



# Anti-inflammatory Activity and Major Compounds of the Traditional Thai Medicines, Triphala, Trikatuk, and their Combined Formulae

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## ABSTRACT

Inflammation is a common factor leading to pathogenesis of several chronic diseases, including cardiovascular diseases, chronic metabolic syndromes, arthritis, and cancer. Trikatuk and Triphala are traditional poly-herbal preparations widely used in Ayurvedic and Thai traditional medicine. The present study aimed to investigate various combinations of these preparations and their mixing methods to optimize product efficacy. Samples were extracted by boiling in water and maceration with 95% ethanol. All samples were tested for inhibitory effects on *LPS*-induced nitric oxide (NO) production, and on *LPS*-induced prostaglandins (PGE<sub>2</sub>) from RAW 264.7 cells. Contents of major markers of the extracts were analyzed with HPLC. Trikatuk ethanolic extract showed anti-inflammatory activity through inhibition of NO production but Triphala had no effect on neither NO nor PEG<sub>2</sub> production. A combined formula of Triphala and Trikatuk (1:1) provided moderate anti-inflammatory activity in both pathways. The present study reported the anti-inflammatory effects of various combinations and mixing methods of Trikatuk and Triphala, the results of which could be used to develop optimal combinations for future use as dietary supplements and other health products.

**Keywords:** Inflammation; Thai traditional medicine; Triphala; Trikatuk

## 1. Introduction

Inflammation is a biological response system that acts to remove harmful stimuli

from the body and to restore normal physiological functions of tissues and organs. Inflammation can be categorized as either

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acute or chronic. Acute inflammation starts rapidly and becomes severe within a short period. However, the body will recover to normal conditions shortly thereafter, unlike chronic inflammation which can eventually cause progressive inflammation, leading to many chronic diseases [1-2]. Inflamed cells play an important role in inflammatory processes, such as in macrophages, leukocytes, mast cells, etc. The inflammatory mediators and cytokines released from these cells, such as nitric oxide (NO), tumor necrosis factor-alpha (TNF- $\alpha$ ), and prostaglandins (PG), act as biological defenders neutralizing foreign agents [3]. Inflammation is a common factor leading to pathogenesis of several chronic diseases including cardiovascular diseases, chronic metabolic syndromes, arthritis, and cancer [4]. NO, a free radical released from macrophages, is synthesized by members of the nitric oxide synthase (NOS) enzyme family. Inducible nitric oxide synthases (iNOS) play an important role in producing large amounts of NO during inflammation. Long-term NO exposure of tissue or organs is involved in many diseases such as asthma, rheumatoid arthritis, and atherosclerosis [5-6]. PGs are inflammatory mediators derived from arachidonic acid and metabolized by the cyclooxygenase (COX) pathway [1]. PGs have been detected in many inflammatory diseases including osteoarthritis [7-8]. Prostaglandin E2 (PGE<sub>2</sub>) is one of the most abundant PGs produced in the body. PGE<sub>2</sub> is an important mediator of many biological functions, such as immune response regulation, blood pressure modulation, gastrointestinal integrity, and fertility [9].

Traditional medicines derived from medicinal plants have been used for treatments of disease since ancient times. Nowadays, healthcare personnel in modern medicine are increasingly interested in traditional medicines as a new source of treatments for disease. Trikatuk and Triphala are traditional poly-herbal preparations widely used in Ayurvedic and Thai

traditional medicine. Trikatuk ('Trikatu' in Ayurveda) is composed of *Piper nigrum* (PN), *Piper chaba* (PC), and *Zingiber officinale* (ZO) at a ratio of 1:1:1. In Ayurvedic medicine, Trikatuk has been used to treat a wide range of illnesses and to increase the bioavailability of other drugs or herbal preparations [10]. Doss and co-workers studied the potential therapeutic effects of Trikatuk in rats with adjuvant-induced arthritis. The results showed that oral administration of Trikatuk suspension (1,000 mg/kg/bw) suppressed the production of pro-inflammatory cytokines (tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin (IL)-1 $\beta$ , IL-6, and monocyte chemoattractant protein (MCP)-1) and down-regulated the expression of related mRNA [11].

