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Full Length Research Paper

Anti-oxidative effects of flavonoids enriched Corni fructus extract and the mechanism

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We tested to determine if Corni fructus extract has antioxidant activities and explored its potential mechanism in terms of iNOS and COX-II involvement. Anti-oxidative actions were explored by measuring free radical (NO, DPPH) scavenging activity, and TBARS levels. The mechanism of anti-oxidative action of Corni fructus extract was determined by performing Western blot analysis for iNOS and COX-II expression in lipopolysaccharide (LPS) stimulated Raw cells. 70% methanolic extract of Corni fructus exerted significant DPPH free radical scavenging activity in a dose-dependent manner. The DPPH-free radical scavenging activity was stronger than that of vitamin E. It also markedly inhibited TBARS formation. Strikingly, the Corni fructus extract has dramatic reducing power with maximal activity observed as 231-fold over control. Production of iNOS induced by LPS was significantly inhibited by the Corni fructus extract, suggesting it inhibits NO production by suppressing iNOS expression. However, COX-2 induced by LPS was not significantly altered by the Corni fructus extract. The Corni fructus extract contains anti-oxidant components including phenolics, flavonoids and anthocyanin at the concentration of 18.07, 2.31 and 7.83 mg/g, respectively. The subsequent chloroform fraction enriched with flavonoids almost completely blocked the iNOS induction and NO production caused by LPS in the Raw cells.

Key words: Corni fructus, free radical, iNOS, NO, COX-II, lipid peroxidation.

INTRODUCTION

Corni fructus is the dryed fruit of *Cornus officinalis* SIEB and Zucc which belongs to the Cornaceae family. These trees grow wild in China and the central and southern part of our country. With a diversity of efficacy and flavor, 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 contains cornin that is known to stimulate parasympathetic nerves, glycosides such as morroniside, loganin, tannin and saponin, organic acids such as gallic acid, linolic acid, palmitic acid and ursolic acid, and vitamin A plus a considerable amount of sugar. It also contains 18 kinds of minerals and abundant K, Ca, Mg and amino acid (Ding, 2007). It increases the secretion of acetylcholine, thereby promoting the secretion of insulin (Hsu et al., 2006). A number of reports ave been published regarding its pharmacological effects: corniside separated from Corni fructus extracts causes relaxation of the smooth muscles of blood vessels (Kang et al., 2007); Corni fructus extracts has anticancer effects against hepatoma cells or leukemic cells (Chang et al., 2004). In addition, it has been found that polysaccharides in Cornus officinalis modulate immunologic functions (Du et al., 2008), and morroniside had the ability to prevent neurotoxicity caused by oxidative stress (Wang et al., 2009). It has been found that *C. officinalis* (has the ability to enhance cognitive action (Lee et al., 2009) and to inhibit melano-genesis (Nawa et al., 2007). Antioxidants play important roles for us to maintain health and they are mainly ingested through foods such as fruits or vegetables. They serve the roles of suppressing or delaying lipid peroxidation and removing free radicals (active oxygen). Antioxidants such as vitamin C, vitamin E, phenolic acid, flavonoids can reduce the risk of chronic inflammations such as tumors or heart diseases and they are helpful in neutralizing active oxygen which is unstable molecules

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that are involved in the onsets of those diseases (Sanbe et al., 2007). 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 (Conforti and Menichini, 2011; Codener-Franch et al., 2011); it has been suggested that COX-II is also take part in the same processes (Uchida, 2008). In this study, we explored to determine if Corni fructus extracts have anti-oxidative activites and tested whether iNOS, NO and COX II are involved in anti-oxidative actions exerted by Corni fructus extracts.

MATERIALS AND METHODS

Preparation of plant extracts

Authentic samples of Corni fructus 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. 4N-006) was deposited at the Herbarium of the Department of Pharmacology, School of Dentistry, Kyung Hee University. Corni fructus (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 rotary evaporator and dried by freeze- dryer, yielding approximately 25.7 g. Then 10 g of freeze-dried 70% methanol extract was resuspended with 200 ml methanol-water (1:1.5) solution, adjusted to pH 3 with 2M H_2SO_4, further extracted with 200 ml of chloroform (three times). The chloroform fraction was concentrated using the rotary evaporator, and followed by freeze-drying, yielding 0.2 g (Fraction C).

