SAFETY EVALUATION OF THAI TRADITIONAL MEDICINE REMEDY: BEN-CHA-LO-KA-WI-CHIAN

Chatubhong Singharachai[1], Chanida Palanuvej[1], Hiroaki Kiyohara[2, 3], Haruki Yamada[2, 3], Nijsiri Ruangrungsi[1, 4, *]

[1]College of Public Health Sciences, Chulalongkorn University, Bangkok 10330, Thailand
[2]Kitasato Institute for Life Sciences, Kitasato University, Tokyo 108-8641, Japan
[3]Graduate School of Infection Control Sciences, Kitasato University, Tokyo 108-8641, Japan
[4]Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand

ABSTRACT: Ben-Cha-Lo-Ka-Wi-Chian remedy (BLW remedy) is a Thai traditional medicine that has long been used as an antipyretic drug by traditional practitioner and has been notified in the List of Medicine Products of the National List of Essential Drugs A.D. 2006. It is used as a mixed powders of the roots of *Capparis micracantha* DC., *Clerodendrum petasites* S. Moore, *Harrisonia perforata* (Blanco) Merr., *Ficus racemosa* L. and *Tiliacora triandra* (Colebr.) Diels, in equal part by weight. No scientific evidence of safety of this remedy and its component herbs still has been reported. This study aimed to assess the safety of the remedy and each species extracts of Ben-Cha-Lo-Ka-Wi-Chian ingredients. The experiments were investigated through cytotoxicity, free radical scavenging activity, mutagenicity and anti-mutagenicity using *Artemia salina*, DPPH and *Salmonella typhimurium* TA98 and TA100 as targets life forms respectively. The fingerprint profile of the BLW remedy was also carried out by 3D-HPLC. The results demonstrated that most of samples are non-toxic except for the ethanol extract of *T. triandra* (LC_{50} 44 µg/ml). Most of samples also showed good scavenging activity particularly in the ethanol extract samples. Along with a non-direct mutagenic activity, however most of the extracts exhibited indirect mutagenic activity when combined with nitrosation. Nevertheless, the remedy extracts and the components herb extracts strongly inhibited mutagenicity when nitrite-treated 1-aminopyrene was used as a mutagen. Three-Dimensional HPLC showed clear twelve high major peaks in BLW remedy. The present study provided scientific evidences on biological activities of Thai traditional medicine: Ben-Cha-Lo-Ka-Wi-Chian remedy and its component herbs. Nevertheless, consumers should be advised on the adverse effects of using the remedy with nitrite containing foods.

Keywords: Ben-Cha-Lo-Ka-Wi-Chian remedy, *Artemia salina* lethality, DPPH, Ames test, Nitrite, 3D-HPLC

INTRODUCTION

The elementary principles in the stipulation of traditional medicine or herbal products are the efficacy and safety. The belief that herbal remedies are safe and less damaging to the human body than synthetic drugs, has been a misassumption that "natural means safe" [1-3]. Moreover, interest in medicinal plant practiced has been increasing greatly. Therefore, the biological activities regarding to either efficacy or safety of traditional medicine are crucial scientific evidences to continue the usage and consumer protections.

In Thailand, varieties of medicinal plants species are usually combined to prepare a multiherbal remedy. Ben-Cha-Lo-Ka-Wi-Chian remedy (BLW remedy) as well is a mixed powder from the roots of *Capparis micracantha* DC. (CAPPARIDACEAE), *Clerodendrum petasites* S. Moore (VERBENACEAE), *Harrisonia perforata* (Blanco) Merr. (SIMAROUBACEAE), *Ficus racemosa* L. (MORACEAE) and *Tiliacora triandra* (Colebr.) Diels (MENISPERMACEAE) in equal proportions by weight. This remedy is registered as general antipyretic in the List of Herbal Medicine Products of the National List of Essential Drugs A.D. 2006 for use in Primary Health Care services [4].

