Mongolian Academy of Sciences

Mongolian Journal of Chemistry

The Institute of Chemistry & Chemical Technology

Hepatoprotective effects of *Paeonia anomala* against acetaminophen-induced cell damage through activation of anti-oxidant system

O. Sarangerel[1,2], K. Kang[1], S.B. Lee[3], J.H. Yun[1], D. Batsuren[4], J. Tunsag[4], C.W Nho[1]

[1] *Functional Food Center, Korea institute of Science and Technology, Gangneung, Gangwon-do, Republic of Korea*

[2] *School of Pharmacy, Health Sciences University of Mongolia, Zorig Street-3, Ulaanbaatar 210648, Mongolia*

[3] *Emerging Technology Research Center, Samsung Advanced Institute of Technology, Gyeonggi-do, Republic of Korea*

[4] *Institute of Chemistry and Chemical Technology, Peace ave., Ulaanbaatar 13330, Mongolia*

ARTICLE INFO: 3 October 2013; revised 7 October 2013; accepted 8 October 2013

Abstract: Overdose of the analgesic and anti-pyretic acetaminophen causes a potentially fatal hepatic necrosis due to a high toxicity and depletion of cellular defense mechanisms. In the present work, the potential hepatoprotective effect of the fruit extract of *Paeonia anomala* against acetaminophen induced cell damages was evaluated in cultured HepG2 cells and compared to the root extract. The fruit extract showed a potent protection against acetaminophen induced cell death, while the root extract showed a weak protection. Particularly, the pre-treatment of lower doses of the fruit extract, 10 μg/mL and 20 μg/mL, significantly enhanced cell viability. The level of total glutathione in HepG2 cells treated with the fruit extract prior to the treatment of 40 mM acetaminophen was enhanced, however, the root extract failed for this activity. In addition, activities of quinone reductase, glutathione peroxidase and glutathione reductase were increased and protein levels of glutathione peroxidase 1 and superoxide dismutase 1 were enhanced in the cells treated with 10-20 μg/mL of the fruit extract. Furthermore, the protein level of Nrf2, a crucial regulator for detoxifying and antioxidant systems, was increased by the fruit extract treatment. These results suggest that the fruit extract of *P. anomala* exerts protective effects against acetaminophen-induced toxicity through activation of key antioxidant systems.

Keywords: Paeonia anomala, acetaminophen, hepatoprotection, anti-oxidation, detoxification

INTRODUCTION

Although considered safe at therapeutic doses, in overdose, acetaminophen (AP) produces a fatal toxicity to the liver. As delineated from urinary metabolites and accumulated evidences, AP is metabolized primarily by glucuronidation catalyzed by UDP-glucuronosyl transferase (UGT) and sulfation by sulfotransferase. The major conjugates are eliminated from the liver and blood mainly via urine (both) and a little via bile (AP-glucuronide). About 30% and 55% of administered AP is excreted in urine as AP-sulfate and AP-glucuronide, respectively [1]. A small amount of acetaminophen is metabolically activated by cytochrome P450 to form a reactive metabolite known as N-acetyl-p-benzoquinone imine (NAPQI), which is formed by direct two electron oxidation [2]. Cytochrome 2E1, 1A2, 3A4, and 2A6 have been reported to oxidize acetaminophen to the reactive metabolite. Also it was reported that NAPQI is detoxified by GSH to form an acetaminophen conjugate via non-enzymatically and enzymatically reaction catalyzed by glutathione S-transferase (GST) [3]. In overdose of acetaminophen, sulfation and glucuronidation pathways are saturated, and GSH is depleted by overproduction of NAPQI as much as 90% of total GSH, and it causes NAPQI covalently to bind to cysteine groups on proteins, forming acetaminophen-protein adduct. Also acetaminophen was reported to have an ability to bind covalently to DNA or lipids triggering lipid peroxidation [2].

Although the precise mechanism by which AP or its metabolites cause cell injury is unknown, the cell damage and liver failure are seemed the result from oxidative damage to essential macromolecules, depressed mitochondrial function and distribution of calcium homeostasis [4]. In overdose of AP it was shown that NAPQI reacts very rapidly with GSH, thus GSH concentration is very low in centrilobular cells, and glutathione peroxidase (GPx), a major peroxide detoxification enzyme, functions very insufficiently under a condition of GSH depletion [5]. In addition, during formation of NAPQI by cytochrome P450s, the superoxide anion is formed, after dismutation it leads to a formation of peroxide [6]. Also, it was suggested that peroxidation of AP to semiquinone free radical may lead to increased superoxide and toxicity [7].

