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In Vitro and Cellular Antioxidant Activity of a Water Extract of *Saururus chinensis*

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Abstract The water extract of *Saururus chinensis* was investigated for oxygen radical absorbance capacity (ORAC), reducing capacity, metal chelating activity, and intracellular antioxidant activity using HepG2 cell. When 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) was used for the generation of peroxyl radicals *in vitro*, *S. chinensis* extract (SC-E) showed the strong and concentration-dependent scavenging activity through donating protons which could be explained by its reducing property. When hydroxyl radicals were generated *in vitro* through the addition of Cu^{2+} and H_2O_2, SC-E demonstrated the antioxidant activity depending on its concentration. In HepG2 cell model, most of intracellular oxidative stress generated by AAPH was efficiently removed by SC-E. However, when Cu^{2+} without H_2O_2 was used as an oxidant in the intracellular assay, SC-E partially reduced the oxidative stress caused by Cu^{2+} in cellular antioxidant activity assay system. These results indicate that SC-E could be utilized for the development of functional foods as antioxidant resource in the near future.

Key words: cellular antioxidant activity, *Saururus chinensis*, HepG2 cell

Introduction

Reactive oxygen species (ROS) including superoxide anion radical ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$), singlet oxygen (O_2^{1}), and hydrogen peroxide (H_2O_2) are generated as byproducts of normal cellular metabolism or results of exogenous factors including smoking and air pollution (1). Oxidative stress is the term referring the imbalance between the generation of ROS and the activity of the antioxidant defenses. Severe oxidative stress has been implicated in aging and such chronic diseases as cancer and coronary heart disease due to the damage of many biological molecules such as lipids, proteins, carbohydrates, and DNA (2). Especially, the chemical damage in DNA as modification of DNA bases and DNA strand breakage induced by ROS may result in the development of cancer if there is an inefficient DNA repair capacity. A possible scavenger of these ROS may be used as a preventive tool to control oxidative stress-related diseases. Many polyphenolic compounds from plant materials including herb extracts have shown the antioxidant activity against ROS (3,4).

Saururus chinensis is a perennial herbaceous plant that has been used in folk medicine to care for edema, gonorrhea, jaundice, and inflammation in Korean traditional medicine. Previous studies have reported that *S. chinensis* and its lignan constituents possess physiological activities such as anti-inflammatory (5), hepatoprotective (6,7), neuroleptic (8), anti-asthmatic (9), hypercholesterolemia (10), and hypoglycemic activities (11) attributable to their antioxidant effects for scavenging ROS and chelating transition metal ions (12-15). A triene, 12, 13-dehydrogeranylgeraniol, has been demonstrated to inhibit intracellular ROS-catalyzed oxidation within HL-60 cells (16).

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In this study the antioxidant effect of *S. chinensis* extract using *in vitro* and cellular antioxidant assay was evaluated and discussed what kind of the mechanism might be involved in its antioxidant activity.

Materials and Methods

Materials 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) was obtained from Wako Pure Co. (Osaka, Japan). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and Hank's balanced salt solution (HBSS) were purchased from Gibco BRL. (Calsbad, CA, USA). Folin-Ciocalteu reagent, 1,10-phenanthroline, fluorescein, 2',7'-dichlorofluorescin diacetate (DCFH-DA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), phosphate buffered saline (PBS, pH 7.4), Trolox, dimethyl sulfoxide (DMSO), neocuproine, ferrozine, and heat-inactivated fetal serum albumin were purchased from Sigma-Aldrich (St. Louis, MO, USA). HepG2 cell was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA).

Preparation of *S. chinensis* extract A volume of 5 g of dried *S. chinensis* purchased from a local market in Daejeon, Korea, was extracted by autoclaving for 15 min at 115°C or by reflux at 85°C for 2 hr with 100 mL distilled water, freeze-dried, and kept at −20°C until analysis.

Total soluble phenolics assay Total soluble phenolics were determined according to the method of Chandler and Dodds (17) based on that of Singleton and Rossi (18). A volume of 1 mL of *S. chinensis* extract solution was transferred to a test tube and mixed well with 1 mL of 95% ethanol and 5 mL of distilled water. To each sample was added 0.5 mL of 50% Folin-Ciocalteu reagent and after 5 min, 1 mL of 5% Na_2CO_3. The samples were mixed and allowed to stand for 60 min. The absorbance was read at 725 nm with 95% ethanol as a blank using a spectro-

photometer (Shimadzu Inc., Kyoto, Japan). A standard curve was established using various concentrations of gallic acid in 95% ethanol. Absorbance values were converted to mg of total soluble phenolics/g *S. chinensis* extract.