Some biological activities for each component herb of BLW remedy have been previously studied such as: the inhibition of lung cancer and anti-tuberculosis of *C. micracantha* crude drugs [5-7], the antibacterial activity [8], bronchodilator effect [9] and relaxation of tracheal smooth muscle of *C. petasites* [10], exhibiting anti-inflammatory, hepatoprotective and hypoglycemic effects of *F. racemosa* [11-13]. The extracts of the leaves and the branches of *H. perforata* showed *in vitro* antimalarial activity against *Plasmodium falciparum* [14]. Whereas for *T. triandra*, from the study of Sareeratawong et al. [15], the root extract had antimalarial activity against *P. falciparum in vitro*.

Besides, the lacks of biological activities of these plant roots as BLW remedy still have been exhibited.

**Correspondence to: Nijsiri Ruangrungsi*
E-mail: nijsiri.r@chula.ac.th
Tel. +66 (0) 2218 8152, Fax. +66 (0) 2253 2395

Therefore the current study aimed to investigate the safety of the remedy and each species extracts through cytotoxicity and mutagenicity evaluation. In addition, the investigation was expanded to carry out antioxidant and anti-mutagenicity of the remedy by using DPPH and *Salmonella typhimurium* TA98 and TA100 as targets respectively. Furthermore the fingerprint profile of the BLW remedy was carried out using three-dimensional high performance liquid chromatography (3D-HPLC).

MATERIALS AND METHODS

Collection of plant material

Each root of five plant species was collected from Nong-kai province, Thailand and avoided from the contamination of upper ground plants. All set of crude drugs were authenticated by Ruangrungsi N. Voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University, Bangkok, Thailand.

Preparation of plant extracts and the BLW remedy

After the authenticated five root species were sliced into small pieces and shade dried, all dried crude materials were pulverized to a course powdered by Universal Cutting Mill.

Each coarsely powdered of five roots species was macerated in ethanol for 24 h, filtered through filter paper no.1 with Buchner funnel. The ethanol extract was evaporated *in vacuo*. The marc was re-extracted until exhausted. The filtrates were evaporated as above manner. The pooled dried residue was weighed and stored at -20^{o} C.

The marc successively extracted with ethanol was dried, then macerated gently with boiling distilled water for 1 h, and allowed to standing for 24 h at room temperature (24°C), filtered through filter paper no.1 with Buchner funnel. The whole process was repeated until exhausted. The pooled water extract was lyophilized to dryness, weighed and stored at-20^{o} C.

The remedy extract was prepared by mixing each extract in according to their yields as to make a mixture of powder of component herbs in equal proportions by weight

Three-dimensional (3-D) HPLC analysis of Ben-Cha-Lo-Ka-Wi-Chian remedy

The 3-D HPLC system for BLW remedy fingerprint analysis was obtained according to Ito et al. [16] using Agilent 1100 3D-HPLC system by Agilent Technologies, Tokyo, Japan equipped with a photodiode-array detector by using a column 4.6 x 250 mm TSK gel ODS-80Ts (Tosoh Corp. Tokyo, Japan) and kept at 40° C. The elution of mobile phase was performed by 10 mM phosphoric acid-acetonitrile linear gradient (95:5-5:95) by 60 min at a flow rate of 0.8 ml/min. The UV spectrum used was monitored by a range of 200-400 nm. Assignments of all major peaks were supposed by comparing the UV spectrum patterns of each peak with the data registered in The Dictionary of natural products Program (DNP 19.1) provided by Tayle & Francis Group.

