

# Anti-inflammatory Effects of Extract from *Eucommia ulmoides* Oliv. Leaves on Macrophage Cells

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**Abstract** This study investigated the anti-inflammatory effects of *Eucommia ulmoides* Oliv. (EUO) leaf extracts on RAW264.7 macrophages. Cell toxicity was determined by MTT assay. We evaluated the anti-inflammatory effects of EUO extracts by measuring nitric oxide (NO), inducible nitric oxide synthase (iNOS) production, and cyclooxygenase-2 (COX-2) expression by western blotting. EUO ethanolic extracts (0.1, 1, 5, and 10 µg/mL) significantly suppressed LPS-stimulated production of NO. EUO ethanolic extracts reduced the expression of iNOS and COX-2 proteins. The present results show that EUO ethanol extract has potent anti-inflammatory effects on RAW264.7 macrophages. These results also suggest that the anti-inflammatory effects of EUO extracts may be related to the inhibition of LPS-stimulated NO production. Therefore, ethanolic extracts of EUO leaves may be utilized as a good source of functional food and pharmaceutical applications for protection against inflammatory diseases.

**Keywords:** *Eucommia ulmoides* Oliv., inflammation, macrophage cells, iNOS, COX-2

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## 1. Introduction

Proinflammatory cytokines work in a synergistic manner through a cytokine transcription factor regulatory loop, thereby augmenting the inflammatory response and tissue damage [1]. Nitric oxide (NO) is an important inflammatory mediator produced by NOS under physiological and pathophysiological conditions and downregulation of NO is necessary for the treatment of the latter [2]. The production of NO and prostaglandins by inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), respectively, is considered to be the most prominent molecular mechanism in inflammatory processes [3,4], and is also involved in multistage carcinogenesis, especially at the promotion stage [5]. Excessive and prolonged NO generation caused by overexpression of iNOS has been implicated in inflammatory tumorigenesis, while COX-2-mediated prostaglandin production has been shown to stimulate cell proliferation, invasion, and angiogenesis in cancer development [6].

*Eucommia ulmoides* Oliver (EUO) is one of the oldest known and used tonic herbs in Asia. It is an elm-like deciduous and dioecious tree belonging to only one species, one genus (*Eucommia*) and one family (Eucommiaceae) [7]. The tree EUO belongs to the family Eucommiaceae, its cortex and leaves are traditional oriental medicines [8]. In traditional Chinese medicine, roasted *Eucommia* cortex is utilized to reinforce the muscles and lungs, lower blood pressure, prevent miscarriages, improve the tone of the liver and kidneys

and increase longevity [9,10]. The EUO contain various bioactive substances, including lignans, iridoids, phenols, steroids, triterpenes, organic acids, polysaccharides, flavonoids, amino acids, and microelements [11] as well as nutrients, i.e., amino acids, vitamins and minerals [12].

Our results suggest that EUO extract could have potential applications as a natural anti-inflammation supplement in the food industry.

## 2. Materials and Methods

### 2.1. Materials

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay kit, and LPS from *Escherichia coli* (serotype 0127:B8) were procured from Sigma Aldrich (St. Louis, MO, USA). Tissue culture plates and culture dishes were purchased from Nunc, Inc. (North Aurora Road, IL, USA). The antibodies against COX-2, iNOS, and  $\beta$ -actin were supplied by Cell Signaling (Danvers, MA, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Hyclone (Logan, UT, USA). Reagents for ECL western blot detection were purchased from GE Healthcare Biosciences (Piscataway, NJ). All other reagents were of the highest grade available commercially.

### 2.2. Cell Culture

Mouse macrophage cell line, RAW264.7, was obtained from American Type Culture Collection (ATCC, Rockville,

MD, USA) and cultured in DMEM supplemented with penicillin, streptomycin, and 10% heat-inactivated FBS in 5% CO<sub>2</sub> and 95% humidified air at 37°C.

