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Research Report

Antioxidant and Nitric Oxide Release Inhibition Activities of Methanolic Extract from *Clerodendrum cyrtophyllum* Turcz

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Abstract. Antioxidant and anti-inflammatory activities of methanolic extract of *Clerodendrum cyrtophyllum* Turcz (ECT) were investigated in the present study. ECT showed high phenolic content (223 μg·GAE^{-1} equivalent) and strong 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging (IC_{50} = 52.74 μg·mL^{-1}) and reduced power ability. Furthermore, ECT exhibited significant protective ability in H_2O_2/Fe^{3+}/ascorbic acid-induced DNA and protein damage. Moreover, ECT inhibited NO production by 47.15% at 0.8 mg·mL^{-1} in lipopolysaccharides-stimulated RAW264.7 macrophages. Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis showed that the expression of iNOS and COX-2 in RAW264.7 cells was down-regulated after treatment with increasing concentration of ECT. In conclusion, ECT has shown potent antioxidant and anti-inflammatory activities could be considered as a source of health promoting antioxidant and complementary medicinal supplement.

Additional key words: 1,1-diphenyl-2-picrylhydrazyl (DPPH), anti-inflammatory, DNA damage, protein damage, semi-quantitative RT-PCR

Introduction

Reactive oxygen species (ROS) has been considered to be a major cause of oxidative damage to biological molecules in the human body (Song et al., 2006). The oxidative stress can damage cellular substances including DNA, membrane lipids and proteins. Many diseases such as cardiovascular disease, arthritis, Alzheimer's, Parkinson's, diabetes mellitus, gastric ulcers, and cancer are related to the oxidative stress (Je et al., 2009). Although most organisms possess antioxidant defense and repair systems but their functions are limited especially during oxidative stress. Antioxidants have diverse functions in biological systems as defense against oxidative damage and also participate in the major signaling pathways of the cell (Scandalios, 2005). Several synthetic antioxidants like butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and *tert*-butylhydroquinone (TBHQ) are commercially available, however, their applications has been restricted due to potential health risks (Wanasundara and Shahidi,1998). Therefore, recently, people put more attention on natural antioxidants. Inflammation is the sequelae of pain (Osadebe and Okoye, 2003) and that are related to the expression of proinflammatory mediators (Yoo et al., 2008). Pro-inflammatory cytokines activates the COX-2 pathways leading to inflammation and pain that are controlled by the activity of iNOS (Shu et al., 2006). Reactive oxygen and nitrogen species attack on some outstanding biological molecular, including proteins, polyunsaturated fatty acids, and DNA (SchÖneich, 1999). This study showed that ECT can provide some protection to reduce these risks.

Clerodendrum cyrtophyllum Turcz belongs to Verbenaceae family, have been cultivating for centuries in Asia which have the ability to remove heat and toxic material produced during enteritis (Li et al., 2008). *C. cyrtophyllum* Turcz has potent anti-inflammatory activity, which was reported by Zhang et al. (1993), and that has been used against painful conditions. Therefore, the present study was designed to evaluate the potential characteristic effects of *C. cyrtophyllum* Turcz and its application in pharmaceutical industry. ECT could be considered as a potential food resource and complementary medicine.

Materials and Methods

Preparation of Extracts

C. cyrtophyllum Turcz were obtained from China and dried in the shade at room temperature. Two hundred grams of extract was obtained from the entire plant with absolute methanol at 60℃ for 3 hr. The extract was filtered and evaporated under reduced pressure using a vacuum rotary evaporator and lyophilized. The dried samples were weighed and kept at -20℃ in a refrigerator until further analysis.

Chemicals

DPPH, butyrated hydroxyanisole (BHA), Folin-Ciocalteu reagent, ethylenediamine tetraacetic acid (EDTA), 3-(2-pyridyl) -5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine (feerozine), tannic acid, quercetin, ascorbic acid, and lipopolysaccharides (LPS) were purchased from Sigma-Aldrich (St. Louis, MO). These chemicals were analytical and HPLC grades.

