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Anti-oxidant and Hepatoprotective Effect of White Ginsengs in H_2O_2-Treated HepG2 Cells

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Abstract – The antioxidant activity of white ginseng was not recorded in Korea Functional Food Code, while its activity of red ginsengs was recorded. The aim of this study was to evaluate the antioxidant and hepato protective effect of different ginsengs in H_2O_2-treated HepG2 cells. White and red ginseng were prepared from longitudinal section of the same fresh ginseng (4-year old). The whole parts of white and red ginsengs were separately extracted with 70% ethanol and distilled water respectively, at 70 ºC to obtain therapeutic ginseng extracts namely, WDH (distilled water extract of white ginseng), WEH (70% ethanol extract of white ginseng), RDH (distilled water extract of red ginseng) and REH (70% ethanol extract of red ginseng). In this work, we have investigated the DPPH, hydroxyl radical, Fe^{2+}-chelating activity, intracellular ROS scavenging capacity and lipid peroxidation of different ginsengs. All these extracts showed a dose dependent free-radical scavenging capacity and a ROS generation as well as lipid peroxidation was significantly reduced by treatment with bioactive extracts of white ginsengs (WDH) than red ginsengs. Additionally, white ginseng extracts (WDH) has dramatically increased intracellular antioxidant enzyme activities like superoxide dismutase and catalase in H_2O_2-treated HepG2 cells. All these results explain that administration of white ginseng is useful as herbal medicine than red ginseng for chemoprevention of liver damage.
Keywords – White ginseng, Antioxidant, Cell viability, Chemoprevention

Introduction

Oxidative stress has played a vital role for the variety of pathophysiological conditions including cardiovascular dysfunctions, atherosclerosis, inflammation, carcinogenesis, reperfusion injury and several neurodegenerative diseases.[1] A state of oxidative stress has been defined as the increasing level of reactive oxygen species (ROS) and/or a decrease in the level of antioxidant defense mechanisms.[2] ROS are also called as free radicals, including superoxide radicals, hydroxyl radicals and hydrogen peroxide are often produced as by-products of the biological reactions and mostly removed by endogenous antioxidants such as superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and catalase (CAT). Biologically, some of this free radical is important for body's normal metabolic process. However, their increased generation has been shown to cause damage to cell membranes, DNA, liver (fatty liver, hepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma), inducing oxidation that causes membrane lipid peroxidation, decreased membrane fluidity, DNA mutations leading to cancer and various neurodegenerative diseases.[3] Hepatocellular carcinoma is predominantly considered as the fifth most common cancer in the world and being countable for 4.1% of newly diagnosed human cancers. A large number of deaths by lung, stomach, and liver cancer in 2006 were noticeable - as large as, that cardiovascular and stroke disease is no longer responsible for the top cause of death.[4] Most importantly, an incidence of post recurrence among these critical cases is between 20 - 25%, developing a novel therapeutic and chemopreventive nondrug materials which target anti-ROS stress could be exerts an antioxidant property in the protection of liver damage and various metabolic syndromes.[5] For all these reasons, the interest in health/functional foods which have antioxidant property particularly ginseng, may be helpful for the purpose of reducing the risk of oxidative damage and increasing the healthcare promotion.[6]

Ginseng has been considered as one of the most widely

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used natural tonic and being strongly acclaimed as medicinal herbs in the world. Many researchers have confirmed that ginseng roots possess antioxidative, anti-diabetic, neuroprotective, anti-tumor and hepatoprotective effects.[7-9] There are two different types of ginseng (white and red) have particularly been used for commercial applications. A steamed and dried (red) ginseng has been considered to be biologically more active than simply dried (white) ginseng for the notable respects. For example, the antioxidant activity was only recorded in red ginsengs of Korea Functional Food Code.

Enormous differences in the white and red ginsengs with respect to their biological efficiency are due to the structural and chemical changes of ginsenosides after the steaming process.[10] In normal steaming condition, ginsenosides are partially converted to deglycoslated derivatives that possess higher anti-cancer activity (e.g., ginsenoside Rg3, Rg5 and Rh2).[11] Many researcher have reported that red ginseng is long-term preservable and has various bioactivities than white ginseng. Notably, red ginseng has been exerted a protective effects on hepatotoxicity induced by carbon tetrachloride (CCl_4), high fat diets, and alcohol in animal models consumption has been largely growing in East Asia particularly in South Korea.[12]

Though few researchers have demonstrated that ginseng roots were considerably effective to improve hepatic damages due to toxic substances, however a comparative understanding between different extracts of red and white ginsengs on hepatoprotective effect is not clearly studied. Therefore, our present study has demonstrated that pharmacological differences with respect to antioxidant property between white ginseng and red ginseng on hydrogen peroxide (H_2O_2) - induced oxidative stress in HepG2 hepatoma cells.

