Antioxidative Properties of Sachil-Tang Extract

Hyo Seung Yi, Jin Young Moon*

Department of Acupoint, College of Oriental Medicine, Dongguk University

Sachil-Tang (SCT) has been traditionally used as a prescription of spasm of the esophagus by stress, pectoralgia and oppressive feeling of the chest in Oriental medicine. This study was carried out to investigate the antioxidant activities of the ethanol extract of SCT and its inhibitory effect on intracellular oxidation and vascular cell adhesion molecule-1 expression in human umbilical vein endothelial cells (HUVECs) using various methods. The SCT extract showed a strong inhibitory effect on free radical generating model systems, including DPPH radical, superoxide anions, hydroxyl radical, peroxynirite and nitric oxide. Besides, the SCT extract exhibited a strong inhibitory effect on lipid peroxidation in rat liver homogenate induced by $FeCl_2$-ascorbic acid, and protected plasmid DNA against the strand breakage in a Fenton's reaction system. The SCT extract also inhibited copper-mediated oxidation of human low-density lipoprotein (LDL), and repressed relative electrophoretic mobility of LDL. Furthermore, the SCT extract protected intracellular oxidation induced by various free radical generators and inhibited expression of vascular cell adhesion molecule-1 (VCAM-1) in HUVECs. These results suggest that SCT can be an effective natural antioxidant and a possible medicine of atherosclerosis.

Key words : Sachil-Tang, antioxidant, human low-density lipoprotein, intracellular oxidation, human umbilical vein endothelial cells

Introduction

Sachil-Tang (SCT) has been known as a prescription of spasm of the esophagus, pectoralgia and oppressive feeling of the chest caused by stress in Korean traditional medicine[1]. A few studies have reported the effects of SCT, which include plasma and uterus changes in rats in a state of immobilization stress[2] and treatment for vomiting during pregnancy[3]. In Korean and Chinese traditional medicine, it has been thought that emotional instability or stress have close relation to heart. And pectoralgia and oppressive feeling of the chest are important clinical symptoms of cardiovascular atherosclerosis[4]. However, no study has so far been examined about the phytochemical and pharmacological effects of SCT with regard to cardiovascular atherosclerosis. So, we tried to evaluate a new possibility of SCT in the role of natural antioxidants at the early phases of the management of atherosclerosis. Oxidative stress caused by free radicals can raise pathogenesis in cardiovascular diseases, including atherosclerosis, heart failure, diabetic vascular complications, and hypertension[5,6]. Reactive oxygen species (ROS) such as superoxide anion ($O_2\cdot$), hydrogen peroxide (H_2O_2), hydroxyl radical (·OH) and singlet oxygen ($1O_2$), as well as reactive nitrogen species (RNS) such as peroxynitrite (ONOO-) and nitric oxide (·NO), are concerned in modifying phospholipids, especially in low-density lipoprotein (LDL)[7,8]. Previously, it has been reported that oxidized-LDL is incorporated into monocytes and macrophages to form foam cells, which may promote the atherosclerotic process[9,10]. Moreover, ROS are involved in vascular cell signaling correlated with migration and proliferation[11-13]. Endothelium is an important place concerned with atherosclerosis because oxidative injury of the endothelium accompanies acute inflammation[14] and adhesion of monocytes to endothelium initiate the development of atherosclerosis[15]. Interaction between monocytes and endothelial cells is mediated by cell adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), which also play an important role in leukocyte adhesion and transendotheilal migration at sites of inflammation[16]. On the other hand, accumulating evidence shows that antioxidant agents protect lipid peroxidation related to macrophage adhesion and activation, in biological systems by scavenging free radicals[17] and

* To whom correspondence should be addressed at : Jin Young Moon, Department of Acupoint, College of Oriental Medicine, Dongguk University, Sukjang-Dong 707, Kyungju, Kyungbuk, Korea
· E-mail : ampmoon@dongguk.ac.kr, · Tel : 054-770-2665
· Received : 2009/06/10 · Revised : 2009/06/25 · Accepted : 2009/07/15

minimizing the risk of myocardial infarction and atherosclerosis[18-20]. In this report, we investigated the antioxidant activity of SCT extract in different types of free radical generating systems, lipid peroxidation, DNA nicking and human LDL oxidation. Furthermore, since oxidative injury and expression of adhesion molecules in endothelium play key roles in the process of atherosclerosis, this study also aimed to ascertain whether the SCT extract can inhibit oxidation and expression of adhesion molecules in human umbilical vein endothelial cells (HUVECs).

Materials and Methods

1. Chemicals

2,2-diphenyl-1-picryl-hydrazyl (DPPH), ethylenediaminetetraacetic acid (EDTA), (-)-epigallocatechin gallate (EGCG), butylated hydroxytoluene (BHT), dimethyl sulfoxide (DMSO), L-ascorbic acid, α-tocopherol, deoxyribose, hydrogen peroxide (H_2O_2), nitro blue tetrazolium (NBT), trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA), hypoxanthine, xanthine oxidase, bicinchoninic acid kit, DL-penicillamine, diethylenetriaminepentaacetic acid (DTPA), 4,5-Diaminofluorescein (DAF-2), 3-morpholinosydnonimine hydrochloride (SIN-1), copper(II) sulfate ($CuSO_4$), human low-density lipoprotein (LDL), sodium nitroprusside and gallic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Dihydrorhodamine 123 (DHR 123) and 6-carboxy-2′,7′-dichlorofluorescein diacetate (DCFH-DA) were from Molecular Probes (Eugene, OR). Peroxynitrite was from Cayman Chemical Co. (Ann Arbor, MI). Folin-Ciocalteu's phenol reagent and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl -2H-tetrazolium bromide (MTT) was obtained from Merck (Merck KGaA, Germany). Blue/Orange 6X loading dye, agarose (LE, analytical grade) and ethidium bromide were obtained from Promega (Madison, WI). Ferric chloride ($FeCl_3$), ferrous chloride ($FeCl_2$) and 2,2′-azobios(2-amidinopropane) dihydrochloride (AAPH) were obtained from Wako Pure Chemical Industries LTD. (Osaka, Japan). All other chemicals used were analytical grade.

2. Preparation of SCT Extract

The prescription of SCT was prepared with Pinellia ternata (50 g), Poria cocos (40 g), Magnolia officinalis (30 g) and Perilla frutescens (20 g). These plant materials were obtained from the Dongguk Oriental Medicine Hospital (Kyungju, Kyungbuk). Extract of SCT was prepared by incubation of 70% ethanol (100 g/L) for 12, 24 and 48 hours followed by each step. After filtration, the extract was evaporated under vacuum condition and dried using a Freeze Drier (Freezone 6. Labconco, USA). The yield of the ethanol extract was 1.93% of the initial material.

3. Free Radical Scavenging Activities

1) DPPH Scavenging Activity

DPPH radical scavenging activity of SCT extract was determined by the method according to Gyamfi et al.[21] with a slight modification. Briefly, different concentrations of SCT extract (50 μl) were mixed with 1 ml of 0.1 mM DPPH ethanol solution and 450 μl of 50 mM Tris-HCl buffer (pH 7.4). The mixture was shaken and incubated for 30 min at room temperature. The absorbance was measured at 517 nm and DPPH radical scavenging activity was calculated as follows : % inhibition=[(absorbance of control-absorbance of sample)/absorbance of control]×100. Ascorbic acid and BHT were used as positive control.