* corresponding author: moosaraa_o@yahoo.com

The transcription factor Nrf-2 is a member of the "cap 'n' collar" family of basic leucine zipper transcription factors. Among six members of the family, Nrf-1 and Nrf-2 were shown to play crucial roles in the cellular detoxification process via the trans-activating reporter genes linked to anti-oxidant response elements and their sites of expression coincide with those of many phase II detoxifying genes. Nrf-2 mediates the induction of detoxifying genes including quinone reductase (QR or NQO1), GST, heme oxygenase (HO), catalase (CAT), superoxide dismutase (SOD), and UGT. Therefore, Nrf-2 plays an important role for detoxification of AP and its metabolites [8].

Numerous natural products have been found to have hepatoprotective effects against AP-induced toxicity. Moutan Cortex (cortex of *P. suffruticosa*) pre-exposure attenuated acetaminophen induced GSH depletion, cytochrome P450 2E1 activity and hepatic DNA damage in mice [9]. Also, sulforaphane found in cruciferous vegetables have been proven to have hepatoprotective effect against AP-induced liver damage through the induction of expression of the anti-oxidant response element (ARE) related genes, which attribute the activation of GSH and other detoxifying and anti-oxidative enzymes [10].

In this study, the protective effect of the root and seed cases extracts of *P. anomala* against AP-induced toxicity was investigated in HepG2 cells. Sulforaphane (5μM) was used as a positive control.

EXPERIMENTAL

Plant material: Fruits (seed cases without seeds) and roots of *P. anomala* were collected in Bayan chandmani Sum, Tuv province, located at 70 km northwest from Ulaanbaatar city, Mongolia in October 2011. A voucher specimen (2011/60) was deposited in the Flora and Plant Systematic Laboratory, Institute of Botany, Mongolian Academy of Sciences.

Cell culture: Human hepatoma HepG2 cells were obtained from the American Type Culture Collection (Rockville, MD,U.S.A.). HepG2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) *(HyClone Laboratories, Inc., Utah, USA) supplemented with 10% fetal bovine serum (HyClone Laboratories, Inc., Utah, USA),* 100 units/mL of penicillin and 100 μg/mL of streptomycin. These cells were maintained at subconfluence in 95% air, 5% CO_2 humidified atmosphere at 37°C.

Cell viability assay: The cell viability was evaluated by using the Cell Counting Kit (CCK-8) produced by Dojindo Laboratories (Tokyo, Japan) as described in 2.3.3 Section with minor modification. In brief, HepG2 cells ($1.5Ч10^4$ cells per well) were plated in 96-well plates, incubated at 37°C for 24 hours, and the cells were treated with indicated concentrations of the extracts acquired from roots and fruits of *P. anomala* and pure compounds dissolved in DMSO. DMSO concentration in DMEM was not exceeded than 0.2% and was equal in all groups including control group. For cellular treatments, HepG2 cells were cultured in DMEM without fetal bovine serum to reduce direct interaction between the phytochemicals and fetal bovine serum. After 24 hours incubation at 37°C the cells were washed by Dulbecco's phosphate buffered saline (DPBS) two times and treated with 40 mM of AP for 24 hours. Then 10μL of CCK-8 solution was added and the absorbance was measured at 450 nm by using the Synergy HT Multi-Mode Microplate Reader(Bio-Tek Instruments, Winooski, VT, U.S.A.) after additional 1 hour incubation.

Quantification of total Glutathione (t-GSH): Total glutathione (glutathione and glutathione disulfide) in the cell extract was determined in 96-well plates by using a recycling enzymatic assay as previously described [11] with some modification. HepG2 cells were pretreated with various concentrations of the extracts for 24 hours, then washed by DPBS two times, and treated with 40 mM of AP for additional 24 hours. After washing by ice-cold DPBS two times, the cells were lysed with ice-cold 143 mM sodium phosphate buffer (pH 7.5) containing 0.1% Triton-X and 8 mM EDTA. Protein concentration was measured in the cell lysate according to Bradford method by using Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA, USA.). The cell lysate was extracted with 5% sulfosalicylic acid for 5 minute and total glutathione was determined in the extract. 120 μL freshly prepared solution containing 1.68 mM 5,5'-dithiobis-2 -nitrobenzoic acid and 3.33 u/ml glutathione reductase was added to 25 μL of the cell extract prior to exposure of 60 μL fresh solution of 895 μM β-NADPH. Change in absorbance at 450 nm was immediately measured by using of Synergy HT Multi-Mode Microplate Reader (Bio-Tek Instruments, Winooski, VT, U.S.A.). Total glutathione concentration in the cell extract was calculated by using rate values of 5-thio-2-nitrobenzoic acid formation obtained from GSH standards.