Experimental

Chemical reagents – All chemicals for the extraction from different red and white ginseng extracts, 1,1-diphenyl-2-picryl hydrazyl (DPPH) and 20,70-dichlorofluorescein diacetate (DCF-DA) were obtained from Sigma Chemicals Co. (St. Louis, USA). All other reagents were of the highest quality generally available.

Sample preparation – Korean fresh ginseng (*Panax ginseng* C.A. Meyer, 4-year old) was purchased from local market (Geumsan, Korea). White and red ginsengs were prepared from longitudinal section of the same fresh ginseng. A part of fresh ginseng was dried at 50 ºC for 4 days in air dryer for white ginseng and another part of same fresh ginseng was dried prior to steaming at 90 ºC for 6 h in the case of red ginseng. Roots of white and red ginseng (100 g) were extracted separately with 70% ethanol and distilled water for three times at 70 ºC along with high and room temperature using microwave for 4 h. The ethanol and water extract were filtered and concentrated *in vacuo* to dryness. The extracts of four white and red ginseng extracts were named as WDH (white ginseng extract by distilled water at 70 ºC), WEH (white ginseng extract by ethanol at 70 ºC), RDH (red ginseng extract by distilled water at 70 ºC) and REH (red ginseng extract by ethanol at 70 ºC). Each 20 mg of white and red ginseng extract were dissolved in water and filtrated for further experiment.

Analysis of ginsenosides – Ten mg of white and red ginseng extract were melted by 1 ml of methanol (MeOH) and filtered out by 0.45 μm membrane filter after extraction with ultrasonic waves for 30 min, and then finally analyzed in HPLC. The HPLC system was Waters 1525 (Waters, USA) with PDA detector (Water, 2998). Waters X-bridgeTM C18 column (250 mm × 4.6 mm, 5 μm, Waters, USA) was also used. The detection wavelength, flow rate, injection volume, and column oven temperature were set at 203 nm, 1.0 ml/min, 20 μl and 40 ºC, respectively. The mobile phase consisted of purified water (A) and acetonitrile (B) using the following gradient program: 0 min 18% B, 0 - 42 min 24% B, 42 - 46 min 29% B, 46 - 75 min 40% B, 75 - 100 min 65% B, 100 - 135 min 85% B, and 135 - 180 min 18% B.

DPPH radical scavenging activity – The antioxidant activity of the extracts was evaluated first by monitoring its ability in quenching the stable free radical DPPH.[13] The reaction mixture containing 100 μl of varying concentrations of the test samples (125, 250, 500 and 1000 μg/ml dissolved in DMSO) and 100 μl of 135 μM DPPH ethanol solution in a 96 well plate. The reaction mixture was vortexed and left in the dark for 30 min at 37 °C. The absorbance of the mixture was measured at 515 nm according to the increasing concentrations of the extracts. All determination was carried out in triplicate. Ascorbic acid was used as a positive control. The percentage inhibition of the DPPH free radical was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = ((A_0 - A_1)/A_0) \times 100.$$

Where, A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the test sample. The actual decrease in absorption induced by the test was compared with the positive controls.

Hydroxyl radical scavenger assay – Hydroxyl radical scavenging ability was measured according to a literature

procedure[14] with a few modifications on 96 well plates. Hydroxyl radical was generated from Fenton reaction between 1.5 mM $FeSO_4$ and 1 mM H_2O_2 (1.4:1, *v/v*) at 37 °C for 30 min before the assay and detected by their ability to hydroxylate salicylate. The reaction mixture contained 152 μl of hydroxyl radical, 100 μl of varying concentrations of the test samples (125, 250, 500 and 1000 μg/ml dissolved in DMSO) and 50 μl of sodium salicylate (20 mM). After a reaction for 1 h at 37 °C, the absorbance of the hydroxylated salicylate complex was measured at 562 nm. Hydroxyl radical scavenging ability was calculated from the percentage of inhibition. All determination was carried out in triplicate. Ascorbic acid was used as a positive control. The percentage inhibition of the hydroxyl radical scavenger ability was calculated using the following equation:

$$\text{Hydroxyl radical scavenging ability } (\%) = ((A_0 - A_1) / A_0) \times 100.$$

Where, A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the test sample. The actual decrease in absorption induced by the test was compared with the positive controls.

