

# The Effect of Ayurved Siriraj Wattana Recipe (AVS073) on LPS Induced COX-2 Expression in Human PBMC

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## ABSTRACT

**Objective:** To investigate the anti-inflammatory effects of AVS073 via the expression of COX-2 in human peripheral blood mononuclear cells (PBMC) induced by LPS.

**Methods:** PBMC from healthy volunteers were treated with LPS alone, AVS073 alone (0.1-10 µg/ml) and LPS plus AVS073 for 18 hours. Cell proliferation was measured using MTT assay, while COX-2 mRNA, COX-2 protein and PGE<sub>2</sub> production were assessed using real time RT-PCR, western blot and enzyme immunoassay (EIA), respectively.

**Results:** It was found that neither LPS nor AVS073 has effects on cell proliferation. The optimum time of LPS-induced COX-2 expression was 18 hours. Even the expression of COX-2 mRNA showed trends toward decrease in all concentrations of AVS073 treatment, it was not significantly different. However, the expression of COX-2 protein was significantly decreased by 10 µg/ml AVS073. Surprisingly, endogenous PGE<sub>2</sub> was significantly increased, whereas exogenous PGE<sub>2</sub> was not significantly changed.

**Conclusion:** AVS073 has some anti-inflammatory effects, probably at the level of post translational modification. Increase in endogenous PGE<sub>2</sub> production, in spite of the decrease in the expression of COX-2 protein, may be due to the other factors or isoforms of COX.

**Keywords:** Ayurved Siriraj Wattana Recipe, AVS073, COX-2, PBMC

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## INTRODUCTION

Ayurved Siriraj Wattana Recipe (AVS073) has long been used for prevention of age-related problems and health promotion. It is one of the Thai traditional folk medicines used at the Center of Applied Thai Traditional Medicine, Faculty of Medicine Siriraj Hospital,

Mahidol University. Recently, it has been reported to relieve pain of knee osteoarthritis (OA) in phase II clinical trial<sup>1</sup>. Nonsteroidal anti-inflammatory drugs (NSAIDs) are generally drugs of choice for the treatment of OA. NSAIDs possibly cause serious side effects after long term usage. In this case, AVS073 may be used in place of NSAIDs to minimize side effects. Although the anti-inflammatory effect of AVS073 has not been studied yet, the pharmacological properties (including immunomodulatory, anti-inflammatory, and antioxidant) of its components was reported in *in vitro*, *in vivo*, and clinical studies. AVS073

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consists of 18 kinds of medicinal plants, including *Boesenbergia rotunda* (L.) Mansf, *Aucklandia lappa* DC., *Ligusticum sinense* Oliv. cv. Chuanxiong, *Cinnamomum illicioides* A. Chev., *Carthamus tinctorius* L., *Anamirta cocculus* (L.) Wight & Arn., *Cladogynos orientalis* Zipp. ex Span., *Derris scandens* (Roxb.) Benth., *Cryptolepis dubia* (Burm.f.) M.R.Almeida, *Tinospora crispa* (L.) Hook.f. & Thomson, *Caesalpinia sappan* L., *Piper nigrum* L., *Ferula assa-foetida* L., *Putranjiva roxburghii* Wall., *Aegle marmelos* (L.) Corrêa, *Citrus sinensis* (L.) Osbeck, *Terminalia chebula* Retz., *Cyperus rotundus* L. Antioxidant and anti-inflammatory activities have been reported among these plant components such as *P. nigrum* L.<sup>2</sup>, *T. chebula* Retz.<sup>3</sup> *B. rotunda*<sup>4,5</sup>, *C. tinctorius* L.<sup>6</sup>, *A. cocculus*<sup>7</sup>, *D. scandens*<sup>8</sup>, *P. nigrum* L.<sup>9,10</sup> and *C. rotundus*<sup>11</sup>. These evidences possibly contribute to the efficacy of AVS073 in relieving pain of knee osteoarthritis (OA). In this study, the model of COX-2 expression in LPS-stimulated human PBMC, inflammatory mediator cells, was undertaken to investigate the anti-inflammatory activity of AVS073.