Determination of GPx and GR activity: GPx activity was measured based on the oxidation of NADPH by the method of Flohe and Gunzler [12]. Briefly, $5Ч10^4$ HepG2 cell were plated in 100-ϕ dish and sequentially treated by the extracts and AP for 24 hours. The cells were collected by scrubbing in ice-cold DPBS and after centrifugation the cells were lysed in 0.1% Triton X-100 solution in 50 mM potassium phosphate (pH7.5) buffer containing 1 mM EDTA and sonicated for 4 sec with 10% amplitude. 10 μL of the cell extract was transferred into 96-well plate containing 100 μL solution of 1 mM GSH, 0.5 U/ mL glutathione reductase, and 0.3 mM NADPH in 50 mM potassium phosphate buffer (pH7.5) containing 1mM EDTA and 2 mM sodium azide. After adding 20 μL 1mM *t*-BHP, the rate of NADPH consumption was monitored at 340 nm every 2 min for 8 min.

GR activity was quantified by NADPH oxidized to form reduced GSH from oxidized GSH. Briefly, 10 µL of the cell extract prepared in the same way described above was transferred into 96-well plate containing 100 µL solution with 2 mM oxidized GSH and 1.5 mM NADPH in 50 mM potassium phosphate buffer (pH7.5) containing 1 mM EDTA. The rate of NADPH consumption was measured at 340 nm.

Determination of QR activity: The QR (NQO1) activity was measured as described previously [13] with minor modifications. Hepa1c1c7 cells ($1*10^4$ cells per well) were plated into 96-well plate and incubated for 24 hours prior to treatment. The media containing the indicated concentrations of the fruit extract were added and incubated for an additional 24 hrs. Five µM SFN was used as a positive control of QR inducer. The cells were rinsed with DPBS, lysed with 80 µL of 0.08% digitonin solution with 2 mM EDTA, and incubated for 30 min at room temperature, then QR activity was measured in the cell lysate. Two hundred µL of mixed solution containing 49 mL of 25 mM Triss buffer, 34mg of BSA, 0.34 mL of 1.5% Tween 20 solution, 0.34mL of thawed co-factor solution (150 mM glucose -6-phosphate, 4.5 mM NADH, 0.75 mM FAD in Tris buffer), 100 U of glucose-6-phosphate dehydro-genase, 15 mg of MTT (3-(4.5-dimethylthiazo-2-yl)-2.5 -diphenyltetrazolium bromide) and 25 µL of 50 mM menadione in acetonitrile was added into a 50 µL of aliquot of the cell lysate. Absorbance at 610 nm was measured for 5 times with 5 sec apart using a Synergy HT Multi-microplate reader. The induction of QR was expressed as CD values, which is a concentration required to double QR specific activity. The CI (chemopreventive index) is obtained by dividing IC50 value to CD value [14].

Western blot analysis: The treated cells were lysed in the cell lysis buffer (1M Tris, pH 7.4, 2M NaCl, 1M EDTA, 10% NP40 and protease inhibitor cocktail) by sonication. Total cell lysate was obtained following centrifugation at 12,000xg for 30 min at 4^0C. After denaturation of an equal concentration of total cell lysate, the protein samples were separated by electrophoresis on 10% NuPAGE gels using a Xcell Sure Lock Mini-cell, then transferred to a polyvinylidene diflouride (PVDF) membrane using Trans-BlotR Semi-Dry cell. The membrane was blocked with 5% skim milk in PBST buffer (8g/L NaCl, 0.2 g/L KlCl, 1.44 g/L Na_2HPO_4, 0.24 g/L NaH_2PO_4 and 0.1% Tween-20) by incubation for 1 hour at room temperature, then incubated for overnight at 4ɵC with primary anti-bodies against SOD1, CAT, GPx1, QR and β-actin. After washing PBST the membrane was incubated for 1 hour with horseradish peroxide-conjugated secondary anti-body at room temperature. Immunoreactive proteins were detected using enhanced chemiluminescence reagents.

Statistical analysis: The data are expressed throughout as the mean ± standard deviation, the statistical significance among the groups was determined by Student's *t*-test. P value of less than 0.005 was considered statistically significant.

RESULTS

Cytoprotective effects of the fruit and the root extracts were examined in HepG2 cells stressed with AP for 24 hours. The cell viability was decreased significantly by 40 mM of AP treatment for 24 hours (Fig. 1A). However, this decrease of cell viability was recovered by a pre-treatment of the fruit extract, especially, at its lowest dose, 10 µg/mL, the cell viability was increased until the level of AP-free control. The root extract showed a weak protective effect against AP-induced cell death. Twenty µg/mL of the root extract showed a significant protective effect, while the other doses failed to recover the cell viability. Also, the cell protective effects of the fractions from the extracts were examined (Fig. 1B).

