



## Development and optimization of Benjakul microemulsion formulations for enhancing topical anti-inflammatory effect and delivery

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### Abstract

**Background and purpose:** Benjakul (BJK) is a combination of five botanical herbal constituents widely used in Thai traditional medicine as an anti-inflammatory remedy. This study aimed to develop a novel topical microemulsion containing BJK for clinical use.

**Experimental approach:** The microemulsions were produced by a phase inversion temperature (PIT) methodology. Physicochemical properties and stability were evaluated to determine an optimal formula. The stable BJK-loaded microemulsion formulas were then subjected to *in vitro* studies for their anti-inflammatory activity, skin cell toxicity, drug permeation, and stability.

**Finding/Results:** Two novel formulations containing isopropyl myristate (ME1-BJK and ME2-BJK) passed the compendial stability test. BJK constituents were completely dissolved in the oil phase and incorporated into the microemulsion base Transcutol<sup>®</sup> and Labrasol<sup>®</sup> avoiding the use of alcohol, both microemulsion formulations demonstrated high anti-inflammatory activity with IC<sub>50</sub> values of 3.41 ± 0.36 and 3.95 ± 1.73 µg/mL, respectively. However, dissolution of ME1-BJK showed a superior release profile through both lipophilic and hydrophilic membranes with the highest accumulated amount at 4 h of 25.13% and 38.06%, respectively. All tested formulations of BJK extract demonstrated no apparent skin cell toxicity at concentrations up to 50 µg/mL. After six-month storage under accelerated conditions, there were no significant changes in anti-inflammatory activity.

**Conclusions and implications:** A novel and stable BJK-loaded microemulsion formulation was successfully developed with excellent release and stability properties. Further clinical research to evaluate pain reduction, edema, and skin irritation using this formulation in animal models is ongoing.

**Keywords:** Anti-inflammatory activity; Benjakul; Microemulsion; Phase inversion temperature.

### INTRODUCTION

Chronic inflammatory arthropathies such as osteoarthritis are a common problem particularly in the elderly and its underlying pathophysiology involves many factors such as nitric oxide (NO), prostaglandins, and tumor necrosis factor (1). Contemporary Western

medicines are often taken orally to treat such conditions such as non-steroidal anti-inflammatory drugs (NSAIDs).

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Prescribed oral NSAIDs have had a black box warning required by the FDA since 2005. This warning includes both serious adverse gastrointestinal and serious adverse cardiovascular effects. Fatal events are included as a potential consequence for both the serious adverse gastrointestinal and cardiovascular effects (1). Patients at the highest risk for adverse effects from oral NSAIDs are also those at highest risk for osteoarthritis, the development of additional safe topical anti-inflammatory alternatives that can effectively treat osteoarthritis is critically important.

Topical administration is popular due to its site-specific local action and lower first-pass metabolism and reduced systematic exposure (2). However, drugs need to be able to reach the site of action through the stratum corneum which is the main barrier of the skin (3). Drug penetration depends on its physical and chemical properties and formulation as well as the properties of the skin (4). Advanced drug delivery methods including novel penetration enhancers and surfactants are used in the pharmaceutical industry in both oral and transdermal drug delivery systems. Research has demonstrated that the use of such technology can increase drug absorption through the skin, enhance drug stability, and thus produce better treatment outcomes (5,6). There are two major methods for emulsion preparation, the high-energy and the low-energy emulsification methods (7). We previously utilized a high-energy method to develop a nanoemulsion of Benjakul (BJK) for exploratory work. However, only a small amount of nanoemulsion can be prepared by a high-energy method, limiting further work such as clinical studies or scale-up studies. Therefore, this work will investigate new BJK loaded formulations suited for a low-energy preparation method, which does not require expensive laboratory equipment and is easily scaled up for clinical studies.

BJK, a Thai herbal remedy on the Thailand National List of Essential Medicines (NLEM), is commonly used in Thai traditional medicine (TTM) to treat inflammation. It comprises five herbs: *Piper chaba* Hunt. (PCH), *Piper sarmentosum* Roxb. (PSR), *Piper interuptum* Opiz. (PIO), *Plumbago indica* Linn. (PIL), and

*Zingiber officinale* Roscoe (ZOR). The remedy and its plant constituents have been previously reported to have cytotoxic activities against cancer cells (8,9), anti-allergic activity (10), and anti-inflammatory effects (10-13). A phase 2 clinical trial of oral BJK ethanolic extract capsules was assessed for clinical efficacy for treating primary osteoarthritis of the knee (14). BJK remedy was demonstrated to provide equal clinical efficacy in alleviating symptoms of osteoarthritis in knees when compared with the NSAID diclofenac. The anti-inflammatory effects of BJK were apparent without demonstrable liver or kidney toxicity. However, the patients who received both BJK extract capsules and diclofenac did have some abdominal discomfort including gastric pain (14).

