

Characterization of anti-oxidative effects of Mori Cortex Radicis

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SUMMARY

We tested to determine if Mori Cortex Radicis extract has antioxidant activities and its potential mechanism of action was explored. Anti-oxidative effects were tested by measuring free radical and nitric Oxide (NO) scavenging activity, and reducing power. Since iNOS and COX-2 are important enzymes responsible for the production of free radicals in the cell, Mori Cortex Radicis extract was tested as to whether it could inhibit iNOS and COX-2 expression in LPS stimulated Raw cells. 70% methanolic extract of Mori Cortex Radicis exerted significant DPPH free radical and NO scavenging activities. In addition, the Mori Cortex Radicis extract exerted dramatic reducing power with maximal activity observed at 1 mg/ml (11-fold over control). Production of iNOS induced by LPS was significantly inhibited by the Mori Cortex Radicis extract, suggesting it could inhibit NO production by suppressing iNOS expression. COX-2 induced by LPS was also significantly inhibited by the Mori Cortex Radicis extract. The extract contains well known antioxidant components including phenolics, flavonoids and anthocyanin at the concentration of 0.23 mg/g, 42.97 mg/g and 12.08 mg/g, respectively. These results suggest that 70% methanolic extract of Mori Cortex Radicis exerts significant anti-oxidant activity via inhibiting iNOS and COX-2 induction.

Key words: Mori cortex radicis; free radical; iNOS; NO; COX-2

INTRODUCTION

Mori Cortex Radicis has been widely used as an herbal folk medicine in oriental countries. It is derived from the root bark of *Morus alba* L. It has been found that it contains prenylflavone including mulberrin and a number of other compounds such as scopoletin, β -tocopherol, umbelliferone, and 5,7-dihydroxychromone. In addition, moran A, isolated from the root of Mori cortex radicis, has hypoglycemic action. Morasan, an interferon-inducing polysac-

charide, is also identified in the root (Tang and Esisenbrand, 1992). A variety of pharmacological effects have been suggested. It has been used for the control of inflammation, diabetes and bronchial asthma (Nanba, 1981). In addition, it has been reported that it could exert hypotensive activity (Nomura, 1988), antiviral activity (Ishitsuka *et al.*, 1982), inhibit cAMP phosphodiesterase activity (Nikaido *et al.*, 1984), regulate arachidonate metabolism (Kimura *et al.*, 1986) and inhibit histamine release from mast cells (Chai *et al.*, 1999). Interestingly, its anti-tumor activity has been reported (Nomura *et al.*, 1988). Anti-cancer action of the Mori Cortex Radicis was also reported (Nam *et al.*, 2002).

Reactive oxygen species (ROS) and nitric oxide

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(NO) induce oxidative stress which has been implicated in aging process and various diseases such as diabetes, cancer, neurodegenerative diseases and inflammation. iNOS is a critical enzyme responsible for the production of nitric oxide (NO) and it play an important role in oxidative stress-induced diseases and inflammation; it is well known that COX-2 (cyclooxygenase-2) takes part in inflammation processes. Recently, it has been suggested that iNOS is essential in mediating bone development and P. gingivalis-induced alveolar bone loss (Gyurko *et al.*, 2005). Interestingly, it has been claimed that COX-2 is involved in osteoclast formation and bone destruction (Ono *et al.*, 2002). In this study, we explored to determine if Mori Cortex Radicis extract has anti-oxidative activities and explored whether iNOS and COX 2 are involved in anti-oxidative actions exerted by Mori Cortex Radicis.

MATERIALS AND METHODS

Preparation of plant extracts

Authentic samples of Mori Cortex Radicis 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. 00-15) was deposited at the herbarium of the Department of Pharmacology, School of Dentistry, Kyung Hee University, Seoul, South Korea. Mori Cortex Radicis (100 g) was cut into small pieces and extracted with 70% methanol (300 mL) for 3 hrs three times. The resulting methanol extract was concentrated by rotary evaporator and dried by freeze-dryer.

Reagents and materials

The iNOS and COX-2 antibodies were purchased from Cell Signaling (Danvers, MA, USA) and Santa Cruz Biotechnology Co. (Santa Cruz, CA, USA), respectively. The ECL kit was purchased from Amersham Co. (Piscataway, NJ, USA) All other

reagents were purchased from Sigma Co. (St. Louis, MO, USA). Cell culture media were purchased from Gibco Co. (Grand Island, NY, USA).

