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Phytochemical Constituents and Anti-Inflammatory Activities of *Clerodendrum Petasites*

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***Abstract*---**Inflammation is process of immune responses to microbial infections and cell damage. Macrophages are pro-inflammatory leukocytes crucial for initiation, maintenance, and resolution of inflammation. Root extract of a medicinal plant *Clerodendrum petasites* (CP), has been widely used as a component of antipyretic and anti-inflammatory remedies. In the present study, we investigated whether CP aerial part (leaf and stem) extracts had the same contents with the root extracts as well as their cytotoxicity and inflammatory effects in lipopolysaccharide (LPS)-stimulated murine RAW 264.7 monocyte/macrophage-like cell model. Analysis of the methanol-extracted CP contents with the high-performance liquid chromatography ultra-violet spectroscopy (HPLC-UV) showed that major phenolic compounds found in the root extract were verbascoside and quercetin, while the aerial parts also additionally contained rosmarinic acid and hispidulin. These CP extracts were not toxic to the cells and able to inhibit production of pro-inflammatory markers, nitric oxide (NO) and prostaglandin E2 (PGE2). In addition, the aerial CP extracts also inhibited the pro-inflammatory cytokines, interleukin 6 (IL-6), and tumor necrosis factor-α (TNF-α), suggesting additional role of rosmarinic acid and hispidulin. In conclusion, this study showed that the aerial parts of the CP contained phytochemical

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constituents and could be used as anti-inflammatory medicinal plants.

Keywords---*Clerodendrum petasites,* inflammation, phytochemicals, phenolic compounds, medicinal plants.

Introduction

Inflammation is a biological process of the immune responses to harmful stimuli such as microbial infections, damaged cells, and toxic compounds that cause repairing, reconstituting, and healing the damaged tissue [1, 2]. It is depicted by tissue reaction and microcirculation as clinically manifested by pain (through chemical mediators and nerves), swelling (exudation), redness (erythema), heat (hyperemia), and loss of function [3]. It occurs place by vasoconstriction thenceforward vasodilation, stasis, hyperemia, white blood cell (WBC) accumulation, exudation of fluid, and fibrin deposition. Macrophages are pro-inflammatory WBCs that play a critical role in the initiation, maintenance, and resolution of inflammation. Macrophages express a set of pattern recognition receptors whose ligands include pathogen-associated molecular patterns (PAMPs) such as aldehyde-derivatized proteins, surface phosphatidylserine, and LPS [4, 5]. The LPS is a part of cell walls of gram-negative bacteria (GNB), common causes of pneumonia, meningitis, and sepsis [6, 7]. The LPS-activated macrophages overproduce pro-inflammatory mediators such as nitric oxide (NO) and cyclooxygenase 2 (COX-2), which stimulate the conversion of arachidonic acid (AA) to various types of lipid signaling molecules, including prostaglandin E2 (PGE2) [8]. PGE2 is a principal mediator of inflammation that acts on four PGE2 receptor (EP) subtypes (EP1-EP4) to provoke inflammation by increasing the expression of the pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6) [9].

In clinical practice, broad-spectrum antibiotics have been used for treatment of GNB infections [10]. In some settings; however, it has been reported that adjunctive treatments of GNB infections with anti-inflammatory agents like corticosteroids and nonsteroidal anti-inflammatory drugs (NSAIDs) can reduce morbidity and systematic symptoms [11]. Besides, phytochemicals extracted from various medicinal plants like phenolic compounds have been reported to have anti-inflammatory activities in macrophages. Hsu and co-workers found that phenolic compounds extracted from yellow water lily (*Nymphaea hapsus* Zucc.) were able to inhibit NO, COX-2, and TNF-α synthesis in murine RAW 264.7 monocyte/macrophage-like cells [12]. Recently, Calabrese and colleagues reported that phenylethanoid glycosides (PhEGs), water-soluble phenolic compounds extracted from leaves of the great mullein (*Verbascum hapsus* L.) suppressed NO production in RAW 264.7 cells [13]. Root-extracted phenolic compounds from *Clerodendrum petasites* S. Moor (CP) have been used as complementary herbal medicine for treatment of inflammation [14]. On top of that, other studies also reported the presence of phenolic compounds in other parts of the CP. Hazekamp and colleagues found that aerial parts (leaves and stems) of the CP contained a bronchodilator hispidulin, which was able to reduce histamine-induced bronchospasm [15]. Interestingly, together with other PhEGs, verbascoside