Thus, topical BJK formulations were developed in this study by focusing on the low energy method using phase inversion temperature (PIT) which relies on the spontaneous formation of an emulsion under a specific composition (15). The physicochemical and drug-releasing properties and the stability of the preparations were also evaluated to determine an optimal formulation for clinical use with adequate penetration and stability.

## MATERIALS AND METHODS

### Materials

Standard piperine was purchased from Merck (Thailand). High-pressure liquid chromatography (HPLC)-grade water, methanol, and acetonitrile were procured from Labscan (Bangkok, Thailand). Chemicals used in emulsion preparations including isopropyl myristate (IPM), capryol-90, caprylocaproyl polyoxyl-8 glycerides, Transcutol<sup>®</sup>, Labrasol<sup>®</sup> and polyglyceryl-3 dioleate, Plurol<sup>®</sup> Oleique were supplied by Gattefosse (France). Human keratinocytes (HaCaT) were purchased from CLS cell lines service (Eppelheim, Germany). Murine macrophage cells (RAW 264.7) were from ATCC (Manassas, USA). 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and fetal bovine serum (FBS) were purchased from Sigma-Aldrich (USA). Dulbecco's modified eagle's medium (DMEM) was purchased from Gibco (Grand Island, NY).

Hydrophilic and lipophilic membranes were from Merck Millipore (Darmstadt, Germany).

### **Plant materials**

Five plant ingredients of BJK were bought from Thailand and authenticated by the herbarium curator of Southern Centre of Thai Medicinal Plant, Faculty of Pharmaceutical Science, Prince of Songkla University, Thailand. The voucher specimens of each plant were PCH: SKP 146160301, PSR: SKP 146161901, PIO: SKP 146160901, PIL: SKP 148160901, and ZOR SKP: 206261501.

### **Preparation of BJK extract**

The five herbs were dried and ground into powder and mixed in equal amounts. The mixture was macerated with 95% ethanol for three days (liquid:solid, 2:1). The macerate was filtered and the filtrate was collected. The residue was re-macerated twice, and all filtrates were combined and evaporated to dryness in a rotary evaporator. Rotary evaporation was performed at 45 °C and 50 mBar.

### **Determination of piperine in BJK extract**

Five concentrations of standard solutions were prepared by dissolving 10 mg of dried BJK extract in methanol and filtered through a 0.45 µm membrane filter. The BJK piperine content analysis was performed by using HPLC with a photodiode array (PDA) detector (2). A reverse-phase column (Phenomenex), Luna® 5 µm C18, 100 Å, 250 × 4.6 mm, was used. Ten µL solution samples were injected and gradient eluted with 0.1% phosphoric acid and acetonitrile with gradient elution as follows: 0 min, 60:40; 30 min, 50:50; 50 min, 5:95; 60 min, 0:100, a flow rate of 1.0 mL/min. Piperine

was determined at 340 nm. The concentration range was linear between 20-400 µg/mL

### **Microemulsion preparation**

#### *Experimental design*

In the PIT method, it is necessary to properly adjust the amounts of the oil, surfactant, co-surfactant, and water. IPM as an oil phase, Labrasol® as a surfactant, and Transcutol® as a solubilizer and co-surfactant were utilized. Firstly, the ratio of water phase to oil phase was fixed at 1:1 (50% water in the preparation). The three components in the oil phase were optimized according to the mixture design (Minitab 18): IPM, Labrasol®, Plurol® Oleique, and Transcutol®. A total of 9 formulas, all of which avoided the use of alcohol, were prepared (Table 1).

#### *Ternary-phase diagram formation*

The percentage of transmittance of the formulations using UV-Vis spectrophotometer (Thermo Scientific®, Genesys 10S, USA) at 650 nm was evaluated against distilled water as a blank (100% transmission). A pseudo-ternary-phase diagram was then calculated using Minitab 18 software.

#### *Microemulsion and BJK-loaded emulsion preparation*

The basis of the PIT method is to vary the heating temperature while the formula composition is fixed. Firstly, IPM was added into a premixed Labrasol® and Transcutol® followed by the addition of water and heating to 65 °C to form an emulsion. The emulsion was stirred for 20 min, then cooled rapidly to 25 °C. The percent components of each formulation are listed in Table 1.

**Table 1.** Microemulsion formulas proposed following the mixture design and percentage of transmittance in each formula.

Formula	Water phase	Components in the oil phase		
		Isopropyl myristate	Labrasol	Transcutol
1	50	7.5	35	7.5
2	50	15	30	5
3	50	12.5	25	12.5
4	50	5	30	15
5	50	5	40	5
6	50	7.5	30	12.5
7	50	12.5	30	7.5
8	50	10	30	10
9	50	15	20	15

### **Physicochemical evaluation of microemulsion formulas**

#### *Physical appearance*

The preparations were examined visually for color, homogeneity, and phase separation.

#### *Percentage of transmittance*

The clarity of the preparations was measured by percent transmittance (transparent formula = 100% transmittance) using UV-Vis spectroscopy at 638.2-650 nm.

