

Original Research Article

Methanol Extract of *Codonopsis pilosula* Inhibits Inducible Nitric Oxide Synthase and Protein Oxidation in Lipopolysaccharide-Stimulated Raw Cells

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Abstract

Purpose: To evaluate the mechanism of antioxidant activity of the methanol extract of *Codonopsis pilosula*.

Methods: Anti-oxidative properties were assessed by measuring free radical scavenging activity, nitric oxide (NO) levels, protein oxidation and reducing power, while the mechanism of antioxidative effect of *Codonopsis pilosula* extract was determined by measuring iNOS and COX-2 expression in lipopolysaccharide (LPS)-stimulated raw cells.

Results: *Codonopsis pilosula* extract (CPE) exerted significant DPPH free radical and NO-scavenging activities. Protein oxidation was decreased by 30 % by the CPE (1mg/ml). The extract (1mg/ml) enhanced reducing power 16-fold (compared with control). LPS-induced production of iNOS was significantly inhibited by the extract (60 %), suggesting that it inhibits NO production by suppressing iNOS expression. However, LPS-induced production of COX-2 was not significantly ($p < 0.05$) inhibited by the extract. The levels of total phenolics, total flavonoids, and total anthocyanin in CPE were 0.38, 2.10, and 2.47 mg/g, respectively.

Conclusion: These results suggest that CPE exerts marked antioxidant activity via inhibition of iNOS and protein oxidation.

Keywords: *Codonopsis pilosula*, Lipopolysaccharide, Cyclooxygenase-2 (COX-2), Free radicals, Inducible nitric oxide synthase (iNOS), Nitric oxide (NO)

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INTRODUCTION

Codonopsis pilosula has been widely used as an Oriental medicine. It has been found to contain a wide variety of phytochemicals including hesperidin, β -sitosterol, loberyolinin, β -daucosterol [1], triterpenyl esters, codonopilates A-C [2], phenylpropanoid glycosides [3] and β -carboline alkaloid [4]. Historically, it has long been used for the treatment of digestive disorders such as indigestion, loose stool, fatigue and pulmonary insufficiency including shortness of breath, coughing [5]. Recent studies also showed that *Codonopsis pilosula* is

capable of ameliorating peptic ulcer, promoting bowel movement, enhancing learning and memory, and increasing red blood cells and hemoglobin [6].

Imbalance between reactive oxygen species (ROS) and anti-oxidative system causes oxidative stress. The production of ROS such as superoxide anion, hydrogen peroxide, and hydroxyl radical occurs via mitochondrial respiratory chain [7]. Nitric oxide (NO) synthesized by iNOS in response to ROS-induced oxidative stress play important roles in many diseases such as cancer, diabetes,

cardiovascular diseases, neurodegenerative disease and oral diseases. COX-2 (cyclooxygenase-2) is known to produce prostaglandins which are essential mediators for inflammatory reaction in the body, and it is regarded as an important molecular target of antioxidative drug development. The objective of the present study was to determine whether *Codonopsis pilosula* extract has anti-oxidative activities and whether iNOS and COX-2 are involved in its anti-oxidant actions.

EXPERIMENTAL

Preparation of plant extracts

Authentic samples of *Codonopsis pilosula* collected during October, 2010 were purchased from Kyung-Dong Oriental Market in Seoul. They were authenticated by Emeritus Professor Chang-Soo Yok, Department of Oriental Pharmacy, College of Pharmacy, Kyung Hee University, Seoul, Korea. A voucher specimen (no. 8N009) was deposited at the herbarium of the Department of Pharmacology and Toxicology, School of Dentistry, Kyung Hee University, Seoul, Korea. The plant (100 g) was cut into small pieces and extracted 3 times with 300 ml of 70 % methanol for 3 h. The resultant extract was concentrated by a rotary evaporator and dried by a freeze-dryer.

Reagents and materials

The iNOS and COX-2 antibodies were purchased from Cell Signaling and Santa Cruz Biotechnology Co, respectively while ECL kit was purchased from Amersham Co., USA. All other reagents were purchased from Sigma Co., USA. Cell culture media were purchased from Gibco Co.

