCTCATC	NM_006164.3
	R: TGCCTCCAAAGTTCAATCA	
HMOX1 (HO-1)	F: GCATGCCCCAGGATTTGTCA	NM_002133.2
	R: GCTCTCCTTGTTGCGCTCAA	
GAPDH	F: CCAGCGCTGCTTTAACTC	NM_001256799.1
	R: GCTCCCCCCTGCAAATGA	

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase, HO-1: Heme oxygenase-1, Nrf2: Nuclear factor erythroid 2-related factor 2

for 30 s, 95°C for 5 s, and 60°C for 30 s for 40 cycles followed by 95°C for 60 s and 55°C and 95°C for 30 s. The level of each target gene was normalized to that of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene. Primers were designed using the Universal Probe Library Assay Design Center (https://lifescience.roche.com/webapp) and likely target gene specificity was examined using a Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) search of the NCBI GenBank database.

Effects of PN Extract on Nrf2 and HO-1 Protein Expression

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis were performed to assess the effects of PN extract on expression of Nrf2 and HO-1 proteins. HepG2 cells were seeded into Petri dishes (1×10^6 cells/dish) and incubated for 24 h. Then, the cells were treated with 0, 0.01, or 0.1 mg/ml PN extract for 3 h before incubation with 500 μM H_2O_2 for 24 h. After this treatment, the cells were harvested and washed twice with ice-cold phosphate buffer saline (PBS). Cytosolic and nuclear protein fractions were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific, Rockford, IL, USA). Protein concentrations in the cytosolic and nuclear fractions were assessed using a Pierce® bicinchoninic acid protein assay kit (Thermo Scientific, Waltham, MA, USA). The fractions (20 μg protein/lane) were separated by 10% SDS-PAGE. Proteins were transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA) using a semidry transfer system, blocked overnight with 5% skimmed milk in PBS-T, and then probed with rabbit polyclonal anti-Nrf2 antibody (Catalog No. sc-722) and anti-HO-1 antibody (Catalog No. sc-10789) (both from Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membrane was then incubated with HRP-conjugated secondary antibody (Abcam, Cambridge, MA, USA). The loading control, GAPDH, was detected using the anti-GAPDH antibody (Catalog No. sc-32233, Santa Cruz Biotechnology). The band intensity was measured with an Amersham Imager 600 (GE Healthcare, Freiburg, Germany).

Statistical Analysis

Half maximal inhibitory concentrations (IC_{50}) were calculated using probit analysis. All results are presented as mean ± standard deviation of three independent experiments. Statistical analysis was performed using SPSS version 18. Differences among means were analyzed by one-way analysis of variance followed by a Tukey test for multiple comparisons. Differences were considered significant at $P < 0.05$.

RESULTS

DPPH Radical Scavenging Activity of PN Extract

In the DPPH scavenging assay, PN extract was evaluated for its free radical scavenging activity using ascorbic acid as the standard control. It was demonstrated that the scavenging effect increased with the increasing concentrations of PN extract and the IC_{50} value for the PN extract and ascorbic acid were 57.00 ± 0.02 and 43.50 ± 6.07 μg/ml, respectively [Table 3].

Cytotoxicity of PN Extract and H_2O_2 in HepG2 Cells

PN extract showed no toxic effects in HepG2 cells at concentrations of 0.0001–0.1 mg/ml, with cell viabilities of >80%. However, a significant decrease in cell viability to about 50% was found at 1 mg/ml PN [Figure 1a]. The calculated IC_{50} for PN extract in HepG2 cells was 0.93 mg/ml. Treatment of HepG2 cells with H_2O_2 resulted in a significant and concentration-dependent decrease of cell viability, with an IC_{50} of 789.80 μM [Figure 1b]. Therefore, concentrations of PN of up to 0.1 mg/ml were used for further studies of the protective effect of PN extract against H_2O_2.