## MATERIALS AND METHODS

### Chemicals and reagents

The high quality grade of chemicals and reagents were purchased from Sigma (USA and Germany), Merck (Darmstadt, Germany), Bio-rad (Germany), Cayman (USA) and Gibco (USA).

### Preparation of plant extract

The crude extract of AVS073 was obtained from Manufacturing Unit of Herbal Medicines and products, manufactured under GMP, Center of Applied Thai Traditional Medicine (CATTM), Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand. AVS073 was extracted with 80% ethanol and then resuspended in Roswell Park Memorial Institute (RPMI) medium to obtain final concentrations of 1-100 ng/ml and 1-100 µg/ml.

### Isolation of peripheral blood monocytes (PBMC)

Fresh heparinized blood obtained from healthy volunteers was mixed with phosphate-buffered saline and centrifuged on Ficoll-Hypaque

density gradient. The layers of PBMC were collected and washed twice in RPMI 1640 then maintained in a humidified atmosphere with 5% CO<sub>2</sub> at 37 °C for 1 hour before treatment.

### Cell treatment

To find the optimum time course of LPS induced COX-2 expression, PBMC were incubated with 10 µg/ml LPS for 2-24 hours. After those times, cells at each time point were harvested for real time RT-PCR and western blot analysis. The optimum time point was selected. Then, PBMC were treated with AVS073, 1-100 ng/ml and 1-100 µg/ml, in conditions with or without 10 µg/ml LPS according to the selected time point. Control cells were PBMC incubated under the same conditions without AVS073. Indomethacin, a well-known anti-inflammatory agent, was used as a positive control for measurement of the COX activity.

### Measurement of cell viability

Cell respiration is an indicator of cell viability which can be assessed by the mitochondrial dependent reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) to formazan<sup>12</sup>. Therefore, after treatment with AVS073, cell respiration using a conventional colorimetric assay (MTT assay) was performed. Briefly, after 18 hours of cell treatment, MTT 0.2 mg/ml in medium was added and further incubated for an additional 2 hours. The medium was then removed and cells were solubilized in DMSO. The extent of reduction of MTT to formazan within cells was quantitated by the measurement of optical density at a wavelength of 595 nm using a microplate reader (Synergy HT, USA). Viability of treated cells was assessed as percent of control.

### Measurement of COX mRNA using real time RT-PCR

Total RNA of PBMC was prepared and converted into complementary DNA (cDNA) using the illustra RNAspin Mini RNA Isolation Kit (GE Healthcare) and ImProm-II™ Reverse Transcription System (Promega), respectively, according to the manufacturer's recommendation. Sets of primers to amplify GAPDH and COX-1 mRNA were designed using primer express ver-

sion 3.0 software (Applied Biosystem, USA) while to amplify COX-2 mRNA, sets of primers were designed as described by Zhao et al. (2005)<sup>13</sup>. These sequences of primer sets were as follows. GAPDH forward primer: 5'-GACCACTTTGT-CAAGCTCATTTCC-3'

GAPDH reverse primer: 5'-TGAGGGTCTCTCT-CTTCCTCTTGT-3'

COX-1 forward primer: 5'- GACCCGCCT-CATCCTCATAG-3'

COX-1 reverse primer: 5'- CCACCGATCTT-GAAGGAGTCA-3'

COX-2 forward primer: 5'- CAAAAGCTGGG-AAGCCTTCT-3'

COX-2 reverse primer: 5'- CCATCCTTGAAA-GGCGCAG-3'

Amplification program of COX-1 mRNA reaction was 95°C, 10 min for initial denaturation followed by 40 cycles of 95°C, 15 s for denaturation, 50°C, 40 s for primer annealing and 72°C, 40 s for extension whereas for COX-2 and GAPDH mRNA the reaction was the same except primer annealing temperature was 60°C for 40 s. ABI Prism 7500 Real-Time PCR System (Applied Biosystems, USA) was used with the FastStart Universal SYBR Green Master kit (Roche). The COX values were normalized again for analysis.