Reagents and materials

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

Free radical scavenging activity

Free radical scavenging activity was measured by evaluating the ability to remove DPPH under the principle of reduction reactions of DPPH radical solutions in the presence of hydrogen-donating anti-oxidants (Brand-Williams et al., 1995). Briefly, Corni fructus extract dissolved in 1 ml MeOH was missed with 1 ml DPPH solution at 23°C and optical density was measured in 30 min at 515 nm.

Measurement of lipid peroxidation (TBARS measurement)

The final products of polyunsaturated fatty acid oxidation reaction will react with thiobarbituric acid (TBA) to form red floaters. Thiobarbituric acid reactive substances (TBARS) were measured by the Sroka's method (2003). The TBA reagent was prepared as follows. Reagent A: 375 mg of thiobarbituric acid (Fluka chemical) and 30 mg of tannic acid were melted into 30 ml of hot water. Reagent B: 15 g of thiobarbituric acid was melted into 70 ml of 0.3 M hydrogen chloride solution. Then, 30 ml of reagent A and 70 ml of reagent B were mixed up. Rapeseed oil (5.2 µl) was suspended in 2 ml of 0.2 M Tris-HCL buffer (pH 7.4) containing 15.6 mg of Tween-40. The produced suspension was irradiated with UV light for 60 min at 25°C and then 100 µl of Corni fructus extracts were mixed with 1 ml of the suspension. This mixture was irradiated with UV light for 30 min. 2 ml of TBA reagent was put into the mixture and then the mixture was made to react for 15 min in boiling water and centrifuged for 3 min at 100 x g and the supernatant fluid was taken and the optical density was measured at 532 nm. The suppression of lipid and oxidation reactions was measured as the percentage to the control group not containing Corni fructus extracts.

Reducing power

The reducing power of Corni fructus extract was measured using the Oyaizu's (1986) method. 2.5 ml of Corni fructus extract (0.2 M phosphate buffer, pH 6.6) was put into 2.5 ml of potassium ferricyanide (10 mg/ml) solution and made to react for 15 min at 30°C. 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 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 (NO)

Raw cells were cultured with DMEM and 10% FBS. NO was measured by measuring the amount of NO in the cell supernatant as nitrite and nitrate. The safe form of nitrite after being reduced to nitrate was measured using the Greiss reagent (Sigma, USA). 2 x 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 and 0.1 mg/ml for experiments. Four hours later, LPS of the final concentration 1 mg/ml was put into all wells except for the well for the control group to stimulate the samples. The amounts of NO generated were measured by gathering the supernatant around 18 h later, having them react with the Greiss reagent for 10 min after shading and then measuring the optical density at 540 nm.

Measurement of iNOS and COX-II expression by Western blotting

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 x 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 (1976). Protein (50 µg/lane) was electrophoretically separated in 10% polyacrylamide gels containing SDS. Proteins were transferred to nitrocellulose membranes was carried out for 1 h at 100 V (constant) as described by Towbin et al. (1979). The filter papers were preincubated for 1 h at 23°C with PBS containing 0.1% Tween

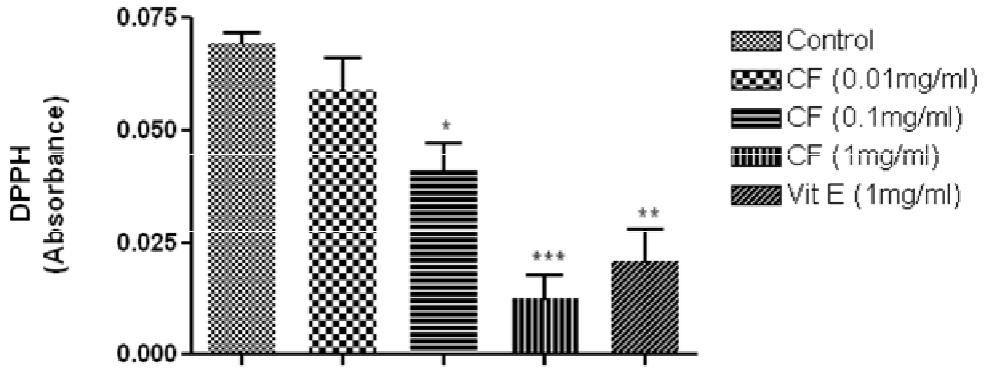

Figure 1. DPPH scavenging activity by Corni fructus extract. DPPH scavenging activity of Corni fructus extracts was measured as described in the Methods. All the measured values were shown as means ± S.E.M of three experiments and showed significant differences from those of the control group at the levels of P<0.05 and **P<0.01.