isolated from the leaves of *Clerodendrum chinense* also had an anti-inflammatory action in carrageenan-induced rat paw edema model [16]. Verbascoside was also present in the aerial CP-extracted solution [17]; however, an anti-inflammatory effect of the aerial CP-extracted constituents has not been investigated. This present study was aimed to investigate CP-extracted phytochemical constituents and their anti-inflammatory effects in LPS-treated macrophages.

Materials and Method

Plants and extractions

All samples were collected from Northeast region of Thailand and identified by Dr.Nuttapong Wichai, Faculty of Pharmacy, Mahasarakham University (Fig. 1). Leaves, stems, and roots of the CP were dried at 50°C for 24, 48, 72 h, respectively; and powdered. The portion of 5-g powder was extracted with 50 ml methanol (MeOH) at room temperature, filtrated with Whatman filter paper No.1 for 4 times. The extracts of all repeats were dried at 50°C by rotary evaporator, stored at room temperature in desiccator until their weights were stable, and transferred to store at -20°C with closed tubes.

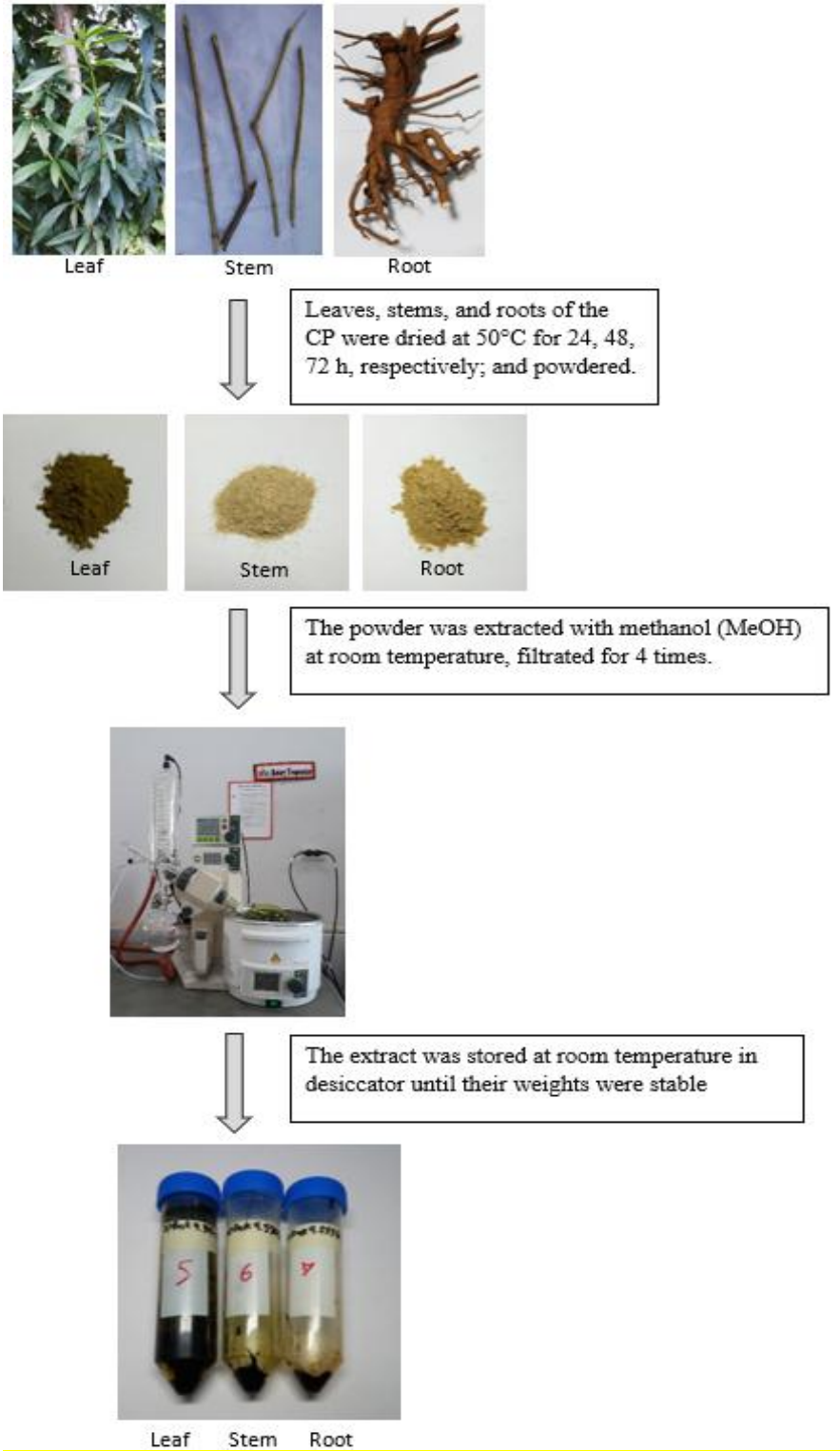

Figure 1 *Clerodendrum petasites* S. Moor leaf and stems extraction process.

High-performance liquid chromatography ultra-violet spectroscopy (HPLC-UV) system

HPLC method determining chemical constituents of CP consisted of column C18 LiChrospher phase (5µm), 125×4 mm with LiChroCART® guard column, HPLC Spectra-Physics (SpectraSYSTEM P4000) with quaternary gradient pumps and UV-detector (UV2000) at 330 nm (Fig. 2). The mobile phase was modified from the method of Thitilertdecha and co-workers by adjusting acetonitrile and 2.5% acetic acid solution in a gradient mode [17]. Combination of solvent started from 2.5% acetic acid 81% and acetonitrile 19% for 10 min at flow rate of 0.5 ml/min, then adjusted 2.5% acetic acid to 75% and acetonitrile 25% with flow rate of 1 ml/min and maintained for 25 minutes, adjusted 2.5% acetic acid to 20% and acetonitrile 