Triphala is composed of *Phyllanthus emblica* (PE), *Terminalia chebula* (TC), and *Terminalia bellirica* (TB) at a ratio of 1:1:1. Researchers have confirmed its anti-inflammatory [12], antimutagenic, radioprotective [13], anticariogenic [14], gastrointestinal health [15], and antineoplastic [16] activities. Recent studies have further demonstrated that it also possesses biological activities such as antiretinopathy [17], anti-cancer cell proliferation [18], skin protection [19], and inhibition of protein fibrillation [20].

Thai Traditional doctors use Trikatuk and Triphala as adaptogenic drugs and prescribe them in different seasons. Trikatuk is prescribed in the rainy season while Triphala is prescribed in the summer. A combination formula of Triphala and Trikatuk has been suggested for use during all seasons. Traditionally, Trikatuk and Triphala are used in a decoction solution form. Nowadays, both Triphala and Trikatuk are sold worldwide as herbal medicines and dietary supplements in various forms such as decoction solution, capsule, and tablet [21-22]. The present study aimed to compare the effects of various preparation methods and to find optimized methods and suitable formulate for anti-inflammatory activity of

Trikatuk and Triphala. It was expected that the results would be useful for further development of traditional medicines or dietary supplements.

## 2. Materials and Methods

### 2.1 Chemicals and reagents

Fetal bovine serum (FBS) and trypsin-EDTA were purchased from Gibco® (OK, USA). Dulbecco's Modified Eagle's Medium (DMEM), penicillin-streptomycin (P/S), and phosphate buffer saline (PBS) were purchased from Biochrom (MA, Germany). 3-(4, 5-dimethyl -2- thiazolyl)-2, 5-diphenyl -2H-tetrazolium bromide (MTT) was purchased from Sigma (MO, USA). Phosphate buffer saline (PBS) was also purchased from Amresco (OH, USA). Dimethyl sulfoxide (DMSO) was purchased from Fluka (Munich, Germany). Prednisolone, piperine, gallic acid, and ellagic acid were purchased from Sigma (MO, USA). Acetonitrile and methanol (HPLC grade) were purchased from RCI LabScan (Bangkok, Thailand). Purified water was prepared by Milli Q® system from Millipore (Bedford, MA, USA). PGE2 Enzyme Immuno-Assay Kit was purchased from Cayman Chemical (MI, USA).

### 2.2 Plant materials

*Piper nigrum* L. (fruits; SKP 146 16 14 01), *Piper chaba* Hunt. (fruits; SKP 146 16 03 01 ), *Zingiber officinale* Roscoe. (rhizomes; SKP 206 26 15 01), *Phyllanthus emblica* Linn. (fruits; SKP 071 16 05 01), *Terminalia chebula* (fruits; SKP 049 20 03 01), *Terminalia bellirica* (fruits; SKP 049 20 02 01) were collected from Kanchanaburi, Thailand. Plant materials were authenticated by comparison with specimens deposited at the herbarium of the Southern Center of Thai Medicinal Plants at the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand.

### 2.3 Preparation of crude extracts

Plant materials were cleaned, sliced into small pieces, and dried at 50°C for 24-48 hours. The dried plants were ground into powder with an electric grinder and sieved through a size 60 mesh sieve. The plant powders were stored in air-tight containers at room temperature until use.

Crude extracts of Trikatuk and Triphala were prepared as described in Table 1. The extraction methods used were decoction and maceration with 95% ethanol (3 times x 3 days). The mixing methods for Trikatuk, Triphala, and their combined preparations were as follows. First method, each plant powder was weighed and mixed homogeneously according to the proportion of each formula before extraction. Second method, each plant powder was extracted separately to produce crude extracts which were then weighed and combined according to the formula's proportion (Table 1).