Total flavonoid assay Total flavonoid was measured by using the method of Moreno *et al.* (19). To 0.1 mL of *S. chinensis* extract solution was added 0.9 mL of 80% ethanol. Half mL of mixture solution was mixed with 0.1 mL of 10% aluminum nitrate, 0.1 mL of 1 M potassium acetate, and 4.3 mL of 80% ethanol, and allowed to stand for 40 min at room temperature. The absorbance was read at 415 nm using spectrophotometer (Shimadzu Inc.). Standard curve was established using various concentrations of quercetin. Absorbance values were converted to total flavonoids which were expressed as mg of quercetin equivalent/g *S. chinensis* extract.

Oxygen radical absorbance capacity (ORAC) assay The ORAC assay was carried out on a Tecan GENios multi-functional plate reader (Salzburg, Austria) with fluorescent filters (excitation wavelength: 485 nm and emission filter: 535 nm). In the final assay mixture, fluorescein (40 nM) was used as a target of free radical attack with either AAPH (20 mM) as a peroxyl radical generator in peroxyl radical scavenging capacity ($ORAC_{ROO\cdot}$) assay (20) or H_2O_2-$CuSO_4$ (H_2O_2, 0.75%; $CuSO_4$, 5 μM) as mainly a hydroxyl radical generator in hydroxyl radical scavenging capacity ($ORAC_{OH\cdot}$) assay (21). Trolox (1 mM) was used as a control standard and prepared fresh daily. The analyzer was programmed to record the fluorescence of fluorescence every 2 min after AAPH or H_2O_2-$CuSO_4$ was added. All fluorescence measurements were expressed relative to the initial reading. Final results were calculated based on the difference in the area under the fluorescence decay curve between the blank and each sample. $ORAC_{ROO\cdot}$ and $ORAC_{OH\cdot}$ were expressed as μM of Trolox equivalents (TE). One ORAC unit is equivalent to the net protection area provided by 1 μM of Trolox.

Determination of reduction capacity The reducing ability of *S. chinensis* extract was determined according to the method of Aruoma *et al.* (22). The 40 μL of different concentrations of *S. chinensis* extract in distilled water were mixed with 160 μL of the mixture containing 0.5 mM $CuCl_2$ and 0.75 mM neocuproine in 10 mM phosphate buffer, pH 7.4. The absorbance was measured with a microplate reader at 454 nm for 1 hr. Increased absorbance of the reaction mixture indicates increased reducing power.

Metal chelating activity The metal chelating activity of *S. chinensis* extract was measured by the method of Decker and Welch (23). A volume of 200 μL of different concentration of *S. chinensis* extract were mixed with 20 μL of $FeCl_2$ solution (2 mM in H_2O). The reaction was initiated by the addition of 20 μL of 2.4 mM ferrozine, and the mixture was shaken vigorously and stood at room temperature for 10 min. The absorbance of the mixture (the ferrous ion-ferrozine complex) was measured with a microplate reader at 562 nm. The metal chelating activity of the test sample on ferric ions was calculated below formula:

$$\text{Metal chelating activity (\%)} = [(A_0 - A_1)/A_0] \times 100$$

where A_0 was the absorbance of the control, and A_1 was the absorbance of the sample.

Cell viability assay The tetrazolium dye colorimetric MTT test was used to determine the viability of HepG2 cells. The MTT assay is based on the ability of functional mitochondria to catalyze the reduction of 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide to insoluble purple formazan, the concentration of which can be measured spectrophotometrically. HepG2 cells were first cultured in 12-well plates (5×10^5/mL) for 24 hr, washed twice using PBS, and pretreated with different concentrations of sample tested. After 4 hr incubation, MTT reagent was added to each well, and the plate was incubated at 37°C for 1 hr. The media was removed, and the plate was washed twice with PBS (pH 7.4). The intracellular insoluble formazan was dissolved in DMSO. The absorbance of each cell was then measured at 570 nm using enzyme-linked immunosorbent assay (ELISA) reader, and the % viability was calculated.

