

Research Article

Inhibition of iNOS and DNA Oxidation by Methanol Extract of *Schizonepeta tenuifolia*

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Abstract

Purpose: To investigate the antioxidant properties of the methanol extract of *S. tenuifolia* as well as its effect on inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression in lipopolysaccharides (LPS)-induced cell damage in macrophage cells.

Methods: The antioxidant activities of the plant extract were explored by measuring free radical scavenging activity, viz, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and nitric oxide (NO), and deoxyribose oxidation levels. The mechanism of antioxidant action of the extract was determined by Western blot analysis for iNOS and COX-2 expression in LPS-stimulated RAW 264.7 cells.

Results: The extract contained antioxidant components, including phenolics, flavonoids and anthocyanin and exerted significant radical scavenging activity in a dose-dependent manner. It also produced deoxyribose oxidation and dramatic reducing power. Production of iNOS induced by LPS was significantly inhibited by the extract, suggesting that the extract inhibits nitric oxide (NO) production by suppressing iNOS expression. However, COX-2 induced by LPS was not significantly affected by the extract.

Conclusion: These results suggest that the methanol extract of *S. tenuifolia* exerts significant antioxidant activity via inhibition of free radicals, iNOS and DNA oxidation.

Keywords: *Schizonepeta tenuifolia*, NO, iNOS expression, COX-2, Deoxyribose oxidation, Antioxidant

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INTRODUCTION

The dried aerial parts of *Schizonepeta tenuifolia* (Benth.) Briq. (family: Labiatae) are widely used in Oriental medicine to treat fever, sore throat, allergic dermatitis, eczema and psoriasis [1]. In addition, it has been reported that water extract of *S. tenuifolia* has immunomodulatory responses by reducing the release of Th1 and Th2 cytokines from T cells [2]. It contains flavonoids such as hesperidin and luteolin, as well as phenolics such as rosmarinic acid, and terpenes such as pulegone, ursolic acid [3].

Oxidative stress is caused by imbalance between free radical productions and their scavenging anti-oxidative defense system in the body, leading to damage to cellular components such as lipid, protein and DNA [4]. Most free radicals and their reactive metabolites such as reactive oxygen species (ROS) are generated by the mitochondrial respiratory chain during cellular metabolism and play essential roles in cellular signaling pathways. The mitochondrial respiratory chain can also produce nitric oxide (NO) and thus reactive nitrogen species (RNS) [5]. ROS and RNS induce excessive lipid peroxidation, producing other reactive species including malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) [6].

Antioxidants are essential for maintenance of normal cellular integrity and play important roles in preventing lipid peroxidation and removing free radicals. It has been well accepted that a number of antioxidants such as vitamin C, vitamin E, phenolic acid and flavonoids can reduce the risk of tumors and heart diseases and they are helpful in scavenging ROS which involved in the onsets of those diseases [7]. Inducible nitric oxide synthase (iNOS), is a key enzyme responsible for the production of nitric oxide (NO) and it plays an important role in the oxidative stress and inflammation [7]. It has been well known that cyclooxygenase-2 (COX-2) is implicated in inflammation via production of various prostaglandins [8].

To evaluate the antioxidative activity of the methanol extract of the aerial parts of *S. tenuifolia*, as well as the total phenolics, flavonoids and anthocyanin of methanolic extract of *S. tenuifolia* and their *in vitro* radical scavenging abilities were investigated in this study. Furthermore, the effects of the extract on the expression of NO, iNOS and COX-2 in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells were evaluated.

EXPERIMENTAL

Preparation of plant extracts

Dried aerial parts of *Schizonepeta tenuifolia* collected in autumn were purchased from Kyung-Dong Oriental Market, Seoul, Rep. of Korea. They were authenticated by Professor Chang-Soo Yok, Department of Oriental Pharmacy, Kyung Hee University, Seoul, Republic of Korea. A voucher specimen (no. 8N-008) was deposited at the herbarium of the Department of Pharmacology and Toxicology, School of Dentistry, Kyung Hee University, Seoul, Republic of Korea. The plant (100 g) was cut into small pieces and extracted three times with 70 % methanol (300 ml) for 3 h. The resulting methanol extract was concentrated in a rotary evaporator and freeze-dried.

Measurement of total phenolics

Total phenolic content (TPC) was determined using the Folin-Ciocalteu reagent with gallic acid as a standard [9]. Basically, 0.5 ml test sample was mixed with 0.5 ml Folin-Ciocalteu reagent and 1.5 ml 10 % sodium carbonate solution. After the mixture was reacted for 10 min at 75 °C, the absorbance was read at 760 nm. The results were expressed in milligram gallic acid equivalent per gram of dried sample.

