Antioxidative effects of Cinnamomi cortex: A potential role of iNOS and COX-II

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ABSTRACT

Background: Cinnamomi cortex has wide varieties of pharmacological actions such as anti-inflammatory action, anti-platelet aggregation, and improving blood circulation. In this study, we tested to determine whether the Cinnamomi cortex extract has antioxidant activities. **Materials and Methods:** Antioxidative actions were explored by measuring free radical scavenging activity, NO levels, and reducing power. The mechanism of antioxidative action of Cinnamomi cortex was determined by measuring iNOS and COX-II expression in lipopolysaccharide (LPS) stimulated Raw cells. **Results:** Seventy percent methanolic extract of Cinnamomi cortex exerted significant 1,1diphenyl-2-picrylhydrazyl (DPPH) free radicals and NO scavenging activities in a dose-dependent manner. More strikingly, the Cinnamomi cortex extract exerted dramatic reducing power activity (13-fold over control). Production of iNOS induced by LPS was significantly inhibited by the Cinnamomi cortex extract, suggesting that it inhibits NO production by suppressing iNOS expression. Additionally, COX-2 induced by LPS was dramatically inhibited by the Cinnamomi cortex extract. **Conclusion:** These results suggest that 70% methanolic extract of Cinnamomi cortex exerts significant antioxidant activity via inhibiting iNOS and COX-II induction.

Key words: Cinnamomi cortex, COX-II, free radical, iNOS, NO

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INTRODUCTION

Cinnamomi cortex has been widely used as a herbal medicine in Asia and Europe. It has long been used as a medicinal stuff in traditional oriental medicine and folk remedies and is widely used as a material of liquor, tea, or herb medicines. It has been found that it contains cinnamaldehyde, phellandrene, eugenol, and methyleugenol. A variety of pharmacological effects have been suggested: anti-inflammatory action via cyclooxygenase-II, sedative effects, and anti-bacterial activity against *Escherichia coli* and *Pseudomonas aeruginosa* are among them.[1-3] In traditional Chinese medicine, Cinnamomi cortex has been claimed for improving blood circulation. In addition, it has been found that Cinnamomi cortex has the ability to inhibit platelet aggregation via suppressing release of arachidonic acid.[4] Recently, an interesting report has been published that cinnamaldehyde, a main component of Cinnamomi cortex, induces endothelium-dependent and -independent vasorelaxant action on isolated rat aorta.[5]

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; it has been suggested that COX-II is also take part in the same processes. In this study, we explored to determine if whether Cinnamomi cortex extracts have antioxidative activities and tested whether iNOS and COX II are involved in anti-oxidative actions exerted by Cinnamomi cortex.

MATERIALS AND METHODS

Preparation of plant extracts
Authentic samples of Cinnamomi cortex 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. Cinnamomi cortex (100 g) was cut into small pieces and extracted with 70% methanol (300 ml) for 3 h, three times. The resulting methanol 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

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Signaling and Santa Cruz Biotechnology Co., respectively. The ECL kit was purchased from Amersham Co. All other reagents were purchased from Sigma Co. Cell culture media were purchased from Gibco Co.

Free radical scavenging activity

Free radical scavenging activity was measured by evaluating the ability to remove 1,1diphenyl-2-picrylhydrazyl (DPPH) under the principle of reduction reactions of DPPH radical solutions in the presence of hydrogen-donating antioxidants.[6] DPPH (Fluka Chemie, Buchs, Switzerland) solutions show an optical density at 515 nm and show a very deep violet color. Once DPPH. has been reduced by antioxidants, the optical density and the degree of color development are reduced. Briefly, Cinnamomi cortex extract was dissolved in 1 ml MeOH, and then was mixed with 1 ml DPPH. solution at room temperature. Then, optical density was measured at 515 nm.

Reducing power

The reducing power was measured using the Oyaizu's method.[7] An aliquot of 2.5 ml of Cinnamomi cortex extract (0.2 M phosphate buffer, pH 6.6) was put into 2.5 ml of potassium ferricyanide (10 mg/ml) solution and subjected to react for 15 min at 30 °C. An aliquot of 2.5 ml of trichloroacetic acid (100 mg/ml) was put into the reactant and mixed up and 2.5 ml of the mixture was again mixed with 2.5 ml of distilled water and 0.5 ml of ferric chloride (1.0 mg/ml) and the optical density was measured at 700 nm.

