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Original Article

ANTI- INFLAMMATORY RESPONSE OF *ZANTHOXYLUM MYRIACANTHUM* WALL. EX HOOK.F. FRUIT EXTRACT

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Abstract

Background

Zanthoxylum myriacanthum, known as Ma-khwaen, is widely used in Thai folk medicine and as a culinary spice. The whole fruits, including pericarp and seeds, extracted with 95% ethanol has anti-inflammatory potential, but the mechanisms of action need to be validated.

Aims

To investigate the anti-inflammatory effect of fruit ethanolic extract.

Methods

The extract, prepared via 95% ethanolic maceration, underwent HPLC analysis for quality control using L-asarinin as a standard. Its anti-inflammatory effects were evaluated using the cyclooxygenase inhibitor assay and RAW 264.7 macrophage cells through MTT cytotoxicity, COX inhibition, and RT-PCR assays, measuring inflammatory gene expression (COX-1, COX-2, IL-1β, IL-6, TNF-α). Statistical analysis (One-way ANOVA) assessed the significance of findings.

Results

ZL1 extract, obtained with a 14.32% yield and containing 0.040 % w/w L-asarinin, demonstrated an IC_{50} of 102.46 µg/mL on RAW 264.7 cells. Notably, ZL1 inhibited COX-2 activity in a dose-dependent manner, while showing no effect on COX-1. Gene expression analysis in LPS-inflamed RAW 264.7 cells further revealed that ZL1 down-regulated COX-2, IL-1β, IL-6, and TNF-α at concentrations as low as 12.5 µg/mL, indicating its selective anti-inflammatory potential.

Conclusion

Zanthoxylum myriacanthum ethanolic extract (ZL1) selectively inhibited COX-2. It significantly suppressed inflammatory genes (COX-2, IL-1β, IL-6, TNF-α) in LPS-induced RAW264.7 cells at 12.50, 25 and 50 µg/mL, demonstrating its potent anti-inflammatory effects.

INTRODUCTION

Inflammation is part of the body's defense mechanism. Inflammatory response can be triggered by a variety of physical, biological, and chemical etiological causes, including trauma, overuse, chemicals, poisons, and viruses. Inflammation also plays a complex role in athlete recovery, where muscles are damaged by exercise, by repairing and regenerating stronger muscles requires temporary, regulated inflammation (Cerqueira *et al*., 2020). In elderly people, Sarcopenia is a disorder where the body's capacity to gain and preserve muscle mass is impaired by persistent, low-grade inflammation (Dalle *et al*., 2017). Inflammatory cytokines, especially the well-known interleukin 6 (IL-6) and Tumor Necrosis Factor Alpha (TNF-α), are produced by NK cells, monocytes, T and B lymphocytes, and other immune cells, as well as non-immune cells such as glial cells, smooth muscle cells, chondrocytes, and astrocytes when they are stimulated (Moldoveanu *et al*., 2001). Prostaglandin E2 (PGE2) plays a major role in acute inflammation at the site of tissue injury and induces heat, vasodilation, edema, and severe pain (Ricciotti and FitzGerald, 2011). Inflammatory cytokine TNF-α binds to PGE2 and activates phospholipase A2, releasing eicosanoids from the lipoxygenase and cyclooxygenase pathways in the metabolism of arachidonic acid (Martel-Pelletier *et al*., 2003). PGE2 is a significant byproduct of cyclooxygenase. The preferred medications for inhibiting the generation of PGE2 linked to COX-2 are NSAIDs. On the other hand, prolonged use of NSAIDS has been linked to severe and occasionally fatal adverse effects (Marcum and Hanlon, 2010; Davis and Robson, 2016; Sohail *et al*., 2023). Finding a different treatment plan with comparable effectiveness but fewer adverse effects is therefore crucial. Numerous inflammatory diseases have been treated with natural ingredients that have therapeutic qualities (Attiq *et al*., 2018). These conventional anti-inflammatory treatments eventually served as the basis for the creation of aspirin; the first synthetic anti-inflammatory medication developed from a natural substance (Maroon *et al*., 2010).