Generation of Intracellular ROS and

Table 3: DPPH free radical scavenging activity of PN extract and ascorbic acid

Test compound	IC_{50} (μg/ml)
PN extract	57.00±0.02
Ascorbic acid (standard control)	43.50±6.07

Data are mean±standard deviation of three independent experiments.
PN: Phikud Navakot, DPPH: 2,2-diphenylpicrylhydrazyl

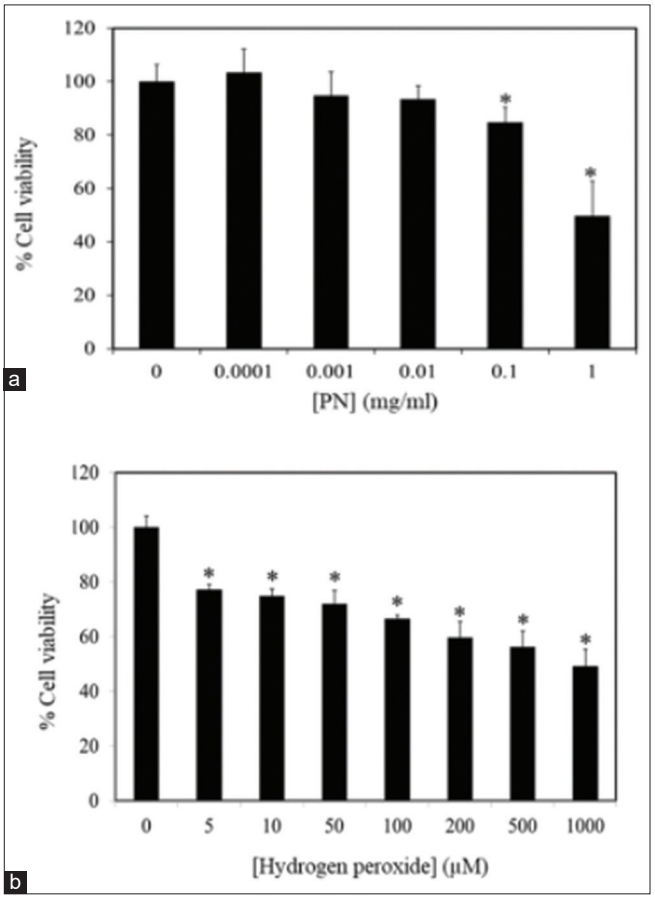

Figure 1: Cytotoxicity of Phikud Navakot (PN) extract and hydrogen peroxide (H_2O_2) in HepG2 cells. The cells were treated with various concentrations of PN (a) and H_2O_2 (b) for 24 h. Cell viability was determined by 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay. Data are mean ± standard deviation of three independent experiments. *$P < 0.05$ versus non-treated control

Inhibition of Intracellular ROS by PN Extract

Consistent with the cytotoxicity results, PN extract did not generate intracellular ROS at concentrations of 0.0001–0.1 mg/ml, but there was a significant increase of ROS at 1 mg/ml of PN extract. A significant increase of ROS of 6.6-fold occurred with 500 μM H_2O_2 compared with the untreated cells [Figure 2a]. Pre-treatment with both NAC (positive control) and PN at 0.001–0.1 mg/ml significantly attenuated intracellular ROS induction by H_2O_2. However, the ROS-scavenging effect of PN was significantly lower than that of 5 mM NAC [Figure 2b].

Cytoprotective Effects of PN Extract against H_2O_2-induced Cytotoxicity

Similar to the ROS scavenging effects, pre-treatment of HepG2 cells with PN extract at 0.001–0.1 mg/ml resulted in concentration-dependent attenuation of H_2O_2-induced cytotoxicity [Figure 2c]. The cytoprotective effects of PN extract at these concentrations were clearly less than that of NAC, which restored cell viability (90%) almost to that in non-treated control cells [Figure 2c].

Effects of PN Extract on GSH Levels and Activities of Antioxidant Enzymes (CAT, GPx, GR, and SOD)

H_2O_2 induced oxidative stress by decreasing total GSH and GSH but accumulating glutathione disulfide (GSSG). Therefore, the GSH/GSSG ratio decreased as compared to the non-treated group [Table 4]. Pre-treatment of HepG2 cells with PN extract at up to 0.1 mg/ml improved all of these markers. PN extract had no effect on GSH, but diminished GSSG, thus increased the GSH/GSSG ratio in a concentration-dependent manner [Table 4]. PN extract also increased the activities of antioxidant enzymes CAT, GPx, GR, and SOD compared to the non-treated control. Pre-treatment with PN prevented depletion of these enzymes by H_2O_2 [Table 5].