Intracellular ROS measurement using DCFH-DA assay Cellular oxidative stress owing to ROS generating by AAPH or Cu^{2+} was measured by spectrofluorometrically by the DCFH-DA method (24). DCFH-DA diffuses through the cell membrane and is enzymatically hydrolyzed by intracellular esterase to the non-fluorescent DCFH, which can be rapidly oxidized to the highly fluorescent DCF in the presence of ROS. HepG2 cells were first cultured in 96-well plates (5×10^5/mL) for 24 hr. After the cell was incubated with different concentration of *S. chinensis* for 30 min, media was discarded, and wells were gently washed twice using PBS. Instead of media, HBSS which is stable to fluorescence, was added to each well. AAPH was used as an inducer of peroxyl radical oxidative stress, and Cu^{2+} were used as an inducer of another oxidative stress. After wells were treated with 100 μM AAPH or 20 mM Cu^{2+} for 30 min, DCFH-DA was added to the culture plates at a final concentration of 40 μM and incubated for 30 min at 37°C in the darkness. DCF fluorescence intensity was measured with excitation wavelength at 485 nm and emission wavelength at 535 nm using Tecan GENios fluorometric plate reader.

Statistical analysis All data are presented as means± standard deviation (SD). Statistical analyses were done using statistical package SPSS (Statistical Package for Social Science, SPSS Inc., Chicago, IL, USA) program, and significance of each group was verified with the analysis of one-way analysis of variance (ANOVA) followed by the Duncan's test of $p<0.05$.

Results and Discussion

Content total soluble phenolics and total flavonoid The extraction yields of *S. chinensis* with reflux and autoclave method were 10.68 and 19.43%, respectively (Table 1). The contents of total soluble phenolics of freeze-dried extracts prepared by reflux and autoclaving method were 7.0 and 6.9%, respectively, and the amount of total flavonoid of *S. chinensis* extract with these methods were

Table 1. Extraction yield, total soluble phenolics, and total flavonoid of *S. chinensis* extract

Extract method	Yield (%, w/w)	Total soluble phenolics (%, w/w)	Total flavonoid (%, w/w)
Reflux	10.68±1.2[1)]	7.0±0.1	3.7±0.0
Autoclave	19.43±2.0	6.9±0.1	2.7±0.1

[1)]Values with different superscripts within a column are significantly different at $p<0.05$ by Duncan's test.

3.7 and 2.7%. It suggests that an autoclaving method is a more efficient way to extract solid materials such as soluble phenolics and flavonoid from *S. chinensis* than a reflux method. Therefore, the water extract of *S. chinensis* produced by autoclaving method was used for further analysis.

***In vitro* antioxidant activity of *S. chinensis* extract** The ORAC assay developed by Cao *et al.* (25) has been proved to be a widely accepted method for evaluating antioxidant capacity of various foods and biological samples (26,27). Antioxidant activity of *S. chinensis* extract was investigated for their peroxyl radical scavenging capacity using ORAC assay system, where AAPH was used as a generator of peroxyl radicals. Figure 1 demonstrates that the scavenging activity of *S. chinensis* extract on peroxyl radicals generated from AAPH was found to be dose-dependent between 1 and 5 μg/mL. The $ORAC_{ROO\cdot}$ of *S. chinensis* extract in 1 μg/mL was 2.6 TE (μM), indicating that its scavenging capacity was similar to that of green tea extract (3.0 TE)