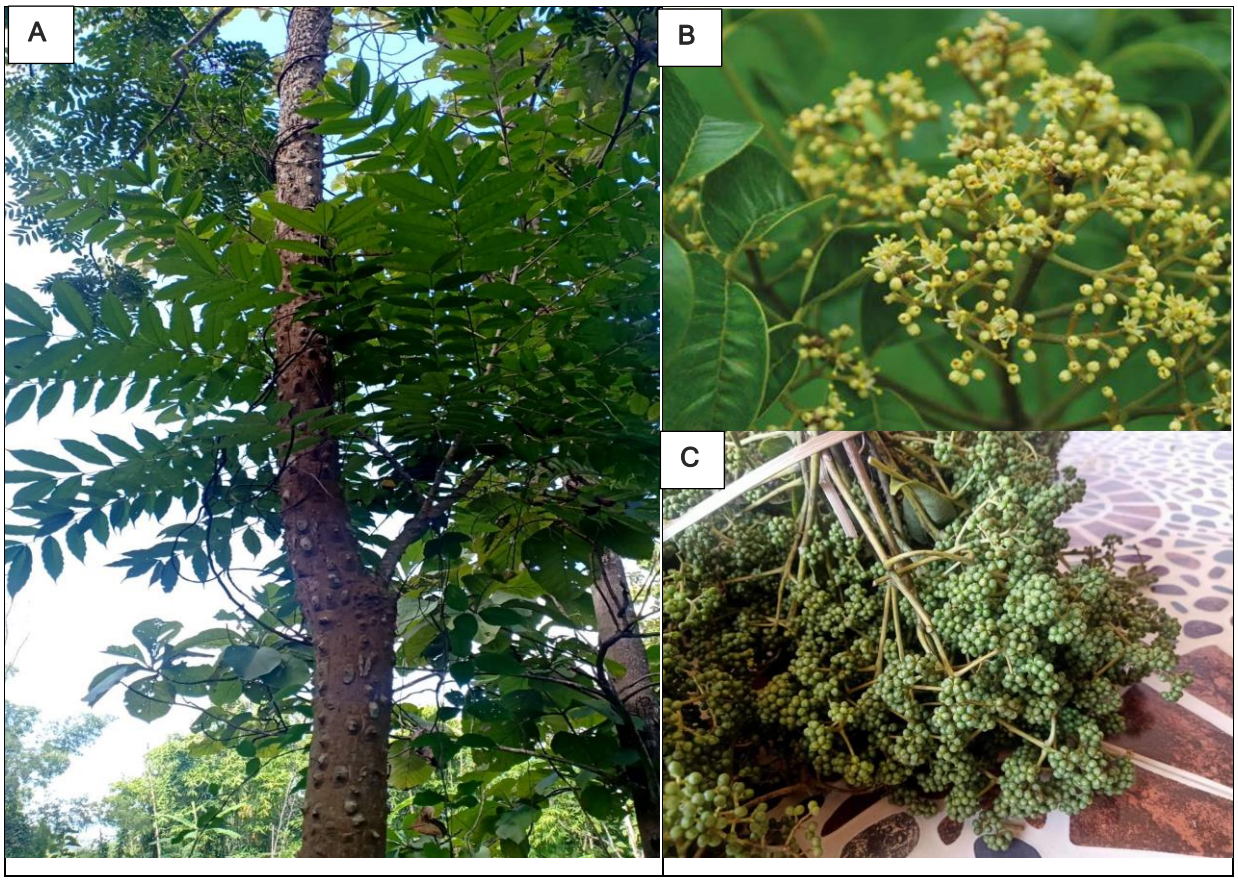

Figure 1 *Zanthoxylum myriacanthum* Wall. ex Hook.f., tree(A), flowers(B) and fruits(C).

The plant, *Zanthoxylum myriacanthum* Wall. ex Hook.f. (Figure 1) belongs to the Rutaceae family and has a strong scent. The entire fruit, which consists of the seed and pericarp, is consumed in the northern region

of Thailand (Imphat *et al*., 2021; Sriwichai *et al*., 2021). The essential oil of *Z. spp* fruit pericarp was found to possess several compounds such as L-limonene, β-phellandrene, β-philandrene and terpinen-4-ol (Sriwichai *et al*., 2021; Sriwichai *et al*., 2019). The pharmacological actions of these extracts included anti-inflammatory (Li *et al*., 2014; Adam *et al*., 2023), antimicrobial (Li *et al*., 2014), antioxidant (Yamazaki *et al*., 2007), antidiabetes, antigout, and antileukemia properties (Quan *et al*., 2022). Moreover, an extensive study that examined the 95% ethanolic extract of pericarp and seeds separately showed a notable anti-inflammatory effect on TNF-α, NO production and PGE2 (Imphat *et al*., 2021). This research, therefore, aimed to investigate the anti-inflammatory effect of *Z. myriacanthum* whole fruit ethanolic extract.