80% for 10 minutes with flow rate of 0.5 ml/min. Finally, 2.5% acetic acid was changed to 81% with 19% acetonitrile for 5 minutes with flow rate of 0.5 ml/min. 10 µl of each standard compound (10-µg/ml verbascosides, rutin, rosmarinic acid, quercetin, apigenin, and hispidulin) was injected with AS3000 autosampler.

Figure 2 High-performance liquid chromatography ultra-violet spectroscopy (HPLC-UV) system

Cell viability assay

RAW 264.7 cells were cultured in 96-well plates at the density of 2×10^5 cells/well. After 24-h incubation, the adhered cells were treated with the final concentrations of 15.625, 31.25, 62.5, 125, 250, and 500 µg/ml MeOH-extracted CP solution. The cell control group was treated by 5 µl/ml dimethyl sulfoxide (DMSO) in the medium. After 24 h, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was added at a final concentration of 0.5 mg/ml and incubated for 30 min at 37 °C and 5% carbon dioxide (CO_2). The medium was discarded, and the formazan precipitate was solubilized in DMSO. The absorbance was measured at 550 nm on a microplate reader. Cell viability assay was repeated for 8 times and calculated as previously described [18]. The

concentrations of the extracts with more than 80% cell viability were used for assays of anti-inflammatory activities.

Pro-inflammatory marker assays

RAW 264.7 cells were treated with LPS 200 ng/ml and CP extracts at concentrations of 5, 83, and 167 µg/ml for 24 h. The negative control group was treated with 5 µl/ml DMSO Supernatants were collected and stored at -20 °C for pro-inflammatory marker assays. The enzyme-linked immunosorbent assays (ELISA) kits for PGE2 (KGE004B), IL-6 (ab100712), and TNF-α (ab100747) were purchased from Bio-Techne and Abcam PLC. The assay procedures were performed according to manufacturer's manuals and determined optical density with a microplate reader at 450 nm. NO concentration was measured as previously described [19]. The assays were performed in triplicates and data were averaged.

