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Antioxidant Properties of Ginseng (*P. ginseng* C.A. Meyer) Extracts by Organic Solvent Fractionation

– Research Note –

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Abstract

The objective of this study was to investigate antioxidant activities of *Panax ginseng* extracted with various solvents including *n*-hexane, chloroform, EtOAC, *n*-butanol and water. Among the various ginseng extracts, ethyl acetate (EtOAC) extracts showed the most powerful scavenging activities against DPPH radicals. Among the other solvent extracts, the butanol extract seemed relatively more effective in scavenging activity, followed by chloroform, water and hexane extracts. Moreover, the highest reducing power and ferrous ion chelating activity were found in the EtOAC extract followed by other extracts of ginseng. EtOAC extracts, which exhibited the best antioxidant activities of all solvent extracts of ginseng, possessed higher concentrations of total phenolics (777.61 mg/100 g) than other extracts. These results suggest that EtOAC extracts of ginseng (*P. ginseng* C.A. Meyer) have the most effective antioxidant capacity compared to *n*-hexane, chloroform, *n*-butanol and water tested in this study, and has important applications for the pharmaceutical and food industries.

Key words: antioxidant properties, ginseng, organic solvent fractionation, polarity

INTRODUCTION

The roots or rhizome of Panax plants (such as *Panax ginseng*, *P. notoginseng*, *P. japonicus*, and *P. quinquefolius*) have long been used in traditional oriental medicine. Among those Panax species, *P. ginseng* C.A. Meyer is one of the most widely used medicinal plants in Far Eastern countries including Korea, China and Japan. It is most often used as a general tonic, and it involves a wide range of pharmacological actions, such as antiaging, adaptogen-like effects to foreign deleterious substances, immunoenhancement, antistress, antitumor, etc (1,2). Many of its effects have been attributed to its antioxidant action (3,4). In general, the pharmacological effects of ginseng roots have been attributed to ginsenosides, which are widely considered to be major components that contribute to the medicinal activities (5,6). Besides saponin, *P. ginseng* also accumulates other secondary metabolites (phenolics compounds) and the growth of the plant for 4～6 years is needed for proper accumulation of secondary metabolites (7). Especially, traditional medicinal herbs such as ginseng are rich in flavonoids and polyphenols (8). Many antioxidant compounds, naturally occurring from plant sources, have been identified as a free radical or active oxygen scavengers (8). Among various natural antioxidants, phenolic compounds are reported to have oxygen-derived free radical quenching properties by donating a hydrogen atom or an electron to a free radical (9). Also, phenolic compounds of plant materials have demonstrated capacity to neutralize free radicals in various model systems (10). The antioxidant effect of polyphenols has been demonstrated in many systems from in vitro studies (human low density lipoprotein, liposomes) to investigations of normal human subjects (11), although their effects remain controversial (12). However, few studies have been conducted to compare the food components and pharmacological activities of ginseng (*Panax ginseng* C.A. Meyer) extracts by organic solvent fractionation. In this study, 6 year old ginseng was examined for antioxidant activities, total phenolics, DPPH, ferrous ion chelating activity and reducing power.

MATERIALS AND METHODS

Plant materials

The 6-year-old ginseng plants were collected from Gaesung Ginseng Cooperative Association (Gyunggi-do, Korea). The average temperature and precipitation of the area of Gyunggi-do throughout the year was 10.5°C and

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1,300 millimeters, respectively. Soon after collection, the ginseng was freeze-dried and stored at -80°C in a freezer.

Sample extraction

Each solvent extract of freeze-dried of ginseng (60 mesh particle size) were obtained as follows. Powdered ginseng (100 g) was suspended and extracted with 200 mL of methanol at 80°C for 2 hrs. The extracts were filtered through Whatman #2 filter paper (Whatman International Limited, Kent, England) and evaporated to dryness. The dry materials were re-dissolved in 100 mL of distilled water. The solution was consecutively partitioned in a separatory funnel with the equivalent amount of *n*-hexane, chloroform, EtOAC, *n*-butanol and water. Each fraction was concentrated in a vacuum evaporator at 40°C. Water filtrate was frozen and lyophilized. The extracts were placed in a glass bottle and stored at -20°C until used. The lyophilized extracts were re-dissolved in methanol to a concentration of 10 mg mL^{-1}.