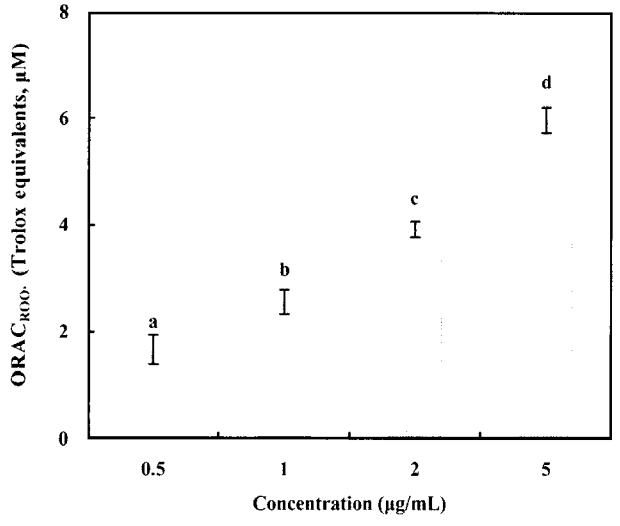

Fig. 1. $ORAC_{ROO\cdot}$ activity (Trolox equivalent, μM) of *S. chinensis* extract. The ORAC value is calculated by dividing the area under the sample curve by the area under the Trolox curve, with both areas being corrected by subtracting the area under the blank curve. One ORAC unit is assigned as the net area of protection provided by Trolox at a final concentration of 1 μM. The area under the curve of the sample is compared to the area under the curve for Trolox, and the anti-oxidative value is expressed in μM of Trolox equivalent/L. The results represent the means±SD of values obtained from 3 measurements. The significance of differences at $p<0.05$ was determined with the Duncan's test.

which has been known to scavenge effectively peroxyl radicals produced *in vitro* (28).

The ability of *S. chinensis* extract to stimulate the reduction of copper ion was investigated measuring the concentration of Cu^{+} formed from Cu^{2+} through the reduction by electrons whether $ORAC_{ROO\cdot}$ activity of *S. chinensis* extract could result from its reduction capacity donating electrons or hydrogens to peroxyl radicals. The reduction capacity of *S. chinensis* extract appears to increase with increasing the concentration from 5 to 40 μg/mL (Fig. 2), which is very consistent with its result of the $ORAC_{ROO\cdot}$ assay. These results suggest that the $ORAC_{ROO\cdot}$ of *S. chinensis* extract could be attributed to the fact that its reducing ability reduces peroxyl radicals by donating electrons or hydrogens to make them into relatively stable compounds. The previous studies have reported that the water extract of *S. chinensis* leaves and its flavonol glycosides showed a dose-dependent free radical scavenging activity of DPPH (12,13).

ORAC assay system has been used successfully to determine the reaction capacity with hydroxyl radical, one of most harmful and ROS in biological system. Hydroxyl radicals were generated by the Fenton reaction of Cu^{2+} with H_2O_2. *S. chinensis* extract showed the dose dependency of hydroxyl radical scavenging activity between 50 and 200 μg/mL (Fig. 3). The $ORAC_{OH\cdot}$ of *S. chinensis* extract increased with increasing the concentration to 200 μg/mL, demonstrating the excellent capacity to scavenge hydroxyl radical generated by the reaction of H_2O_2 and $CuSO_4$.

The $ORAC_{OH\cdot}$ of antioxidant in ORAC assay system has been known to be dependent on 2 mechanisms, the metal chelating activity and the scavenging activity on hydroxyl radical itself. The *S. chinensis* extract can block cupric ions from the interaction with hydrogen peroxide in $ORAC_{OH\cdot}$ assay system by chelating them to inhibit the generation of hydroxyl radicals and then contribute to its $ORAC_{OH\cdot}$ activity. The metal chelating activity of *S. chinensis* extract was determined by measuring the inhibition percentage of ferrous ion-ferrozine complex formation. The metal chelating activity of *S. chinensis* extract increased with increasing the concentration between 2.5 and 5.0 mg/mL

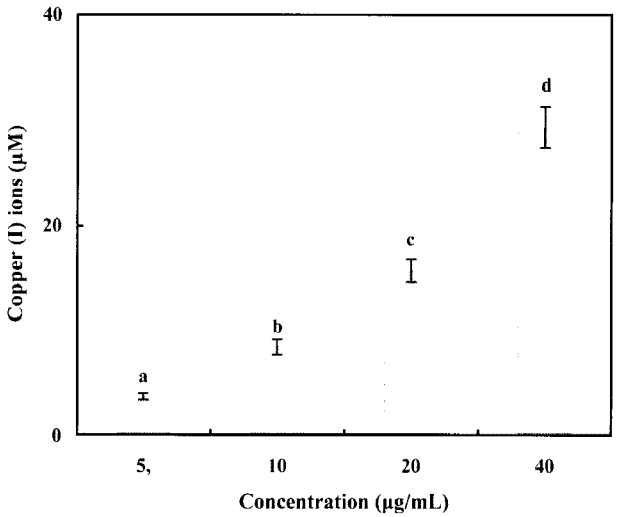

Fig. 2. Ability of *S. chinensis* extract to reduce Cu^{2+} ions. Each bar represents means±SD of 3 measurements. Different letters indicate significant differences at $p<0.05$ of the Duncan's test.