Free radical scavenging activity

Free radical scavenging activity was measured by evaluating reduction reactions of DPPH radical in the presence of hydrogen-donating antioxidants [8] and NO. DPPH (Fluka Chemie, Buchs, Switzerland) solutions show a peak absorption at 515 nm and present deep violet color. Briefly, *Codonopsis pilosula* extract (0.01mg/ml, 0.1mg/ml, 1mg/ml) was dissolved in 1 ml MeOH, mixed with 1 ml DPPH solution at room temperature and optical density measured at 515 nm (BioRad Model 550). NO generated from sodium nitroprusside (SNP) was measured using Griess reagent at 540 nm.

Briefly, *Codonopsis pilosula* extract (0.01, 0.1 or, 1mg/ml) in a volume of 100 μ l was added to 200

μ l SNP (10 mM) and 1.8ml of phosphate buffer (pH 7.4). The reaction mixture was allowed to incubate at 37 °C for 3 h. Thereafter, 1.0 ml of the reaction mixture was mixed with 0.5 ml of Griess reagent and the absorbance measured at 540 nm.

Determination of protein oxidation

The effect of *Codonopsis pilosula* extract on protein oxidation was determined as previously described [9]. The reaction mixture (1.2 mL) in phosphate buffer (20 mM, pH 7.4), containing the *Codonopsis pilosula* extract, bovine serum albumin (20 mg/mL), FeCl₃ (400 μ M), H₂O₂ (1 ml, 3mM), and ascorbic acid (400 μ M) was incubated at 37 °C for 1 h. Dinitrophenyl hydrazine (20 mM in 2N HCl) and trichloroacetic acid (20% w/v) were added to the reaction mixture and subjected to centrifugation at 650 g for 10 min. The supernatant was discarded and the resulting pellet was washed three times with 2.0 mL of EtOH/EtOAc (1:1) solution, dissolved in 2.0 mL of 6M guanidine-HCl (pH 6.5) and monitored spectrophotometrically at 370 nm.

Determination of reducing power

Reducing power was measured by Oyaizu method [10]. *Codonopsis pilosula* extract (2.5 ml, in 0.2M phosphate buffer, pH 6.6) was placed in 2.5 ml of potassium ferricyanide (10 mg/ml) solution and incubated for 15 min at 30 °C; 2.5 ml of trichloroacetic acid (100 mg/ml) was added to the reaction mixture. To 2.5 ml of the mixture, 2.5 ml of distilled water and 0.5 ml of ferric chloride (1.0 mg/ml) were added. Then, optical density was read at 700 nm.

Cell culture

Murine RAW 264.7 macrophage cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) (Gibco BRL, Grand island, NY) with 10% heat-inactivated fetal bovine serum in 5% humidified CO₂ atmosphere at 37 °C.

Measurement of nitric oxide

The Raw cells were cultured with DMEM and 10% FBS. NO was measured with cell supernatant as nitrite and nitrate. The safe form of nitrite after being reduced to nitrate was measured using Griess reagent (Sigma, USA). A total of 2 × 10⁶ Raw cells were seeded into a 6 well plate for 24 h before the extract treatment. and the cells were incubated with the samples at different concentrations (1.0, 0.1, and 0.01 mg/ml) Four hours later, lipopolysaccharide (LPS, final concentration 10 μ g/ml) was put into

all wells except for the well for the control group to stimulate the cells. The amount of NO generated was determined as nitrite, a major stable product of NO, by the Griess reagent assay. The absorbance was measured spectrophotometrically 18 h later at 540 nm.