Measurement of total phenolic content

Total phenolic substances in each solvent extract were determined with Folin-Ciocalteu's reagent according to the method of Singleton et al. (13). Dried solvent extracts (100 mg) were extracted with 40 mL of 80% acetone at room temperature for 25 min in a sonication bath followed by centrifugation at 3,800 *g* for 10 min. After the supernatant was collected, the precipitate was re-extracted as above. After 1st and 2nd extraction, the supernatants were combined and the volumes brought up to 100 mL with 80% acetone. One milliliter of extracts and 1.0 mL of diluted Folin-Ciocalteu's reagent were mixed. After 3 min, 1.0 mL of 10% sodium carbonate was added to the mixture and was allowed to stand for 1 hr. Absorbance of the extract was measured at 760 nm and the reading was compensated to standard gallic acid. The concentration of total phenolic compounds in the extracts was calculated using the following linear equation based on the calibration curve:

$$Y = 0.0012X + 0.0412.\ R^2 = 0.973$$

where, Y is the absorbance and X is the total phenolic contents in microgram gallic acid equivalents per milliliter extract.

DPPH free radical scavenging activity

The free radical scavenging activity of ginseng extracts were determined by the 1,1-diphenyl-2-picryl-hydrazil ($DPPH^{\bullet}$). This activity was measured by the procedure described by Yen and Hsieh (14) wherein the bleaching rate of a stable free radical, $DPPH^{\bullet}$ is monitored at a characteristic wavelength in the presence of the sample. In its radical form, $DPPH^{\bullet}$ absorption at 517 nm decreases upon reduction by an antioxidant or a radical species. Briefly, 0.12 mM solution of $DPPH^{\bullet}$ in methanol was prepared daily and protected from light. An aliquot of 2 mL of this solution was added to 80 μL of the sample at various concentrations and 320 μL of distilled water. The solution was then mixed vigorously and allowed to stand at room temperature in the dark for 30 min. The absorbance of mixtures was measured at 517 nm using a spectrophotometer (Shimadzu UV 160A, Shimadzu Co., Kyoto, Japan). The control was prepared in the same manner, except that distilled water was used instead of ginseng extracts solution. For the blank, the assay was conducted in the same manner but methanol was added instead of DPPH solution. The percentage of $DPPH^{\bullet}$ scavenging activity was calculated as follows (15):

$$DPPH^{\bullet}\ \text{scavenging activity}\ (\%) = (1 - A_{sample(517\ nm)} / A_{control(517\ nm)}) \times 100$$

where $A_{sample\ (517\ nm)}$ was the absorbance of sample and $A_{control\ (517\ nm)}$ was the absorbance of the control.

Ferrous (Fe^{2+}) metal ions chelating activity

The chelating of ferrous ions (Fe^{2+}) by ginseng extracts and standards were estimated by the method of Dinis et al. (16). Ginseng extracts (100 μL) were added to 600 μL of distilled water and 100 μL of 0.2 mM $FeCl_2 \cdot 4H_2O$. The mixture was allowed to rest at room temperature for 30 s. The reaction mixture thus obtained was later added to 200 μL of 1 mM ferrozine and changes in color were monitored at 562 nm with a spectrophotometer (Shimadzu UV 160A, Shimadzu Co., Kyoto, Japan), after a 10 min resting time at room temperature. BHT and Trolox were used to compare the chelating activity. The percentage of inhibition of ferrozine -Fe^{2+} complex formation was calculated in the following equation:

$$\text{Ferrous ion chelating activity} = [(A_{control} - A_{sample}) / A_{control}] \times 100$$

where, $A_{control}$ was the absorbance of the control and A_{sample} was the absorbance with the sample.

Total ferric ions (Fe^{3+}) reduction capability

The Fe^{3+} reducing powers of ginseng extracts were determined by the method of Oyaizu (17). One mL of ginseng extracts were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL, 1%). The mixture was incubated at 50°C for 20 min. An aliquot (2.5 mL) of trichloroacetic acid (10%) was added to the mixture. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL)

and $FeCl_3$ (0.5 mL, 0.1%), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicates an increase of reduction capability.