### Measurement of COX protein using Western blot analysis

PBMC were lysed in ice-cold extraction buffer. Total protein concentration was determined using the Bio-Rad protein assay reagent. An equal amount of total protein in each sample was loaded onto 10% polyacrylamide gel electrophoresis (SDS-PAGE). Immunoblotting using anti-COX-1 and COX-2 mouse monoclonal antibody was performed overnight at 4°C. The relative protein amount of gene expression was determined using the Image Lab software (Bio-Rad).

### Measurement of COX activity (Prostaglandin E<sub>2</sub>) using enzyme immunoassay

COX activity was measured by the production of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) in the replaced fresh medium containing exogenous arachidonic acid. Briefly, PBMC were washed once with phosphate-buffered saline (PBS). Subsequently,

20 μM arachidonic acid, dissolved in medium, was added and further incubated for 10 minutes. Then, the supernatant was collected to determine PGE<sub>2</sub> using an enzyme immunoassay, according to the manufacturer's protocol (EIA kit, Amersham, GE Healthcare UK).

### Statistical analysis

All data were expressed as means ± standard error of mean (SEM) from triplicate determinations. Using GraphPad Prism version 5 software, data were analyzed with one-way ANOVA followed by Dunnett's multiple comparison test and Bonferroni's multiple comparison test. A p-value of less than 0.05 was considered statistically different.

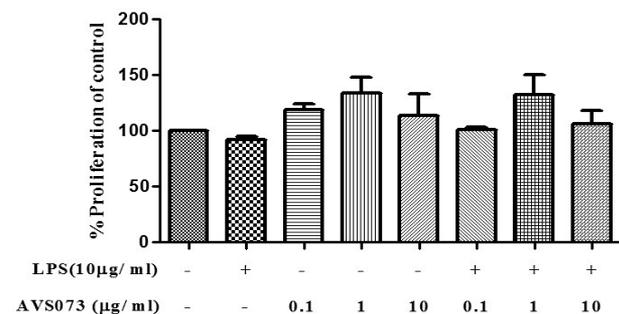
## RESULTS

### Effect of AVS073 on cell viability

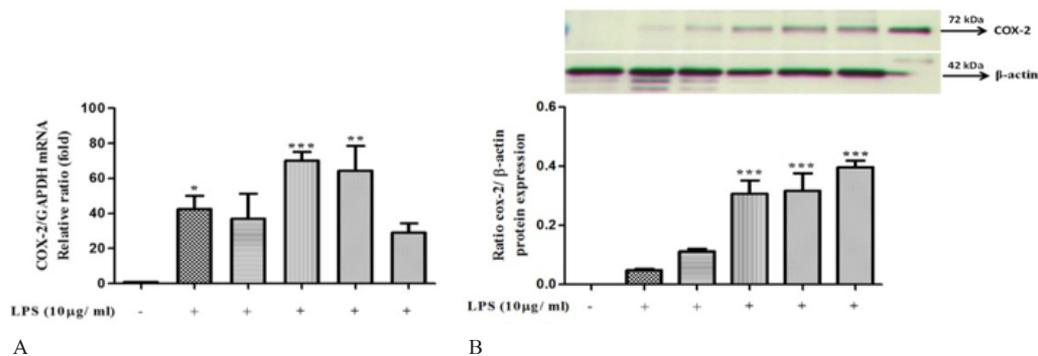
There was no significant difference in cell proliferation at all doses of AVS073, neither with nor without LPS. AVS073 and LPS, therefore, did not affect viability of PBMC (Fig 1).