2) Superoxide Anions Scavenging Activity

Scavenging activity of SCT extracts on superoxide anion radical (O_2-) was estimated by the method of Gotoh and Niki[22]. The SCT extracts of different concentrations were added to a reaction solution containing 100 μl of 30 mM EDTA (pH 7.4), 10 μl of 30 mM hypoxanthine and 200 μl of 1.42 mM NBT. After preincubation for 3 min at room temperature, l00 μl of 0.5 U/ml xanthine oxidase was added and then the final volume was supplemented with 50 mM phosphate buffer (pH 7.4) up to 3 ml. After the solution was incubated for 20 min at room temperature, the absorbance was measured at 560 nm.

3) Hydroxyl Radical Scavenging Activity

The hydroxyl radical (·OH) scavenging activity of SCT extract was assessed by the method reported by Halliwell and Gutteridge[23]. Various concentrations of extract were mixed with 1.25 mM H_2O_2 and 0.2 mM $FeSO_4$ by using 96-well microplate. After the mixture was incubated at 37℃ for 5 min, esterase-treated 2 μM H2DCFDA was added, and the final volume was 250 μl/well. The changes in fluorescence were measured using a microplate fluorescence spectrophotometer (GENios-basic, TECAN, Austria), with excitation and emission wavelengths of 485 and 528 nm for 30 min. Trolox was used as a positive control.

4) Nitric Oxide Scavenging Activity

To measure nitric oxide (·NO) scavenging ability, the method of Nagata et al.[24] was used. At first, 1 mg of DAF-2 was dissolved in 0.55 mL of DMSO, which was diluted with 50 mM phosphate buffer (1:400, v/v) to prepare DAF-2 solution. And then, different concentrations of SCT extract (10μl) were mixed with 130 μl of 50 mM phosphate buffer (pH 7.4), 10 μl of 40 mM SNP and 50 μl of DAF-2 solution. After

incubation at room temperature for 10 min, the fluorescence signal caused by the reaction of nitric oxide and DAF-2 was measured by the fluorescence microplate reader at excitation and emission wavelengths of 495 and 515 nm after 10 min at room temperature.

5) Peroxynitrite Scavenging Activity

The peroxynitrite (ONOO-) scavenging activity of SCT extract was determined by the method of Kooy et al.[25] with a slight modification. Briefly, 10 µl of the SCT extracts of different concentrations were mixed with 175.8 µl of rhodamine buffer (50 mM sodium phosphate dibasic, 50 mM sodium phosphate monobasic, 90 mM sodium chloride and 5 mM potassium chloride) containing 4 µl of 5 mM DTPA and 0.2 µl of 5 mM DHR 123. The reaction was started by adding 10 µl of 10 µM peroxynitrite. After 10 min at room temperature, fluorescent intensity of the mixture was monitored at excitation and emission wavelengths of 480 and 530 nm, respectively. The scavenging effect of extract was expressed as the percent inhibition of DHR 123 oxidation, and penicillamine was used as a positive control. All experiments were carried out in triplicate and the free radical scavenging activities of SCT extract were expressed as IC_{50}. The IC_{50} value was defined as the final concentration (in µg/ml) of samples that inhibits 50% of the free radical formation.

4. Preparation of Rat Liver Homogenate

Male Sprague-Dawley rats aging 4-5 weeks old were purchased from Oriental Bio. (Seongnam, Korea). They were kept in an air-conditioned room with needed temperature and humidity (24±2℃, 60±5%). After one week of adaptation, rats were sacrificed and the hepatic tissue was homogenized in 0.15 M KCl buffer (pH 7.0) with Teflon Plotter Elvehiem Homogenizer (Teflon Plotter Elvehiem, Glas-Col Co., USA). The amount of protein was determined using a bicinchoninic acid protein kit. Rat liver homogenate was stored at -86℃ until use.

5. $FeCl_2$-ascorbic Acid Stimulated Lipid Peroxidation in Rat Liver Homogenate

Antioxidant activity of SCT extract on $FeCl_2$-ascorbic acid stimulated lipid peroxidation in rat liver homogenate was determined by the method of Patro et al.[26]. Different concentrations of SCT extract were added to the reaction mixture [0.5 ml of 7.5 mg/ml rat liver homogenate, 0.1 ml of 50 mM Tris-HCl buffer (pH 7.2), 0.05 ml of 0.1 mM ascorbic acid and 0.05 ml of 4 mM $FeCl_2$]. After incubation at 37℃ for 1 h, 0.9 ml of distilled water and 2 ml of 0.6% TBA were added to the mixture, which was shaken vigorously and heated at 100℃ for 30min. After cooling continuously, 5 ml of n-butanol was added and the mixture was shaken vigorously and centrifuged for 10 min at 1000×g to obtain the n-butanol layer. Absorbance of the supernatant at 532 nm was determined using a spectrophotometer (UltroSpec 6300 Pro, Amersham, UK). The inhibitory effect of SCT extract on lipid peroxidation was calculated by using the following equation: % inhibition= [(absorbance of control－absorbance of sample)/absorbance of control]×100. α-tocopherol was used as positive control.

6. DNA Nicking Assay

Protective effect of SCT extract against DNA nicking was assessed by the method described by Lee et al.[27] with a slight modification. Various concentrations of SCT extract were mixed with 2.0 µg of supercoiled pBR322 plasmid DNA in the presence of 50 µM ascorbic acid, 300 mM H_2O_2 and 80 µM FeCl3. After the mixture was incubated for 30 min at 37℃, Blue/Orange 6X loading dye was added and loaded onto 1% agarose gel in 1X TAE buffer containing ethidium bromide. The gel was run at 100 V for 1 h, and photographed under UV illuminator.

7. Antioxidant Effect on Human Low-density Lipoprotein (LDL)

1) Copper-Mediated LDL Oxidation

The inhibitory effect of SCT extract on the copper-mediated human LDL oxidation was determined spectrophotometrically by measuring the amount of TBARS[28]. Briefly, 100 µg/ml of human LDL in PBS (pH 7.4) was mixed with SCT extract and the oxidation was initiated by the addition of 10 µM $CuSO_4$. After incubation at 37℃ for 4h, 1 ml of 20% TCA was added to the mixture and then centrifuged at 1500 × g for 10 min. The supernatant (1 ml) was mixed with 1 ml of 0.67% TBA in 0.05 M NaOH, and then heated at 90℃ for 45 min. After cooling, the absorbance was measured at 532 nm. The inhibition ratio (%) of the sample was calculated by the following equation : % inhibition=[(absorbance of control-absorbance of sample)/absorbance of control]×100. Ascorbic acid and caffeic acid were used as positive control.

2) Relative Electrophoretic Mobility (REM) Assay

The REM of LDL was determined by agarose gel electrophoresis according to the method of Yoon et al.[29]. The LDL (120 µg/ml) in PBS (pH 7.4) was mixed with different concentrations of SCT extract and 10 µM $CuSO_4$, afterwards incubated at 37℃ for 12 h. After incubation, 3 µg of LDL was loaded using 0.7% agarose gel for 1 h at constant voltage of 85 V in TAE buffer (40 mM Tris, 40 mM acetic acid, and 1 mM

EDTA). The gel was dried and stained with Coomassie brilliant blue R-250. As positive controls, 20 μM EDTA and 2 μg/ml ascorbic acid were used.