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 (1:1,000), COX-II (1:2,000), 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 (1:1,000 for iNOS and GAPDH; 1:2,000 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 (Singleton and Rossi, 1965). 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 Miliauskasa et al. (2004) and was expressed as the rutin eqivalent (mg rutin acid/ g extract) using rutin as a standard flavonoid. 1 ml of Corni fructus extracts was mixed with aluminum thichloride 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 (Piccolella et al., 2008). 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 expresssed as kouromanin equivalent (mg kouromanin / g extract).

Statistical analysis

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

RESULTS

DPPH free radicals were decreased by approximately 20% at 0.01 mg/ml of Corni fructus extract and about 40% at 0.1 mg/ml as compared to the control. At 1.0 mg/ml, the DPPH free radicals decreased by around 80% and this is much stronger than that of same concentration of Vitamin E (Figure 1). Degree of lipid peroxidation is used as an index for oxidative stress and analyzed by measuring thiobarbituric acid reactive substances (TBARS). Lipid peroxides are unstable and are decomposed into various kinds of compounds including carbonyl compounds. The TBARS production was significantly suppressed by the Corni fructus extract at the concentration of 0.1 ~ 1 mg/ml and its action was equal to that found in Vitamin E (Figure 2) . Regarding reducing power of Corni fructus extracts, it had the reducing power of 12.8 and 29.8 fold as compared to control at 0.1 and 1 mg/ml, respectively. It exerted 5.1 fold stronger reducing power at the concentration of 1 mg/ml than that of vitamin E (Figure 3). The amount of nitric oxide production markedly increased when the raw cells were treated with lipopolysaccharide (LPS) to activate the macrophages whereas when the cells were pretreated with the Corni fructus extract, NO production was significantly decreased by 19 and 52% in response to 0.1 and 1 mg/ml extract, respectively, as compared to the control (Figure 4).

To elucidate mechanisms of antioxidant actions by the Corni fructus extract, the 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 significantly. On the other hand, when the cells were pretreated with the Corni fructus

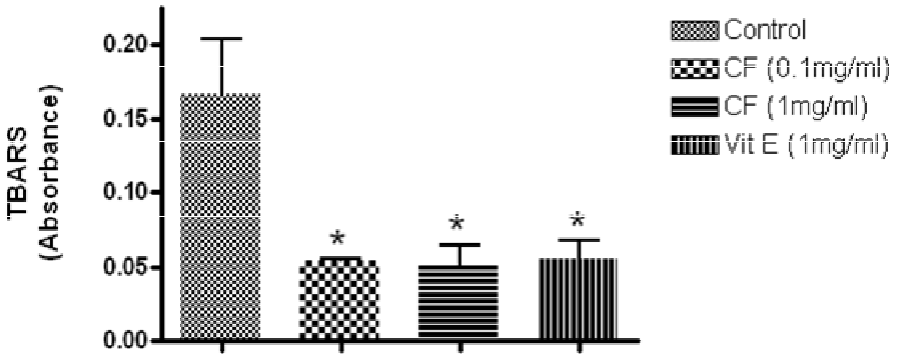

Figure 2. TBARS inhibition by Corni fructus extract. Lipid peroxidation activity of Corni fructus extracts was analyzed by measuring TBARS as described in the Methods. All the measured values were shown as means ± S.E.M of three experiments and showed significant differences from those of the control group at the level of *P<0.05 .

