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Research article

In vitro Anti-inflammatory effects of Thai herb essential oils

Nancharee Homnan[1],
Suchitra Thongpraditchote[2],
Mullika Chomnawang[1],
Krit Thirapanmethee[1*]

[1] Department of Microbiology, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand
[2] Department of Physiology, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand

***Corresponding author:**
krit.thi@mahidol.edu

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ABSTRACT

Inflammation is a common mechanism of the immune system that protects the host body from injuries caused by physical wounds and non-self-invaders, while chronic inflammation might cause tissue damages and is associated with many diseases such as rheumatoid arthritis, Alzheimer's disease, and various physical dysfunctions. The non-steroidal anti-inflammatory drugs (NSAIDs) are widely used because of cyclooxygenase-2 (COX-2) inhibitory function. However, the drugs can also act on COX-1 leading to an unfavorable condition such as irritation of the stomach and increase the risk of bleeding. The aim of this study was to evaluate anti-inflammatory effects of 11 Thai herb essential oils on lipopolysaccharide (LPS)-induced inflammation in phorbol 12-myristate 13-acetate (PMA) differentiated human monocyte cell-line (THP-1) cells., The COX-2 activity and level of prostaglandin E2 (PGE2) were examined. The results showed that among 11 Thai herb essential oils, lesser galanga essential oils exhibited the significant COX-2 and PGE2 inhibitory activities. β-ocimene, the major compound of lesser galanga essential oil, was further evaluated for the anti-inflammatory effects. It was clearly demonstrated that β-ocimene effectively inhibited COX-2 activity and lowered PGE2 level in a dose-dependent manner with IC_{50} of 75.64 and less than 20 µg/mL, respectively. It was noteworthy that lesser galanga essential oil possessed higher inhibitory effect on COX-2 activity and PGE2 levels than its major compound, β-ocimene. In conclusion, essential oil of lesser galanga and its major compound could be potentially developed as the anti-inflammatory agents.

1. INTRODUCTION

Inflammation is a common mechanism of the immune system that protects against injuries caused by physical wounds and non-self-invaders. The inflammation response is lead to vascular changes and white blood cell recruitment at the site which has been invaded or can be stimulated throughout the body. During inflammation, white blood cells secrete many inflammatory cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) and inflammatory mediators such as nitric oxide (NO) and prostaglandin E_2 (PGE_2), which are generated by inducible nitric

oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), respectively[1]. Nowadays, numerous evidence have pointed out that PGE2 plays an important role in the inflammatory response because it was related to the classic signs of inflammation: redness, warmth, swelling, and pain[2]. As mention above, PGE2 is lipid mediators that produced from arachidonic acid via both isoforms of COX enzymes (COX-1 and COX-2). Arachidonic acid released from the phospholipid membranes by phospholipase A2 (PLA2) was converted by COX-2 into prostaglandin H2 (PGH2) and finally prostaglandin E synthase (PGES) metabolite into PGE2. Then, PGE2 acts as vasodilators to promote vascular permeability and increased blood flow into the inflamed tissue leading to redness, swelling and edema. Furthermore, warmth and pain resulted from the action of PGE2 stimulates peripheral sensory neurons and the preoptic area of the hypothalamus to promote pyrogenic effects[2]. Although, the purpose of the inflammatory process is to eradicate the invaders and start repairing process to maintain general physiological functions, long-term inflammation might cause tissue damages and associated with many diseases such as rheumatoid arthritis, inflammatory bowel disorders, Alzheimer's disease and also cause of various physical dysfunctions. Non-steroidal anti-inflammatory drugs (NSAIDs) are common medications used to treat inflammation. Their mechanisms were inhibited COX activity lead to decrease prostaglandins biosynthesis. The anti-inflammatory mechanism of NSAIDs was COX-2 inhibition, while inhibition of COX-1 can cause unfavorable conditions such as irritation of the stomach lining and increase the risk of bleeding[3].

