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Free radical scavenging and antioxidant enzyme fortifying activities of extracts from *Smilax china* root

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Abbreviations: DPPH, 1,1-diphenyl-2-picrylhydrazyl; SOD, superoxide dismutase; CAT, catalase; GPX, glutathione peroxidase

Abstract

The extract from *Smilax china* root has been used as medicinal remedy and reported to retain antimicrobial and antimutagenic acitivities. In this study, a possible presence of antioxidant activity of *Smilax china* root extract was investigated. Methanol extract (Me) revealed the presence of high 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity (IC_{50} 7.4 μg/ml) and protective property of cell's viability. Further fractionation with various solvent extraction and assay showed high levels of DPPH free radical scavenging activity in the ethyl acetate, butanol and water extracted fractions. In addition, V79-4 cells treated with Me of *Smilax china* root induced an increase of superoxide dismutase, catalase and glutathione peroxidase activities in a dose-dependent manner between 4-100 μg/ml. These results suggest that the medicinal component of the root of *Smilax china* extracts also contains antioxidant activity.

Keywords: Antioxidant activity, antioxidant enzymes, lipid peroxidation, radical scavenging, *Smilax china* root

Introduction

The reactive oxygen species (ROS) such as O_2^-, OH^- and H_2O_2, together with unstable intermediates in the peroxidation of lipids are well known inducer of cellular and tissue pathogenesis leading to numerous disease states including cardiovascular disease (Witztum, 1993) and age-related degenerative conditions (Finkel and Holbrook, 2000). Neurodegenerative diseases such as Alzheimer's disease (Richardson, 1993) and cancer (Borek, 1991) are also linked to damage from ROS as a result of an imbalance between the rate of generation of radicals and scavenging of radicals.

The cellular radical-scavenging systems include the enzymes such as superoxide dismutase (SOD), which scavenges the superoxide ion by speeding up its dismutation, catalase (CAT), a haeme enzyme, which removes hydrogen peroxide and glutathione peroxidase (GPX), a selenium-containing enzyme, which scavenges other peroxides as well as hydrogen peroxide (Blake *et al*., 1987). Other molecules that can counteract ROS include glutathione, flavonoids, ubiquinol-10, glucose and albumin (Halliwell and Gutteridge, 1998). The discovery by McCord and Fridovich (1969) of the SOD activity of erythrocuprein together with the finding that almost all mammalian cells contain SOD suggests the physiological importance of at least the central ROS, superoxide. External sources of antioxidative protection include antioxidant vitamins C, E, β-carotene and carotenoids as well as minerals such as selenium and zinc (Halliwell and Gutteridge, 1998).

Great efforts have been made in an attempt to find safe and potent natural antioxidants from plant resources. These include seeds (Deiana *et al*., 1999), beans (Duh *et al*., 1997), herbs (Kitts *et al*., 2000), tea (Roedig-Penman and Gordon, 1997), berry crops (Wang and Jiao, 2000), *Ginkgo biloba* (Gohil *et al*., 2000), *Panax ginseng* (Keum *et al*., 2000) and fruits and vegetables (Cao *et al*., 1996; Wang *et al*., 1996; Brown and Rice-Evans, 1998). In this study, *Smilax china* was selected based on its traditional use in Chinese medicine for inflammatory diseases and ischuria. Several reports describe the antimicrobial activity (Song *et al*., 1998) and antimutagenic activity (Lee and Lin, 1988) of this medicinal plant. Song *et al*. (1998) showed methanol, chloroform, ethyl acetate and butanol extracts of *Smilax china* exhibited potent antimicrobial activities. The extracts of *Smilax china* inhibited the mutagenicity of benzo[a]pyrene (Lee and Lin, 1988). However, little research has been done on antioxidant activity of this plant.

In the present study, the antioxidative actions of extract from *Smilax china* root are evaluated by their abilities to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals, protect cell viability, and inhibit the formation of lipid peroxides. The effect of extracts from *Smilax china* root on the activity of antioxidant enzymes such as SOD, CAT and GPX was also investigated.