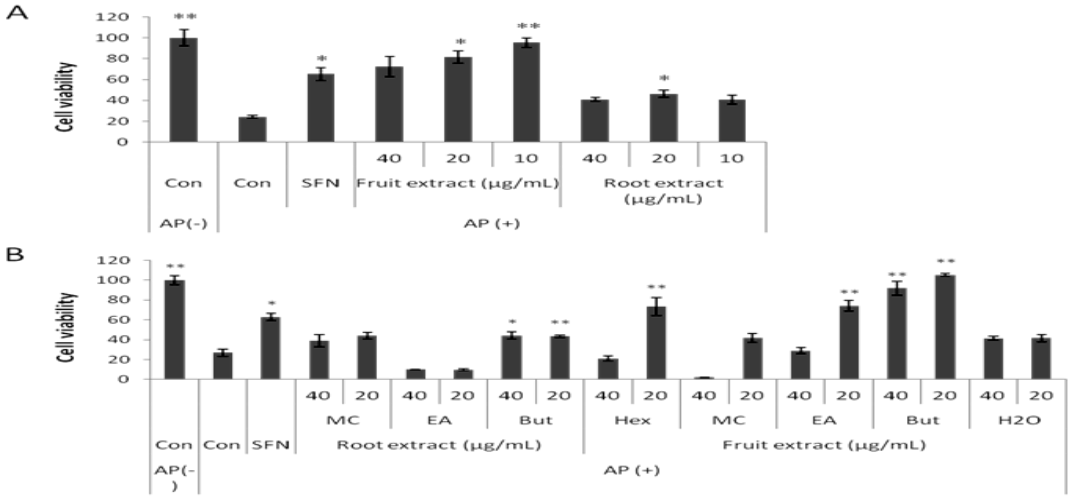

Fig. 1. Protective effect of the root and fruit extracts and fractions on cell viability. Each value represents the mean ±S.D. of three independent experiments with triplicate. Student's *t*-test compared with AP treated group: *$p < 0.005$, **$p < 0.001$

Hexane, ethyl acetate and butanol fractions of seed cases of *P. anomala* showed potent cytoprotective effects. In addition, the cell protective effects of the compounds isolated from the ethyl acetate and butanol fractions of the seed cases extract were measured in HepG2 cells. Among the compounds ellagic acid, methyl gallate, ethyl gallate, quercetin derivatives showed a significant cytoprotective effect (Fig. 2).

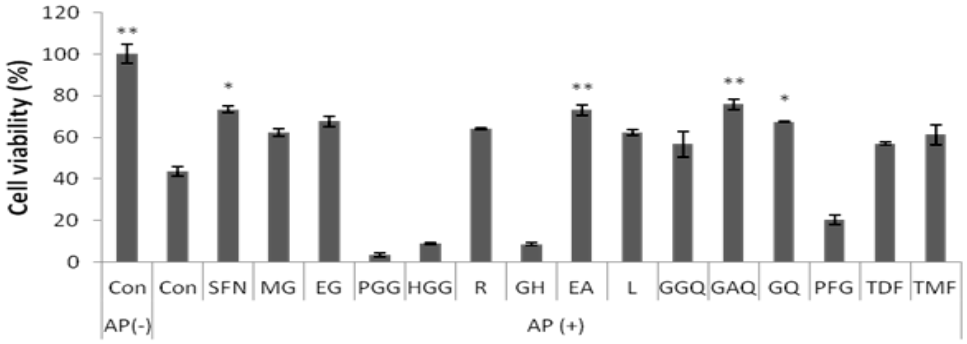

Fig. 2. Protective effect of compounds from fruit extract of *P. anomala* on cell viability.
Each value represents the mean ±S.D. of three independent experiments with triplicate. Student's *t*-test compared with AP treated group: **p* < 0.005, ***p* < 0.001

Cellular total GSH was measured in the HepG2 cells after sequential treatments of the extracts and 40 mM AP. T-GSH concentration was depleted by the AP treatment for 24 hours, but 2.5 µg/mL to 20 µg/mL doses of the seed cases extract enhanced t-GSH level (Fig. 3).