Thai herbs had been used for medicinal purposes for a long time. Some of them were traditionally used as anti-fever and topical anti-inflammatory drugs. There are several reports about the anti-inflammatory effect of Thai herbs, such as extract of *Gynura pseudochina* var. hispida (Asteraceae) and *Oroxylum indicum* (Bignoniaceae) showed anti-inflammatory activities via inhibiting nuclear factor kappa B (NF-κB) and PGE2 in HeLa cells. *Muehlenbeckia platyclada* (Polygonaceae) extract also inhibited pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) in HeLa cells as well[4]. The essential oil of *Cinnamomum osmophloeum* twigs displayed inhibitory effects on PGE2 production in LPS-activated RAW 264.7 macrophages[5]. *Ocimum americanum* Linn. (Lamiaceae) essential oil was contained linalool and 1,8-cineole that blocked leukocyte influx into the synovial space, reduced paw edema and inhibited interferon-γ levels in zymosan-induced paw edema[6]. *Vetiveria zizanioides* (Poaceae) essential oils demonstrated anti-inflammatory effect of LPS-induced RAW 264.7 macrophages by regulating the expression of the inflammation related enzymes heme oxygenase-1, iNOS and COX-2 and the inflammatory cytokines. Moreover, *V. zizanioides* essential oil shown the anti-inflammatory activity correlated with its antioxidant activity of decreasing LPS-induced superoxide anion production and malondialdehyde levels[7]. Even though several studies have reported the various anti-inflammatory effect of essential oil but the reports on actions of Thai herbs essential oil on the inflammatory activity in human cells have been limited. In addition, most of the *in vitro* anti-inflammatory experiments were performed in mouse monocyte cell line (RAW264.7), which probably shows different results compared with human cell line. Therefore, the purpose of this study was to evaluate the anti-inflammatory effects of 11 Thai herbs essential oils on lipopolysaccharide (LPS) induced inflammation in human monocyte (THP-1) cell line.

2. MATERIALS AND METHODS

2.1. Chemical reagents

Phorbol 12-myristate 13-acetate (PMA), Lipopolysaccharide (LPS) from *Escherichia coli* 055:B5, ocimine, and celecoxib were purchased from Sigma Chemical, USA. RPMI1640 medium and penicillin/streptomycin solution were obtained from GIBCO BRL, USA. 2-Mercaptoethanol was purchased from BDH, UK. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from USB, USA. Dimethyl sulfoxide (DMSO) and fetal bovine serum (FBS) were purchased from Fluka chemical, Germany and PAA, Germany respectively.

2.2. Culture of THP-1 cells

The human monocytes cell line, THP-1, was obtained from American Type Culture Collection

(ATCC). This cell was cultured in RPMI 1640 medium containing 0.05 mM 2-mercaptoethanol, 2.2 g/L sodium bicarbonate and 0.99 g/L glucose supplemented with 10% fetal bovine serum and penicillin/streptomycin at 37 °C and 5% CO_2 in a humidified incubator. The cell was subculture every 2 or 3 days. Recommended used less than 20 passages. THP-1 was differentiated to macrophage by incubating with PMA at concentration 40 ng/mL for 48 hours prior to all experiments.

2.3. Preparation of essential oils and test compounds

Eleven essential oils were purchased from Thai-China Flavours and Fragrances Industry Co., Ltd. Scientific name and part used of each herb was shown in Table 1. All essential oils were dissolved in DMSO while ocimene was dissolved in absolute ethanol and store at -20ºC until used. The working solutions of test compounds were freshly prepared by diluting with FBS free RPMI 1640 medium to achieve the desired concentrations for each experiment. The final DMSO and ethanol concentration in the working solution would not exceed 0.1% v/v.

2.4. Cytotoxic assay

THP-1 at concentration 1 x 10^6 cells/well were cultured in RPMI 1640 medium containing 3% FBS and 40 ng/mL of PMA in the 24-well plates for 48 hours. After wash with PBS and cultured in PMA- free medium for 24 hours, each test compound was added and further incubated for 1 hour. After that, 1 µg/mL of LPS was added to each well and incubated at 37°C, 5% CO_2, and 90% relative humidity incubator for 24 hours. Then, cell was washed with PBS and 500 µl of 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide solution (MTT, 5 mg/mL) was added and further incubated for 2 hours at a 37°C, 5% CO_2 and 90% relative humidity incubator. After incubation, the formazan crystal was completely solubilized by adding 500 µl of DMSO to each well and gently shaking for 30 minutes at room temperature. The absorbance value at wavelength 570 nm was measured in a microplate reader (Tecan) using wavelength 650 nm as a reference value. The treated cells, untreated cells and blank were assayed in triplicate for three individual experiments. The relative cell survival was calculated by the following formula:

$$\text{Relative cell survival (\%)} = (\text{(A treated-A blank)})/((\text{A untreated-A blank}))\times 100$$

A treated = Absorbance of wells containing THP-1 and test compound 1

A untreated = Absorbance of wells containing THP-1 and solvent

A blank = Absorbance of wells containing only test compound

Table 1. List of Thai herb essential oils and part used.