Materials and Methods

Preparation of plant extracts

The root of *Smilax china* (100 g) extracted for 3 h in 70% methanol at 80°C was filtered, concentrated with a vacuum rotary evaporator (Eyela, Japan) under the reduced pressure, and freeze-dried (Ilsin, Korea). This methanol extract (ME) was kept at -70°C. The methanol extract was further fractionated by additional extraction with n-hexane, dichloromethane, ethyl acetate and n-butanol, in a stepwise manner. Solvent fractions from each step were removed by rotary evaporation. The extract powder(s) was dissolved in dimethyl sulfoxide (DMSO) and diluted with phosphate buffered saline (PBS, pH 7.4) to give final concentrations in the range of 0.8 to 100 μg/ml.

Cell culture

Chinese hamster lung fibroblast, V79-4 (ATCC CCL-93) cells were maintained at 37°C in an incubator with a humidified atmosphere of 5% CO_2/95% air. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, USA) containing 5% fetal bovine serum (FBS, BioWhittaker, USA), 100 μg/ml of streptomycin, 100 unit/ml of penicillin (Gibco BRL) and 2 mM L-glutamine (Gibco BRL).

Measurements of antioxidant activity

DPPH free radical scavenging activity

In order to measure antioxidant activity, DPPH free radical scavenging assay was carried out as described by Blosi (1958). Methanol extract (ME) and solvent extracted fractions (SEF) of *Smilax china* root at various concentrations (0.8, 4, 20, and 100 μg/ml) were added to a solution of 1.5 x 10^{-4} M DPPH (Sigma, USA) in methanol and the reaction mixture was shaken vigorously. The amount of DPPH remaining was determined at 520 nm, and the radical scavenging activity was obtained from the equation:

$$\text{Radical scavenging activity (\%)} = \{(OD_{control} - OD_{sample})/OD_{control}\} \times 100$$

The antioxidant activity of *Smilax china* root extract was expressed as IC_{50}. The IC_{50} value was defined as the concentration (μg/ml) of the extract required for inhibiting the formation of DPPH radical by 50%.

Cell viability

Cell viability was estimated by the MTT assay, which is based on the cleavage of the tetrazolium salt by mitochondrial dehydrogenases in viable cells (Hansen *et al*., 1989). V79-4 cells were seeded in a 96 well plate at a concentration of 1.2 x 10^4 cells / well and incubated for 16 h. After treatment with various concentrations of ME (4, 20 and 100 μg/ml, respectively) and 100 mM H_2O_2 was added to the culture 1 h later. Cells were incubated for an additional 24 h at 37°C. During the last 4 h, cells were incubated with 20 μl of MTT stock solution (5 mg/ml) in 200 μl medium at 37°C. Samples were then extracted with acidic isopropanol and the absorbance was measured with the ELISA reader (Bio-Rad, USA) at 570 nm. The relative cell viability was determined by the amount of MTT converted to the insoluble formazan salt. The data are expressed as mean percentage of viable cells as compared to the respective control cultures.

Lipid peroxidation inhibitory activity

Lipid peroxidation was assayed by the measurement of malondialdehyde (MDA) according to the method of Ohkawa *et al*. (1979). The cells were exposed to ME of *Smilax china* root at various concentrations (4, 20 and 100 μg/ml) in the incubation medium for 60 min, followed by 1 mM of H_2O_2 for 60 min. Cells were then washed with cold PBS, scraped and homogenized in ice-cold 1.15% KCl. Samples containing 100 μl of cell lysates were combined with 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid adjusted to pH 3.5 and 1.5 ml of 0.8% thiobarbituric acid. The mixture was brought to a final volume of 4.0 ml with distilled water and heated to 95°C for 120 min. After cooling to room temperature, 5.0 ml of a mixture of n-butanol and pyridine (15:1, v/v) was added to each sample and the mixture was shaken vigorously. After centrifugation at 1500 rpm for 10 min, the supernatant fraction was isolated and the absorbance was measured at 532 nm. Inhibitory activity towards lipid peroxidation was expressed as IC_{50}.