Results

CP roots, leaves, and stems contained different concentrations of phytochemical contents

The main substances found in CP roots were verbascoside and quercetin. When sorting the quantity of verbascoside in different parts of the plants, the roots had the highest amount of verbascoside, followed by stem and leaves, respectively (1.724±0.130, 0.354±0.030 and 0.199±0.018 mg/g dried samples, respectively) (Table 1). In addition, the root was found to have the highest amount of quercetin, followed by stem and leaves, respectively (0.918±0.008, 0.320±0.045, and 0.155±0.018 mg/g dried samples, respectively). In contrast, leaves and stems extracts contained rosmarinic acid and hispidulin, which were not present in the root extracts.

Table 1 Phytochemical contents in dried samples

Sample	Verbascoside (mg/g)	Rosmarinic acid (mg/g)	Quercetin (mg/g)	Hispidulin (mg/g)
Root	1.724±0.130	NS	0.918±0.008	ND
Leaf	0.199±0.018	7.197±0.331	0.155±0.018	0.067±0.002
Stem	0.354±0.030	0.679±0.069	0.320±0.045	0.037±0.009

NS = not separated, ND = not detected, mean±SD, n=3

CP extracts were not toxic to macrophage cells

After RAW 264.7 cells were treated with various concentrations of CP root, leaf, and stem extracts (15.63, 31.25, 62.5, 125, 250, and 500 µg/ml), results showed that cell viability was higher than 80% in all groups (Fig. 3). This finding indicated the selected concentrations of the extracts were not toxic to the cells.

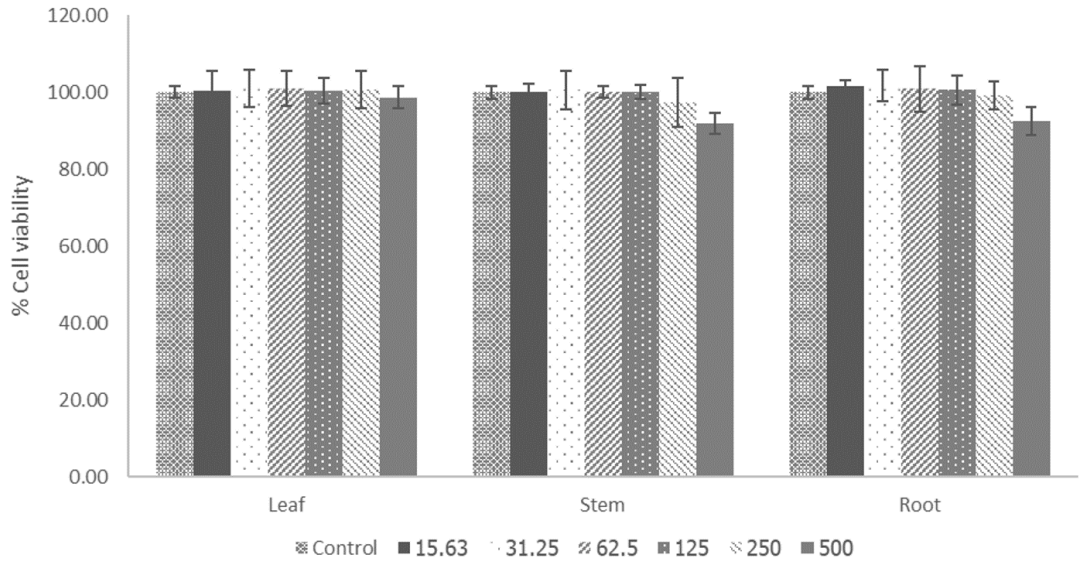

Figure 3 Percentage of RAW 264.7 cell viability after treatments with CP extracts.

CP extracts from roots, leaves, and stems differentially affected pro-inflammatory marker contents

We investigated effects of the extracts on nitric oxide (NO) inhibition. Results showed that the CP leaf, stem, and root extracts at concentration of 5 µg/ml inhibited NO production in the RAW 264.7 cells by 24.50, 22.08, and 23.26%, respectively; at 83 µg/ml by 100, 76.41, and 73.60%; and at 167 µg/ml by 100, 100, and 95.33%, respectively. ($p < 0.05$) (Fig. 4).