Essential oil	Scientific name	Part used
Kaffir lime	*Citrus hystrix* DC.	leaves
Phlai	*Zingiber cassumunar* Roxb.	rhizomes
Galanga	*Alpinia galanga* (L.) Wild.	rhizomes
Holy basil	*Ocimum tenuiflorum* L.	leaves and flowering shrub
Hairy basil	*Ocimum americanum* L.	leaves
Guava	*Psidium guajava* L.	leaves
Lesser galanga	*Boesenbergia pandurata* (Roxb.) Schltr.	rhizomes
Betel	*Piper betle* L.	leaves
Sweet basil	*Ocimum basilicum* L.	leaves and flowering shrub
Lemongrass	*Cymbopogon citratus* (DC.) Stapf.	leaves
Turmeric	*Curcuma longa* Linn.	rhizomes

2.5. Assay of COX-2 activity

THP-1 cells (1×10^7 cells/mL) were seeded in 100 mm culture dish with RPMI 1640 medium containing 3% FBS and 40 ng/mL of PMA for 48 hours. The differentiated cell was washed with PBS and culture in PMA- free medium for 24 hours. The desired concentration of test compounds and essential oil was adding to each dish and further incubated for 1 hour. The inflammation was induced by adding 1 µg/mL of LPS and incubated for 24 hours. DMSO and Celecoxib were used as negative and positive control, respectively. After incubation, cultured medium was collected and kept at -80°C for PGE_2 determination and treated cells were harvested and lysed using Soniprep 150 Ultrasonic in cold Tris-HCl pH 7.8 containing 1 mM EDTA and centrifugation at 10,000g for 15 minutes at 4°C. Then, all supernatant were subjected to measure COX-2 level by COX Activity Assay kit (Cayman Chemical, Ann Arbor, MI, USA). The measurement was carried out according to the manufacturer's manual. The COX-1 inhibitor (SC-560) was added to subtract absorbance from total COX activity. The total COX and COX-2 activity were determined as percent inhibition which calculated by the formula from kit's instruction.

$$\text{Total COX Activity} = \frac{(\triangle\ \text{A590/5min})}{(0.0826\ \mu\text{M} - 1)} \times \frac{0.21\ \text{mL}}{0.04\ \text{mL}} \div 2 = \text{nmol/min/mL (U/mL)}$$

$$\%\ \text{Inhibition} = 100 - \%\ \text{Activity}$$

While

After that, the IC50 was determined using Graph Pad Prism software (Graph Pad Inc, San Diego, USA) with Equation: log concentration (inhibitor) vs. normalized response -- Variable slope.

2.6. Assay of Prostaglandin E2 (PGE2) production

The PGE2 level in the cultured medium of treated THP-1 was determined by enzyme Immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA). The measurement was performed according to the manufacturer's instructions. Briefly, PGE2 in the supernatants of each sample and PGE2-acetylcholinesterase conjugate (PGE2 Tracer) in constant concentration were competitive binding to PGE2 monoclonal antibody that pre-coated on microplate well. The plate was washed by wash buffer to remove all unbound reagents and added Ellman's reagent into each well. Then, the absorbance at a wavelength between 405 to 420 nm was measured. The effects on PGE2 productions were determined by comparison with a standard curve and represent as PGE2 concentration. Finally, PGE2 level was calculated and reported as percent inhibition.

2.7. Statistical analysis

All results were expressed as mean± standard deviation (SD) from 3 independent experiments. The data were statistical analysis by one way ANOVA.

3. RESULTS AND DISCUSSION

3.1. Cytotoxicity of 11 species Thai herb essential oils

In this study, 11 Thai herbs that traditionally used for anti-inflammatory was selected to evaluate their activity *in vitro*. The cytotoxicity concentrations of 11 species Thai herbs essential oils on differen tiated THP-1 were examined by MTT assay. Differentiated THP-1 was cultured in 96-well plate with different concentrations (25–200µg/mL) of each essential oil for 24 h. followed by incubation with the MTT reagent and solubilizing detergent. The results showed that all essential oils did not significantly decrease the viability of differentiated THP-1 cells (Fig. 1). At concentration 200 µg/mL of kaffir lime leave and galanga, Holy basil essential oils demonstrated no obvious toxicity (percent viability more than 90%), while cell treated with Phlai, Hairy basil, Guava leave, Lesser galanga, Turmeric, Betel, Sweet basil and Lemongrass essential oils were survived at least 76.82 % compared with untreated cells. In addition, all essential oils at concentration 100 µg/mL demonstrated more than 95% viability (Fig. 1). Therefore, 100µg/mL of essential oils was selected as the optimal treated concentration and used in the further study.