Assays for antioxidant enzymes

The cells were treated with 4, 20 and 100 μg/ml of ME of *Smilax china* root for 60 min. The cells were then lysed in a lysis buffer appropriate for the requirements of each assay, as described below. The method of Bradford (1976) was used to determine protein concentrations. Results are expressed as enzyme activity per mg protein compared with corresponding control cultures.

Superoxide dismutase (SOD) activity

SOD activity was assayed by the nitroblue tetrazolium (NBT) method of Beauchamp and Fridovich (1971). NBT is reduced to blue formazan by O_2^-, which has a strong absorbance at 560 nm. The presence of SOD inhibits this reaction. The cells were homogenized in 0.05 M sodium carbonate buffer (pH 10.2). The assay mixture consisted of 0.05 M sodium carbonate buffer (pH 10.2) containing 3 mM xanthine, 0.75 mM NBT, 3 mM EDTA, 1.5 mg/ml BSA and 50 μl of homogenate. The reaction was initiated by the addition 50 μl of xanthine oxidase (0.1 mg/ml) and incubated for 30 min at room tempar-

ature. The reaction was stopped by adding 6 mM $CuCl_2$ and centrifuged at 1,500 rpm for 10 min. The absorbance of blue formazan in the supernatants was measured at 560 nm.

Catalase (CAT) activity

The reaction mixture contained 12 µl of 3% (v/v) H_2O_2 and 100 µl of cell lysates in 50 mM phosphate buffer (pH 7.0) at a final volume of 1.0 ml. Samples were incubated at 37°C for 2 min and the absorbance of the samples were monitored for 5 min at 240 nm. The change in absorbance is proportional to the breakdown of H_2O_2 (Carrillo *et al*., 1991).

Glutathione peroxidase (GPX) activity

GPX was assayed by the method of Paglia and Valentine (1967). The reaction mixture contained 0.1 M phosphate buffer (pH 7.0), 1 mM EDTA, 10 mM glutathione (GSH), 1 mM NaN_3, 1 unit of glutathione reductase, 1.5 mM NADPH and 0.1 ml of cell lysate. After incubation for 10 min at 37°C, H_2O_2 was added to each sample at a final concentration of 1 mM. GPX activity was measured as the rate of NADPH oxidation at 340 nm.

Results

Antioxidant activity of methanol extract (ME) and other solvent extracted fractions (SEF) of *Smilax china* root

The antioxidant activities of *Smilax china* root extract were examined by DPPH free radical scavenging activity, the protective effect on cell viability, and inhibition of lipid peroxidation. The DPPH radical scavenging activities of ME and SEF are presented in Figure 1. All samples showed the DPPH radical scavenging activity in a dose-dependent manner. ME of *Smilax china* root showed relatively high DPPH radical scavenging activity, with an average IC_{50} value of 7.4 µg/ml. The DPPH radical scavenging activity of SEF samples was found to be in the order of ethyl acetate, butanol and water fractions, with IC_{50} values of 4.6, 8.7 and 9.6 µg/ml, respectively. The DPPH free radical scavenging activity of hexane and dichloromethane fractions was not significant. IC_{50} values of hexane and dichloromethane fractions were >100 and 16 µg/ml, respectively. The DPPH free radical scavenging activity of ME was comparable to that of ethyl acetate, butanol and water extracted fractions, which showed significantly high free radical scavenging activities in this study. Therefore, we used ME to investigate overall antioxidant activity of *Smilax china* root.

The effect of *Smilax china* on V79-4 cell proliferation

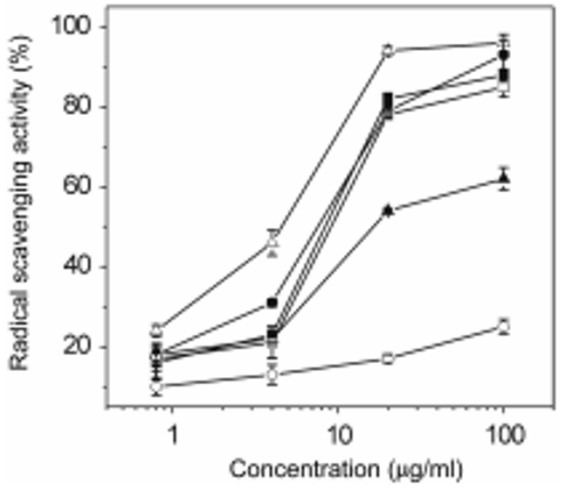

Figure 1. DPPH radical scavenging activity of total extracts and fractions of *Smilax china* root. Each experiment was performed at least 3 times and data are expressed as average percent change from control ± S.D. ● -Total extracts, ○ -Hexane fractions, ▲ -Dichloromethane fractions, △ -Ethyl acetate fractions, ■ -Butanol fractions and □ -Water fractions.