### 2.4 Inhibitory activity on LPS-induced nitric oxide production from RAW 264.7 cells

The inhibition of NO production from RAW 264.7 cells was evaluated following a modified method [23]. Briefly, the cells were seeded in 96-well plates at a density of  $1 \times 10^5$  cells per well (100  $\mu$ l) and incubated at 37°C in 5% CO<sub>2</sub> for 24 hrs. The medium was replaced with fresh medium containing 5 ng/ml of LPS (100  $\mu$ l). Each crude extract was dissolved in DMSO and was diluted to various working concentrations before applying into each well, then the plate was again incubated for 24 hrs. NO production was analyzed by Griess reagent. Aliquots (100  $\mu$ l) of supernatant in each well were transferred into another 96-well plate to which the same volume of Griess reagent (1% sulfanilamide and 0.1% *N*-(1- naphthyl) ethylenediamine dihydrochloride in 2.5% phosphoric acid) was added. The optical density (OD) was measured by using a microplate reader at a wavelength of 570 nm. The %inhibition of the NO production was

calculated using the following equation, and the IC<sub>50</sub> values were calculated using a Prism software.

$$\%Inhibition = \left[ \frac{OD_{control} - OD_{sample}}{OD_{control}} \right] \times 100,$$

where  $OD_{control}$  = mean of control media (+LPS) - mean of control media (-LPS)  
 $OD_{sample}$  = mean of control sample (+LPS) - mean of control sample (-LPS).

## 2.5 Inhibitory activity on LPS-induced prostaglandins (PGE<sub>2</sub>) from RAW 264.7 cells

The RAW264.7 cells were seeded into 96-well plates,  $1 \times 10^5$  cells/well, and 80 ng/ml of LPS was added to stimulate macrophages, cells were allowed to adhere for 24 hours at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. After incubation, the supernatant was collected, and the amount of PGE<sub>2</sub> was determined using a PGE<sub>2</sub> enzyme immunoassay kit (Cayman Chemical, MI, USA). The amount of PGE<sub>2</sub> was measured relative to that of the positive control. The %inhibition was calculated using the following equation:

$$\%Inhibition = \left[ \frac{OD_{control} - OD_{sample}}{OD_{control}} \right] \times 100.$$

## 2.6 HPLC analysis of the contents of major markers of the crude extracts

HPLC analysis was done with Agilent 1200 HPLC system (Agilent Technologies, USA) equipped with a solvent degasser (G1322A), a quaternary solvent pump (G1311A), an autosampler (G1329A), a column oven (G1316A), and a photodiode array detector (G1315D). The chromatographic data were processed by Chemstation® software revision B.04.01 SP1.

Chromatographic separation was conducted along a C18 column (4.6 × 150

mm, 5 micron). The mobile phase consisted of acetonitrile (A) and 0.1%v/v phosphoric acid (B) using a linear gradient program as follows: 0-5 min, 5%B; 5 – 40 min, 5%B - 50%B; 40 - 50 min, 50%B - 95%B, 50 -55 min, 95-100%B; 55-60 min, 5%B. The flow rate was set at 1.0 mL/min. Samples of 10 µl each were injected into the HPLC system and detected with diode array detector using a wavelength of 256 nm.

Gallic acid, ellagic acid, and piperine were the major chemical components of Triphala and Trikatuk. All compounds were separated from other peaks showing the specificity of the method. The quantitative analyses of these three compounds were conducted using the constructed calibration curve of standard compounds. The correlation coefficient ( $r^2$ ) of calibration curves was more than 0.99.

## 2.7 Data analysis

The results of the anti-inflammatory activity are reported as mean ± standard error of means (SEM), measurements were done in triplicate. IC<sub>50</sub> values were calculated using regression analysis. Mean differences among groups were analyzed by ANOVA and LSD test. Statistical analysis was conducted using Graphpad Prism software (CA, USA).