Materials and Methods

Plant Materials

Fresh mature fruits of *Z. myriacanthum* (Table 1) *were* collected from Nan province, Thailand, in October 2023. The plants were identified by Mr. Sakwichai Ontong, a botanist and some voucher specimens were collected as DMSC5370 and deposited at the DMSC International Herbarium, Department of Medical Sciences, Ministry of Public Health. The origin and identity of all samples are summarized in Table 1.

Plant Extraction

The whole fruits were cleaned up with tap water, their pedicels and sub-twigs were removed. For the dried plant extract, the whole fruits to be prepared were dried in a hot air oven at 45-50 ºC for 24-48 h. The dried plant was ground and macerated with 95% ethyl alcohol (250 g of the powder: 1 L of ethyl alcohol), The plant material was filtered and further extracted with ethyl alcohol one more time. The ethanolic extract was then pooled, concentrated and dried by rotary evaporation yielding ZL1 extract (Table 2). The dried extract was collected and kept in a light protected bottle at -20 ºC until used.

Table 1| Identity and origin of *Zanthoxylum myriacanthum* materials used in this work.

Code	Scientific name	Part Used	Location (GPS Coordinates)	Date (YYYY/MM/DD)	Quantity
ZM	*Zanthoxylum myriacanthum**	Fruits	19.050083, 100.787473	2023/10/05	5.0 g

*Identified by Sakwichai ONTONG, Nonthaburi, Thailand, 5 September 2022.

Table 2 | Extraction method of *Zanthoxylum myriacanthum* whole fruits

Code	Scientific name	Part Used	Extraction method	Solvent/Volume	Yield (%)
ZL1	*Zanthoxylum myriacanthum*	Fruits	Maceration	Ethyl alcohol	14.32

High-Performance Liquid Chromatography (HPLC)

The extract was analyzed for its phytochemicals for quality control using HPLC by applying the conditions from *Shu et al*. (2024). L-Asarinin was used as a standard. The extract (10 mg) was dissolved in 1 mL of methanol, vortexed, then sonicated for 5 min and finally centrifuged to produce the sample solution. L-asarinin was purchased from Phytochem, China, and the standard solution was prepared at a concentration of 0.75 mg/mL by dissolving the compounds in methanol. The HPLC analysis was conducted on a Waters Acquity Ultra Performance LC and separation was achieved on a Hypersil GOLD C18 column (2.1 x 100 mm, particle size: 1.9 µm) connected to an Acquity UPLC BEH C18 guard column (20 × 4 mm, 1.7 µm in

diameter) at room temperature. The mobile phases were acetonitrile(A) and 0.1% formic acid in water (B) using a gradient elution of 6% A at 0-4 min, 25% A at 5-8 min, 55% A at 9-13 min, 70% A at 14-16 min and 6% A at 17-18 min and was pumped at a flow rate of 0.3 mL/min with the injection volume 1 µL (Table 3). The detection was set at the wavelength of 235 nm.

Table 3| HPLC conditions used in this study

Code	Column Injection Volume	Elution Conditions				
HPLC		Time	Acetonitrile (mobile phase A)	0.2% acetic acid in water (mobile phase B)	Flow ($mLmin^{-1}$)	Temp.
Waters Acquity Ultra Performance LC	1 µL	0-4 min	6%	94%	0.3	RT
		5-8 min	25%	75%	0.3	RT
		9-13 min	55%	45%	0.3	RT
		14-16 min	70%	30%	0.3	RT
		17-18 min	6%	94%	0.3	RT

Cell culture

RAW 264.7, mouse macrophage cell lines (ATCC, NY, USA), were used in the gene expression assay. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, NY, USA) cell culture medium containing 2 mM L-glutamine, 0.1 mM non-essential amino acid, 1 mM sodium pyruvate and 10% foetal bovine serum at 37 ^{o}C in a 5% CO_2 incubator.

Standards

Indomethacin and Aspirin were from Sigma-Aldrich, Germany.

MTT cytotoxicity assay

RAW 264.7 cell lines, the macrophage immune cells, were used to evaluate the inflammatory gene response to the compound. The cells were seeded at $5x10^3$ cells in 100 µL medium per well of 96-well plates and left in the incubator for 24 h. The medium was removed and the cells were treated with eight different concentrations of extract solution in triplicate and then incubated for 48 h. The medium was replaced with 200 µL 0.5 µg/mL MTT reagent and then continued to be incubated for 4 h. The MTT solution was discarded and 200 µL DMSO was added to each well to dissolve the purple formazan product. The absorbance of the formazan product of viable cells was read using the microplate reader at 570 nm. The background absorbance was reduced by the blank and % viability was calculated compared to the control.

