

# Antioxidant and Anti-Adipogenic Activities of *Trapa japonica* Shell Extract Cultivated in Korea

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**ABSTRACT:** *Trapa japonica* shell contains phenolic compounds such as tannins. Studies regarding the antioxidant and anti-adipogenic effects of *Trapa japonica* shell cultivated in Korea are still unclear. Antioxidant and anti-adipogenic activities were measured by *in vitro* assays such as 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activity, ferric reducing ability of plasma assay, reducing power, superoxide dismutase-like activity, and iron chelating ability in 3T3-L1 cells. We also measured the total phenol and flavonoids contents (TPC and TFC, respectively) in *Trapa japonica* shell extract. Our results show that TPC and TFC of *Trapa japonica* shell extract were  $157.7 \pm 0.70$  mg gallic acid equivalents/g and  $25.0 \pm 1.95$  mg quercetin equivalents/g, respectively. *Trapa japonica* shell extract showed strong antioxidant activities in a dose-dependent manner in DPPH and ABTS radical scavenging activities and other methods. Especially, the whole antioxidant activity test of *Trapa japonica* shell extract exhibited higher levels than that of butylated hydroxytoluene as a positive control. Furthermore, *Trapa japonica* shell extract inhibited lipid accumulation and reactive oxygen species production during the differentiation of 3T3-L1 preadipocytes. *Trapa japonica* shell extract possessed a significant antioxidant and anti-adipogenic property, which suggests its potential as a natural functional food ingredient.

**Keywords:** *Trapa japonica* shell, antioxidant activity, anti-adipogenic activity, natural antioxidant

## INTRODUCTION

*Trapa japonica*, commonly known as water caltrop, is a dicotyledonous annual aquatic plant belonging to the family of Trapaceae. It is a free floating plant grown in shallow wetlands, ponds or swampy lands in tropical and sub-tropical Asian countries (1). The grown *Trapa japonica* has one pair of spines in the shoulder and one pair of short spines in the middle section (2). The outer shell of *Trapa japonica* is hard and difficult to peel off to obtain the white edible pulp inside (3). The pulp of *Trapa japonica* is consumed primarily in a cooked form and is eaten raw at the young age. The pulp contains about 80% starch, 5% protein, and significant amount of vitamins, and the shell contains phenolic compounds such as tannins (4,5). It has been reported that phenolic compounds usually accumulate in the outer parts of plants such as shells (6). Therefore, the information on the phenolic content of *Trapa japonica* shell can be taken as an important source.

Phenolics are compounds possessing one or more aromatic rings with one or more hydroxyl groups. They are

the most important groups of secondary metabolites and bioactive compounds in plants and good sources of natural antioxidants in human diets. Phenolics are also natural products and antioxidant substances capable of scavenging reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide, hydroxyl radical, and peroxynitrite, reducing the incidence of cancer and protecting biological systems against the detrimental effects of oxidative processes on macromolecules, such as enzymes, carbohydrates, proteins, lipids, and DNA (7,8). ROS cause more than one hundred disorders in humans including atherosclerosis, arthritis, and cancer (9). Recently, phenolics have been considered powerful antioxidants *in vitro* (10,11). Moreover, ROS play a critical role in the differentiation of preadipocytes by accelerating mitotic clonal expansion (12). During adipogenesis, ROS production markedly increased in parallel with fat accumulation. Recent studies suggested that accumulated fat in adipocytes is associated with increased oxidative stress (13). The inverse relationship between fruit and vegetable intake and the risk of oxidative stress associated diseases such

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as cardiovascular diseases, cancer, or osteoporosis has been partially ascribed to phenolics (14,15).

The objective of this study was to investigate the total phenolic and flavonoids contents (TPC and TFC, respectively), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activity, ferric reducing ability of plasma (FRAP) assay, reducing power assay, superoxide dismutase (SOD)-like activity, iron chelating ability, and major phenolic compounds of *Trapa japonica* shell cultivated in Korea. Moreover, we assessed lipid accumulation and ROS production during 3T3-L1 adipogenesis.

## MATERIALS AND METHODS

### Chemicals

Folin-Ciocalteu reagent, gallic acid, quercetin, DPPH, ABTS, 2,4,6-tri(2-pyridyl)-S-triazine (TPTZ), ferrozine [3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-*p,p'*-disulfonic acid monosodium salt hydrate], trizma base, trichloroacetic acid, and ascorbic acid were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), bovine calf serum (BCS), fetal bovine serum (FBS), penicillin-streptomycin (P/S), phosphate-buffered saline (PBS), and trypsin-ethylenediaminetetraacetic acid (EDTA) were purchased from Gibco (Gaithersburg, MD, USA). Dexamethasone (DEX), 3-isobutyl-1-methylxanthine (IBMX), insulin, Oil red O, nitroblue tetrazolium (NBT), and *N*-acetyl cysteine were also purchased from Sigma-Aldrich Co.. All other chemicals were of reagent grade.