Effect of PN Extract on Nrf2 and HO-1 Expression

To obtain further insights into the molecular mechanisms underlying the protective effect of PN extract against H_2O_2, the possible involvement of transcription factors Nrf2 and HO-1 was assessed. There was no induction of Nrf2 mRNA at any time point after 0.1 mg/ml PN extract exposure for up to 12 h [Figure 3a]. While HO-1 mRNA was significantly induced by treatment with 0.1 mg/ml PN extract for 3 h and 6 h, with an increase of about 3-fold compared with 0 h ($P < 0.01$) followed by a decrease after 12 h of PN extract exposure [Figure 3b]. For the expression of these proteins, treatment of the cells with PN extract caused a significant increase in both cytosolic Nrf2 [Figure 4a and b] and nuclear Nrf2 [Figure 4c and d] compared to the control. PN extract had no significant effect on HO-1 [Figure 4e and f]. H_2O_2 caused significant decreases of Nrf2 and HO-1 levels in the cells. As expected, pre-treatment of the cells with PN extract (0.01 and 0.1 mg/ml)

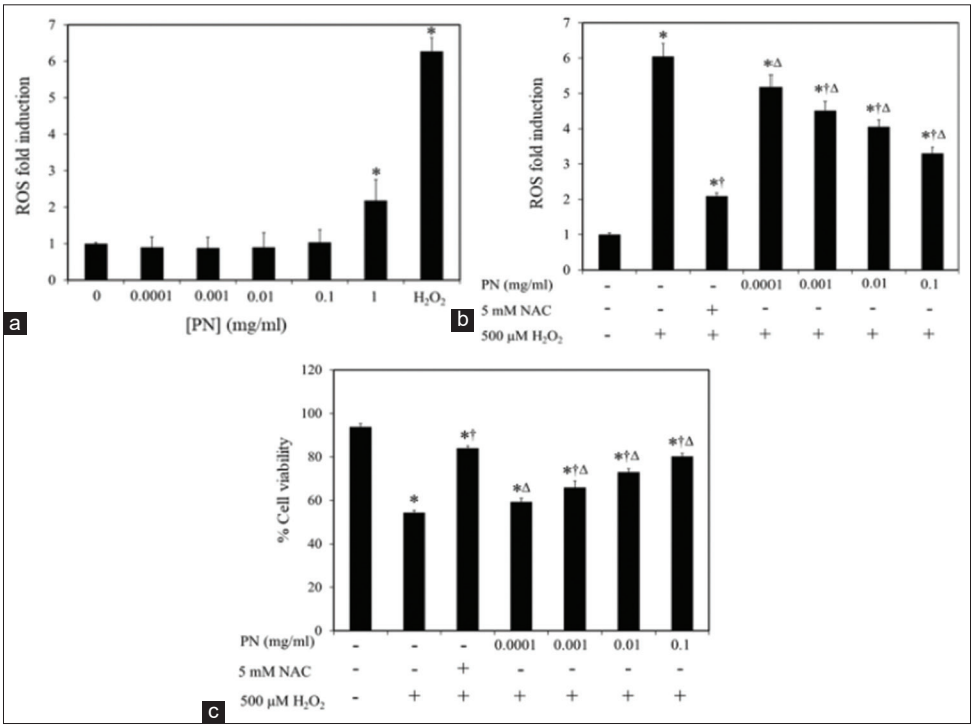

Figure 2: Protective effects of Phikud Navakot (PN) on reactive oxygen species (ROS) formation and HepG2 cell viability. To investigate the effect of PN on induction of intracellular ROS, the cells were treated with various concentrations of PN for 3 h. Hydrogen peroxide (H_2O_2) at the concentration of 500 μM was used as a positive control. Intracellular ROS was measured by 2′,7′-dichlorofluorescein diacetate (DCFH-DA) assay (a). In the other experiments, HepG2 cells were pretreated with various concentrations of PN or 5 mM N-acetylcysteine (NAC) before treatment with 500 μM H_2O_2. Intracellular ROS was measured by DCFH-DA assay (b) and cell viability was determined by 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay (c). Data are mean ± standard deviation of three independent experiments. *$P < 0.05$ versus non-treated control; †$P < 0.05$ versus H_2O_2-treated control; Δ $P < 0.05$ versus NAC with H_2O_2