Cytotoxicity by Artemia salina lethality assay

The cytotoxicity screening of all extracts was investigated by the *Artemia salina* lethality test according to Meyer et al. [17] with some modifications. Briefly, *Artemia salina* L. (Brine shrimp larva) eggs were hatched in shallow rectangular dish filled with artificial sea water. A plastic divider with several 2 mm holes was clamped in the dish to make two equal compartments. The eggs were sprinkled into the dark compartment, while another compartment was illuminated. After 24 hours the phototropic nauplii were collected by Pasteur pipette from light side. Ten brine shrimp aged 48 hours were transferred to each vial, and 5 ml artificial sea water was added. The vials were maintained under illumination. Difference concentrations of samples in methanol and control (methanol only) were impregnated into 0.5x2 cm filter paper and air dried before being placed in the vial filled with *A. salina*. Five replicated were prepared for each concentration. The survivors were counted after 6 and 24 hours and the concentrations that kill 50% of the *Artemia salina* or the medium lethal concentrations (LC_{50}) were determined.

Scavenging activity by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical

The free radical scavenging activity of each extracts and remedy were evaluated using the DPPH assay described by Brand-William et al. [18] with some modifications. Briefly, 6×10^{-5} M solution of DPPH in methanol was prepared. A 100 µl of DPPH solution was added to 100 µl of sample or positive control (Quercetin and Buthylated hydroxytoluene: BHT) prepared in methanol in different concentrations. The assay was carried out in a 96 well microplate at room temperature for 30 minutes and escaped from light. Each concentration was performed in triplicate. The percentage of radical scavenging activity against DPPH was determined from % decreasing of absorbance at 517 nm. The concentration of the extracts led to 50% inhibition (IC_{50}) was determined from the plotted graph of % scavenging activity against the concentration of the extract.

Mutagenic and Anti-mutagenic activity by Ames test

The ethanol extracts of each species and the remedy extract were diluted in dimethylsulfoxide (DMSO) whereas the water extracts were diluted in sterile distilled water at the adequate doses. All aliquots were filtered through 0.45 µm sterile membrane filter discs. Ames tests [19] were performed on *Salmonella typhimurium* strains TA98 for frame-shift mutation and TA100 for base-pair substitution

mutation in an acidic condition (pH 3 - 3.5) without metabolic activation. Both direct and in-direct mutagenicity were assayed in the condition without and with sodium nitrite. The anti-mutagenicity against standard mutagen (nitrite treated 1-aminopyrine) was also investigated. Each assay was investigated in triplicate.

Nitrite treatment

An aliquot of ethanol and water extracts of each species and BLW remedy in responding to 5, 10, 20, 40 mg/ml were performed into a sterile test tube. The volume was adjusted to 200 µl with DMSO or sterile distilled water. Two-hundred fifty µl of 2M sodium nitrite and 550 µl of 0.25 N hydrochloric acid were added respectively for acidify the reaction mixture to pH 3-3.5 [20, 21]. The mixtures were incubated at 37°C in shaking water bath for 4 hr then placed for 1 min into the ice bath to stop the reaction and 250 µl of 2 M ammonium sulfamate was added. All tubes were allowed to standing 10 min in the ice bath again.

Ames mutagenicity assay

S. typhimurium (His^-) strains TA98 and TA100 were grown in nutrient broth (NB) liquid medium for 16 h at 37°C in agitation (90 rpm). One-hundred µl of untreated or nitrite treated mixture was transferred into sterile test tubes and mixed with 500 µl of 0.5 M phosphate buffer (pH 7.4), followed by 100 µl of TA98 or TA100 strains suspension. The final volume was 700 µl. The mixtures were incubated at 37°C for 20 min. Next, 2 ml of top agar, which consisted of 0.5 mM L-histidine and 0.5 mM D-biotin at 45°C was added to the mixture and poured onto a minimal glucose agar plate. The plates were incubated at 37 °C in darkness for 48 h and the numbers of his+ revertant colonies were manually counted.

1-Aminopyrine (1-AP) treated with nitrite in acid solution at 0.06 and 0.12 µg/plate on strains TA98 and TA100 was used as positive mutagenic respectively. Dimethyl sulfoxide or sterile distilled water was used as a spontaneous reversion.