## 3. Results and Discussion

### 3.1 Inhibitory activities on NO and PGE<sub>2</sub> production from RAW 264.7 cell lines

Results of the inhibitory activity of the ethanolic extracts on the production of NO from RAW264.7 are shown in Fig 1. All sample showed less than 30% cytotoxic effect on RAW 264.7 cells except those of ethanolic extract of TK (TKE and TKEm) and *P. nigrum* (PNE) which showed more than 30% cytotoxicity in the PGE assay at concentrations above 25 µg/ml and 50 µg/ml, respectively. Among the formulae, ethanolic extract of Trikatuk had the greatest efficacy with an IC<sub>50</sub> of 19.64±9.31 µg/ml, and 26.53±1.39 µg/ml for TKE and TKEm, respectively. However, all crude extracts

exhibited an inhibitory effect less than the positive control, prednisolone ( $IC_{50}$   $0.11 \pm 0.02$   $\mu\text{g/ml}$ ). Among the plant extracts, that of ZOE showed the highest inhibitory activity with an  $IC_{50}$  of  $17.94 \pm 6.32$   $\mu\text{g/ml}$ .

Inflammation is a complex process in which immune cells and their soluble factors work together to generate protective responses against infection and cell damage. Further, the inflammatory process involves pro-inflammatory cytokines, chemokines, and inflammatory immune cells [24]. Activation of macrophages is related to the release pro-inflammatory mediators and cytokines, such as NO,  $PGE_2$ , interferon-gamma ( $IFN-\gamma$ ),  $TNF-\alpha$ ,  $IL-1\beta$ , and  $IL-6$ . Therefore, over-expression of these mediators and cytokines can lead to tissue injury, cell death, and multiple organ failure [25] and are related to many diseases including asthma, cancer, osteoarthritis, rheumatoid arthritis, and diabetes [26].

TKE showed potent inhibitory activity on NO production ( $19.64 \pm 9.30$   $\mu\text{g/ml}$ ); however, in the assay of the inhibition of  $PGE_2$  production, TKE at concentration  $>25$   $\mu\text{g/ml}$  showed toxicity to the cultured cells. From the results, the combination of Triphala and Trikatuk provide beneficial effects on both anti-inflammatory activities. All combined formulae exhibited moderate inhibitory activity on NO production with  $IC_{50}$  values in the range of 30 – 80  $\mu\text{g/ml}$ . The combination of Trikatuk and Triphala provided advantages in terms of efficacy and safety. The anti-inflammatory activity of the combined formula was greater than that of Triphala alone. Although the activity of the combination was less than that of Trikatuk, its toxicity was also less than Trikatuk. As shown in Table 1, all combined formulae did not show cytotoxicity to RAW264.7 cells treated with LPS (80 ng/ml).