#### *Mean particle size and polydispersity index analyses*

Mean particle size (MPS) and polydispersity index (PDI) were analyzed by photon correlation spectroscopy using Malvern Zetasizer Nano ZS90, with water as the dispersant. It is based on the principle of light scattering at a fixed angle of 173°, 25 °C.

#### *Emulsion-type confirmation study*

In order to confirm that the microemulsion produced was an oil-in-water system, the following tests were conducted. Conductivity measurement: the emulsion conductivity was measured using the Accumet® XL20 conductivity meter together with a 1.0 Accumet® probe. Only oil-in-water emulsions show conductivity. Dye test: water-soluble dye was used as the indicator. Only oil-in-water emulsions are miscible with the dye.

### **Stability testing**

#### *Centrifugation method*

Emulsion samples were centrifuged at 50,000 rpm for 15 min using Beckman® air-driven ultracentrifuge. The physical appearance of each sample was evaluated.

#### *Heating-cooling method*

Emulsion samples were kept at 45 °C for 24 h followed by 4 °C for 48 h (1 cycle). The test was repeated for six cycles. The physical appearance of each sample was then evaluated.

### **In vitro anti-inflammatory effect of BJK-loaded microemulsion**

#### *In vitro anti-inflammatory effect of BJK-loaded microemulsion by inhibition of NO production assay*

The inhibition of BJK extract on NO production was evaluated with the following

modified method of Tewtrakul (16). Murine macrophage cells (RAW264.7) were cultured in 96-well plates for 24 h in 5% CO<sub>2</sub> at 37 °C. The cells were stimulated with 5 ng/mL of lipopolysaccharide and treated with sample solution for 24 h. The supernatant was transferred into a new plate and Griess reagent was added. The absorbance was measured at 570 nm. The anti-inflammatory activity of the extracts was reported as IC<sub>50</sub>.

#### *Evaluation of cell damage as a result of anti-inflammatory tests*

RAW 264.7 cell damage was determined by the MTT assay. After separating the supernatant from the incubated plate, 5 mg/mL MTT solution was added to each well and incubated at 37 °C, 5% CO<sub>2</sub> for two h. Subsequently, the old medium was removed and 100 µL of 0.04 M hydrochloric acid (HCl) in isopropanol was added to dissolve the formazan product and the optical density (OD) was measured at 570 nm. The percentage of cell survival was compared against the control.

### **In vitro permeation of piperine through synthetic membranes**

The permeation study was performed using the Franz diffusion cell apparatus. One g of sample was added on synthetic lipophilic and hydrophilic membranes (Merck Millipore®, USA) (2.08 cm<sup>2</sup>, d = 1.63 cm). The receptor chambers were filled with phosphate buffer (pH 7.4), the temperature and stirring rate were controlled at 37 °C and 600 rpm, respectively. The solution was withdrawn and replaced with buffer at six different time points: 15, 30, 60, 120, 240, and 360 min. The accumulative amount of piperine was calculated as percentage permeation (% permeation) and plotted against time.

### **In vitro skin cell toxicity of BJK-loaded microemulsion**

Microemulsion formulas that passed the stability test were selected for the skin toxicity study. A concentration of 5,000 µg/mL was prepared and further diluted with water yielding 100, 50, 10, and 1 µg/mL. The emulsion base was treated in the same manner. The skin toxicity study was evaluated based on human adult keratinocytes (HaCaT) viability after exposure to the microemulsion. Cells were

seeded in 96-well plates at a density of  $2.5 \times 10^5$  cells/mL in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin and incubated at 37 °C under 5% CO<sub>2</sub> for 24 h. The diluted microemulsion formulas were then added to the wells and incubated further for 24 h. Subsequently, the cell viability was determined using the MTT assay and compared with the untreated control. MTT dye solution was added to each well and incubated at 37 °C for another 2 h. The formazan crystals formed were solubilized with 100  $\mu$ L DMSO and its absorbance was measured at 570 nm and cell viability was calculated using the following equation. The toxicity was noted when the survival of HaCaT cells was less than 70%.

$$\text{Cell viability (\%)} = \frac{\text{Sample OD} - \text{Control OD}}{\text{Control OD}} \times 100$$

#### **Chemical and biological stability under accelerated storage conditions**

The accelerated stability study was carried out according to International Conference on Harmonisation (ICH) guidelines (climatic zone IVb). The highest piperine permeation formula was selected and its biological stability was determined by monitoring in glass vials under accelerated storage conditions at temperature  $40 \pm 2$  °C and  $75 \pm 5\%$  relative humidity for