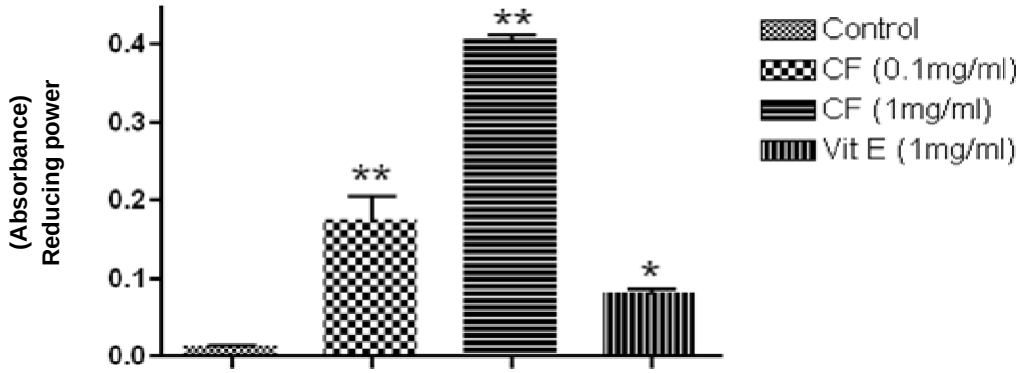

Figure 3. Reducing power by Corni fructus extract. The reducing power of Corni fructus extracts was measured as described in the methods. All the measured values were shown as means ± S.E.M of three experiments and showed significant differences from those of the control group at the levels of *P<0.05, **P<0.01, ***P<0.001.

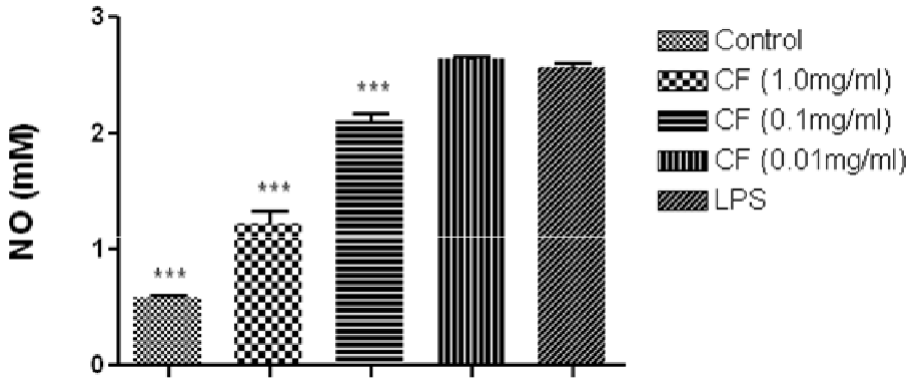

Figure 4. NO scavenging Activity by Corni fructus extract. Raw 264.7 cells were pretreated with Corni fructus extracts at the concentrations of 0.01, 0.1 and 1.0 mg/ml for four hours and then treated with LPS 10 mg/ml for 18 to 24 h and the amount of NO was measured using the Greiss reagent. All the measured values were shown as means ± S.E.M of three experiments and showed significant differences from those of the LPS-treated group at the levels of ***P<0.001.