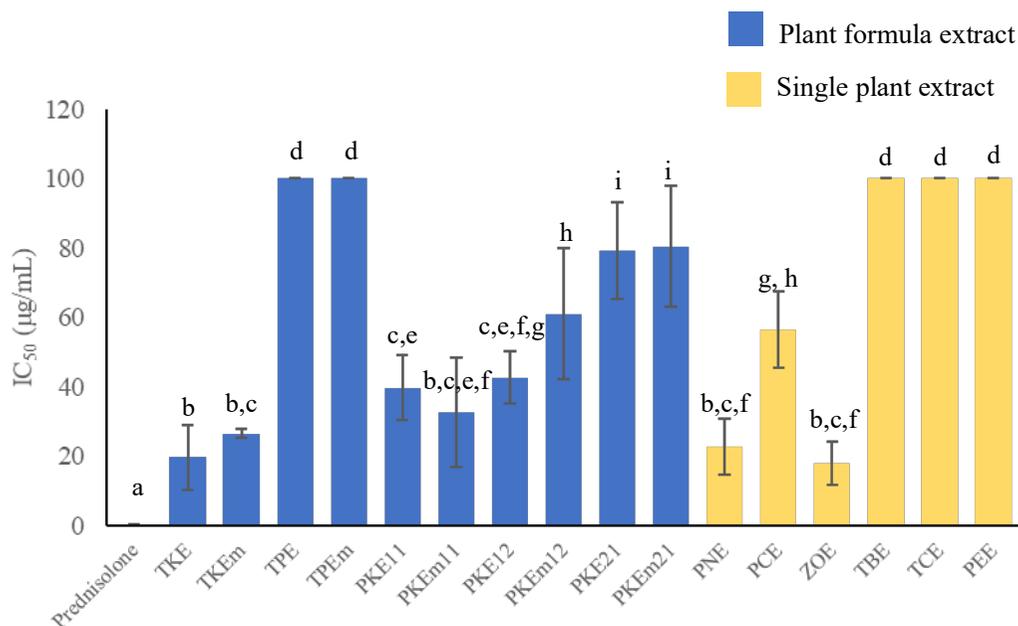
Regarding the extracts of the plant components of the formulae, ethanolic extract of ZO (ZOE) showed the strongest inhibitory activity on NO and  $PGE_2$  production with  $IC_{50}$  values of  $17.94 \pm 6.32$

$\mu\text{g/ml}$  and  $19.53 \pm 0.40$   $\mu\text{g/ml}$ , respectively. A previous study by Maged and co-workers showed that ethanolic extract of ZO inhibited NO production in RAW 264.7 cells with an  $IC_{50}$  of 10  $\mu\text{g/ml}$  [27]. Another study showed inhibitory activity of NO production comparable to our study with an  $IC_{50}$  of  $20.32 \pm 3.23$   $\mu\text{g/ml}$  [28]. The traditional Thai Trikatuk medicine uses *Z. mekongense* instead of ZO. However, the present study used ZO because it is more commonly found compared to *Z. mekongense* and it is commonly used in the Thai Trikatuk found in Thai markets. In addition, PN and PC which are plant components of Trikatuk also showed high to moderate inhibitory activity; however, PE, TB, and TC, which are plant components of Triphala, did not show any inhibition.

### 3.3 Contents of major components of crude extracts

HPLC analysis was a modification of the method used for Trikatuk determination [29]. The gradient mobile phase was optimized to enhance selectivity in the separation of the analytes along the HPLC column. The calibration curves of all compounds showed  $r^2$  greater than 0.99. The retention times of gallic acid, ellagic acid, and piperine were 5, 21, and 42 minutes, respectively. The major compounds of all crude extracts are shown in Table 1. In Triphala, gallic acid and ellagic acid were the two major components, while piperine was the major component in Trikatuk. Therefore, the combined formulae of Triphala and Trikatuk contained these three markers in the chromatogram. The highest content of gallic acid was found in the aqueous extracts of PE (PEW and PEWm), while ellagic acid was the highest in ethanolic extracts of TB (TBE and TBE<sub>m</sub>). Therefore, in Triphala there were high contents of gallic acid and ellagic acid but not piperine. The highest content of piperine was found in ethanolic extract of PN, which was one of the plant components in Trikatuk, contributing to the high content of

piperine in the ethanolic extract of Trikatuk (TKE and TKEm). This study is the first investigation on combined formulae of Triphala and Trikatuk and on the contents of the major markers and their inhibitory effects on NO and PGE<sub>2</sub> production in RAW264.7 cells.



**Fig. 1.** Inhibition of various crude extracts on NO production in LPS-induced RAW 264.7 cells. Mean and SEM values from n=3 are shown. Statistical analysis was performed using ANOVA with LSD test to compare means of each group. Samples labeled with different letters showed significant difference. ( $p < 0.05$ ).

