Research Article

Anti-Inflammatory Investigations of Extracts of Zanthoxylum rhetsa

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Zanthoxylum rhetsa has been consumed in the diet in northern Thailand and also used as a medicament in ancient scripture for arthropathies. Thus, this study aimed to evaluate the activity of various extracts from differential parts of Z. rhetsa via inhibition of inflammatory mediators (NO, TNF-α, and PGE2) in RAW264.7 macrophages. The chemical composition in active extracts was also analyzed by GC/MS. The parts of this plant studied were whole fruits (F), pericarp (P), and seed (O). The methods of extraction included maceration in hexane, 95% ethanol and 50% ethanol, boiling in water, and water distillation. The results demonstrated that the hexane and 95% ethanolic extract from pericarp (PH and P95) and seed essential oil (SO) were the most active extracts. PH and P95 gave the highest inhibition of NO production with IC₅₀ as 11.99 ± 1.66 μg/ml and 15.33 ± 1.05 μg/ml, respectively, and they also showed the highest anti-inflammatory effect on TNF-α with IC₅₀ as 36.08 ± 0.55 μg/ml and 34.90 ± 2.58 μg/ml, respectively. PH and P95 also showed the highest inhibitory effect on PGE2 but less than SO with IC₅₀ as 13.72 ± 0.81 μg/ml, 12.26 ± 0.71 μg/ml, and 8.61 ± 2.23 μg/ml, respectively. 2,3-Pinanediol was the major anti-inflammatory compound analyzed in PH (11.28%) and P95 (19.82%) while terpinen-4-ol constituted a major anti-inflammatory compound in SO at 35.13%. These findings are the first supportive data for ethnomedical use for analgesic and anti-inflammatory activity in acute (SO) and chronic (PH and P95) inflammation.

1. Introduction

Pain is a common symptom and sign of inflammation and tissue damage [1–3]. Etiology including physical, biological, and chemical factors such as trauma, overuse, chemical, toxins, and pathogens can activate inflammatory response [1]. Inflammation is a response to protect and restore cells and tissues to a normal state [4]. The stimulus activates leukocytes to produce inflammatory cytokines such as tumor necrosis factor-α (TNF-α) [1]. In a site of tissue injury, prostaglandin E₂ (PGE₂) plays an important role in acute inflammation and causes vasodilatation edema, acute pain, and fever [5]. TNF-α is an inflammatory cytokine that is intertwined with PGE₂ as it stimulates phospholipase A₂ and releases eicosanoids from the cyclooxygenase and lipooxygenase pathways in arachidonic acid metabolism [5]. The important product from cyclooxygenase is PGE₂ [5]. Additionally, high levels of TNF-α can trigger fever and activate endothelial cells to express adhesion molecules resulting in leukocytes adherence and prolonged inflammation [6].
Macrophages trigger production of TNF-α cytokines causing pain and fever, loss of cell function, or loss of mobility in joints [2]. TNF-α can also activate macrophages to produce nitric oxide (NO) [7]. NO is a free radical derived from L-arginine and oxygen by inducible nitric oxide synthase (iNOS) enzyme from macrophages [8]. NO induces toxicity by interaction with superoxide and produces peroxynitrite which is highly toxic to microorganisms and normal neighboring cells [8]. Cells and tissues are gradually destroyed by excessive NO production, and as a result, the perception of pain remains.

Although the outcome of inflammatory responses involves physiological functions to protect and restore cells and tissues to a normal state, excessive inflammatory response is the cause of persistent inflammation and leads to chronic inflammation and pain [9, 10]. The impact of chronic inflammation involvement in chronic diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis, osteoarthritis, cancer, and cardiovascular diseases is well known [10].

Herbal remedies have been used for their anti-inflammatory and pain-relieving properties according to folk wisdom and in traditional ethnomedicine for centuries. According to Thai traditional medicine principles, herbs which have a spicy taste and pungent aroma such as cap-sicum, ginger, and plai (Zingiber cassumunar) are often used for pain relief [11].

The chemotaxonomy study of some Zanthoxylum species such as Z. anacanthodium, Z. nitidum, and Z. myricacanthum are found in Northern Thailand [12, 13] or Z. budrunga, Z. bungeanum, and Z. schinifolium, all have shown anti-inflammatory and antinociceptive action [14–17]. Zanthoxylum rhetsa is a pungent plant and a member of the Rutaceae family. Its whole fruit consists of pericarp and seed and is used in the diet in the Northern part of Thailand. Both pericarp and fruit are described in Pra-O-Sod-Pra-Narai scripture and Thai Traditional Household Remedy for muscle spasm, a pain relief from swelling of muscle and tendons and also as pain relief from abscesses and hemorrhoids [11, 18]. Z. rhetsa fruit is also extensively used as an anti-inflammatory agent and anti-septic in India [19]. Z. rhetsa fruit and seed are also used as a pain relief treatment from toothache, digestion problems, inflammation, and infection in Southeast Asia [19]. Z. rhetsa activity is a mosquito repellent, and its larvicidal, antimicrobial, antioxidant, and antitumor activities have been characterized [20]. Additionally, major chemical compounds in pericarp, fruit, and seed of Z. rhetsa as monoterpenes such as limonene, terpinen-4-ol, sabinene, and α-pinene [21–30] have been reported for their anti-inflammatory activity [31–33].

Therefore, the present study compared and investigated the anti-inflammatory activity of various anatomical parts such as whole fruits, pericarp, and seed of Z. rhetsa extracts through the inhibition of lipopolysaccharide- (LPS-) induced NO, TNF-α, and PGE2 in RAW264.7 macrophages. Additionally, chemical compositions of the active extracts were also delineated as anti-inflammatory, and pain relief activity of Z. rhetsa has been poorly studied [21–23]. Furthermore, the analysis of chemical constituents in pericarp, fruit, and seed of Z. rhetsa of various extractions and characterizing the anti-inflammatory activity has not been undertaken [31–33].

2. Materials and Methods

2.1. Plant Materials. Z. rhetsa was collected from its natural habitat in Ban Mae Khaw Tom Thasud village, Muang district, Chiang Rai province, Thailand. The voucher specimen was identified by using important characteristic of the morphology of both flower and fruit. After that, the scientific name of plant material was identified by botanists in the Department of National Parks, Wildlife and Plant Conservation, Bangkok, Thailand. The voucher specimen BKF number 193835 was preserved in the office of the Forest Herbarium, Bangkok, Thailand.