### Preparation of 70% ethanol extract of *Trapa japonica* shell

*Trapa japonica* was bought from Biodepot in Korea and divided into pulp and shell. The *Trapa japonica* shell was extracted with 10 volumes (v/w) of 70% ethanol at 70°C for 24 h, and the extraction was repeated three times. The extracts were filtered through Whatman filter paper (No. 2), concentrated with a vacuum evaporator, and completely dried in a freeze drier (DC1316, Ilshin Lab. Co., Ltd., Gyeonggi, Korea).

### Determination of TPC and TFC

TPC of extracts from *Trapa japonica* shell was determined by the modified method of Gutfinger (16). The sample solution (1 mL) was placed in a test tube with Folin-Ciocalteu reagent (1 mL) and sodium carbonate solution (1 mL). After incubation for 1 h at 25°C, the absorbance was measured at 750 nm, and TPC was calculated as gallic acid equivalents (mg GAE/g). TFC of extracts from *Trapa japonica* shell was determined according to the method of Moreno et al. (17). The sample solution (0.5

mL) was mixed with 0.1 mL aluminum nitrate (10%). After incubation, 4.3 mL ethanol (80%) and 0.1 mL of potassium acetate (1 M) were added. After incubation at room temperature for 40 min, the absorbance was measured at 415 nm, and TFC was calculated as quercetin equivalents (mg QE/g).

### DPPH radical scavenging activity

The antioxidant activity of *Trapa japonica* shell extract was measured on the basis of the electron donating ability (EDA) of the stable DPPH as previously described, with slight modifications (18). One milliliter of ethanolic DPPH solution ( $4 \times 10^{-4}$  M) was added to the samples at various concentrations (10~1,000 µg/mL). The samples were vortexed and incubated in the dark for 10 min at room temperature. DPPH radical scavenging activities were measured by a spectrophotometer at 490 nm and were expressed as a percentage using the following formula:

$$\text{DPPH radical scavenging activity (\%)} = 1 - \frac{A}{B} \times 100$$

where A is absorbance value of testing solution and B is absorbance value of control solution.

### ABTS radical scavenging activity

ABTS radical scavenging activity was measured by the method of Re et al. (19). Before analysis, the stock solution was prepared by stirring ABTS (7 mM) and potassium persulfate (2.45 mM) in water at room temperature for 16 h. The ABTS solution was diluted with ethanol to achieve an absorbance of  $0.70 \pm 0.02$  at 734 nm. Then, 1 mL ABTS solution was added to 10 µL of different concentrations (10~1,000 µg/mL) of *Trapa japonica* shell extract. The samples were vortexed and incubated in the dark for 6 min. ABTS radical scavenging activities were measured by a spectrophotometer at 734 nm, and the results were expressed as a percentage using the following formula:

$$\text{ABTS radical scavenging activity (\%)} = 1 - \frac{A}{B} \times 100$$

where A is absorbance value of testing solution and B is absorbance value of control solution.

### FRAP assay

The ferric reducing ability of *Trapa japonica* shell extract was measured by the method of Benzie and Strain, with modifications (20). The FRAP reagent was prepared from 300 mM acetate buffer (pH 3.6), 10 mM TPTZ in dimethyl sulfoxide, and 20 mM iron chloride in proportions of 10:1:1 (v/v), respectively. Different concentrations (10~

1,000 µg/mL) of sample solution (50 µL) was mixed with distilled water (150 µL) and FRAP reagent (1.5 mL) was added. The absorbance of the reaction mixture was then measured at 593 nm after 4 min.

#### Reducing power

The reducing power of *Trapa japonica* shell extract was evaluated by the method of Jayaprakasha et al. (21). Different concentrations (10~1,000 µg/mL) of *Trapa japonica* shell extract in distilled water (0.5 mL) were mixed with 2.5 mL of sodium phosphate buffer (pH 6.6) and 2.5 mL of potassium ferricyanide (1%). The mixtures were incubated at 50°C for 20 min. Then, 2.5 mL of trichloroacetic acid (10%) was added, and the mixture was centrifuged at 1,790 g for 10 min. Then, 2.5 mL of the supernatant was taken out and mixed with 2.5 mL of distilled water. After adding 0.5 mL of iron (III) chloride (0.1%), the absorbance was measured by a spectrophotometer at 750 nm.