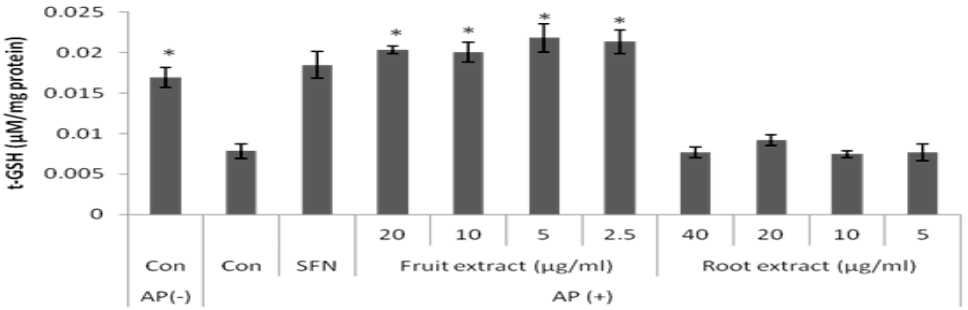

Fig. 3. Effects of the root and the fruit extracts on t-GSH.
Each value represents the mean ±S.D. of three independent experiments with triplicate.
Student's *t*-test compared with AP treated group: **p* < 0.005, ***p* < 0.001

The positive control - sulforaphane also induced the t-GSH level in HepG2 cells, but statistical significance was not observed. The root extract at its all dosages failed to increase t-GSH level in the cells stressed with AP.

QR enzyme activity was measured in Hepa1c1c7 cells treated with the fruit extract of *P. anomala* (Table1). The fruit extract showed 9.4 µg/ mL CD value, which is a concentration required to double QR specific activity, and chemopreventive index (21.3) of the extract was similar to that of sulforaphane (19.49).

Table 1. Effect of the fruit extract on QR activity

Treatments	CD[1]	IC_{50}[2]	CI[3]
SFN (µM)	0.67	13.1	19.49
Fruit extract (µg/ml)	9.4	>200	21.3

[1]Concentration required to double QR activity
[2]Concentration required inhibiting cell growth by 50%.
[3]Chemoprevention index is obtained by dividing IC_{50} value with CD value.

Compounds are abbreviated as followings; MG: methyl gallate, EG: ethyl gallate, EA: ellagic acid, PGG: 1,2,3,4,6-penta-*O*-galloyl-β-glucopyranoside, HGG: 6-m-digalloyl-1,2,3,4-tetra-*O*-galloyl-β-glucopyranoside, R: resveratrol, GGQ: 3-*O*-(6"-*O*-galloyl-β-glucopyranosyl)quercetin, GAQ: 3-*O*-(2"-*O*-galloyl-β-arabinopyranosyl)quercetin, GQ: 3-*O*-(β-glucopyranosyl)quercetin, L: luteolin, O: onopordin, MO: 3'-*O*-methylonopordin, PFG: paeoniflorigenone, GH: gnetin H, FB: fischeroside B, β-S: β-sitosterol, α-L: α-linolenic acid.

Activity of GPx and GR enzymes were measured in HepG2 cells. The fruit extract slightly induced GPx enzyme activity, especially at 10 µg/mL dose it showed the highest induction (Fig. 4). The root extract did not show any effect on GPx induction at its all doses. GR enzyme activity was enhanced by the fruit extract treatment, particularly, at its lower concentration, 2.5 µg/mL, it showed slightly inductive effect, but the root extract showed the effect only at its highest dose 40 µg/mL.

The protein level of anti-oxidative and detoxifying enzymes were detected in cell lysates of HepG2 cells treated with the fruit extract of *P. anomala* (Fig. 5A). GPx and SOD levels were increased by the treatment of the fruit extract at 20 µg/mL and 10 µg/mL doses. CAT level was slightly influenced by the treatment at 20 µg/mL dose. QR level was increased by 5 µg/mL of the fruit extract treatment. Transcription factor Nrf-2 protein level was significantly increased by the treatment of 20 µg/mL and 10 µg/mL dosages of the fruit extract along with the positive control sulforaphane (Fig.5B).

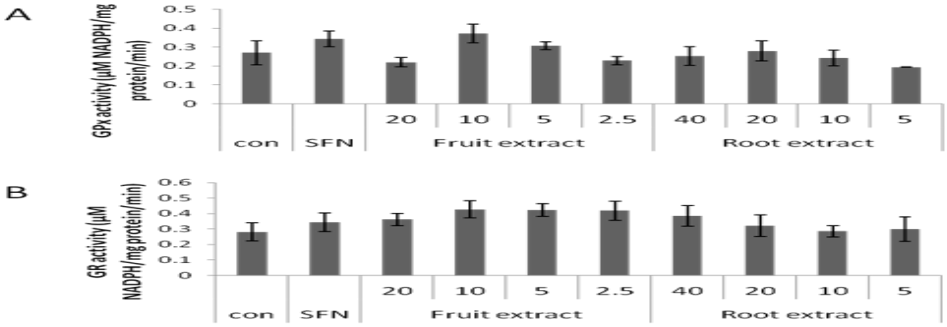

Fig. 4. Effect of the root and the fruit extracts on GPx and GR enzymes activity.
Each value represents the mean ±S.D. of three independent experiments with triplicate.