Fe^{2+}-chelating activity assay – This assay was measured according to a literature procedure [15] with a few modifications on 96 well plates. The reaction mixture contained 24 μl of 2 mM $FeCl_2$, 100 μl of varying concentrations of the test samples (125, 250, 500 and 1000 μg/ml dissolved in DMSO) and 128 μl of distilled water. The mixture was shaken vigorously and left at room temperature for 30 min. After 30 min, 30 μl of 5 mM ferrozine was added and mixed. The absorbance of the Fe^{2+}-ferrozine complex was measured at 562 nm. Fe^{2+}-chelating activity assay was calculated from the percentage of inhibition. All determination was carried out in triplicate. Deferoxamine was used as a positive control. The percentage inhibition of the Fe^{2+}-chelating activity was calculated using the following equation:

$$Fe^{2+}\text{-chelating activity ability } (\%) = ((A_0 - A_1) / A_0) \times 100.$$

Where, A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the test sample. The actual decrease in absorption induced by the test was compared with the positive controls.

Cell culture – HepG2 hepatoma cells were obtained from Korean Cell Line Bank (South Korea) and maintained in minimum essential medium (MEM, high glucose) supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C.

Cell viability – The HepG2 hepatoma cells (Korea Cell Line Bank) were grown in minimum essential medium supplemented with 10% (*v/v*) fetal bovine serum (FBS) (Hyclone, Logan, UT) and 10 μg/ml gentamycin at 37 °C in an incubator at 5% carbon dioxide (CO_2). Cells were first grown on a 96-well plate at a density of 1×10^4 cells/well overnight before treatment. After overnight culture, the concentrations ranging from 125 to 1000 μg/ml were used to study cytotoxic test of both red and white ginseng extracts and 1 mM of hydrogen peroxide was applied to the cells in 100 μl of MEM/10% FBS, and cells were incubated for additional 24 h at 37 °C. After 24 h of sample treatment to cells, 10 μl of water soluble tetrazolium salts (WST-1) was added and incubated for 1 h at 37 °C. The absorbance of the resulting formazan product converted by the viable cells was read in an enzyme-linked immunosorbent assay (ELISA) plate reader at 450 nm with a 620 nm reference. Cell viability was expressed as a percentage of the absorbance seen in the untreated control cells.

Intracellular ROS scavenging activity – Intracellular ROS scavenging activity was carried out by DCF-DA which is used to detect the levels of intracellular ROS. HepG2 cells were seeded on 96-well plate at 1×10^4 cells/well. Sixteen hours after plating, the cells were treated with varying concentrations of the test samples (125, 250, 500 and 1000 μg/ml dissolved in DMSO) and 30 min later, 1 mM of H_2O_2 was added to the plate for 1 h. After 1 h, 100 μM of DCF-DA solution was added for 45 min and then the fluorescence of 2, 7-dichlorofluorescein was detected at 485 nm excitation and at 535 nm emission using the spectrofluorometer. For the image analysis for the generation of intracellular ROS, HepG2 cells were seeded on a six-well plate containing a coverslip at 1×10^4 cells/well. Sixteen hours after plating, the cells were treated with WDH extracts (1000 μg/ml dissolved in DMSO) and 30 min later, 20 mM of H_2O_2 was added to the plate for 1 h. After 1 h, the medium was changed, 300 μM of DCF-DA was added to each well and then the plate was incubated for an additional 30 min at 37 °C. Next, after washing with PBS, the stained cells were mounted onto a microscope slide in a mounting medium. The images were collected using a confocal microscope.

Superoxide anion scavenging activity (SOD) assay – The enzymatic antioxidant activity of the extract was determined using the SOD assay Kit-WST purchased from Sigma-Aldrich. The different concentrations of the test samples (125, 250, 500 and 1000 μg/ml dissolved in DMSO) was used for the experiment. This assay was done using 96 wells micro titer plate. Sample solution (20

μl) was added to sample well and blank well, and 20 μl of dd H_2O (doubled distilled water) was added to $blank_1$ and $blank_3$ wells. A chemical such as, WST (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) working solution (20 μl) was then added to each well and 20 μl of enzyme working solution was added to the sample well and the $blank_1$ well. The resultant mixtures were then mixed thoroughly. The plate was then incubated at 37 °C for 20 min. After incubation, the absorbance was read at 450 nm using an ELISA microplate reader. The superoxide anion scavenging activity was calculated according to the following equation:

$$\text{SOD activity (inhibiton rate,\%)} = \{[(Ablank_1 - Ablank_3) - (Asample - Ablank_2)] / (Ablank_1 - Ablank_3)\} \times 100$$

Where $Ablank_1$, $Ablank_2$, $Ablank_3$ and Asample are absorbances of $blank_1$, $blank_2$, $blank_3$, and sample wells. One unit of SOD activity was defined as the amount of enzyme having a 50% inhibitory effect on WST-1. The experiment was conducted in triplicates.