#### SOD-like activity

The SOD-like activity of *Trapa japonica* shell extract was evaluated using the method of Ling et al. (22). Different concentrations (100~1,000 µg/mL) of *Trapa japonica* shell extract in distilled water (0.2 mL) were mixed with 2.6 mL of Tris-HCl buffer (50 mM Tris+10 mM EDTA, pH 8.5) and 0.2 mL of pyrogallol (7.2 mM). After incubation at room temperature for 10 min, 0.1 mL of 1 N HCl was added. SOD-like activities were measured by a spectrophotometer at 420 nm, and the results were expressed as a percentage using the following formula:

$$\text{SOD-like activity (\%)} = 1 - \frac{A}{B} \times 100$$

where A is absorbance value of testing solution and B is absorbance value of control solution.

#### Iron chelating ability

The iron chelating ability of *Trapa japonica* shell extract was measured using the method of Brand-Williams et al. (23). Different concentrations (0.5~10 mg/mL) of *Trapa japonica* shell extract in distilled water (0.5 mL) were placed in test tubes with 0.1 mL of iron (II) chloride (0.6 mM) and 0.9 mL methanol. After incubation at room temperature for 5 min, 0.1 mL of ferrozine (5 mM in methanol) was added. The mixtures were vortexed and incubated for 10 min at room temperature. Iron chelating abilities were measured by a spectrophotometer at 550 nm, and the results were expressed as a percentage using the following formula:

$$\text{Iron chelating ability (\%)} = 1 - \frac{A}{B} \times 100$$

where A is absorbance value of testing solution and B is absorbance value of control solution.

#### Cell culture

3T3-L1 preadipocytes (CL-173) obtained from the American Type Culture Collection [American Type Culture Collection (ATCC), Manassas, VA, USA], were cultured, maintained, and differentiated as described by Lee et al. (24). In brief, cells were plated and grown in DMEM with 3.7 g/L sodium bicarbonate, 1% P/S and 10% BCS. Adipocyte differentiation was induced via 2 days of treatment with post-confluent cells with 10% FBS and a hormonal mixture consisting of 0.5 mM IBMX, 1.0 µM DEX, and 1.67 µM insulin (MDI). Two days after the initiation of differentiation, the culture medium was replaced with DMEM supplemented only with 1.67 µM insulin and 10% FBS. This medium was then replenished every other day. For the treatments, 2 day post-confluent cells were differentiated with or without *Trapa japonica* shell extract.

#### Determination of lipid accumulation and ROS production

The extent of differentiation reflected by the amount of lipid accumulation was determined on day 8 via Oil red O staining. In brief, the cells were fixed in 10% formaldehyde in PBS for 1 h, washed with distilled water, and dried completely. Cells were stained with 0.5% Oil red O solution in 60:40 (v/v) isopropanol : H<sub>2</sub>O for 30 min at room temperature and washed four times in water, then dried. Differentiation was also monitored under a microscope and quantified via elution with isopropanol and optical density (OD) measurements at 490 nm (25). ROS production was detected via a NBT assay. NBT is reduced by ROS to a dark-blue, an insoluble form of NBT called formazan (13). On day 8 after induction, the cells were incubated for 90 min in PBS containing 0.2% NBT. Formazan was dissolved in 50% acetic acid, and the absorbance was determined at 570 nm.

#### Analysis of phenolic compounds using high-performance liquid chromatography (HPLC)-photodiode array

HPLC analysis of *Trapa japonica* shell extract was determined by the method of Dyrby et al. (26) with slight modifications. Ten mg of extracts were dissolved in 1 mL of water : MeOH (50:50, v/v, %) and filtered with a Millipore membrane filter (0.45 µm) prior to HPLC analysis. The HPLC equipment was a Waters 2690 (Waters, Milford, MA, USA) with a photodiode array detector at 280 nm. HPLC analysis was carried out using a C<sub>18</sub> column (4.6×250 mm, 5 µm, Shiseido, Tokyo, Japan). The mobile phase consisted of water : acetic acid (97:3, v/v, %) (A) and water : acetonitrile : acetic acid (70:27:3, v/v/v, %) (B). The flow rate was 1.0 mL/min with the following isocratic method. The standards used were gallic acid, catechin, chlorogenic acid, caffeic acid, *p*-coumaric acid,

**Table 1.** Extraction yield, total phenol content (TPC), and total flavonoids content (TFC) of *Trapa japonica* shell extract cultivated in Korea

Items	<i>Trapa japonica</i> shell extract
Extraction yield (%)	15.80
TPC (mg GAE/g)	157.7±0.70
TFC (mg QE/g)	25.0±1.95

The values are mean±standard deviation from three replications. GAE, gallic acid equilibrium; QE, quercetin equilibrium.

and ferulic acid.