2.2. Chemicals and Reagents. Ethanol 95% (EtOH) (commercial grade) was purchased from C.M.J. Anchor Company (Thailand). Analytical grade dimethyl sulfoxide (DMSO), hexane, hydrochloric acid (HCl), and isopropanol were purchased from RCI Labscan (Thailand). Distilled water was produced by Milli-Q water purification system from Millipore (USA). Griess reagent (1% sulfanilamide and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride in 2.5% phosphoric acid), thiazolyl blue tetrazolium bromide (MTT), lipopolysaccharide (LPS) from E.coli (O55:BS), and prednisolone were purchased from Sigma-Aldrich (USA). Fetal bovine serum (FBS), penicillin-streptomycin (P/S), RPMI 1640 medium, and Dulbecco’s modified eagle medium (DMEM) were purchased from Gibco (USA). The prostaglandin E2 ELISA kit was purchased from Cayman Chemical (USA), and Mouse TNF-α Quantikine ELISA test kit was purchased from R&D System Inc (USA).

2.3. Preparation of Extracts. After plant materials were sun-dried, they were separated into pericarp, fruit, and seed. Each part was ground to coarse powder and then was extracted by 3 methods consisting of maceration with hexane, 95% EtOH and 50% EtOH, water distillation, and decoction.

For maceration: each part powder (1 kg) was extracted by maceration with different solvent for three days (solvent: powder ratio = 2:1) and filtered through Whatman no.1 filter paper. The marc was remacerated twice, and the combined filtrate was evaporated by rotary evaporator to give the hexane extract, 95% ethanolic extract, and 50% ethanolic extract of pericarp (PH, P95, and P50), fruit (FH, F95, and F50), and seed (SH, S95, and S50), respectively.

For water distillation: each part powder (500 g) was distilled in a Clevenenger apparatus for 100 minutes and the essential oil was collected and gave the essential oil from pericarp (PO), fruit (FO), and seed (SO).

For decoction: each part of powder (500 g) was boiled in distilled water for 15 minutes and filtered. The residue had twice repeated decoction, and the combined filtrate was reduced to 1/3 by boiling then freeze dried to give the water extract from pericarp (PW), fruit (FW), and seed (SW). All crude extracts showed percentage of yield on Figure 1. The
2.4. Cell Culture and Culture Media. RAW 264.7 macrophages from mouse (Mus musculus) were purchased from American Type Culture Collection (ATCC®TIB-71) (USA). Cells were cultured in two types of media according to assays: (1) RPMI 1640 medium for the assay of inhibition of LPS-induced nitric oxide (NO) and tumor necrosis factor-α (TNF-α) production following the established method [34] and the procedure in the manufacturer’s manual [35], respectively, and (2) DMEM medium for the assay of inhibition of LPS-induced prostaglandin E₂ (PGE₂) production following the method of the procedure in the manufacturer’s manual [36]. Each medium was supplemented with 10% FBS and 1% P/S (100 unit/ml) and incubated in an incubator at 37°C, 5% CO₂, and 95% humidity.

2.5. Determination of Cell Viability. Cell viability was done in triplicate by using MTT assay [34]. Briefly, after 1 × 10⁵ cells/well of RAW 264.7 macrophages were seeded in sterilized 96 well-plate (100 μl/well) and incubated for 24 h, the medium was removed and replaced with 100 μl/well of fresh medium. Various dilutions of samples were added (100 μl/well) and incubated for another 24 h. Subsequently, the supernatants (100 μl/well) were removed, and the viable cells were determined by adding 10 μl/well of the MTT solution (5 mg/ml) and further incubated for 2 h. The medium was then removed and replaced with 100 μl/well of isopropanol containing 0.04 M HCl to dissolve formazan in the cells. The absorbance was measured by microplate reader at 570 nm. Cell viability that was higher than 70% compared with control (control medium for water extracts and control solvent: 0.2% DMSO of final concentration for crude extracts, essential oils, and prednisolone) indicated that the activity of the tested samples was not due to cytotoxicity [34]. The percentage of cell viability was calculated by using the following equation:

\[
\text{% cell viability} = \left( \frac{\text{OD sample}}{\text{OD control}} \right) \times 100,
\]

where OD = optical density; OD sample = mean of sample ODs; OD control = mean of control ODs.

2.6. Anti-Inflammatory Activities

2.6.1. Determination of Inhibition of LPS-Induced NO Production. The determination of inhibitory effect of LPS-induced NO production was done in triplicate following the protocol of an established method [34]. Briefly, 100 μl/well of RAW 264.7 macrophages (1 × 10⁵ cells/well) were seeded in sterilized 96 well-plate and incubated for 24 h, and then the medium was removed and replaced with 100 μl/well of fresh medium containing LPS (2 ng/ml of final concentration). Various dilutions of samples were added (100 μl/well) and incubated for another 24 h. Subsequently, a 100 μl/well of supernatant was transferred into a nonsterilized 96 well-plate and added with Griess reagent (100 μl/well). The absorbance of the mixed solution was measured by microplate reader at 570 nm. The result of the tested sample was compared with that of prednisolone, a positive control. The percentage of the inhibition of LPS-induced NO production was calculated by using the following equation, and IC₅₀ values were calculated by using GraphPad Prism software (CA, USA):

\[
\text{% inhibition} = \left( \frac{\text{OD control} - \text{OD sample}}{\text{OD control}} \right) \times 100,
\]

where OD = optical density; OD control = mean of control ODs (+LPS) – mean of control ODs (−LPS); OD sample = mean of sample ODs (+LPS) – mean of sample ODs (−LPS).
2.6.2. Determination of Inhibition of LPS-Induced TNF-α Production. The inhibition of LPS-induced TNF-α production was determined by using Mouse TNF-α Quantikine ELISA test kit following the procedure in the manufacturer’s manual [35]. Briefly, RAW 264.7 macrophages (1 × 10^6 cells/well) were seeded in sterilized 96 well-plate (100 μl/well) and incubated for 24 h; then, the medium was removed and replaced with 100 μl/well of fresh medium containing LPS at 2 ng/ml final concentration. Various dilutions of samples were added (100 μl/well) and incubated for another 24 h. After incubation, the supernatant (50 μl/well) was transferred into 96 well-plate of ELISA kit and it was carried out according to the method in the manufacturer’s manual [35]. The absorbance was measured at 450 nm by using the microplate reader. The result of the tested sample was compared with that of prednisolone, a positive control. The experiment was conducted in triplicate. The percentage of the inhibition of LPS-induced TNF-α production was calculated by using the following equation, and IC_{50} values were calculated by using GraphPad Prism software (CA, USA):

\[
\text{% inhibition} = \left( \frac{\text{OD control} - \text{OD sample}}{\text{OD control}} \right) \times 100, \quad (3)
\]

where OD = optical density; OD control = mean of control ODs (+LPS) − mean of control ODs (−LPS); OD sample = mean of sample ODs (+LPS) − mean of sample ODs (−LPS).