Determination of the Total Phenolic Content

An aliquot of each sample (1 mL) was mixed with 1.8 mL of 50% Folin-Ciocalteu reagent. The mixture was then allowed to stand at 25℃ for 5 min before the addition of 1.2 mL of 15% sodium carbonate solution. Tannic acid was used as the standard to create a calibration curve. The total phenolic content was expressed as tannic acid $mg \cdot g^{-1}$. The absorbance at 765 nm was measured (Shimadzu UV-1601, Tokyo, Japan).

Determination of total Flavonoid Concentration

0.5 mL solution of extract ($1 mg \cdot mL^{-1}$) was aliquoted and was added to the test tubes containing 0.1 mL of 10% aluminiumchloride hexahydrate, 0.1 mL of 1 M potassium acetate, 2.8 mL of deionized water and 1.5 mL 95% ethanol. The mixture was held for 40 min at room temperature and the absorbance was determined at 415 nm (Shimadzu UV-1601, Tokyo, Japan). Quercetin was used as a standard. Quercetin is a type of plant-based chemical, or phytochemical, known as a flavonoid. The concentrations of flavonoid compounds were calculated using a standard curve.

DPPH free Radical Scavenging Activity

Briefly, 0.5 mL of 0.1 mM DPPH in methanol was added to the test tube containing 0.5 mL of the extract at various concentrations (12.5-200 $\mu g \cdot mL^{-1}$). The mixture was then shaken vigorously for 1 min and kept at room temperature for 30 min in the dark. The absorbance of each sample solution was subsequently measured at 515 nm with plate reader (Bio-Tek, Winooski, VT). The extract concentration that could scavenge 50% of the DPPH radicals (EC_{50}) was calculated from the plot of scavenging activity against the concentration of extract. The ability to scavenge DPPH radicals was calculated using the following equation: $I (\%) = [1-(A_i - A_j) /A_c] \times 100\%$. In the equation, Ac is the absorbance of the DPPH solution without sample (0.5 mL DPPH solution + 0.5 mL of ethanol), A_i is the absorbance of the test sample mixed with DPPH solution (0.5 mL sample + 0.5 mL DPPH solution) and A_j is the absorbance of the sample without DPPH solution (0.5 mL sample + 0.5 mL methanol).

Ferric-ion Reducing Power Assay

The method described by Nandita and Rajini (2004) was used to determine the reducing power of the extracts. Total 1 mL aliquot of the extract ($300 \mu g \cdot mL^{-1}$), 2.5 mL of 0.2 M phosphate buffer pH 6.6 and 2.5 mL of 1% (w/v) $K_3Fe(CN)_6$ were added and incubated at 50℃ for 30 min. Further, 10 % (w/v) trichloroacetic acid (2.5 mL) was added to the mixture and was centrifuged at 3000 rpm for 10 min. 2.5 mL of the supernatant was collected, then added to 2.5 mL of distilled water and 0.5 mL of 0.1 % (w/v) $FeCl_3$ solution. The absorbance was measured at 700 nm. Ascorbic acid was determined as positive control.

Ferrous-ion Chelating Activity

Ferrous ion chelating capacity was measured according to Dinis et al. (1994). Sample solution (0.1 mL) along with 0.1 mL of 0.2 mM $FeCl_2 \cdot 4H_2O$ was added to 3 mL of deionized water and then was stored at room temperature for 30 s. Later on, the reaction mixture was added with 5 mM ferrozine (0.1 mL). The mixture was kept at room temperature for 10 min. The Fe^{2+}-ferrozine complex formation was observed by monitoring the absorbance at 562 nm (Shimadzu UV-1601, Tokyo, Japan). Ethylenediaminetetraacetic acid (EDTA) was used as a positive control.

Genomic DNA Isolation

Genomic DNA was isolated from HEK 293 cells as per the method described by Li et al. (2010). Cells were harvested, washed with PBS and the pelleted cells were lysed further in in extraction buffer (1 M Tris-HCl, 0.5 M EDTA, 5 M NaCl and 20% sodium dodecylsulfate, SDS) at 64℃ for 10 min. The DNA sample was added with phenol:chloroform:isoamylalcohol (25:24:1) before centrifugation at 13000 rpm for 15 min for 3 times. DNA was diluted with distilled water after washed by 70% ethanol. After centrifugation, the supernatant was collected and was incubated with isopropanol and was centrifuged at 13000 rpm for 10 min. Finally, DNA was collected after 70% ethanol wash.