8. Determination of Total Phenolics

The content of total phenolic compounds was determined using Folin-Ciocalteus reaction[30] using gallic acid as standard. Forty microliters of SCT extract (1 mg/ml) was mixed with 200 μl of Folin-Ciocalteus reagent and 1160 μl of distilled water. And then, 3 min later at room temperature, 600 μl of 20% sodium carbonate was added to the mixture. After shaking for 2 h at room temperature, the absorbance was measured at 765 nm. The concentration of total phenolic compounds was expressed as gallic acid equivalents (mg of gallic acid/g of sample).

9. Cell Culture and Viability

Human umbilical vein endothelial cells (HUVECs) were purchased from MCTT (MC1133, Seoul, Korea) and cultured with endothelial cell basal medium-2 (EBM-2) from Cambrex (Walkersville, MD) in a humidified atmosphere of 5% CO_2 at 37℃. Cell viability was measured by using a colorimetric assay with MTT. Briefly, HUVECs were seeded into 96-well culture plates at a density of 10^4 cells/well and cultured for 24 h. And then, cells were treated for 12 h with different concentrations of SCT extract and after incubation, stock solution of MTT (1 mg/ml in phosphate buffered saline) was added to each well for 4 h. At the end of the treatment the incubation medium was removed and the insoluble formazan crystals were dissolved by 150 μl solution of DMSO. The absorbance of each well was measured on an ELISA micro-plate reader (VERSAmax, Molecular Devices Corp., USA) at 570 nm. The cells without sample treatment were served as controls.

10. Antioxidant Effect on Cellular Oxidative Stress

In an attempt to determine antioxidant effect of SCT extract in a cell model, we used dichlorofluorescein (DCF) assay[31]. HUVECs (10^4 cells/well) were seeded into 96-well culture plates 1 day before the experiment. On the day of the experiment, after removing the medium, SCT extract (200 μg/ml in serum free EGM-2) were added to wells. After incubation for 12 h at 37℃ in 5% CO_2 following the removal of SCT extract, plates were incubated with 10 μM DCFH-DA diluted in PBS buffer for 45 min. And then, cells were washed twice with PBS buffer and were treated with different kinds of free radical generators: 1 mM AAPH, 50 μM SIN-1, 10 mM SNP and 4 mM H_2O_2. The fluorescent intensity was monitored at excitation and emission wavelengths of 485 and 535 nm using a fluorescence microplate reader. The fluorescence reading was taken at intervals of 10 min up to 100 min. The DCF fluorescence increase (%) was calculated every 10 min as following equation : % increase=[(fluorescent intensity of sample-fluorescent intensity of control)/ fluorescent intensity of control]×100. The cells, which were incubated without free radical generators or sample treatment, served as controls and the % increase of sample was compared with that of group which was only treated free radical generators.

11. Western Blot Analysis for VCAM-1 and ICAM-1 Expression

Western blot analysis was performed by using a previously described method[32]. HUVECs were pretreated with SCT extract (10, 100, 200 μg/ml) for 12 h and then incubated with serum free medium containing TNF-α (10 ng/ml) for 6 h. After treatment, cells were washed with ice-cold PBS (pH 7.4), scraped with a rubber policeman and centrifuged at 4℃ for 10 min at 10000 × g. Cell lysates were extracted using PRO-PREP (iNtRON Biotechnology, Korea) protein extract solution. The sample was centrifuged at 12000 × g for 20 min and protein concentration was determined by bicinchoninic acid assay with BSA as standard. Aliquot of 20-50 μg of protein were boiled for 5 min with a gel-loading buffer [125 mM Tris-HCl, 4% SDS, 10% 2-mercaptoethanol (pH 6.8), 0.2% bromophenol blue]. Each sample were separated by SDS-polyacrylamide gel electrophoresis for 1.5 h at 90 V and transferred to PVDF (Pall, USA) membrane in Semi-dry transfer Cell (Bio-Rad Lab, Hercules, CA). After blocking the membrane with 5% fat-free dry milk powder dissolved in TBS (50 mM Tris, 0.5 M NaCl, pH 7.4) containing 1% Tween 20 (TBS-T) for 2h at room temperature, we incubated the membranes with primary antibodies against VCAM-1 (1:1000, Santa Cruz Biotechnology) and ICAM-1 (1:500, Santa Cruz Biotechnology), each supplemented with anti β-actin antibody (1:1000, Cell signaling technology) over night at 4 ℃. The membranes were washed with TBS-T and then incubated for 1 h with secondary antibodies (horseradish peroxidase conjugated anti-goat or anti-rabbit, 1:10000, Santa Cruz Biotechnology). The signal was detected by enhancer chemiluminescent assay (Amersham, Berkshire, UK) detection system. Pre-stained protein markers (iNtRON Biotechnology) were used for molecular weight determinations.

12. Statistical Analysis

The experiments shown in this paper are a summary of the data from at least three experiments. Results are expressed as mean ± standard deviation and analyzed by SPSS (version 14.0 for Windows, SPSS Inc.). Statistical significance was

evaluated by Student's t-test. Values of $p < 0.05$ were considered statistically significant.

Results

1. Free Radical Scavenging Activity

At first, we evaluated the antioxidant activity of SCT extract through various free radical generating systems. Table 1 shows the scavenging activities of SCT extract against DPPH radical, superoxide anions and hydroxyl radical compared to those for reference antioxidants. In the DPPH radical scavenging assay, ascorbic acid exhibited the highest activity (IC_{50}=6.14±1.01 μg/ml). On the other hand, SCT extract showed better scavenging activity (IC_{50}=14.00±0.85 μg/ml) than that of BHT (IC_{50}=18.33±0.88 μg/ml). In the superoxide anions scavenging test, SCT extract showed much stronger activity (IC_{50}=6.02±0.11 μg/ml) than epigallocatechin gallate (IC_{50}=14.28±0.97 μg/ml) and caffeic acid (IC_{50}=16.96±1.49 μg/ml), which were used as reference compounds. In the hydroxyl radical scavenging test, SCT extract showed a bit lower activity (IC_{50}=34.78±0.24 μg/ml) than that of trolox ((IC_{50}=14.45 ±0.33 μg/ml). Table 2 shows the scavenging activities of SCT extract on RNS including nitric oxide and peroxynitrite. The SCT extract effectively scavenged nitric oxide (IC_{50}=2.25±0.09 μg/ml) and peroxynitrite (IC_{50}=0.92±0.20 μg/ml). In nitric oxide scavenging activity case, SCT extract is comparable to well known nitric oxide scavenger, resveratrol (IC_{50}=1.76±0.10 μg/ml). And, in the peroxynitrite scavenging activity case, SCT showed better scavenging activity than that of penicillamine (IC_{50}=1.37±0.23 μg/ml).

Table 1. Scavenging activity of SCT extract on free radical.