Cell culture

Murine RAW 264.7 macrophage cells were cultured in Dulbecco's modified Eagle's Medium (Gibco BRL, Grand island, NY) with 10% heat-inactivated fetal bovine serum in 5% humidified CO_2 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 the Greiss reagent (Sigma, USA). A total of 2×10^6 Raw cells were put into a 6 well plate and washed two times with PBS when the confluence was 80% and then cultured for at least 24 h and the samples were made into the final concentrations of 10, 1.0, 0.1 mg/ml for experiments. Four hours later, lipopolysaccharide (LPS, final concentration 1 mg/ml) was put into all wells except for the well for the control group to stimulate the cells. The amounts of NO generated were measured with the supernatant 18 h later at 540 nm.

Measurement of iNOS and COX-II expression by Western blot analysis

Raw cells were cultured with DMEM and 10% FBS. When the cells reached confluence, the DMEM culture medium was removed and replaced by the EMEM culture medium which is a serum-free culture medium and then the cells were treated with Corni fructus extracts and cultured for 24 h. The cells were washed two times with PBS and scraped into a buffer containing 10 mM Tris–HCl, pH 7.4, 50 mM NaCl, 5 mM EDTA, 30 mM NaF, 0.1 mM Na_3VO_4, 1% triton X-100, 0.5% NP-40, 1 μg/ml leupetin, 1 μg/ml aprotinin. Then, the cells were disrupted by passing them through a 1-ml tuberculin syringe five times. The cell lyaste was subjected to centrifugation at $10,000 \times g$ for 10 min and the supernatant was used for western blot analysis. The protein content of the soluble fraction was assessed by the method of Bradford.[8] Protein (50 μg/lane) was electrophoretically separated in 10% polyacrylamide gels containing SDS. Proteins were transferred to nitrocellulose membranes (Schleicher and Schuell), which was carried out for 1 h at 100 V (constant) as described by Towbin et al. The filter papers were preincubated for 1 h at 23°C with PBS containing 0.1% Tween 20 and 5% skim milk and washed with PBS containing 0.1% Tween 20 three times for 10 min each. Followed by the blots were probed with primary antibody directed against iNOS (1:1000), COX-II (1:2000), GAPDH (1:1000) for 2 h at room temperature or overnight at 4 °C diluted in blocking buffer. The blots were then incubated with HRP-conjugated anti-rabbit IgG (1:1000 for iNOS and GAPDH; 1:2000 for COX-II) for 1 h at room temperature and washed with PBS containing Tween 20 three times for 10 min each. The detection of immobilized specific antigens was carried out by ECL (NEN). The images analysed using Image J software.

Component analysis (anthocyanin, phenolics, flavonoids)

Measurement of total phenolics

The total phenolics content was measured using the Folin-Ciocalteau procedure at 725 nm.[9] Gallic acid was used as a standard for phenolic compounds and the phenolic concentration was calculated by using a gallic acid standard calibration curve. The total phenolics content was expressed as the gallic acid equivalent (mg gallic acid/g extract).

Measurement of total flavonoids

The total flavonoids existing was measured using the method of Miliauskas et al,[10] and was expressed as the rutin equivalent (mg rutin acid/g extract) using rutin as a standard flavonoid. One milliliter of the Cinnamomi cortex extract was mixed with aluminum trichloride in ethanol (20 mg/ml) and diluted to 25 ml. After incubation for 40 min at 20 °C, the optical density was measured at 415 nm.

Measurement of total anthocyanin

The total anthocyanin was measured using color reactions.[11] Corni fructus extracts were dissolved in 1 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 the GraphPad Prism 5 with one-way ANOVA followed by Turkey's multiple comparison test. $P < 0.05$ was considered as significant.

RESULTS

DPPH free radicals were decreased by approximately 26% at 0.01 mg/ml of the Cinnamomi cortex extract and about 77% at 0.1 mg/ml as compared to the control. At 1.0 mg/ml, the DPPH free radicals decreased by around 57% [Figure 1]. The Cinnamomi cortex extract also decreased the level of NO by 11%, 15% and 20% at 0.01, 0.1, and 1 mg/ml, respectively [Figure 2]. Regarding reducing power of Cinnamomi cortex extracts, it stimulated the reducing power by 3.1-fold and 13-fold as compared to control, respectively [Figure 3]. Its reducing power appeared almost the same as that of vitamin E. The amount of nitric oxide production markedly increased (2.3-times over basal) when the Raw cells were treated with LPS to activate the macrophages whereas when the cells were pretreated with the Cinnammoi cortex extract, NO production was significantly decreased by 33%, 37%, and 49% in response to 0.01, 0.1, and 1 mg/ml extract, respectively, as compared to the control [Figure 4].