DNA Damage Protection Assay

Mixture of 3 μL of phosphate buffer (pH 7.4), 0.4 μg genomic DNA isolated from HEK 293 cells, 10 μL of extract ($250, 500, 1{,}000 \mu g \cdot mL^{-1}$), and 3 μL of 2 mM $FeSO_4$ was

made. 4 μL of 30% H_2O_2 was added to this mixture and was incubated at 37℃ for 30 min. Finally, 10 μL of the mixture was separated by 1% agarose gel electrophoresis. Densitometric analysis was done by software (Quantity one; Bio-rad, Hercules, CA).

Protein Damage Protection Assay

Protein damage was measured using the method of Hu et al. (2009). BSA was oxidized by a H_2O_2/Fe^{3+}/ascorbic acid system. The reaction mixture (1.0 mL) containing sample, phosphate buffer (20 mM pH 7.4), BSA (1 mg·mL^{-1}), $FeCl_3$ (50 μM), H_2O_2 (1 mM) and ascorbic acid (100 μM) were incubated for 3 hr at 37℃. After incubation, the reaction mixture was analyzed by electrophoresis in 10% SDS polyacrylamide gel. The gel was stained for 2 hr with brilliant blue R, distained later and then photographed.

Cell Line and Cell Culture

RAW 264.7 cell line was purchased from the Korean Cell Line Bank (Seoul, Korea). RAW 264.7 cells were grown in RPMI 1640 medium supplemented with 10% FBS, 100 U· L^{-1} penicillin and 100 μg·mL^{-1} streptomycin. Cells were incubated at 37℃ in a humidified atmosphere of 95% air and 5% CO_2.

Measurement of NO Inhibitory Activity

RAW 264.7 cells (1×10^6) were plated in 96-well cell plate and stimulated with LPS (2 μg·mL^{-1}) in the presence or absence of 0.2, 0.4, and 0.8 mg·mL^{-1} of ECT for 24 hr. 100 μL of cell culture medium were mixed with 100 μL Griess reagent. The absorbance was measured with a spectrophotometer (Bio-Tek; Winooski, VT) at 550 nm.

Semi-quantitative RT-PCR Analysis iNOS and COX-2 Expression

RAW 264.7 cells (1×10^6) were plated in 6-well cell plate and stimulated with LPS (2 μg·mL^{-1}) in the presence or absence of 250, 500, and 1,000 μg·mL^{-1} of ECT for 6 hr. Total RNA was then isolated from the cells using a Trizol RNA isolation kit (Invitrogen, Carlsbad, CA). Subsequently, one μg of the RNA was reverse-transcribed to cDNA and used as the template for PCR amplification. The products were separated by 1% agarose gel electrophoresis. The gels were stained with ethidium bromide and photographed by a Mini BIS image analysis system (DNR Bio-Imaging Systems Ltd., Jerusalem, Israel).

Statistical Analysis

All experiments were conducted in independent triplicate (n = 3) and data were expressed as the mean ± standard deviation (SD). All analyses were performed using SPSS 11.5 (SPSS Institute, Cary, NC). The level of significance was set at less than 5%.

Results and Discussion

Total Phenolic and Flavonoid Contents

Phenolic compounds play an important role in varieties of medicinal applications (Shetty, 2004). Phenolics also show properties such as antihepatotoxic, antitumor and antimicrobial in several health activities (Luthria, 2008). In plants, phenolic antioxidants are produced as a result of secondary metabolism and are good sources of natural antioxidants in human diets (Chun et al., 2005). They are quite powerful in protecting inflammatory and several heart disease conditions (Wang et al., 2008). Phenolic and flavonoids contents of ECT have been expressed as tannic acid and quercetin equivalent, respectively (Table 1). Phenolic content of ECT (223.05 tannic mg·g^{-1} extract) was found to be very high as compared with others species of verbenaceae family. The phenolic content of *Vitex trifolia* L. var. *simplicifolia* Cham fruit in methanolic extracts and aqueous extracts were 0.41 and 1.03 g·100 g^{-1} dry weight, respectively (Cai et al., 2004). The phenolic content of *Gmelina arborea* Roxb. and *Dremna herbacea* Roxb. were 8.8 and 9.9 mg·g^{-1} on dry weight basis, respectively. However, the content of flavonoid of ECT was very low (0.50 quercetin mg·g^{-1} extract). ECT has more potential as an antioxidant compound most due to the high phenolic content. Total phenolic content was observed to be higher in *Clerodendrum myricoides* leaves (0.2 mg·g^{-1}) as compared to its stems (0.1 mg·g^{-1}) when catechin as equivalents per gram of dry matter (Aremu et al., 2010).