	IC_{50} values ± SD(μg/mℓ)[a]		
	DPPH	Superoxide	Hydroxyl radical
SCT extract	14.00 ± 0.85	6.02 ± 0.11	34.78 ± 0.24
Vit. C[b]	6.14 ± 1.01	-	-
BHT[b]	18.33 ± 0.88	-	-
Caffeic acid[b]	-	16.96 ± 1.49	-
EGCG[b]	-	14.28 ± 0.97	-
Trolox[b]	-	-	14.45 ± 0.33

a) The results are expressed as IC50 values, and each value represents the mean of three separate experiments. b) Used as a positive control.

Table 2. Scavenging activity of SCT extract on reactive nitrogen species.

	IC50 values ± SD(μg/mℓ)[a]	
	Nitric Oxide	Peroxynitrite
SCT extract	2.25 ± 0.09	0.92 ± 0.20
Resveratrol[b]	1.76 ± 0.10	-
Penicillamine[b]	-	1.37 ± 0.23

a) The results are expressed as IC50 values, and each value represents the mean of three separate experiments. b) Used as a positive control.

2. Inhibitory Effect of SCT extract on Lipid Peroxidation in Rat Liver Homogenate

Fig. 1 shows the antioxidant effect of SCT extract compared with α-tocopherol, used as a control antioxidant. In the $FeCl_2$-ascorbic system, SCT extract and α-tocopherol showed a dose-dependent inhibition of lipid peroxidation. At the 500 μg/ml concentration, the percentage inhibition of SCT extract and α-tocopherol on lipid peroxidation was 83.70±2.01% and 71.13±2.63%, respectively. Moreover, in terms of IC_{50} value, SCT extract (IC_{50}=51.76±1.14 μg/ml) was lower than that of α-tocopherol (IC_{50}=81.12±7.24 μg/ml).

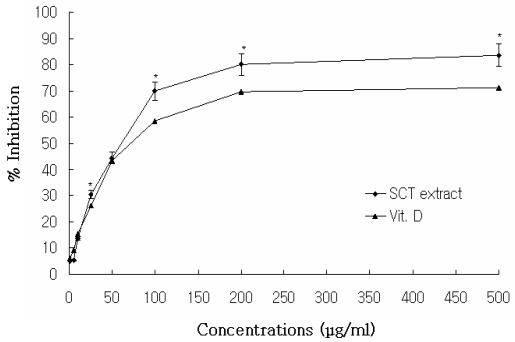

Fig. 1. Inhibitory effects of SCT extract and α-tocopherol on MDA production in rat liver homogenate induced by $FeCl_2$-ascorbic acid in vitro. The concentration of SCT extract and α-tocopherol test ranged from 1 to 500 μg/ml. The results are the means of three separate experiments (* : $p < 0.05$ compared with reference sample, vit. D).

3. Effect of SCT Extract on DNA Nicking.

The protective effect of SCT extract on hydroxyl radical induced DNA nicking was investigated by incubating supercoiled pBR322 plasmid DNA in presence of H_2O_2 and $FeCl_3$ for 30 min at 37℃. As shown in Fig. 2, the addition of Fenton's reagents to the pBR322 plasmid increased the conversion of DNA from supercoiled (SC) form to an open circular (OC) form, indicating increased DNA nicking by hydroxyl radicals (lane 2). However, SCT extract (10, 25, and 50 μg) revealed a constant inhibition of DNA nicking (lanes 5, 6, and 7), and this inhibitory effect was similar to that of 5 U of catalase (lane 3) and 100 mM EDTA (lane 4). This result indicates that SCT extracts protect DNA damage in Fenton reaction system.

4. Inhibitory effect of SCT Extract on LDL Oxidation.

Fig. 3 shows the inhibitory effect of SCT extract and commercial reference antioxidants on copper-mediated human LDL oxidation. At the 2-20 μg/ml concentrations, the percentage inhibition of SCT extract was the highest among test samples. And, in terms of IC_{50} value, SCT extract (IC_{50}=1.56±0.08 μg/ml) was comparable to ascorbic acid (IC_{50}=1.54±0.20 μg/ml) and caffeic acid (IC_{50}=0.53± 0.09 μ

g/ml). Fig. 4 shows the inhibitory effect of SCT extract on REM of LDL. Incubation with 10 μM $CuSO_4$ alone enhanced REM of LDL (lane 2), compared to that of native LDL (lane 1). In contrast, SCT extract (2, 5, 10 μg/ml) revealed dose-dependent inhibition of LDL oxidation (lanes 3, 4, 5). When treated with 10 μg/ml of SCT extract (lane 5), the protective effect of LDL oxidation was better than that of reference antioxidant compounds including EDTA and ascorbic acid (lanes 6, 7).

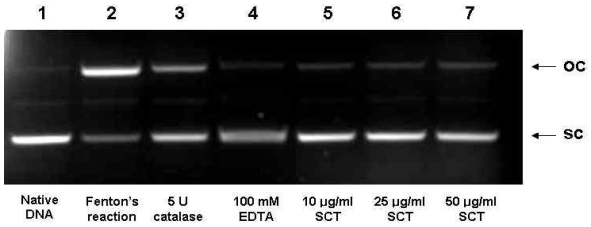

Fig. 2. Inhibitory effect of SCT extract on DNA nicking caused by hydroxyl radicals. The DNA nicking reaction was initiated by adding 500 ng of supercoiled pBR 322 plasmid DNA to Fenton's reaction solution in the presence of SCT extract for 30 min at 37℃. Lanes 1 and 2 show native plasmid DNA and Fenton's reaction alone, respectively. Lanes 3 and 4 show the result for reaction mixture containing 5 U of catalase and 100 mM EDTA, respectively. Lanes 5-7, supercoiled DNA with reaction mixture containing SCT extract (10, 25, and 50 μg/ml).

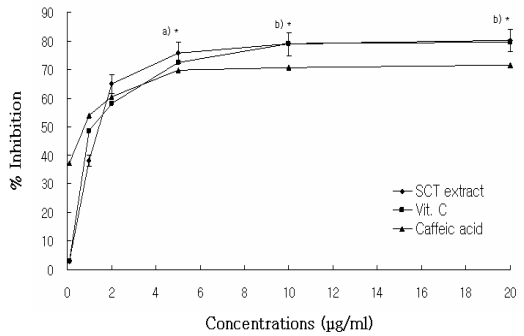

Fig. 3. Values of antioxidant effects of SCT extract, vit. C and caffeic acid on Cu+-mediated LDL oxidation. The level of LDL oxidation was measured by TBARS assay and results expressed as mean±SD of three separate experiments. a) Significantly different from vit. C. b) Significantly different from caffeic acid (* : $p < 0.05$).

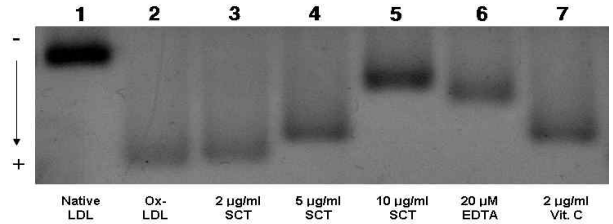

Fig. 4. Effect of SCT extracts on the relative electrophoretic mobility of LDL. Human LDL (120 μg protein/mL) was added to SCT extracts and reference antioxidants. The LDL oxidation was initiated by adding 10 μM $CuSO_4$. After incubation for 12 h at 37 ℃, about 3 μg of LDL protein was loaded on 0.7% agarose gel for 1 h, and the gel was stained with Coomassie brilliant blue R-250. Lane 1, native LDL; lane 2, oxidized LDL; lanes 3-5, SCT extract (2, 5, 10 μg/ml); lanes 6, EDTA (20 μM); lane 7, ascorbic acid (2 μg/ml).

