

ANTI-INFLAMMATORY AND ANTIOXIDANT ACTIVITIES OF THAI TRADITIONAL REMEDY CALLED “YA-HA-RAK”

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ABSTRACT: Benjalokawichien (BLW) or Ya-Ha-Rak remedy (HR) is the drug list in herbal medicinal products A.D 2011 of Thailand and used as antipyretic drug and treat rash in Thai Traditional Medicine. Its plant ingredients consists with five plant roots such as *Ficus racemosa* Linn., *Capparis micracantha* DC., *Clerodendrum petasites* S. Moore., *Harrisonia perforata* Merr., *Tiliacora triandra* Diels. This study are investigation anti-inflammatory and antioxidant, activities of the extract of HR and its plant components by inhibitory activity on the release of inhibitory activities against lipopolysaccharide (LPS) induced nitric oxide (NO) production in RAW 264.7 cell lines and DPPH scavenging assays respectively. HR extract possessed the highest NO inhibitory activity with an IC₅₀ value of 40.4µg/ml. The ethanolic extract of *Ficus racemosa* exhibited the highest antioxidant activity (IC₅₀= 4.9 µg/ml) and HR extract also show antioxidant activity (EC₅₀=40.9µg/ml). This study can support the use of HR in Thai Traditional Medicine for treatment inflammatory diseases.

Keywords: Benjalokawichian remedy, Ya-Ha-Rak remedy (HR), *Ficus racemosa*, anti-inflammatory, antioxidant

INTRODUCTION

Nitric oxide (NO) is one of the inflammatory mediators causing inflammation in many organs and it is an inorganic free radical which has been implicated in physiological and pathological processes, such as vasodilation, non-specific host defense and acute or chronic inflammation [1, 2]. NO and its derivatives, including reactive nitrogen intermediates (nitrite and nitrate), produced by iNOS have been identified as important effector molecules that restrict pathogen growth in infected hosts, and monocytes and macrophages, endothelial cells, hepatocytes, and neutrophils can synthesize reactive nitrogen intermediates by constitutive pathways, inducible pathways, or both [3]. However, excessive production of this free radical is pathogenic to the host tissue itself, since NO can bind with other superoxide radicals and acts as a reactive radical which directly damages the function of normal cells [4, 5]. LPS is one of the major constituents of the outer membrane of Gram-negative bacteria, and the immune system is constantly exposed to low levels of LPS through infections. LPS recognition and signal transmission are the key events aimed at eliminating an invading pathogen.

The LPS-induced activation of macrophages results in the production of bioactive lipids, reactive oxygen species, and in particular, inflammatory cytokines to fight and clear the bacterial infection [6, 7]. Thus, the evaluation of anti-inflammation of drug used the inhibitory nitric oxide production assay as the one model of antiinflammation.

Benjalokawichian or Ya-Ha-Rak is a Thai Traditional medicine preparation which is in the list of Herbal Medicinal Products A.D 2011 Thailand. It consist of five plant roots such as *Ficus racemosa* Linn., *Capparis micracantha* DC., *Clerodendrum petasites* S.Moore., *Harrisonia perforata* Merr., *Tiliacora triandra* Diels. It has been commonly used to reduce fever which is relative with the inflammatory mechanism and also used to apply to inflammation for allergic skin in Thai traditional medicine long time ago [Rehabilitation Foundation for the Promotion of traditional Thai medicine. Ayurvedic College (Cheevakomalapaj) [8]. Although BLW or Ya-Ha-Rak (HR) remedy is widely used as an antipyretic by many traditional practitioners in Thailand, there have been only three scientific studies that support its use. Konsue et al. demonstrated that the root powder of BLW formula showed the antipyretic efficacy by using a Baker's yeast-induced fever model in rats [9]. Jongchanapong et al. initially determined the

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antipyretic activity of the root extract of BLW remedy using lipopolysaccharide (LPS)-induced fever in rats compared to that of acetylsalicylic acid (ASA) [10]. Recently, Singharachai et al. diagnosed and distinguished of five roots in Thai Traditional Medicine remedy: Ben-ja-lo-ka-wi-chian or Ya-Ha-Rak from the morphological and histological characters [11]. Surprisingly, there is no previous report for investigation on anti-inflammatory activity by inhibitory effect on NO production by Mouse Macrophage Leukemia-like RAW264.7 cells on these five root extract and HR. Thus, the results antiinflammatory testing of HR and its plant components should be support using this drug for development as the modern anti-inflammatory drug from Thai traditional remedy in the future.