Statistical analysis

All data were performed in triplicate. Duncan's multiple range test in the SPSS statistical package (SPSS 12.0 for windows, SPSS Inc, Chicago, IL, USA) was used to detect significance of differences ($p<0.05$).

RESULTS AND DISCUSSION

Total phenolic compound contents

Total phenolic compound contents of ginseng extract was determined spectrophotometrically using the Folin-Ciocalteu's method and compared with those of the different fractions of ginseng extract according to the polarity of the solvents, because phenolic compounds are commonly found in both edible and inedible plants, and have been reported to have multiple biological effects, including antioxidant activities (18). As shown in the Fig. 1, EtOAC extracts, which exhibited the best antioxidant activities among all solvent extracts of ginseng, possessed the highest concentrations of polyphenols. EtOAC extracts of ginseng had higher concentrations of total phenolics (777.61 mg/100 g) than other extracts. These results suggest that the more efficient antioxidant activity of ginseng EtOAC extract, compared to other extracts, at the same concentration, is due to the presence of higher concentrations of such potent antioxidant compounds, such as phenolic compounds. In similar reports, the enrichment of phenolic compounds within plant extracts is correlated with their enhanced antioxidant activity (19,20). The antioxidant activity of phenolic compounds is mainly due to their redox activities, which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (21). Therefore, the results of the present study indicate that the EtOAC extract of ginseng possess high antioxidant activity and free radical scavenging activity. These assays are useful for establishing the ability of phenolics to chelate and reduce Fe^{3+} according to polarity for extraction solvent and have important applications for the pharmaceutical and food industries. However, further investigation of individual phenolic compounds, their in vivo antioxidant activity and the different antioxidant mechanisms is needed.

DPPH radical scavenging activity

The free radical scavenging activities of ginseng extracts are shown in Fig. 2. DPPH is one of the chromogen radical compounds that can directly react with antioxidants (22). When the DPPH radical is scavenged by antioxidants through donation of hydrogen to form a stable DPPH-H molecule, the color changes from purple to yellow (4,23). In the present study, the DPPH radicals were dose dependently scavenged by the various solvent extracts of ginseng in doses dependant ranging from 2 to 8 mg/mL. As a positive control, BHT and Trolox were also examined for DPPH radical scavenging activities. EtOAC extracts of ginseng showed the best results through all concentrations. Among the other solvent extracts, butanol extract was a relatively effective scavenger, followed by chloroform, water and hexane extracts. At the concentration of 2 mg/mL, remarkable differences in scavenging activities were observed between control and the EtOAC extract of ginseng. However, as concentration of EtOAC extracts in the assay system increased, the differences in scavenging activities between control and EtOAC extracts decreased.

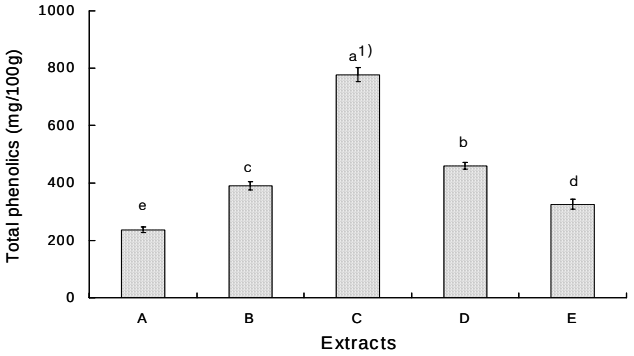

Fig. 1. Comparison of phenolic contents of ginseng extracts by organic solvent fractionation (mg/100 g, dry basis). (A), *n*-Hexane fraction; (B), Chloroform fraction; (C), Ethyl acetate fraction; (D), *n*-Butanol fraction; (E), Water fraction. Data are mean±standard deviation (bars) values of triplicate experiments. [1)]Values sharing the same superscript letter are not significantly different at $p<0.05$.