5. Content of Total Phenolics

Content of total phenolics of SCT ethanol extract was determined by the method of Folin-Ciocalteu reaction using gallic acid as standard, and compared it with that of the SCT water extract. As shown in Table 3, the amount of total phenolic compounds of SCT ethanol extract was 147.47±9.00 mg/g, which was about 2.3-fold higher than that of the SCT water extract (65.13±3.29 mg/g).

Table 3. Total phenolic contents of the water and ethanol extract from SCT.

Samples	Total Phenolics (mg/g)[a]
Water extract of SCT[b]	65.13 ± 3.29
Ethanol extract of SCT	147.47 ± 9.00

Content of total phenolics was determined according to the method of Folin-Ciocalteu reaction, using gallic acid standard. a) Total phenolic amount was expressed as gallic acid equivalents(GAE) in milligrams per gram sample. b) For preparation of the water extract, the grounded SCT was mixed with boiling water for 3h. After filtration, the filtrate was evaporated and then frozen. Each experiment was performed in triplicate and results are the mean ± SD.

6. Effect of SCT Extract on Cell Viability in HUVECs

The effect of SCT extract on cell survival was examined by MTT assay. Fig. 5 shows the percentage of viability after 12 h incubation with different concentrations (10-400 μg/ml) of SCT extract. SCT extract at 1-200 μg/ml had nearly no significant effect on cell viability (over 95%). However, 400 μg/ml of SCT extract revealed over 15% of cell cytotoxicity.

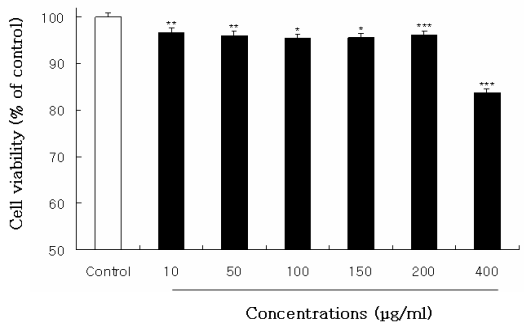

Fig. 5. Effects of SCT extract on cell viability in HUVECs. Cells were treated for 12 h with indicated concentrations of SCT extract, and cell viability was measured by MTT assay, as described in Materials and Methods section (* : $p < 0.05$, ** : $p < 0.01$, *** : $p < 0.005$ compared with untreated cells).

7. Antioxidant Effect on Cellular Oxidative Stress in HUVECs

Fig. 6 shows the effect of SCT extract on cellular oxidative stress of HUVECs. HUVECs were preincubated with 200 μg/ml of SCT extract for 12 h. After removal of SCT, plates were incubated with DCFH-DA for 45 min and various radical generators were added. The percentage increase of DCF fluorescence was measured for 100 min at 10 min intervals. When AAPH, a noted peroxyl radical generator was added, a definite time-dependent percentage increase of DCF fluorescence was observed. The DCF fluorescence was

increased over 600% for 100 min. However, cells that were preincubated with SCT extract showed only about 460% increase for 100 min (Fig. 6a). In case of SIN-1, a well known peroxynitrite generator, cells that were not pre-treated SCT extract showed over 700% increase of DCF fluorescence for 100 min, whereas cells that were pre-treated SCT extract showed about 500% increase (Fig. 6b). In case of SNP, a nitric oxide generator, time-dependent DCF fluorescence increase of SCT pre-treated cells was also lower than that of not treated cells (Fig. 6c). For the last time, cells that were not pre-treated SCT extract showed over 250% increase of DCF fluorescence for 100 min, whereas cells that were pre-treated SCT extract showed about 70% increase, when H_2O_2 was added(Fig. 6d).

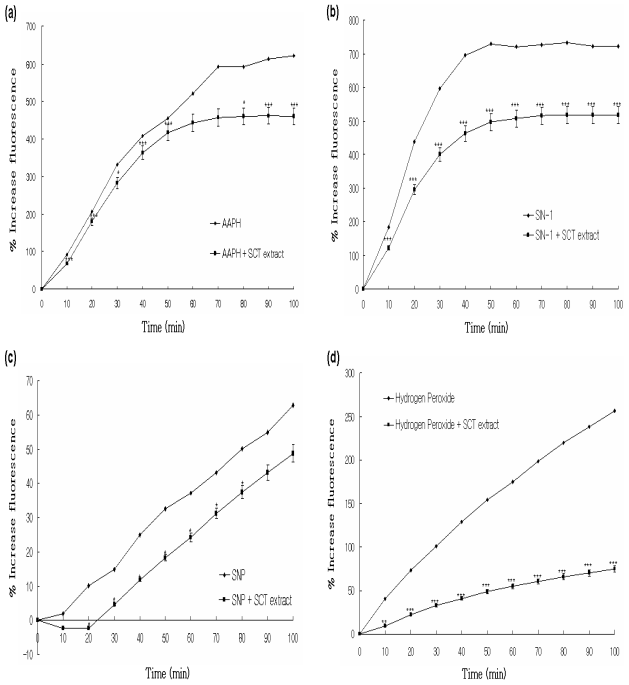

Fig. 6. Effect of SCT extract on intracellular oxidation in HUVECs induced by various kinds of radical generators. HUVECs were preincubated with 200 μg/ml of SCT extract for 12 h at 37℃ in 5% CO_2. The percentage increase of DCF fluorescence was measured for 100 min at 10 min intervals. Each data point represents the mean of data from eight wells and experiments were performed in triplicate. *Significant difference from the not-treated SCT extract (* : $p < 0.05$, ** : $p < 0.01$, *** : $p < 0.005$). (a) Time-response curve of percentage increase of DCF fluorescence in HUVECs for 100 min exposure to 1 mM AAPH. (b) Time-response curve of percentage increase of DCF fluorescence in HUVECs for 100 min exposure to 50 μM SIN-1. (c) Time-response curve of percentage increase of DCF fluorescence in HUVECs for 100 min exposure to 10 mM SNP. (d) Time-response curve of percentage increase of DCF fluorescence in HUVECs for 100 min exposure to 4 mM H_2O_2.

8. Effects of SCT Extract on VCAM-1 and ICAM-1 Expression in HUVECs

The effect of SCT extract on TNF-α-mediated VCAM-1 and ICAM-1 expression was analyzed by Western blot analysis. Pre-incubation with SCT extract (10, 100 and 200 μg/ml) suppressed VCAM-1 expression in a concentration-dependent manner (Fig. 7a). However, SCT extract had no significant effect on TNF-α-mediated ICAM-1 expression under our experimental conditions(Fig. 7b).

