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EFFECT OF *MORUS ALBA* STEM EXTRACT ON NITRIC OXIDE PRODUCTION IN LIPOPOLYSACCHARIDE-INDUCED RAW 264.7 MACROPHAGE CELLS

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KEYWORDS: *Morus alba* L., Oxyresveratrol, Macrophage 264.7 cell, Nitric oxide, Osteoarthritis

INTRODUCTION

Nitric oxide (NO) is a cellular signaling molecule known to mediate key physiologic processes in host defense, inflammation and immunity. The role of NO in some diseases such as cancer[1] and osteoarthritis (OA)[2] has been reported. For OA, NO has been reported to play an important role in inflammation and pain[3,4]. The significant amount of NO was found to be produced in cartilage obtained from patients with OA[3]. It was shown to mediate the expression of proinflammatory cytokines and inhibit collagen and proteoglycan synthesis, leading to the breakdown of the extracellular matrix[2,4]. It also found to induce chondrocyte apoptosis.

Morus alba L., known as white mulberry, has been widely cultivated in Thailand under support of Queen Sirikit. Oxyresveratrol, the bioactive compound found in *M. alba* extract, has shown to have the anti-oxidant activity with IC_{50} value of 3.6 ± 0.0 μM and 15.1 ± 2.3 μM determined by lipid peroxidation and DPPH assay, respectively[5]. Chung *et al.*[5] reported the anti-inflammatory activity of oxyresveratrol by inhibiting the production of nitric oxide (NO) and prostaglandin (PG) E_2, inducible nitric oxide synthase (iNOS) expression, and NF-κB activation in the LPS-activated RAW 264.7 macrophage cells[5]. Thongsuk[6] found that the amount of oxyresveratrol in various parts of mulberry tree was different and the ethanolic extract obtained from stems has the highest amount of oxyresveratrol compared to the extract obtained from twigs and leaves[6]. Recently, the extract prepared from the stems of *M. alba* was investigated for the efficacy on pain reduction using rat model of OA which was induced in male Wistar rats by anterior cruciate ligament transection (ACLT)[7]. The pain-related behavior was determined using hind limb weight bearing tester. The results showed that MSE significantly attenuated joint pain in dose-dependent manner[7]. The reduction on inflammation and pain may be related to various mediators. The present study was aimed to investigate the effect of *M. alba* stem ethanolic extract (MSE) on NO production in LPS-induced RAW 264.7 cells.

MATERIALS AND METHODS

Plant materials and preparation of plant extract *M. alba* stems were obtained from the Queen Sirikit Sericulture Center (Tak), Tak Province, Thailand. The preparation of MSE was performed as described previously[8]. Briefly, the fresh stems were chopped and dried. Then, the dried plant was extracted by maceration technique using 80% ethanol for 2 cycles at room temperature. After filtration, the filtrate was evaporated under reduced pressure using a rotary evaporator (BUCHI Rotavapor R-114, Switzerland) and continued drying using a water bath (M25 LAUDA, Germany). Then, the dried powder was stored in tight, light-protected container.

Cell culture Mouse macrophage cell line (RAW 267.4) obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagles' medium (DMEM) (HyClone, Logan, UT) supplemented with 10% fetal bovine serum (FBS) (HyClone) and 1% L-glutamine (Biological industries, Kibbutz Beit Haemek, Israel). The cultures were incubated at 37°C under 5% CO_2 atmosphere and were subcultured every 2 days.

Cell viability assay RAW 264.7 cells were seeded in a 6-well plate at a concentration of 1.9×10^5 cell/mL. After overnight incubation, cells were treated with various tested samples in the presence of heat-killed *E. coli* for 24 h. After treatment, cell viability was determined using trypan blue dye exclusion by mixing with trypan blue dye solution (Gibco, Grand Island, NY, USA). Then, the unstained cells were counted using hemocytometer (Reichert, Buffalo, NY, USA) under a light microscope. The percentage cell viability was calculated by the following equation:

$$\% \text{ Cell viability} = (\text{viable cells/total number of cells}) \times 100$$

Treatment of mouse macrophage cell line RAW 264.7 cells (1.875 x 10^5 cell/mL) were seeded in a 6-well plate. After overnight incubation, cells were resuspended in 1 mL of supplemented DMEM before pretreated with various concentrations of samples for 2 h and lipopolysaccharide (LPS) was then added and incubated for another 16 h. After that, the cell supernatants were collected to 1.5 mL microcentrifuge tube as subjected to NO production analysis.

Determination of nitric oxide (NO) production The nitrite concentration in cell supernatant was measured as an indicator of NO production following the Griess Reagent Kit (Molecular Probes, Inc, Eugene, OR). Briefly, 150 µL of each supernatant was combined with 20 µL of Griess reagent (1% sulfanilamide in 5% phosphoric and 0.1% naphthylethylenediamine dihydrochloride in water) and distilled water 130 µL in a 96-well plate, incubated at room temperature for 30 min. After incubation, the absorbance of each well was determined at 548 nm using a microplate reader (Molecular Devices). The amount of nitrite in samples was back-calculated from a sodium nitrite calibration curve (0-100 µM). The percentage inhibition of NO production was calculated relative to control, i.e. LPS-stimulated untreated cells. Diclofenac sodium (DCS) was used as a positive control.

