DEVELOPMENT OF TOPICAL NANOEMULSION PRODUCT CONTAINING BENJAKUL REMEDY EXTRACT FOR ANTI-INFLAMMATION

BY

MISS CHUNYIKA THUMMAWAN

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF MASTER OF SCIENCE
IN APPLIED THAI TRADITIONAL MEDICINE
FACULTY OF MEDICINE
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ENTITLED

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ABSTRACT

Benjakul is a Thai traditional remedy and used to be adaptogen. It is composed with five plants such as *Piper chaba* Hunt., *Piper sarmentosum* Roxb., *Piper interruptum* Opiz., *Plumbago indica* Linn., and *Zingiber officinale* Roscoe. It has ever been reported to treat Osteoartitis knee patients. It showed good efficacy equally NSAID drug (Diclofenac). However it has side effect such as stomach pain. Thus, the aim of this research was to develop to be a topical nanoemulsion product containing BKE for anti-inflammation, to reduce side-effect and to increase the efficient delivery of the drug.

The ethanolic extract of Benjakul before adding to the NE-base showed anti-inflammatory activity with IC_{50} value of 19.72 ± 0.72 µg/ml. and it had piperine content of 88.89 mg/g. Stress testing found that the extract was treated under thermolysis, hydrolysis, acid hydrolysis, base hydrolysis, and oxidation condition had anti-inflammatory activity with IC_{50} values of 21.17±2.09, 15.97±1.4, 19.47±3.2, 20.87±3.2, 15.35±1.8 µg/ml respectively and piperine content in Benjakul extract of every conditions were not decreased significantly. In the development of nanoemulsion the solubility of piperine in BKE was studied by dissolving in oleic acid,

myritol, isopropyl myristate, oil mix and tween80. The results showed that piperine content in every solvent were 31.00, 34.16, 41.41, 44.86, 51.30 mg/g, respectively.

The development of a topical nanoemulsion product containing BKE involved the study of the various formulations, i.e. oleic acid, myritol, isopropyl myristate, tween80, and other compositions with properties in the acceptable range were selected for the study. BKE3-NE-T8-x0.2 contains BKE and oil mixed (oleic acid: myritol: isopropyl myristate), and aqueous phase (propylene glycol, paraben cocn.) contains the surfactant (tween80), water and xanthan gum. Properties of the best formula of Benjakul nanoemulsion (BKE3-NE-T8-x0.2) was tested and showed particle size, PDI, zeta potential, and viscosity as 587.6 ± 25.0 nm, 0.6 ± 0.09 and -5.7 ± 0.2 mV respectively. The viscosity had a flow curve exhibiting non-Newtonian flow behavior (pseudoplastic) and the release of BKE from nanoemulsion was 0.0855 µg/min.

BKE3-NE-T8-x0.2 showed anti-inflammatory activity by inhibitory effect on nitric oxide induced by LPS in RAW264.7 cells, with IC_{50} of 8.36±1.0 µg/ml. and cytotoxicity activity by using MTT assay in HaCaT cells showed that the percentage of cell viability was more than 70% at every concentration which indicated that they were not toxic to human skin. The stability test of BKE3-NE-T8-x0.2 indicated it was unstable but still anti-inflammatory and piperine content was not reduced, surprisingly BKE3-NE-T8-x0.2 showed increasing anti-inflammatory activity indicating that it can be stored more than two years.

In conclusion, BKE3-NE-T8-x0.2 is an effective anti-inflammatory product and it is also a non-toxic on keratinocytec cell line. Although, the product was unstable but it still showed higher anti-inflammatory activity when it was keep long time. Therefore, these results can conclude that a topical nanoemulsion product containing BKE has potential as an anti-inflammatory drug. The further research BKE3-NE-T8-x0.2 should be studied on anti-inflammatory activities and, irritation test in the animal model and normal volunteers for safety.

Keywords: Topical nanoemulsion, Benjakul extract, Anti-inflammatory activity, Transdermal drug delivery, Release of Benjakul

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Miss Chunyika Thummawan

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LIST OF ABBREVIATIONS

Symbols/Abbreviations	Terms
ATCC	American type culture collection
BKE	Benjakul extract
cm	Centimeter
CO2	Carbon dioxide
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
DLS	Dynamic Light Scattering
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethy sulfide
EDTA	Ethylendiamintetraacetic acid
EtOH	Ethanol
g	Gram
$H_2NC_6H4SO_2NH_2$	Sulfanilamide
H_3PO_4	Phosphoric acid
HaCaT	Immortalized human keratinocyte cell line
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
hr	Hour
IC_{50}	Concentration causing 50% inhibition
IL-6,12	Interlukin-6,12

LIST OF ABBREVIATIONS

Symbols/Abbreviations	Terms
iNOS	Inducible nitric oxide
LPS	Lipopolysaccharide
m	Meter
M	Molar (concentration)
mg	Milligram
mg/kg	Milligram per kilogram
mg/ml	Milligram per milliliter
min	Minute
ml	Milliliter
mm	Millimeter
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromine or Thiazolyl blue tetrazolium bromine
mV	Millivolts
N	Normality
$NaH_2PO_42H_2O$	Monosodium dihydrogenortho-phosphate
$NaHCO_3$	Sodium bicarbonate
$NaHPO_42H_2O$	Disodium hydrogenortho-phosphate dihydrate
NaOH	Sodium hydroxide
ng	Nanogram
nm	Nanometer
NO	Nitric oxide
NSAIDs	Non-steroidal anti-inflammatory drugs
P/S	Penicillin/Streptomycin solution