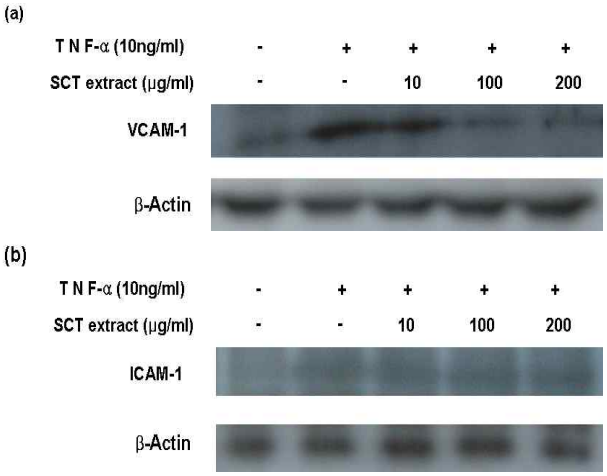

Fig. 7. Effects of SCT extract on VCAM-1 and ICAM-1 expression in HUVECs. Cells were preincubated for 12 h with various concentrations (10, 100, 200 μg/ml) of SCT extract, and then stimulated for 6 h with 10 ng/ml of TNF-α. Western blotting for VCAM-1 expression (a) and ICAM-1 expression (b) were carried out, as described in Materials and Methods section.

Discussion

Damages caused by free radical-induced oxidative stress is the key causative agent of many disorders including cancer, atherosclerosis, tissue injury, aging and rheumatoid arthritis[33-35]. Lately, antioxidants derived from natural resources, like plant or herb, have been gradually used to prevent oxidative damages. Natural antioxidants have some advantages over synthetic stuffs because they can be obtained easily and have slight side effects. There are many plants and herbs that have been widely known to possess antioxidant activities[36,37], and SCT also can be a useful natural antioxidant. Antioxidants transform the purple colored DPPH radical (DPPH·) into a colorless compound (DPPH-H) by donating hydrogen. To evaluate a direct scavenging activity of SCT extract on free radical, we used DPPH radical scavenging assay. The SCT extract revealed a potent free radicals scavenging activity, and this activity was better than that of BHT and similar to that of ascorbic acid (Table 1). These results indicate that SCT extract have an excellent hydrogen donating action. Superoxide anion, also called the superoxide radical, can be formed during normal matabolism, in autoxidation of organic compounds, in phagocytosis, and in enzymatic reactions[38]. Moreover, superoxide anions are the precursors of hydrogen peroxide and hydroxyl radical via Fenton reaction[39]. Recently, vascular cell studies have shown that superoxide radicals generated by NAD(P)H oxidase and

xanthine oxidase play a critical role in the early stages of atherosclerosis[40,41]. In particular, xanthine oxidase generates superoxide anions by catalyzing hypoxanthine to uric acid[42], and these superoxide radicals convert NBT to diformazan. To test the scavenging activity of SCT extract on superoxide anions, we used an hypoxanthine-xanthine oxidase system and measured the level of the reduction of NBT induced by superoxide anions (Table 1). In this assay, the SCT extract showed a potent scavenging activity in superoxide radical generating system, suggesting that this extract may be useful in preventing oxidative damage by superoxide radicals. The hydroxyl radical scavenging activity of SCT extracts was tested using the fluorescent probe dichlorodihydrofluorescein diacetate (DCFH-DA) after exposure to H_2O_2 and $FeSO_4$, and assessed by monitoring the fluorescent intensity of DCF (Table 1). In this assay system, H_2O_2 reacts with $FeSO_4$ to produce hydroxyl radical, and it oxidize DCFH to fluorescent product DCF. In this test, trolox, a well-known antioxidant compound, revealed better scavenging activity than SCT extract. However, hydroxyl radical scavenging activity of SCT was also significant. To confirm the scavenging property of SCT extract on hydroxyl radical in depth, we used $FeCl_2$-ascorbic acid-induced lipid peroxidation system. In this assay system, ascorbic acid can exert pro-oxidant action, leading to generation of hydroxyl radicals. The ascorbic acid reacts with oxygen to produce superoxide and superoxide radical undergoes transformation into hydrogen peroxide, subsequently. The reaction of hydrogen peroxide with ferrous ions, namely Fenton reaction, generates hydroxyl radicals which play a key role in initiating the lipid peroxidation. In rat liver homogenate, lipid peroxidation was significantly inhibited by SCT extract, and this inhibitory effect was higher than α-tocopherol (Fig. 1). Furthermore, SCT extract showed efficient protective effect against the DNA nicking, induced by hydroxyl radical. It was reported that hydroxyl radical generated by the Fenton reaction stimulated DNA nicking[43], and this oxidative DNA damage was inhibited by EDTA and catalase, which chelates iron ions and inactivates H_2O_2[44]. In this assay, hydroxyl radical enhanced oxidative damage of DNA. SCT extract inhibited hydroxyl radical-induced DNA nicking markedly, and this inhibitory activity was similar to that of EDTA and catalase (Fig. 2). These results indicate that SCT extract is a efficient scavenger of ROS including sueroxide anions and hydroxyl radical, and acts as a iron chelating agent. To investigate the antioxidant ability of SCT extract on RNS, we examined the scavenging activity using peroxynitrite and nitric oxide generating systems, respectively. Peroxynitrite, a reactive nitrogen species, is the product of the reaction between superoxide and nitric oxide in vivo. According to former studies, vascular endothelial cell produces both superoxide and nitric oxide to generate peroxynitrite within the vascular wall[45], which can cause LDL modification and endothelial cell damage[46]. This modified LDL can be recognized and taken up by the macrophages via scavenger receptor, leading to the formation of foam cells[47,48], which may be a critical event in the development and progression of atherosclerosis. However, the biological system lacks endogenous protectors against peroxynitrite despite that peroxynitrite is much more reactive than superoxide and hydrogen peroxide. Thus, antioxidant agents derived from natural plant or medicinal herb could be beneficial in the prevention of oxidative damage by peroxynitrite. In our assay, the scavenging activity of SCT extract on peroxynitrite was similar to that of penicillamine which is well known as a peroxynitrite scavenger. In addition, scavenging property of SCT extract on the nitric oxide was also distinguished (Table 2). These data indicates that SCT extract is a potent scavenger of RNS including nitric oxide and peroxynitrite and we assumed that SCT extract may be useful in preventing oxidative modification of LDL which may play a key role in atherogenesis. To ascertain the possible antioxidant activity of SCT extract against LDL oxidation, we used copper-mediated human LDL oxidation model system. In the experiment, the result demonstrate that SCT extract is effective in copper-mediated human LDL oxidation (Fig. 3). Moreover, from the REM assay, another model of LDL oxidation, SCT extract effectively suppressed the electrophoretic mobility during exposure of LDL to copper-induced oxidation (Fig. 4). Therefore, SCT extract may be effective to prevent the human LDL oxidation via metal ions chelating and radical scavenging actions. It has been reported that antioxidant activity of natural plant materials is related to their total phenolic contents[49]. We determined total phenolic amount in SCT extract using Folin-Ciocalteu assay. As shown in Table 3, the amount of total phenolic compounds of SCT ethanol extract was much higher than that of the water extract, suggesting that the potent antioxidative and antiradical capacity of SCT ethanol extract was probably due to the high level of phenolic compounds. It is known that the oxidative modifications of LDL can potentiate the tumour necrosis factor (TNF)-α-induced expression of adhesion molecules on endothelial cells[50] and these adhesion molecules increase interaction of monocytes with endothelial cells which is a consequence early event in the initiation of atherosclerosis[51]. The signal pathways for these adhesion molecules include the translocation of the transcriptional factor NF-κB[52] and intracellularly generated