Table 1. Total phenolic and flavonoid contents, and DPPH radical scavenging activity of *Clerodendrum cyrtophyllum* Turcz.

	Total phenolic (tannic mg·g^{-1})	Total flavonoid (quercetin mg·g^{-1})	DPPH (IC50:mg·mL^{-1})	Reducing power[z]
ECT	223.05 ± 3.99[y]	0.50 ± 0.47	52.74 ± 2.70	0.449 ± 0.01
Ascorbic acid	-	-	6.99 ± 0.19	1.18 ± 0.00
BHA	-	-	9.09 ± 0.87	-

[z]Reducing power assay: the absorbance was measured at 700 nm as the concentration was 300 μg·mL^{-1}.
[y]Each value is expressed as mean ± SD (n = 3).

DPPH Radical Scavenging Activity Assay

DPPH has been described as a stable free radical and that accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Soares et al., 1997). DPPH has been often used as a substrate to evaluate antioxidative activity of antioxidants (Duh et al., 1999) and therefore its scavenging ability was determined by decrease in its absorbance at 517 nm. The scavenging effect of ECT and standards on the DPPH radical was shown in Table 1. The IC_{50} values of ECT and positive controls (ascorbic acid and BHA) were 52.74, 6.99, and 9.09 μg·mL^{-1}, respectively. The results mean ECT may act as a DPPH radical scavenger for further research.

Reducing Power Assay

For measuring the reductive ability, we investigated Fe^{3+}/Fe^{2+} transformation in the presence of ECT. Iron (III) was widely used to measure ferric-reducing antioxidant power as it has role in the measurement of total antioxidant capacity area (Berker et al., 2007). The reductive capabilities of ECT and ascorbic acid were shown in Table 1. The reducing power ability of ECT was found to be lower than positive control (ascorbic acid). Reducing power ability of leaf ethanol extract of *Clerodendrum infortunatum* L was observed to be stronger than ECT (Gouthamchandra et al., 2010).

Ferrous-ion Chelating

Iron has been described as a fundamental metal that helps the brain in neural functioning and has pertinent role in the protection of neural cells in various neurodegenerative diseases (Armstrong et al., 2001). Iron has important role to play in oxygen free radical transition (Mandel et al., 2007). Protein transport in the presence of higher levels of iron causes accumulation and toxicity of metal ions as it gets bounded by proteins. So, in order to facilitate the protein transport, iron needs to be chelated (Martell et al., 1999). In our study, potentiality of metal chelation by ECT was increased with the increasing concentration of ECT from 0.25 mg·mL^{-1} to 2 mg·mL^{-1}, indicating the potentiality of ECT for health.

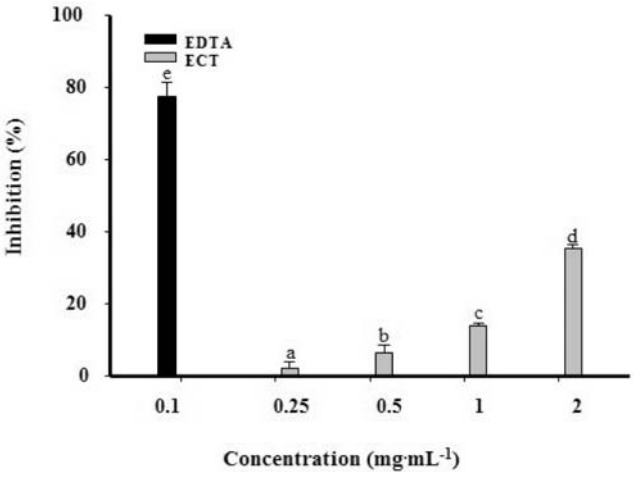

Fig. 1. Iron chelating activity of *Clerodendrum cyrtophyllum* Turcz. EDTA was used as the positive control. Values in the same column are significantly different by Duncan's multiple range test ($P < 0.05$).