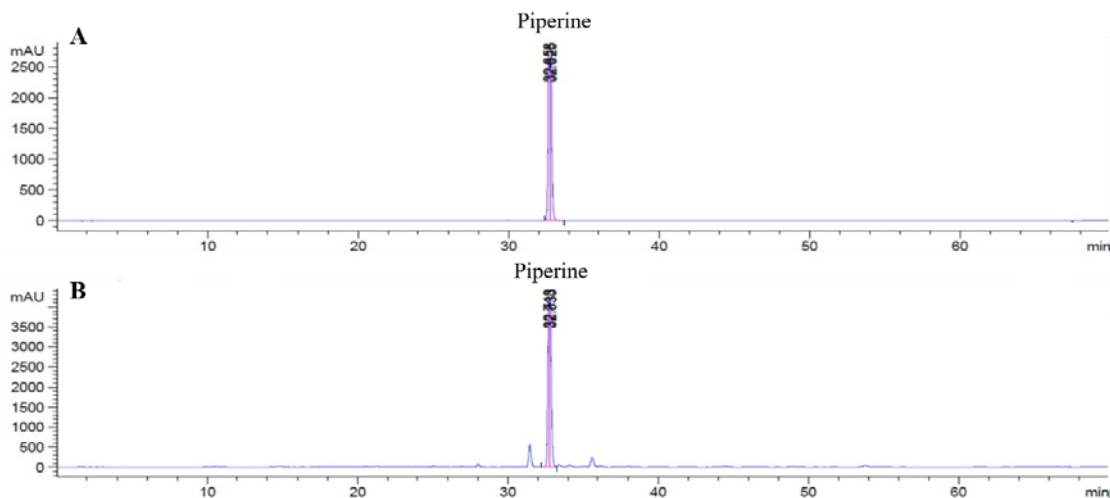
6 months. Aliquot samples were taken every 30 days. A total of eight samples were tested for the piperine content and inhibition of NO production in comparison with day 0.

#### **Statistical analysis**

All experiments of emulsion preparation were performed in triplicate. The data were presented as mean  $\pm$  standard deviation (SD) while the tested of biological activity were presented as mean  $\pm$  standard error of the mean (SEM). The stability results were expressed as mean  $\pm$  SEM compared with the control group at each time interval. Statistical significance was determined by one-way analysis of variance (ANOVA) at the 95% confidence interval.

## **RESULTS**

The HPLC chromatogram of BJK extract is shown in Fig. 1 and the calibration curve of standard piperine was obtained using HPLC with the retention time of 32 min. The correlation coefficient was calculated from  $y = 57040x + 525770$ , giving  $R^2 = 0.9996$ . One g of BJK extract was found to contain  $111.4 \pm 2.26$  mg of piperine, which could be used as an indication of 100% solubility of BJK extract.



**Fig. 1.** HPLC chromatograms of (A) piperine standard and (B) Benjakul extract detected using diode array at 340 nm.

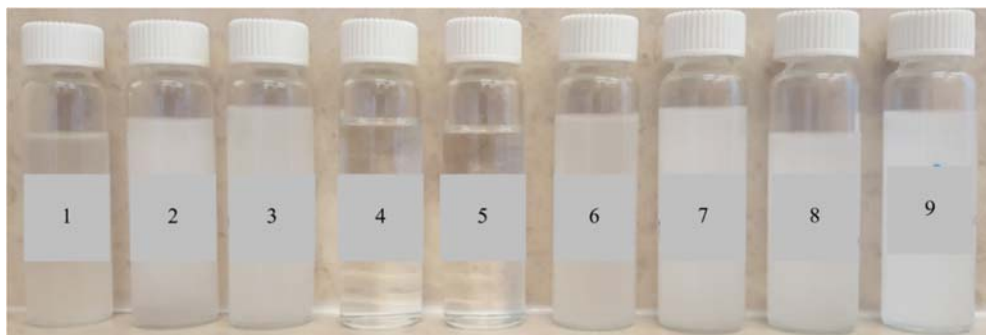


Fig. 2. Physical appearance of nine microemulsion formulas.

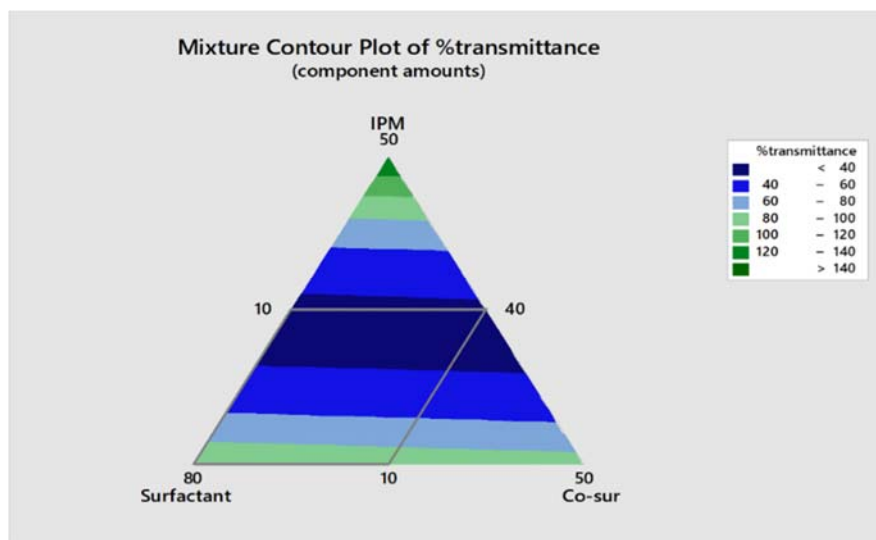


Fig. 3. Pseudo-ternary phase diagram of microemulsions (based on the percentage of transmittance).