### The optimum time course of LPS-induced COX-2 expression

The expression of COX-2 mRNA induced by LPS was detected as early as 2 hours through 18 hours and it was slightly declined at 24 hours while the expression COX-2 protein was detected as early as 8 hours through 24 hours, in a time-dependent manner (Fig 2). The optimum time point, therefore, should be 18 hours.



**Fig 1.** The effects of AVS073 and LPS on cell proliferation of PBMC. Around  $2 \times 10^5$  cells/well were treated with different concentrations of AVS073 coincubated with or without LPS for 18 h. Percent proliferation was determined using MTT assay of treated PBMC with untreated PBMC.



**Fig 2.** COX-2 mRNA (A) and protein (B) expressed in PBMC were measured after 2, 4, 8, 18, and 24 hour incubation with LPS (10 µg/ml). Data were shown as mean ± SEM and analyzed with one-way ANOVA followed by Dunnett's multiple comparison test. \* $p < 0.05$ , \*\* $p < 0.01$  as compared with untreated PBMC.

### The effects of AVS073 on COX mRNA and protein expression

Expression of LPS-induced COX-2 protein was significantly inhibited by 10 µg/ml AVS073 as well as 10 µg/ml indomethacin while of COX-2 mRNA was not significantly changed (Fig 3; panel A and B). However, 10 µg/ml AVS073 inhibited neither constitutive COX-1 mRNA nor protein while 10 µg/ml indomethacin could significantly inhibit only mRNA (Fig 4; panel A and B).

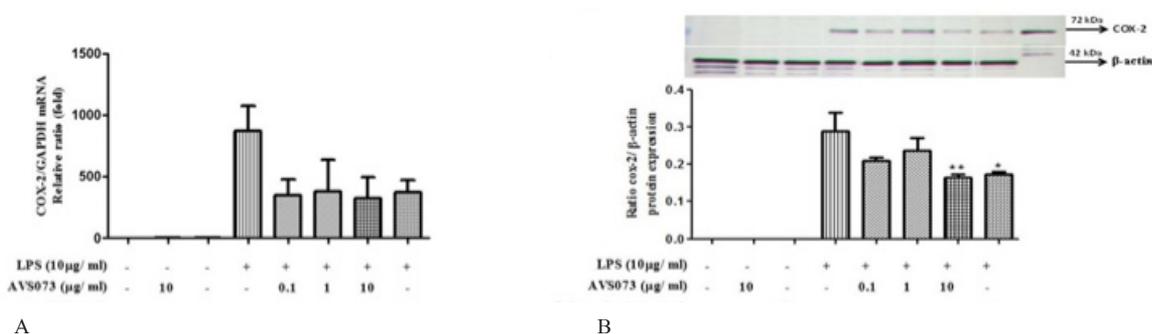
### The Effects of AVS073 on COX activity

COX activity was measured as PGE<sub>2</sub> released in the conditioned medium of PBMC in the absence of exogenous arachidonic acid (endogenous activity Fig 5A) or presence of exogenous arachidonic acid (exogenous activity, Fig 5B). AVS073 at all concentrations used in the present study has