oxidative stress, which play a key role in this process[53]. We thought that SCT extract could have protective abilities on intracellular oxidation and expression of adhesion molecules in HUVECs because of its preventive effect on the human LDL oxidation. 2,2′-azobios(2-amidinopropane) dihydrochloride (AAPH; a peroxyl radical generator), 3-morpholinosydnonimine hydrochloride (SIN-1; a peroxynitrite generator), sodium nitroprusside (SNP; a nitric oxide generator) and hydrogen peroxide (H_2O_2) were reported to induce oxidative stress in cells, and the level of oxidation can be quantified by using DCF assay. The SCT extract showed remarkable protective effects against all free radical generators (AAPH, SIN-1, SNP and H_2O_2), especially against H_2O_2 (Fig. 6a, b, c and d). Furthermore, SCT extract prevented induction of expression of VCAM-1, one of important adhesion molecules, in a concentration manner after stimulation with the inflammatory cytokine, TNF-α, at the level of protein (Fig. 7a). However, SCT extract had no significant effect on TNF-α -mediated ICAM-1 expression under our experimental conditions (Fig. 7b).

Conclusion

In summary, our studies provide that SCT extract is effective in scavenging free radicals including DPPH, ROS and RNS, preventing lipid peroxidation and protecting against human LDL oxidation. In addition, SCT extract showed notable protective effect on intracellular oxidation and expression of VCAM-1 in human endothelial cells. These results suggest that SCT, a prescription of Korean traditional medicine, may play a beneficial role in protecting against ROS- or RNS-involved diseases including atherosclerosis. Therefore, further researches will be required to isolate and determine the active components with concerning anti-oxidative or anti-atherosclerotic activity, and finally, clinical demonstration of SCT extract will be preceded.

References

1. Hur, J. Dongeuibogam. Seoul, Bupin Publishing. pp 639-663, 1999.
2. Choi, E.J., Lee, I.S. Effect of SACHIL-TANG on the plasma and uterus changes of rats in immobilization stress. The Journal of Oriental Gynecology 13(1):447-468, 2000.
3. Jung, B.M., Jin, C.S. A Study on prescriptions and single herb for treatment of vomiting during pregnancy. The Journal of Oriental Gynecology 13(2):370-398, 2000.
4. Yoo, H.J. Sclerosis of arteries. Monthly Korea Forum 142(1):166-169, 2001.
5. Minuz, P., Fava, C., Lechi, A. Lipid peroxidation, isoprostanes and vascular damage. Pharmacol Rep. 58: 57-68, 2006.
6. Dandona, P., Ghanim, H., Brooks, D.P. Antioxidant activity of carvedilol in cardiovascular disease. J. Hypertens. 25: 731-741, 2007.
7. Trostchansky, A., Batthyany, C., Botti, H., Radi, R., Denicola, A. and Rubbo, H. Formation of lipid-protein adducts in low-density lipoprotein by fluxes of peroxynitrite and its inhibition by nitric oxide. Arch. Biochem. Biophys. 395: 225-232, 2001.
8. Asmis, R., Begley, J.G., Jelk, J., Everson, W.V. Lipoprotein aggregation protects human monocyte-derived macrophages from OxLDL-induced cytotoxicity. J. Lipid. Res. 46: 1124-1132, 2005.
9. de Vries, H.E., Buchner, B., van Berkel, T.J., Kuiper, J. Specific interaction of oxidized low-density lipoprotein with macrophage-derived foam cells isolated from rabbit atherosclerotic lesions. Arterioscler. Thromb. Vasc. Biol. 19: 638-645, 1999.
10. Tabata, T., Mine, S., Kawahara, C., Okada, Y., Tanaka, Y. Monocyte chemoattractant protein-1 induces scavenger receptor expression and monocyte differentiation into foam cells. Biochem. Biophys. Res. Commun. 305: 380-385, 2003.
11. Wang, Z., Castresana, M.R., Newman, W.H. Reactive oxygen and NF-kappaB in VEGF-induced migration of human vascular smooth muscle cells. Biochem. Biophys. Res. Commun. 285: 669-674, 2001.
12. Moon, S.K., Thompson, L.J., Madamanchi, N., Ballinger, S., Papaconstantinou, J., Horaist, C., Runge, M.S., Patterson, C. Aging, oxidative responses, and proliferative capacity in cultured mouse aortic smooth muscle cells. Am. J. Physiol. Heart Circ. Physiol. 280: 2779-2788, 2001.
13. Luczak, K., Balcerczyk, A., Soszynski, M., Bartosz, G. Low concentration of oxidant and nitric oxide donors stimulate proliferation of human endothelial cells in vitro. Cell Biol. Int. 28: 483-486, 2004.
14. Rong, Y., Geng, Z., Lau, B.H. Ginko biloba attenuates oxidative stress in macrophages and endothelial cells. Free Radic. Biol. Med. 20: 121-127, 1996.
15. Stein, O., Thiery, J., Stein, Y. Is there a genetic basis for resistance to atherosclerosis? Atherosclerosis 160: 1-10, 2002.
16. Mo, S.J., Son, E.W., Lee, S.R., Lee, S.M., Shin, D.H., Pyo, S. CML-1 inhibits TNF-α-induced NF-κB activation and adhesion molecule expression in endothelial cells through inhibition of IκBα kinase. J. Ethnopharmacol. 109: 78-86, 2007.
17. Hafeman, D.G., Hoekstra, W.G. Lipid peroxidation in vivo