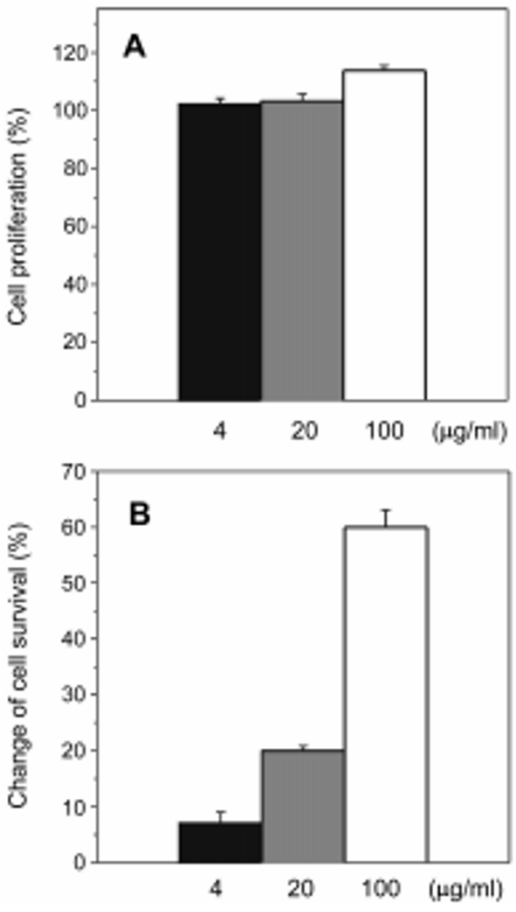

Figure 2. (A) Effect on cell proliferation of *Smilax china* root extracts in V79-4 cells. (B) Protective effect of total extracts of *Smilax china* root against H_2O_2-induced oxidative damage in V79-4 cells. Each experiment was performed at least 3 times and data are expressed as mean±S.D. (A) or average percent change from control ± S.D. (B) Black, gray and white bars indicate 4, 20 and 100 µg/ml of samples, respectively.

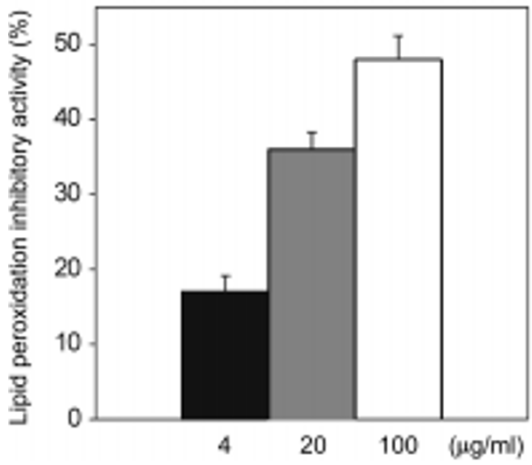

Figure 3. Lipid peroxidation inhibitory activity of total extracts of *Smilax china* root against H_2O_2-induced oxidative damage in V79-4 cells. Each experiment was performed at least 3 times and data are expressed as average percent change from control ± S.D. Black, gray and white bars indicate 4, 20 and 100 μg/ml of samples, respectively.

was evaluated by MTT assay. *Smilax china* treatment at concentration from 4 to 100 μg/ml caused slight stimulation of cell proliferation and did not affect any cell morphology. Thus, all subsequent experiments were carried out with ME concentration range of 4-100 μg/ml.