### 2.3. MTT Assay

For evaluating the cytotoxicity of extracts from EUO (0.1, 1, 5, and 10 µg/mL) with LPS (100 ng/mL), the cells were harvested using phosphate buffered saline (PBS) containing 0.15% trypsin and 0.08% EDTA. Cells were incubated in well plates at a density of  $1 \times 10^5$  cells/well. MTT solution was added to each well. Following incubation for 4 h at 37°C in 5% CO<sub>2</sub>, the supernatant was removed. The medium was removed and the cells were washed by PBS, and the formazan crystals produced in viable cells were solubilized in 200 µL DMSO. The absorbance was measured using a microplate reader (Tecan Trading AG, Männedorf, Switzerland) at 550 nm. All experiments were performed with three replicates [13].

### 2.4. NO Assay

The RAW264.7 cells were plated in a 96-well plate at a density of  $5 \times 10^4$  cells/well in 100 µL of culture medium. Following attachment, cells were pretreated for 30 min with predetermined concentration of extract from EUO (0.1, 1, 5, and 10 µg/mL) and then stimulated with LPS, 100 ng/mL, for 24 h. Following LPS treatment, 50 µL of supernatant from each well was transferred to a corresponding well in another plate containing 50 µL of Griess reagent and incubated for 10 min at room temperature and absorbance measured at 540 nm using a microplate reader (Tecan Trading AG). Experiments were performed in triplicate [14].

### 2.5. Western Blot Analysis

To obtain the total cell lysate, 50 µL of RIPA buffer was added to the changed cells ( $3 \times 10^5$  cells/mL) cultured in 6-well plates. The cells were harvested, incubated for 10 min on ice, and centrifuged at 14,000 g for 10 min at 4°C. The protein concentration was determined using a DC protein assay from Bio-Rad, and 20 µg of whole cell lysate was separated on 12% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE). Electrophoresis was performed and the proteins were transferred to Polyvinylidene difluoride (PVDF) membranes using an electro blotting apparatus. The membranes were blocked for 1 h in a mixture of Tris-Buffered Saline and Tween 20 (TBS-T), containing 0.1% Tween-20 and 5% dry skim milk; the membranes were incubated overnight with primary antibodies (1:1000) followed by incubation for 1 h with horseradish peroxidase-conjugated secondary antibodies (1:1000). The optical densities of the antibody-specific bands were analyzed by a Luminescent Image Analyzer, LAS-3000 (Fuji, Tokyo, Japan) [15].

### 2.6. Statistical Analysis

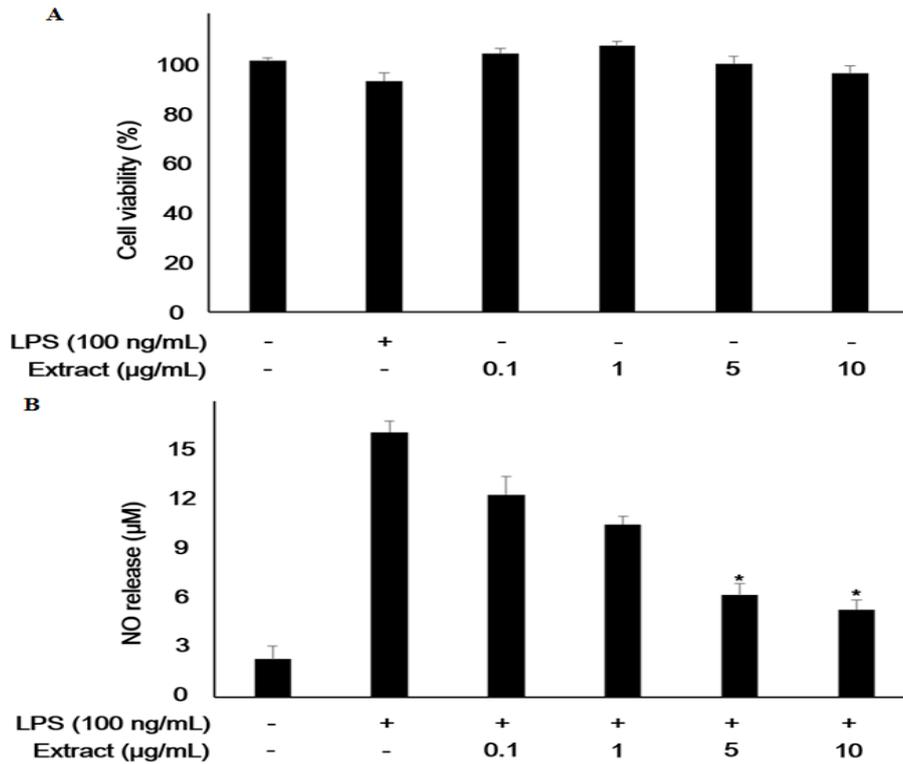
Statistical analyses were performed 3 times for all the experiments. The data are expressed as the mean  $\pm$  one standard error of mean (SEM). Statistical analyses were assessed by Student's *t*-test for paired data. Graph Pad