Cyclooxygenase inhibitor screening assay

The cyclooxygenase (COX) inhibitor screening kit (Cayman Chemical, Michigan, USA) measured the peroxidase component of COXs. The peroxidase activity of COXs (COX-1 and COX-2) is assayed calorimetrically by monitoring the appearance of oxidized *N, N, N′, N′*-tetramethyl-*p*-phenylenediamine (TMPD) at 590 nm using spectrophotometry. In brief, the extracts were prepared and diluted to various concentrations in assay buffer and pipetted 10 µL into each well. A positive control (inhibitor) was aspirin or indomethacin. According to the manufacturer's instructions, the enzyme (COX-1 or COX-2) was added to each well. The plate was shaken and incubated for 5 min at 25 °C. The reaction was initiated by adding 20 µL of arachidonic acid and 20 µL of the substrate to each well. The plate is shaken to mix the substances and incubated at 25 °C for 2 min. The absorbance at 590 nm was measured using a microplate reader. The blank well had no enzyme and inhibitor, while the 100% initial activity well had no inhibitor.

Gene expression using real-time reverse-transcription polymerase chain reaction (RT-PCR)

RAW 264.7 cell lines were seeded at 5 x 10^5 cells/well in 6-well plates for 24 h, then treated with the compounds or extracts at concentrations that were not toxic to the cells for an h and then 1 µg/mL lipopolysaccharides (LPS) were added and continued incubation for 3 h. Then the cell was rinsed with 1 mL phosphate buffer saline (PBS) in each well. Total RNA of each sample was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany). According to the manufacturer's protocol, RNA was re-suspended in 30 µL of nuclease-free water and then the products were run on agarose gels to check the quality of the RNA. cDNA was synthesized using QuantiTect Rev. Transcription Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. Briefly, 2-µg template RNA was added to the reverse-transcription master mix and then the sample tubes were incubated at 42 ºC for 15 min. The cDNA samples were tested in triplicate with quantitative PCR using a QuantiTect SYBR Green PCR Reagents kit (Qiagen, Valencia, CA, USA). Total 2 µL of each sample was mixed with SYBR Green PCR Master Mix and 10x QuantiTect Primers which were cyclooxygenase1 (COX-1), cyclooxygenase2 (COX-2), Interleukin-1 beta (IL-1β), Interleukin 6 (IL-6) and Tumor Necrosis Factor Alpha (TNF-α) (Qiagen, Valencia, CA, USA), then performed the real-time PCR (RT-qPCR) followed the manufacturer's protocol in Thermal cycler (PCR) (Analytik Jena GmbH, Valencia, Jena, Germany). mRNA ratios relative to β-actin (ACTB), the housekeeping gene were calculated for the standardization of gene expression levels. A melting curve analysis was also performed to verify the specificity and identity of PCR products. For selected genes, the data were analyzed using the equation described by Livak and Schmittgen (Livak and Schmittgen, 2001) as follows: the amount of target =$2^{-\Delta\Delta Ct}$. The average ΔCt from the untreated cells is a calibrator for each gene tested. This assay was conducted at the Toxicology Laboratory, Medicinal Plant Research Institute, Department of Medical Sciences.

Statistical analysis

The total percentage of the COX inhibition assay was expressed as the mean of duplicated experiments. The percentage of relative COX-1, COX-2, IL-1β, IL-6 and TNF-α inhibition and relative quantity of mRNA expression were expressed as mean ± SD (n=3). The differences of the mean between the LPS-induced cells and the cells treated with different concentrations of ZL1 were analysed using One-way ANOVA (GraphPad Prism Version 9.5.1, CA, USA).

RESULTS AND DISCUSSIONS

Zanthoxylum myriacanthum (800 g) yielded 114.56 g (14.32% w/w) of ZL1 extract. From the results of HPLC analysis, it was found that L-asarinin presented in ZL1 was 0.040 ± 0.001 % w/w. The chromatograms of ZL1, consisting of L-asarinin are in Figure 2.