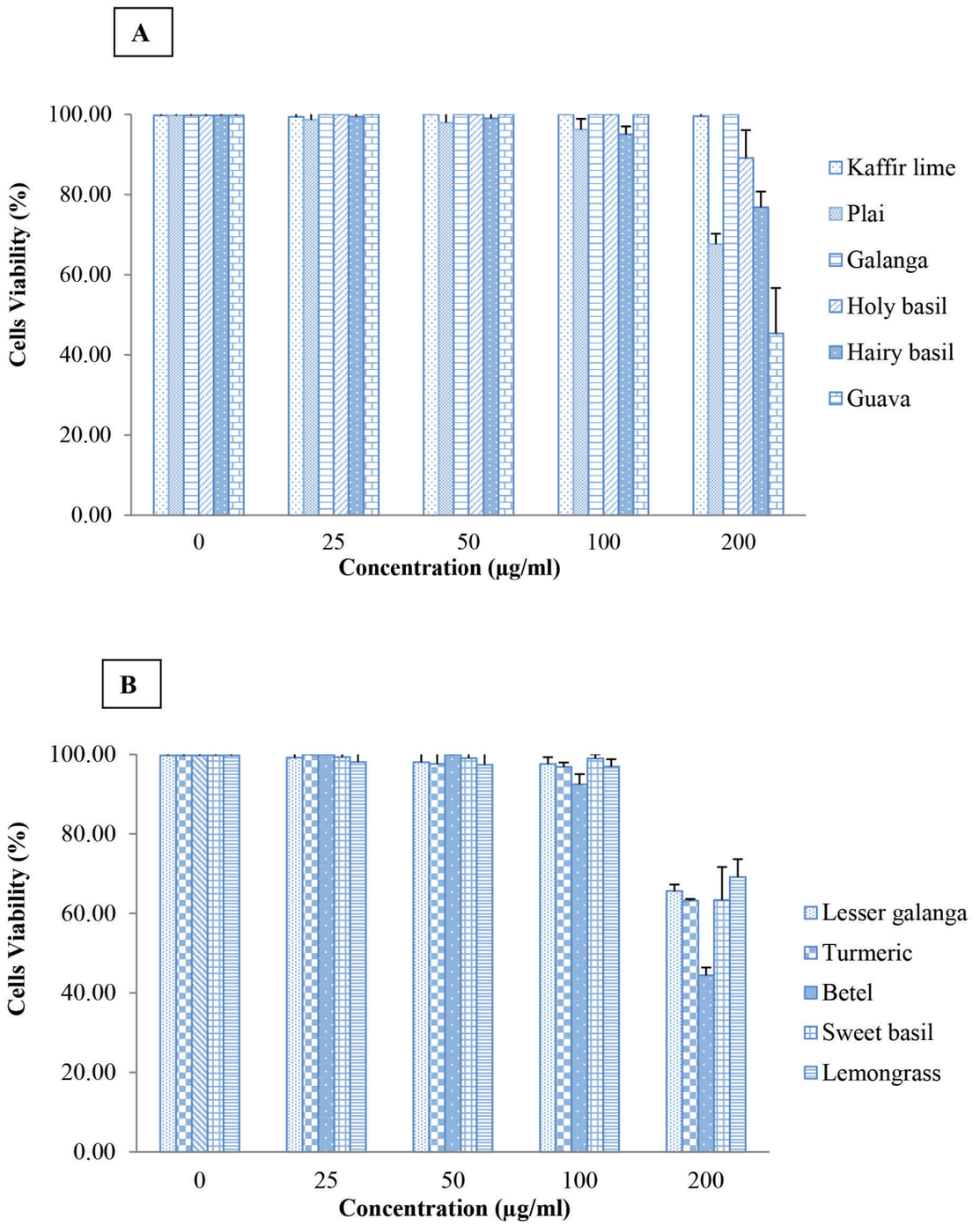

Figure 1. The effects of 11 species Thai herbs essential oils on differentiated THP-1 viability were evaluated by MTT assay. A) kaffir lime leave oil, phlai oil, galanga oil, holy basil oils, hairy basil oil, and guava oil; B) lesser galanga oil, turmeric oil, betel vine oil, sweet basil oils, and lemongrass oil. Each data presents the mean ±SD from 3 independent experiments

3.2. Effects on cyclooxygenase 2 (COX-2) activity

To investigate the anti-inflammatory effect of 11 species Thai herbs essential oils on LPS induced inflammation in PMA differentiated THP-1 macrophage, we first examined the effect on COX-2 activity. The differentiated THP-1 was treated with each Thai herb essential oils (100µg/mL) for 1 hour. After that, inflammatory response was induced by incubated with 1 µg/mL of LPS for 24 hours. Treated cells were harvested to determine COX-2 levels by COX activity assay kit.