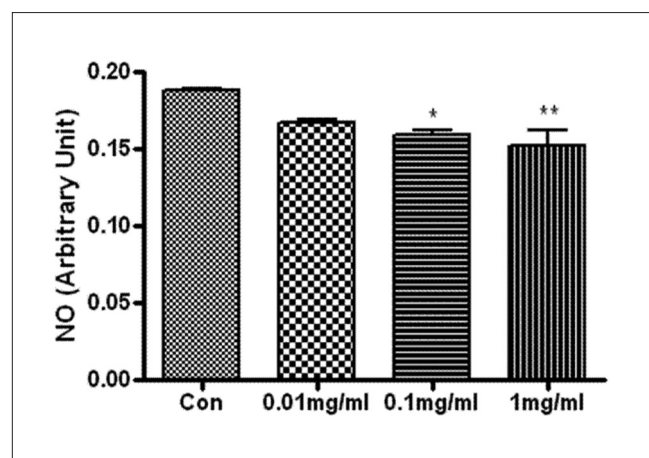To elucidate mechanisms of antioxidant actions by the
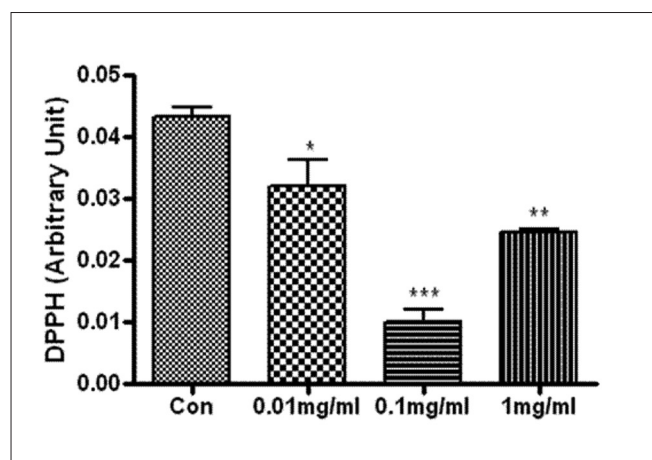
Cinnamomi cortex extract, expression of iNOS which is a key enzyme for the generation of NO was examined by western blot analysis using a specific iNOS antibody. When LPS was administered to the Raw cells, the expression of iNOS increased by 19-fold over basal. On the other hand, when the cells were pretreated with Cinnamomi cortex extract, iNOS expression levels were markedly decreased by 34%, 77%, and 96% in response to 0.01, 0.1, and 1 mg/ml, respectively [Figure 5]. Cyclooxygenase-2 (COX-2) was also explored to see if it is a target of Cinnamomi cortex extract. When LPS was administered, the expression of the COX-2 enzyme was induced by 6.6-fold over basal. The Cinnamomi cortex extract had little effect on the expression of LPS-induced COX-II at 0.01 mg/ml; however, it was dramatically inhibited by 82% and 100% at 0.1 and 1 mg/ml, respectively [Figure 6].

When antioxidant components present in the Cinnamomi cortex extract was analyzed, total phenolics, total flavonoids, and total anthocyanin were determined as 0.36, 38.73, and 24.65 mg/g, respectively [Table 1].

DISCUSSION

A significant body of evidence has accumulated to indicate that oxidative stress plays an important role in the development of many diseases such as diabetes, Parkinson's disease, cardiovascular diseases, and periodontal diseases. In this study, we have provided evidence that the Cinnamomi cortex extract exerts antioxidative actions via inhibition of iNOS and COX-II induction.

Oxygen free radicals are produced in a large amount by

Figure 1: 1,1diphenyl-2-picrylhydrazyl (DPPH) Scavenging Activity by the Cinnamomi cortex extract. DPPH scavenging activity of the Cinnamomi cortex extract was measured as described in the Materials and Methods section. All the measured values were shown as means ± SEM and showed significant differences from those of the control group at the levels of *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$


