

## **Anti-inflammatory activity of 4-methoxycinnamyl *p*-coumarate isolated from *Etlingera pavieana* rhizomes in lipopolysaccharide-induced macrophages**

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### **ABSTRACT**

Nitric oxide (NO) and prostaglandins E2 (PGE<sub>2</sub>) play a critical role as inflammatory mediators but overproduction can lead to inflammatory diseases. Regulation of NO and PGE<sub>2</sub> secretion is a particular target in the development of anti-inflammatory agents. 4-methoxycinnamyl *p*-coumarate (MCC) was isolated from rhizomes of *Etlingera pavieana* by NO inhibitory activity-guided isolation. In this study, we evaluated the inhibitory effect of MCC on NO and PGE<sub>2</sub> production as well as enzymatic activities of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in lipopolysaccharide (LPS)-induced RAW 264.7 macrophages. MTT assay showed that MCC at 6.25 to 25 μM has no significant cytotoxicity compared to unstimulated control cells. MCC significantly reduced NO and PGE<sub>2</sub> production with IC<sub>50</sub> values of 8.5±0.4 and 26.2±3.7 μM, respectively. MCC at concentrations to 25 μM significantly inhibited iNOS enzyme activity but it did not suppress COX-2 enzyme activity. Interestingly, MCC itself did not reduce COX-1 expression and reversed the suppressive effect on COX-1 expression in LPS-stimulated RAW 264.7 macrophages. Collectively, these results suggest that MCC inhibits NO and PGE<sub>2</sub> production, as well as iNOS enzyme activity. MCC exhibits potent anti-inflammatory action with selective inhibitory effect on COX expression and might be used in the development of an anti-inflammatory agent with fewer side effects than current drugs.

**Keywords:** Anti-inflammatory, Nitric Oxide, Prostaglandins E<sub>2</sub>, Cyclooxygenase (COX), 4-methoxycinnamyl *p*-coumarate

### **INTRODUCTION**

Inflammation is a complex physiological process in response to pathogens by activation of immune cells. Macrophages play an important role in inflammation by secretion of mediators such as nitric oxide (NO) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) (Medzhitov 2008). However, overproduction of NO and PGE<sub>2</sub> in a state of chronic inflammation leads to pathological conditions including rheumatoid arthritis (Choy and Panayi 2001), osteoarthritis (Sokolove and Lepus 2013), cardiovascular diseases, kidney diseases (Nasrallah et al. 2016) and neurological disorders (Teeling and Perry 2009). Therefore, inhibition of NO and PGE<sub>2</sub> along with the activation of iNOS and COX-2 is a therapeutic approach for treatment of inflammatory diseases.

Large amount of NO and PGE<sub>2</sub> are catalyzed by inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), respectively (Sherwood and Toliver-Kinsky 2004). There are two main COX isozymes, COX-1 and COX-2. The former is a constitutive enzyme expressed in most tissues and is involved in maintenance of homeostasis. In contrast, COX-2 is an inducible enzyme, undetectable in most normal tissues but up-regulated by various pathological conditions and potentially induced by lipopolysaccharide (LPS) (Simmons et al. 2004). Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly and widely used to relieve inflammation. Although NSAIDs are developed to selectively inhibit COX-2, several side effects (such as renal failure, gastric damage and ulceration) have been reported due to COX-1 inhibition (Micklewright et al. 2003). Novel anti-inflammatory agents with lower adverse side effects are still needed (Rao and Knaus 2008). Natural products are an alternative resource of therapeutic compounds with anti-inflammatory potential and lower toxicity (Debnath et al. 2013).

*Etlingera pavieana* (Pierre ex Gagnep.) R.M.Sm of the family Zingiberaceae is found in Eastern Thailand and used in traditional medicine for treatment of dyspepsia, carminative, nausea and diuretic (Phonsena 2007). Recently, our group has shown anti-inflammatory activity in *E. pavieana* rhizome and isolated 4-methoxycinnamyl *p*-coumarate (MCC) by bioassay-guided isolation (Srisook et al. 2017). In the present study, we investigated further the effect of 4-methoxycinnamyl *p*-coumarate on inhibition of NO and PGE<sub>2</sub> production and iNOS and COX-2 activity in LPS-induced RAW 264.7 cells.