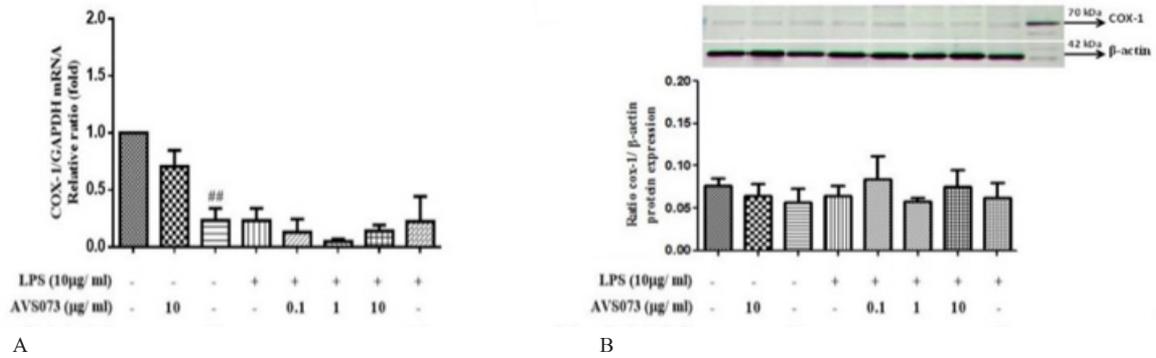
no effects on COX activity in PBMC activated with LPS, except for 10 µg/ml AVS073 which significantly increased endogenous COX activity. In contrast, both of them were significantly inhibited by indomethacin.

## DISCUSSION

The effects of AVS073 on COX expression are very interesting because most of its components exhibit various pharmacological properties including anti-inflammatory activities<sup>3,5,14,15,16</sup>. COX is a major target for non-steroidal anti-inflammatory drugs because it is a key enzyme that catalyzes transformation of arachidonic acid to several biological active prostanoids involving inflammation. COX exists in two isoforms, one of which, COX-2, is primarily responsible for



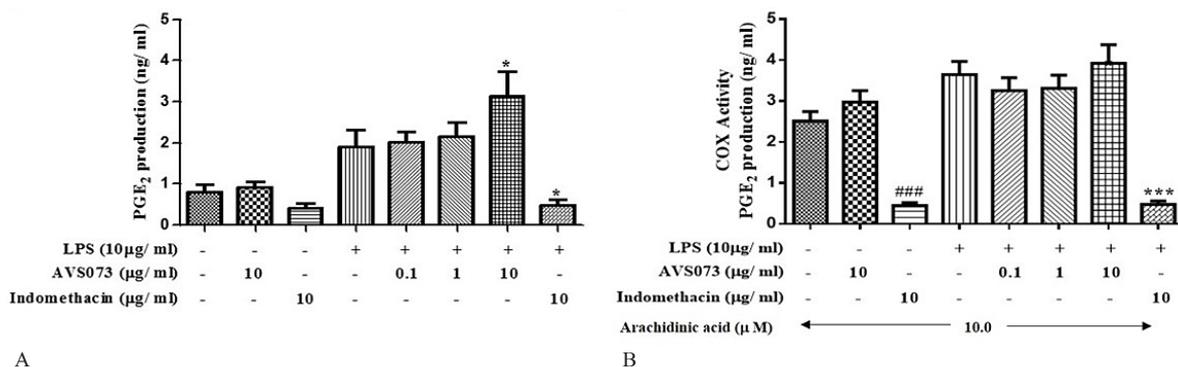
**Fig 3.** The effects of AVS073 on COX-2 mRNA (A) and protein (B) expressed in PBMC induced by LPS. PBMC were treated with different concentration (0.1, 1, and 10 µg/ml) of AVS073 with and without LPS for 18 hours. Indomethacin was used as a positive control. Data were shown as mean ± SEM and analyzed with one-way ANOVA followed by Bonferroni's multiple comparison test. (\* $p < 0.05$ , \*\* $p < 0.01$  as compared with treated LPS).



**Fig 4.** The effects of AVS073 on COX-1 mRNA (A) and protein (B) expressed in PBMC induced by LPS. PBMC were treated with different concentration (0.1, 1, and 10 µg/ml) of AVS073 with and without LPS for 18 hours. Indomethacin was used as a positive control. Data were shown as mean ± SEM and analyzed with one-way ANOVA followed by Bonferroni’s multiple comparison test. ###p < 0.01 as compared with untreated LPS.