Nine emulsion bases were formulated according to the mixture design (Table 1). Formulas 4 and 5 gave transparent solutions (Fig. 2). A pseudo-ternary phase diagram (based on the percentage of transmittance) was created (Fig. 3). Suitable components in emulsions in this study were prepared with transmittance above 95% (green region followed Fig. 3). The optimized formula by DOE experiment design was: IPM, Labrasol® and Transcutol® 5, 30, 5-10%, respectively.

To find the best formula suitable for BJK extract, the effects of oil were evaluated. Four clear emulsion bases (ME1-ME4) were formulated with particle sizes below 60 nm. A 0.5% of dark brown and viscous BJK extract was dissolved in the oil phase of each emulsion base coded ME<sub>x</sub>-BJK, x = 1-4. The formulas were shown in Table 2. All of the eight formulas were oil-in-water emulsions.

Physicochemical evaluation of emulsion formulas was conducted and is shown in Table 3. However, after BJK was loaded, the particle sizes in ME3-BJK and ME4-BJK increased and became unstable after the completion of the 6<sup>th</sup> heating-cooling cycle of stability testing. Therefore, only ME1-BJK and ME2-BJK were further studied.

A study was conducted to compare emulsions prepared by PIT and phase inversion composition (PIC) methods. No differences were found between formulas produced with the PIT or PIC method (Table 4). However, the PIC method was obtained by changing the water phase added into the oil phase. The rate of adding the water phase required strict control as it affected the particle size of the finished products, which could affect the solubility of BJK. Therefore, PIT was selected as the appropriate microemulsion method for BJK-loaded formulations.

**Table 2.** Microemulsion formulas. Each formula was made up to 100% with deionized water.

Formula	Oil		Surfactant	Co-surfactant	
	Isopropyl myristate	Capryol-90	Labrasol®	Transcutol®	Plurol oleique
ME1	5	-	30	10	-
ME2	5	-	30	5	5
ME3	-	5	30	10	-
ME4	-	5	30	5	5
ME1-BJK	5	-	30	10	-
ME2-BJK	5	-	30	5	5
ME3-BJK	-	5	30	10	-
ME4-BJK	-	5	30	5	5

BJK, Benjakul.

**Table 3.** Physicochemical properties of the microemulsion.

Formula	Appearance	Physicochemical properties				
		Transmittance (%)	pH	MPS (nm)	PDI	Conductivity (mV)
ME1	Colorless	99.76 ± 0.08	3.84 ± 0.13	42.53 ± 12.12	0.43 ± 0.02	12.73 ± 0.35
ME2	Colorless	99.90 ± 0.01	3.69 ± 0.18	34.80 ± 2.47	0.42 ± 0.01	16.7 ± 0.25
ME3	Colorless	99.90 ± 0.01	3.72 ± 0.12	55.18 ± 0.23	0.25 ± 0.01	19.03 ± 1.81
ME4	Colorless	99.84 ± 0.02	3.9 ± 0.25	44.25 ± 2.19	0.18 ± 0.03	26.23 ± 0.19
ME1-BJK	Dark green	N/A	3.78 ± 0.11	278.8 ± 42.24	0.39 ± 0.09	45.70 ± 0.24
ME2-BJK	Dark green	N/A	3.72 ± 0.15	188.8 ± 20.51	0.19 ± 0.08	53.20 ± 0.43
ME3-BJK	Dark green	N/A	3.81 ± 0.10	344.9 ± 3.25	0.46 ± 0.01	43.88 ± 0.28
ME4-BJK	Dark green	N/A	3.81 ± 0.02	356.8 ± 16.15	0.62 ± 0.06	55.11 ± 0.43

BJK, Benjakul; MPS, mean particle size; PDI, polydispersity index.

**Table 4.** Comparison of microemulsions prepared by PIT and PIC methods.

Formula	Method	Physicochemical evaluation of microemulsion			
		Appearance	Transmittance (%)	pH	MPS (nm)
ME1-Base	PIT	Transparent	99.76 ± 0.08	3.84 ± 0.13	42.53 ± 12.12
ME1-Base	PIC	Transparent	99.82 ± 0.08	3.79 ± 0.14	59.69 ± 29.83
ME2-Base	PIT	Transparent	99.90 ± 0.01	3.69 ± 0.18	34.80 ± 2.47
ME2-Base	PIC	Transparent	99.90 ± 0.06	3.72 ± 0.11	42.03 ± 11.31

PIT, Phase inversion temperature; PIC, phase inversion composition; MPS, mean particle size.