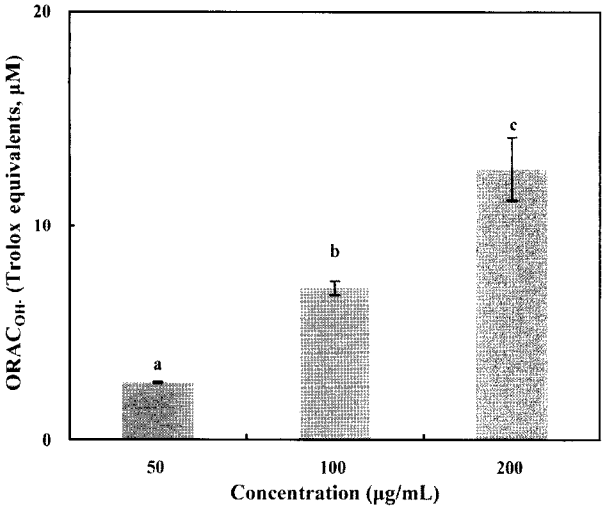

Fig. 3. $ORAC_{OH}$ activity of *S. chinensis* extract. The results represent the means±SD of values obtained from 3 measurements. The significance of differences at $p<0.05$ was determined with the Duncan's test.

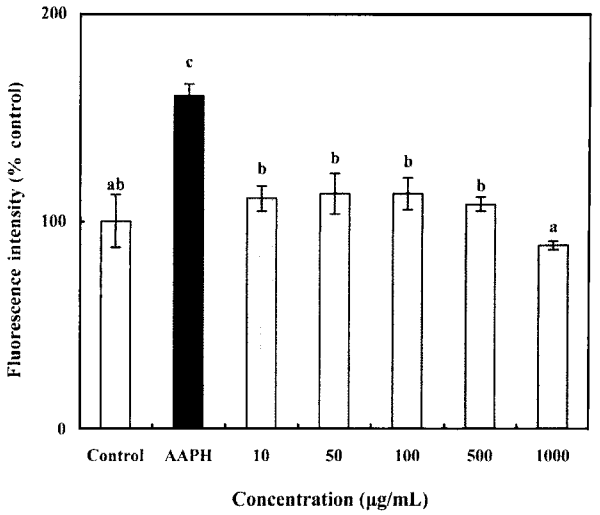

Fig. 5. Intracellular peroxyl radical scavenging activity of *S. chinensis* extract. The results represent the means±SD of values obtained from 3 measurements. The significance of differences at $p<0.05$ was determined with the Duncan's test.

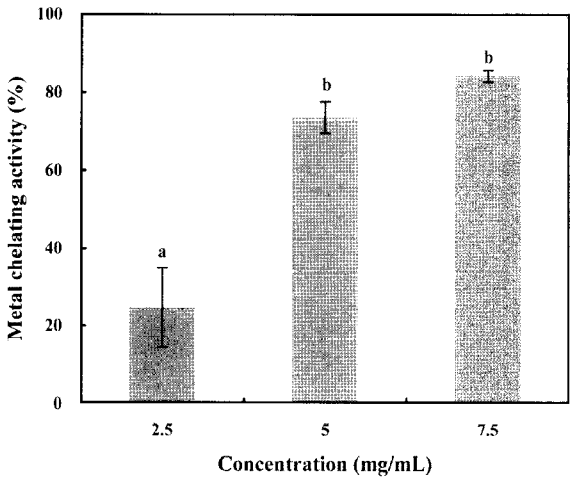

Fig. 4. Metal chelating activity of *S. chinensis* extract against auto-oxidation process. Each bar represents means±SD of 3 measurements. Different letters indicate significant differences at $p<0.05$ of the Duncan's test.