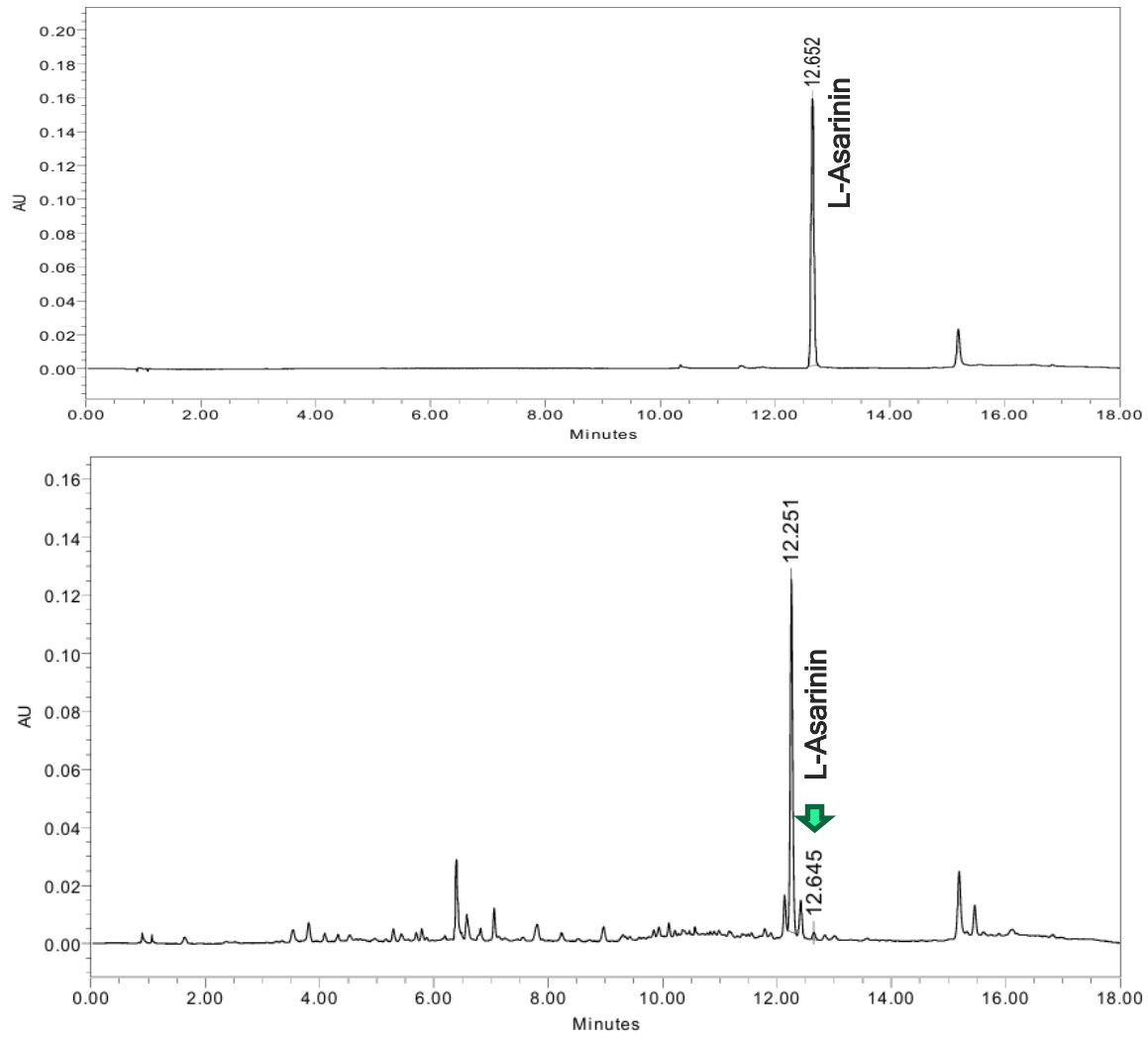

Figure 2. Chromatograms of 0.01 mg/mL L-asarinin and 1.00 mg/mL ZL1 extract, inj. vol. of 1 µL, monitored at 235 nm.

The cytotoxicity of ZL1 extract to RAW 264.7 cell lines was evaluated (Figure 3). The cells were used as a model of anti-inflammatory response in the gene expression assay. The percentage of cell viability was calculated by comparing it with the negative control (without treatment). ZL1 extract showed the IC_{50} of 102.46 µg/mL.