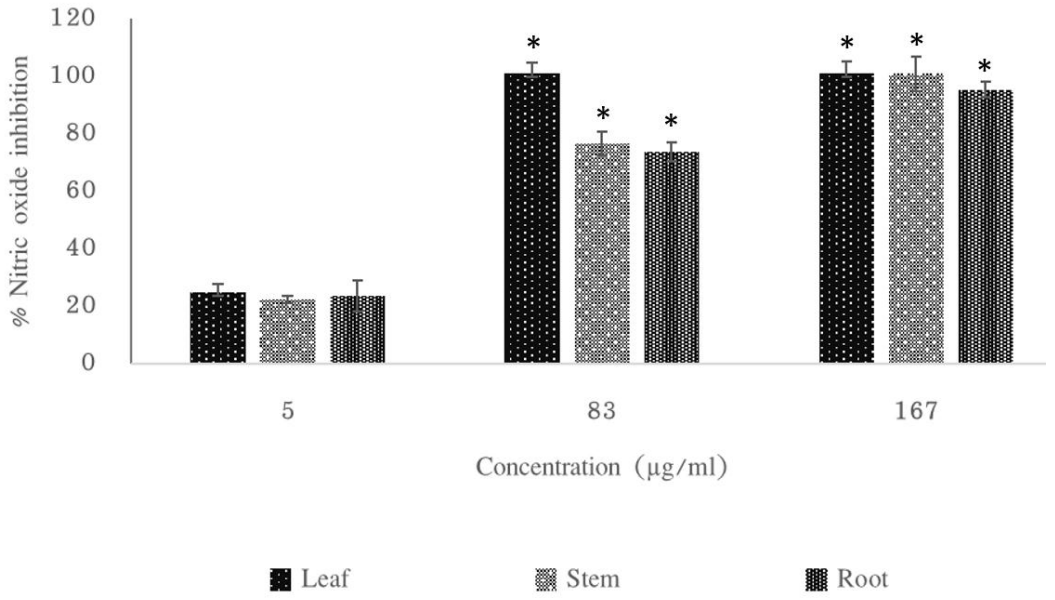

Figure 4 Inhibitory activity of CP extracts on nitric oxide production. *$p < 0.05$ compared to negative control (LPS alone treated cell) (n=3).

PGE2 concentration in the negative control group was 2,440.69±50.23 pg/ml (Fig. 5). At concentration of 5 μg/ml, CP extracts from leaves, stems, and roots had no significant effect on PGE2 concentrations 2,199.00±144.03, 2,348.03±216.11, 2037.50±175.46 pg/ml, respectively. In contrast, at concentration of 83 μg/ml, CP extracts from leaves, stems, and roots significantly reduced PGE2 concentrations to 1,832.50±212.7, 2,049.35±82.19, and 1,258.50±22.87 pg/ml, respectively ($p < 0.05$). Finally, at concentration of 167 μg/ml, CP extracts from leaves, stems, and roots further reduced PGE2 concentrations to 941.90±54.16, 907.30±32.51, and 524.60±58.13 pg/ml, respectively ($p < 0.05$).