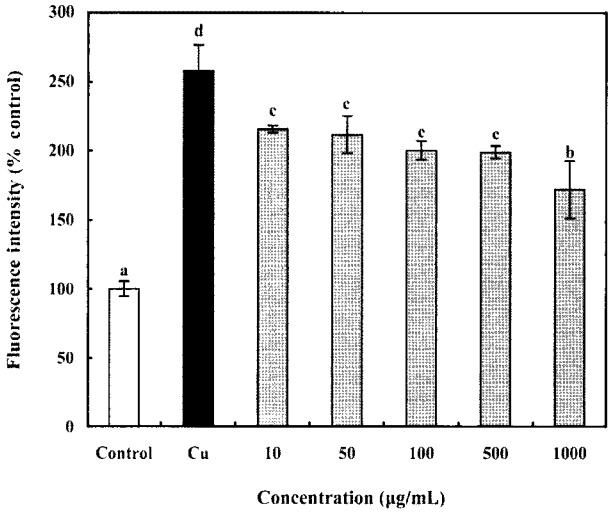

Fig. 6. Intracellular antioxidant activity of *S. chinensis* extract against the oxidative stress generated by Cu^{2+} in HepG2 cells. The results represent the means±SD of values obtained from 3 measurements. The significance of differences at $p<0.05$ was determined with the Duncan's test.

(Fig. 4), suggesting the existence of some bioactive compounds to chelate with transition metal ions. Therefore, the $ORAC_{OH}$ activity of *S. chinensis* can be partially explained by its metal chelating activity. The hydroxyl radical scavenging activity of *S. chinensis* seems to result from the combination effect of scavenging activity on hydroxyl radical itself and chelating activity with transition metal ions.

Intracellular antioxidant activity in HepG2 cells
Hepatoma HepG2 cells were pre-incubated with different concentrations of *S. chinensis* extract for 30 min. After incubation, cells were exposed to 100 μM AAPH for 30 min and treated with DCFH-DA for 30 min which is a fluorescent probe for detecting ROS to measure intracellular oxidative stress. *S. chinensis* extract at concentrations between 10 and 500 μg/mL showed the antioxidant activity reducing the extent of intracellular oxidative stress due to peroxyl radicals, which was very strong and almost same (Fig. 5). It means that most of intracellular oxidative stress caused by peroxyl radicals (78-86%) was removed by bioactive compounds of *S. chinensis* extract permeated into HepG2 cell membrane, indicating that its active compounds scavenging the peroxyl radical may easily permeate into HepG2 cell membrane even in low concentration of 10 μg/mL and play as an antioxidant to scavenge peroxyl radical. Furthermore, the dose-dependent intracellular peroxyl radical scavenging activity of *S. chinensis* extract was observed at only high concentration between 500 and 1,000 μg/mL.

HepG2 cells were pre-incubated with 3 different concentrations of *S. chinensis* extract for 30 min prior to the treatment of 10 μM Cu^{2+}. The protecting activity of *S. chinensis* extract against Cu^{2+}-induced oxidative stress was

shown in Fig. 6. From 10 and 500 μg/mL, *S. chinensis* extract reduced the oxidative stress induced by Cu^{2+} by about 26-38%. Even the high concentration of *S. chinensis* extract as 1,000 μg/mL could not completely remove the Cu^{2+}-induced oxidative stress of the experimental condition tested, suggesting that an only small amount of active compounds to chelate with transition metal ions or to scavenge hydroxyl radicals might be permeable into HepG2 cells. Therefore, the protection activity of *S. chinensis* extract against Cu^{2+}-induced oxidative stress in HepG2 cells could not be resulted from the antioxidant effect such as metal chelating activity and radical scavenging activity, but from both the permeability into cell membrane and the combined effect of metal chelating activity and radical scavenging activity. These results obtained from intracellular antioxidant activity assay of *S. chinensis* extract could be useful for the prediction about its effect as an antioxidant *in vivo* model system.

In conclusion, this study has shown that *S. chinensis* extract prepared by autoclave method scavenged peroxyl and hydroxyl radical *in vitro* assay, peroxyl radical in cellular antioxidant activity assay and partially reduced the oxidative stress caused by Cu^{2+} in cellular antioxidant activity assay system. The protection activity of *S. chinensis* extract against Cu^{2+}-induced oxidative stress in HepG2 cells could not be resulted from the antioxidant effect such as metal chelating activity and radical scavenging activity, but from both the permeability into cell membrane and the combined effect of metal chelating activity and radical scavenging activity. *S. chinensis* extract could be utilized for the development of functional foods as an anti-oxidative resource in the near future. However, further study may be required to identify some bioactive compounds to perform the antioxidant activity such as peroxyl or hydroxyl radical scavenging activity and metal chelating activity inside of HepG2 cells.

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