## METHODOLOGY

### Preparation of 4-methoxycinnamyl *p*-coumarate

Compound 4-methoxycinnamyl *p*-coumarate or MCC was isolated from rhizomes of *E. pavieana* by the method described by Srisook et al. (2017). It was dissolved in dimethyl sulfoxide (DMSO) and filtered through a 0.22 µm sterile filter.

### Cell culture

Murine macrophage cell line RAW 264.7 was obtained from American-Type Culture Collection (ATCC). Cells were suspended in DMEM containing 100 U/ml of penicillin, 100 µg/ml of streptomycin, and 10% heat-inactivated fetal bovine serum (FBS) in 5% CO<sub>2</sub> atmosphere at 37°C. Cells were subcultured and incubated overnight before treated with LPS or other chemicals at indicated times.

### MTT assay for test cell viability

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Srisook et al., 2015). Briefly, RAW 264.7 macrophages ( $1.5 \times 10^5$  cells/well) were plated in each well of 24-well plates and exposed to MCC at concentrations 3.12-100 µM for 24 h. Then MTT (5 mg/mL in PBS) was added to each well and incubated for another 2 h. before solubilizing formazan crystals in DMSO. Absorbance of each well was read at a wavelength 550 nm using a microplate reader.

### Determination of NO and PGE<sub>2</sub> production

RAW 264.7 macrophage cells ( $1.5 \times 10^5$  cells/well) were cultured in a 24-well plate for 24 h followed by co-treatment with LPS (1  $\mu\text{g}/\text{mL}$ ) and MCC in phenol red-free DMEM for 24 h. Concentration of nitrite, an oxidation product of NO, in the conditioned media was used an index of NO amount. Nitrite concentration was determined by Griess reagent. PGE<sub>2</sub> concentrations in the culture medium were quantified using a Prostaglandins E<sub>2</sub> enzyme immunoassay kit (Arbor Assays, USA) according to the manufacturer's instructions. Quantities of PGE<sub>2</sub> were calculated from a standard curve with known concentrations of PGE<sub>2</sub>. Percentage inhibition of NO and PGE<sub>2</sub> production of each treatment was determined in comparison to LPS-stimulated cells.

### iNOS activity assay

iNOS activity assay was modified from Hu and Kitts (2004). Macrophage RAW 264.7 ( $1.5 \times 10^5$  cells/well) was cultured in a 24-well-plate. After 24 h, cells were exposed to LPS (1  $\mu\text{g}/\text{mL}$ ) for 24 h to activate iNOS protein expression, then culture medium was removed and cells were washed twice with Hank's balanced salt solution (HBSS). Cells were treated with MCC at various concentrations for 6 h. Culture media were collected and quantified by Greiss reaction.

### COX-2 activity assay

COX-2 activity was measured as described by Laing et al., (1999) with a slight modification. Cells ( $1.5 \times 10^5$  cells/well) were cultured in a 24-well plate for 24 h. COX-2 expression was induced by exposure of macrophages to LPS for 24 h. Then cells were washed twice with HBSS, treated with MCC for 30 min and followed by incubation with the exogenous substrate of arachidonic acid 1  $\mu\text{M}$  for 1 h. PGE<sub>2</sub> concentrations in the culture medium were examined by a Prostaglandins E<sub>2</sub> enzyme immunoassay kit as described above.