2.6.3. Determination of Inhibition of LPS-Induced PGE_2 Production. The inhibition of LPS-induced PGE_2 production was determined by using prostaglandin E_2 ELISA Kit-Monoclonal following the procedure in the manufacturer’s manual [36]. Briefly, RAW 264.7 macrophages (1 × 10^6 cells/well) were seeded in sterilized 96 well-plate (100 μl/well) and incubated for 24 h, and then the medium was removed and replaced with 100 μl/well of fresh medium containing LPS at 5 μg/ml final concentration. Various dilutions of samples were added (100 μl/well) and incubated for another 24 h. After incubation, the supernatant (50 μl/well) was transferred into 96 well-plate of ELISA kit and the procedure carried out according to the method in the manufacturer’s manual [36]. The absorbance was measured at 412 nm by using the microplate reader. The result of tested sample was compared with that of prednisolone, a positive control. The experiment was conducted in triplicate. The percentage of the inhibition of LPS-induced PGE_2 production was calculated by using the following equation, and IC_{50} values were calculated by using GraphPad Prism software (CA, USA):

\[
\text{% inhibition} = \left( \frac{\text{mean OD sample (+LPS)} - \text{mean OD control (+LPS)}}{\text{mean OD control (−LPS)} - \text{mean OD control (+LPS)}} \right) \times 100, \quad (4)
\]

where OD = optical density.

2.6.4. Chemical Composition Analysis by Gas Chromatography/Mass Spectrometry (GC/MS). The chemical compositions of the active extracts were analyzed by using a Thermo Focus GC, Polaris Q with an autoinjector and a capillary column TG-5 slims (30 m × 0.25 mm × 0.25 μm) (Thermo Fisher Scientific). Column oven temperature was programmed using the initial temperature at 60°C and 5 min initial time and then heated at the rate of 5°C/min to 300°C and held for 5 min. The injector temperature was 200°C, helium (He) was used as the carrier gas with constant flow rate of 1.0 ml/min, and the injection volume was 2 μl (splitting ratio 1:50). The ionization energy was 70 eV. Mass spectrum of the GC/MS peak was detected by mass spectrometry and compared with library database of the National Institute of Standards and Technology (NIST 08, MD, USA) which matches the score for all compounds analyzed more than 870 would be selected [37]. Chemical composition analysis was carried out by the Herb and Thai Traditional Medicine Division, Thailand Science Park.

2.6.5. Statistical Analysis. Cell viability, percentage of the inhibition of LPS-induced NO, TNF-α and PGE_2 production, and IC_{50} were presented as mean ± standard error of means (SEM). Comparison of means between control and treatment groups was done by one-way analysis of variance followed by Dunnett’s multiple comparison test. Comparison of means in between independent treatment groups (2 groups) was analyzed by using unpaired t test. Comparison of means in multiple treatment groups (≥3 groups) was analyzed by using one-way analysis of variance followed by one-way ANOVA. The level of significant difference was p < 0.05.

3. Results

3.1. Preparation of Extract. The percentage yields of extracts and essential oils are shown in Figure 1. The pericarp showed the highest yield of extraction by three methods such as 50% ethanol, oil part, and water extract (16.47%, 14.30%, and 13.37%, respectively). The seed showed the highest yield of extraction by hexane and 95% ethanol.

3.2. Determination of Cell Viability. Cell viability after exposure to the various extracts of Z. rhetsa and prednisolone (Pred) (positive control) is presented in Figure 2(a) for inhibition of LPS-induced NO and TNF-α production and in Figure 2(b) for inhibition of LPS-induced PGE_2 production. The various extracts of Z. rhetsa and prednisolone...
showed greater than 70% cell viability at all concentrations when they were tested.

3.3. Determination of Inhibition of LPS-Induced NO Production. Anti-inflammatory activity of the various extracts of *Z. rhetsa* via the inhibition of NO production by the induction of LPS in RAW 264.7 macrophages compared with prednisolone (positive control) is shown in Table 1. PH and P95 at 50 μg/ml gave the highest %inhibition of NO production (97.15% ± 0.37 and 97.66% ± 1.12, respectively) while FH, F95, F50, and S50 at 100 μg/ml gave the highest %inhibition of NO production (91.55% ± 0.04, 93.36% ± 2.33, 82.62% ± 1.26, and 81.94% ± 2.79, respectively). These results were not significantly different from prednisolone at 50 μg/ml (96.82% ± 0.34) (Figure 3).

The extract results showed that PH and P95 had an inhibitory effect on NO production with IC₅₀ values as 11.99 ± 1.66 μg/ml and 15.33 ± 1.05 μg/ml, respectively. They were significantly different (p value < 0.01 and p value < 0.001, respectively) from prednisolone (IC₅₀ = 0.07 ± 0.001 μg/ml or 0.19 ± 0.001 μM). However, the pericarp
Table 1: Inhibitory effect and IC50 values of LPS-induced nitric oxide (NO) production in RAW264.7 macrophages of various *Zanthoxylum rhetsa* extracts.