The percentage inhibition of COX-2 in each treatment was demonstrated in Figure 2. The results showed that lesser galanga and turmeric essential oil at concentration 100µg/mL were significantly inhibited COX-2 activity with 100% inhibition compared with negative control (DMSO), whereas kaffir lime, phlai, galanga, holy basil, hairy basil, guava, betel, sweet basil, and lemongrass essential oil gave 0, 34.53, 89.09, 39.99, 30.89, 54.54, 26.53, 22.08 and 50.57% inhibition, respectively.

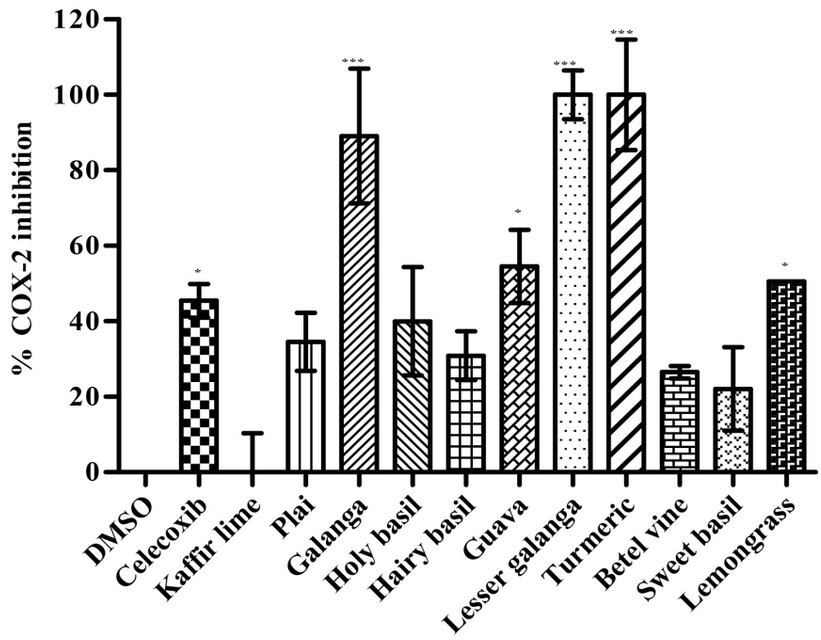

Figure 2. Effect of 11 Thai herb essential oils on COX-2 levels in LPS-stimulated THP-1 cells. Each data presents the mean ± SD from 3 independent experiments (*p<0.05, **p<0.01 and ***p<0.001, when compared to DMSO and LPS treated cell)

3.3. Effects on Prostaglandin E2 (PGE2) levels

The effect of 11 species Thai herb essential oils on prostaglandin E2 (PGE2) levels were also investigated. In this experiment, the culture media of treated cells were collected to measure PGE2 levels using PGE2 enzyme immunoassay kit. The quantity of PGE2 was determined by comparison to the PGE2 standard curve and calculated in percent inhibition.

As shown in Figure 3, at concentration 100 µg/mL most of 11 Thai herb essential oils were inhibited PGE2 production more than 50% except sweet basil oil. betel vine and holy basil essential oil showed the most potent inhibitor of PGE_2 production with up to 99% inhibition while phlai, lesser galanga, and turmeric exhibited 94.30%, 93.12% and 90.6% inhibition, respectively. Together with the previous experiment, lesser galanga and turmeric essential oil showed the outstanding COX-2 and PGE2 inhibitory effect.