Measurement of total flavonoids

Total flavonoids was measured using the method of Miliauskasa *et al* [10]. To summarize this, 1.0 ml test sample was

mixed with aluminum chloride in ethanol (20 mg/ml) and diluted to 20 ml. Following incubation for 40 min at 20 °C, the absorbance of the reaction mixture was measured at a wavelength of 415 nm. The total flavonoid content of the extract was expressed in milligram rutin equivalent per gram of dried sample.

Measurement of total anthocyanins

Total anthocyanins was measured by colorimetric assay [11]. Essentially, the extract (1.0 mg) was dissolved in acetate buffer (25 mM, pH 4.5, 1.0 ml). Sample absorbance was read at 520 nm against deionized water. Anthocyanin content was expressed as milligrams of kouroumanin (cyanidin-3-O-glucoside; KO) equivalent per gram of dried sample.

DPPH radical scavenging activity

The ability of the extract to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals was assessed [12]. When a solution of DPPH is mixed with a substance that can donate a hydrogen atom, the reduced form of the radical is generated, accompanied by loss of color. This delocalization is also responsible for the deep violet color, characterized by an absorption band in ethanol solution at about 515 nm. Briefly, 1.0 ml of aliquot of extract or control was mixed with 1.0 ml of methanolic DPPH (0.1 mM/l) solution at 23 °C. After a 30-min reaction, the absorbance was recorded at 515 nm. All the experiments were performed in triplicate.

Nitric oxide (NO) radical scavenging activity

NO generated from sodium nitroprusside (SNP) was measured using the Griess reagent [13]. The test sample (0.5 ml) was added to 0.2 ml of SNP (10 mM) and 1.8 ml 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 containing nitrite was mixed with 1.0 ml of

Greiss reagent and allowed to stand for 30 min in diffused light. The absorbance of the pink colored chromospheres was measured at 540 nm against the corresponding blank solution.

Determination of the effect of extract on oxidation of deoxyribose

The determination was carried out as described by Halliwell et al [14]. The reaction mixture (1.4 ml) containing deoxyribose (6 mM), hydrogen peroxide (H₂O₂, 3 mM), ferric chloride (FeCl₃, 400 μM), ethylenediamine tetra acetic acid (EDTA, 400 μM) and ascorbic acid (400 μM) in phosphate buffer (20 mM, pH 7.4) was mixed with 0.2 ml of test sample and incubated for 1 h at 37 °C. 1.0 ml of thiobarbituric acid (TBA, 1 %w/v) and 1.0 ml of trichloroacetic acid (TCA, 2.8 %w/v) were added to the mixture, which was heated in a water bath at 90 °C for 20 min. The absorbance of the mixture was read spectrophotometrically at 532 nm.

Reducing power

The reducing power of the extract was measured according to the method of Oyaizu [15]. The test sample in phosphate buffer (2.5 ml, 0.2 M, pH 6.6) was added to potassium ferricyanide (2.5 ml, 10 mg/ml) solution and the mixture incubated at 15 min at 30 °C. Trichloroacetic acid (TCA, 2.5 ml, 100 mg/ml) was added to the mixture, and 2.5 ml of it mixed with 2.5 ml of distilled water and 0.5 ml of ferric chloride (1.0 mg/ml). The absorbance was read at 700 nm. Higher absorbance of the reaction mixture indicates greater reducing power.

Cell culture and measurement of nitric oxide

The murine macrophage RAW 264.7 (ATCC®TIB-71) cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's modified essential media (DMEM; Gibco, Invitrogen, Carlsbad, CA, USA)

supplemented with 10 % heat-inactivated fetal bovine serum (FBS; Gibco, Invitrogen, Carlsbad, CA, USA), streptomycin (100 µg/ml) and penicillin (100 U/ml) at 37 °C in a 5 % CO₂ atmosphere. Cells were seeded at a density of 1×10^6 onto each well in a 6-well plate for 24 h before drug treatment. The cells were incubated with test sample at different concentrations (1.0, 0.1 and 0.01 mg/ml). Four hours later, cells were stimulated with lipopolysaccharide (LPS, 10 µg/ml; Sigma Chemical Co., St. Louis, MO, USA) for 18 h. The concentration of NO in culture supernatants was determined as nitrite, a major stable product of NO, by the Griess reagent assay. The absorbance of the pink colored chromospheres was measured at 540 nm against the corresponding blank solution and the results expressed as mM nitric oxide.