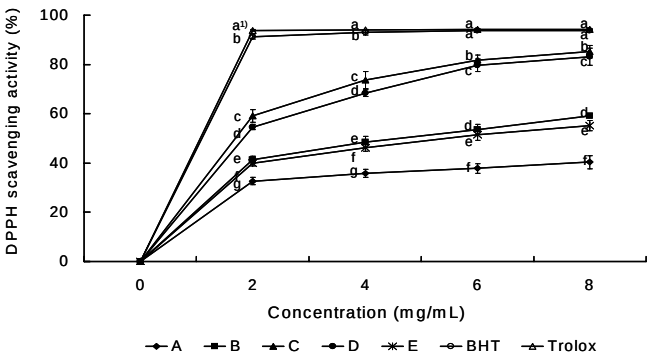

Fig. 2. DPPH radical scavenging activities of ginseng extracts by organic solvent fractionation. (A), *n*-Hexane fraction; (B), Chloroform fraction; (C), Ethyl acetate fraction; (D), *n*-Butanol fraction; (E), Water fraction. Data are mean±standard deviation (bars) values of triplicate experiments. [1)]Values sharing the same superscript letter are not significantly different at $p<0.05$.

Therefore, although various solvent extracts of ginseng showed DPPH scavenging activity, EtOAC extract was a significantly ($p<0.05$) stronger scavenger of DPPH radicals. This indicated that DPPH radical scavenging activities of all solvent extracts of ginseng were related to the amount of antioxidants extracted from ginseng by various solvents. The stable radical DPPH has been widely used for the determination of primary antioxidant activity, that is, the free radical scavenging activities of pure antioxidant compounds, plant and fruit extracts and food materials (24). An almost linear correlation between DPPH radical scavenging activity and concentrations of polyphenolic compounds in various vegetable and fruits have been reported (25,26). The anti-radical activity of phenolic compounds depends on their molecular structure, that is, on the availability of phenolic hydrogens and on the possibility of stabilization of the resulting phenoxyl radicals formed by hydrogen donation (27,28). These results revealed that ginseng EtOAC extracts are free radical scavengers, acting possibly as primary antioxidants. EtOAC extracts of ginseng might react with free radicals, which are the major initiators of fatty acid autoxidation, thereby terminating the chain reaction (29).

Ferrous (Fe^{2+}) metal ions chelating activity

The production of highly reactive oxygen species (ROS), such as superoxide anion radicals, hydrogen peroxide, and hydroxyl radicals, are catalysed by free iron through the Haber-Weiss reaction (30):

$$O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + OH^- + OH^{\bullet}$$

Ferrous ion chelating activities of ginseng extracts are shown in Fig. 3. Transition metals, such as ferrous ion,

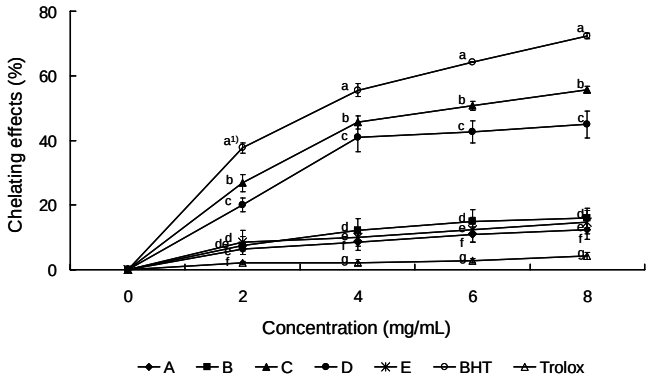

Fig. 3. Ferrous ion chelating activities of ginseng extracts by organic solvent fractionation. (A), *n*-Hexane fraction; (B), Chloroform fraction; (C), Ethyl acetate fraction; (D), *n*-Butanol fraction; (E), Water fraction. Data are mean±standard deviation (bars) values of triplicate experiments. [1)]Values sharing the same superscript letter are not significantly different at $p<0.05$.

can stimulate lipid peroxidation by generating hydroxyl radicals through the Fenton reaction and accelerate lipid peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals, therefore drive the chain reaction of lipid peroxidation (31). The Fenton reaction is:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$$