The results data were assessed by mean and standard deviation of histidine (His^+) reverstants per plate. The mutagenic index (MI) was also calculated for each concentration. MI is the average number of revertants per plate divided by the average number of the spontaneous revertants per plate. The mutagenic effect of each sample was pronounced if the number of His^+ revertants per plate was higher than twice of spontaneous revertants (MI > 2) and also a concentration-response relationship was shown [22].

Anti-mutagenicity with modification by nitrite treated 1-aminopyrene

One-hundred µl of *S. typhimurium* strain suspension was added into the sterile test tube containing 500 µl 0.5 M phosphate buffer (pH 7.4), 0.15 µg of nitrite-treated 1-AP and 5, 10 and 15 mg/ml of each sample solutions. Dimethylsulfoxide (DMSO) or sterile distilled water was added to adjust the final volume to 700 µl. Subsequently, the mixtures followed the protocol as described in *Ames mutagenic assay.* The percent modification was calculated by the following formula:

$$\% \text{ Inhibition} = [(A - B) / (A - C)] \times 100$$

Where A is the number of histidine revertants per plate induced by nitrite treated 1-AP, B is the number of histidine revertants per plate induced by nitrite treated 1-AP in the presence of extract and C is the number of spontaneous histidine revertants per plate. The percentage of inhibition was classified as strong (higher than 60%), moderate (60-41%), weak (40-21%) and negligible (20-0%) [23].

RESULTS

Three-dimensional (3D) HPLC fingerprint

Figure 1 showed 3D-HPLC fingerprint of BLW remedy. Twelve components (Cappine B, Yanangine, Tiliacosine, Tiliacorinine, Tiliasine, Nevedencin 7-rutinoside, Hispidulin 7-methyglucoside, Hispidulin, Heteropeucenin 7-Me-ether, Perforatin G, Racemosa and Isowingtione) were illustrated.

Cytotoxicity by Artemia salina lethality assay

The ethanolic extract of *T. triandra* and *H. perforata* exhibited lethality effect to *A. salina* with the LC_{50} of 44 and 600 µg/ml respectively. The water extracts of *T. triandra* and *H. perforata* also showed toxicity to the *A. salina* with the LC_{50} 200 and 560 µg/ml respectively. Both ethanolic and water extracts of *C. petasites* and *F. racemosa* showed LC_{50} more than 10,000 µg/ml. Finally, Ben-Cha-Lo-Ka-Wi-Chian remedy extract demonstrated LC_{50} of 265 µg/ml (Table 1). According to Meyer et al. [18], who classified crude extracts into toxic (LC_{50} value < 1,000 µg/ml) and non-toxic (LC_{50} value > 1000 µg/ml), Ben-Cha-Lo-Ka-Wi-Chian remedy, *H. perforata* and *T. triandra* had potential to be toxic to *A. salina*.

Scavenging activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical

The ethanol extracts of *H. perforata* and *T. triandra* showed the scavenging activity with IC_{50} of 71.46 and 83.64 µg/ml respectively, whilst BLW remedy extract showed the IC_{50} of 83.53 µg/ml. Only ethanol extract of *C. micracantha* exhibited weak free radical scavenger (IC_{50} > 1,000 µg/ml). Most of the water extracts showed weak radical scavenging activities including *C. micracantha*, *C. petasites* and *T. triandra* (IC_{50} > 1,000 µg/ml), excepted *F. racemosa* (IC_{50} 93.15µg/ml) (Table 1).