Figure 2: NO scavenging activity by the Cinnamomi cortex extract. NO scavenging activity of the Cinnamomi cortex extract was measured as described in the Materials and Methods section. All the measured values were shown as means ± SEM and showed significant differences from those of the control group at the levels of *$P < 0.05$ and **$P < 0.01$
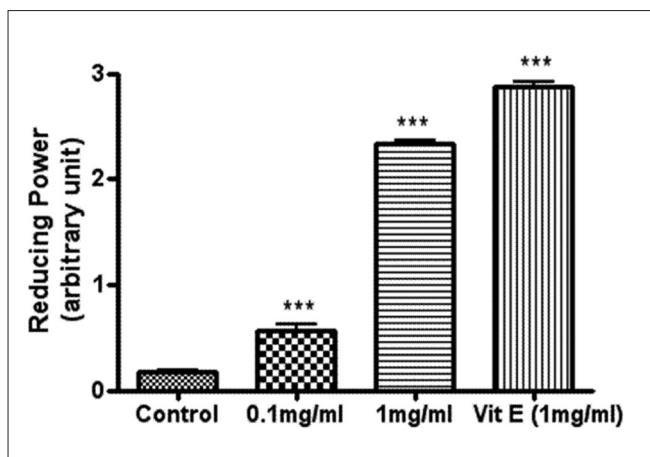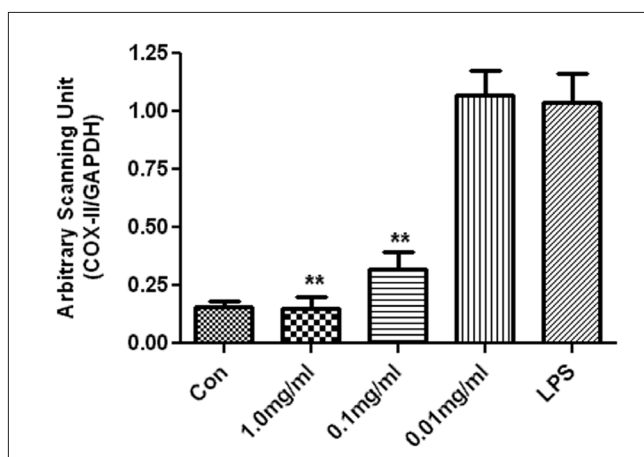
Figure 3: Inhibition of NO production by Cinnamomi cortex in Raw cells. Raw 264.7 cells were pretreated with the Cinnamomi cortex extract at the concentrations of 0.01, 0.1 and 1.0 mg/ml for 4 h and then treated with LPS 10 mg/ml for 18–24 h and the amount of NO was measured using the Greiss reagent. All the measured values were shown as means ± SEM and showed significant differences from those of the control group at the level of ***$P < 0.001$
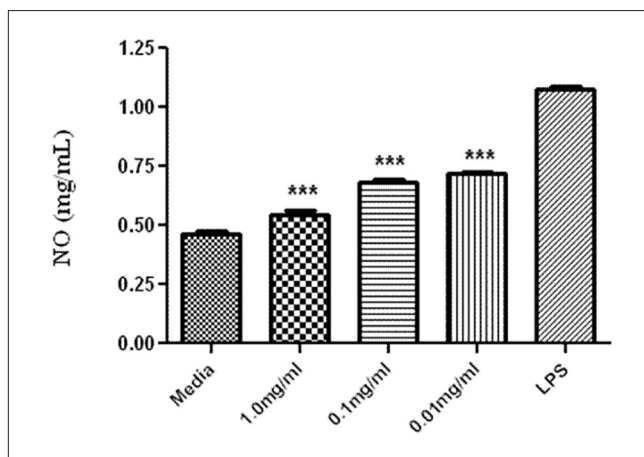
Figure 4: Reducing power by the Cinnamomi cortex extract. The reducing power of the Cinnamomi cortex extract was measured by the Oyaizu's method as described in the Materials and Methods section. All the measured values were shown as means ± SEM and showed significant differences from those of the control group at the level of ***$P < 0.001$
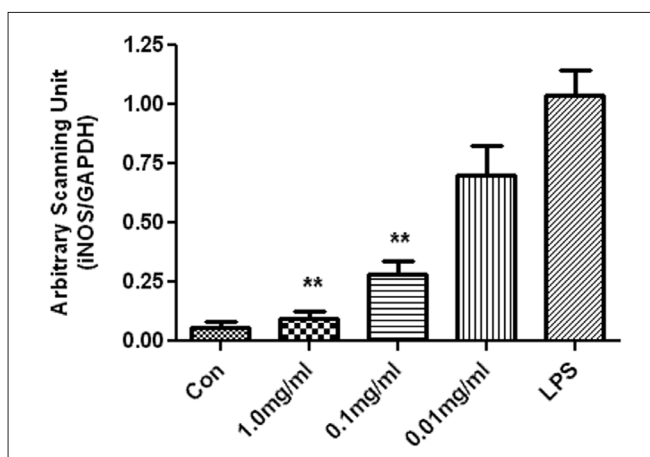
Figure 5: Effect of the Cinnamomi cortex extract on LPS-induced iNOS induction. Raw 264.7 cells were pretreated with the Cinnamomi cortex extract at the concentrations of 0.01, 0.1, and 1.0 mg/ml for 4 h and then treated with LPS 10 mg/ml for 18–24 h and then western blot analysis with a specific antibody against iNOS and GAPDH as a internal control as described in the Materials and Methods section (B). The blots were subjected to scanning densitometry and expressed as a ratio of iNOS to GAPDH (A). **$P < 0.01$