Fe^{2+} ion is the most powerful pro-oxidant among the various species of metal ions (32). The method of assaying chelating activity is based on chelating of Fe^{2+} by the reagent ferrozine (16). In the presence of other chelating agents, the complex formation is disrupted with a concomitant decrease in the formation of the red color (33). In the present study, the formation of the red-colored complex was inhibited in the presence of ginseng extracts, indicating chelating activity. The chelating activities of all solvent extracts increased with increasing concentration, the highest chelating activity was found in the EtOAC extract of ginseng. Chelating activity of EtOAC extracts was 26.78% at 2 mg/mL and significantly increased up to 55.66% at 8.0 mg/mL. As concentration of extracts in the assay system increased, the differences in chelating activity among chloroform, hexane and water extracts became less significant. Whereas ginseng extracts and BHT have shown strong ferrous ion chelating activity, as a positive control, Trolox did not show any chelating activity at the concentrations tested. The metal chelating activities were dependent upon not only the unique phenolic structures of the compounds, but also the number and position of the hydroxyl groups (34). Ferrous ions are the most powerful pro-oxidants in food system (35), therefore, high Fe^{3+} ions chelating abilities of EtOAC extracts from ginseng would be beneficial.

Total ferric ions (Fe^{3+}) reduction capability using the potassium ferricyanide reduction method

The results obtained for the reducing power in ginseng extracts are shown in Fig. 4. In the reducing power assay, the presence of reductants (antioxidants) in the test samples reduce Fe^{3+}/ferricyanide complex to the ferrous form (Fe^{2+}), which can be monitored by measuring the formation of Perl's Prussian blue color at 700 nm (36). In the present study, the reducing power of all solvent extracts of ginseng increased with increasing concentration, EtOAC extract showed a strong reducing power for Fe^{3+} in a dose dependent manner. On the other hand, hexane extract had the weakest reducing power among all extracts examined. The reducing powers of the different extracts were significantly different from one another. From these observations, it is suggested that

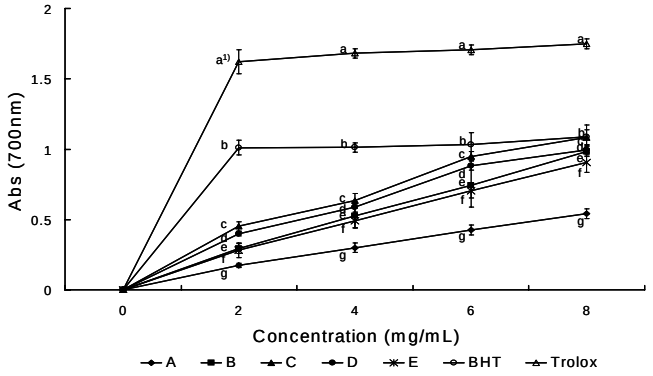

Fig. 4. Reducing power of ginseng extracts by organic solvent fractionation. (A), *n*-Hexane fraction; (B), Chloroform fraction; (C), Ethyl acetate fraction; (D), *n*-Butanol fraction; (E), Water fraction. Data are mean±standard deviation (bars) values of triplicate experiments. [1)]Values sharing the same superscript letter are not significantly different at $p<0.05$.

EtOAC extract has a remarkable potency to react with free radicals to convert them into more stable non-reactive species and to terminate radical chain reaction.

Many studies have observed a direct correlation between antioxidant activity and reducing power of certain plant extracts (37,38). The reducing activities are generally associated with the presence of reductones (37), which have been shown to use antioxidant activity to break the free radical chain by donating a hydrogen atom (39). Reductones are also reported to react with certain precursors of peroxide, and thus prevent peroxide formation (40). In the present study, EtOAC extract of ginseng exhibited good reducing potential, while hexane extract had the weakest reducing power among all extracts examined (Fig. 4). Otherwise, water extract of *P. notoginseng* exhibited no reducing power in a Fe^{3+}-Fe^{2+} system (41). The ginseng extract examined in this study demonstrated good reducing capacity thereby acting as efficient reductones. The results on reducing power demonstrate the electron donor activities of ginseng extracts thereby neutralizing free radicals by forming stable products.

In conclusion, we investigated various solvent extracts of ginseng for their effectiveness to extract antioxidant compounds from freeze-dried ginseng and compared them to BHT and Trolox. Overall, among the solvents tested in this study, EtOAC extracts of ginseng had the highest antioxidant activities although the effectiveness as an antioxidant was not superior to the chemical antioxidants BHT and Trolox.

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