MATERIALS AND METHODS

1. Reagents

Animal cell lines, Chemicals and Reagents

RAW 264.7 murine macrophage leukemia cell lines were established and kindly provided by Assoc. Prof. Dr. Supinya Tewtrakul, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand. RPMI Medium 1640 (RPMI 1640) Medium powder with L-glutamine, Fetal Bovine Serum (FBS), Penicillin-Streptomycin (P/S), trypsin-EDTA and trypan blue were purchased from Gibco, USA. Phosphate Buffer Saline (PBS) was from Amresco, USA. Sodium bicarbonate was from BDH, England. Lipopolysaccharide (LPS, from *Escherichia coli*), Dimethyl sulfoxide (DMSO) and 1,1-Diphenyl-2-picrylhydrazyl (DPPH) were from Fluka, Germany.

2. Plant materials and extraction

The parts of plants were collected from Dan-Chang, Suphanburi in Thailand. The voucher specimens were deposited at the herbarium of Southern Center of Thai Medicinal Plants at Faculty of Pharmaceutical Science, Prince of Songkla University, Songkhla, Thailand. Five plant materials were dried at 50°C. One hundred gram of each plants was to provide homogeneous combined as Benjalokawichian or Ha-Rak preparation, each roots was also ground and macerated with 95% ethanol, then filtered and concentrated by rotary evaporator (under reduced pressure) to obtain the ethanolic extracts. The percentage of yields of each extract were calculated.

3. *In vitro* assay for Anti-inflammatory activity

Inhibitory effect on NO production by Mouse

Macrophage Leukemia-like RAW264.7 cells was evaluated using a modified method Tewtrakul and Subhadhirasakul [12]. RAW 264.7 cells line was cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (100 µg/ml). The cells were seeded in 96-well plate (cell concentration 1×10^5 cells/well) and incubated in CO₂ incubator at 37°C for 1 hour. 100 µl/well of RPMI medium containing 100µg/ml of LPS was added into control and sample wells, whereas only RPMI medium was added into a blank well. 100µl/well of different sample concentrations (1-100 µg/ml) were added into sample wells and their corresponding blank sample wells. Then cells were incubated at 37°C for 48 hours. Supernatant (100µl) was added in another 96-well plate and followed by the addition of 100µl/well of Griess reagent. The color was detected at a wavelength of 570 nm.

Cytotoxicity was also determined using the MTT method. After 48 h incubation with the test samples, MTT solution (10 µl, 5 mg/ml in PBS) was added to the wells and incubated at 37°C for 2 hours. The medium was removed, and isopropanol containing 0.04 M HCl was then added to dissolve the formazan production in the cells. The formazan solution was measured with a micro-plate reader at 570 nm. The test sample was considered to be cytotoxic when the optical density of the sample-treated group was less than 70-80% of that in the control (vehicle-treated) group. Indomethacin was used as positive controls.

4. DPPH radical scavenging activity

The antioxidant activity of all extracts and fractions was determined according to the modified method of Yamasaki et al. [13]. Samples for testing were prepared by dissolution in absolute ethanol and prepare sample solution (4 concentration as two-fold dilution) to be 100, 50, 10, 1 µg/ml. A portion of sample solution (0.1 ml) was mixed with the same volume of 6×10^{-5} M DPPH in absolute ethanol. After the mixture had been allowed to protected from light for 30 minutes at room temperature, its absorbance was measured at 520 nm using a spectrophotometer.

RESULTS

1. The percent yield of extracts

The percent yield of HR and each extract showed in Figure 1. The HR extract showed the highest (4.26 %) percentage of yields whereas FR showed the lowest (2.01%).