LIST OF ABBREVIATIONS

Symbols/Abbreviations	Terms
Pa	Pascal
PBS	Phosphate buffer saline
PDI	Polydispersity index
PGE2	Prostaglandin E2
pH	Potential of hydrogen ion
RAW 264.7	Murine macrophage leukemia
RH	Relative humidity
RPM	Revolution per minute
RPMI 1640	Roswell Park Memorial Institute 1640
s-1	Revolutions per minute
SD	Standard deviation
SEM	Standard error of mean
TEM	Transmission electron microscopy
TNF-**α**	Tumor necrosis factor-**α**
w/v	Weight by volume
µl	Microliter
µg/ml	Microgram per milliliter
µm	Micrometer or micron

CHAPTER 1
INTRODUCTION

1.1 Introduction

Inflammation is a process by which the body's immune system responds to the stimulus from infection with foreign organisms, such as bacteria and viruses or external injuries like bruises. There are signs that may indicate inflammation such as pain swelling, redness, heat, loss of function. One of the most common signs of inflammation is the pain. Currently, there are a lot of people who have muscle pain and bone pain such as back pain, leg pain, knee pain and the other areas. Emerging markets predict sales of medicine sorted by symptoms in 2018 put pain symptom as the primary symptom. Thus, analgesic use is increasing the value of medicine imports in 2012 for Thailand, the values of NSAIDs use for the musculoskeletal system was 7,427.41 million baht (Bureau of Drug Control, 2012). In the future, if pain symptoms increase, NSAIDs drugs will be also imported and used. However, NSIADs drugs were used long-term, the side effects of NSAIDs can lead to stomach ulcers, gastrointestinal bleeding, and other disorders.

Benjakul is a Thai traditional medicine in National List of Essential Medicines. It is composed of five plants; *Piper chaba* Hunt., *Piper sarmentosum* Roxb., *Piper interruptum* Opiz., *Plumbago indica* Linn., and *Zingiber officinale* Roscoe. The plants, which are ingredients of Benjakul have different properties. However, when the five plants are combined, and it showed promoting balanced health in Thai traditional medicine used because it helps control abnormalities of the earth, water, wind, and fire elements in the body. The previous research revealed that BKE was safe for medical use and has anti-inflammatory activities for Osteoarthritis Knee patients and it showed equally effective when compared with Diclofenac (Rachawat *et al*., 2017). It is used to relieve muscle and bone pain and may be used as a substitute for NSAIDs because the active ingredients of Benjakul such as *Piper chaba* Hunt and *Plumbago indica* Linn have an anti-inflammatory mechanism similar to

NSAIDs (Kakatum, 2011). Therefore, promoting the use of Benjakul to relieve musculoskeletal pain, and inflammation is encouraged. It reduces the use of non-steroidal anti-inflammatory drugs in patients with inflammatory and musculoskeletal pain and reduces the importation of chemicals in pharmaceutical production. However, Benjakul tablets must be taken daily. Occasionally, its extract capsules can also cause adverse reactions in the patient, such as mild stomach pain, dry lips and throat (Rachawat *et al*., 2017). It is also used with caution in patients who have problems with peptic ulcer, and in acid reflux children. Therefore, it is compelling to develop medicinal products containing BKE for topical medicine. It will help reduce the problem of such adverse reactions because the drug is absorbed through the skin into the inflammatory site without going through the gastrointestinal route. However, the dosage form of the product is an important part of its development. Several forms of suspension provide different efficacy in drug delivery. The dosage forms of topical products for muscle pain already in the market include patches, creams, gels, and lotions (Barel *et al*., 2001; Fun, 2006). Patch form is not popular,being stuck on the skin for a long time and does not feel good on the skin. Cream, gel, and lotion forms are popular but may be sticky and or wash out easily (Zurdo Schroeder *et al*., 2007:1). This will cause the concentration of the drug and active ingredients on the skin to be decreased.

The nanoemulsion system is emulsion with a small internal particle size range. It is used as a delivery system for skin emulsions (Prow *et al*., 2011). The delivery of the substance to the skin is faster and more effective than the old emulsion. Nanoemulsions also have a high physical stability because there is a small internal particle size. It does not cream. The key has excellent sensory properties as a cosmetic. Surfactants are in low concentration, so often nanoemulsion does not cause skin irritation. Adherence on the skin can be increased by adding the appropriate amount of viscosity. Nanoemulsion system is widely accepted by users (Tadros *et al*., 2004).

Thus, nanoemulsion form is interesting because it is a suspension of ultra-small particles of active ingredient and as such, is one of the most effective

forms which can deliver transdermal drug into the skin layers. The objectives of this research are to evaluate the efficacy of a topical nanoemulsion containing Benjakul remedy extract for anti-inflammatory activity, and to study the effect of the formulation components on the physicochemical properties, to evaluate the chemical stability, and to evaluate the toxicity on the skin cells and the release of Benjakul extract from the finished product.