### Statistical analysis

The results are expressed as the mean±standard deviation of triplicate experiments.

Statistical analysis was performed using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). An analysis of variance (ANOVA) and Duncan's multiple range tests were used to determine the significance of difference, and  $P<0.05$  was considered statistically significant.

## RESULTS

### TPC and TFC of *Trapa japonica* shell

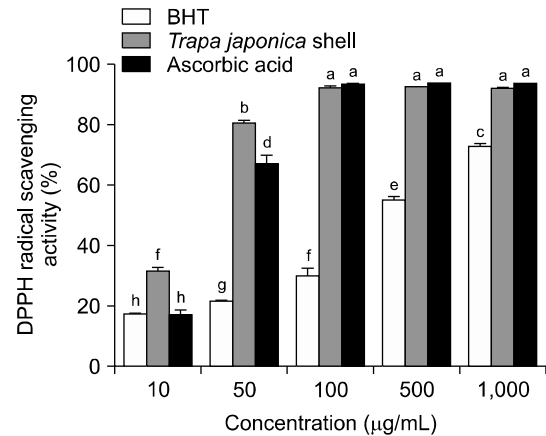
The extraction yield of *Trapa japonica* shell was 15.80%. In addition, the TPC of *Trapa japonica* shell, determined using a regression equation of the calibration curve ( $y=13.33+0.0004x$ ,  $R^2=0.9985$ ) and expressed as gallic acid equivalents, was found to be  $157.7\pm0.70$  mg GAE/g (Table 1). TFC of *Trapa japonica* shell extract, determined using a regression equation of the calibration curve ( $y=2.5303+0.037x$ ,  $R^2=0.9995$ ) and expressed as quercetin equivalents, was found to be  $25.0\pm1.95$  mg QE/g (Table 1).

### DPPH radical scavenging activity

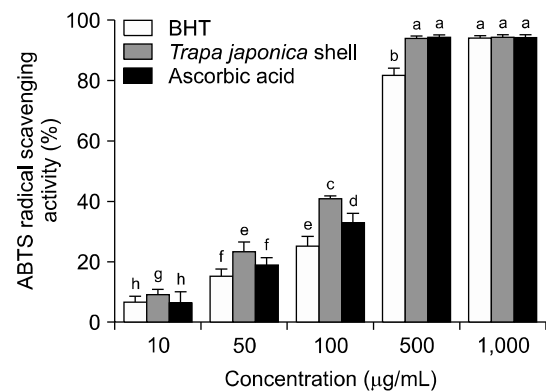
The DPPH radical scavenging activities of *Trapa japonica* shell extract, ascorbic acid and butylated hydroxytoluene (BHT) were evaluated in the following order: *Trapa japonica* shell extract > ascorbic acid > BHT in the presence of the same concentration of test samples (10 and 50 µg/mL) (Fig. 1). The results of *Trapa japonica* shell at different concentrations (100, 500, and 1,000 µg/mL) were not significantly different compared with ascorbic acid. The DPPH radical scavenging percentages of various concentrations (10, 50, and 100 µg/mL) of *Trapa japonica* shell extract were 31.7, 80.9, and 92.4%, respectively.

### ABTS radical scavenging activity

The ABTS radical scavenging activity of *Trapa japonica* shell, BHT and ascorbic acid showed the following order: *Trapa japonica* shell extract > ascorbic acid > BHT in the



**Fig. 1.** 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of *Trapa japonica* shell extract. Values are mean±standard deviation (n=3). Means not sharing a common letter (a-h) are significantly different ( $P<0.05$ ) by Duncan's multiple range test.

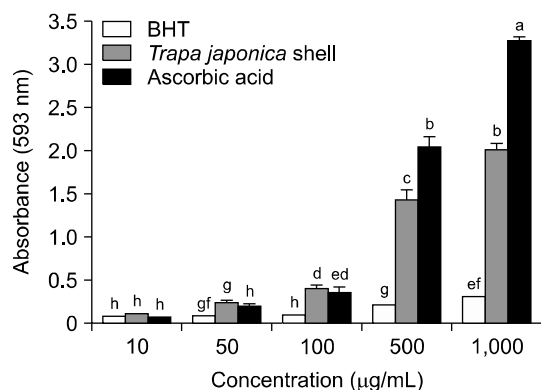


**Fig. 2.** 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activity of *Trapa japonica* shell extract. Values are mean±standard deviation (n=3). Means not sharing a common letter (a-h) are significantly different ( $P<0.05$ ) by Duncan's multiple range test.