**Table 5.** Piperine content in microemulsions.

Vehicle	Piperine content in 100 g formulation (mg), mean ± SD	Solubility (%)
BJK in MeOH	50.75 ± 1.26	100
ME1-BJK	50.41 ± 0.397	99.33
ME2-BJK	50.73 ± 9.96	99.96

The BJK marker, piperine, was analyzed in each formula and compared with methanol. Both ME1-BJK and ME2-BJK showed > 99% piperine content (Table 5). Two BJK-loaded emulsion formulas were tested for anti-inflammatory activity using the NO production inhibition assay. The results showed no significant difference between ME1-BJK and ME2-BJK with IC<sub>50</sub> values of 3.41 ± 0.36 and 3.95 ± 1.73 µg/mL, respectively (Fig. 4). ME1-

BJK and ME2-BJK showed higher activity than BJK extract possibly because ME-based formulation induces cell death. Therefore, cell viability at each concentration of the emulsions was evaluated and found that cell survival was still > 70%. The micro emulsion-based formulas were further tested for NO inhibition which showed their IC<sub>50</sub> values to be > 20 µg/mL with no cell toxicity. However, at 50 µg/mL concentration, the cells could not

attach to the well plates, therefore the results could not be interpreted. The IC<sub>50</sub> results of these emulsions are five times as potent as the preliminary emulsion reported by Thummawan et al. (12)

The *in vitro* permeation profile of ME1-BJK and ME2-BJK was reported as % of piperine released against time through lipophilic and hydrophilic membranes (Figs. 5 and 6). Piperine was detected even after the first 15 min. ME1-BJK showed higher % piperine release through both membranes than ME2-BJK, with the highest accumulated amounts at 4 h of 25.13 and 38.06%, respectively. ME2-BJK showed piperine release of not more than 15%. Therefore,

ME1-BJK was selected for further skin cell toxicity and biological stability tests.

Skin cell toxicity was determined as the survival of HaCaT cells > 70% at various sample concentrations. We found that the survival pattern of HaCaT cells treated with the blank ME1-base and ME1-BJK showed the same pattern. Both ME1-base and ME1-BJK showed cell toxicity at concentrations above 20 µg/mL while BJK extract was safe up to 50 µg/mL. These results were similar to those of macrophage cells tested for NO inhibition which demonstrated that at high concentrations cells could not attach to the wells and thus the results could not be interpreted.

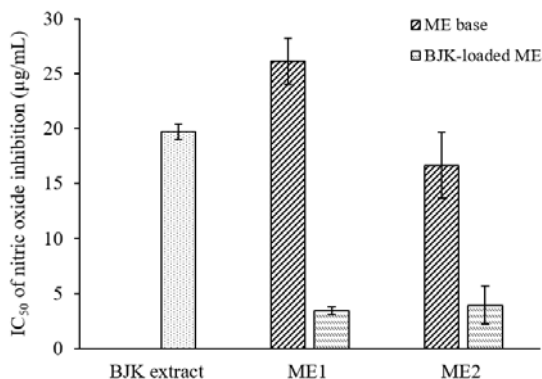


Fig. 4. IC<sub>50</sub> of the stable microemulsion formulas based on *in vitro* nitric oxide inhibition. BJK, Benjakul.

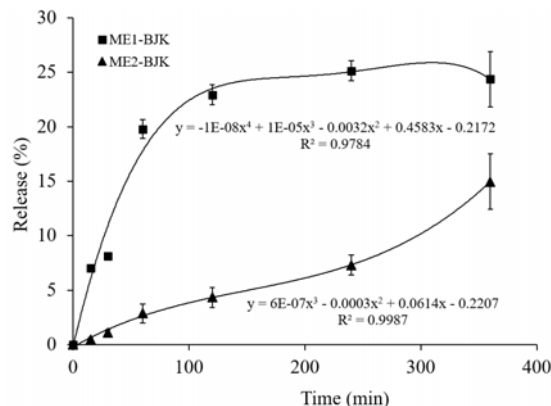


Fig. 5. Percent of permeation of piperine through the lipophilic membrane of ME1-BJK compared with ME2-BJK. BJK, Benjakul.

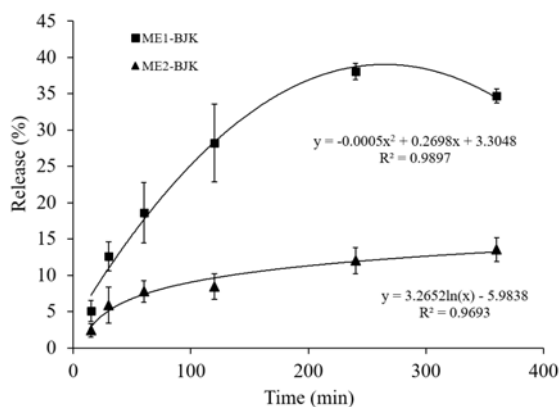


Fig. 6. Percent of permeation of piperine through the hydrophilic membrane of ME1-BJK compared with ME2-BJK. BJK, Benjakul.