1.2 Objectives of this study

1.2.1 Overall objectives

The overall objectives of this research are to evaluate the efficacy of a topical nanoemulsion containing Benjakul extract for anti-inflammatory activity.

1.2.2 Specific objectives

1.2.2.1 To study the effect of the formulation components on the physicochemical properties of topical nanoemulsion containing Benjakul extract.

1.2.2.2 To evaluate the chemical stability of topical nanoemulsion containing Benjakul extract.

1.2.2.3 To evaluate the release of Benjakul extract from the prepared product.

1.2.2.4 To evaluate the toxicity of topical nanoemulsion containing Benjakul extract on the skin cells.

CHAPTER 2
REVIEW OF LITERATURE

2.1 Benjakul remedy

2.1.1 *Piper retrofractum* Vahl., *Piper chaba* Linn. or *Piper longum* Linn. (PIPERACEAE)

Figure 2.1 *Piper retrofractum* Vahl.

Common names: Di-pli,Prik-hang,Dipli-chueak (Thai) and Long pepper (English)

Family: PIPERACEAE

Botanical characteristics

Piper retrofractum Vahl is monoecious, climber, many parts finely powdery pubescent when young. Stem often flexuous. Petiole 1-3 cm long. Leaves on creeping branch and epiphytic branches blade ovate or elliptic. Leaves on free branches blade ovate to ovate-oblong. Leaf blade membranous, dark green, 3-5 cm wide, 7-10.5 cm long; apex acuminate; base cordate or oblique; veins 5, one pair basal, one pair arising 1.5 cm apart from base, opposite or alternate. Male spike straight up, 5-8 cm long, 0.3-0.7 cm in diameter; peduncle 0.5 cm long; bract orbicular, stalked; stamens. Female spike erect, 0.6-2 cm long, ca. 0.2 cm in diameter; peduncle 0.5 cm long; bract circular, peltate; stigmas.

Fruiting spike straight up, 0.7-2.5 cm long; drupe globose, sessile, arranged densely on rachis. Flowering from May to September. (Chaveerach *et.al.*,2006).

Part used: Fruits

Chemical constituents

The fruits of *Piper retrofractum* Vahl. contain alkaloids, piperine, piperidine, piplartine,sesamin and essential oils. Essential oils are about 0.7%, including thujene, terpinolene, p-cymene, dihydrocarveol, zingiberine.

Traditional used

Fruits are stimulant, carminative, anthelmintic and expectorant; used in cough, cold, asthma, bronchitis, fever, piles. The root is useful against asthma, bronchitis and tuberculosis (Medicinal plants of Bangladesh).

2.1.2 *Piper sarmentosum* Roxb. (PIPERACEAE)

Figure 2.2 *Piper sarmentosum* Roxb.

Common names: Cha-phlu, Nom wa, Phlu ling (Thai)

Family: PIPERACEAE

Botanical characteristics

Piper sarmentosum Roxb is a monoecious, normally small shrubs, 30 cm tall, sometimes climber, all parts glabrous. Petiole 1-2.5 cm long; leaf blade thin to thick chartaceous or papery, light to dark green, broadly ovate to elliptic, 4.5-6 cm wide, 7.5-9.5cm long; apex acute; leaves on epiphytic branches basedeeply equally cordate with rounded lobes, leaves on free branch base cuneate to subtruncate; veins 7, all basal. Spike with male and female flowers together straight up, cylindrical, 1-1.5 cm long, 0.3-0.5 cm in diameter; peduncle ca. 1.5 cm long; bract rounded; stamen 1; stigmas 3-4. Female spike white cylindric, other characters are as above. Fruiting spike 1-2 cm long, 0.5-1 cm in diameter. Flowering on year round, many in rainy season (Chaveerach *et.al.*, 2006).

Part used: Root

Chemical constituents

The essential oil was analyzed by gas chromatography/mass spectrometry and 41 components were identified. Myristicin (65.22%) and trans-caryophyllene (13.89%) were the major components. (Qin *et.al.,*2010)

Traditional use

All parts are expectorant; relieve flatulence, cough, and cold.(Ridtitid *et.al.,*2007)

2.1.3 *Piper interruptum* Opiz. (PIPERACEAE)

Figure 2.3 *Piper interruptum* Opiz

Common names: Sa-kan, Sa-kan-lek and Sa-kan-youak (Thai)

Family: PIPERACEAE

Botanical characteristics

Piper interruptum Opiz is a dioecious climber. Stems 2-4.5 mm thick, ridged, glabrous. Petiole 1-2.5(-4) cm, glabrous, sheathed at base only; leaf blade ovate to long ovate, 6-13 × 4-7 cm, ± membranous or papery, without evident glands, both surfaces glabrous, base rounded or shortly tapered, ± symmetric, apex acute or shortly acuminate; veins 5(-7), all basal; reticulate veins abaxially prominent, lax. Spikes leaf-opposed. Male spikes 11- 27 cm × 1.5-3 mm; peduncle ca. as long as petioles, glabrous; bracts oblong, 3-4 × ca. 1 mm, adnate to rachis, margin free, apex ± rounded. Stamens 2(or3). Female spikes 7-17 cm, flowers unevenly developed, sparse or interrupted in fruit; peduncle nearly as long as opposite leaves, glabrous; rachis and bracts as in male spikes. Ovary distinct, ovoid, apex acute; stigmas 4 or 5. Drupe ovoid or ovoid-globose, 3-6 × 2-4 mm, smooth (Cheng *et.al*., 1999).