Western Blotting Analysis – The whole cell extracts for immunoblotting, SOD and catalase were isolated from H_2O_2-stimulated HepG2 cells. These sample proteins were separated in a 12% polyacrylamide gel at 100 V and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA) using a semi-dry transfer system. The membranes were blocked with 5% non-fat dry milk in 1 × PBST buffer (0.1% Tween 20 in PBS) overnight at 4 °C and incubated with rabbit polyclonal antibody in PBST solution containing 5% non-fat dry milk. After washing 3 times with PBST, the membranes were incubated for 1 h at room temperature with anti-rabbit atibodies with horseradish peroxidase for 3 h at room temperature, and washed with PBST 3 times. Final detection was performed with enhanced chemiluminescence (ECLTM) Western blotting reagents (Santa Cruz).

Lipid peroxidation assay – This assay was carried out according to the literature procedure[16] with some modifications. The HepG2 cells were cultured in a 6-well plate at 1×10^4 cells/well for 16 h. Sixteen hours after plating, the cells were treated with the varying concentrations of test samples (125, 250, 500 and 1000 μg/ml dissolved in DMSO) for 30 min. After 30 min, 1 mM H_2O_2 and $FeCl_2$ were added to the plate and incubated for 12 h. The cells were then washed with cold phosphate-buffered saline (PBS), harvested, and homogenized in an ice cold 1.15% KCl. One hundred microlitre of the cell lysates were mixed with 0.1 ml of 8.1% sodium dodecylsulfate, 0.75 ml of 20% acetic acid (adjusted to pH 3.5), and 0.75 ml of 0.8% thiobarbituric acid (TBA). The mixture was made up to a final volume of 4 ml with distilled water and heated to 95 °C for 2 h. After cooling to room temperature, 2.5 ml of an *n*-butanol/pyridine mixture (15:1, *v/v*) was added and the mixture was shaken. After centrifugation at 1000 g for 10 min, the supernatant fraction was isolated and the absorbance was measured spectrophotometrically at excitation wavelength of 532 nm and emission wavelength of 553 nm using ELISA microplate reader.

Statistical analysis – Data is expressed as mean ± SD. Statistical analysis were performed using SPSS version 20. One-way ANOVA followed by Duncan's multiple comparison was used to compare the values among samples. A p value < 0.05 was regarded as indicating significant differences. Each treatment was replicated 3 times and each experiment was repeated at least twice. Different letters (a - g) showed significant difference ($p < 0.05$) from all samples and control.

Results and Discussion

Distribution of ginsenoside components – In order to determine the amount (mg/g) of different ginsenosides in ginseng extracts, we performed HPLC analysis. Major ginsenosides (in order) were obtained as, Rb1 > Rc > Re > Rb2 > Rg1 > Rd > Rb3 > S-Rg3 > R-Rg3 > Rh4. Earlier studies reported that these ginsenosides were possess beneficial effect in terms of antioxidation and anti-carcinogenesis. Rb1 and Rg1 are the most pharmacologically active components. Interestingly, ginsenoside Rb1 prompted the release of nitric oxide (NO) in rat ventricular myocytes and depressed tumor necrosis factor-α (TNF-α) in human endothelial cells in order to protect against cardiovascular disease.[17] Ginsenoside Re, also showed an antioxidative effect in cardiomyocytes and a neuroprotective effect in PC12 cells.[18] Experimentally, Kang *et al*. reported that lipopolysaccharides in ginseng are more active antioxidants than saponin as a scavenger of such free radicals as H_2O_2 and O_2.[19] Even though there have been few *in vivo* and *in vitro* studies regarding liver damage, previous studies of ginsenoside on antioxidant actions in other cells can be applied. In this study, the total amounts (mg/g) of all the ginsenosides in different white and red ginsengs were significantly different. Among them, ethanol extract of white ginsengs possesses higher contents of ginsenosides than red ginsengs. Conversely, ginsenosides contents in red and white ginsengs of water extracts were shown similarity (Table 1).

Anti-oxidant activity of the different ginseng extracts – The antioxidant activities of the extracts from red (RDH, REH) and white (WDH, WEH) ginseng extracts

Table 1. Distribution of various ginsenosids in different white and red ginsengs.

Different ginseng extracts	Ginsenoside content (mg/g)											
	Rg1	Re	Rf	Rb1	Rc	Rb2	Rb3	Rd	Rh4	S-Rg3	R-Rg3	Total
WDH	8.4	7.4	2.2.	14.9	10.1	5.2	0.7.	2.2	ND	ND	ND	51.1
WEH	7.9	13.1	3.9	26.8	17.2	12.2	1.8	4.6	ND	ND	ND	87.3
RDH	6.7	8.0	2.4	18.0	10.7	7.6	1.1	2.6	0.1	0.3	0.1	57.6
REH	6.7	8.2	3.3	27.1	17.2	10.5	1.5	3.5	0.8	1.3	0.9	80.9

ND: not determined; WDH: white ginseng extract by distilled water at 70 °C; WEH: white ginseng extract by ethanol at 70 °C; RDH: red ginseng extract by distilled water at 70 °C; REH: red ginseng extract by ethanol at 70 °C.