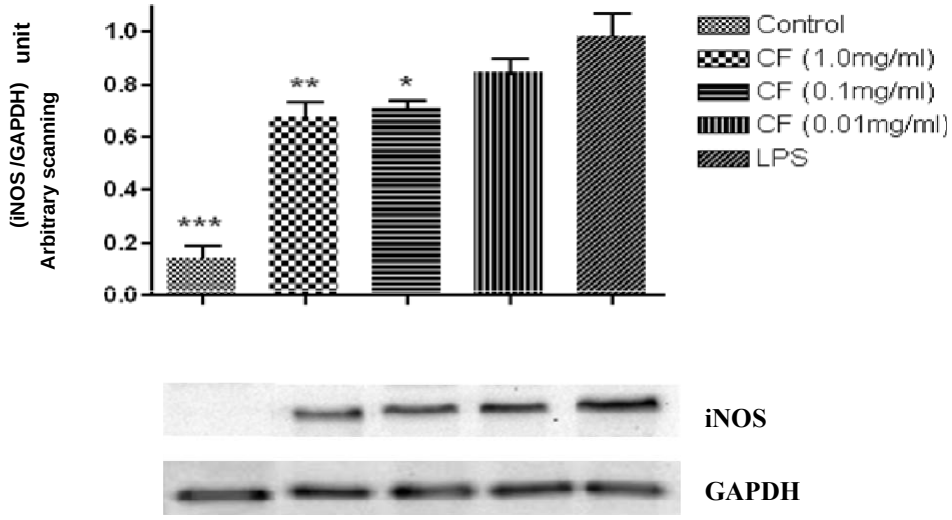

Figure 5. Effect of Corni fructus extract on LPS-induced iNOS induction. Raw 264.7 cells were pretreated with Corni fructus extracts at the concentrations of 0.01, 0.1 and 1.0 mg/ml for four hours and then treated with LPS 10 mg/ml for 18 to 24 h and then Western blot analysis with a specific antibody against iNOS and GAPDH as a internal control as described in the methods (B). The blots were subjected to scanning densitometry and expressed as a ratio of iNOS to GAPDH (A). All the measured values were shown as means ± S.E.M of three experiments and showed significant differences from those of the LPS-treated group at the levels of *P<0.05, **P<0.01, ***P<0.001.

extract, iNOS expression levels were markedly decreased by 14, 27, and 31% in response to 0.01, 0.1 and 1 mg/ml, respectively (Figure 5). COX -2 (cyclooxygenase-2) was also explored to see if it is a target of the Corni fructus extract. When LPS was administered, the expression of the COX-2 enzyme was markedly induced; however, the Corni fructus extracts failed to inhibit the expression of LPS-induced COX-II (Figure 6).

When antioxidant components present in the 70% methanolic extract of Corni fructus (T) were analyzed, total phenolics, total flavonoids and total anthocyanin were determined as 18.07, 2.31 and 7.83 mg/g, respectively (Figure 7). In an effort to fractionate phenolic compound from the total extract (T), subsequent extraction with chloroform under pH 3 was performed. The chloroform fraction (C) contained total phenolics, total flavonoids and anthocyanin were determined as 7.98, 6.53 and 5.14 mg/g, respectively. NO production by LPS was inhibited by 25, 83 and 84% in response to 0.01, 0.1 and 1 mg/ml the chloroform fraction (C), respectively (Figure 8). The chloroform fraction had the ability to inhibit iNOS induction by LPS by 3, 94 and 99% in response to 0.01, 0.1 and 1 mg/ml, respectively (Figure 9).

DISCUSSION

A significant body of evidence has accumulated to indicate that oxidative stress plays an important role in the development of many diseases. In the present study, we have provided evidence that Corni fructus extracts exert remarkable anti-oxidative actions possibly through iNOS inhibition, not through COX-II.

During *in vivo* oxygen metabolism, free radicals are unceasingly produced and take part in destroying the structure and functions of cell membranes. Accordingly, protein synthesis is interfered and mitochondrial functions are lost, eventually leading to cell damages. Oxygen free radicals are also excessively produced by 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 (Kim et al., 1997; Evereklioglu et al., 2003).

The DPPH free radical removing action of the 70% methanolic Corni extract was clearly observed in a dose-dependent manner and its activity at 1mg/ml was stronger than that of Vitamin E. Its effect on the TBARS formation was also clearly observed and it showed almost same potent as vitamin E. Moreover, the Corni

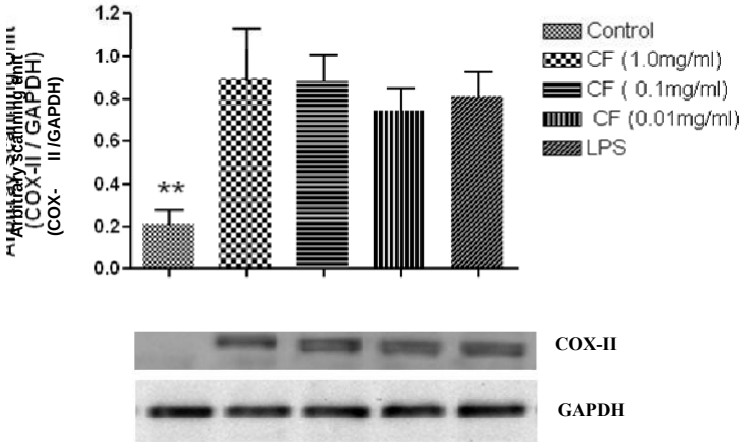

Figure 6. Effect of Corni fructus extract on LPS-induced COX-II induction. Raw 264.7 cells were pretreated with Corni fructus extracts at the concentrations of 0.01, 0.1 and 1.0 mg/ml for four hours and then treated with LPS 10 mg/ml for 18 to 24 h and then Western blot analysis with a specific antibody against COX-II and GAPDH as a internal control as described in the methods (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 and showed significant differences from those of the LPS-treated group at the levels of **P<0.01.