Part used: Vine

Traditional use

Vines release wind in the intestine and relieve colic. The stem is used as carminative, antiflatulant, and element tonic (Pichiensunthon & Jeerawongs, 2004)

2.1.4 *Plumbago indica* Linn. (PLUMBAGINACEAE)

Figure 2.4 *Plumbago indica* Linn.

Common names: Chettamun-phloeng-daeng, Pit-piu-daeng, Fai-tai-din (Thai)

Family: PLUMBAGINACEAE

Botanical characteristics

Plumbago indica Linn is a shrub up to 1.5 m tall, branched from the base, stems drooping, sometimes rooting; leaves oblong, 5-15 cm x 2-8 cm, petiole not auriculate; inflorescence a rather sparsely flowered spike, not corymbose, rachis glabrous, 10-30 cm long; flowers with calyx about 1 cm long, covered in glands, red, corolla tube 2.5-4 cm long, lobes 2-3 cm in diameter, distinctly mucronate, red; fruit unknown. Plumbago indica is found in the vicinity of (former) anthropogenic localities, locally semi- 13 spontaneous, often persistent in abandoned cultivation, also in teak forest, up to 1000 m elevation (Chuakul *et.al*., 1999).

Part used: Root

Traditional use

The root is used as carminative and emmenagogue for treatment of hemorrhoids. It contains plumbagin which stimulates uterine and intestinal contraction, increases digestive enzyme secretion and stimulates appetite. Because plumbagin can irritate mucous membranes, it should be used cautiously (Saralamp *et.al*., 1996).

2.1.5 *Zingiber officinale* Roscoe. (ZINGIBERACEAE)

Figure 2.5 *Zingiber officinale* Roscoe.

Common names: Khing (Thai), Ginger (English)

Family: ZINGIBERACEAE

Botanical characteristics

Zingiber officinale Roscoe is a perennial herb with a subterranean, digitately branched rhizome producing. Stems up to 1.50 m in height with linear lanceolate sheathing leaves (5–30 cm long and 8–20 mm wide) that are alternate, smooth and pale green. Flower stems shorter than leaf stems and bearing a few flowers, each surrounded by a thin bract and situated in axils of large, greenish yellow obtuse bracts, which are closely arranged at end of flower stem forming collectively an ovate-oblong spike. Each flower shows a superior tubular calyx, split part way down one side; an orange yellow corolla composed of a tube divided above into 3 linearoblong, blunt lobes; 6 staminodes in 2 rows, the outer row of 3 inserted at mouth of corolla; the posterior 2, small, horn-like; the anterior petaloid, purple and spotted and divided into 3 rounded lobes; an inferior, 3-celled ovary with tufted stigma. Fruit is capsule with small arillate seeds (WHO,1999).

Part used: Rhizome

Chemical constituents

Volatile oil from rhizome of ginger contains menthol, borneol, fenchone, 6-shogaol and 6-gingerol. Menthol is carminative. Borneol, fenchone and 6-gingerol increase bile secretion and promote fat digestion (Saralamp *et.al*., 1996).

Traditional use

The rhizomes are used as carminative, antiemetic, expectorant, antispasmodic and diaphoretic (Saralamp *et al*., 1996).

2.2 Anti-inflammatory activities of ingredients of Benjakul remedy

Table 2.1 Anti-inflammatory activities of ingredients of Benjakul remedy

Scientific Name	Activities	Part used/Bioactive compounds	Detail on biological activities	References
***Piper retrofractum* Vahl., *Piper chaba* Linn. or *Piper longum* Linn.**	Anti-inflammatory	Fruit	95% Ethanol extract inhibits nitric oxide release in RAW 264.7 cell line (IC_{50} = 3.09 µg/ml)	Itharat *et.al.,*2010
			Piperine inhibited adhesion of neutrophils to endothelial monolayer due to its ability to block TNF-α induced expression of cell adhesion molecules	Kumar *et.al.,*2007
***Piper sarmentosum* Roxb.**	Anti-inflammatory	Aerial	n-Hexane extract was found to possess COX-1 and 5-LO inhibitory activity (COX-1 IC_{50}=19 µg/ml, 5-LO IC_{50}=10 µg/ml)	Stöhr *et.al.,*1999
		Leaves	The extract at the dose of 200 mg/kg exhibited inhibitory effect on carrageenan induced rat paw edema comparable to that of aspirin at the dose of 200 mg/kg	Ridtitid *et.al.,*2007

Table 2.1 Anti-inflammatory activities of ingredients of Benjakul remedy (Continued)