were evaluated by DPPH radical, hydroxyl radical scavenging and Fe^{2+}-chelating assay in non-cellular system. An important function of an antioxidant is to remove free radicals. The mechanism for antioxidants to remove free radicals involves donating hydrogen to a free radical and hence its reduction to an unreactive species through removing the odd electron feature which is responsible for radical reactivity.[20] In DPPH radical scavenging assay, WDH (white ginseng extract by distilled water at 70 °C) extract had a strong scavenging activity which removed DPPH radical by 51.2% at 125 μg/ml, 53.5% at 250 μg/ml, 57.4% at 500 μg/ml, 65.0% at 1000 μg/ml, showing an excellent scavenging activity of DPPH radical than the other extracts (Fig. 1A). The order obtained in relation with the DPPH scavenging activity was significantly conferred increasing activity as WDH > REH > WEH > RDH. Hence, WDH extract showed the higher level of radical scavenging as the concentration increased, while others show moderate antioxidant properties.

Hydroxyl radical scavenging is an important antioxidant activity because of very high reactivity of hydroxyl radicals which enables it to react with a wide range of molecules found in living cells such as sugars, amino acids, lipids and nucleotides. Hydrogen peroxide itself is not very reactive, but it can sometimes be toxic to cells, since it may give rise to hydroxyl radicals inside the cell. The Fenton reaction between Fe^{2+} and hydrogen peroxide generates Fe^{3+} and hydroxyl radical.[21] The hydroxyl radical generated from the Fenton reaction can cause oxidative DNA damage that has been shown to play a key role in carcinogenesis. With this assay, WDH extracts showed a significantly higher scavenging activity which scavenged the hydroxyl radical by 15.6% at 125 μg/ml, 16.5% at 250 μg/ml, 16.8% at 500 μg/ml, 18.3% at 1000 μg/ml, respectively (Fig. 1B). On the basis of hydroxyl radical scavenging assay, the antioxidant activity of the investigated extracts showed significant increase in accordance with the followed order; WDH > REH > WEH > RDH. The extracts which showed the strongest hydroxyl radical scavenging activity is WDH, whereas other extracts showed lower activity; thereafter, hydroxyl radical scavenging activity increased with the concentration of the extract.

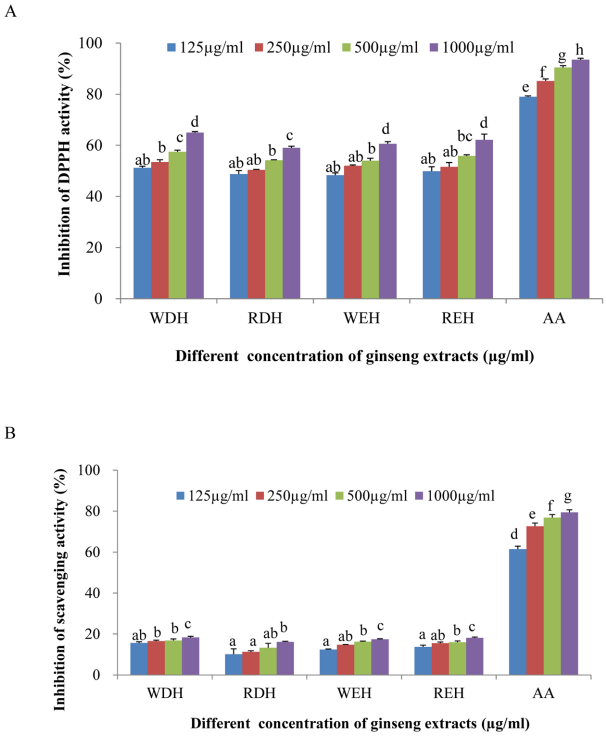

Fig. 1. DPPH free radical (A) and hydroxyl radical scavenging activities (B) of the extracts from different red and white ginseng extracts. Values are means ± SD; $n = 3$. AA: Ascorbic acid used as positive control.

Iron and copper are essential transition metal elements in the human body required for the activity of a wide range of enzymes and for some proteins that are involved in cellular respiration, O_2 transport and redox reactions. Unfortunately, they contain unpaired electrons that enable them to participate in one-electron transfer reactions. Hence, they are powerful catalysts of auto-oxidation reactions. With this assay, WDH extracts strongly chelated Fe^{2+} by 19.1% at 125 μg/ml, 26.5% at 250 μg/ml, 29.6% at 500 μg/ml, 42.1% at 1000 μg/ml, respec-