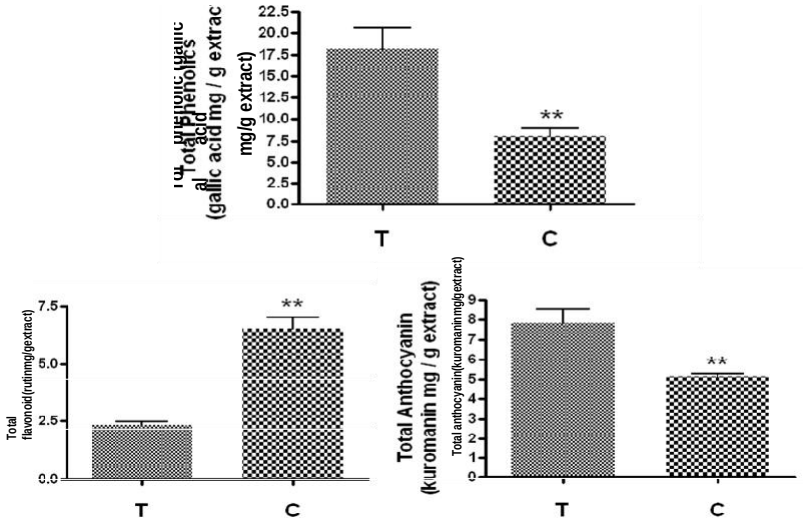

Figure 7. Antioxidant component of Corni fructus. To elucidate the antioxidant components of Corni fructus extracts, the contents of total phonolics, total flavonoids and total anthocyanin were analyzed as described in the methods. All the measured values were indicated as means ± S.E.M of three experiments and showed significant differences from those of the control group (T) at the levels of **P<0.01.

fructus extract has dramatic reducing power activity. Thus, it can not only remove various kinds of free radicals generated through oxidative stress but also suppress the lipid peroxidation triggered by the generation of free radicals. In addition, the marked reducing power of the extract will be beneficial in removing toxic peroxides

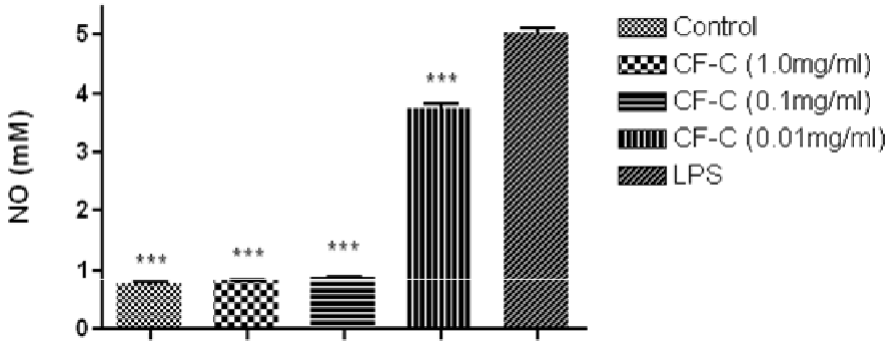

Figure 8. NO scavenging activity by chloroform fraction of Corni fructus extract. Raw 264. 7 cells were pretreated with Corni fructus extracts at the concentrations of 0.01, 0.1 and 1.0 mg/ml for four hours and then treated with LPS 10 mg/ml for 18 to 24 h and the amount of NO was measured using the Greiss reagent. All the measured values were shown as means ± S.E.M of three experiments and showed significant differences from those of the LPS-treated group at the levels of ***P<0.001.