| Part of plant | Extract and positive control | Code | Percentage of inhibition at various concentrations | IC50 (μg/ml) |
|---------------|------------------------------|------|----------------------------------------------------|--------------|
|               |                              | 100 μg/ml | 50 μg/ml | 10 μg/ml | 1 μg/ml | 0.10 μg/ml | 0.01 μg/ml |              |
| Pericarp       | Hexane                       | PH   | 97.15 ± 0.37† | 38.47 ± 8.69 | 11.70 ± 3.94 | −12.38 ± 3.61 | − | 11.99 ± 1.66** |              |
|               | 95% ethanol                  | P95  | − | 97.66 ± 1.12† | 24.46 ± 2.71 | 15.60 ± 3.58 | −15.65 ± 2.27 | − | 15.33 ± 1.05*** |              |
|               | 50% ethanol                  | P50  | 72.96 ± 1.04** | 35.78 ± 1.83 | 3.10 ± 2.50 | −1.68 ± 4.25 | − | 67.55 ± 2.22*** |              |
|               | Essential oil                | PO   | 47.75 ± 6.07*** | 15.29 ± 3.43 | −14.02 ± 1.79 | −15.51 ± 2.41 | − |              |              |
|               | Water                        | PW   | 16.23 ± 4.25*** | 8.75 ± 1.53 | − | − | − | 100*** |              |
| Fruit         | Hexane                       | FH   | 91.55 ± 3.04† | 60.95 ± 0.84 | 8.69 ± 3.68 | −5.20 ± 4.00 | − | 39.81 ± 0.53*** |              |
|               | 95% ethanol                  | F95  | 93.36 ± 3.23† | 72.35 ± 4.53 | 11.88 ± 1.01 | −14.05 ± 6.36 | − | 29.42 ± 3.05*** |              |
|               | 50% ethanol                  | F50  | 82.62 ± 1.26† | 48.32 ± 0.51 | 4.02 ± 1.80 | −2.95 ± 0.87 | − | 51.63 ± 0.43*** |              |
|               | Essential oil                | FO   | 42.23 ± 10.48*** | 15.29 ± 7.11 | −13.02 ± 3.56 | −11.27 ± 3.42 | − |              |              |
|               | Water                        | FW   | 20.97 ± 2.36*** | 8.77 ± 1.86 | − | − | − | 100*** |              |
| Seed          | Hexane                       | SH   | 45.92 ± 1.91*** | 22.75 ± 1.03 | −1.17 ± 5.23 | −11.93 ± 8.02 | − | 73.10 ± 1.55*** |              |
|               | 95% ethanol                  | S95  | 62.56 ± 0.98*** | 35.63 ± 1.36 | 2.52 ± 7.24 | −3.69 ± 8.17 | − |              |              |
|               | 50% ethanol                  | S50  | 81.94 ± 2.79† | 46.88 ± 1.10 | 5.86 ± 3.64 | −10.45 ± 8.58 | − | 54.36 ± 1.21*** |              |
|               | Essential oil                | SO   | 76.57 ± 1.91*** | 35.40 ± 3.07 | −6.95 ± 2.64 | −13.79 ± 2.95 | − | 65.34 ± 3.18*** |              |
|               | Water                        | SW   | 18.83 ± 4.06*** | 10.88 ± 3.05 | − | − | − | 100*** |              |
| Prednisolone   | Pred                         | − | 96.82 ± 0.34† | 89.32 ± 0.31 | 81.49 ± 6.98 | 72.90 ± 2.26 | 5.16 ± 1.25 | 0.07 ± 0.001 (0.19 ± 0.001 μM) |              |

The results are shown as mean ± standard error of mean (SEM) (n = 3). LPS: lipopolysaccharide; IC50: the half maximal inhibitory concentration; †: the % inhibition was not different significantly from prednisolone; **p value < 0.01, ***p value < 0.001 compared with prednisolone as a positive control; * not significantly different between PH and P95; ** significantly different (p value < 0.01) between PH, P95, and P95; † significantly different (p value < 0.05) between F95 and FH; (–) not tested.
was macerated in hexane and 95% ethanol. The results demonstrated with the whole fruits macerated in hexane and 95% ethanol showed higher activity than decoction in water and maceration in 50% ethanol. For seeds which underwent water distillation, significant anti-inflammatory activity on NO production was demonstrated compared to other extraction means. The method of extraction revealed the most activity in the pericarp on the inhibition of NO production which was demonstrated with maceration in 95% ethanol and hexane. All water extracts (PW, FW, and SW), the essential oil of both pericarp (PO) and fruits (FO), and the hexane extract of seed (SH) were not active (IC\textsubscript{50} > 100 \mu g/ml).

### 3.4. Determination of Inhibition of LPS-Induced TNF-α Production

PH and P95 at 50 \mu g/ml gave the highest % inhibition of TNF-α production (64.79% ± 0.26 and 60.46% ± 3.07, respectively) which were significantly different (\textit{p}-value < 0.001) from prednisolone at 50 \mu g/ml (89.00% ± 0.70) as the same as other extracts at 100 \mu g/ml which gave the highest %inhibition of TNF-α production which were significantly different (\textit{p} value < 0.001) from prednisolone at 50 \mu g/ml (Figure 3).

The results of IC\textsubscript{50} on inhibitory effect of TNF-α production are shown in Table 2. The pericarp which was macerated in hexane and 95% ethanol maintained inhibitory effects of NO production. PH and P95 were 36.08 ± 2.58 \mu g/ml and 34.90 ± ± 2.58 \mu g/ml, respectively, but were significantly different (\textit{p} value < 0.001) from prednisolone (IC\textsubscript{50} = 0.08 ± 0.003 \mu g/ml or 0.22 ± 0.003 \mu M). The IC\textsubscript{50} of SO (49.85 ± 4.29 \mu g/ml) was significantly different (\textit{p} value < 0.05) from PH and P95. All water extracts (PW, FW and SW) and all extracts of the seed (except for the essential oil of the seed: SO) did not have the activity on LPS-induced TNF-α production inhibition (IC\textsubscript{50} > 100 \mu g/ml).

### 3.5. Determination of Inhibition of LPS-Induced PGE\textsubscript{2} Production

SO at 100 \mu g/ml gave the highest %inhibition of PGE\textsubscript{2} production (83.70% ± 0.22) which were not significantly different from prednisolone at 50 \mu g/ml (93.20% ± 3.80), while PH and P95 at 50 \mu g/ml gave the highest %inhibition of PGE\textsubscript{2} production (71.83% ± 7.51 and 67.44% ± 2.53, respectively) which were significantly different (\textit{p} value < 0.001) from prednisolone (Figure 3).