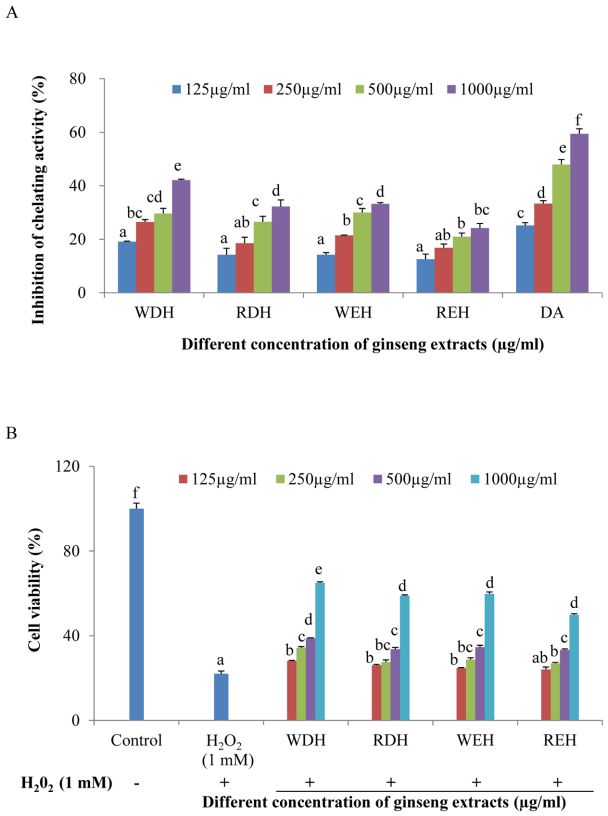

Fig. 2. Fe^{2+}-chelating activities (A) and cell viability (B) of HepG2 cells by treatment with different red and white ginseng extracts. Values are means ± SD; $n = 3$. DA: Deferoxamine is used as positive control.

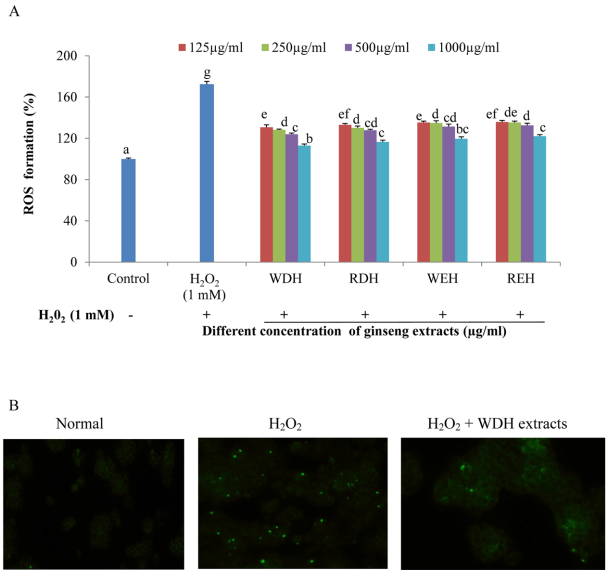

Fig. 3. Effect of the extract from different red and white ginseng extracts on scavenging intracellular ROS (A) and the data plotted as the mean of replicate inhibitory effects (%) values ± S.D. (n = 3). The intracellular ROS generated was detected by the DCF-DA method (B). Representative confocal images illustrate the increase in green fluorescence dots of DCF produced by ROS in H_2O_2 treated cells as compared to the control and of the cells treated with WDH (1000 μg/ml) extract in presence of H_2O_2.

tively (Fig. 2A). Hence, the reducing power of each extracts in decreasing order was significantly followed as WDH > WEH > REH > RDH. Hence, WDH extracts showed the significant increase in Fe^{2+}-chelating activity as concentration increased than other extracts. Epidemiologic studies have shown the effectiveness of antioxidants in reducing the risks of cancer and other diseases.[22] ROS damage can be reduced by two antioxidant factors such as scavenging of radicals formed during reaction and inhibiting the radical generation. The results for scavenging DPPH radical, hydroxyl radical and Fe^{2+}-chelating activity suggest that WDH can reduce ROS damage by scavenging generated radical during the reaction.

Cell viability – As shown in Fig. 2B, in cell viability assay, the treatment of hydrogen peroxide caused the cell death by about 22% while the different extract treatment prevented the cells from the death. Our ginseng samples not showed cell toxicity even the concentration of 1000 μg/ml in H_2O_2-treated HepG2 cells. These results suggest that all of white and red ginseng extract may strongly prevent cell death caused by ROS and have a cancer chemopreventive effect through inhibiting lipid peroxidation that function as tumor initiator. On the basis of cell viability assay, the percentage of live cell viability of the investigated extracts have significantly followed the order; WDH > WEH ≥ RDH ≥ REH.