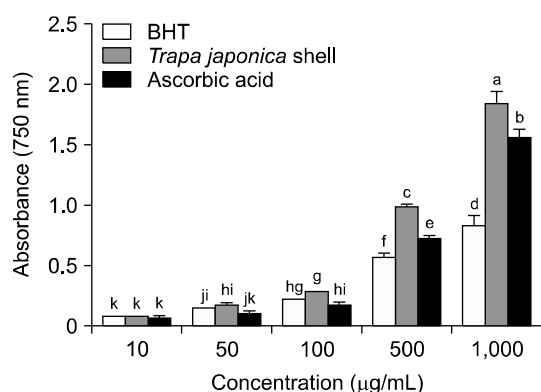
presence of the test samples at all concentrations (Fig. 2). The ABTS radical scavenging percentages of various concentrations (10~1,000 µg/mL) of *Trapa japonica* shell extract were 9.0, 23.5, 41.1, 94.4, and 94.6%, respectively. In contrast, the ABTS radical scavenging percentages at concentrations of ascorbic acid were 6.7, 19.2, 32.9, 94.4, and 94.4%, respectively. These results show that *Trapa japonica* shell extract had the highest ABTS radical scavenging activity than the other positive controls.

### FRAP of *Trapa japonica* shell extract

The results for ferric ion reducing activities of *Trapa japonica* shell extract, BHT and ascorbic acid are shown in Fig. 3. For *Trapa japonica* shell extract, the absorbance was increased due to the formation of the  $Fe^{2+}$ -TPTZ complex with increasing concentrations (Fig. 3). *Trapa japonica* shell extract at a concentration of 1,000 µg/mL had similar FRAP as 500 µg/mL of ascorbic acid and higher than that of BHT.



**Fig. 3.** Antioxidative activity of *Trapa japonica* shell extract using ferric reducing ability of plasma (FRAP) assay. Values are mean±standard deviation (n=3). Means not sharing a common letter (a-h) are significantly different ( $P<0.05$ ) by Duncan's multiple range test.



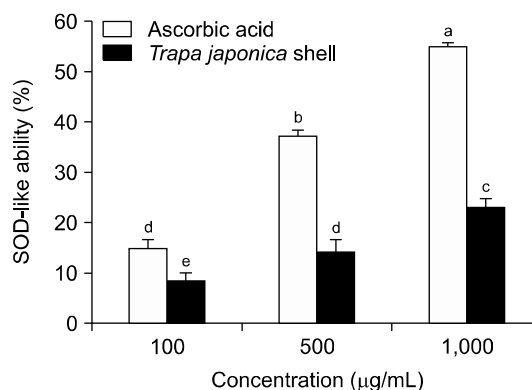
**Fig. 4.** Antioxidative activity of *Trapa japonica* shell extract using reducing power assay. Values are mean±standard deviation (n=3). Means not sharing a common letter (a-k) are significantly different ( $P<0.05$ ) by Duncan's multiple range test.

#### Reducing power of *Trapa japonica* shell extract

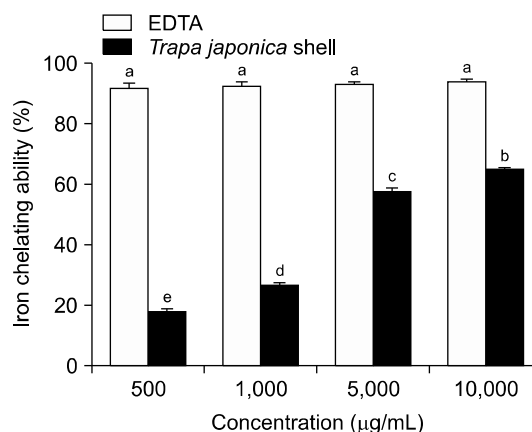
The *Trapa japonica* shell extract at various concentrations (10~1,000 µg/mL) had the highest reducing power (about 0.71 at 750 nm). These results suggest that *Trapa japonica* shell had a better electron donation capacity than the positive controls, vitamin C and BHT (Fig. 4).