**Table 6.** Chemical and biological stability of ME1-BJK, based on nitric oxide inhibition under accelerated storage conditions.

Testing	Piperine content Mean (mg/100 g formula) ± SD		Inhibition on nitric oxide production (%) (mean ± SEM)	Toxicity (%) (mean ± SEM)
	Content <sup>a</sup> (mg/g)	Remaining (%)		
Day 0	50.69 ± 3.47	100.00	39.13 ± 5.23	-5.32 ± 2.67
Day 15	50.05 ± 1.34	98.74	39.21 ± 1.41	-7.13 ± 2.31
Day 30	50.31 ± 0.64	99.25	36.76 ± 3.7	-14.11 ± 0.45
Day 60	48.98 ± 1.88	96.63	39.36 ± 1.38	-7.26 ± 1.88
Day 90	48.99 ± 0.88	96.66	36.88 ± 3.48	-3.85 ± 1.00
Day 120	49.26 ± 2.07	97.18	35.94 ± 1.11	-3.63 ± 2.39
Day 180	48.44 ± 0.5	95.56	39.76 ± 1.06	-0.83 ± 5.63

<sup>a</sup> Data of piperine content were calculated following the standard linear equation:  $y = 22.452x - 1500.8$ ,  $R^2 = 0.9997$ ; BJK, Benjakul.

After 6-month storage under accelerated conditions, ME1-BJK was tested for piperine content and NO inhibition. One-way ANOVA was used to evaluate the mean difference of % inhibition. No significant change was detected compared with day 0 at a 95% confidence interval (Table 6). According to the Thai FDA regulations, drugs that pass the 6-month accelerated storage test can claim two years of stability for drug registration approval.

## DISCUSSION

In traditional folk medicine practice, physicians often use a combination of herbs as a remedy rather than a single herb to provide the desired effect. In Thailand, Thailand's National List of Essential Medicines (NLEM) has approved six traditional remedies as medicines for muscle and joint pain. Each of the remedies contains a complex mixture of herbs, which in turn imposes limitations on the use of these traditional remedies. BJK is potentially a suitable remedy for drug development because it is composed of only five plants, whereas other remedies such as the Sahastra remedy are composed of more than 20 single plants (17). In this study, we were interested in further investigation of BJK which is the most well-known traditional remedy comprising five spicy herbs. A central tenet of TTM principles is to utilize spicy herbs to reduce pain and inflammation. In addition, the TTM principle believes that three *Piper* species in BJK remedy can distribute throughout the whole body. Piperine as an alkaloid from three *Piper* species in BJK has been reported to have anti-inflammatory activity and enhance skin

permeation related to TTM principles (18). Ginger as a plant component in BJK has been successfully investigated for the treatment of joint inflammation (19). Knowledge of TTM suggests that ginger will increase the wind element and therefore increase blood circulation. Thus, this BJK as a combination herbal therapeutic in TTM principles has both anti-inflammatory and skin-enhancing permeation to the target site. In addition, a previous report found the ethanolic extract of BJK also showed anti-inflammatory effects both *in vitro* and *in vivo* consistent with TTM principles (10,13).

Due to its simple plant combination, together with the defined anti-inflammatory activity, BJK ethanolic extract development as an anti-inflammatory medicine to be applied for acute and chronic inflammation demonstrated inhibitory effects on nitric oxide and PGE<sub>2</sub> production (10,13). The ethanolic extract of BJK inhibits inflammation in rats both topically and orally (13). In additional studies, a double-blind, randomized controlled trial of efficacy and safety of BJK remedy extract was investigated in primary osteoarthritis of knee compared with diclofenac. BJK remedy extract showed equivalent clinical efficacy in relieving symptoms of osteoarthritis of the knee when compared with diclofenac (14). However, 7.14% of patients administered the BJK extract orally had significant gastric pain (14). To reduce local irritant gastric as well as systemic effects of BJK, topical formulas are being developed. BJK cream was developed and tested in primary knee osteoarthritis patients compared with diclofenac cream. BJK cream was stable under accelerated condition testing.

BJK cream reduced knee pain in volunteers after 100 meters of walking and reduced the time required to complete the distance (20). The volunteers demonstrated significantly better quality of life when evaluated by WOMAC index scores (20). However, a significant compliance issue was apparent because of the suboptimal greasy feel of the BJK cream. Thus, the aim of the current investigations was to develop a significantly improved emulsion with greater stability and increased skin permeation with better efficacy.