The results on inhibitory effect on PGE\textsubscript{2} production are shown in IC\textsubscript{50} values (Table 3); SO exhibited the highest anti-inflammatory effect on PGE\textsubscript{2} with IC\textsubscript{50} as 8.61 ± 2.23 \mu g/ml and was significantly different (\textit{p} value < 0.05) from prednisolone (IC\textsubscript{50} = 0.07 ± 0.003 \mu g/ml or 0.19 ± 0.003 \mu M). The inhibitory effect on PGE\textsubscript{2} production of PH and P95 (IC\textsubscript{50} = 13.72 ± 0.8 and 12.26 ± 0.71 \mu g/ml) were not significantly different with SO but they were significantly different with prednisolone. However, its pericarp demonstrated higher anti-inflammatory activity on the inhibitory effect of PGE\textsubscript{2} production than whole fruit and seed accept only seed oil (SO). All water extracts (PW, FW, and SW) and all extracts of the seed (except the essential oil of the seed: SO) did not have the activity on LPS-induced TNF-α production inhibition (IC\textsubscript{50} > 100 \mu g/ml).

### 3.6. Chemical Composition Analysis by Gas Chromatography/ Mass Spectrometry (GC/MS)

PH and P95 showed the highest production inhibition of LPS-induced NO, TNF-α, and PGE\textsubscript{2} while SO showed the highest production
Table 2: Inhibitory effect and IC50 values of LPS-induced tumor necrosis factor-α (TNF-α) production in RAW264.7 macrophages of various Zanthoxylum rhetsa extracts.

| Part of plant | Extract and positive control | Code | Percentage of inhibition at various concentrations | IC50 (μg/ml) |
|---------------|------------------------------|------|---------------------------------------------------|-------------|
|               |                              |      | 100 μg/ml | 50 μg/ml | 10 μg/ml | 1 μg/ml | 0.1 μg/ml | 0.01 μg/ml |
| Pericarp       | Hexane                       | PH   | —         | 64.79 ± 0.26*** | 22.26 ± 1.19 | 4.24 ± 9.04 | −34.86 ± 12.07 | —         | 36.08 ± 0.55*** | a |
|                | 95% ethanol                  | P95  | —         | 60.46 ± 3.07*** | 30.49 ± 10.19 | 15.66 ± 9.88 | −16.05 ± 6.31 | —         | 34.90 ± 2.58*** | a |
|                | 50% ethanol                  | P50  | 64.53 ± 1.14*** | 45.57 ± 2.06 | 26.86 ± 0.76 | 17.22 ± 1.13 | —         | —         | 63.15 ± 3.82*** |
|                | Essential oil                | PO   | 56.24 ± 3.61*** | 30.24 ± 4.30 | 1.03 ± 0.48 | −7.37 ± 7.59 | —         | —         | 85.05 ± 3.24*** |
|                | Water                        | PW   | 21.33 ± 1.57*** | —         | —         | —         | —         | —         | >100***    |
| Fruit          | Hexane                       | FH   | 58.77 ± 2.00*** | 26.36 ± 0.55 | 0.59 ± 4.06 | −11.31 ± 8.34 | —         | —         | 88.11 ± 1.85*** |
|                | 95% ethanol                  | F95  | 58.81 ± 4.68*** | 21.74 ± 7.55 | −1.13 ± 15.71 | −16.78 ± 3.36 | —         | —         | 91.12 ± 3.42*** |
|                | 50% ethanol                  | F50  | 54.42 ± 2.59*** | 30.00 ± 4.20 | 16.52 ± 4.33 | 0.53 ± 3.97 | —         | —         | 93.54 ± 4.02*** |
|                | Essential oil                | FO   | 64.54 ± 0.70*** | 43.48 ± 3.46 | 12.06 ± 2.08 | −16.44 ± 1.98 | —         | —         | 73.22 ± 3.85*** |
|                | Water                        | FW   | 15.88 ± 0.60*** | —         | —         | —         | —         | —         | >100***    |
| Seed           | Hexane                       | SH   | 17.92 ± 2.03*** | —         | —         | —         | —         | —         | >100***    |
|                | 95% ethanol                  | S95  | 24.69 ± 1.25*** | —         | —         | —         | —         | —         | >100***    |
|                | 50% ethanol                  | S50  | 27.53 ± 1.47*** | —         | —         | —         | —         | —         | >100***    |
|                | Essential oil                | SO   | 60.41 ± 1.24*** | 50.23 ± 1.13 | 29.93 ± 0.19 | 20.31 ± 0.80 | —         | —         | 49.85 ± 4.29*** | b |
|                | Water                        | SW   | 12.02 ± 0.83*** | —         | —         | —         | —         | —         | >100***    |
| Prednisolone   |                              | Pred | —         | 89.00 ± 0.70 | 77.18 ± 0.69 | 71.01 ± 2.74 | 56.05 ± 0.08 | 28.59 ± 2.59 | 0.08 ± 0.003 (0.22 ± 0.003 μM) |

The results are shown as mean ± standard error of mean (SEM) (n = 3). LPS: lipopolysaccharide; IC50: the half maximal inhibitory concentration. *** p value < 0.001 compared with prednisolone as a positive control; a not different significantly statistic between PH and P95; b different significantly statistic (p value < 0.05) between PH, P95, and SO; (–) not tested.
Table 3: Inhibitory effect and IC50 values of LPS-induced prostaglandin E2 (PGE2) production in RAW264.7 macrophages of various *Zanthoxylum rhetsa* extracts.