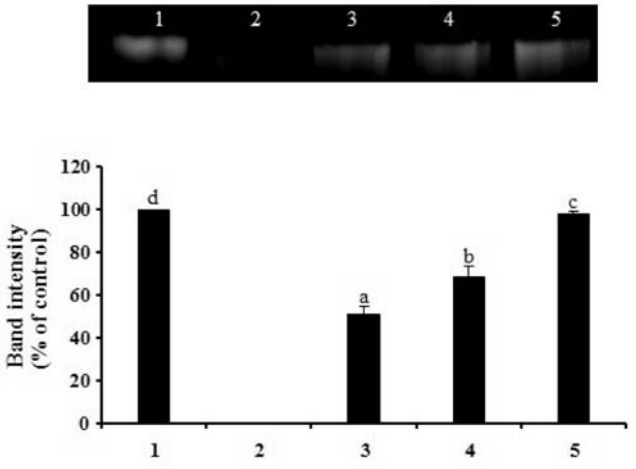

Fig. 2. Protective qualities against free radical-induced DNA damage by densitometric measurement. Line 1, DNA treated without Fenton's reagent; Line 2, DNA treated with Fenton's reagent; Line 3, 4, and 5, DNA incubated with Fenton's reagent after treatment with different concentration of *Clerodendrum cyrtophyllum* Turcz as 0.25, 0.5, 1 mg·mL^{-1}, respectively. Values in the same column are significantly different by Duncan's multiple range test ($P < 0.05$).

Protective Qualities against Free Radical-induced DNA Damage

DNA damage effect the stability of genome and the normal life cycle of the cell during development, growth, differentiation, and maintenance. Cell cycle regulation, repair pathways, and cell death of variety mechanisms has been related to DNA damage (Barzilai et al., 2008). ECT protected DNA in a dose-depended manner (Fig. 2) in our experiments. ECT (1 mg·mL^{-1}) showed 98.1% protection of DNA as compared to the one which DNA was damaged by free radicals. The results in our study suggested that ECT possess the ability to protect DNA from injury generated by free radicals.

Protein Damage Protection Assay

The oxidative damage of protein causes protein dysfunction and that influences the functions of enzymes, receptors, and membrane transporters (Mayo et al., 2003). The protein protection abilities were investigated by induced oxidation of bovine serum albumin (BSA). As shown in Fig. 3, BSA was destroyed when incubated with free radical-induced. In our study, protein was protected from damage about 40.3% after treated by ECT of 0.5 mg·mL^{-1}.

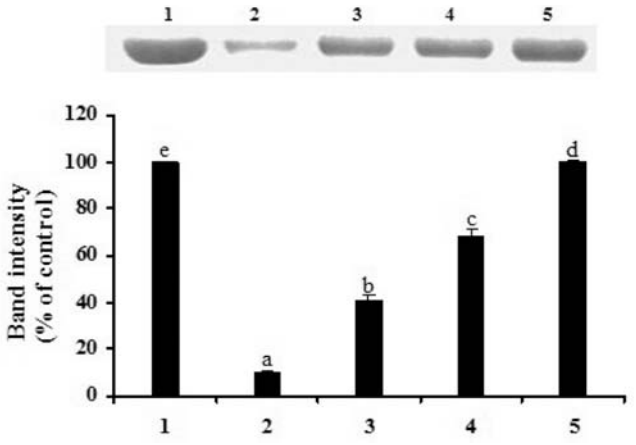

Fig. 3. Protein damage protection assay. Line 1, protein (BSA) treated without free radical-induced; Line 2, protein incubated with free radical-induced; Line 3, 4, and 5, protein treated with free radical-induced after treatment with different concentration of *Clerodendrum cyrtophyllum* Turcz as 0.5, 1, 2 mg·mL^{-1} respectively. Values in the same column are significantly different by Duncan's multiple range test (P < 0.05).