Western blot analysis

Cells were treated as described in the cell culture and measurement of nitric oxide section, and washed twice with 1x PBS. The cell pellets were re-suspended in an appropriate volume of lysis buffer containing 10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 5 mM EDTA, 30 mM NaF, 0.1 mM Na₃VO₄, 1 % triton X-100, 0.5 % NP-40, 1 µg/ml leupetin, 1 µg/ml aprotinin and incubated for 30 min on ice. The lysates were then centrifuged at 12,000 rpm for 10 min at 4 °C and the supernatants used as the total cell lysates. The protein concentration of each sample was determined by the Bradford assay (Bio-Rad, CA, USA) [16]. The protein (50 µg) was separated on 10 % sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH, USA) as described by Towbin *et al* [17]. Each membrane was incubated for 1 h with 5 % skimmed milk in 0.1 M Tris-buffered saline-0.1% Tween 20 (TBS-T) buffer (pH 7.4) to block non-specific binding and then incubated with primary antibodies that recognized iNOS (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), COX-2 (1:2000; Santa Cruz

Biotechnology, Santa Cruz, CA, USA) and GAPDH (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Each protein was detected by using a chemiluminescence detection system according to the manufacturer's protocol (ECL, Amersham, UK). The band intensity was quantified by NIH ImageJ densitometric analysis.

Statistical analysis

Data analysis was performed using Graphpad Prism 5.0 software. Data are expressed as mean ± standard error mean (S.E.M). Significance level of treatment effects was determined by one-way analysis of variance (ANOVA) followed by Tukey's post hoc analysis; *p*-values < 0.05 were considered statistically significant.

RESULTS

Total phenolics, flavonoids and anthocyanins quantities

Table 1 shows the proximate composition analyses of *S. tenuifolia* methanol extract. Total phenolic content of the extract was found 80.9 mg/g while the yield of total flavonoids and total anthocyanins was 16.1 mg/g and 28.3 mg/g, respectively. The level of antioxidant components in the extract closely follows the corresponding antioxidant and free radical scavenging activity.

Table 1: Content of antioxidants of *S. tenuifolia* extract

Compounds	Content (mean±SEM)
Total phenolics (mg of gallic acic equivalents/g of dried sample)	80.90±20.76
Total flavonoids (mg of rutin equivalents/g of dried sample)	28.25±4.06
Total anthocyanins (mg of kuromanin equivalents/g of dried sample)	16.10±0.96

DPPH radical scavenging ability

Fig 1 shows the dose-response results of the DPPH radical scavenging activities of the extract. and they indicate that the DPPH radical scavenging ability of the extract at 0.1 and 1.0 mg/ml were 41 and 59 %, respectively, compared to the control. Vitamin C, used as standard, decreased the DPPH radicals by 36 and 38% at 0.1 and 1.0 mg/ml, respectively.

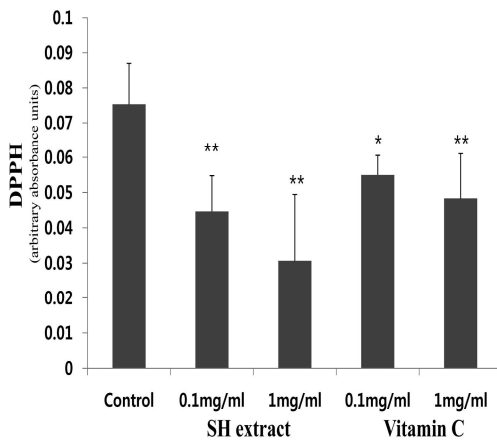


Fig 1: DPPH scavenging activity of *S. tenuifolia* extract (Error bars denote SEM; * $p < 0.05$; ** $p < 0.01$ compared with control)

NO radical scavenging ability

Fig 2 depicts that the extract had the ability to scavenge nitric oxide radicals by 17 and 19 % (compared to control) at 0.1 and 1.0 mg/ml, respectively. Vitamin C (0.1 mg/ml) exerted only 3 % decrease in nitric oxide radicals.

Deoxyribose oxidation assay

Fig 3 shows that the extract suppressed deoxyribose oxidation up to 20 % at a concentration of 1.0 mg/ml. The ability of the extract to quench reactive hydroxyl radical species has potential application to extend the shelf life of products.