Figure 6: Effect of the Cinnamomi cortex extract on LPS-induced COX-II induction. Raw 264.7 cells were pretreated with the Cinnamomi cortex extract at the concentrations of 0.01, 0.1, and 1.0 mg/ml for 4 h and then treated with LPS 10 mg/ml for 18–24 h and then western blot analysis with a specific antibody against COX-II and GAPDH as a internal control as described in the Materials and Methods section (B). The blots were subjected to scanning densitometry and expressed as a ratio of COX-II to GAPDH (A). The data shown are the mean values ± SEM of three experiments. **$P < 0.01$

Table 1: Component analysis of the Cinnammi cortex extract

Component	Concentration (mg/g)
Total flavonoids (mg rutin/g extract)	38.73 ± 3.24
Total Phenolics (mg gallic acid/g extract)	0.38 ± 0.02
Anthocyanin (mg kuromanin/g extract)	24.65 ± 0.06

Total flavonoids, total phenolics, and anthocyanin were analyzed as described in the Materials and Methods section. Data are expressed as mean ± SEM ($n = 3$)

various environmental factors, such as air pollution or smoking, ultraviolet rays, stress, and intensive exercises and they play an essential role in damaging lipid, proteins, and nucleic acid, thereby promoting various kinds of diseases and aging.[12,13]

The DPPH free radical removing action and nitric oxide scavenging action of the Cinamomi cortex extract are

clearly demonstrated in this study. Strikingly, it showed approximately 13 times potent reducing power as compared to control. These results strongly suggest that the Cinnamomi cortex extract has the ability to remove various kinds of free radicals generated due to oxidative stress. In addition, the marked reducing power of the extract will be beneficial in removing toxic peroxides generated by oxidizing reactions.

Wahl *et al*,[14] proposed that decreasing the levels of nitric oxide in the synovial fluid of patients with chronic temporomandibular diseases could result in reducing pain and tissue damages. Interestingly, it has been suggested that oxidative stress could affect the progress of periodontal diseases and antioxidant substances such as flavonoids or vitamin C could suppress such progresses.[15-17] Nitric oxide (NO) is a substance that plays an important role in various kinds of inflammatory responses *in vivo* and it is generated by the iNOS enzyme that is induced by inflammation. Plant extracts have been shown to affect iNOS and COX-II, thereby inducing anti-inflammatory actions.[18-21] In this study, it has been found that the Cinnamomi cortex extract significantly reduces NO generated by LPS in the Raw cells with dramatic inhibition of iNOS expression. Similarly, COX-II was also affected by the Cinnamomi cortex extract. These results suggest that the anti-oxidative action of the Cinnamomi cortex extract is mediated by the inhibition of the iNOS-NO system as well as COX-II enzyme. Taken together, anti-oxidative actions of the Cinnamomi cortex extract could be useful in the prevention and treatment of various inflammatory diseases including chronic temporomandibular diseases.

Recently, it has been identified that a number of plant extracts containing phenolics, anthocyanin, and flavonoids exert remarkable antioxidative actions.[22-24] It has been identified that the Cinnamomi cortex extract has diverse components including cinnamaldehyde. The Cinnamomi cortex extract contains significant amounts of phenolics including anthocyanins and flavonoids, suggesting that these components could be major contributors to the iNOS and COX-II inhibitory action of the Cinnamomi cortex extract. However, it will be necessary to isolate the real chemical constituents of Cinnamomi cortex to better understand the mechanism of its anti-oxidative actions.

CONCLUSION

We propose that the antioxidant activities of the Cinnamomi cortex extract are due to the inhibition of iNOS and COX-II, and scavenging of NO and free radical. This property could be applied to the prevention and treatment of various kinds of diseases triggered by oxidative stress

such as temporomandibular joint diseases, periodontal diseases, ulcers, arthritis, hepatitis, and nephritis. In-depth component analyses of the Cinnamomi cortex extract and the identification of active principle(s) will give us insight into the development of new therapeutics having antioxidant actions.

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