Scientific Name	Activities	Part of used/Bioactive compounds	Detail on biological activities	References
***Piper sarmentosum* Roxb.**	Anti-inflammatory	Root	*P.sarmentosum* extract inhibited ethyl phenylpropiolate-induced ear edema as well as carrageenan-induced hind paw edema in rats. The extract reduced transudative and granuloma weights of the chronic inflammatory model using the cotton pellet-induced granuloma formation in rats.	Sireeratawong *et.al.,*2010
***Piper interruptum* Opiz or *Piper rostratum* Roxb.**	Anti-inflammatory	Stem	Topical application of EPP on rat ears produced edema PI extract at the dose of 1 mg/ear inhibited the ear edema formation. As a positive control, phenylbutazone (1 mg/ear) exhibited inhibitory activity on the ear edema formation at all determination times.	Sireeratawong *et.al.,*2012

Table 2.1 Anti-inflammatory activities of ingredients of Benjakul remedy (Continued)

Scientific Name	Activities	Part of used/Bioactive compounds	Detail on biological activities	References
***Plumbago indica* Linn.**	Anti-inflammatory	Roots	*In-vitro* anti-inflammatory models were studied; as inhibition of protein denaturation, effect of membrane stabilization of human RBC. *Plumbago indica* was extracted with methanol exhibited anti-inflammatory activity at different concentrations.	Raju *et.al.,*2014
***Zingiber officinale* Roscoe.**	Anti-inflammatory	Rhizome	95% Ethanol extract inhibited nitric oxide release in RAW 264.7 cell line (IC_{50} = 13.76 µg/mg)	Itharat *et.al.,*2010
			95% Ethanol extract (100mg/ml) reduced inflammation (inhibited the function of TNF-α and COX-2)	Frondoza *et.al.,*2004
			[6]-gingerol inhibited the production of proinflammatory cytokines (TNF-α, IL-12, and IL-1β) production from LPS stimulated macrophages.	Tripati *et.al.,*2007

2.3 Anti-inflammatory activities and Clinical research of Benjakul remedy

2.3.1 Anti-inflammatory

The ethanolic extract of BJK showed potent NO inhibitory effect in RAW 264.7 cells by lipopolysaccharide (LPS) and COX-2 with IC_{50} values of 18.23 µg/ml and 5.82 µg/ml respectively (Itharat *et.al*.,2010).

Plumbagin and 6-shogaol exhibited higher activity than crude BJK extract with IC_{50} values of 0.002 and 0.92 µg/ml, respectively. In particular, plumbagin also showed higher anti-inflammatory power than prednisolone, positive control, with IC_{50} value of 0.59 µg/ml. 6-Shogaol also showed inhibitory effect on TNF-**α** release IC_{50} was 9.16 µg/ml (Makchuchit *et.al*., 2017).

2.3.2 Clinical research

The ethanolic extract of Benjakul at concentration 0.5 % caused no irritation or allergic reaction on human skin, so this may be safe and suitable for drug preparation and the other products for external skin application but Plumbago indica root showed irritation at low concentration so it is not safe to use as active ingredient for topical preparation for skin. (Triyasut, 2015)

Clinical efficacy and safety of Benjakul recipe in treating primary Osteoarthritis of knee compared with diclofenac showed all patients from both group reported a decrease in the VAS pain score. For safety outcome, can also cause adverse reactions in Benjakul extract group were mild stomach pain, dry lips, and throat. However, the Benjakul remedy extract no toxicity in renal and liver functions after taking Benjakul extract for 28 days (Rachawat *et al*., 2017).

2.4 Anti-inflammatory activities and solubility of piperine

2.4.1 Anti-inflammatory activities of piperine

Piperine (10, 50 and 100 μg/ml) inhibited the production of NO and PGE2 on human osteoarthritis chondrocytes and reduced the gene expression of iNOS and COX-2 induced by IL-β. Piperine has the ability to inhibit the release of TNF-α, and NO production significantly in RAW264.7 cells (Ying *et.al.,*2013). In addition, piperine showed anti-inflammatory effect in the arthritis model of rats (Damanhouri and Ahmad, 2014) and inhibited the expression of IL6 and reduced the production of PGE2 which was significantly inhibited even at 10 μg/ml (Bang *et al.*,2009).

2.4.2 Solubility of piperine

Piperine forms monoclinic needles. Despite its excellent therapeutic properties, piperine is only slightly soluble in water (40 mg/L, or 1g/25L (18°C)) and more so in alcohol (1g/15mL), ether (1g/36mL) or chloroform (1g/1.7mL) (Vasavirama.et *al.,* 2014).

2.5 Nitric oxide (NO)

Pro-inflammatory cytokines are produced predominantly by activated macrophages and are involved in the up-regulation of inflammatory reactions and pro-inflammatory cytokines such as NO, IL-1β, IL-6, and TNF-α are involved in the process of pathological pain. (Zhang and An, 2007) Nitric oxide (NO) is a free radical gas, which is a product of a reaction between molecular oxygen and L-arginine. (Habib *et al.*, 2011).

Nitric oxide generated by iNOS has antimicrobial, antitumor, cytostatic and cytotoxic effects that are associated mainly with the production of free radicals (superoxide, peroxynitrite). Increased iNOS activity plays a protective role in various bacterial and parasitic infections and in response to inflammation and controls many

processes in the immune system. Cytotoxic effect of NO is not only expressed in immune processes but in some cases nitric oxide acts as an important pro-inflammatory molecule. Nitric oxide (NO) has basically dual action in biological systems. Low optimal concentrations regulate a lot of physiological processes, however, overproduction of nitric oxide takes a role in the pathogenesis of many diseases, and is also involved in the pathogenic process of septic shock or chronic inflammatory diseases (Antosova *et al*., 2012). The synthesis of nitric oxide is catalyzed by the enzyme nitric oxide synthase (NOS) induced by inflammatory stimuli such as cytokines or lipopolysaccharides (LPS) (Arzumanian *et al.,* 2003).