**Table 1.** Major components and IC<sub>50</sub> of anti-inflammatory effect of the extracts.

Sample	Code	Ratio of Name mixing	Solvent	Code of Sample	Content of compounds mg/g			Inhibitory effects (IC <sub>50</sub> ) (µg/ml)	
					Gallic acid	Ellagic acid	Piperine	Nitric oxide	PGE <sub>2</sub>
<i>P. embelica</i>	PE	-	95% Ethanol	PEE	59.74 ±0.53	38.35 ±1.54	-	>100 <sup>a</sup>	>100 <sup>a</sup>
				PEW	62.11 ±0.39	6.12 ±0.10	-	>100 <sup>a</sup>	>100 <sup>a</sup>
<i>T. Chebula</i>	TC	-	95% Ethanol	TCE	30.02 ±2.14	12.73 ±1.94	-	>100 <sup>a</sup>	>100 <sup>a</sup>
				TCW	19.55 ±0.48	-	-	>100 <sup>a</sup>	>100 <sup>a</sup>
<i>T. bellerica</i>	TB	-	95% Ethanol	TBE	25.82 ±2.37	33.63 ±4.00	-	>100 <sup>a</sup>	>100 <sup>a</sup>
				TBW	18.54 ±0.60	4.82 ±0.16	-	>100 <sup>a</sup>	>100 <sup>a</sup>
<i>Z. officinale</i>	ZO	-	95% Ethanol	ZOE	-	-	-	17.94±6.32 <sup>b,c,d</sup>	19.53±0.40 <sup>b</sup>
				ZOW	-	-	-	>100 <sup>a</sup>	>100 <sup>a</sup>
<i>P. nigrum</i>	PN	-	95% Ethanol	PNE	-	-	241.62 ±3.06	22.54±8.05 <sup>b,c,d</sup>	>50 <sup>(t)</sup>

<i>P.chaba</i>	PC	-	Water	PNW	-	-	-	>100 <sup>a</sup>	>100 <sup>a</sup>
			95% Ethanol	PCE	-	-	156.49 ±2.82	56.51±11.00 <sup>e,f</sup>	>100 <sup>a</sup>
			Water	PCW	-	-	-	>100 <sup>a</sup>	>100 <sup>a</sup>
PE:TC:TB (Triphala)	-	1:1:1	95% Ethanol Water	TPE	39.48 ±1.10	27.15 ±2.37	-	>100 <sup>a</sup>	>50 <sup>(t)</sup>
				TPW	30.34 ±1.05	2.36 ±0.35	-	>100 <sup>a</sup>	>100 <sup>a</sup>
ZO:PN:PC (Trikatuk)	-	1:1:1	95% Ethanol Water	TKE	-	-	165.03 ±4.07	19.64±9.30 <sup>b</sup>	>25 <sup>(t)</sup>
				TKW	-	-	-	>100 <sup>a</sup>	>100 <sup>a</sup>
PEE:TCE:TBE	-	1:1:1	95% Ethanol Water	TPEm	44.59 ±2.61	37.46 ±3.15	-	>100 <sup>a</sup>	>100 <sup>a</sup>
				TPWm	30.79 ±0.46	5.27 ±0.64	-	>100 <sup>a</sup>	>100 <sup>a</sup>
ZOE:PNE:PCE	-	1:1:1	95% Ethanol Water	TKEm	-	-	143.32 ±4.03	26.53±1.39 <sup>b,c</sup>	>25 <sup>(t)</sup>
				TKWm	-	-	-	>100 <sup>a</sup>	>100 <sup>a</sup>
TPE:TKE	-	1:1	95% Ethanol Water	PKE11	26.18 ±0.97	14.05 ±0.89	81.50 ±3.26	39.62±9.41 <sup>c,g</sup>	>100 <sup>a</sup>
TPW:TKW	-			PKW11	17.08 ±0.39	-	-	>100 <sup>a</sup>	>100 <sup>a</sup>
TPEm:TKEm	-	1:1	95% Ethanol Water	PKEm11	23.95 ±1.27	15.89 ±2.26	76.26 ±1.94	32.60±15.79 <sup>b,c,d,g</sup>	41.39±0.66 <sup>b</sup>
TPWm:TKWm	-			PKWm11	16.15 ±0.81	-	-	>100 <sup>a</sup>	>100 <sup>a</sup>
TPE:TKE	-	1:2	95% Ethanol Water	PKE12	18.31 ±0.28	6.32 ±0.89	95.86 ±0.38	42.57±7.54 <sup>c,d,e,g</sup>	>100 <sup>a</sup>
TPW:TKW	-			PKW12	10.42 ±0.14	-	-	>100 <sup>a</sup>	>100 <sup>a</sup>
TPEm:TKEm	-	1:2	95% Ethanol Water	PKEm12	18.90 ±0.20	5.93 ±0.72	106.37 ±2.53	60.96±18.91 <sup>f</sup>	>100 <sup>a</sup>
TPWm:TKWm	-			PKWm12	11.65 ±0.15	-	-	>100 <sup>a</sup>	>100 <sup>a</sup>
TPE:TKE	-	2:1	95% Ethanol Water	PKE21	18.31 ±0.28	6.32 ±0.89	95.86 ±0.38	79.35±13.95 <sup>h</sup>	51.00±1.97 <sup>c</sup>
TPW:TKW	-			PKW21	10.42 ±0.14	-	-	>100 <sup>a</sup>	>100 <sup>a</sup>
TPEm:TKEm	-	2:1	95% Ethanol Water	PKEm21	18.90 ±0.20	5.93 ±0.72	106.37 ±2.53	80.40±17.35 <sup>h</sup>	62.22±0.24 <sup>d</sup>
TPWm:TKWm	-			PKWm21	11.65 ±0.15	-	-	>100 <sup>a</sup>	>100 <sup>a</sup>
Prednisolone								0.11±0.02 <sup>i</sup>	0.95±0.19 <sup>e</sup>