### Western blot analysis

Cells were treated with MCC in the presence or absence of LPS for 24 h. Cellular protein was extracted using iced-cold RIPA lysis buffer. The protein concentrations were quantified using Bradford reagent (Bio-Rad, USA). Protein lysates were electrophoresed in 10% SDS-polyacrylamide gels. The separated proteins were transferred onto PVDF membranes. Non-specific binding was blocked with 5% nonfat dried milk solution. Membranes were incubated further with primary antibodies against COX-1 (1:1000) incubated at 4°C overnight and GAPDH (1:1000) at room temperature for 1 h. Afterwards, blots were incubated with goat anti-rabbit IgG horseradish peroxidase conjugated antibodies (1:5000) at room temperature for 1 h. Protein bands were visualized on X-ray film activated by chemiluminescence using SuperSignal West Pico Chemiluminescent substrate. Band signal intensities were determined by densitometry using BIOPROFIL Bio-1D version 11.9 (Vilber Lourmat, France). Image densities of specific band of COX-1 were normalized with that of the respective GAPDH band.

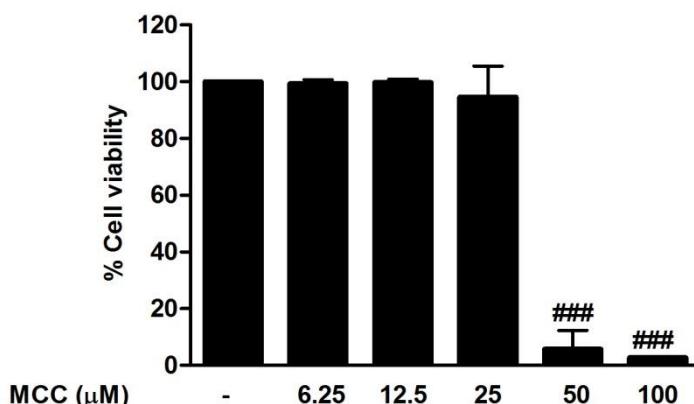
### Statistical analysis

Results are presented as means $\pm$ SD of at least three independent experiments and analyzed by Minitab 16 for Windows. Statistical significance was determined via analysis of variance (ANOVA), followed by Tukey's method for multiple comparison. A value of  $p<0.05$  was considered significant.

## RESULTS AND DISCUSSION

### Effect of MCC on cell viability in LPS-induced RAW 264.7 cells

The cytotoxic effect of MCC was evaluated using MTT assay as shown in Figure 1. MCC at a concentration of 6.25-25  $\mu$ M did not significantly change in cell viability compared to control resting cells. However, MCC at concentrations of 50 and 100  $\mu$ M caused significant cytotoxicity. Thus, the concentration of MCC used in the further experiments was <50  $\mu$ M.



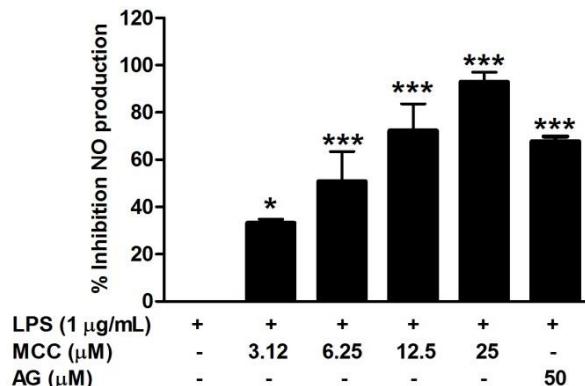
**Figure 1** Effect of MCC on cell viability of RAW 264.7 macrophages. Each column represents a mean $\pm$ SD of three experiments with triplicate samples.

##  $p<0.001$  significantly different from control group.

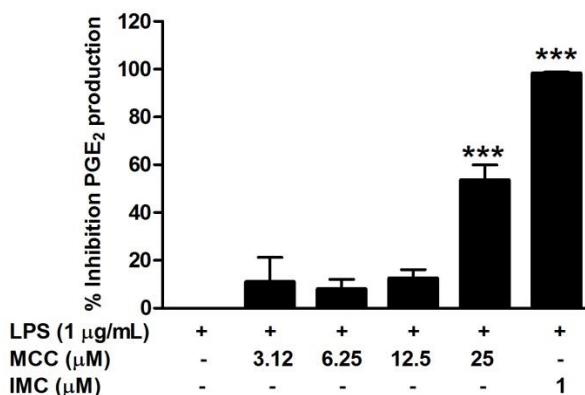
### MCC inhibited NO and PGE<sub>2</sub> production in LPS-induced RAW 264.7 cells