Measurement of iNOS and COX-II expression

Raw cells were cultured with DMEM and 10% FBS. When the cells reached confluence, the DMEM culture medium was removed and replaced by the Eagle's Minimum Essential Medium (EMEM) culture medium which is a serum-free culture medium and then the cells were treated with *Codonopsis pilosula* extracts and cultured for 24 h. The cells were washed two times with PBS and scraped into a 10 mM Tris-HCl buffer, pH 7.4, containing 50 mM NaCl, 5 mM EDTA, 30 mM NaF, 0.1 mM Na₃VO₄, 1 % triton X-100, 0.5 % NP-40, 1 ug/ml leupeptin, and 1 ug/ml aprotinin. The cells were disrupted by passing them 5 times through a 1-ml tuberculin syringe. The cell lysate was subjected to centrifugation at 10,000 × g for 10 min and the supernatant used for Western blot analysis. The protein content of the soluble fraction was assessed by the method of Bradford [11]. After addition of sample loading buffer, equivalent amounts of protein samples (50ug/lane) were electrophoresed on a 10% sodium dodecyl sulfate denaturing polyacrylamide slab gels (SDS-PAGE) and subsequently transferred to nitrocellulose transfer membrane (Whatman PROTRAN) for 1 h at 100 V (constant) as described by Towbin *et al* [12]. The membrane was incubated in fresh blocking buffer (0.1% Tween 20 in Tris-buffered saline, TTBS, pH 7.4, containing 5% non fat dried milk) at room temperature for 60 min and then probed with the iNOS (1:1000), COX-II (1:2000), GAPDH (1:1000) antibodies overnight in blocking buffer at 4°C. The membrane was washed three times for 10 min each using TTBS. After that it was incubated in the appropriate Horse radish peroxidase (HRP)-conjugated secondary antibody (1:1000 for iNOS and GAPDH; 1:2000 for COX-II) at room temperature for another 2 h and washed again three times in TTBS buffer. The membrane was developed using the enhanced chemiluminescence reagents (ECL) for 5 min according to the manufacturer's instructions and was exposed to Amersham Hyperfilm ECL (GE Healthcare Limited, UK). The results obtained from the immunoblot assay were calculated using IMAGEJ imaging software (NIH, USA) as the integrated density.

Measurement of total phenolics

Total phenolic content was measured spectrophotometrically by Folin-Ciocalteu method at 725 nm [13]. The extract (200 ul, 2 mg/ml) was mixed with 1.8 ml water and 200 ul of Folin-Ciocalteu reagent. After 3 min, 400 ul of Sodium carbonate (35%, w/v) and 1.4 ml of water were added to the mixture and followed by the measurement of absorbance at 725 nm. Gallic acid was used as a standard for phenolic compounds and the phenolic concentration was calculated using a gallic acid standard calibration curve. The total phenolic content was expressed as the gallic acid equivalent (mg gallic acid/g extract).

Measurement of total flavonoids

Total flavonoids was measured by the method of Miliuskas *et al*. [14] and was expressed as the rutin equivalent (mg rutin acid/g extract) using rutin as a standard flavonoid; 200 ul of the extract (1 mg/ml) was mixed with 200 ul of aluminum trichloride in ethanol (20 mg/ml) and diluted to 5 ml. After incubation for 40 min at 20 °C, the optical density was read spectrophotometrically at 415 nm.

Measurement of total anthocyanin

Total anthocyanin was measured as described previously [15]. The extract (2 mg) was dissolved in 3 ml of acetate buffer (25 mM, pH 4.5) and the optical density was measured at 520 nm. The content of anthocyanin was expressed as kuromanin equivalent (mg kuromanin/ g extract).

Statistical analysis

All data were expressed as mean ± SEM. Statistical analysis was performed using GraphPad Prism 5 by one-way ANOVA followed by Tukey's multiple comparison test. *P* < 0.05 was considered as significant.

RESULTS

DPPH free radicals were decreased approximately 7 % by 0.01 mg/ml of *Codonopsis pilosula* extract and by about 3 % at 0.1 mg/ml, compared to control. At a concentration of 1.0 mg/ml, DPPH free radicals decreased by 19 % (Figure 1).

Taurine did not show any significant effect on DPPH scavenging activity whereas butylated hydroxytoluene (BHT) caused 46 % reduction of DPPH free radical levels. The extract also

decreased the level of NO by 6, 6 and 13 % at extract concentrations of 0.01, 0.1, and 1 mg/ml, respectively, while taurine decreased NO levels by 6 and 4 % at 0.01 mg/ml and 0.1mg/ml, respectively (Figure 2).