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 antioxidants at 515 nm (Brand-Williams, 1995).

Reducing power

The reducing power was measured at 700 nm using the Oyaizu's method (1988).

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 CO₂ atmosphere at 37°C.

Measurement of Nitric Oxide (NO)

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 Chemical Co., St. Louis, MO, USA). 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 hrs and the samples were made into the final concentrations of 1.0, 0.1, 0.01 mg/ml for experiments. Four hours later, 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-2 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 Mori Cortex Radicis extracts and cultured for 24 h. The cells were washed two times with PBS and disrupted by passing them through a 1-ml tuberculin syringe five times. The cell lysate was subjected to Western blot analysis. Protein (50 mg/lane) was electrophoretically separated in 10% polyacrylamide gels containing SDS. Proteins were transferred to nitrocellulose membranes (Schleicher & Schuell) was carried out for 1 hr 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 (1:1,000), COX-2 (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 1:2,000 for COX-2) 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, Waltham, MA, USA). 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-Ciocalteu 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 Miliauskas *et al.* (2004) and was expressed as the rutin equivalent (mg rutin acid/g extract) using rutin as a standard flavonoid. 1 ml of Mori Cortex Radicis extract was mixed with aluminum thichloride in ethanol (20 mg/ml) and diluted to 25 ml. After incubation for 40 min at 20, the optical density was measured at 415 nm.

Measurement of total anthocyanin

The total anthocyanin was measured using color reactions (Piccolella *et al.*, 2008). Mori Cortex Radicis 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 kouromanin equivalent (mg Kouromanin/g extract).

Statistical analysis

All data were expressed as mean \pm S.E.M. Statistical analysis was performed using the GraphPad Prism 4 (GraphPad Software, La Jolla, CA, USA) 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 22% at 0.01 mg/ml of Mori Cortex Radicis extract

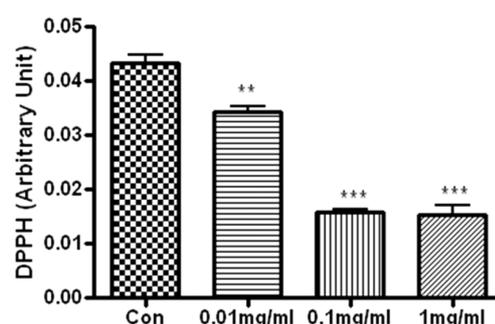


Fig. 1. DPPH Scavenging Activity by Mori Cortex Radicis extract. DPPH scavenging activity of Mori Cortex Radicis extract was measured as described in the Methods. All the measured values were shown as means \pm S.E.M and showed significant differences from those of the control group at the levels of ** $P < 0.01$ and *** $P < 0.001$.

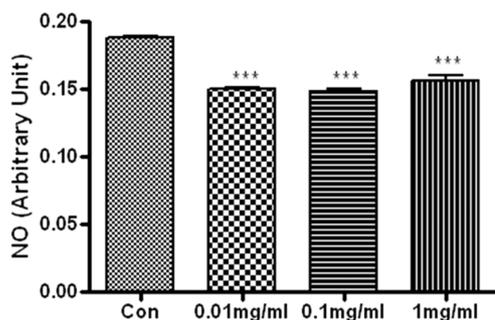


Fig. 2. NO scavenging Activity by Mori Cortex Radicis extract. NO scavenging activity of Mori Cortex Radicis extract was measured as described in the Methods. All the measured values were shown as means \pm S.E.M and showed significant differences from those of the control group at the levels of $***P < 0.001$.

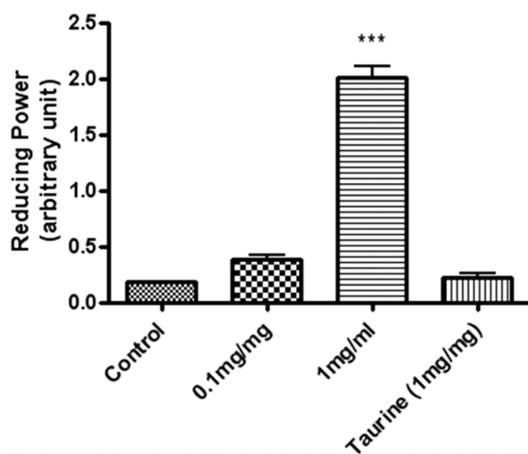


Fig. 3. Reducing Power by Mori Cortex Radicis extract. The reducing power of Mori Cortex Radicis extract was measured by the Oyaizu's method as described in the Methods. All the measured values were shown as means \pm S.E.M and showed significant differences from those of the control group at the level of $***P < 0.001$.