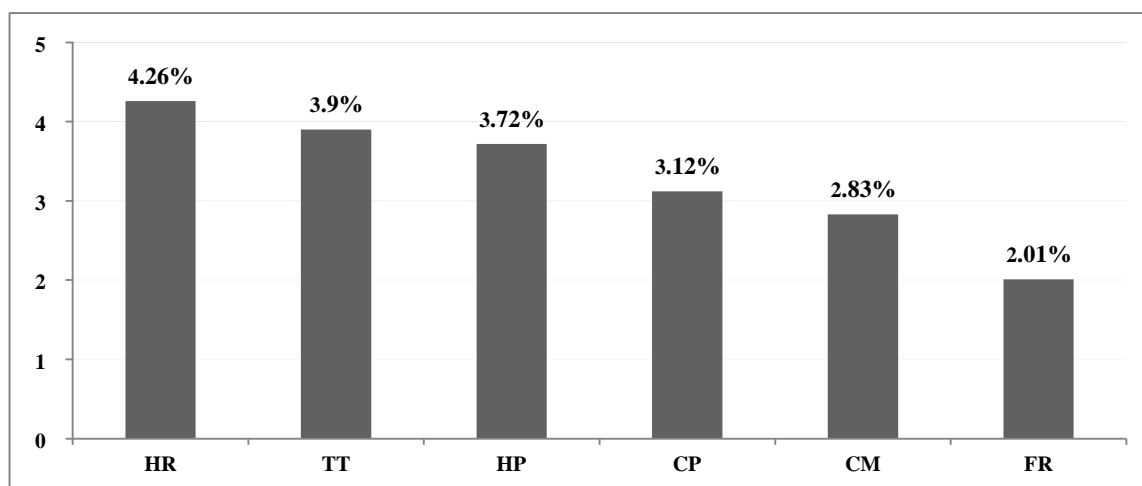


Figure 1 The percent yield of Ya-Ha-Rak remedy and each component extracts by maceration with 95% ethanol

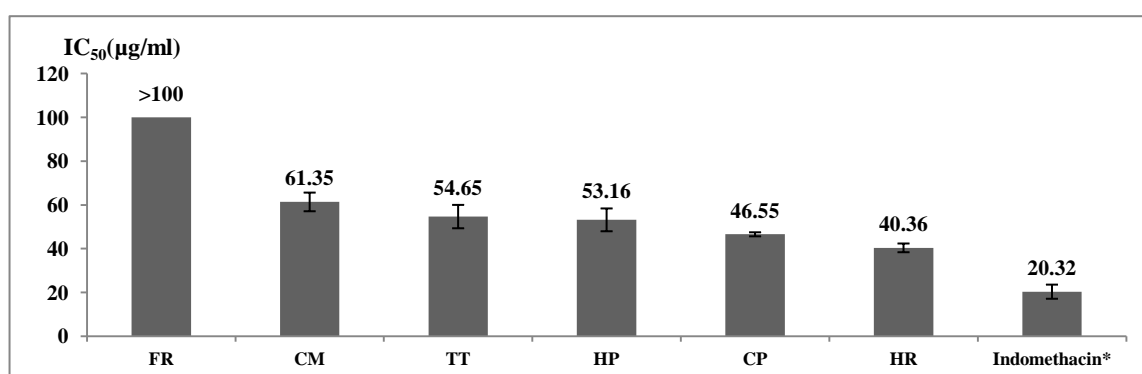


Figure 2 Anti-inflammatory Activity of Benjalokawichian Remedy (Ya-Ha-Rak) and its plant ingredients (n=3), *Positive control

Table 1 The results of Anti-Inflammatory Activity of Benjalokawichian Remedy (Ya-Ha-Rak) and its ingredients (n=3)

Sample (Ethanolic extracts)	%Inhibition (µg/ml)/ (percentage of viable cells) at various concentration					IC ₅₀ ±SEM
	100	50	30	10	1	
<i>Ficus racemosa</i> Linn. Z (FR)	47.94±6.90 (85.37±5.14)	14.91±4.47 (105.74±2.57)	2.50±5.09 (112.84±6.29)	-0.57±5.21 (120.47±3.98)	-5.63±4.31 (128.34±1.72)	>100
<i>Capparis micracantha</i> DC. (CM)	79.68±2.05 (116.97±20.22)	40.87±3.12 (127.93±16.61)	25.76±1.88 (142.69±21.64)	15.25±2.53 (106.18±9.75)	2.97±1.07 (127.79±20.48)	61.35±4.26
<i>Tiliacora triandra</i> Diels. (TT)	79.63±4.00 (109.22±5.84)	48.05±5.43 (90.9±1.19)	28.19±2.20 (99.73±4.11)	15.80±1.45 (106.87±10.98)	0.30±2.17 (112.56±7.74)	54.65±5.34
<i>Harrisonia perforata</i> Merr. (HP)	83.45±1.78 (57.51±8.01)*	46.55±6.96 (83.62±7.09)	19.96±7.86 (106.79±5.61)	6.14±8.52 (100.44±2.50)	-1.60±5.20 (109.35±9.87)	53.16±5.21
<i>Clerodendrum petasites</i> S.Moore (CP)	88.00±1.46 (137.46±27.28)	55.18±1.79 (150.23±24.65)	26.35±3.20 (159.32±18.69)	8.60±5.15 (141.00±21.93)	-2.12±4.51 (155.25±12.64)	46.55±0.92
Benjalokawichian (BJW, Ya-Ha- Rak, HR)	86.50±2.76 (95.15±0.64)	62.77±1.30 (92.53±4.97)	36.42±1.81 (119.09±6.79)	17.51±13.60 (126.10±12.10)	4.63±5.78 (122.42±10.03)	40.36±1.99
Indomethacin	63.70±1.50 ^a (91.26±1.15)	38.60±3.80 ^a (82.46±4.87)	-9.15±3.40 ^a (79.66±1.76)	-	-32.95±15.70 ^a (84.27±11.96)	20.32±3.23