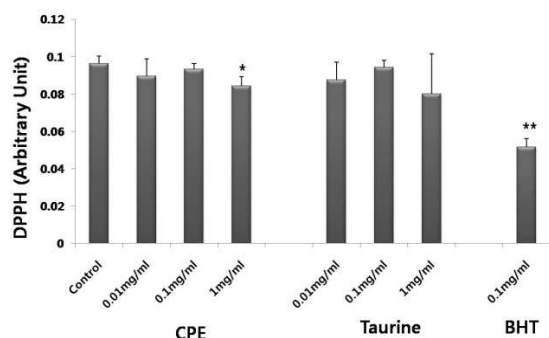


Fig 1: DPPH radical scavenging activity of *Codonopsis pilosula* extract. **Note:** value are mean ± SEM; **p* < 0.05, ***p* < 0.01

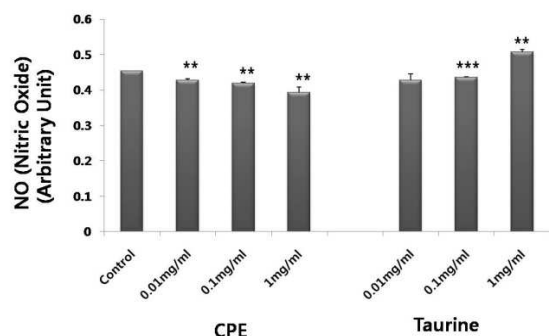


Fig 2: Nitric oxide (NO) radical scavenging activity of *Codonopsis pilosula* extract. **Note:** value are mean ± SEM; ***p* < 0.01, ****p* < 0.001

Protein oxidation was also decreased by 30 and 40 % at concentrations of 0.1mg/ml and 1mg/ml, respectively, the extract (Figure 3).

Table 1: Antioxidant components of *Codonopsis pilosula* extract

CP extract	Mean±SEM
Phenolics (gallic acid, ug/mg)	0.376±0.118
Anthocyanins (kuromanin, ug/mg)	2.47± 0.753
Flavonoids (rutin, ug/mg)	2.100±0.265

The extracts stimulated the reducing power by 6.7-fold and 16.1-fold (compared to control) at 0.1 and 1mg/ml, respectively, whereas taurine stimulated it by 1.8-fold at 1mg/ml. (Figure 4).

The amount of nitric oxide production was markedly increased (5.0-fold over basal) when the Raw cells were treated with LPS to activate the macrophages whereas when the cells were pretreated with the *Codonopsis pilosula* extract, NO production was significantly decreased by 4.5, 34.5, and 52.7 % in response to 0.01, 0.1,

and 1 mg/ml extract, respectively, compared to LPS-induced stimulation (Figure 5).

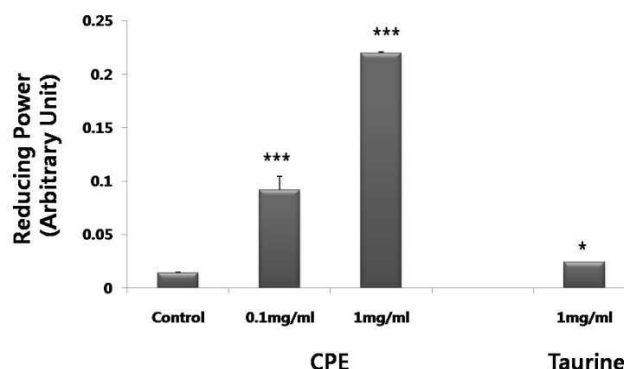


Fig 3: Reducing power of *Codonopsis pilosula* extract. **Note:** Values are mean ± SEM; **p* < 0.05, ****p* < 0.001

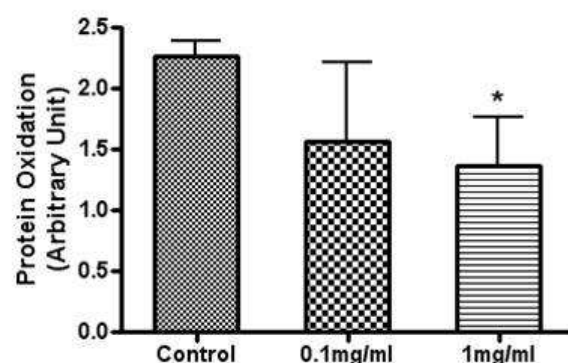


Fig 4: Effect of *Codonopsis pilosula* extract on protein oxidation. **Note:** Values are mean ± SEM; **p* < 0.05