during vitamin E and selenium deficiency in the rat as monitored by ethane evolution. J. Nutr. 107: 666-672, 1977.
18. Halliwell, B. Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? Lancet. 344(8924):721-724, 1994.
19. Suarna, C., Wu, B.J., Choy, K., Mori, T., Croft, K., Cynshi, O., Stocker, R. Protective effect of vitamin E supplements on experimental atherosclerosis is modest and depends on preexisting vitamin E deficiency. Free Radic. Biol. Med. 41: 722-730, 2006.
20. Baskol, G., Atmaca, H., Tanrıverdi, F., Baskol, M., Kocer, D., Bayram, F. Oxidative stress and enzymatic antioxidant status in patients with hypothyroidism before and after treatment. Exp. Clin. Endocrinol. Diabetes 115(8):522-526, 2007.
21. Gyamfi, M.A., Yonamine, M., Aniya, Y. Free-radical scavenging action of medicinal herbs from Ghana. Gen. Pharmacol. 32: 661-667, 1999.
22. Gotoh, N., Niki, E. Rates of interactions of superoxide with vitamin E, vitamin C and related compounds as measured by chemiluminescence. Biochim. Biophys. Acta. 1115: 201-207, 1992.
23. Halliwell, B., Gutteridge, J.M. Role of free radicals and catalytic metal ions in human disease: an overview. Method Enzymol. 186: 1-85, 1990.
24. Nagata, N., Momose, K., Ishida, Y. Inhibitory effects of catecholamines and anti-oxidants on the fluorescence reaction of 4,5-diaminofluorescein, DAF-2, a novel indicator of nitric oxide. J. Biochem.(Tokyo) 125: 658-661, 1999.
25. Kooy, N.W., Royall, J.A. Ischiropulos, H., Beckman, J.S. Peroxynitrite-mediated oxidation of dihydrorhodamine 123. Free Radical Biol. Med. 16: 149-156, 1994.
26. Patro, B.S., Bauri, A.K., Mishra, S. Chattopadhyay, S. Antioxidant activity of Myristica malabarica extracts and their constituents. J. Agric. Food Chem. 53: 6912-6918, 2005.
27. Lee, J.C., Kim, H.R., Kim, J., Jang, Y.S. Antioxidant property of an ethanol extract of the stem of Opuntia ficus-indica var. Saboten. J. Agric. Food Chem. 50: 6490-6496, 2002.
28. Xu, M.Z., Lee, W.S., Han, J.M., Oh, H.W., Park, D.S., Tian, G.R., Jeong, T.S., Park, H.Y. Antioxidant and anti-inflammatory activities of N-acetyldopamine dimers from Periostracum Cicadae. Bioorg. Med. Chem. 14: 7826-7834, 2006.
29. Yoon, M.A., Jeong, T.S., Park, D.S., Xu, M.Z., Oh, H.W., Song, K.B., Lee, W.S., Park, H.Y. Antioxidant effect of quinoline alkaloid and 2,4-di-tert-butylphenol isolated from Scolopendra subspinipes. Biol. Pharm. Bull. 29(4):735-739, 2006.
30. Singleton, V.L. Naturally occurring food toxicants: phenolic substances of plant origin common in foods. Adv. Food Res. 27: 149-242, 1981.
31. Wang, H., Joseph, J.A. Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. Free Radic. Biol. Med. 27: 612-616, 1999.
32. Habib, A., Créminon, C., Frobert, Y., Grassi, J., Pradelles, P., Maclouf, J. Demonstration of an inducible cyclooxygenase in human endothelial cells using antibodies raised against the carboxyl-terminal region of the cyclooxygenase-2. J. Biol. Chem. 268(31):23448-23454, 1993.
33. Hussain, S.P., Hofseth, L.J., Harris, C.C. Radical causes of cancer. Nat. Rev. Cancer. 3(4):276-285, 2003.
34. Violi, F., Cangemi, R. Antioxidants and cardiovascular disease. Curr. Opin. Investig. Drugs. 6(9):895-900, 2005.
35. Basaga, H.S. Biochemical aspects of free radicals. Biochem. Cell Biol. 68(7-8):989-998, 1990.
36. Ahmad, R., Ali, A.M., Israf, D.A., Ismail, N.H., Shaari, K., Lajis, N.H. Antioxidant, radical-scavenging, anti-inflammatory, cytotoxic and antibacterial activities of methanolic extracts of some Hedyotis species. Life Sci. 76(17):1953-1964, 2005.
37. Jo, Y., Kim, M., Shin, M.H., Chung, H.Y., Jung, J.H., Im, K.S. Antioxidative phenolics from the fresh leaves of Ternstroemia japonica. J. Nat. Prod. 69: 1399-1403, 2006.
38. Nageswara, R.M., Aleksandr, V., Marschall, S.R. Oxidative stress and vascular disease. Arterioscler. Thromb. Vasc. Biol. 25: 29-38, 2005.
39. Kujala, T.S., Loponen, J.M., Klika, K.D., Pihlaja, K. Phenolics and betacyanins in red beetroot (Beta vulgaris) root: distribution and effect of cold storage on the content of total phenolics and three individual compound. J. Agric. Food Chem. 48: 5338-5342, 2000.
40. Wassmann, S., Wassmann, K., Nickenig, G. Modulation of oxidant and antioxidant enzyme expression and function in vascular cells. Hypertension 44: 381-386, 2004.
41. Warnholtz, A., Nickenig, G., Schulz, E., Macharzina, R., Brasen, J.H., Skatchkov, M., Heitzer, T., Stasch, J.P., Griendling, K.K., Harrison, D.G., Bohm, M., Meinertz, T., Munzel, T. Increased NADH-oxidase-mediated superoxide production in the early stages of atherosclerosis: evidence for involvement of the renin-angiotensin system. Circulation 99: 2027-2033, 1999.
42. Madamanchi, N.R., Hakim, Z.S., Runge, M.S. Oxidative stress in atherogenesis and arterial thrombosis: the disconnect between cellular studies and clinical outcomes. Thrombosis and Haemostasis 3: 254-267, 2004.

43. Lindqvist, C. and Nordstrom, T. Generation of hydroxyl radicals by the antiviral compound phosphonoformic acid (foscarnet). Pharmacol. Toxicol. 89: 49-55, 2001.
44. Badisa, V.L., Latinwo, L.M., Odewumi, C.O., Ikediobi, C.O., Badisa, R.B., Ayuk-Takem, L.T., Nwoga, J., West, J. Mechanism of DNA damage by cadmium and interplay of antioxidant enzymes and agents. Environ. Toxicol. 22: 144-151, 2007.
45. Hogg, N., Darley-Usmar, V.M., Wilson, M.T., Moncada, S. Production of hydroxyl radicals from the simultaneous generation of superoxide and nitric oxide. Biochem. J. 281: 419-424, 1992.
46. Steinbrecher, U.P., Parthasarathy, S., Leake, D.S., Witztum, J.L., Steinberg, D. Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. Proc. Natl. Acad. Sci. USA 81: 3883-3887, 1984.
47. Yla-Herttuala, S., Palinski, W., Rosenfeld, M.E., Parthasarathy, S., Carew, T.E., Butler, S., Witztum, J.L., Steinberg, D. Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. J. Clin. Invest. 84: 1086-1095, 1989.
48. Holvoet, P., Collen, D. beta-VLDL hypercholesterolemia relative to LDL hypercholesterolemia is associated with higher levels of oxidized lipoproteins and a more rapid progression of coronary atherosclerosis in rabbits. Arterioscler. Thromb. Vasc. Biol. 17: 2376-2382, 1997.
49. Ioannides, C., Yoxall, V. Antimutagenic activity of tea: role of polyphenols. Curr. Opin. Clin. Nutr. Metab. Care 6: 649-656, 2003.
50. Khan, B.V., Parthasarathy, S.S., Alexander, R.W., Medford, R.M. Modified low density lipoprotein and its constituents augment cytokine-activated vascular cell adhesion molecule-1 gene expression in human vascular endothelial cells. J. Clin. Invest. 95: 262-270, 1995.
51. Joris, I., Zand, T., Nunnari, J.J., Krolikowski, F.J., Majno, G. Studies on the pathogenesis of atherosclerosis. I. Adhesion and emigration of mononuclear cells in the aorta of hypercholesterolemic rats. Am. J. Pathol. 113(3):341-358, 1983.
52. Voraberger, G., Schäfer, R., Stratowa, C. Cloning of the human gene for intercellular adhesion molecule 1 and analysis of its 5′-regulatory region. Induction by cytokines and phorbol ester. J. Immunol. 147(8):2777-2786, 1991.
53. Schreck, R., Rieber, P., Baeuerle, P.A. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. EMBO J. 10(8):2247-2258, 1991.