The protein level of anti-oxidative and detoxifying enzymes were detected in cell lysates of HepG2 cells treated with the fruit extract of *P. anomala* (Fig. 5A). GPx and SOD levels were increased by the treatment of the fruit extract at 20 µg/mL and 10 µg/mL doses. CAT level was slightly influenced by the treatment at 20 µg/mL dose. QR level was increased by 5 µg/mL of the fruit extract treatment. Transcription factor Nrf-2 protein level was significantly increased by the treatment of 20 µg/mL and 10 µg/mL dosages of the fruit extract along with the positive control sulforaphane (Fig. 5B).

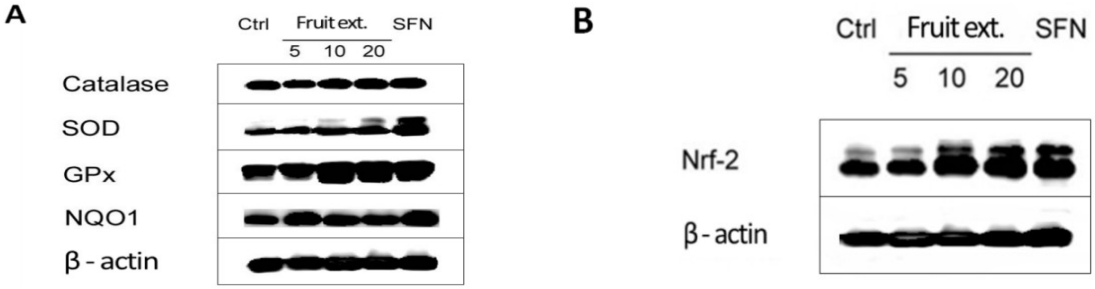

Fig. 5. A - Effect of the fruit extract on CAT, SOD, GPx and QR enzymes levels. B - Effect of the fruit extract on Nrf-2 protein level. The data represents the three independent experiments.

Cytoprotective effect of the fruit and the root extracts of *P. anomala* against AP-induced toxicity was investigated in this study. The results of the present study demonstrate that the pre-treatment of the fruit extract markedly prevented against the AP toxicity. However, the root extract showed a weak protection against AP induced toxicity, although strong protective effects were reported for root and cortex parts from *Paeonia* species such as Moutan Cortex, which was reported to have a protective effect against AP-induced toxicity in mouse [9]. A study on the mechanisms of AP-induced toxicity showed that GSH play a key role for the detoxification of AP and its highly reactive metabolites, and the liver necrosis results from the depletion of GSH [15]. Under the condition of AP overdose, the sulfation and glucuronidation pathways of AP metabolism are saturated in cells, which results to enhancement the generation of NAPQI from cytochrome P450 system. The increased generation of NAPQI conjugates to GSH, which triggers a depletion of GSH concentration in the cells and alters cellular redox state. Excessive NAPQI binds to proteins and DNA covalently, which further leads to necrosis. In this study, the pre-treatment of fruit extract retained t-GSH concentration in HepG2 cells, especially, at its lowest dose, 2.5 µg/mL, the fruit extract showed an excellent effect for enhancement of t-GSH in the cells treated by AP. But the root extract did not increase the t-GSH contents in HepG2 cells. This result is probably due to the up-regulation of AP metabolizing enzymes activity such as UTP and sulfotransferase and t-GSH synthesis by the fruit extract of *P. anomala*, and the root extract does not have this activity. The enzymes involved to the "safe" metabolism of AP are sulfotase and glucuronidase, which belong to a family called phase II detoxifying enzymes. Transcriptional activation of phase II enzymes is mediated by a cis-acting element called antioxidant-responsive element (ARE). The ARE has been detected in the promoter or upstream promoter region of the genes coding several anti-oxidative and detoxifying enzymes such as UGT, GPx,

GST and γ-glutamylcysteine synthatase, which is a rate limiting enzyme for the GSH synthesis [16]. A hallmark of phase II detoxification enzymes is QR, which can be measured easily and shows a large inducer response [8]. In this study, the QR enzyme activity was measured in Hepa1c1c7 cells treated with the fruit extract of *P. anomala*. The fruit extract concentration, which can induce QR by 2 folds (CD value) was 9.4 μg/mL, and the chemopreventive index (CI) of the extract (21.3) was almost similar to sulforaphane, which was 19.49. Also the fruit extract enhanced activities of GPx and GR enzymes, although the statistically significance was not observed. In addition, the protein levels of CAT, SOD, GPx and QR enzymes checked by western blotting assay were induced by the treatment of fruit extract of *P. anomala* in HepG2 cells. These results suggest that the fruit extract of *P. anomala* can be a potent inducer of phase II detoxification and anti-oxidative enzymes.