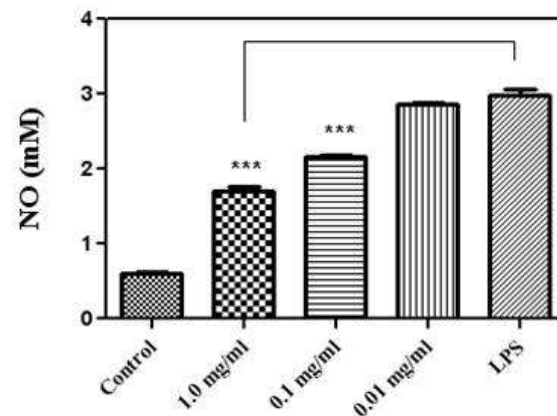


Fig 5: Inhibition of nitric oxide (NO) by *Codonopsis pilosula* extract. **Note:** Values are mean ± SEM; ****p* < 0.001

When LPS was administered to the Raw cells, the expression of iNOS increased by 9.6-fold over basal. On the other hand, when the cells were pretreated with *Codonopsis pilosula* extract, iNOS expression levels were markedly decreased by 11, 13, and 82 % in response to concentrations of 0.01, 0.1, and 1 mg/ml, respectively, compared to the LPS-induced stimulation (Figure 6).

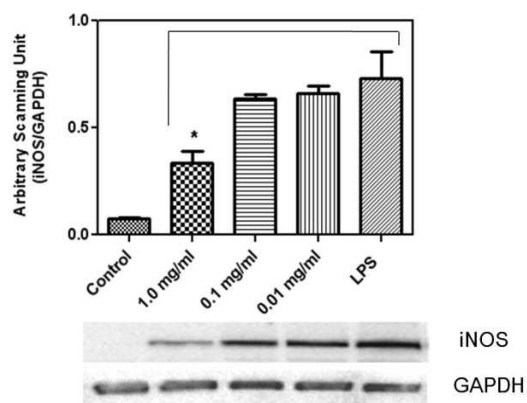


Fig 6: Effect of *Codonopsis pilosula* extract on LPS-induced iNOS induction. **Note:** value are mean \pm SEM; * $p < 0.05$,

The *Codonopsis pilosula* extract had little or no effect on the expression of LPS-induced COX-2 (Figure 7).

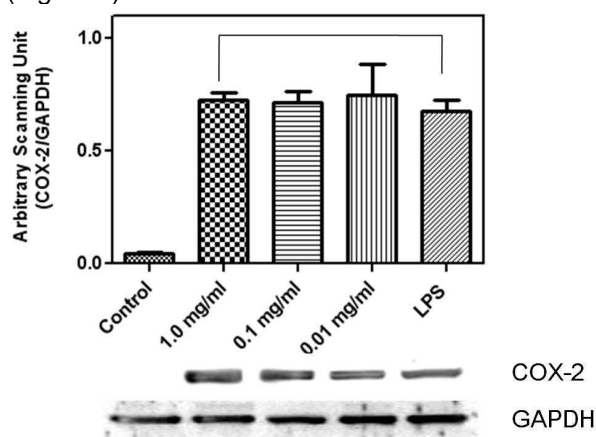


Fig 7: Effect of *Codonopsis pilosula* extract on LPS-induced COX-2 induction. **Note:** Values are mean \pm SEM

When antioxidant components present in the *Codonopsis pilosula* extract was analyzed, total phenolics, total flavonoids, and total anthocyanin were determined as 0.38, 2.10, and 2.47 mg/g, respectively (Table 1).

DISCUSSION

It has been widely accepted that oxidative stress plays an important role in the development of many diseases such as cancer, diabetes, Parkinson's disease, cardiovascular diseases, and periodontal diseases [16-18].