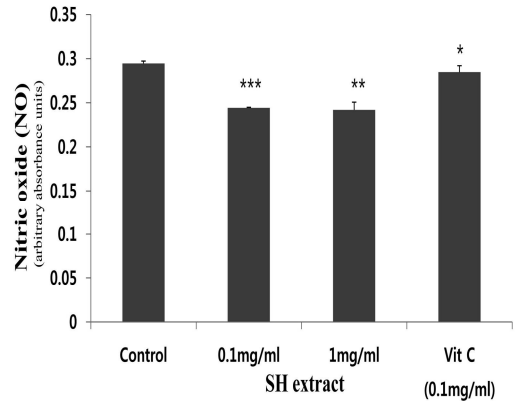


Fig 2: NO scavenging activity of *S. tenuifolia* (Error bars denote SEM; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared with control)

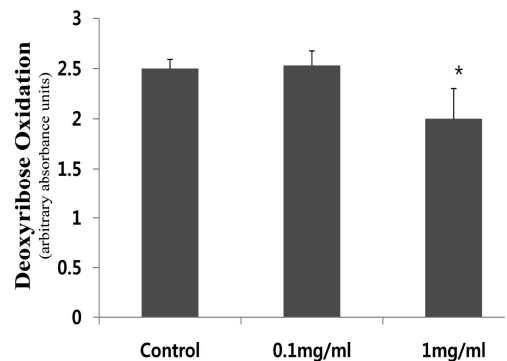


Fig 3: Effect of *S. tenuifolia* extract on deoxyribose oxidation (Error bars denote SEM; * $p < 0.05$ compared with control)

Reducing power

Fig. 4 shows the antioxidant activity of the extract based on its ability to reduce ferric (Fe^{3+}) to ferrous (Fe^{2+}) ion through the donation of an electron. The results indicate that the extract had the strongest reducing power (14.7-fold) compared to control and vitamin E (1 mg/ml).

Effect of *S. tenuifolia* extract on NO production induced by LPS in RAW 264.7 cells

Fig 5 shows that treatment with LPS induced a high release of NO to the culture medium.

Pretreatment with the extract resulted in the inhibition of the NO production by 14, 51 and 87 % in response to a concentration of 0.01, 0.1 and 1.0 mg/ml, respectively, compared to LPS-induction.

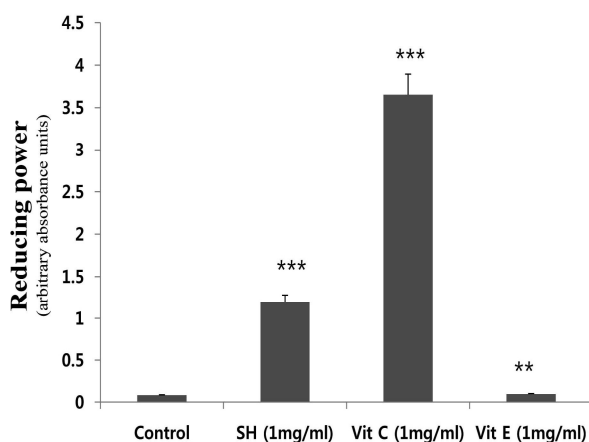


Fig 4: Reducing power of *S. tenuifolia* extract (Error bars denote SEM; ** $p < 0.01$ compared with control)

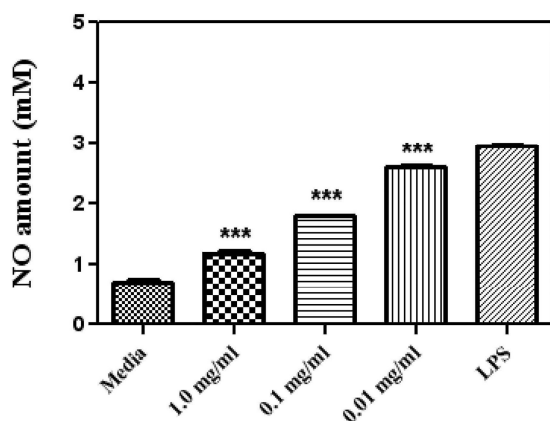


Fig 5: Effect of *S. tenuifolia* extract on NO production induced by LPS in RAW 264.7 cells (Error bars denote SEM; $p < 0.001$ compared with LPS alone)

Effect of *S. tenuifolia* extract on iNOS and COX-2 proteins by LPS in RAW 264.7 cells

Fig. 6 shows that the protein expressions of iNOS and COX-2 were significantly increased (7.4 fold in iNOS and 11.9 fold in COX-2)

upon LPS treatment as compared to control in RAW 264.7 cells. However, *S. tenuifolia* prominently suppressed the LPS-induced iNOS protein expression (Fig. 6A) in a dose-dependent manner (50%, 76% and 78% in response to 0.01, 0.1 and 1.0 mg/ml, respectively) as compared to the LPS-induced stimulation. The *S. tenuifolia* extract failed to inhibit the expression of LPS-induced COX-2 in RAW264.7 cells (Fig. 6B).