Protective effect of ME on the cell viability was assessed by treating cells with ME for 1 h prior to the addition of H_2O_2. Control cells were treated with H_2O_2 in the presence of vehicle only. The final concentration of DMSO was below 0.1% in this study. There was no cytoprotective effect of DMSO at this concentration. The relative cell survival was determined 24 h later by the MTT assay. As shown in Figure 2B, treatment with ME of *Smilax china* root induced a dose-dependent increase in cell survival. Cell viability was increased definitively at a dose level of 100 μg/ml ME.

A possible effect of ME on lipid peroxidation in H_2O_2-treated V79-4 cells was also assessed (Figure 3). ME did inhibit lipid peroxidation but a lot less than DPPH radical scavenging activity, with IC_{50} value of >100 μg/ml.

Effect of Methanol Extract of *Smilax china* root on antioxidant enzyme activity

In order to investigate whether these antioxidant activities of *Smilax china* root extract are mediated by an increase in antioxidant enzymes, SOD, CAT and GPX activities in V 79-4 cells were examined following ME treatment (Figure 4). Treatment of cells with ME at doses of 4, 20 and 100 μg/ml induced 10, 16 and 25% increase in SOD levels, respectively (Figure 4A). Increments of SOD activity in a dose dependent manner over ME concentration range of 4 to 100 μg/ml was detected. The activity of SOD in control cells untreated with ME

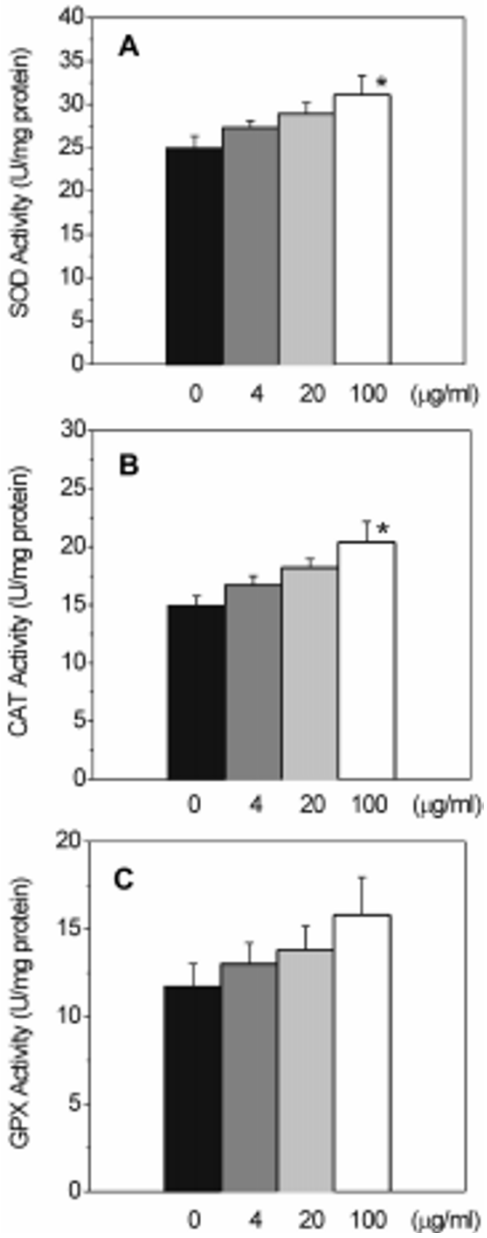

Figure 4. Effect of total extracts of *Smilax china* root on SOD (A), CAT (B) and GPX (C) activity in V79-4 cells. Each experiment was performed at least 3 times and data are expressed as average enzyme unit per mg protein ± S.D. Black, gray, light gray, and white bars indicate 0, 4, 20, and 100 μg/ml of samples, respectively. *statistically significant vs. control ($p < 0.05$).

was 24.9 ± 1.5 U/mg protein. The relatively higher increment of CAT than SOD activities was observed when cells were treated with *Smilax china* root ME (Figure 4B). The CAT activity was also dose-dependently increased upon exposure of cells to various ME concentrations. At doses of 4, 20 and 100 μg/ml, ME activated CAT by 12, 22 and 37%, respectively. The CAT activity of control cells untreated with extracts was 14.9 ± 1.9 U/mg protein.