Intracellular ROS scavenging activity of the different ginseng extracts – Intracellular ROS scavenging activity of the extracts was done by using DCF-DA staining (Fig. 3A). The oxidative damage caused by ROS may generate various diseases in humans such as aging, arthritis, cancer, inflammation, and heart diseases.[23] Hydrogen peroxide (H_2O_2) is produced endogenously from microsomes and peroxisomes and also by several physiological processes such as inflammatory respiratory burst and oxidative phosphorylation. H_2O_2 has often been used as a model to investigate the mechanism of cell injury by oxidative stress.[23] When HepG2 cells were challenged with 1 mM H_2O_2, ROS were generated at higher level compared to the unchallenged control and the pretreatment with different ginseng extracts dose-dependently decreased the H_2O_2-mediated ROS formation. Among the ginseng extracts WDH extracts showed a statistically significant inhibitory activity of ROS formation was 130.8% at 125 μg/ml, 128.2% at 250 μg/ml, 123.9% at 500 μg/ml, 112.99% at 1000 μg/ml, respectively. These results imply that WDH may have an ability to directly scavenge ROS and/or free radicals. As shown in Fig. 3B, the treatment of the WDH

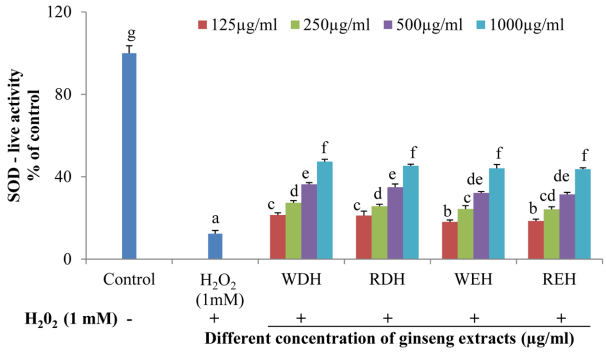

Fig. 4. Effects of different ginseng extracts on superoxide dismutase (SOD)-like activities in H_2O_2-treated HepG2 cells. Data are mean ± SD; $n = 3$.

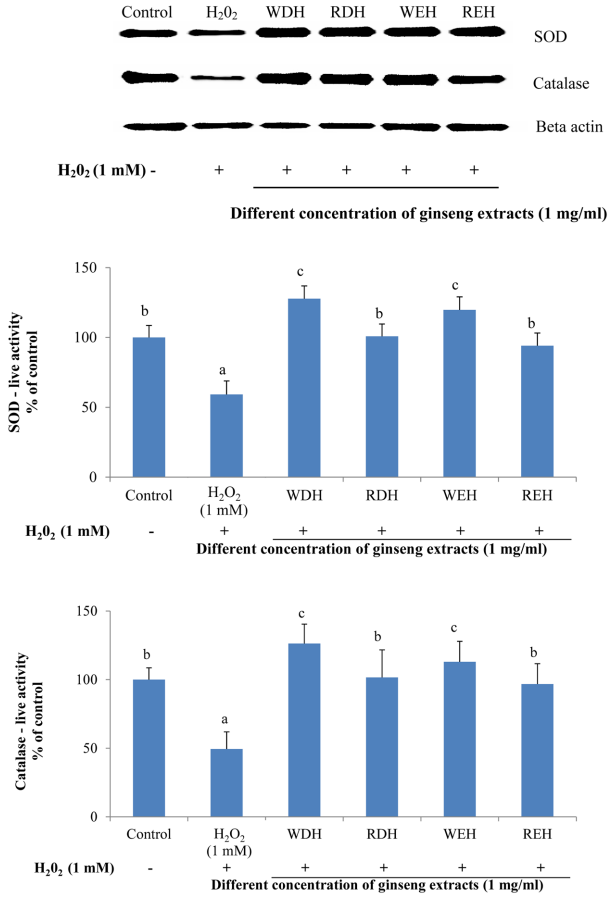

Fig. 5. Western blotting analysis of different ginseng extracts (1 mg/ml) on SOD and catalase protein expression. Data are mean ± SD; $n = 3$.

extracts (1000 μg/ml) reduced the green fluorescence upon H_2O_2 treatment alone, which reflects a reduction of ROS generation. It is evident from these results that endogenous antioxidants of WDH may be useful in preventing the deleterious consequences of oxidative stress and candidates for the prevention of oxidative damage caused by ROS.