* Cytotoxic effect was observed, ^a Value in µM

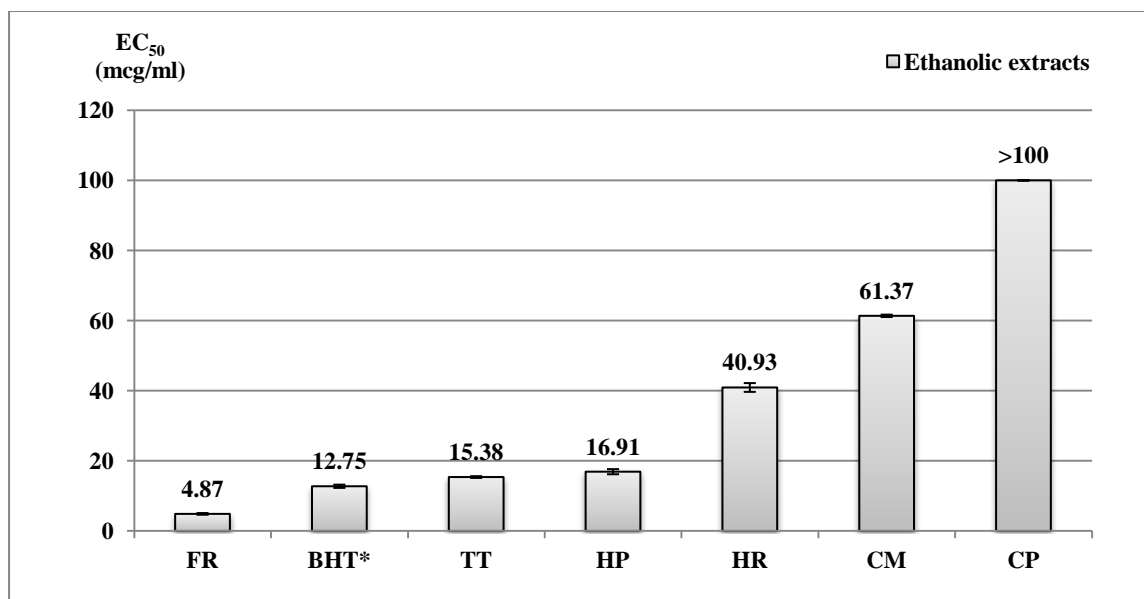


Figure 3 The results of DPPH radical scavenging assay of Benjalokawichian Remedy (Ya-Ha-Rak) and its ingredients (n=3), *Positive control

2. Anti-inflammatory activity by NO inhibitory effect and cytotoxicity test in RAW264.7 cells

The ethanolic extract of Ya-Ha-Rak (HR) remedy exhibited the most potent inhibitory activity in this test ($IC_{50} = 40.36 \mu\text{g/ml}$) but it exhibit NO production inhibitory effect lower than Indomethacin ($IC_{50} = 20.32 \mu\text{g/ml}$). Moreover, the ethanolic extract of *Clerodendrum petasites*, *Harrisonia perforata*, *Tiliacora triandra*, *Capparis micracantha* showed moderate inhibition activity ($IC_{50} < 60 \mu\text{g/ml}$). The *Ficus racemosa* extracts showed no inhibition activity ($IC_{50} > 100 \mu\text{g/ml}$) (Figure 2). The cytotoxicity test, all of extracts showed anti-inflammatory and no toxicity in RAW 264.7 cells. There are showed survival more than 80% in Table1.

3. DPPH radical scavenging activity

The ethanolic extract of *Ficus racemosa* exhibited higher DPPH radical scavenging activity ($EC_{50} = 4.87 \mu\text{g/ml}$) than BHT which is the standard ($EC_{50} = 12.75 \mu\text{g/ml}$). The *Tiliacora triandra* and *Harrisonia perforata* showed strong antioxidant activity ($EC_{50} = 15.38, 16.91 \mu\text{g/ml}$, respectively). The results of Benchalokawichian remedy and *Capparis micracantha* showed antioxidant activity ($EC_{50} = 40.93, 61.37 \mu\text{g/ml}$, respectively) whereas *Clerodendrum petasites* showed no antioxidant activity ($EC_{50} > 100 \mu\text{g/ml}$) (Figure 3).