Reactive oxygen species (ROS) generated by oxidative stress like smoking, ultraviolet rays, stress, and aging are important signaling molecules in the cell [16, 17]. They damage lipids, proteins and nucleic acids, thereby promoting many diseases and aging. DPPH free radical-removing and nitric oxide scavenging

properties of *Codonopsis pilosula* extract have been demonstrated in this study. In addition, the extract showed strong reducing power relative to control. These results suggest that the extract has the ability to neutralize various reactive oxygen species generated by oxidative stress. For instance, the marked reducing power of the extract could be beneficial in removing toxic peroxides generated by oxidative stress.

Oxidative stress causes protein oxidation and thereby disturbing the normal protein structure and function. Metabolism-related proteins, mitochondrial proteins, chaperones and protein members of ubiquitin-proteasome system are known to be sensitive to oxidation [18]. Protein oxidation is associated with human degenerative diseases, supranuclear palsy and age-related diseases [19]. Interestingly, it has been reported that oxidative stress could inactivate many proteins such as Cu,Zn superoxide dismutase [20]. Taking these into consideration, *Codonopsis pilosula* extract may be beneficial to prevent oxidative damages to proteins.

NO has been implicated in pain and tissue damage [21]. Oxidative stress is essential in the progress of periodontal diseases while antioxidants such as flavonoids or vitamin C can prevent such progresses. NO is an important signaling molecule responsible for various inflammatory responses *in vivo* [22,23]. It is generated by the activation of iNOS enzyme induced by inflammation. Plant extracts with the ability to regulate iNOS and COX-II also have anti-inflammatory actions [24-26]. In the present study, it has been shown that the *Codonopsis pilosula* extract significantly reduces the concentration of NO generated by LPS in the Raw cells and shows strong inhibition of iNOS expression. In contrast, COX-II was not affected by the *Codonopsis pilosula* extract. These results suggest that the anti-oxidative action of the *Codonopsis pilosula* extract may be mediated by the inhibition of the iNOS-NO system, rather than COX-II enzyme.

Plant extracts with antioxidative effects are known to contain phenolics, anthocyanin, and flavonoids [27]. The *Codonopsis pilosula* extract contains significant amounts of phenolics including anthocyanins and flavonoids, suggesting that these components may be major contributors to the iNOS inhibitory action of the extract.

CONCLUSION

We propose that *Codonopsis pilosula* extract could exert antioxidant effect due to the

inhibition of iNOS, and scavenging of NO and free radical, as well as inhibition of protein oxidation.