NO is a free-radical molecule generated from L-arginine by iNOS and serves as a regulatory effector of innate immune response that plays a critical role in the inflammation process (Guzik et al. 2003). Nitrite concentration in unstimulated cells was  $1.5\pm0.4$   $\mu$ M. After treatment with LPS, cells produced a high level of nitrite ( $65.9\pm11.2$   $\mu$ M). MCC significantly inhibited NO production in a dose-dependent manner with an IC<sub>50</sub> value of  $8.5\pm0.4$   $\mu$ M. Aminoguanidine, a specific iNOS inhibitor, was used as a positive control (at 50  $\mu$ M) and strongly inhibited NO production ( $67.9\pm2.0\%$ ) (Figure 2). PGE<sub>2</sub> is produced by COX that is one important inflammatory mediators which causes classic signs of inflammation including, redness, swelling, and pain (Ricciotti and FitzGerald 2011). The inhibitory effect of MCC on PGE<sub>2</sub> production was examined. It showed significant suppression of PGE<sub>2</sub> production at 25  $\mu$ M (Figure 3). Its IC<sub>50</sub> value was  $26.2\pm3.7$   $\mu$ M. Indomethacin, a COX inhibitor, used as a positive control at 1  $\mu$ M dramatically inhibited PGE<sub>2</sub>

production ( $98.2 \pm 0.3\%$ ). As shown in Figure 1, MCC at concentration to 25  $\mu\text{M}$  did not significantly affect cell viability and indicated that the inhibitory effect of MCC on NO and PGE<sub>2</sub> production was not due to cytotoxicity.



**Figure 2** Inhibitory effect of MCC on NO production. Each column represents a mean $\pm$ SD for three experiments with triplicate samples. \* $p<0.1$ , \*\*\* $p<0.001$  significantly different from LPS group. AG; aminoguanidine

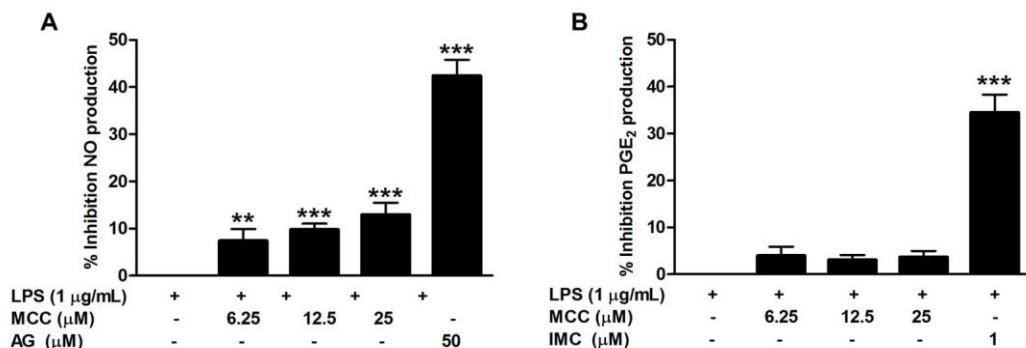


**Figure 3** Inhibitory effect of MCC on PGE<sub>2</sub> production. Each column represents a mean $\pm$ SD of three experiments with triplicate samples. \*\*\* $p<0.001$  significantly different from LPS group. IMC; indomethacin

#### Effect of MCC on iNOS and COX-2 enzymatic activity in LPS-induced RAW 264.7 cells

NO and PGE<sub>2</sub> generations were regulated by iNOS and COX-2 expression and/or enzyme activity (Chen et al. 2001). Thus we determined whether the inhibitory effects of MCC on NO and PGE<sub>2</sub> production were due to its direct effect on enzyme activity of iNOS and COX-2, respectively. MCC at 6.25-25  $\mu\text{M}$  slightly