Mutagenic and Anti-mutagenic activity by Ames test

Ben-Cha-Lo-Ka-Wi-Chian remedy and its component extracts at all doses (5, 10, 20 and 40 mg/plate) were not directly mutagenic (MI < 2) towards *S. typhimurium* TA98 (Figure 2A) and

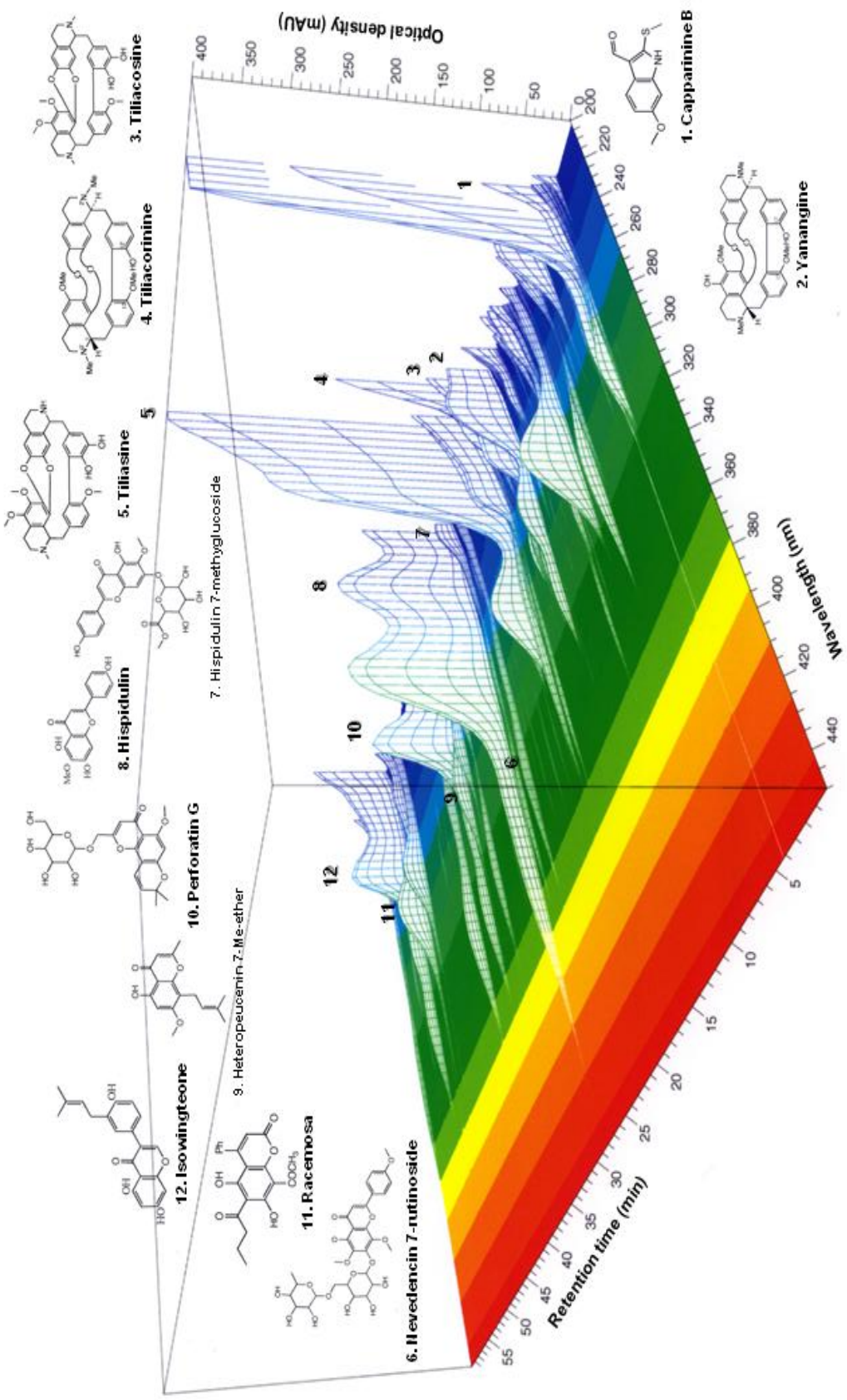


Figure 1 The 3D-HPLC Profile of Ben-Cha-Lo-Ka-Wi-Chian Remedy. The analysis situation was combining from colum: TSK gel ODS-80Ts (4.6x250mm), mobile phase: 10 mM Phosphoric acid-Acetonitrile, Linear gradient: (95:5 → 5:95, 60 min), flow rate: 0.8 mL/min, infusion volume: 5 mL, temperature: 40°C and Wavelength: 200-400 nm.