REFERENCES

1. Qi HY, Wang R, Liu Y, Shi YP. Studies on the chemical constituents of *Codonopsis pilosula*. *Zhong Yao Cai* 2011; 34: 546-548.
2. Wakana D, Kawahara N, Goda Y. Three new triterpenyl esters, *codonopilates A-C*, isolated from *Codonopsis pilosula*. *J Nat Med* 2011; 65: 18-23.
3. Mizutani K, Yuda M, Tanaka O, Saruwatari Y, Jia MR, Ling YK, Pu XF. Tangshenosides I and II from *Chun-Dangshen*, the root of *Codonopsis tangshen* Oliv. *Chem Pharm Bull* 1998; 36: 2726-2729.
4. Liu T, Liang W, Tu G. Peroloyrine: a β -carboline alkaloid from *Codonopsis pilosula*. *Planta Medica* 1988; 54: 472-473.
5. Xu QP. Ed. *Pharmacology of Chinese Herbs (Chinese): Guizhou Science and Technology Publishing Company, Guiyang, Guozhou Province, P.R. China, 1994.*
6. Singh B, Song H, Liu XD, Hardy M, Liu GZ, Vinjamury S, Martirosian CD. *Dangshen (Codonopsis Pilosula)* and *Bai Guo (Gingko Biloba)* enhance learning and memory. *Alternative Therapies* 2004; 10: 52-56.
7. Fridovich I. The biology of oxygen radicals. *Science* 1978; 201: 875-880.
8. Brand-Williams W, Cuvelier ME, Berset C. Use of a free radical method to evaluate antioxidant activity. *Lebenson Wiss Technol* 1995; 28: 25-30.
9. Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz AG, Ahz BW, Shaltiel S, Stadtman ER. Determination of carbonyl content in oxidatively modified proteins. *Methods Enzymol* 1990; 186: 464-478.
10. Oyaizu M. Studies on products of browning reaction: Antioxidative activities of products of browning reaction prepared from glucosamine. *Jpn J Nutr* 1986; 44: 307-314.
11. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; 72: 248-254.
12. Towbin H, Staehelin J, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some application. *Proc Natl Acad Sci USA* 1979; 76: 4350-4354.
13. Singleton VL, Rossi JA. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic reagents. *Am J Enol Vitic* 1965; 16: 144-158.
14. Miliauskasa G, Venskutonis PR, van Beek TA. Screening of radical scavenging activity of some medicinal and aromatic plant extracts. *Food Chem* 2004; 85: 231-237.
15. Piccolella S, Fiorentino A, Pacifico S, D'Abrasca B, Uzzo P, Monaco P. Antioxidant properties of sour cherries (*Prunus cerasus* L.): Role of colorless phytochemicals from the methanolic extract of ripe fruits. *J Agric Food Chem* 2008; 56: 1928-1935.
16. Kim BJ, Kim JH, Kim HP, Heo MY. Biological screening of 100 plant extracts for cosmetic use (II): Antioxidative activity and free radical scavenging activity. *Int J Cosmet Sci* 1997; 19: 299-307.
17. Evereklioglu C, Er H, Doganay S, Cekmen M, Turkoz Y, Otlu B, Ozerol E. Nitric oxide and lipid peroxidation are increased and associated with decreased antioxidant enzyme activities in patients with age-related macular degeneration. *Doc Ophthalmol* 2003; 106: 129-136.
18. Avery SV. Molecular targets of oxidative stress. *Biochem J* 2011; 434: 201-210.
19. Martínez A, Portero-Otín M, Pamplona R, Ferrer I. Protein targets of oxidative damage in human neurodegenerative diseases with abnormal protein aggregates. *Brain Pathol* 2010; 20: 281-297.
20. Costa WMV, Amorim MA, Quintanilha A, Moradas-Ferreira P. Hydrogen peroxide-induced carbonylation of key metabolic enzymes in *Saccharomyces cerevisiae*: the involvement of the oxidative stress response regulators Yap 1 and Skn7. *Free radical Biol.* 2002; 33: 1507-1515.
21. Ndengele MM, Cuzzocrea S, Esposito E, Mazzoni E, Di Paola R, Matuschak GM, Salvemini D. Cyclooxygenases 1 and 2 contribute to peroxynitrite-mediated inflammatory pain hypersensitivity. *FASEB J.* 2008; 22: 3154-64.
22. Condoner-Frnach P, Travarez-Alonso S, Murria-Estal R, Megias-Vericat J, Tortajada-Girbes M, Alonso-Iglesias E. Nitric oxide production is increased in severely obese children and related to markers of oxidative stress and inflammation. *Atherosclerosis*, 2011. Epub ahead of print.
23. Conforti F, Menichini F. Phenolic compounds from plants as nitric oxide production inhibitors. *Curr. Med. Chem.* 2011; 18: 1137-1145.
24. Handunnetti SM, Kumara RR, Deraniyagala SA, Ratnasooriya WD. Anti-inflammatory activity of *Ixora coccinea* methanolic leaf extract. *Pharmacogn Res* 2009; 1: 80-90.
25. Gambhire MN, Wankhede SS, Juvekar AR. Antiinflammatory activity of aqueous extract of *Barleria cristata* leaves. *J Young Pharm* 2009; 1: 220-224.
26. Kumar D, Kumar S, Singh J, Narender, Rashmi, Vashistha BD, et al. Free radical scavenging and analgesic activities of *Cucumis sativus* L. fruit extract. *J Young Pharm* 2010; 2: 365-368.
27. Kähkönen MP, Hopia AI, Vuorela HJ, Rauha JP, Pihlaja K, Kujala TS, Heinonen M. Antioxidant Activity of Plant Extracts Containing Phenolic Compounds. *J. Agric. Food. Chem.* 1999; 47: 3954-3962