#### SOD-like activities of *Trapa japonica* shell extract

The SOD-like activities of *Trapa japonica* shell extract is in the following order: ascorbic acid > *Trapa japonica* shell extract in the presence of test samples at concentrations of 100~1,000 µg/mL (Fig. 5). The SOD-like activities of various concentrations (100~1,000 µg/mL) of *Trapa japonica* shell extract were 8.5, 14.1, and 23.1%, and ascorbic acid were 14.8, 37.2, and 55.1%, respectively. The SOD-like activity of *Trapa japonica* shell extract at a concentration of 500 µg/mL was lower than that of 100 µg/mL of vitamin C, but these results were not significantly different.



**Fig. 5.** Superoxide dismutase (SOD)-like activity of *Trapa japonica* shell extract. Values are mean±standard deviation (n=3). Means not sharing a common letter (a-e) are significantly different ( $P<0.05$ ) by Duncan's multiple range test.



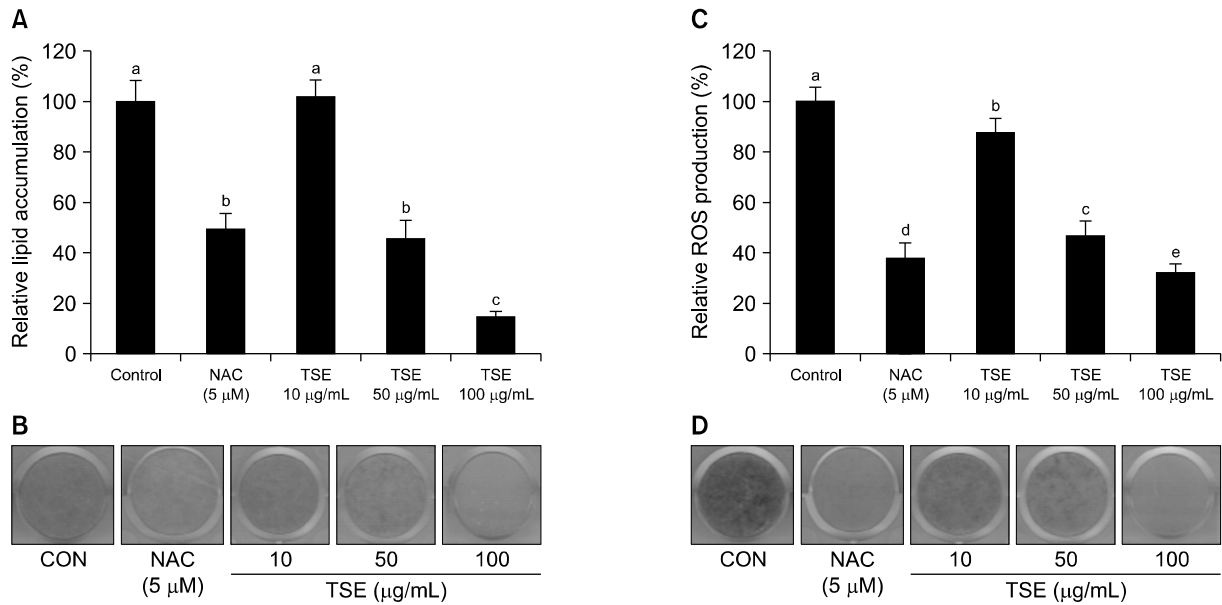
**Fig. 6.** Iron chelating ability of *Trapa japonica* shell extract. Values are mean±standard deviation (n=3). Means not sharing a common letter (a-e) are significantly different ( $P<0.05$ ) by Duncan's multiple range test.

#### Iron chelating ability of *Trapa japonica* shell extract

The chelating effect of various concentrations (0.5~10 mg/mL) of *Trapa japonica* shell extract on  $\text{Fe}^{2+}$  and ferrozine complex formation is shown in Fig. 6. *Trapa japonica* shell extract showed a concentration-dependent activity and chelating ability of 18.0, 26.9, 57.8, and 65.2%, respectively, which is lower than that of the positive control as EDTA. The antioxidant activities may depend on the amount of TPC and TFC.

#### Effects of *Trapa japonica* shell extract on lipid accumulation and ROS production

We evaluated the effect of *Trapa japonica* shell extract on lipid accumulation and ROS production in adipocytes differentiated with MDI. *Trapa japonica* shell extracts significantly inhibited lipid accumulation during the differentiation of 3T3-L1 preadipocytes. The production of dark-blue formazan, which represents ROS production, was decreased in adipocytes treated with *Trapa japonica* shell extract when compared with the control (Fig. 7).