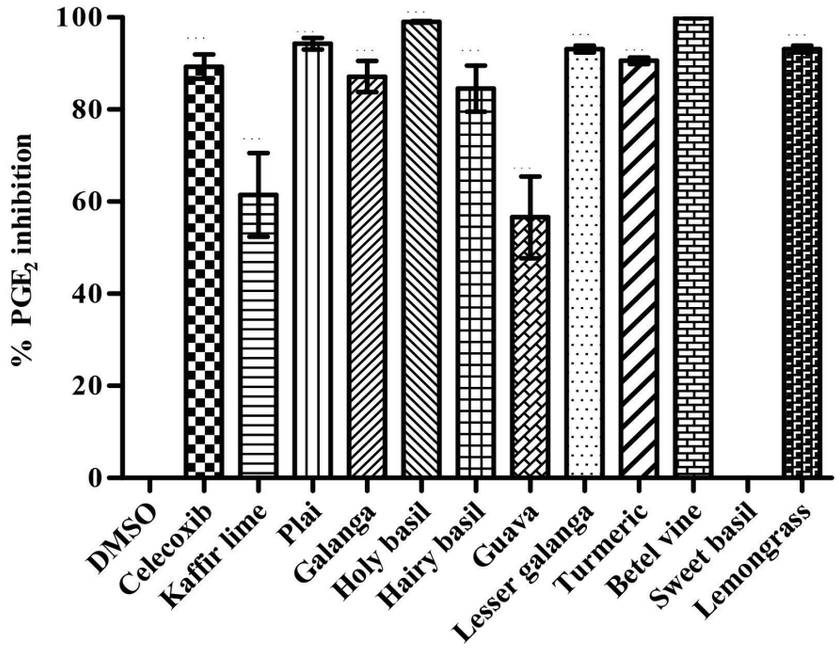

Figure 3. Effect of 11 Thai herb essential oils on PGE2 levels in LPS-stimulated THP-1 cells. Each data presents the mean ± SD from 3 independent experiments (*p<0.05, **p<0.01 and ***p<0.001, when compared to DMSO and LPS treated cell).

Lantz et al. were reported the effect of turmeric extracts on inflammatory mediator production in LPS induced HL-60 cells. Their results showed that turmeric oil was potentially inhibited PGE2[8]. Several studies have reported the major constituent in turmeric oil was curcumin and turmerone, which responsible for its anti-inflammatory effect[9, 10]. There were a few studies mentioned about anti-inflammatory effect of lesser galanga extract but the effects of lesser galanga essential oil were yet to be reported. For this reason, lesser galanga essential oil and their main constitutes were selected to evaluate the anti-inflammatory effects. Chahyadi and colleagues reported that essential oils of lesser galanga were contained several interesting flavonoids that displayed many pharmacological activities[11]. Panduratin A isolated form lesser galanga was potentially inhibited both nitric oxide and PGE2 production and showed concentration-dependent suppressed both inducible nitric oxide synthase (iNOS) and COX-2 enzyme expression in LPS-stimulated RAW 264.7 cells[12, 13].

In this study, lesser galanga essential oil inhibited COX-2 activity and PGE2 levels in a dose-dependent manner with IC_{50} values of 51.2 µg/mL and 41.01 µg/mL, respectively. Lesser galanga essential oil was contained camphene (8.30%), β-ocimene (27.29%), D(+)-camphor (16.23%), geraniol and methyl cinnamate (3.83%)[14]. The anti-inflammatory effects of these pure compounds have never been reported. Since β-ocimene and D(+)-camphor are the major constituents of lesser galangal essential oil, they were expected to be an active compound in anti-inflammatory effect. Then, the effect of ocimene and D(+)-camphor on inflammatory mediators was investigated.

3.4. The anti-inflammatory effects of Ocimene and D(+)-Camphor

The differentiated THP-1 was incubated with various concentrations (20-100µg/mL) of ocimene and D(+)-camphor before exposed to LPS. THP-1 treated with ocimene concentration 20-100 µg/mL displayed 0, 4.2, 9.07, 47.46 and 77.44% COX-2 inhibition, respectively. COX-2 inhibitory effect of D(+)-camphor were 30.87, 69.89, 45.93, 62.52 and 78.34, respectively (Figure 4A). It was remarkable that ocimene was reduced COX-2 activity in a dose-dependent manner but D(+)-camphor was not. The percentage of COX-2 inhibition at concentrations 40 µg/mL

of D(+)-camphor was higher than 80 µg/mL. After that, the log dose-response curve of ocimene was plotted (Figure 4B). The result showed that IC_{50} of ocimene was 75.64 µg/mL.