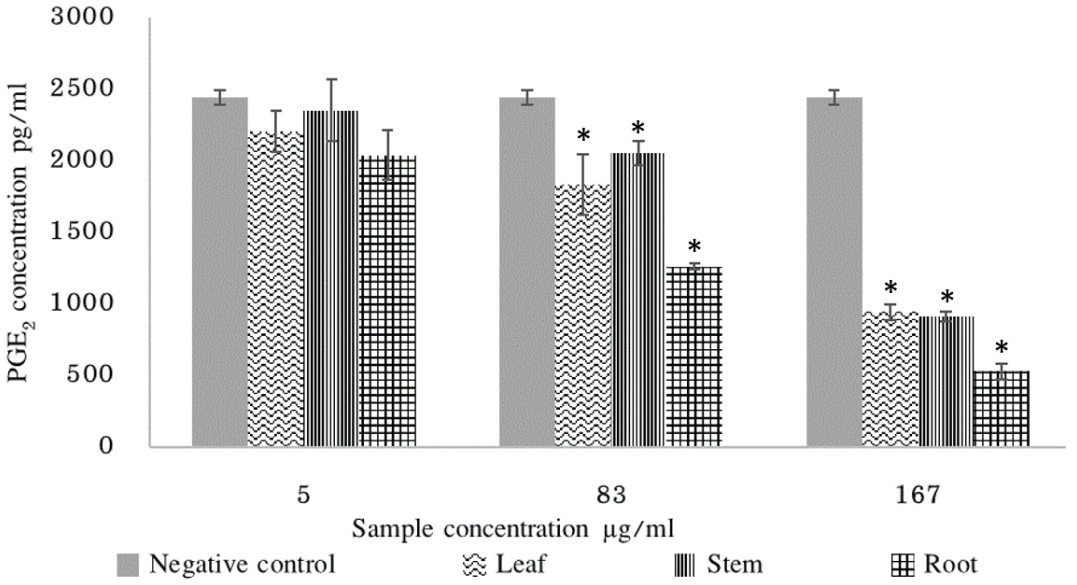

Figure 5 Inhibitory activity of CP and BLW extracts on PGE_2 assay. *$p < 0.05$ compared to negative control (LPS alone treated cell) (n=3).

In contrast to the findings in PGE2, only CP extract from leaves at concentration of 83 and 167 µg/ml decreased IL-6 concentrations from 599.23±11.36 to 145.73±7.42 and 58.97±0.92 pg/ml, respectively ($p < 0.05$) (Fig. 6).

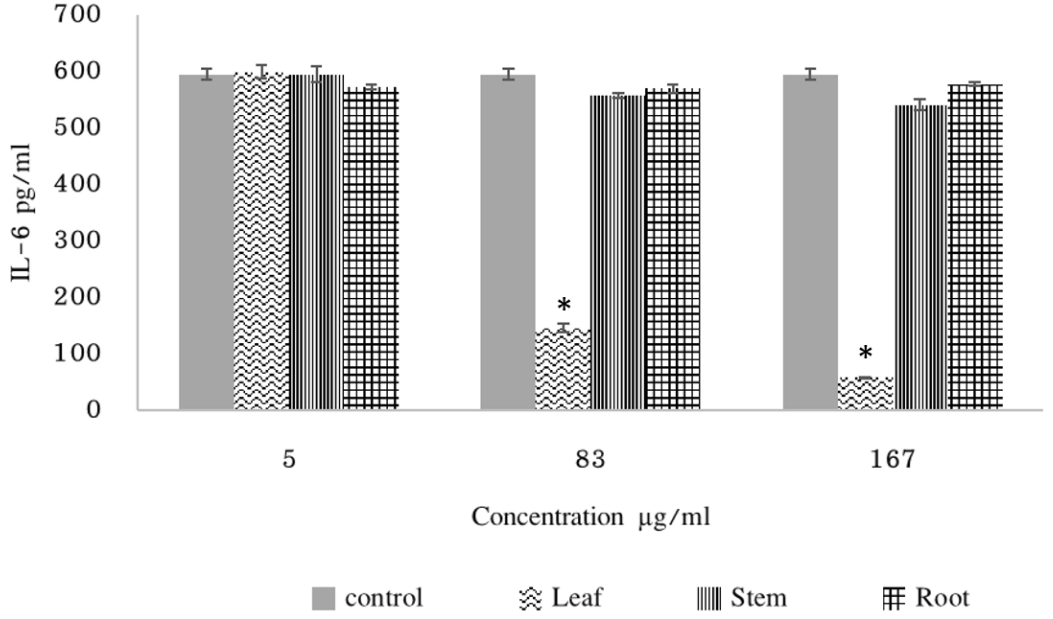

Figure 6 Inhibitory activity of CP extracts on IL-6 concentrations**.** *$p < 0.05$ compared to negative control (LPS alone treated cell) (n=3).