Table 1 Cytotoxic: *Artemia salina* lethality (LC_{50}) and antioxidant; mean inhibition concentration (IC_{50}) of the ethanol, water extract of five root species and BLW remedy

Samples	Cytotoxic *Artemia salina* lethality (LC_{50}: µg/ml)		Antioxidant Mean inhibition concentration (IC_{50}: µg/ml)	
	Ethanol extract	Water extract	Ethanol extract	Water extract
Capparis micracantha DC.	>1,000	>10,000	>1,000	>1,000
Clerodendrum petasites S. Moore	>10,000	>10,000	249.10	>1,000
Harrisonia perforata (Blanco) Merr.	600	560	71.46	404.64
Ficus racemosa L.	>10,000	>10,000	111.87	93.15
Tiliacora triandra (Colebr.) Diels	44	200	83.64	>1,000
Ben-Cha-Lo-Ka-Wi-Chian remedy	265		83.53	
Quercetin	nd		0.45	
Buthylated Hydroxyl toluene(BHT)	nd		3.47	

n=5 for cytotoxic, n=3 for antioxidant, nd=not determined

Figure 2 The mutagenic index (MI) of mutagnenic with-out nitrite effect induced by each plant species and BLW remedy on *S.typhimurium* strains TA98 (2A) TA100 (2B). Abbreviations including E: ethanol extract, W: water extract, CM: *C. micracantha*, CP: *C. petasites*, HP: *H. perforata*, FR: *F. racemosa*, TT: *T. triandra* and BLW: Ben-Cha-Lo-Ka-Wi-Chian Remedy.

Figure 2A (TA98)

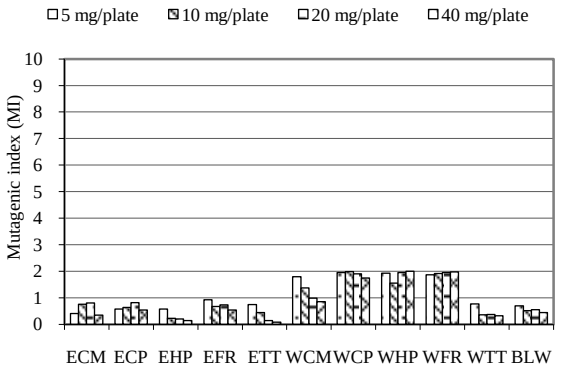


Figure 2B (TA100)

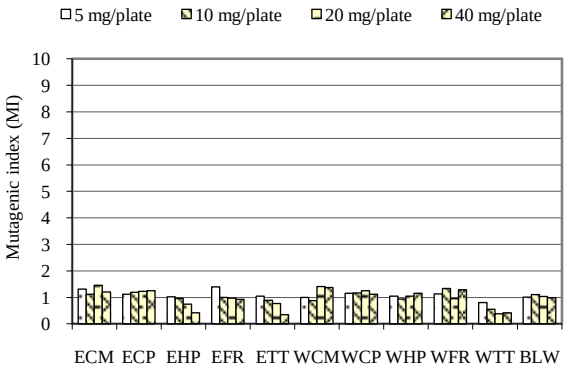


Figure 3. The mutagenic index (MI) of mutagnenic with nitrite effect induced by each plant species and BLW remedy on *S.typhimurium* strains TA98 (3A) TA100 (3B). Abbreviations including E: ethanol extract, W: water extract, CM: *C. micracantha*, CP: *C. petasites*, HP: *H. perforata*, FR: *F. racemosa*, TT: *T. triandra* and BLW: Ben-Cha-Lo-Ka-Wi-Chian Remedy.