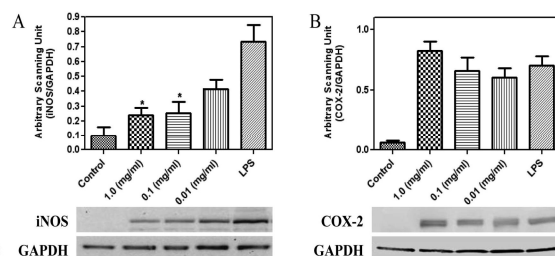


Fig 6: Effect of *S. tenuifolia* extract on iNOS and COX-2 induced by LPS in RAW 264.7 cells (Error bars denote SEM; * $p < 0.05$ compared with LPS alone)

DISCUSSION

Reactive oxygen and nitrogen species can be generated as by-products during oxidative progresses of living organisms [18]. Many diseases, including aging, cancer, cardiovascular disease, neurodegenerative disease and inflammation, are linked to excessive amounts of free radicals [19]. In the present study, we provide evidence that *S. tenuifolia* extract exerts marked antioxidant activity, possibly via free radical scavenging activities, inhibition of NO production and iNOS expression.

S. tenuifolia extract contains significant amount of phenolics including flavonoids and anthocyanins known as antioxidant components and these plant secondary metabolites have been recognized as having the potential to reduce diseases by helping to neutralize free radicals.

Various ways are being developed to measure the potential of natural products with

various antioxidant mechanisms. DPPH is a stable free radical and has been used extensively to test the ability of natural products to act as free radical scavengers or hydrogen donors [20]. Antioxidants either transfer an electron or a hydrogen atom to DPPH, thus neutralizing a number of DPPH molecules equal to that of hydroxyl groups [21]. It appears that *S. tenuifolia* possesses hydrogen-donating abilities to act as an antioxidant. Based on the data obtained from this study, the scavenging abilities increased with increasing concentration of the extract.

In addition to DPPH, the extract also showed nitric oxide scavenging activity. The toxicity and damage caused by NO with superoxide anion is multiplied as they react to produce reactive peroxynitrite (ONOO⁻), which leads to serious toxic reactions with biomolecules such as protein, lipids and nucleic acids [22]. In this study, the mean inhibition was increased with an increasing concentration of the extract and was relatively more pronounced than that of vitamin C (standard). The NO scavenging activity of the *S. tenuifolia* may help to arrest the chain reactions initiated by excess generation of ONOO⁻ that is detrimental to human health.

Hydroxyl radical is known to react with all components of the DNA molecule: the purine and pyrimidine bases as well as the deoxyribose backbone [23] and result in a series of reactions that cause the formation of malondialdehyde (MDA). In this study, the extract would compete with deoxyribose for the availability of hydroxyl radicals, thus reducing the amount of MDA formation. The inhibitory action of the *S. tenuifolia* against hydroxyl radicals mediated DNA oxidation protects against DNA damage as well as ROS generation.

Moreover, *S. tenuifolia* showed remarkable reducing power. The reducing ability generally depends on the presence of reductants [24]. The reductants cause the reduction of the Fe³⁺-ferricyanide complex to the ferrous form by donating a hydrogen

atom and exhibited antioxidative potential by breaking the free radical chain reactions [25].

In the macrophages, the high concentration of nitric oxide radical can be converted into peroxynitrites, which will cause diverse chemical reactions in a biological system including nitration of tyrosine residue of protein, triggering lipid peroxidation, inactivation of aconites, inhibition of mitochondrial electron transport, and oxidation of biological thiol compound [26]. In the present study, it is shown that *S. tenuifolia* has the ability to reduce nitric oxide production by LPS in the RAW 264.7 macrophages.

Results from Western blotting analysis further indicated that the enzyme iNOS is responsible for long-lasting nitric oxide production and it is strikingly induced by LPS as shown in this study. In addition to inhibition of NO release, the extract also significantly inhibited iNOS expression in LPS-stimulated RAW 264.7 macrophages as dose-dependent manner. In contrast, COX-2, which is responsible for proinflammatory prostaglandin formation, has been found increased by LPS was not altered by treatment with the extract. These results suggest that the anti-oxidant activity of the methanol extract of *S. tenuifolia* methanolic is mediated by inhibition of iNOS-NO system, rather than of COX-2 enzyme.

CONCLUSION

The results obtained from this study indicate that *S. tenuifolia* has powerful free radical scavenging capacity which may be due to the presence of phenolics such as flavonoids and anthocyanins. Furthermore, the immunomodulatory activity of *S. tenuifolia* is dependent on the inhibition of NO production and iNOS gene expression.

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