Similarly, the CP leaf extracts at concentration of 83 and 167 µg/ml significantly reduced TNF-α concentrations ($p < 0.05$). Additionally, the CP stem extract also reduced TNF-α concentration to the less extent, but statistically significant (Fig. 7)

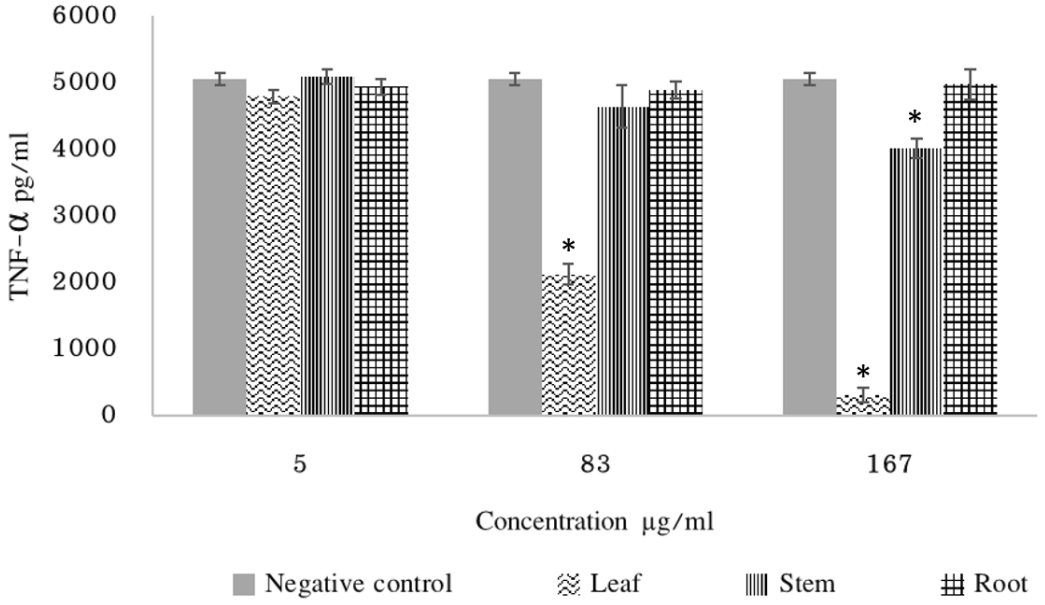

Figure 7 Inhibitory activity of CP extracts on TNF-α. *$p < 0.05$ compared to negative control (LPS alone treated cell) (n=3).

Discussion

Phenolic compounds are phytochemicals present in a many types of foods, plant tissues, and beverages [20]. Phenolic compounds have one (phenolic acids) or more (polyphenols) aromatic rings with hydroxyl groups linked in their structures. Phenolic compounds possess numerous bioactive properties and dietary intake provides health-protective effects [21]. This present study revealed that phenolic compounds extracted from aerial parts of CP had anti-inflammatory effects on macrophages. This conclusion has been drawn from the following findings: 1) CP leaf and stem extracts contained phenolic compounds found in the CP roots, 2) CP leaf and stem extracts were not toxic to macrophages, and 3) CP leaf and stem extracts reduced pro-inflammatory marker production from macrophages.

CP roots have been long used as Thai traditional medicine in treating fever and inflammation [17]. Our findings of verbascoside and hispidulin in the aerial parts of the CP were in line with the study of Thitilertdecha and colleagues [17]. However, to our knowledge, this study was first to report the presence of rosmarinic acid and quercetin in the aerial parts of the CP. Verbascoside is a water-soluble PhEG that exist in many medicinal plants used for anti-inflammation [16, 22]. Recently, Ji and colleagues reported that verbascoside-containing *Odontites vulgaris* Moench extract on reduced NO, TNF-α, and IL-6 production from LPS-treated RAW 264.7 macrophage cells [23]. However, *Odontites vulgaris* Moench extract was toxic to macrophages at the concentration

of 400 μg/ml, while the CP extracts were not toxic at 500 μg/ml [23]. It was not known how verbascoside reduced pro-inflammatory marker synthesis in the RAW 264.7 macrophage cells. By the way, Pesce and co-workers found that verbascoside increased phosphorylation of Src homology region 2 domain-containing phosphatase-1 (SHP-1) which is a redox sensitive protein tyrosine phosphatase capable of inhibiting LPS-induced inflammatory signaling pathways in U937 macrophage-like cells [24].