The induction of all known ARE-regulated genes is under the control of Nrf-2. Many genes encoding detoxifying, anti-oxidant and GSH-biosynthesis enzymes were found to be regulated by Nrf-2 [17]. Mouse lacking Nrf-2 was highly sensitive to AP: lower levels of AP provoked hepatotoxicity in homozygous *nrf2* knockout mice and such a high sensitivity was due to an insufficient expression of detoxifying enzymes. As a consequence, AP-conjugates accumulated in centrilobular hepatocytes causing necrosis [18]. In this study, the fruit extract enhanced the protein level of Nrf-2 in HepG2 cells in dose dependent manner.

The cytoprotective effect of the fruit extract of *P. anomala* could be attributed to a sum effects from its constituents. When the compounds isolated from the fruit extract were examined for their cytoprotective effect against AP-induced toxicity, methyl gallate, ethyl gallate, resveratrol, ellagic acid, luteolin, and quercetin glucosides showed the protecting effects. Flavonoids and polyphenols have been reported to induce phase II enzymes and GSH biosynthesis, which can be regulated by Nrf-2 [19]. Flavonoids and phenolics in the fruit extract showed good protective effect against *t*-BHP induced cell death through direct radical scavenging [20]. Thus, direct antioxidative effect by scavenging free radicals of the compounds also can have contributory effect to the cytoprotection by the fruit extract, since ROS also play an important role for AP toxicity. In addition, hexane fraction of the fruit extract, which was mainly consisted of α-linolenic acid and β-sitosterol showed a potent cytoprotective effect against AP induced toxicity. The protective effect of α-linolenic acid and β -sitosterol could not be tested due to their highly insolubility in water phase and stock solvent DMSO. Fish oil containing n=3 fatty acids reduced AP induced liver centrilobular necrosis, enhanced ALT level in serum, and increased AP-glucuronidation metabolism in mice [21]. Hence α-linolenic acid is a major n=3 fatty acid in fish oil, it can be speculated that α-linolenic acid can have a contribution to the cytoprotective effect of the fruit extract against AP toxicity.

According to the results of cell viability assay and t-GSH quantification, lower concentrations of the extracts showed more potent cell protective effects than higher doses, although the extract at its higher doses showed more apparent SOD, GPx and Nrf-2 induction activity. These results could be explained by pro-oxidative activity of flavonoids and polyphenolics. Flavonoids and polyphenolic are known to have electron-donating anti-oxidative properties however, flavonoid and polyphenolic metabolites have electrophillic activity and can covalently bind to GSH and DNA [22-24]. The major regulator of Nrf-2 is identified to be Keap1 (Kelch-like erythroid-cell-derived protein with CNC homology-associating protein 1), which repress Nrf-2 transcription activation by cytoplasmic sequestration and mediation of degradation of Nrf-2. Several mechanisms of Nrf2 activation resulting the release of Nrf-2 from Keap1 were proposed. One of proposed mechanisms is the direct reaction of oxidative compounds with the Keap1-Nrf-2 complex. Dimeric Keap1 contains multiple cysteine residues in each monomer, certain electrophiles can interact with reactive thiol groups of Keap1 promoters, resulting in intermolecular disulfide formation and conformational changes ultimately resulting Nrf-2 release. Therefore, the release of Nrf-2 from Keap1, leading to the activation of ARE-mediated gene transcription, is reported to be a redox dependent process and activated by ROS and/or electrophiles [23,25,26]. Thus, it can be hypothesized that the moderate concentration of electrophile metabolites from the polyphenolics and flavonoids in the fruit extract of *P. anomala* triggered the signal to induce phase II and anti-oxidative enzymes through Nrf-2 release from Keap1, which conformation was changed due to electrophile binding to its cysteine residues. But, when the concentration of electrophiles generated from flavonoids and polyphenolics are higher, the electrophiles can bind to GSH and can influence negatively to the redox balance of the cell. Therefore, it can be speculated that the lower concentrations of the fruit extract (below than 10 μg/mL) can be appropriate concentrations to protect liver cells against AP induced toxicity.