Figure 3A (TA98)

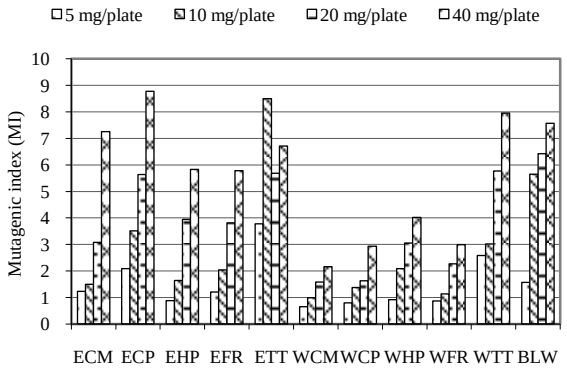
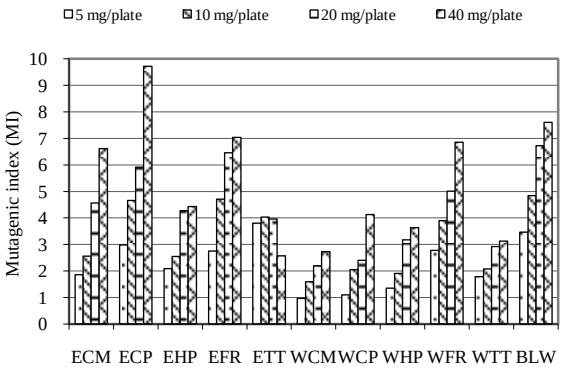


Figure 3B (TA100)



TA100 (Figure 2B). Most of water extracts except *T. triandra* illustrated higher histidine (His^+) reverstants per plate than ethanol extracts.

On the contrary, most of the extracts including BLW remedy extract showed indirect mutagenictiy induced by nitrosation (sodium nitrite treated in acid solution) as shown in Figure 3 (TA 98 in Figure 3A and TA100 in Figure 3B). Most of ethanol extracts demonstrated the mutagenic index higher than the water extracts including the water

Table 2 Percentage of the mutagenicity inhibition on the Ben-Cha-Lo-Ka-Wi-Chian remedy and its components

Sample	Solvent extract	Percentage of inhibition					
		5 mg/ml		10 mg/ml		15 mg/ml	
		TA 98	TA100	TA98	TA100	TA98	TA100
BLW remedy		89.35	75.20	94.19	95.07	96.56	102.18
C. micracantha	Ethanol	79.04	85.83	93.45	83.29	93.09	86.25
	water	72.44	98.52	85.87	103.07	89.81	104.97
C. petasites	Ethanol	71.82	83.85	85.13	99.48	87.01	106.07
	Water	39.40	41.71	50.18	56.36	57.17	57.30
H. perforata	Ethanol	101.48	120.20	102.15	118.36	102.28	124.25
	Water	82.16	99.37	88.71	106.77	96.00	108.31
F. racemosa	Ethanol	97.39	93.74	99.03	110.31	100.75	106.54
	Water	35.13	70.06	61.18	39.22	58.01	52.59
T. triandra	Ethanol	98.98	112.85	101.35	123.12	101.53	121.61
	Water	88.42	101.93	97.21	120.24	98.69	119.02

extract of *T. triandra* and BLW remedy extract. Whereas, ethanol and water extracts of *C. petasites* showed highest mutagenic index in both strains.

For anti-mutagenicity, most of the remedy extract and the components extracts exhibited strongly active inhibition (more than 60% inhibition) against nitrite treated 1-aminopyrine induced mutagenicity in both TA98 and TA100. Particularly ethanol extracts of *H. perforata* and *T. triandra* presented the higher percentage of inhibition (> 100% of inhibition). Whereas, the water extracts of *F. racemosa* and *C. petasites* demonstrated moderately active inhibitor (40 – 60 % inhibition) (Table 2).