Table 4: Effects of PN extract on total GSH, GSH, GSSG, and GSH/GSSG ratio

Treatment	Total GSH (nmol/mg protein)	GSH (nmol/mg protein)	GSSG (nmol/mg protein)	GSH/GSSG ratio
NT	37.75±1.61[a]	35.26±1.78[a]	2.48±0.53[a]	14.65±3.29[a]
0.01 PN	32.21±1.46[a]	30.95±1.28[a]	1.26±0.23[b]	25.03±3.81[b]
0.1 PN	37.05±3.77[a]	36.21±3.68[a]	0.84±0.10[b]	43.37±2.33[c]
H_2O_2	23.70±1.31[b]	6.53±2.20[b]	17.16±3.38[c]	0.41±0.23[d]
0.01 PN+H_2O_2	30.25±2.34[b]	28.77±2.05[a]	1.49±0.29[b]	19.62±2.37[ab]
0.1 PN+H_2O_2	34.02±3.10[a]	33.04±2.97[a]	0.99±0.13[b]	33.65±1.92[e]

Data are mean±standard deviation of three independent experiments, [a,b,c,d]letters indicate a significant difference ($P<0.05$) from values in the same column with other letters. PN: Phikud Navakot, GSH: Glutathione, GSSG: Glutathione disulfide

Table 5: Effects of PN extract on activities of antioxidant enzymes: CAT, GPx, GR, and SOD

Treatment	CAT (nmol/mg protein/min)	GPx (nmol/mg protein/min)	GR (nmol/mg protein/min)	SOD (U/ml)
NT	24.85±1.72[a]	58.07±7.94[a]	50.91±3.97[a]	0.78±0.08[a]
0.01 PN	28.50±1.19[a]	55.27±8.84[a]	59.77±4.67[a]	1.11±0.23[a]
0.1 PN	38.09±3.61[b]	93.85±5.03[b]	70.38±2.37[b]	1.60±0.29[b]
H_2O_2	15.08±0.43[c]	30.31±1.78[c]	37.10±3.64[c]	0.69±0.10[a]
0.01 PN+H_2O_2	24.34±2.28[a]	60.11±9.87[a]	57.90±3.23[a]	2.08±0.05[c]
0.1 PN+H_2O_2	27.34±1.07[a]	75.94±4.38[ab]	65.96±1.27[b]	2.42±0.09[d]

Data are mean±standard deviation of three independent experiments, [a,b,c,d]letters indicate a significant difference ($P<0.05$) from values in the same column with other letters. PN: Phikud Navakot, CAT: Catalase, GPx: Glutathione peroxidase, GR: Glutathione reductase, SOD: Superoxide dismutase

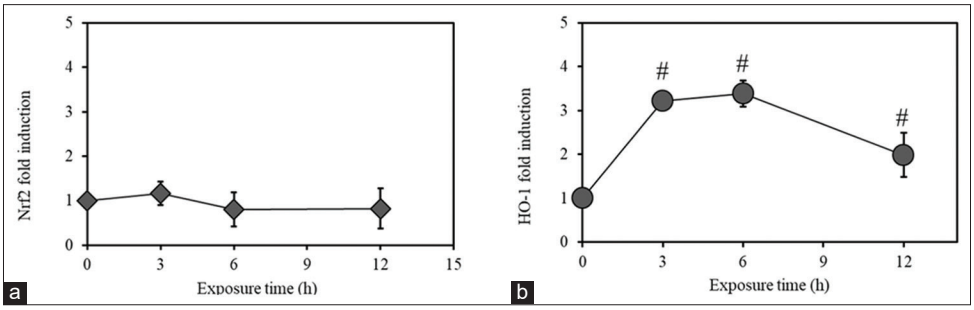

Figure 3: Effect of Phikud Navakot (PN) on nuclear factor erythroid 2-related factor 2 (Nrf2) (a) and heme oxygenase-1 (HO-1) (b) mRNA expression. HepG2 cells were treated with 0.1 mg/ml PN for 0, 3, 6, and 12 h. Nrf2 and HO-1 mRNA levels were determined by quantitative real-time polymerase chain reaction, using glyceraldehyde 3-phosphate dehydrogenase as an internal reference and loading control. Data are mean ± standard deviation of three independent experiments. #$P < 0.01$ versus 0 h

before H_2O_2 treatment attenuated the expression of cytosolic Nrf2 [Figure 4a and b] and even increased the expression of nuclear Nrf2 [Figure 4c and d]. The decrease of expression of HO-1 by H_2O_2 was also prevented by pre-treatment with PN extract [Figure 4e and F].