Cyclooxygenase enzymatic inhibition of ZL1 extract was evaluated at different concentrations. ZL1 showed inhibition activities to COX-2 in a dose-dependent manner. The percentages of inhibition were 32.20, 57.50, 64.40 and 73.30% at the concentration of 31.25, 62.50, 125 and 250 µg/mL, respectively. Whereas, there was no inhibition of ZL1 to COX-1 enzyme at the concentration evaluated. The graph of cyclooxygenase inhibition of ZL1 on COX-1 and COX-2 enzymes was shown in Figure 3. The inhibitory concentration of ZL1 to COX-2 was calculated using non-linear regression as of 62.95 µg/mL. Data are shown in Table 3.

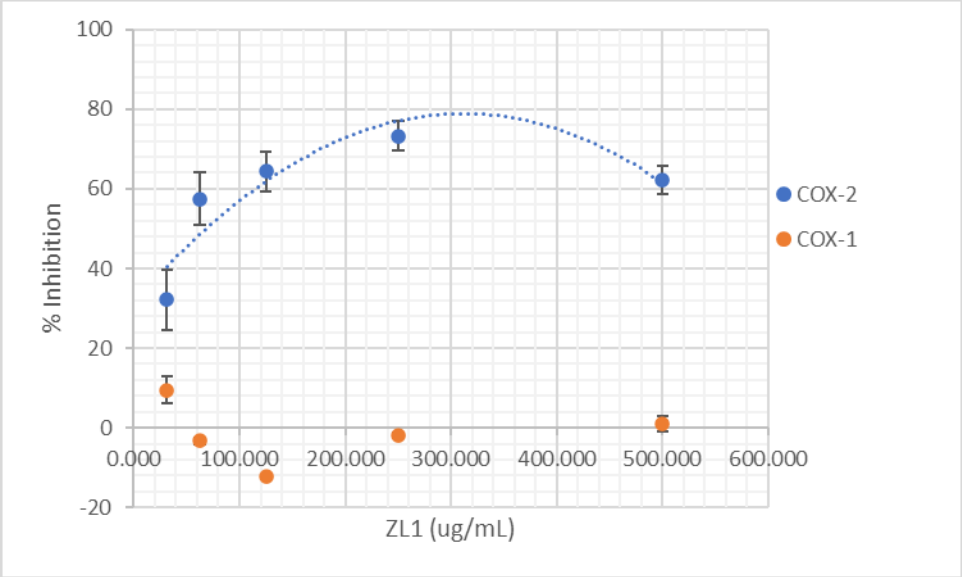

Figure 3 Cyclooxygenase inhibition of ZL1 to COX-1 and COX-2 enzymes. There was no inhibition to COX-1, while it showed the inhibition of COX-2 enzyme.

Table 3 Cyclooxygenase inhibition of ZL1 to COX-1 and COX-2 enzymes. The data were expressed as mean ±SD, n=2. Indomethacin was used as the positive control. The ratio of COX-2: COX-1 to classify cyclooxygenase inhibitors (Lipsky *et al.*, 1998).

Code	Inhibitory concentration (µg/mL)		
	COX-1	COX-2	COX-2: COX-1
Indomethacin (Control)	3.42	21.00	6.14 (Non-specific COX inhibitors)
ZL1	>1000	62.95	<0.01 (Specific COX-2 inhibitors)

Gene expression assay of five main pro-inflammatory response genes, including COX-1, COX-2, IL-1β, IL-6 and TNF-α after treating RAW264.7 cells with LPS was performed. They up-regulated dependently in some extent compared to the untreated control. There were 50 µg/mL Aspirin and Indomethacin as positive controls that targeted at COX-1 and COX-2 genes. Aspirin seems potent in reducing both COX-1 and COX-2 in LPS-reduced cells to about 0.73 and 0.96-fold changes, respectively. Indomethacin reduced the expression of COX-1 and COX-2 in LPS-reduced cells to about 2.10 and 1.41-fold changes, respectively.

The pro-inflammatory response signaling, COX-1, COX-2, IL-1β, IL-6 and TNF-α, were detected in LPS-inflamed RAW264.7 cells. COX-1 was not inhibited by ZL1, whereas COX-2 was down-regulated by ZL-1 in a dose-response manner and decreased significantly at the doses of 25 and 50 µg/mL µg/mL. IL-1β, IL-6 and TNF-α were also down-regulated by the compound at the doses of 12.5, 25 and 50 µg/mL. The mRNA expression data of COX-1, COX-2, IL-1β, IL-6 and TNF-α in Raw 264.7 cells treated with different concentrations of ZL1 are shown in Figure 4.