inflammation. However, it is not constitutively expressed, but rapidly induced in many cell types responding to endotoxin and some other stimuli. PBMC was chosen for this study as a model of LPS-induced COX-2 expression because it is a cell type which plays an important role in the immune system and the inflammatory process<sup>17</sup>. Cell viability was studied to confirm that any possible changes, if they occur, were not due to the differences in cell death. It was found that the AVS073 (1 ng/ml-10 µg/ml) including LPS had no effect on cell proliferation (Fig 1). This is in according to the study that AVS073 up to 1,000 mg/kg was not toxic on splenocytes in Wistar rats<sup>18</sup>. The optimum time course of COX-2 expression was found to be 18 hours because at 24 hours, even the expression of COX-2 protein

still existed, there was a trend toward decreased COX-2 mRNA (Fig 2). This is quite similar to the previous study that the maximum COX-2 expression was found at 19 hours after LPS induction in PBMC<sup>19</sup>. AVS073 probably has some anti-inflammatory properties through the cyclooxygenase pathway at the level of post translational modification, because it was found that AVS073 (10 µg/ml) inhibited COX-2 protein, but not COX-2 mRNA (Fig 3), while it affected neither COX-1 mRNA nor protein (Fig 4). In contrast, PGE<sub>2</sub> production was increased (Fig 5A). This can possibly be explained by the inhibitory properties of COX-2 protein which were not so sufficient to inhibit PGE<sub>2</sub> production through COX activity, because even though PGE<sub>2</sub> is the main product of COX-2<sup>20,21</sup>, it may also be the product of the



**Fig 5.** The effects of AVS073 on endogenous PGE<sub>2</sub> released (A) and exogenous PGE<sub>2</sub> released (B) in PBMC treated with LPS. Data were shown as mean ± SEM and analyzed with one-way ANOVA followed by Bonferroni’s multiple comparison test. ###p < 0.001 vs. no-treated cells; \*p < 0.05, \*\*\*p < 0.001 vs. LPS-treated cells.

other COX isoforms, COX-1, as well<sup>22</sup>. Based on the hypothesis of Bezugla et al<sup>22</sup>, COX-1 probably couples to some newly expressed enzymes such as PGE synthase and contributes to PGE<sub>2</sub> release. Therefore, increased PGE<sub>2</sub> production, is possibly derived from this sustained COX-1. These results are also similar to the study of AVS022 on LPS-induced HUVEC that COX activity was increased while COX-2 protein was decreased<sup>23</sup>. Moreover, as expected as a positive control for measurement of the COX activity, indomethacin, can inhibit constitutive COX-1 mRNA including both endogenous and exogenous PGE<sub>2</sub> production. Nonetheless, considering the inhibitory effects of AVS073 on COX-2 protein expression, some plant components of AVS073 including *A. lappa* DC<sup>24</sup>, *C. rotundus*<sup>25</sup>, *C. tinctorius*<sup>26</sup>, *P. nigrum*<sup>14</sup>, *T. chebula*<sup>27</sup> exhibit the anti-inflammatory effects through the reduction of COX-2 proteins expression. Moreover, the existence of several phenolic compounds including gallic acid (GA) in the AVS073 was preliminarily reported by Panich et al<sup>28</sup>. They suggested that the anti-inflammatory effects of AVS073 were possibly due to the combined activity from the active ingredients of these plant components. However, it still needs many further work to do including screening and identifying lead compounds as well as pharmacokinetic and pharmacodynamic study.

## CONCLUSION

AVS073 is one of the important Thai traditional medicines used for health promotion and prevention of age-related problems. AVS073 was safe with these studied doses on PBMC. The optimum time of LPS-induced COX-2 expression was 18 hours. The highest dose (10 µg/ml) of AVS073 exhibited possible anti-inflammatory effect at the post-translational level by decreasing COX-2 protein. However, endogenous PGE<sub>2</sub> was increased, whereas exogenous PGE<sub>2</sub> was not changed. COX-1, according to the earlier hypothesis, probably contributed the increased endogenous PGE<sub>2</sub> production.

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