Prism software version 4.00 (Graph Pad Software Inc., San Diego, CA) was used. Significant differences ( $p < 0.05$ ) between the mean values of the triplicate samples were determined for various assays.

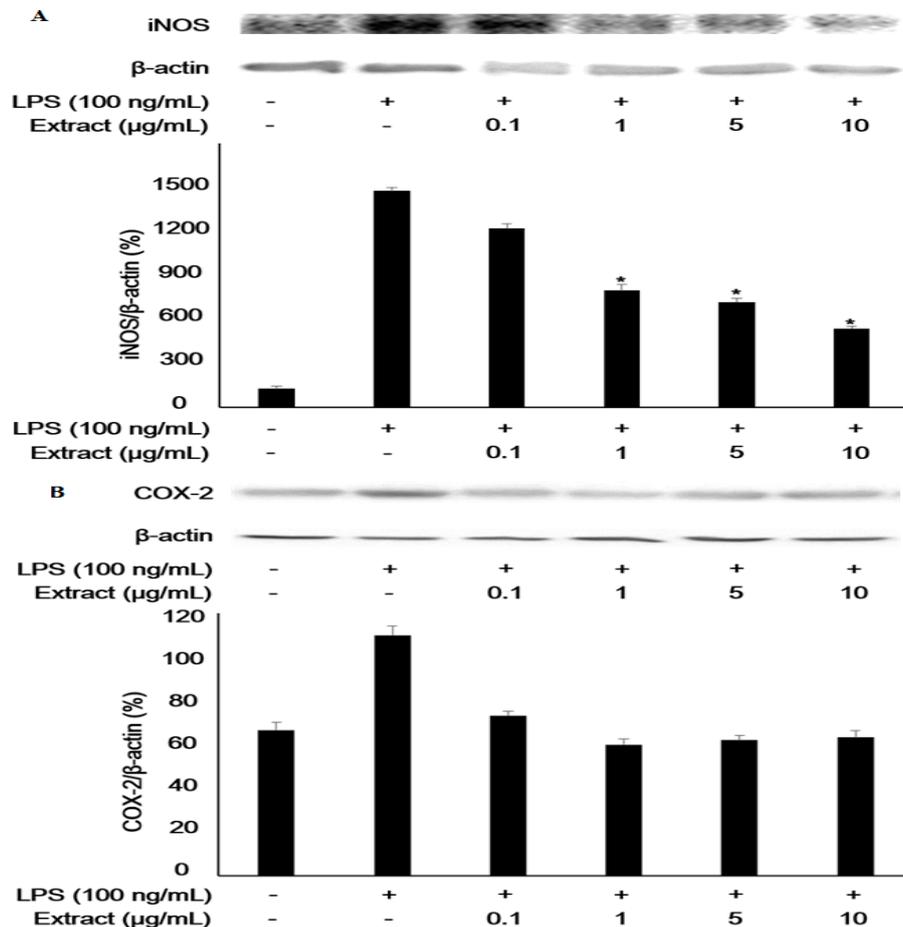
## 3. Results and Discussion

The effect of EUO extract at concentrations of 0.1, 1, 5 and 10 µg/mL on the viability of RAW264.7 cells following exposure for 24 h was examined by performing the MTT assay. As shown in Figure 1A, cell viability was not reduced by various concentration of EUO extract. To investigate whether EUO extract could inhibit LPS-induced NO production, RAW264.7 cells were pretreated for 4 h with various concentration (0.1, 1, 5, and 10 µg/mL) EUO extract followed by stimulation with LPS at 100 ng/mL for 18 h. The level of NO in the culture media was determined using the Griess reagent. Cells treated with LPS alone showed a notable increase in NO production compared to negative control and this upsurge was substantially attenuated in cells that were pretreated with EUO extract (Figure 1B). NO plays a central role in the physiology and pathology of diverse organs including the immune system. Further investigations were carried out to ascertain whether decreased expression of iNOS and COX-2 at the protein level correlated with the EUO extract-mediated suppression of NO production. Western blot analysis of RAW264.7 cells treated as in the NO assay revealed that iNOS (Figure 2A) and COX-2 (Figure 2B) protein levels increased following LPS-stimulation. Moreover, this increase in protein expression was considerably mitigated in the cells pretreated with EUO extract. These results correlate well with the EUO extract-mediated inhibition of NO production in activated macrophages, suggesting that suppression of iNOS and COX-2 at the protein level plays a major role in the anti-inflammatory mechanism of EUO extract.

Excessive and prolonged NO generation caused by overexpression of iNOS has been implicated in inflammatory tumorigenesis, while COX-2-mediated prostaglandin production has been shown to stimulate cell proliferation, invasion, and angiogenesis in cancer development [16]. Early reported that the hypoglycemic activity of EUO extract [17]. Its indicated that EUO could lower hyperglycemia by inhibiting carbohydrate-degrading enzymes and glucose transport during digestion. In addition, another reported that the inhibitory effects on angiotensin converting enzyme (ACE) *in vitro* compared with captopril from EUO extract. Particularly, among active compounds of EUO, (6*R*, 7*E*, 9*R*)-9-hydroxy-4, 7-megastigmadien-3-one-9-O-[ $\alpha$ -L-arabinopyranosyl-(1  $\rightarrow$  6)- $\beta$ -D-glucopyranoside, foliasalacioside B1 and eleganoside A showed the highest anti-hypertensive effects [18]. However, utilization of EUO activities has a little information until now. In the present study, we showed that EUO extract inhibited the production of NO, iNOS, and COX-2 in LPS-stimulated RAW264.7 cells. However, iNOS, COX-2, and pro-inflammatory cytokine expression may be regulated through different pathways in immunoregulatory signaling. Thus, further studies are warranted to elucidate the molecular mechanisms underlying the anti-inflammatory properties of EUO.



**Figure 1.** Effect of extract of EUO and/or LPS on the cell viability of RAW264.7 cells using the MTT assay. RAW264.7 cells were incubated with 0.1, 1, 5, and 10 µg/mL of extract and LPS (100 ng/mL) for 24 h. The results are shown as percentages of control samples (A). Effects of extract of EUO on NO production. RAW264.7 cells were pre-treated with the indicated concentrations of extract (0.1, 1, 5, and 10 µg/mL) for 30 min before incubating with LPS (100 ng/mL) for 24 h (B). Data are presented as the mean ± S.E.M. (n = 3) for three independent experiments



**Figure 2.** Effect of extract of EUO on LPS-induced iNOS (A) and COX-2 (B) protein expression in RAW264.7 cells. Cell lysates were electrophoresed and the protein expression of iNOS and COX-2 was detected by the specific antibody. β-actin was used as an internal control for the western blot analysis. Quantification data are shown in the lower panel. Data are presented as the mean ± S.E.M. (n = 3) for three independent experiments

## 4. Conclusions

The current study demonstrated that EUO extract has an ameliorative effect on the LPS-induced iNOS expression and NO production in activated macrophages. Natural products have been historically used in traditional medicine and are now proving to be a potential source of new drugs and nutraceuticals. In light of this, our study verifying the immunomodulatory activity of EUO extract and the possible underlying mechanism would contribute to the understanding of the biological properties of EUO and its further consideration for therapeutic applications.

## Acknowledgments

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