2.6 Nanoemulsion

Nanotechnology is a science in miniature that involves manufacturing, characterization, and manipulation of materials which have a size range in the nanometer scale. (Sharif *et al*., 2017). Nanoemulsions are emulsions which contain oil, water, and an emulsifier in a biphasic system in which one phase is intimately dispersed in the other phase. The dispersed phase is also known as internal phase or the discontinuous phase while the outer phase is called dispersion medium, external phase or continuous phase. Nanoemulsions are transparent and translucent and size varies from 10 to 1,000 nm. The emulsifier used is generally a surfactant, in which a mixture of oil and water may be separate in two distinct phases due to the coalescence of the dispersed globules. The emulsifier can impart stability to such systems. Oil can be of any type like castor oil, corn oil, coconut oil, evening primrose oil, linseed oil, mineral oil, olive oil, peanut oil, etc. and emulgents are broadly classified as surfactants like spans and tweens, hydrophilic colloids such as acacia and finely divided solids, e.g., bentonite and xanthan gum. (Jaiswal.*et al*., 2015). The Shape of Nanoemulsion is spherical and is thermodynamically unstable and kinetically stable. (Gupta.*et al*., 2016). Preparation of nanoemulsion may be high or low energy method. The high-energy method then uses high pressure homogenizer, ultrasound generator, etc. to reduce size of particles to nanoemulsion (mechanical

shear process). Parameters to describe nanoemulsions are: particle size, particle size distribution, morphology, rheology, zeta potential, and TEM. Advantages of nanoemulsion are improved bioavailability of drug, non-toxic and non-irritant in nature, improved physical stability, small-sized droplets with greater surface area providing greater absorption and they can be formulated in a variety of types such as foams, creams, liquids, and sprays (Jaiswal. *et al*., 2015).

2.6.1 Nanoemulsion ingredients

2.6.1.1 Oleic acid

Oleic acid is the most common monounsaturated fatty acid in human cells and a fatty acid that occurs naturally in various animal and vegetable fats and oils which dietary sources rich in oleic acid include: Olive Oil, Avocado Oil, Almond Oil etc.

Oleic acid is odorless, colorless oil, oily liquid at temperatures above 5-7°C. At 4°C, it solidifies to a crystalline mass. It decomposes when heated to 80-100°C at atmospheric pressure. Oleic Acid has a characteristic lardlike odor. (Liebert, 1987)

2.6.1.2 Myritol 318

Myritol 318 (caprylic/capric triglyceride) caused by the esterification process. It is a clear, slightly yellowish, and odorless. It is moisturizing emollient oil with good cosmetics properties, penetration enhancing solvent and insoluble in water.

In an emulsion form, thickening will occur in the oil phase and emulsion viscosities will be influenced by 3-5% additions. (Nguyen ba, 2018)

2.6.1.3 Isopropyl myristate

Isopropyl myristate (IPM) is colorless synthetic oil that is widely used in a cosmetic formulation. It is an ester of isopropanol and myristic acid. It is a very good emollient property and good absorbed efficiently through the skin which ensures effective delivery of the active ingredient of the formulation. (Unicorn Petroleum Industries, 2018)

2.6.1.4 Tween 80

Tween 80 (polysorbate 80) is a nonionic surfactant solution derived from sorbitan esters. It is used as an emulsifier and dispersing agent. It will be also used as an emulsifier in foods, a surfactant in cosmetics etc. (Acumedia Manufacturers, 2017)

2.6.1.5 Propylene glycol

Propylene glycol (1,2-dihydroxypropane) is a synthetic organic compound with the chemical formula $C_3H_8O_2$. It is a viscous, colorless liquid which is nearly odorless and is miscible with a broad range of solvents, including water, acetone, and chloroform.

Propylene glycol (PG) is not acute toxic. PG is essentially nonirritate to the skin and irritate to the eyes. It is used as an ingredient in cosmetics at concentrations of <0.1% to >50%. (SIDS Initial Assessment Report, 2001).

2.7 Mechanical properties

2.7.1 Particle size, Polydispersity index (PDI) and Zeta potential

The most important physical property of particulate samples is particle size. Dynamic Light Scattering (DLS) is a particle size instrument measuring nanoparticle size distribution. DLS is a non-invasive, well-established technique for measuring the size and size distribution of molecules and particles typically in the submicron region, and with the latest technology, less than 1nm. Typical applications of dynamic light scattering are the characterization of particles, emulsions or molecules which have been dispersed or dissolved in a liquid. Dynamic light scattering technology offers the following advantages such as being accurate, reliable, repeatable particle size analysis in one or two minutes, simple or no sample preparation, high concentration, turbid samples can be measured directly, size measurement from 10µm to < 1nm, low volume requirement (as little as 3µL) etc..