DISCUSSION AND CONCLUSION

The results of this study show hepatoprotective activity of PN extract against oxidative damage induced by H_2O_2 in a human hepatoma cell line, HepG2. In liver cells, mitochondria are targets of xenobiotic toxicity, and dysfunction of mitochondria can cause impairment of energy metabolism and consequent elevation of intracellular oxidative stress and cell death.[20] In this study, an increase of ROS was observed in H_2O_2-treated HepG2 cells and was linked to reduction of cell viability. The mechanism underlying this injury was related to a decrease in both non-enzymatic (GSH) and enzymatic (CAT, GPx, GR, and SOD) antioxidant defenses. These responses are the normal intracellular outcomes of oxidative stress.[21] Interestingly, pre-treatment of PN extract for 3 h before treatment with H_2O_2 could improve cell viability and increased these antioxidant defenses. In addition, we performed the DPPH radical scavenging assay to clarify whether PN extract had direct ROS scavenging activity when cells were treated with H_2O_2. It was demonstrated that PN extract is also effective as an antioxidant through ROS scavenging effect [Table 3]. It should be noted that PN extract itself did not have any effects on ROS generation or cytotoxicity, except at the highest test concentration of 1 mg/ml. The hepatoprotective effect of PN extract was studied using 500 μM H_2O_2 to induce cytotoxicity and oxidative stress.[22] Indeed, it is not possible to measure the blood PN concentrations because the extract comprises several compounds. Therefore, we calculated an approximate

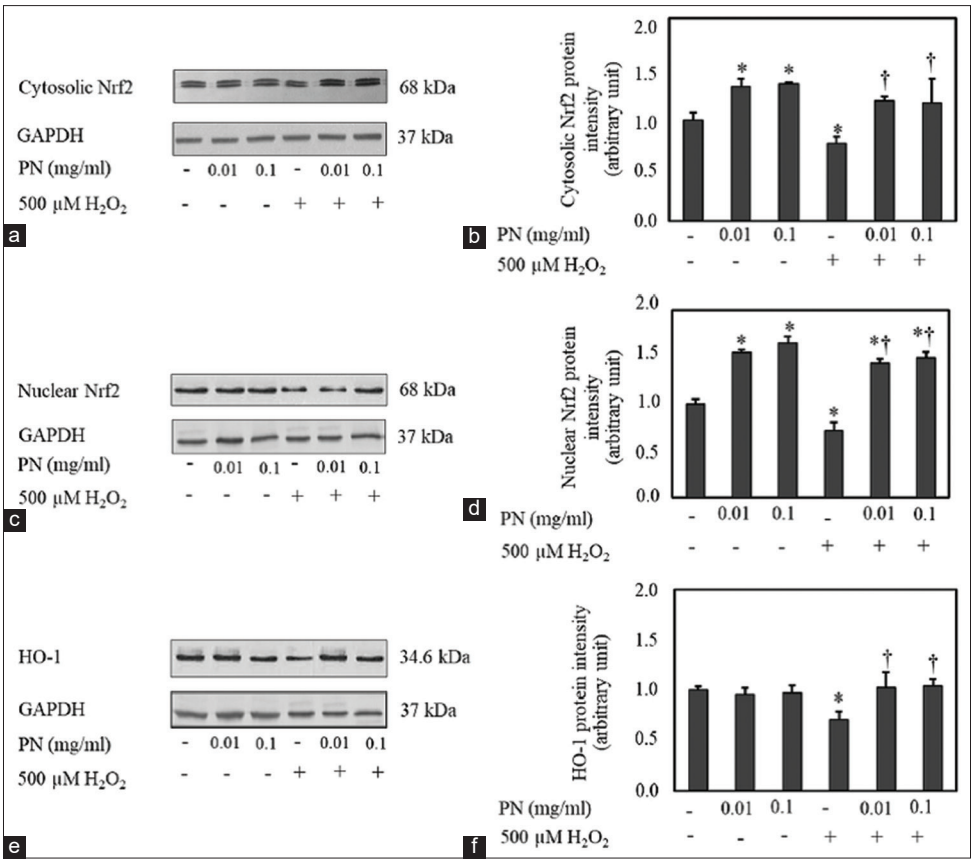

Figure 4: Effect of Phikud Navakot (PN) on nuclear factor erythroid 2-related factor 2 (Nrf2) (cytosolic a and b; nuclear c and d) and heme oxygenase-1 (HO-1) (e and f) protein expression. HepG2 cells were treated with 0.01 or 0.1 mg/ml PN before treatment with 500 μM hydrogen peroxide (H_2O_2). Nrf2 and HO-1 levels were determined by Western blot analyses using an enhanced chemiluminescence system. The intensity of each band was analyzed using a calibrated imaging densitometer and normalized as Nrf2/glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and HO-1/GAPDH ratios. Data are mean ± standard deviation of three independent experiments. *$P < 0.05$ versus non-treated control; †$P < 0.05$ versus H_2O_2-treated control