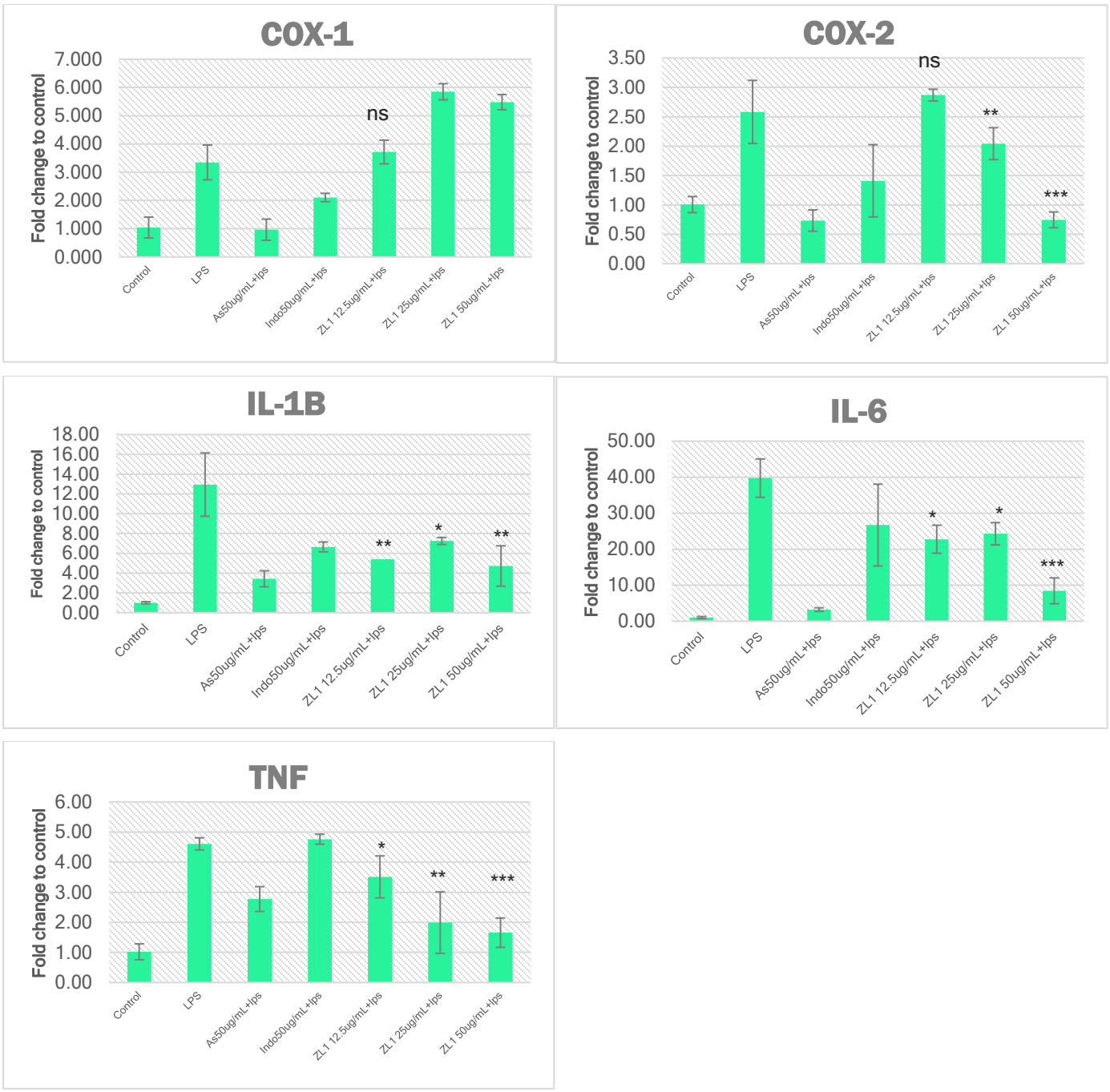

Figure 4 Relative quantity of mRNA expression of COX-1, COX-2, IL-1B, IL-6 and TNF-α in Raw 264.7 cells treated with the extract (ZL1) at 12.5, 25 and 50 µg/mL versus control data. The data were expressed as mean ±SD, n=3. The significant difference was compared relatively to the Lipopolysaccharide (LPS)-treated cells (*$p < 0.05$, **$p < 0.005$, and ***$p < 0.0005$).