DISCUSSION

The results of anti-inflammatory activity showed that HR exhibited maximum NO inhibitory effect but less than indomethacin (positive control). In this study showed results in the same way with

previous study of anti-inflammatory activities, Indomethacin (10 mg/kg) decreased the writhing response by 89% whereas HR doses of 200 and 400 mg/kg decreased the number of writhes induced by acetic acid by 63%) [10]. The *Clerodendrum petasites* extract possessed moderate inhibitory activity on acute phase of inflammation in a dose-related manner as seen in ethyl phenylpropiolate-induced ear edema ($ED_{50} = 2.34 \text{ mg/ear}$) as well as carrageenin-induced hind paw edema ($ED_{30} = 420.41 \text{ mg/kg}$) in rats [14]. Its related with CP extracts showed the second exhibited NO inhibitory effect ($IC_{50} = 46.55 \mu\text{g/ml}$). The ethanolic extract of FR showed no inhibitory activity by NO effect, but on the other hand, the study of *Ficus racemosa* leaves reported that the petroleum ether extract (400 mg / kg) exhibited maximum anti-inflammatory effect, that is 30.4, 32.2, 33.9 and 32.0% at the end of 3 h with carrageenin, serotonin, histamine, dextran-induced rat paw edema, respectively [15]. Furthermore, extracts of the bark from *Ficus racemosa* inhibited COX-1 [16] and 5-LOX enzymatic activities at 90 and $18 \mu\text{M}$ [17]. However, this result showed synergistic antiinflammatory effect of the plant ingredients of HR, so HR exhibited the highest anti-inflammatory activity. The antiinflammatory compound occurred after macerating the mixture of plant ingredients. Thus, it is interesting for isolation anti-inflammatory compound from Ya-Ha-Rak remedy extract.

The FR, TT, HP showed high antioxidant activities with $EC_{50} = 4.87, 15.38, 16.91 \mu\text{g/ml}$, respectively. This results related with the previous report that the

Table 2 Comparison of Percent yield, anti-inflammatory activity by NO inhibitory effect, EC₅₀ values of DPPH radical scavenging activity from Benjalokawichian remedy and it plant ingredients (n=3)

Sample	Part of use	%Yield	IC ₅₀ of NO inhibitory activity (IC ₅₀ ±SEM, µg/ml)	EC ₅₀ values of DPPH radical scavenging activity (EC ₅₀ ±SEM, µg/ml)
<i>Ficus racemosa</i> Linn. (FR)	root	2.01 %	>100	4.87 ± 0.24
<i>Capparis micracantha</i> DC. (CM)	root	2.83 %	61.35 ± 4.26	61.37 ± 0.34
<i>Tiliacora triandra</i> Diels. (TT)	root	3.90 %	54.65 ± 5.34	15.38 ± 0.25
<i>Harrisonia perforata</i> Merr. (HP)	root	3.72 %	53.16 ± 5.21	16.91 ± 0.73
<i>Clerodendrum petasites</i> S.Moore (CP)	root	3.12 %	46.55 ± 0.92	>100
Benjalokawichian(Ya-Ha-Rak, HR)	root	4.26 %	40.36 ± 1.99	40.93 ± 1.25
Indomethacin ^a	-	-	20.32 ± 3.68	-
BHT ^b	-	-	-	12.75 ± 0.46

^{a, b} positive control

stem bark of *Ficus racemosa* ethanolic extract showed high antioxidant activities dependent DPPH, ABTS•-, hydroxyl radical and superoxide radical scavenging and inhibition of lipid peroxidation with IC₅₀ comparable with tested standard compounds [18]. The methanolic extract of *Tiliacora triandra* leaves also showed moderate DPPH radical scavenging activity and total phenolic content compared with positive control [19].

CONCLUSION

In conclusion, the HR extract showed the highest (4.26 %) percentage of yields and anti-inflammatory activity (IC₅₀=40.36 µg/ml), exhibited moderate antioxidant activity (EC₅₀=40.93 µg/ml). The results can support using the Ya-Ha-Rak (HR) remedy to treat the inflammatory conditions which are cause of fever and allergy in Thai traditional medicine.

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