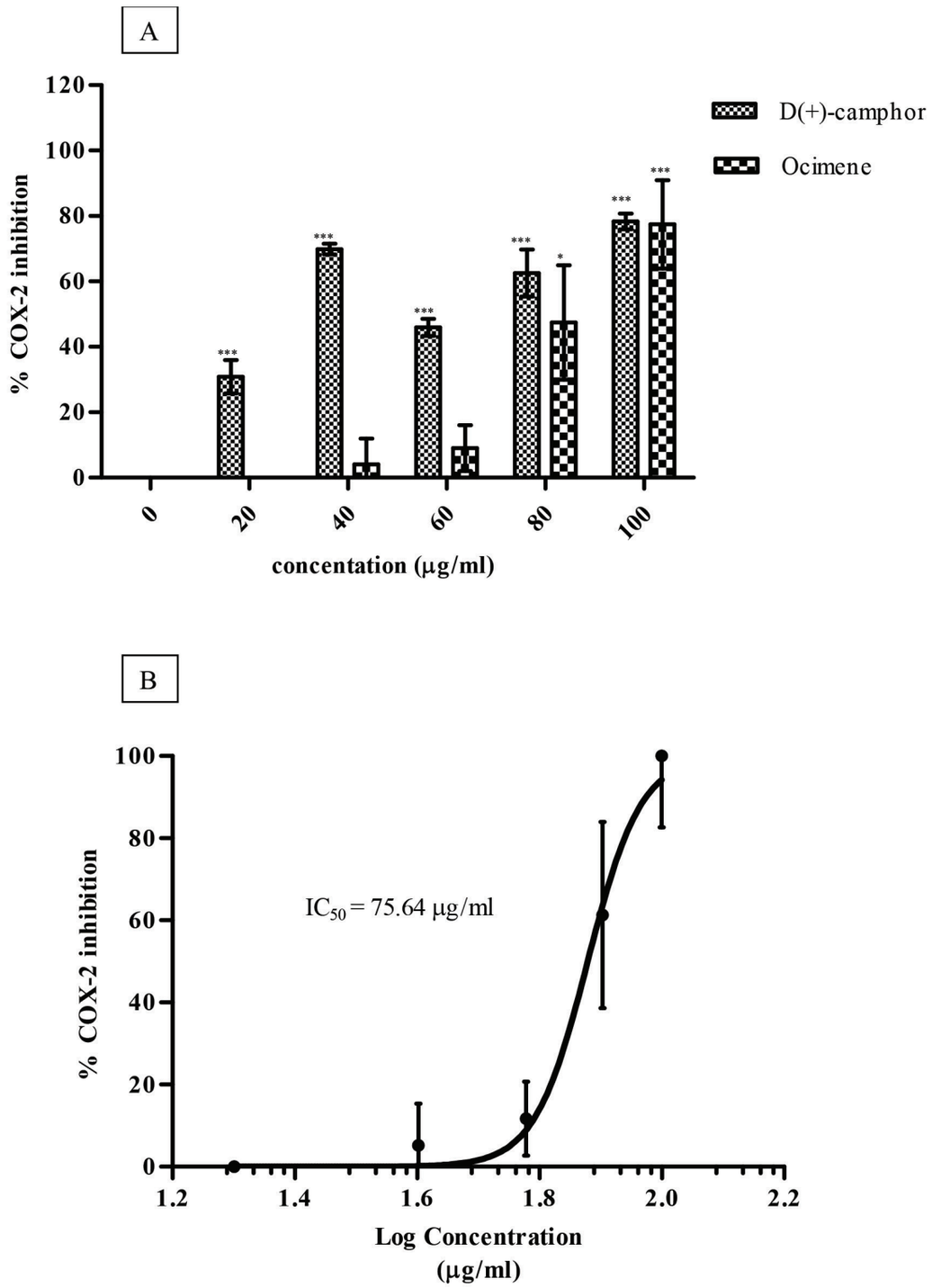

Figure 4. A) COX-2 inhibitory effect of ocimene and D(+)-camphor on LPS-stimulated THP-1 cells. Each data presents the mean ± SD from 3 independent experiments (*p<0.05, **p<0.01 and ***p<0.001, when compared to DMSO and LPS treated cell).
B) Log-dose response curve of ocimene on inhibition of COX-2 activity. The error bar for all experiments was means ± SD (n = 3).

Next, the effect of ocimene and D(+)-camphor on PGE2 level was investigated. THP-1 treated with ocimene 20-100 µg/mL showed 58.57, 68.86, 75.33, 77.24 and 85.67% PGE2 inhibition, respectively (Fig. 5). Whereas, D(+)-camphor exhibited 73.95, 86.73, 72.04, 79.96 and 79.19% PGE2 inhibition (Fig. 5), respectively. All concentration of ocimene and D(+)-camphor was significantly reduced PGE2 level more than 50%. It was remarkable that ocimene was reduced PGE2 level in a dose-dependent manner but D(+)-camphor was not. The IC_{50} of ocimene on PGE2 level was lower than 20 µg/mL. While THP-1 treated with 40 µg/mL of D(+)-camphor exhibited a higher percentage of COX-2 inhibition than 80 µg/mL.