Zanthoxylum fruits, particularly those of species like *Z. myriacanthum,* known as Ma-khwaen, and *Z. rhetsa,* known as Ma-khwuang, are widely used in Thai folk medicine. Fruits are utilized in the form of an oil and an ointment (in the Royal Formulary of King Narai) for the treatment of muscle and joint inflammation (Department of Traditional and Alternative Medicine, 2012). The whole fruits containing pericarp and seeds were extracted with 95% ethanol obtaining a 14.32% yield of the extraction as high as previously reported by Imphat *et al.* (2021). The 95% ethanolic extract of the fruits was also reported to reduce the LPS-induced NO, TNF-α and prostaglandin E2 (PGE2) in RAW264.7 cells. Therefore, the mechanisms of action need to be explored to use the fruit ethanolic extract. Most of the methods analysing chemical compounds in volatile oil used GC-MS (Gas Chromatography-Mass Spectrometry) (Sriwichai *et al*., 2019; Nadon *et al*., 2023. However, the compounds reported to provide anti-inflammatory effect were alkaloids, flavonoids, lignans and other polyphenols (Garcia *et al*., 2020; Nadon *et al*., 2023; Lu *et al*., 2024) LC-diode array detector (DAD) was set up to analyse the chemical profiles of the extract. The compounds showed several peaks at 235 nm, the main peak (higher than 50%) was at the t_R 12.251 min. whereas L-asarinin was used as the marker for quality control of the extract. The extract was first screened for the cyclooxygenase inhibition (COX-1 and COX-2) and lipoxygenase inhibition activities (data not shown) using Cayman Chemical screening kit. This is the first report on the extract showing quite potent inhibition of COX-2, but not COX-1 significantly (shown in Figure 3). ZL1 was classified as a specific COX-2 inhibitor compared to the positive control, Indomethacin, the broad spectrum of a potent nonsteroidal anti-inflammatory drug (NSAID), exerts its primary therapeutic effects by inhibiting the cyclooxygenase (COX) enzymes, notably its greater selectivity for COX-1(Lipsky et al., 1998). The inflammatory gene response, including COX-1, COX-2, IL-1β, IL-6 and TNF-α in LPS-induced RAW 264.7 cells, was analysed and the extract was found to considerably down-regulate the expression of COX-2 but not COX-1 at low doses of 25 and 50 µg/mL. The extract also showed inhibition to the pro-inflammatory response genes, IL-1β, IL-6 and TNF-α, significantly (p values< 0.05).

The results of the cyclooxygenase inhibition assay and the gene expression assay in LPS-induced RAW264.7 cells indicated that ZL1 extract showed potent specific inhibition of COX-2. The inhibition of cyclooxygenase (COX-1 and COX-2) enzymes, converting arachidonic acid into prostaglandin E2 (PGE2) is the rate-limiting step in prostaglandin synthesis. This reduction in prostaglandin levels, particularly PGE2 (which plays a key role in mediating pain, fever, and inflammation), is what provides the anti-inflammatory, analgesic (pain-relieving), and antipyretic (fever-reducing) effects of COX-2 inhibitors (Simon, 1999; Kirkby et al., 2016). The selective COX-2 inhibitors were developed with the explicit aim of improving the safety profile of NSAIDs, particularly by reducing gastrointestinal side effects, including stomach upset, ulcers, and increased risk of bleeding (Attiq *et al*., 2018). However, the serious side effects of COX inhibitors to cardiovascular and renal implications may need to be evaluated. Moreover, the phytochemicals specifically showed strong inhibition to the COX enzyme and pro-inflammatory response needs to be identified.

Z. myriacanthum shows diverse traditional uses in traditional medicines and culinary practices stemming from their aromatic, pungent, and often slightly numbing properties. The research is being conducted to validate and understand the mechanisms behind these traditional claims and new drug or health products. However, the compounds that provided the anti-inflammatory effects need to be identified and the other assays such animal models or clinical trials are the next steps for the efficacy and safety tests.

CONCLUSIONS

Z. myriacanthum ethanolic extract (ZL1) exhibited the specific COX-2 inhibitors using cyclooxygenase of inhibitor assay at the IC_{50} of 102.46 µg/mL. without inhibition of COX-1 at the concentration evaluated. The gene expression assay using specific primers to detect the inflammatory-response genes in LPS-induced RAW264.7 cells using the RT-PCR technique indicated that ZL1 was potent significantly inhibited COX-2 and the pro-inflammatory response genes, including IL-1β, IL-6 and TNF-α at the doses of 12.50, 25 and 50 µg/mL.

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