| Part of plant | Extract and positive control | Code | Percentage of inhibition at various concentrations | IC50 (μg/ml) |
|---------------|-----------------------------|------|-----------------------------------------------|-------------|
|   |                          | 100 | 90 | 50 | 10 | 0.1 | 0.01 | 0.001 |
|   |                          | μg/ml | μg/ml | μg/ml | μg/ml | μg/ml | μg/ml | μg/ml | μg/ml |
| Hexane | PH | — | 71.83 ± 7.51*** | 58.44 ± 2.58*** | 34.46 ± 1.82 | 21.08 ± 0.52 | 12.38 ± 0.71*** | 4.28 ± 1.28*** | 2.27 ± 0.07 |
| 95% ethanol | F95 | 63.39 ± 1.83*** | 40.34 ± 1.77 | 21.30 ± 1.35 | 3.15 ± 0.07 | — | — | — | — |
| 50% ethanol | F0 | 52.48 ± 1.82 | 36.74 ± 0.55 | 21.30 ± 1.35 | 18.84 ± 0.77 | — | — | — | — |
| Essential oil | FO | 42.38 ± 1.28*** | 27.72 ± 1.67 | 12.80 ± 0.77 | 6.32 ± 0.07 | — | — | — | — |
| F | FW | 20.12 ± 1.03*** | 15.34 ± 1.03*** | 9.54 ± 0.10 | 6.18 ± 0.02 | — | — | — | — |
| 95% ethanol | F95 | 66.39 ± 1.03*** | 52.66 ± 0.28 | 39.33 ± 1.31 | 31.50 ± 3.95 | — | — | — | — |
| 50% ethanol | F50 | 67.51 ± 0.82 | 54.22 ± 0.71*** | 39.33 ± 1.31 | 31.50 ± 3.95 | — | — | — | — |
| Essential oil | FO | 67.07 ± 0.94*** | 54.22 ± 0.71*** | 39.33 ± 1.31 | 31.50 ± 3.95 | — | — | — | — |
| Water | FW | 14.19 ± 2.12*** | 9.32 ± 2.12*** | 6.18 ± 0.02 | 6.18 ± 0.02 | — | — | — | — |
| 95% ethanol | F95 | 66.00 ± 1.26 | 52.35 ± 0.28 | 39.33 ± 1.31 | 31.50 ± 3.95 | — | — | — | — |
| 50% ethanol | F50 | 67.51 ± 0.72 | 40.90 ± 2.76 | 22.51 ± 1.75 | 13.23 ± 0.95 | — | — | — | — |
| Essential oil | FO | 67.07 ± 0.94*** | 54.22 ± 0.71*** | 39.33 ± 1.31 | 31.50 ± 3.95 | — | — | — | — |
| Water | FW | 20.06 ± 3.06*** | 16.00 ± 3.06*** | 9.54 ± 0.10 | 6.18 ± 0.02 | — | — | — | — |

LPS: lipopolysaccharide; IC50: the half maximal inhibitory concentration. *The % inhibition was not different significantly from prednisolone; †the % inhibition was not different significantly from prednisolone, but different significantly statistic (p value < 0.01) between PH, P50, and FO; ‡not different significantly statistic between prednisolone, PH, P95, and SO.*

The table shows the results of the experiments with the *Zanthoxylum rhetsa* extracts. The % inhibition values were calculated as the mean of three independent experiments. The IC50 values were calculated using the following formula: IC50 = -log10(1/2) * dilution factor, where 1/2 is the concentration that inhibits the production of PGE2 by 50%.
inhibition of LPS-induced PGE2. Therefore, PH, P95, and SO compositions were analyzed by GC/MS (Table 4) and presented GC/MS chromatogram of PH (Figure 4(a)), P95 (Figure 4(b)), and SO (Figure 4(c)). PH and P95 contained some chemical compounds as in SO; these were γ-terpinene (0.68%, 0.79%, and 4.91%, respectively), terpinen-4-ol (1.07%, 3.38%, and 35.13%, respectively), and terpinenyl acetate (1.57%, 1.62%, and 6.65%, respectively). PH and P95 shared similar composition but different in percentages. Bicyclo(3.1.1)heptane-2,3-diol,2,6,6-trimethyl or 2,3-pinanediol (11.28%), neryl acetate (7.65%), caryophyllene oxide (7.50%), spathulenol (6.65%), and cetanol (3.78%) are constituents in top 5 of PH. Bicyclo(3.1.1)heptane-2,3-diol,2,6,6-trimethyl or 2,3-pinanediol (19.82%), 2,3-camphanediol (5.87%), durene (4.53%), piperitone oxide (4.46%), and spathulenol (4.39%) are in top 5 constituents of P95. Terpinen-4-ol was the major compound (35.13%) in SO; the next top 5 compounds were p-cymene (10.95%), terpinenyl acetate (6.65%), cuminol (5.60%), and limonene (5.48%).

4. Discussion

Pain may be acute or chronic depending on the duration of inflammatory response in the body [38, 39]. Inflammatory mechanisms assist in eliminating pathogens or stimulating wound healing in order to protect and restore cells and tissues into normal physiological functions [4]. Inflammatory responses, resulting in excessive release of inflammatory mediators and cytokines, can lead to tissue damage, chronic disease, and pain [9, 10]. Although medication can be effective for pain relief from inflammation, side effects from medication (i.e., steroid, NSAIDs, opioids, acetaminophen, etc) are significant. Herbal medicine is considered and utilized as a natural alternative for treatment of pain relief with potential to avoid some side-effects [40].

After cell and tissue damage, the body perceives pain. An acute inflammatory mechanism is induced by inflammatory mediators. PGE2 is the one of chemical mediators: histamine, substance P, bradykinin, acetylcholine, leukotrienes, and prostaglandins, resulting in heat, redness, swelling, and nociception. PGE2-induced vasodilatation in the first step of acute inflammatory mechanism leads to increase microvascular permeability and induces pain by acting on peripheral sensory neurons [41]. The inhibition of PGE2 production can be effective to reduce heat, redness, edema, and pain. In the present study, PH, P95, and SO of Z. rhetsa were the most potent groups (IC50 < 20 μg/ml) which showed the greatest potency of LPS-induced PGE2 production in RAW264.7 macrophages, while PO was the second most potent group (IC50 < 30 μg/ml); P50, FO, and P95 was in the third group for potency (IC50 < 50 μg/ml), and other extracts of Z. rhetsa were weak to inactive (IC50 > 50 μg/ml). These results indicate that whole Z. rhetsa fruit should be separated into pericarp and seed, and the inhibitory effect of PGE2 production is higher as a consequence. A previous study reported that an ethanolic extract from Z. rhetsa fruit (consisting of pericarp and seed) could inhibit COX-1 (90.80%) and COX-2 (94.40%) [21]. PGE2 is one of the products derived from cyclooxygenase pathway [5]; therefore, PH, P95, and SO may reduce acute pain from an acute inflammatory mechanism through inhibition of COX-1 and COX-2 as well as the eicosanoid product PGE2. Additionally, an in vivo study on a bioadhesive gel containing essential oil from the fruit could inhibit licking behavior, edema, and redness of the buccal cavity in rats [22] which was also due to reduced PGE2 in acute inflammation. In clinical trials, a massage oil containing essential oil from fruit relieved pain in the calf muscle compared with carrier oil (placebo) in healthy volunteers after induction by standing and heel raise [23].