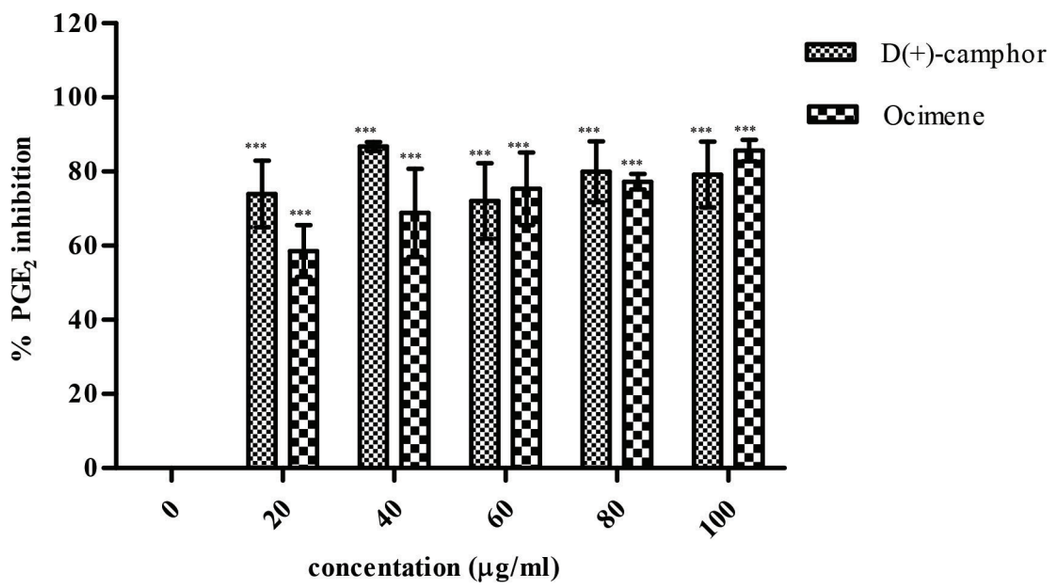

Figure 5. PGE2 inhibitory effect of ocimene and D(+)-camphor on LPS-stimulated THP-1 cells. Each data presents the mean ± SD from 3 independent experiments (*p<0.05, **p<0.01 and ***p<0.001, when compared to DMSO and LPS treated cell).

The result showed that ocimene affected COX-2 activity and PGE2 level in a dose-dependent manner. The IC_{50} of ocimene on COX-2 activity was 75.64 µg/mL while IC_{50} on PGE2 level was lower than 20 µg/mL, which not determined in this experiment. Kim and colleagues were investigated anti-inflammatory activity of Hallabong flower hydro-distilled essential oils which containing 11.07% of β-ocimene. The results showed that hydro-distilled essential oils from Hallabong flower were suppressed the LPS-induced expression of COX-2 protein on LPS-stimulated RAW 264.7. Moreover, it was inhibited PGE2 generation in a dose-dependent manner with IC50 value was less than 0.01% (1). The previous study of Arranz et al., reported that camphor from sage extract was significantly decreased TNF-α, IL-1 β and IL-6 gene expression in THP-1 activated with human ox-LDL[15]. In connection with Yoon et al. reported that camphor from *Artemisia fukudo* oil was capable to inhibit the release of TNF-α, IL-1 β, and IL-6 in LPS-treated RAW 264.7[16]. The present study showed that D(+)-camphor significantly inhibited COX-2 activity in different percent inhibition up to 78.34% but did not show dose-dependent manner. The same result was also found in the PGE2 production. All concentration of D(+)-camphor was significantly inhibited PGE2 level more than 50% but percent inhibition did not depend on concentration. Therefore, the IC_{50} of D(+)-camphor against COX-2 and PGE2 could not be determined. As mentioned above, the IC_{50} of lesser galanga oil and ocimene against COX-2 were 51.2 µg/mL and 75.64 µg/mL, respectively. These results indicated that lesser galanga essential oil had more potential inhibiting activity to COX-2 activity and PGE2 levels than ocimene. We hypothesize whether there are other active compounds in lesser

galanga essential oil that responsible for anti-COX-2. These results suggested that D(+)-camphor inhibited COX-2 with different mechanisms, compared with ocimene.

In summary, this study was performed only in an *in vitro* model. The further studies of lesser galanga essential oil, D(+)-camphor and ocimene should be performed on other mediators and action investigate *in vivo* model.

4. CONCLUSION

Among 11 Thai herb essential oils, lesser galanga and turmeric essential oils exhibited the most COX-2 and PGE2 inhibitory activity which are the key mediators of inflammation. Turmerones was well known as an anti-inflammatory compound of turmeric essential oil. However, the active compound that responsible for anti-inflammatory effects of lesser galanga essential oil has not been reported yet. Therefore, lesser galanga essential oil and their main constitutes were selected to evaluate for the anti-inflammatory effects. The results showed that lesser galanga essential oil was significantly inhibited COX-2 activity and PGE2 levels in a dose-dependent manner. The main constituent of lesser galanga essential oil included β-ocimene and D(+)-camphor which possibly responsible for its anti-inflammatory effect in this study. These results indicated that lesser galanga essential oil had more potential inhibiting activity to COX-2 activity and PGE2 levels than ocimene. This study demonstrated that essential oil of lesser galanga and its major compound could be potentially developed as the anti-inflammatory agents.

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Conflict of interest (If any)

The authors declare that there are no conflicts of interest.

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Ethical approval

None to declare

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