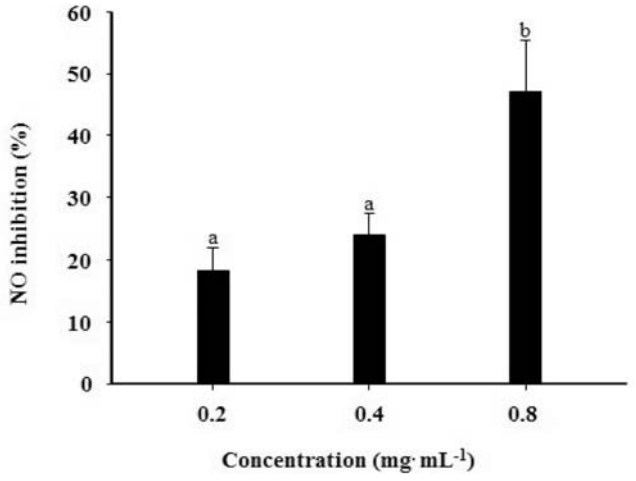

Fig. 4. NO inhibition of *Clerodendrum cyrtophyllum* Turcz on LPS-simulated RAW 264.7 cells. RAW 264.7 was treated with LPS of 2 μg·mL^{-1} and incubated at 37°C for 24 hr. Values in the same column are significantly different by Duncan's multiple range test (P < 0.05).

Inhibition of Nitrite Production

Nitric oxide has effect on the immune system and the functions of a variety cells. The activation of macrophages releases many inflammatory mediators, such as cytokines and NO. Over-induction of NO leads to various harmful responses including tissue injury, septic shock (Xie et al., 1992), apoptosis, and necrosis (Nagai et al., 2003). The inhibition of NO production was measured in LPS induced in RAW 264.7 cells (Fig. 4). The result indicated that ECT inhibited the activity of NO production in a dose-dependent manner.

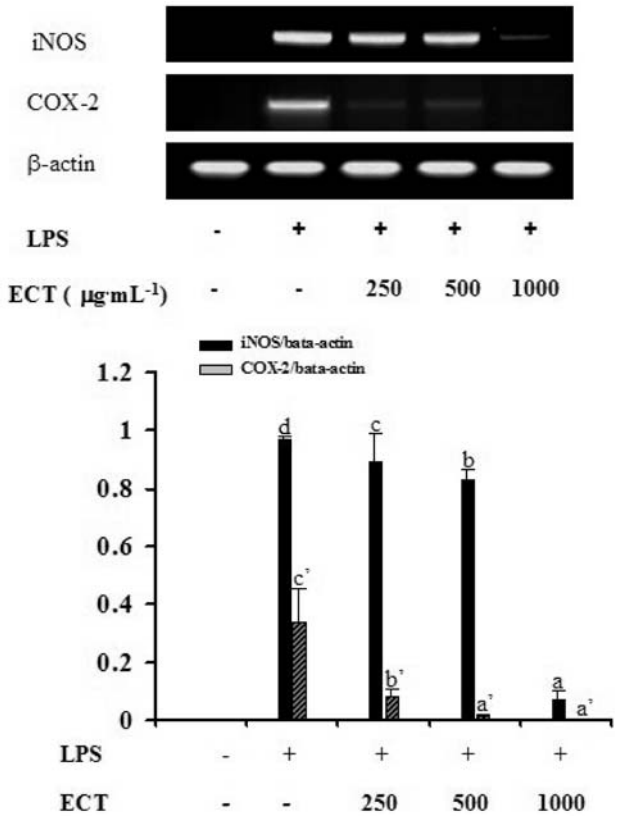

Fig. 5. RT-PCR analysis of COX-2 and iNOS mRNA level after treatment by *Clerodendrum cyrtophyllum* Turcz on LPS-simulated (2 μg·mL^{-1}) RAW 264.7 cells. GAPDH was used as control. Significant gender analysis between the black and gray bar diagram was in progress respectively. Values in the same column are significantly different by Duncan's multiple range test (P < 0.05).

Effects of ECT on iNOS and COX-2 mRNA Expression Level

Since ECT has been known to inhibit the release of nitrite oxide, we investigated whether the decrease in NO production was relative to iNOS and COX-2 expression at mRNA level. The RT-PCR results showed that ECT suppressed the expression of iNOS and COX-2 induced by LPS in RAW264.7 macrophage cells in a dose-dependent manner. These findings revealed that ECT inhibited the NO release by down-regulating the iNOS and COX-2 expression (Fig. 5).

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