and about 64% at 0.1 mg/ml as compared to the control. At 1.0 mg/ml, the DPPH free radicals decreased by around 65% (Fig. 1). Mori Cortex Radicis extract also decreased level of NO by 20%, 21% and 17% at 0.01 mg/ml, 0.1 mg/ml and 1 mg/ml, respectively (Fig. 2). Regarding reducing power of Mori Cortex Radicis extracts, it stimulated the reducing power by 1.1-fold and 11.1-fold as compared

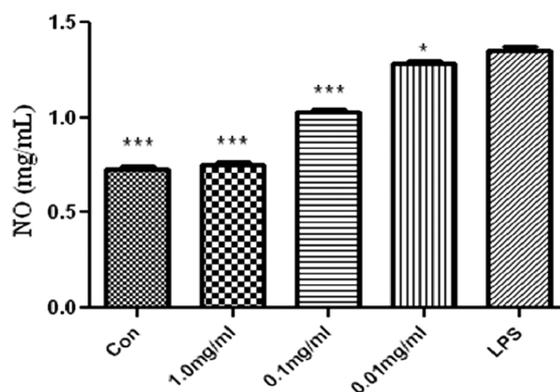


Fig. 4. Inhibition of NO production by Mori Cortex Radicis extract in Raw cells. Raw 264.7 cells were pretreated with Mori Cortex Radicis extract at the concentrations of 0.01, 0.1 and 1.0 mg/ml for 4 h and then treated with LPS 10 mg/ml for 18 - 24 h and the amount of NO was measured using the Greiss reagent. All the measured values were shown as means \pm S.E.M and showed significant differences from those of the control group at the levels of $*P < 0.05$ and $***P < 0.001$.

to control at 0.1 mg/ml and 1 mg/ml, respectively (Fig. 3). Its reducing power appeared 11.8-fold potent as compared to taurine at 1 mg/ml. The amount of nitric oxide production markedly increased (1.9-times over basal) when the Raw cells were treated with LPS (lipopolysaccharide) to activate the macrophages whereas when the cells were pretreated with Mori Cortex Radicis extract, NO production was significantly decreased by 11%, 52% and 96% in response to 0.01 mg/ml, 0.1 mg/ml and 1 mg/ml extract, respectively, as compared to the control (Fig. 4).

To elucidate mechanisms of antioxidant actions by Mori Cortex Radicis 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 6.7-fold over basal. On the other hand, when the cells were pretreated with Mori Cortex Radicis extract, iNOS expression levels were markedly decreased by 30%, 68% and 99% in response to 0.01 mg/ml, 0.1 mg/ml and 1 mg/ml, respectively (Fig.

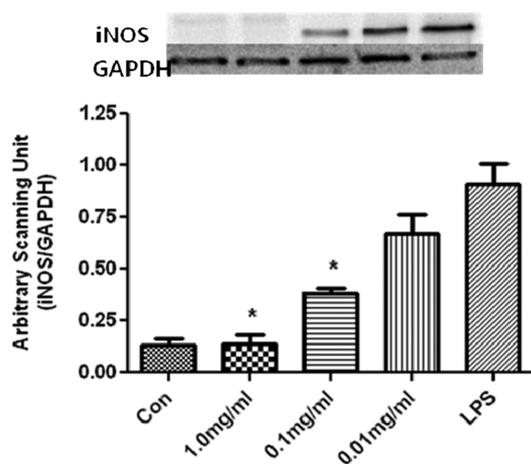


Fig. 5. Effect of Mori Cortex Radicis extract on LPS-induced iNOS induction. Raw 264.7 cells were pretreated with Mori Cortex Radicis extract at the concentrations of 0.01, 0.1 and 1.0 mg/ml for four hours and then treated with LPS 10 mg/ml for 18 - 24 h and then Western blot analysis with a specific 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). * $P < 0.05$.