CONCLUSIONS

In conclusion, the fruit extracts of *P. anomala* exerted a potent protective effect against AP- induced toxicity in HepG2 cells via enhancing total GSH concentration and up-regulating anti-oxidative and detoxifying enzymes including SOD, CAT, QR, GPx and GR.

Furthermore, the fruit extract increased the protein level of a crucial transcription factor, Nrf-2, in HepG2 cells. However the root extract showed a weak protective effect against AP induced cytotoxicity. Therefore, the fruit extract of *P. anomala* can be a promising candidate for development of the functional food or other supplements to prevent the liver from xenobiotics such as AP-induced toxicity.

REFERENCES

1. Tone Y., Kawamata K., Murakami T., Higashi Y., Yata N. (1990) *Journal of pharmacobio-dynamics,* **13**, 327-335.
2. Dahlin D.C., Miwa G.T., Lu A.Y., Nelson S.D. (1984) *Proc. Natl. Acad. Sci.,* **81**, 1327-1331.
3. Chen W.Q., Koenigs L.L., Thompson S.J., Peter R.M., Pettie A.E., Trager W.F., Nelson S.D. (1998) *Chem.Res.in Tox.,* **11**, 295-301.
4. Bessems J.G.M., Vermeulen N.P.E. (2001) *Crit.Rev.in Tox.* **31**, 55-138.
5. Nakamura, W., Hosoda, S., Hayashi, K. (1974) *Biochimica Et Biophysica Acta* **358**, 251-261.
6. Dai Y., Cederbaum A.I. (1995) *Journal of Pharmacology and Experimental Therapeutics,* **273**, 1497-1505.
7. Devries J. (1981) *Biochemical Pharmacology,* **30**, 399-402.
8. Talalay P., Fahey J.W., Holtzclaw W.D., Prestera T., Zhang Y.S. (1995) *Toxicology Letters* **82-3**, 173-179.
9. Shon Y.H., Nam K.S. (2004) *J. Ethnopharmacol,* **90**, 415-419.
10. Kim B.R., Hu R., Keum Y.S., Hebbar V., Shen G.X., Nair S.S., Kong A.N.T. (2003) *Cancer Research,* **63**, 7520-7525.
11. Tietze F. (1969) *Analytical biochemistry,* **27**, 502-522.
12. Flohe L., Gunzler W.A. (1984) *Methods in Enzymology,* **105,** 114-121.
13. Fahey J.W., Dinkova-Kostova A.T., Stephenson K.K., Talalay P. (2004), *PtB,* **382**, 243-258.
14. Kang Y.H., Pezzuto J.M. (2004) *Pt B,* **382**, 380-414.
15. James L.P., Mayeux P.R., Hinson J.A. (2003), *Drug Metabolism and Disposition,* **31**, 1499-1506.
16. Mulcahy R.T., Gipp J.J. (1995) *Biochem. Bioph. Res. Co.,* **209**, 227-233.
17. Kobayashi M., Yamamoto M. (2006) *Advances in Enzyme Regulation Proceedings,* **46**, 113-140.
18. Enomoto A., Itoh K., Nagayoshi E., Haruta J., Kimura T., O'Connor T., Harada T., Yamamoto M. (2001) *Toxicological Sciences, **59***, 169-177.
19. Na H.K., Surh Y.J. (2008) *Food and Chemical Toxicology,* **46**, 1271-1278.
20. Oidovsambuu S., Kim C.Y., Kang K.S., Dulamjav B., Jigjidsuren T., Nho C.W. (2013) *Planta Medica* ,**79**, 116–122
21. Speck R.F., Lauterburg B.H. (1991) *Hepatology,* **13**, 557-561.
22. Lee-Hilz Y.Y., Boerboom A.M.J.F., Westphal A.H., van Berkel W.J.H., Aarts J.M.M.J.G., Rietjens,I.M.C.M. (2006) *Chemical Research in Toxicology,* **19**, 1499-1505.
23. Talalay P. (2000) *Biofactors,* **12**, 5-11.
24. Van der Woude H., Boersma M.G., Alink G.M., Vervoort J., Rietjens I.M.C.M. (2006) *Chem-Biol. Interact.,* **160**, 193-203.
25. Dinkova-Kostova A.T., Holtzclaw W.D., Wakabayashi N. (2005) *Biochemistry,* **44**, 6889-6899.
26. Velichkova M., Hasson T. (2005) *Mol. Cell. Biol.,* **25**, 4501-4513.