Though the previous study was done on whole fruit, our study has shown that Z. rhetsa pericarp and seed could perform the same pharmacological functions. This is an important finding since it would be the preparation of this herbal medicine from pericarp or seed not only whole fruit. Although the percentage yields of SO was less (0.27%) (Figure 1), the preparation of the distillation of the seed should be studied further in order to increase its yield. The present study also showed the highest %inhibition of PGE2 production of SO at 100 μg/ml (83.70% ± 0.22) which was not significantly different from prednisolone at 50 μg/ml (93.20% ± 3.80) while PH and P95 at 50 μg/ml showed the highest %inhibition of PGE2 production (71.83% ± 7.51 and 67.44% ± 2.53, respectively) which was significantly different (p value < 0.001) from prednisolone (Figure 3), whereas IC50 values of PH and P95 were not significantly different from SO (Table 3). Our result was indicated; the preparation of anagelseic and anti-inflammatory agents in acute inflammation from PH, P95, and SO was apparent. Whereas percentage yields of PH (5.89%) and P95 (13.10%) were higher than SO (0.27%) (Figure 1). Our study is also the first report on anti-inflammatory activity of PH, P95, and SO from Z. rhetsa by the inhibition of PGE2 production in RAW 264.7 macrophages.

TNF-α is an inflammatory cytokine which releases in both acute and chronic inflammation; TNF-α induces pain and fever and plays a role in rheumatoid arthritis, osteoarthritis, and systemic lupus erythematosus [42]. Thai ethnomedicine use of Z. rhetsa was able to demonstrate the anti-inflammatory action in joints. Z. rhetsa fruit is used as an oil (named Pa-Ra-Ti-Tri) and ointment (named Bee-Pra-Sen) for treatment of muscle and joint inflammation in Thai ancient scripture (named Pra-O-Sod-Pra-Na-Rai) [18]. Additionally, our extracts were effective on TNF-α production by PH and P95, whereas SO was less active. Therefore, PH and P95 may relieve pain and inflammation via inhibition of TNF-α production. Our findings could be utilized to improve ethnomedicine use by developing a topical analgesic remedy from PH or P95 which demonstrates clinical utility.

PH and P95 also demonstrated the highest potency in the inhibition of NO production, which is a free radical synthesized by inducible nitric oxide synthase (iNOS) from macrophages with L-arginine as a precursor [8]. Increasing concentrations of nitrite in synovial fluid of joints are related to rheumatoid arthritis and osteoarthrosis [43]. Therefore, PH and P95 could protect cells and tissues from injury due to
| No. | Chemical composition   | Hexane extract from pericarp (PH) | 95% ethanolic extract from pericarp (P95) | Essential oil from seed (SO) |
|-----|------------------------|-----------------------------------|-------------------------------------------|-------------------------------|
|     |                        | RT (min) | % Area | Match score | RT (min) | % Area | Match score | RT (min) | % Area | Match score |
| 1   | Sabinene               | ND       | ND     | ND          | ND       | ND     | ND          | ND       | ND     | ND          |
| 2   | Alpha-phellandrene     | ND       | ND     | ND          | ND       | ND     | ND          | ND       | ND     | ND          |
| 3   | Alpha-terpinene        | ND       | ND     | ND          | ND       | ND     | ND          | ND       | ND     | ND          |
| 4   | p-Cymene               | ND       | ND     | ND          | ND       | ND     | ND          | ND       | ND     | ND          |
| 5   | Limonene               | ND       | ND     | ND          | ND       | ND     | ND          | ND       | ND     | ND          |
| 6   | Gamma-terpinene        | 11.47    | 0.68   | 885         | 11.42    | 0.79   | 885         | 11.47    | 4.91   | 878         |
| 7   | Linalool oxide         | ND       | ND     | ND          | ND       | ND     | ND          | ND       | ND     | ND          |
| 8   | Terpinolen             | ND       | ND     | ND          | ND       | ND     | ND          | ND       | ND     | ND          |
| 9   | 2-Methyl-1-phenylpropene | ND  | ND    | ND          | ND       | ND     | ND          | ND       | ND     | ND          |
| 10  | Linalool               | ND       | ND     | ND          | ND       | ND     | ND          | ND       | ND     | ND          |
| 11  | Terpinen-4-ol          | 15.37    | 1.07   | 898         | 15.36    | 3.38   | 898         | 15.38    | 35.13  | 905         |
| 12  | Terpinenyl acetate     | 15.81    | 1.57   | 892         | 15.80    | 1.62   | 892         | 15.82    | 6.65   | 901         |
| 13  | L-carvone              | 17.24    | 1.07   | 874         | 17.22    | 1.01   | 874         | ND       | ND     | ND          |
| 14  | Cuminal                | ND       | ND     | ND          | ND       | ND     | ND          | ND       | ND     | ND          |
| 15  | p-Cymen-3-ol           | ND       | ND     | ND          | ND       | ND     | ND          | ND       | ND     | ND          |
| 16  | Durenol                | 18.82    | 3.71   | 880         | 18.79    | 4.53   | 880         | ND       | ND     | ND          |
| 17  | Bicyclo(3.1.1)heptane-2,3-Diol, 2,6,6-trimethyl | 19.51    | 11.28 | 896         | 19.48    | 19.82  | 896         | ND       | ND     | ND          |
| 18  | Limonene oxide         | 20.11    | 2.07   | 873         | 20.09    | 4.03   | 873         | ND       | ND     | ND          |
| 19  | Nerol                  | ND       | ND     | ND          | ND       | ND     | ND          | ND       | ND     | ND          |
| 20  | Neryl acetate          | 20.95    | 7.65   | 872         | 20.92    | 4.28   | 872         | ND       | ND     | ND          |
| 21  | 2,3-Camphanediol       | 21.46    | 2.57   | 880         | 21.44    | 5.87   | 880         | ND       | ND     | ND          |
| 22  | 7-Tetradecene          | 21.76    | 1.81   | 873         | 21.73    | 0.91   | 873         | ND       | ND     | ND          |
| 23  | Linoelic acid          | ND       | ND     | ND          | ND       | ND     | ND          | ND       | ND     | ND          |
| 24  | Piperitone oxide       | 22.04    | 3.16   | 872         | 22.00    | 4.46   | 872         | ND       | ND     | ND          |
| 25  | Lauric acid            | 25.60    | 0.87   | 879         | 25.53    | 1.01   | 879         | ND       | ND     | ND          |
| 26  | Spathulenol            | 25.97    | 6.65   | 890         | 25.94    | 4.39   | 890         | ND       | ND     | ND          |
| 27  | Caryophyllene oxide    | 26.09    | 7.50   | 889         | 26.07    | 3.66   | 889         | ND       | ND     | ND          |
| 28  | Cetanol                | 26.60    | 3.78   | 877         | 26.58    | 2.34   | 877         | ND       | ND     | ND          |
| 29  | Ethyl linoleolate      | 31.92    | 1.97   | 871         | 31.91    | 1.12   | 871         | ND       | ND     | ND          |