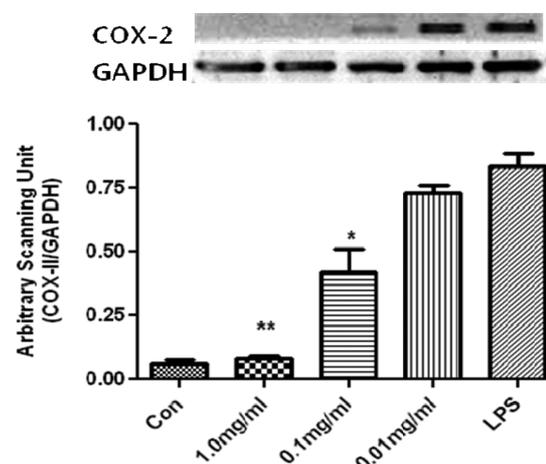


Fig. 6. Effect of Mori Cortex Radicis extract on LPS-induced COX-2 induction. Raw 264.7 cells were pretreated with Mori Cortex Radicis extract at the concentrations of 0.01, 0.1 and 1.0 mg/ml for four hours and then treated with LPS 10 mg/ml for 18 - 24 h and then Western blot analysis with a specific 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-2 to GAPDH (A). The data shown are the mean values \pm SEM of three experiments. * $P < 0.05$ and ** $P < 0.01$.

5). COX-2 was also explored to see if it is a target of Mori Cortex Radicis extract. When LPS was administered, the expression of the COX-2 enzyme was induced by 13.5-fold over basal. Mori Cortex Radicis extract dramatically inhibited the LPS induced COX-2 production by 13%, 54% and 98% at 0.01 mg/ml, 0.1 mg/ml and 1 mg/ml, respectively (Fig. 6).

When antioxidant components present in the Mori Cortex Radicis extract was analyzed, total phenolics, total flavonoids and total anthocyanin were determined as 0.23 mg/g, 42.97 mg/g and 12.08 mg/g, respectively (Table 1).

Table 1. Component analysis of Mori Cortex Radicis extract

Component	Concentration (mg/g)
Total flavonoids (mg rutin/g extract)	42.97 \pm 2.24
Total Phenolics (mg gallic acid/g extract)	0.23 \pm 0.02
Anthocyanin (mg kuromanin/g extract)	12.08 \pm 0.02

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

DISCUSSION

Oxidative stress plays essential roles in the development of many diseases such as diabetes, Parkinson's disease, cardiovascular diseases and periodontal diseases. In the present study, we provided evidence that the Mori Cortex Radicis extract exerts anti-oxidative actions and its potential anti-oxidative mechanism via inhibition of iNOS and COX-2 induction is proposed.

Oxygen free radicals are produced by various

environmental factors, such as air pollution, smoking, stress and intensive exercises and they play an essential role in damaging lipid, proteins and nucleic acid, thereby promoting various kinds of diseases (Kim *et al.*, 1997; Evereklioglu *et al.*, 2003).

The DPPH free radical nitric oxide scavenging actions of Mori Cortex Radicis extract are clearly demonstrated in the present study. Moreover, it showed approximately 13 times potent reducing power as compared to control. These results strongly suggest that Mori Cortex Radicis extract could have the ability to remove various kinds of free radicals generated due to oxidative stress. In addition, the marked reducing power will be of great benefit in removing toxic peroxides generated by oxidizing reactions.

Decreasing of nitric oxide levels in the synovial fluid of patients with chronic temporomandibular diseases has been implicated in reducing pain and tissue damages (Wahl *et al.*, 2003). Considering that oxidative stress could affect the progress of periodontal diseases, antioxidant substances such as flavonoids or vitamin C could suppress such progresses (Tomofuji *et al.*, 2009; Sanbe *et al.*, 2007; Canakci *et al.*, 2006). Nitric oxide (NO) is a key player in the inflammatory responses in vivo and it is generated by the iNOS enzyme that is induced by inflammation. Both iNOS and COX-II are essential players for osteoclast formation and bone loss (Ono *et al.*, 2002; Gyurko *et al.*, 2005). The Mori Cortex Radicis extract significantly reduce NO generated by LPS in the Raw cells with dramatic inhibition of iNOS expression. COX-2 is also affected by Mori Cortex Radicis extract. The Mori Cortex Radicis extract contains significant amount of phenolics including anthocyanins and flavonoids, suggesting that they could be major contributors to the iNOS and COX-2 inhibitory action of Mori Cortex Radicis.

These results suggest that the antioxidative action of Mori Cortex Radicis extract is mediated by the inhibition of iNOS-NO system as well as COX-2

enzyme. Taken together, anti-oxidative actions of Mori Cortex Radicis extract could be useful in the prevention and treatment of various inflammatory diseases including chronic temporomandibular diseases.

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