Induction of super oxide dismutase and catalase activities in HepG2 Cells – In order to investigate whether antioxidant properties of different ginseng extracts are related to the activity induction of super oxide dismutase enzymes, HepG2 cells were treated with various ginseng extracts and the activities of antioxidant enzymes like SOD was measured. Super oxide dismutase enzyme has been regarded as the first line of the antioxidant defense system against ROS generated during oxidative stress.[24] As shown in Fig 4, the activities of the superoxide dismutase enzyme were dramatically down-regulated by H_2O_2 (1 mM) treatment, while treatment with ginseng extracts restored the enzymes activities in a dose dependent manner. Therefore, the ginseng extracts appears to have antioxidant properties by stimulating the activity of endogenous antioxidant enzymes as well as by directly scavenging ROS/free radicals. Among the ginseng extracts WDH extracts significantly showed an increased level of superoxide dismutase enzyme activity at a concentration of 21.4% at 125 μg/ml, 27.3% at 250 μg/ml, 36.3% at 500 μg/ml, 47.3% at 1000 μg/ml, respectively. On the basis of SOD assay, the percentage induction of SOD live activity of the investigated extracts followed the order significantly as; WDH > RDH > WEH > REH. Hence, WDH extracts showed the significant SOD induction activity as the concentration increased, whereas other extracts conferred lower induction activity. As shown in Fig. 5, the activities of the SOD and catalase antioxidant enzymes were dramatically down-regulated by H_2O_2 (1 mM) treatment, while treatment with WDH extract (1 mg/ml) restored the enzymes activities than other extract. Recently, a water-soluble ginsenoside Rb1 was reported to prevent H_2O_2-induced HUVECs senescence through upregulating endogenous antioxidants like SOD and catalase and decreasing vascular lipid peroxidation.[24] This study confirmed for the first time the antioxidant properties of the different red and white ginseng byproducts.

Inhibitory effect of the extracts on the lipid peroxidation caused by H_2O_2 – To evaluate the inhibitory effect of the extracts from different red and white ginsengs on the oxidative cell damage caused by H_2O_2, lipid peroxidation was measured as the formation of MDA in the homogenates of HepG2 cells (Fig. 6). A normal biological system contains a large number of degradation products, such as MDA, are generated and this is found to be an important cause of cell membrane destruction and cell damage.[25] On the basis of lipid peroxidation assay, the percentage inhibition of lipid peroxidation of the investigated extracts followed the

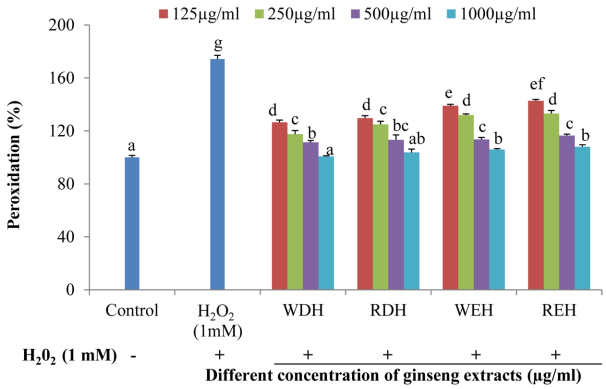

Fig. 6. Lipid peroxidation of HepG2 cells by treatment with different red and white ginseng extracts. The absorbance values were converted to inhibitory effects (%). Finally all the data plotted as the mean of replicate inhibitory effects (%) values ± 1 S.D. (n = 3).

order; WDH > RDH > WEH > REH. In this assay, the WDH extracts has significantly reduced the lipid peroxidation induced by H_2O_2 at 126.5% at 125 μg/ml, 117.5% at 250 μg/ml, 111.4% at 500 μg/ml, 100.7% at 1000 μg/ml, respectively compared with the cells treated with H_2O_2 and $FeSO_4$. Although H_2O_2 is potentially cytotoxic, H_2O_2 generates hydroxyl radical by the reaction between H_2O_2 and transition metal ions such as Fe^{2+} and Cu^{2+} in biological systems. This hydroxyl radical can react with a number of target molecules including proteins, membrane lipids and DNA. Moreover, oxidation of lipids induced by the hydroxyl radical can generate end-products, such as malondialdehyde and unsaturated aldehydes, that can bind to DNA to generate mutagenic adducts.[25] From this result, it is thought that the extracts from white ginseng display a significant protective capability against H_2O_2-induced cell damage.

In conclusion, this study affirmed for the first time the antioxidant properties of the different red and white ginseng byproducts. The present findings indicate that the extracts from white ginsengs (WDH) statistically exhibit powerful antioxidant properties, expressed by its capacity to scavenge DPPH radical, hydroxyl radical and intracellular ROS, and to chelate Fe^{2+} anion. And the extracts reduce H_2O_2-induced lipid peroxidation in the human HepG2 cells via its antioxidant activities. Our data strongly imply that white ginseng (WDH) may participate in cellular protection not only directly as an antioxidant molecule but also indirectly as a stimulator of antioxidant enzymes. Furthermore, white ginseng rather than red ginseng may be useful as a functional biomaterial for the dietary supplement with strong antioxidant activity and hepatoprotective effect. These antioxidant activities and inhibitory effects of the extracts on lipid membrane and cell damage may further explain that white ginsengs (WDH) are useful as an herbal medicine for cancer chemoprevention. Additionally, our findings will require further study in animal experiment which should be carefully designed to optimal dose, duration and administration method need to be optimized for their commercialization.

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