A similar increase of cellular GPX activity was found upon treatment of cells with ME (Figure 4C). ME treatment of cells with 4, 20 and 100 μg/ml resulted increment of GPX activity by 11, 18 and 35%, respectively. The activity of GPX in control cells untreated with extracts was 11.7 ± 1.3 U/mg protein.

Discussion

Beneficial effects of antioxidants are well known in scavenging reactive oxygen species (ROS) before or preventing oxidative damage from spreading, *e.g.*, by interrupting the radical chain reaction of lipid peroxidation (Halliwell and Gutteridge, 1998). Many antioxidants are known to be present in plants, fruits and vegetables. Possible presence of antioxidant activity in many commonly used medicinal plants is not well understood. In this study, methanol extract (ME) of *Smilax china* root, a popular herbal medicinal plant, showed a dose-dependent significant DPPH free radical scavenging activity *in vitro.* ME also enhanced viability in V79-4 cells exposed to H_2O_2. Treatment of cells with ME of *Smilax china* root increased the activity of cellular antioxidant enzymes; SOD, CAT and GPX. Thus, these results indicate that *Smilax china* root extract possesses considerable antioxidant activity. The antioxidant components in *Smilax china* root fractionated by series of various solvent extraction showed the most active fraction was in the hydrophilic solvents; water, methanol, ethyl acetate, and butanol.

Recent studies confirm the presence of antioxidant activity in a small number of medicinal plants. The *Withania somifera* (WS), the ancient Hindu system of medicine, was found to enhance the activities of SOD, CAT and GPX in rat brain frontal cortex and striatum (Bhattacharya *et al*., 2001). The extract of *Amomum xanthoides,* from the fruit of *Amomum vilosum* Lour induced inhibition of alloxan-induced ROS generation (Park *et al*., 2001). Blackberries, raspberries and strawberries are reported to possess potent $ROO^{\cdot}$ scavenging capacity (Wang and Jiao, 2000). Tea polyphenols such as epigallocatechin gallate (EGCG), epigallocatechin (EGC) and epicatechin gallate (ECG) are another antioxidants to react with peroxy radical and terminate lipid peroxidation chain reactions (Li and Xie, 2000). The antioxidant properties of the wild growing sage species, *Salvia reflexa hornem*, were found retain scavenging activity of DPPH free radicals. (Malencic *et al*., 2000). Effect of the fresh leaf pulp extract of Aloe vera was effective in inducing SOD and CAT activities in mice (Singh *et al*., 2000). Treatment with Aloe vera caused a decrease in malondialdehyde (MDA) formation and the activity of lactate dehydrogenase in the liver. Kim *et al*. (1996) found ginsenoside Rb_1, a major saponin from *Panax ginseng*, was able to prevent lipid peroxidation.

Taken together, an increasing body of evidence suggests that many plants have antioxidant activities that could be therapeutically beneficial.

The activities of cellular antioxidant enzymes (SOD, CAT, GPX) are modulated in various disease states by the abundance of free radical species (Halliwell and Gutteridge, 1998). Thus, maintaining the balance between the rate of generation of radicals and scavenging of radicals is an essential part of biological homeostasis. It is of particular interest to note that SOD catalyzes the breakdown of $O_2^{\cdot}$ to O_2 and H_2O_2, prevents formation of OH^{-} and has hence been implicated as an essential defense against the potential toxicity of oxygen. The ROS scavenging activity of SOD is effective only when it is followed by the actions of CAT and GPX, because the dismutase activity of SOD generates H_2O_2, which needs to be further scavenged by CAT and GPX. Extracts of *Smilax china* root activated CAT and GPX to a greater extent than SOD. Taken together, these results also suggest that the antioxidant activity of *Smilax china* root extract may lie in its ability to degrade H_2O_2 and other peroxides.

In conclusion, extracts from *Smilax china* root have high levels of DPPH radical scavenging activity, inhibit lipid peroxidation, promote cell viability, and enhance the effects of various antioxidant enzymes. These findings strongly suggest that extracts of *Smilax china* root have potential chemoprotective activity against oxidative stress.

Demonstration of the high levels of antioxidant components present in *Smilax china* root extract will provide scientific base for further investigation of unknown components.

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