**Note:** Statistical analysis using ANOVA with LSD test comparing means of each group. Samples labeled with different letters showed significant difference. ( $p < 0.05$ ).

(t) The crude extract showed toxicity to RAW264.7 cells at higher concentrations.

### 3.4 Comparison of methods of preparation

In the present study, we designed the experimental procedures to determine the preparation methods that would provide the extract with the highest anti-inflammatory activity. We used decoction according to traditional practice [30] and maceration with 95% ethanol [31]. Regarding the mixing methods, in the first method, we combined the plant powders to produce the formula before it was extracted by decoction and maceration. In the second method, each plant powder was extracted by decoction and maceration separately, then the crude extracts of each plant (with the same extraction method) were combined according to the determined formula ratio. As shown in Table 1, all decoction samples showed low anti-inflammatory activity with IC<sub>50</sub> values of more than 100 µg/ml, while some ethanolic extracts showed moderate to high anti-inflammatory activity. A key limitation of this study was that the formulae combinations were based on the weights of the extracts, not their chemical contents, but the amounts of the chemical components were not equal among the extracts, making it harder to find an optimum combination. Therefore, in future studies, mixing of the extracts based on their extraction yields should be investigated.

### 4. Conclusion

Combined formulae of Triphala and Trikatuk, traditional Thai medicines used as adaptogens during different seasons, were investigated to obtain a combined formula that could be utilized throughout the year. Trikatuk exhibited inhibitory effects on NO production but showed cytotoxicity when tested for anti-PGE<sub>2</sub> production. Triphala did not show anti-inflammatory activity. However, a combined formula of Triphala and Trikatuk provided advantages superior to the traditional formulae. They exhibited moderate inhibition of NO and PGE<sub>2</sub> production and did not show cytotoxicity when exposed to a high level of LPS. These

results provided information for further study of the combined formulae on their protective effects of cells when exposed to hostile environments. The study showed potential anti-inflammatory activity of the combined formulae of Triphala and Trikatuk and provided preparation methods for their future development as traditional medicines or dietary supplements.

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