Zetasizer is the world's most widely used system for nanoparticle, colloid and protein size, zeta potential and molecular weight measurements. It's

used to measure particle and molecular size from less than a nanometer to several microns using dynamic light scattering; zeta potential. Particle size range is 0.3nm - 10µm. (Malvern Panalytical, 2018)

2.7.2 Viscosity

Viscosity is a measure of the resistance of a fluid which is being deformed by either shear stress or tensile stress. Viscosity is thickness or internal friction. Thus, water is thin, having a lower viscosity, while honey is thick, having a higher viscosity. Put simply, the less viscous the fluid is, the greater its ease of movement (fluidity)

Fluids exhibit a more complicated relationship between shear stress and velocity gradient than simple linearity. Thus there exist a number of forms of viscosity (Sayunt and wirat, 2006).

2.7.2.1 Newtonian: It is a flow of fluid that is based on Newton's assumption, at a certain temperature. The fluid has a constant viscosity. Does not change with a shear rate or stirring speed, whether it is stirring fast or slow such as water, oil, syrup, juice, honey, milk, coffee, glycerine, alcohol, etc.

2.7.2.2 Non-Newtonian Fluid: The flow characteristics of fluid that are not according to Newton's assumption are at a certain temperature. Fluid viscosity is not constant. The change depends on shear rate or stirring speed. This flow is divided into 4 types.

(1) Shear thinning (Psuedoplastic): Fluid with reduced viscosity. When increasing the shear rate or even faster stirring, the flow is easier such as juice concentrate, polymer solution from a natural, synthetic polymer solution and emulsion.

(2) Shear thickening (Dilatant): Fluid increased viscosity. When increasing the shear rate or even faster-stirring speed, it showed more viscous.

(3) Bingham Plastic: a material that behaves as a solid at low stresses but flows as a viscous fluid at high stresses, for example, toothpaste, milk chocolate etc.

(4) Plastic: Fluid that is high enough to overcome yield stress to flow and flow in the pseudoplastic or Herschel-Buckley model, for example, tomato sauce mayonnaise etc.

2.7.3 Transmission electron microscopy (TEM)

Transmission Electron Microscopy (TEM) is a vital characterization tool for directly imaging nanomaterials to obtain quantitative measures of particle and grain size, size distribution, and morphology (Nanocomposix, 2012). It's an analytical tool allowing visualization and analysis of specimens in the realms of microspace to nanospace. The TEM reveals levels of detail and complexity inaccessible by light microscopy because it uses a focused beam of high energy electrons. It allows detailed micro-structural examination through high-resolution and high magnification imaging.

Transmission electron microscopy is used to produce images from a sample by illuminating the sample with electrons within a high vacuum and detecting the electrons that are transmitted through the sample. Magnifications of up to 1,000,000x and resolution below 1 nm are achieved routinely. A scale bar is essential on a TEM image, quantitative and qualitative elemental analysis can be provided from features as small as 1 nm. It is also possible to label molecules with electron dense particles such as nano-sized gold spheres that attach to molecules through construct 3-dimensional images of the particle structures (MyScope training for advanced research, 2012).

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Materials and Methodology

3.1.1 Plant materials

Table 3.1 List of plant materials in Benjakul remedy

Scientific Name/Family	Thai name	Part used	Voucher numbers	Source (Province)
Piper retrofractum Vahl., *Piper chaba* Linn. *or Piper longum* Linn. / PIPERACEAE	Di-pli	Fruits	SKP 146160301	Karnchanaburi
Piper sarmentosum Roxb. / PIPERACEAE	Cha-phlu	Root	SKP 146161901	Karnchanaburi
Piper interruptum Opiz. / PIPERACEAE	Sa-kan	Vine	SKP 146160901	Karnchanaburi
Plumbago indica Linn. / PLUMBAGINACEAE	Chettamun-phloeng-daeng	Root	SKP 148160901	Karnchanaburi
Zingiber officinale Roscoe. / ZINGIBERACEAE	Khing	Rhizome	SKP 206261501	Karnchanaburi