DISCUSSION

As a consequence of increasingly interest to herbal medicine, the concern about the safety of all medicinal plants uses are also increasing. Some of Thai traditional remedies, although have been in practice are possible for an adverse effects. Therefore, information on the safety of BLW remedy and its ingredients are important for public health issue. Hence in this study the attempt to study the safety and some bioactivities of BLW remedy extract and each component herb extracts were performed by the cytotoxicity, mutagenicity, antioxidant, antimutagenicity activities including the fingerprint of the remedy was undertaken by using 3D-HPLC as a further evidence base of BLW remedy.

Cytotoxic assay by *A. salina* lethality assay has been used as bench top bioassay for the discovery of bioactive natural products. It is an excellent choice for elementary toxicity investigations of consumer products based on the ability to kill laboratory-cultured *Artemia nauplii* (brine shrimp larva) [17, 24, 25]. Good correlation between the *in vitro* and the *in vivo* test ($r = 0.85$ and $p<0.05$) using LC_{50} of *A. salina* and mice model reported by Perra et al. [26] showed that the *A. salina* is a useful method to predict oral acute toxicity in plant extracts. From the results, BLW remedy extract showed toxicity to *A. salina*, and the toxicity is assume to be due to ingredients in *T. triandra* and *H. perforata*. On the contrary, Paowin et al. [5] reported the different results that the water extract of leaves of *T. triandra* showed no acute or subchronic toxicities in female and male rats. This discrepancy might be due to different parts used of this plant in the present study.

The scavenging activity was used by the ability to scavenge DPPH radical. DPPH (2, 2-diphenyl-1-picrylhydazyl) assay is based on the reaction of metanolic solution of colored free radical DPPH by free radical scavenger. DPPH is a stable free radical with red-purple color (absorbed at 517 nm). If free radical has been scavenged, DPPH will change its color to yellow. Therefore, from this character, the medicinal plant free radical scavenging activity was carried out. The resultant level of reduced DPPH was measured spectrophotometerically [27]. This result showed that BLW remedy had potential to scavenge free radicals. This due to three plant roots ingredients namely *H. perforata*, *T. triandra* and *F. racemosa.* Previously the stem bark of *F. racemosa* was reported of free radical scavenging activity [28]. This study revealed the same antioxidant activity by *F. racemosa* root as well. In addition, the root of *C. micracantha* using Heinz body model has been shown as antioxidant [7].

Mutagenic and anti-mutagenic were undertaken by using *Salmonella typhimurium* as target, TA98 and TA100 strains were used for detected frame-shift and base-paired substitution mutagenicity respectively. The studies of Kato et al. [29] demonstrated that 1-aminopyrine treated with nitrite at pH 3.0 and 37°C for 4 h gave 1-nitropyrene and showed mutagenicity to *S. typhimurium* strain TA98 and TA100 without metabolic enzyme in the system. Whilst 1-Nitropyrene was used as a potent direct mutagen toward *S. typhimurium* strains TA 98 and TA100 with similar condition that occurring in the stomach digestion [20, 21]. International Agency for Research on Cancer (IARC) [30] had revealed

that the mutagenicity of 1-aminopyrine needed to be activated by nitroreductase. From the literature of Thai traditional remedy, only Kangsadalampai et al. [20] had undertaken the investigation of mutagenicity modification of Thai folklore medicine by nitrite in Ames *S.* mutagenicity test. The results of present study showed that all of the extract samples were non mutagenic directly but most of them were mutagenic indirectly under the nitrosation condition (nitrite treated and acidic condition). Nevertheless, for anti-mutagenic property, most of ethanol extract and BLW remedy exhibited strongly active inhibition whereas the water extracts showed moderately active inhibition of mutagenicity. These results were in accordance with the studies of Botting et al. [31] and Wongwattanasathien et al. [32] that the extracts derived from low polar solvents caused higher inhibition of mutagenicity than the crude extracts which derived from high polar solvents.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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