level of PN in blood from the recommended single oral dose of PN (e.g., 100 mg) divided by 5 L of the total body volume of a healthy adult, giving a blood PN concentration of about 20 μg/ml. According to the solubility limitation of the extract, 0–1 mg/ml of the extract were primarily used to investigate the hepatoprotective effects of PN in this study. PN at 0.001, 0.01, and 0.1 mg/ml significantly attenuated ROS induction and improved cell viability against H_2O_2-induced cell injury in a concentration-dependent manner, but the effects were significantly less potent than those of NAC, a positive control antioxidant.[23] These protective effects of PN were likely explained by our findings that PN increased the activities of CAT, GPx, GR, and SOD antioxidant enzymes in normal conditions and under H_2O_2-induced oxidative stress. A concentration-dependent increase in the GSH/GSSG ratio occurred in H_2O_2-induced oxidative stress following PN pre-treatment. Since the GSH/GSSG ratio is regarded as a good indicator of oxidative stress status and DNA damage, and thus cell injury,[24] an increase of this ratio by PN pre-treatment supports our hypothesis that PN possesses a hepatoprotective action associated with its antioxidative effects. These findings are consistent with reports on the beneficial effects of various herbal extracts that are constituents of PN. These extracts attenuated ROS production induced by oxidative stress by enhancing enzymatic and non-enzymatic antioxidant defenses.[7-11,13-15] We further investigated the molecular mechanism of the antioxidant effect of PN by assessing Nrf2 and HO-1 activation. The Nrf2 antioxidant response pathway is the primary cellular defense against the cytotoxic effects of oxidative stress. In general, Nrf2 is localized in the cytoplasm under normal conditions and bound to its inhibitor protein, Kelch-like ECH-associated protein 1 (Keap1). This complex is disrupted by exposure to several stimuli allowing Nrf2 to translocate to the nucleus to form heterodimers with small oncogene family proteins. This leads to selective recognition of the antioxidant response element on target genes, resulting in expression of cellular antioxidant enzymes such as CAT, GPx, GR, and SOD and Phase II detoxifying enzymes such as HO-1, NAD(P)H dehydrogenase (quinone 1), GSH *S*-transferase, and glutamate-cysteine ligase.[25] HO-1 catalyzes degradation of heme, which produces biliverdin, iron, and carbon monoxide.[25] Increased HO-1 activity can attenuate cell apoptosis and decrease superoxide formation.[26] In our study, Nrf2 protein was increased after PN treatment, while HO-1 protein was comparable to the control. H_2O_2-treated HepG2 cells had decreased Nrf2 and HO-1 protein levels, and these proteins were reversed by pre-treatment with PN [Figure 4]. Increased expression of Nrf2 protein by PN explains the translocation of Nrf2 from the cytosol to the nucleus, resulting in the enhancement

of antioxidant enzymes including CAT, GPx, GR, and SOD [Table 4]. Besides, the HO-1 protein was increased in the condition of pre-treatment of PN followed by H_2O_2 treatment. The results reasonably explain the activation of Nrf2 that amends level of HO-1 for cell survival under oxidative stress.[26] The expression level of Nrf2 and HO-1 mRNAs were unlikely associated with their protein expressions. This can be explained by the fact that the relationship between mRNA and protein levels in mammals is quite low, with a Pearson correlation coefficient of about 0.40. The low correlation might be associated with different factors including post-transcriptional or post-translational regulation.[27,28] In oxidative stress conditions, activation of Nrf2 also amends level of HO-1 for cell survival.[26]

PN is widely used by elderly people who are often prescribed multiple medications and the generation of ROS may occur during Phase I metabolism of drugs by the CYP system in the liver through CYP catalytic reaction.[29] With this caveat, we conclude that the administration of PN might be useful for protection against liver injury through modulation of antioxidant defenses and inhibition of ROS generation.

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CONFLICTS OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

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