Six pure compounds such as myristicin, plumbagin, methyl piperate, piperine, 6-gingerol, and 6-shogaol were isolated from the ethanolic extract of BJK (21). Plumbagin and 6-shogaol exhibited the most potent anti-inflammatory activity with  $IC_{50}$  value of  $0.002 \pm 0.002$  and  $0.92 \pm 0.31$  mg/mL, respectively. Piperine, as a major active compound in BJK, showed moderate activity on NO-production inhibition with an  $IC_{50}$  value of  $38.89 \pm 0.79$  mg/mL (10). Moreover, piperine also has been suggested to activate transient receptor potential vanilloid 1 which is related to being analgesic and counterirritant effects (22). However, when BJK was loaded into the formula at the concentration of 0.5% w/w, only piperine was detected with the maximum absorbance at 340 nm. Thus, piperine was chosen as both an analytical and biological marker in this study (Fig. 1).

The clinical trial phase I of BJK ethanolic extract reported that applying 0.5% BJK extract showed no skin irritation while some irritation to the skin was detected when using 1% BJK extract (20). Therefore, an appropriately safe BJK content in all topical formulas was considered to be 0.5%.

Nanotechnology is widely used in the pharmaceutical industry in both oral and transdermal drug delivery systems. The use of nanotechnology can significantly increase drug absorption through the skin, resulting in better treatment outcomes and a more stable drug (23). The particle size of the finished product affects the product permeability through the skin and nanotechnology can produce nano-sized vesicles that enhance skin permeation. Thus, BJK-loaded emulsion formulas were developed and tested for physicochemical

properties, stability and permeation profile. A previous report of a nanoemulsion containing BJK extract and alcohol using high energy probe sonication produced BJK-loaded nanoemulsions with a particle size of  $587.6 \pm 25.1$  nm (12). In this study, we employed low-energy emulsion formulations using the PIT method with varying amounts of oil, surfactant, and co-surfactant. The most suitable formula was: IPM as an oil phase is a polar emollient, Labrasol® is an anionic oil in water surfactant, and solubilizer Transcutol® as a BJK solubilizer and co-surfactant. Transcutol® (diethylene glycol monoethyl ether) is a penetration enhancer and surfactant plays an important role in dissolving BJK to facilitate the extract to be completely dissolved in the oil phase and completely incorporated into the emulsion. The BJK-loaded emulsion prepared following the PIT method produced a stable microemulsion with particle sizes of 150-300 nm. (Table 3).

Two of the BJK-loaded emulsion formulations were tested for anti-inflammatory activity, permeation through the skin, and skin cell toxicity. Though the anti-inflammatory activity and skin cell toxicity of both ME1-BJK and ME2-BJK were similar ( $IC_{50} = 3.41$  and  $3.95$   $\mu$ g/mL, Fig. 4), their permeation results were significantly different (Figs. 5 and 6). ME1-BJK showed higher piperine permeation than ME2-BJK through both lipophilic and hydrophilic membranes with 24.37 and 34.70% (Figs. 5 and 6). ME1-BJK had a greater percentage of Transcutol® than ME2-BJK, so Transcutol® plays a role as a solubilizer that facilitates increasing penetration.

There were no significant differences between formulas produced with either the PIT or PIC method. However, in the PIC method, the rate of adding water phase to the oil phase affected particle size, and might also affect BJK solubility, thus this parameter should be controlled. Therefore, the PIT method was selected as an appropriate emulsion method for BJK-loaded microemulsion.

The stability testing and anti-inflammatory activity of ME1-BJK did not significantly change after 6-months of storage under accelerated conditions (40 °C and 75% relative humidity). Thai FDA regulations stipulate that

drugs that pass a 6-month accelerated storage test can be registered with a tentative shelf-life of two years. Therefore, these results are important for the future registration of BJK-loaded microemulsion as a topical anti-inflammatory drug.

## CONCLUSION

This study provides data to support further development of natural products, especially a combination of herbal extracts that are only partially soluble in water to be able to form stable microemulsions. In this work, we selected BJK, a well-known Thai traditional remedy, which was made completely soluble in the oil phase of an emulsion that contained IPM, caprylocaproyl polyoxyl-8 glycerides, and Transcutol® without using alcohol as a solvent. The emulsions passed a stability test, showed high anti-inflammatory activity with an IC<sub>50</sub> of 3.41 ± 0.36 µg/mL, showed a good release profile through both lipophilic and hydrophilic membranes and were stable under 6-month accelerated storage conditions. A BJK-loaded microemulsion was developed and optimized for topical administration. The product showed anti-inflammatory effects *in vitro*, potentially improved skin permeation, and was stable at room temperature with a tentative shelf-life of two years. Further studies are underway to evaluate irritation on human skin and additional clinical studies in chronic inflammatory diseases.

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## Conflict of interest statement

The authors declared no conflict of interest in this study.

## Authors 'contribution

A. Itharat conceived and supervised the project; P. Kuropakornpong performed the experiments, analyzed and interpreted the data; R. Loebenberg advised the experimental design, planned, and carried out the product development; A. Itharat, R. Loebenberg, B. Ooraikul, and N.M. Davies assisted in the data interpretation; P. Kuropakornpong wrote the manuscript in consultation with A. Itharat, R. Loebenberg, B. Ooraikul, and N.M. Davies . The final version of the manuscript was approved by all authors

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