GC/MS: gas chromatography/mass spectrometry; RT: retention time; min: minutes; ND: not detected.
NO. Our results also demonstrated the highest potency on the % inhibition of NO production by PH (97.15% ± 0.37) and P95 (97.66% ± 1.12) at 50 μg/ml which were not significantly different from prednisolone (96.82% ± 0.34) at 50 μg/ml (Figure 3); therefore, PH and P95 may relieve pain from inflammation. FH and F95 at 100 μg/ml gave the highest % inhibition of NO production (91.55% ± 3.04 and 93.36% ± 3.23, respectively) which were not significantly different from prednisolone (96.82% ± 0.34) at 50 μg/ml (Figure 3). These results indicate the potency of Z. rhetsa pericarp is higher than Z. rhetsa fruit for use as anti-inflammatory agent due to infection. Ethnomedicine use of Z. rhetsa fruit was able to demonstrate the anti-inflammatory action due to infection by a component in the Ma-Ha-Wat-Ta-Nare remedy for the treatment of abscesses in Pra-O-Sod-Pra-Na-Rai ancient scripture [18].
Additionally, both NO and TNF-α have important roles in progressive osteoarthritis and rheumatoid arthritis [44–46]. TNF-α stimulates chondrocytes in cartilages to produce high levels of NO [44]. PH and P95 may reduce pain, swelling, and tissue damage through inhibiting NO and TNF-α production.

All extracts and essential oils from Z. rhetsa and prednisolone (positive control) showed greater than 70% cell viability at all concentrations (Figure 2) when tested, indicating that compounds were not cytotoxic to the cells, and their anti-inflammatory activity via the inhibition of LPS-induced NO, TNF-α, and PGE₂ production in RAW 264.7 macrophages was not due to cytotoxicity [34].

Additionally, our extraction methods and results were supportive data for the Thai traditional preparation of drugs as the extraction by hexane is similar to preparation of the folk method called Hung-Nam-Mun (hot oil extract) [47]. These methods extensively use coconut oil for frying plant materials; however, a rancid odor because of coconut oil is apparent. Whereas maceration in hexane has no odor and a high extraction yield.

Some compounds analyzed in the active extracts, PH, P95, and SO (Table 4), had previously been reported to inhibit inflammatory mediators. Terpinen-4-ol was found in both PH (1.07%), P95 (3.38%), and SO (35.13%), and previous studies reported that terpinen-4-ol could inhibit TNF-α, IL-1β, and PGE₂ production by LPS-activated human blood monocytes [31]. The second most abundant compounds in SO, p-cymene (10.95%), has previously been demonstrated to exhibit analgesic and anti-inflammatory properties in mice [48, 49]. Cuminaldehyde (5.60%) competitively inhibited the activity of 15-lipoxygenase, an enzyme involved in the production of inflammatory mediators such as leukotrienes, using lipoxygenase inhibition assay [50]. Limonene (5.48%) was found to be in the top five compounds of SO and also previously shown to suppress the production of LPS-induced NO, PGE₂, TNF-α, IL-1β, and IL-6 [33]. The occurrence of these compounds in SO was the reasons for its in vitro activities. The compounds in PH and P95, spathulenol (6.65% and 4.39%, respectively) and carophyllene oxide (7.50% and 3.66%, respectively), previously showed that they could inhibit the production of NO, IL-1β, and IL-6 [32]. The major component in PH and P95 was found to be bicyclo(3.1.1)heptane-2,3-diol, 2,6,6-trimethyl or 2,3-pinanediol (11.28% and 19.82%). This compound was earlier reported as an agent that increased microcirculation when applied topically [51]; thus, 2,3-pinanediol could contribute to pain relief when applied in PH and P95 on inflamed areas [52].

5. Conclusions

PH, P95, and SO of Z. rhetsa exerted pain-relieving and anti-inflammatory activity through inhibition of inflammatory mediators via LPS-induced NO, TNF-α, and PGE₂ in RAW264.7 macrophages. Our study suggests that the PH and P95 extract fractions analyzed could provide constituents suitable for pain relief in chronic inflammation due to their activity on NO and TNF-α and SO inhibitory effect on PGE₂ production. Moreover, PH, P95, and SO contained terpinen-4-ol that was previously reported as an inhibitor of LPS-induced PGE₂ and TNF-α. Other components in SO, p-cymene, and limonene have previously been reported for their in vitro and in vivo anti-inflammatory activity. Therefore, SO may have potential for the development into an analgesic and anti-inflammatory product for inflammation, and its active constituents should be further refined or studied further with additional reference standards where possible. A main active constituent determined in PH and P95 which enables inhibition of NO, TNF-α, and PGE₂ appears to be 2,3-pinanediol which comprises almost 20% of P95. These findings are the first foundational supportive data for ethnomedical use as anti-inflammatory and analgesic herbal medicine treatment. Z. rhetsa pericarp that is macerated with hexane and 95% ethanol and seed essential oil are now being studied for analgesic product development in ongoing studies in our laboratories.

Data Availability

The data used to support the findings of this study are available within the article.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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