Statistical analysis Data are expressed as mean ± standard deviation (S.D.) of at least three independent experiments, which were performed in tripicate. Statistical analysis was determined using one-way ANOVA followed by the Tukey's test for multiple comparisons (Graphpad Prism 6.0, Graphpad Software Inc., San Diego, USA). *P*-values less than or equal 0.05 were considered statistically significant.

RESULTS

Cell viability The results of the percentage cell viability are shown in Figure 1. It was shown that MSE at concentrations of 20 and 40 µg/mL as well as oxyresveratrol at concentrations of 5 and 10 µg/mL did not exhibit cytotoxicity to RAW 264.7 cells. The results of cell viability in all tested samples were > 90%.

Table 1 The percentage cell viability of RAW 264.7 cells following incubation with different concentrations of mulberry stem extract (MSE) and oxyresveratrol (oxy) for 24 h

Treatments	%Cell viability
Control	100.00 ± 0.00
MSE 20 µg/mL	98.05 ± 0.30
MSE 40 µg/mL	93.79 ± 1.62
oxy 5 µg/mL	99.91 ± 0.16
oxy 10 µg/mL	97.30 ± 2.58

Each value represents mean ± S.D., N=3 for all groups

Nitric oxide (NO) production LPS-stimulated murine macrophage RAW 264.7 cells model is known to induce iNOS transcription, resulting in NO production. This cell model was therefore selected to evaluate the effect of *M. alba* stem extract on the inhibition of NO production. The production of NO was observed by measuring nitrite (NO_2^-) concentration. The results are shown in Figure 2. Diclofenac, a positive control used in this study, was shown to have inhibitory effect on NO production. This result is in agreement with the result of Bae *et al.*[9]. Both MSE and oxyresveratrol at two different concentrations significantly inhibited the production of NO in a dose-dependent manner compared with LPS-stimulated untreated cells (control). As determined by High Pressure Liquid Chromatography (HPLC), the percentage of oxyresveratrol in MSE used in this study was 17.87±0.61%[8]. Thus, the equivalent concentration of oxyresveratrol in MSE 20 and 40 µg/mL was 3.4 and 6.8 µg/mL, respectively. Therefore, this study suggests that MSE possesses higher inhibitory effect of NO production than oxyresveratrol.

DISCUSSION

MSE had no cytotoxicity to RAW 264.7 cells at the concentrations studied. It inhibited NO production better than oxyresveratrol at an equivalent amount of oxyresveratrol. These results suggest that apart from oxyresveratrol, the other components in MSE such as prenylated flavonoids (i.e. kuwanon E) may have effect on the inhibition of NO production[10]. In this study diclofenac was tested at the lowest concentration, i.e. at 1 µg/mL and it was shown the strongest activities on the inhibition of NO production. The comparable effect on the inhibition of NO production of MSE compared to diclofenac was achieved at 40 µg/mL. However, as a bioactive natural product available in our country, it is still valuable to be used as dietary supplement for reducing inflammation and pain in OA.

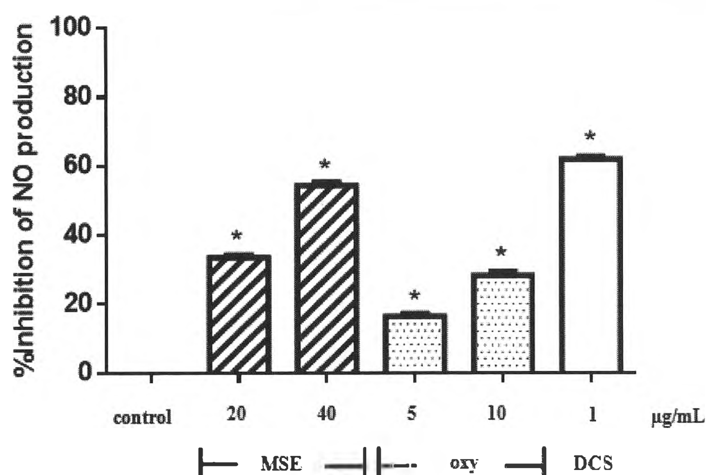

Figure 2 The percentage inhibition on nitric oxide production in RAW 264.7 cells induced by LPS. RAW 264 7 cells (1.875 x 10^5 cells/mL) were treated with 100 ng/mL of LPS for 16 h after pretreating with mulberry stem extract (MSE) at concentrations of 20, 40 µg/mL, oxyresveratrol (oxy) at concentrations of 5, 10 µg/mL or diclofenac sodium (DCS) 1 µg/mL for 2 h. The cell supernatant was then collected and the percentage inhibition on nitric oxide production was determined by Griess reaction assay. ****$p<0.001$ represents significant difference from LPS-stimulated untreated cells.

CONCLUSION

This study clearly demonstrates that mulberry stem ethanolic extract can inhibit NO production in LPS-stimulated cells and has no effect on cell viability. Further investigations on the effect of MSE on the inhibition of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 mRNA expressions are ongoing.

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