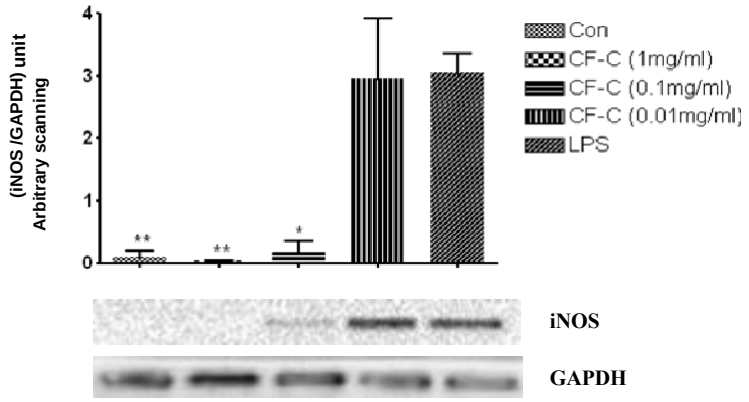

Figure 9. Effect of chloroform fraction of Corni fructus extract on LPS-induced iNOS induction. Raw 264.7 cells were pretreated with Corni fructus extracts at the concentrations of 0.01, 0.1 and 1.0 mg/ml for four hours and then treated with LPS 10 mg/ml for 18 to 24 h and then Western blot analysis with a specific antibody against iNOS and GAPDH as a internal control as described in the Methods (B). The blots were subjected to scanning densitometry and expressed as a ratio of iNOS to GAPDH (A). The data shown are the mean values ± SEM of three experiments and showed significant differences from those of the LPS-treated group at the levels of *P<0.05, **P<0.01.

generated by oxidizing reactions.

Wahl et al. (2003) raised an interesting possibility to reduce pain and tissue damages by decreasing the levels of nitric oxide in the synovial fluid of patients with chronic temporomandibular diseases. It has been reported that oxidative stress could affect the progress of periodontal diseases and antioxidant substances such as flavonoids or vitamin C could suppress such progresses (Tomofuji et al., 2009; Sanbe et al., 2007; Canakçi, 2006). There is an interesting report showing that the degree of lipid peroxidation of cells was higher in an inflammatory disease in the oral cavity such as lichen planus than that in the mucous membranes of normal persons (Agha-Hosseini et al., 2009). Thus, anti-oxidants could be useful for preventing various diseases induced by oxidative stress (Linden et al., 2009).

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. In the present study, it is identified that Corni fructus extracts significantly has the ability to reduce the content of NO generated by LPS in the raw cells with the marked inhibition of iNOS expression. On the contrary, COX-II was not affected by the treatment of Corni fructus extracts. These results suggest that the anti-oxidative action of the Corni fructus extract is mediated by the inhibition of iNOS-NO system, not through COX-II enzyme. Taken together, anti-oxidative actions of the Corni fructus extracts could be useful in the prevention and treatment of various inflammatory diseases including chronic temporomandibular diseases.

Corni fructus have diverse components including saponin, tannin, gallic acid and vitamin A. Their known pharmacological actions include the control of immunelogic functions such as suppressing the evolution of lymphoid cells and the generation of IL-2. It is also known that they have actions such as increasing white blood cell functions and suppressing various kinds of bacteria. Terpenoids and phenolics are known to be recovered in the chloroform-soluble fraction under acidic pH condition (Harborne, 1998). The chloroform fraction of the Corni fructus extract contains significant amount of phenolics including anthocyanins and flavonoids. Interestingly, it has been widely known that plant extract containing phenolics could exert anti-oxidant activity (Kähkönen et al., 1999). Among the phenolics, flavonoids are enriched in the chloroform fraction, suggesting that it could be a major contributor to the iNOS inhibitory action of the Corni fructus extract. In other studies, it has been found that *C. officinalis* contains morroniside, lognin, iridoid total glycoside and ursolic acid, preventing oxidative stress (Xu et al., 2006; Xu and Hao, 2004; Yu et al., 2009).

In conclusion, we propose that the antioxidant activities of the Corni fructus extract is due to the inhibition of iNOS, NO and free radical production. The chloroform fraction of the Corni fructus extract contains significant amount of phenolics including anthocyanins and flavonoids which might be players of the inhibiton of iNOS by the extract. 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 Corni fructus extract and the identification of active principle(s) will give us insight into the development of a new therapeutics having antioxidant actions.

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