**Fig. 7.** Effect of *Trapa japonica* shell extract on (A, B) lipid accumulation and (C, D) reactive oxygen species (ROS) production during adipogenesis in 3T3-L1 cells. Stained Oil red O (lipid accumulation) was dissolved and determined by absorbance at 490 nm. Dark blue formazan (ROS production) was dissolved and the absorbance was determined at 570 nm. Values are mean±standard deviation (n=3). Means not sharing a common letter (a-c) are significantly different ( $P < 0.05$ ) by Duncan's multiple range test.

#### Phenolic compounds in *Trapa japonica* shell extract

Phenolic acids in *Trapa japonica* shell extract were measured by HPLC (Fig. 8). Table 2 shows the results of the

**Table 2.** Phenolic composition of *Trapa japonica* shell extract (unit: mg/g)

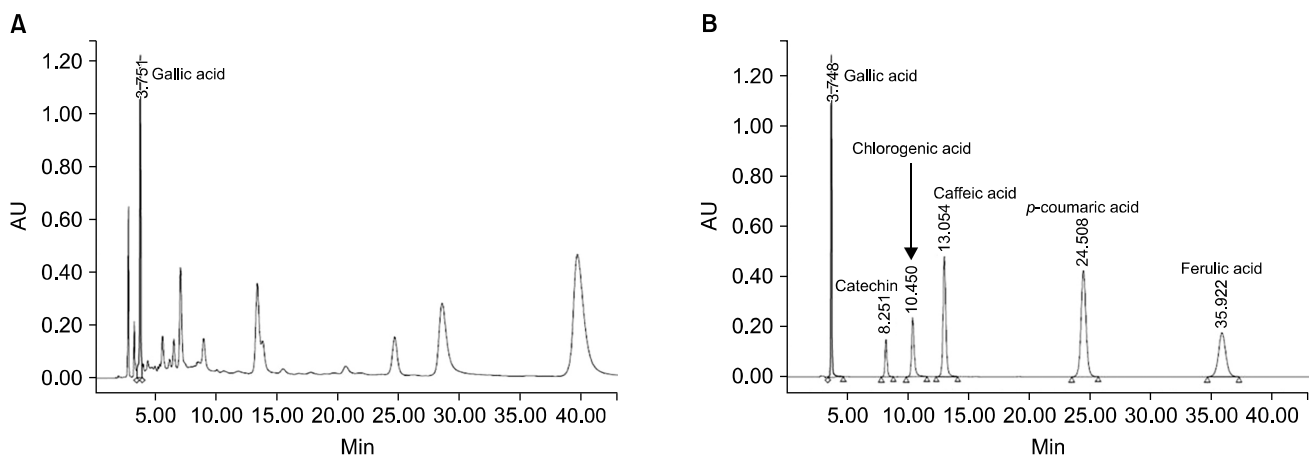
<i>Trapa japonica</i> shell extract	
Gallic acid	27.49
Catechin	N.D.
Chlorogenic acid	N.D.
Caffeic acid	N.D.
<i>p</i> -Coumaric acid	N.D.
Ferulic acid	N.D.

The values are mean±standard deviation from three replications. N.D., not detected.

contents of phenolic acids from *Trapa japonica* shell extract. Only gallic acid was found in *Trapa japonica* shell extract, and the content of gallic acid was 27.49 mg/g. These results show that gallic acid is included in a variety of phenolic acids in *Trapa japonica* shell.

#### Correlation between antioxidant characteristics

TPC and TFC have been reported to be responsible for the antioxidant activities of plant extracts. DPPH, ABTS, FRAP, reducing power, SOD, and iron chelating activities have been used to measure antioxidant activities, and the results should correlate with those of TPC and TFC. *Trapa japonica* shell extract showed a high correlation with total phenol and total flavonoids. In addition, the TPC and TFC in *Trapa japonica* shell extract had a high correlation with other antioxidants assays (Table 3).



**Fig. 8.** HPLC chromatograms (A) major phenolic compound in *Trapa japonica* shell extracts and (B) standard phenolic compounds.

**Table 3.** Correlation coefficient between different antioxidant capacity parameters and TPC and TFC of *Trapa japonica* shell extract

	TPC	TFC	DPPH	ABTS	FRAP	RPA	SOD	ICA
TPC	—	0.9994	0.8243	0.9999	0.9988	0.9927	0.998	0.9517
TFC	—	—	0.8429	0.9999	0.9999	0.9963	0.9951	0.9404
DPPH	—	—	—	0.8475	0.8501	0.8843	0.7888	0.6298
ABTS	—	—	—	—	0.999	0.9971	0.9942	0.9374
FRAP	—	—	—	—	—	0.9975	0.9936	0.9356
RPA	—	—	—	—	—	—	0.9831	0.9087
SOD	—	—	—	—	—	—	—	0.9692
ICA	—	—	—	—	—	—	—	—

TPC, total phenolic content; TFC, total flavonoids content; DPPH, 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical scavenging activity; FRAP, ferric reducing ability of plasma assay; RPA, reducing power activity; SOD, superoxide dismutase-like activity; ICA, iron chelating ability.