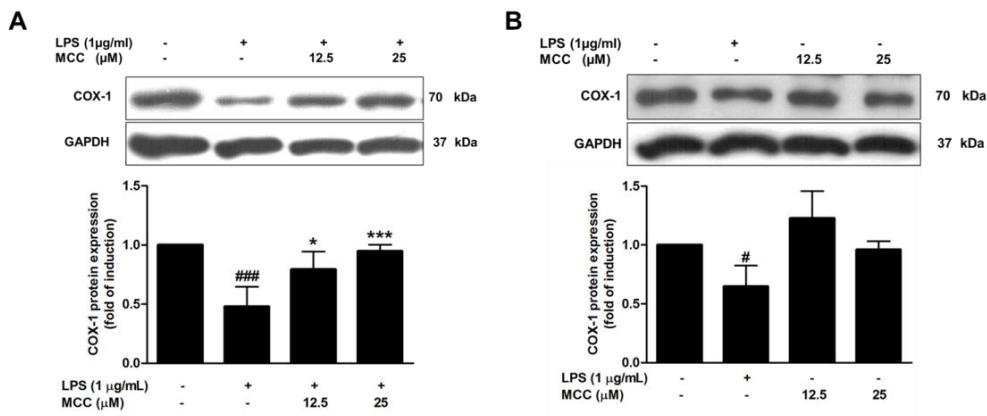
but significantly suppressed iNOS enzyme activity (Figure 4A) while it did not show any significant alteration of COX-2 activity at all concentrations tested. As expected, indomethacin exhibited a significant inhibition on COX-2-generated PGE<sub>2</sub> (Figure 4B). Our data demonstrate that MCC has potent inhibitory effect on NO production, partly, via inactivation of iNOS enzyme activity while its suppressive effect on PGE<sub>2</sub> formation might be due to inhibition of COX-2 expression.



**Figure 4** Effect of MCC on enzyme activity of iNOS (A) and COX-2 (B) activity. Each column represents the mean±SD of three experiments with triplicate samples. \*\*p<0.01, \*\*\*p<0.001 significantly different from LPS.

#### Effect of MCC on COX-1 expression in LPS-induced RAW 264.7 cells

The search for an anti-inflammatory drug, which does not inhibit COX-1 expression now seems feasible. MCC has the potential to be such a novel anti-inflammatory agent since it inhibited the production of NO and PGE<sub>2</sub>. As shown in Figure 5 A, LPS reduced the expression of COX-1 that is correlated with the observation of Chen et al. (2001) and Lee et al. (2011). MCC attenuated the effect of LPS on COX-1 expression through promotion of the COX-1 expression in a dose dependent manner and did not affect the basal level of COX-1 protein compared to control cells p<0.05 (Figure 5B). MCC might be a selective COX-2 inhibitor. This effect is similar to the information reported by Lee et al. (2011). Compound 2,6-Bis(2,5-dimethoxybenzylidene)-cyclohexanone (BDMC33) inhibited PGE<sub>2</sub> production but was not attributed to inhibition of the COX-2 enzyme activities. Moreover, BDMC33 attenuated the suppression COX-1 in IFN-γ/LPS induced cells.



**Figure 5** Effect of MCC on COX-1 protein level expression. Each column is mean $\pm$ SD of three independent experiments. #  $p<0.05$ , ###  $p<0.001$  vs control cells and \* $p<0.05$ , \*\*\* $p<0.001$  significantly different from LPS group.

## CONCLUSIONS

Our results demonstrate that MCC significantly inhibited NO production, at least in part, via inhibition of iNOS activity. It also suppressed PGE<sub>2</sub> production without affecting COX-2 enzyme activity. Moreover, MCC attenuated reduction of COX-1 expression in LPS-stimulated RAW 264.7 macrophages and might be used for development of an anti-inflammatory agent with minimal side effects. However, the precise mechanism underlying anti-inflammatory action of MCC needs further exploration.

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