Hispidulin is a naturally occurring phenolic flavonoid previously found in aerial parts of the CP as bronchodilator [15]. Park and co-workers found that hispidulin extract from *Cirsium japonicum* var. *ussuriense* was able to inhibit NO and TNF-α production in the LPS-stimulated RAW 264.7 cells by downregulating protein synthesis of an inducible nitric oxide synthase (iNOS) [25]. Moreover, Srisook and colleagues also showed that mixed hispidulin with flavones acacetin and diosmetin extracts from *Clerodendrum inerme* leaves dose-dependently inhibited NO synthesis in LPS-stimulated RAW 264.7 cells [26]. Mechanistically, *Clerodendrum inerme* extracts reduced the mRNA and protein expressions of inducible nitric oxide synthase (iNOS) via the blockade of NF-κB DNAbinding activity and JNK pathway. Here we showed an inhibitory action of the aerial part-extracted CP on NO production in the RAW 264.7 cells.

Rosmarinic acid, named after rosemary, is polyphenolic phytochemical with potent anti-inflammatory effects mostly extracted from Lamiaceae family plants [27]. Various species in this family are widely used as culinary herbs, e.g., basil (*Ocimum* spp.), lavender (*Lavandula angustifolia* Mill.), oregano (*Origanum vulgare* L.), peppermint (*Mentha* × *piperita* L.), sage (*Salvia officinalis* L.), thyme (*Thymus vulgaris* L.), and rosemary (*Rosmarinus officinalis* L.). It has been shown that in an *in vivo* model of LPS-induced acute lung injury [28]. In the present study, rosmarinic acid was the most prominent constituent in the CP aerial extracts. Therefore, it can be implied that it was the most bioactive substance accounting for the anti-inflammatory action of the extracts. Our finding was in accordance the study of Rocha and colleagues who demonstrated anti-inflammatory effects of rosmarinic acid of the *Rosmarinus officinalis* L. extract on carrageenin-induced rat paw edema model of local inflammation; and liver ischemia–reperfusion and thermal injury models of systemic inflammation [29]. Furthermore, rosmarinic acid extracted from *Prunella vulgaris* also decreased NO and PGE2 synthesis in the LPS-stimulated RAW 264.7 cells through COX-2 inhibition [30]. This ascertains a possibility of the dominant effects of rosmarinic acid in the present study. Together with rosmarinic acid, quercetin, a plant flavonoid was also exclusively present in the aerial parts of the CP. Overman and co-workers reported that quercetin reduced mRNA expression of inflammatory markers including TNF-α, IL-6, and COX-2 in differentiated U937 macrophage-like cells by attenuating gene transcription regulators – inhibitor of nuclear factor kappa B (IκBα); and phosphorylated c-Jun and Jun N-terminal kinase (JNK) pathways.

Interestingly, we found that the CP-aerial part extracts reduced IL-6 and TNF-α concentration, whereas the root extract had no effect on these cytokines. This discrepancy might be explained by the exclusive presence of rosmarinic and hispidulin in the aerial parts. These phenolic compounds might have direct effects on IL-6 and TNF-α in NO-independent manners as previously shown in other cell

models [31, 32]. Further investigation is still required to unravel exact mechanisms of rosmarinic and hispidulin on IL-6 and TNF-α production in the LPS-stimulated RAW 264.7 cells.

In conclusion, the aerial parts of CP contained the phenolic compounds found in the root parts of CP and other two additional compounds. These phenolic compounds were not toxic to the macrophages and were able to inhibit the pro-inflammatory marker production. These findings pave the way for further treatments of inflammatory diseases with medicinal plants.

Conflict of Interest: The authors have no conflicts of interest to declare.

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