## DISCUSSION

In the present study, we have demonstrated the antioxidant and anti-adipogenic activities of *Trapa japonica* shell extract cultivated in Korea. We also investigated the phenolic acid, TPC and TFC of *Trapa japonica* shell extract. Phenolics have an ordinary structure composed of an aromatic hydroxyl nucleus and there are approximately 8,000 known in nature (27). Phenolic compounds are secondary plant metabolites extensively spread throughout the plant kingdom (28,29). Our results show that the phenolic acids, TPC and TFC in *Trapa japonica* shell extract were 25.49 mg/g as a gallic acid, 157.7±0.70 mg GAE/g and 25.0±1.95 mg QE/g, respectively (Table 1 and 2).

Phenolic compounds and flavonoids are secondary metabolites of plants with poly-hydroxyl group, which possess antioxidant activity (30-32). Phenolic compounds can reduce stable radicals such as DPPH. The DPPH assay is based on the reduction of DPPH in alcoholic solution in the presence of a hydrogen-donating antioxidant. The DPPH assay is an easy way to evaluate antioxidant activity (33). It is a fast screening for radical scavenging activity of diverse natural substances (34). In addition, ABTS is a widely used radical scavenging method (23). The ABTS radical scavenging activity method is based on the ABTS radical cation decolorization assay, which shows similar results to the DPPH reaction. Here we showed that the antioxidant activity *Trapa japonica* shell extract possessed effective radical scavenging ability. At concentrations of 10~100 µg/mL, *Trapa japonica* shell extract was significantly higher in ABTS radical scavenging activity than BHT and vitamin C.

We also showed that *Trapa japonica* shell extract had strong antioxidant activities in a dose-dependent manner in the FRAP assay, reducing power, SOD-like activity, and iron chelating ability. The FRAP assay is based on the ability of the antioxidant to reduce Fe<sup>3+</sup> to Fe<sup>2+</sup> in the presence of TPTZ, forming an intense blue Fe<sup>2+</sup>-TPTZ complex with an absorbance maximum at 595 nm,

which is pH-dependent. The absorbance decrease is proportional to the antioxidant activity (20). The reducing power is also designed to measure general antioxidant activity or reduction potential as well as FRAP assay (35). In the reducing power assay, the presence of reductants (i.e., *Trapa japonica* shell extract), converted the Fe<sup>3+</sup>/ferricyanide complex present in the assay system to the ferrous form. SOD is an enzyme that catalyzes the reaction (2O<sub>2</sub><sup>-</sup>+2H<sup>+</sup>→H<sub>2</sub>O<sub>2</sub>+O<sub>2</sub>) to convert harmful reduced oxygen species (superoxide anion radical) to hydrogen peroxide. The hydrogen peroxide produced by the SOD is finally decomposed into water and oxygen by catalase and peroxidase (8). Ferrozine can quantitatively form complexes with Fe<sup>2+</sup>. However, in the presence of chelating agents, the complex formation is disrupted, and the red color of the complex is decreased. Measurement of color reduction, therefore, allows for the estimation of the chelating activity of the coexisting chelator.

Our results also indicate that *Trapa japonica* shell extracts inhibited lipid accumulation and ROS production during the differentiation of 3T3-L1 preadipocytes. *Trapa japonica* shell extract-treated cells had no indication of cellular toxicity with regards to changes in cell morphology or appearance under phase-contrast microscopy (data not shown). These results suggest that treatment of *Trapa japonica* shell extract reduced the ROS production during adipogenesis in parallel with fat accumulation. The antioxidant and anti-adipogenic activities of *Trapa japonica* shell corresponded to the TFC and TPC, mainly as gallic acid, indicating that the inhibition of radical scavenging activity is due to these active phenolic compounds present in *Trapa japonica* shell extract (Table 2). The radical scavenging effect of *Trapa japonica* shell extract and vitamin C, in high concentrations, was not statistically different, but at low concentrations, *Trapa japonica* shell extract had a better radical scavenging effect than the positive control, vitamin C. Therefore, *Trapa japonica* shell extract is a good source of natural antioxidants and may have a protective effect against oxidative stress related chronic diseases.

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## AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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