3.1.2 Conceptual Framework of Thesis

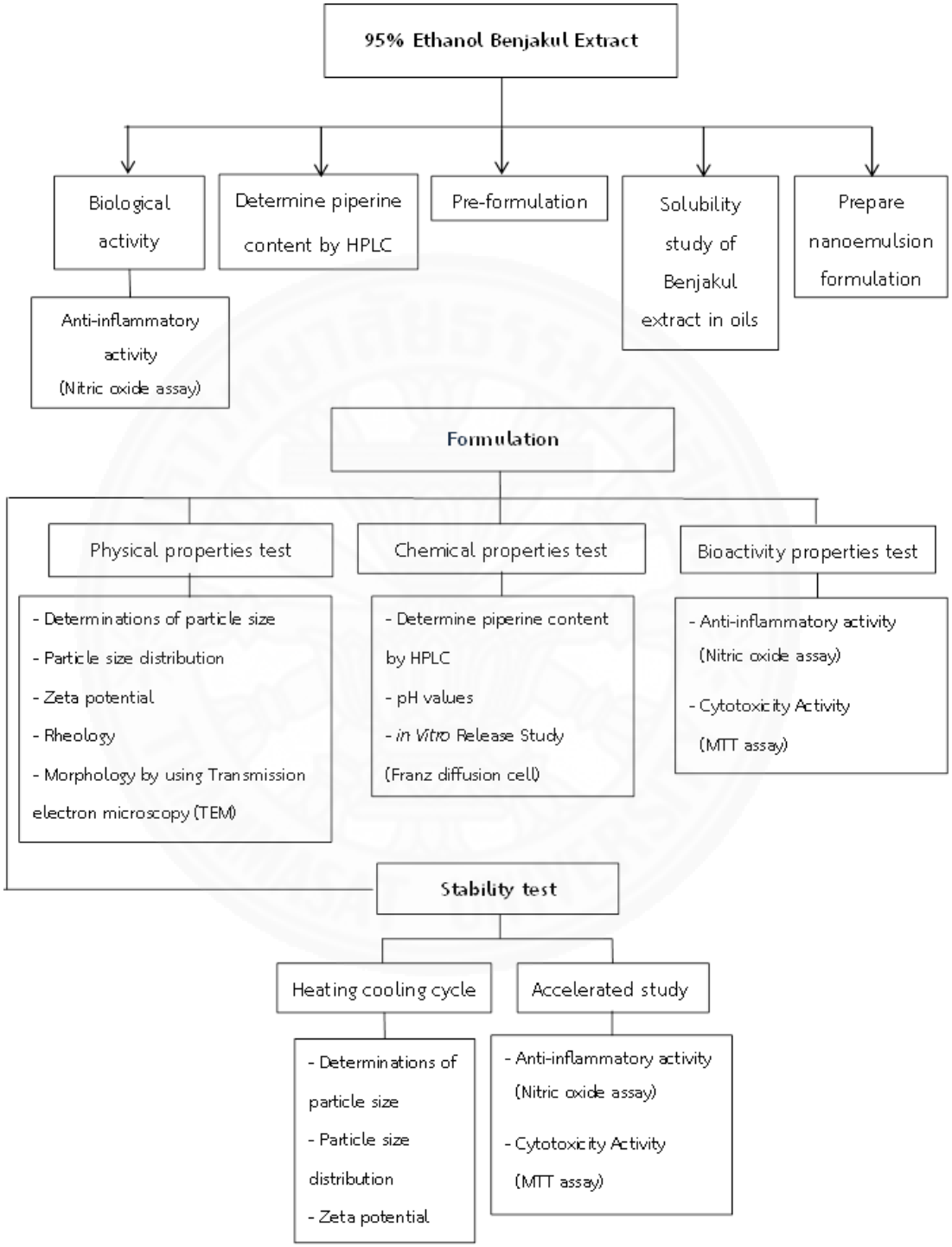

Figure 3.1 Conceptual Framework of Thesis

3.2 Preparation of crude extracts

Benjakul remedy consists of 5 herbs. Plant materials were washed with water to remove contamination and to reduce the microbes. The cleaned plants were sliced into small pieces and dried at 50ºC. Each herb was ground to be crude powder. These plant ingredients were weighed in the same ratio and mixed together to be the Benjakul remedy

Maceration: The crude powder of Benjakul remedy was macerated in 95% ethanol for 3 days and filtered through filter paper. Filtrate was dried by rotary evaporator. The maceration was repeated twice on the residue and percentage of yield calculated. For BKE; the quality control of the extract was also performed by piperine, content testing.

3.3 Quality control of 95%Ethanol Benjakul extract

3.3.1 Study on chemical fingerprint of BKE preparation

3.3.1.1 Apparatus and chromatographic conditions

The content of piperine in BKE was determined by using a High Performance Liquid Chromatography (HPLC) method according to Itharat and Sakpakdeejaroen with some modification. The HPLC system (Agilent® 1200) is composed of 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 are processed by the Chemstation revision B.04.01 SP1 software.

Table 3.2 List of chemicals and reagents for HPLC study on piperine

Name	Source
Standard Piperine	Merck, Thailand
Acetronitrile (HPLC grade)	Labscan, Thailand
Ultra-Pure water	Labscan, Thailand

Chromatography was carried out using a ZORBAX® Eclipse, XDB-C18, analytical 4.6 x 250 mm, 5 microns machine. The sample volume of 10 µl is injecting into the HPLC system and the samples are eluted using gradient mobile phase composed of water (A) and acetonitrile (B) with various ratios as follows: 0-30 min, 40%B -50%B; 30-50 min, 50%B -95%B; 50-60 min, 95%B -100%B; 60.1-70 min, 40%B at flow rate of 1.0 ml/min. The diode array detector is set at a wavelength of 256 nm.

3.3.1.2 Preparation of standard piperine and Benjakul extract

Standard piperine was prepared by weighing one milligram and dissolving in acetonitrile to concentrations of 40, 60, 100, 200, 300 and 400 µg/ml. These solutions were analyzed by HPLC.

BKE was dissolved in acetonitrile at concentrations 10 mg/ml and filtered through a 0.45 µm membrane filter. These solutions were analyzed by HPLC. The standard curve of piperine was constructed and used to calculate piperine content of BKE.

3.3.2 Determination of anti-inflammatory activity

Animal cell lines: Murine leukemia macrophage cell line (RAW 264.7) was obtained from American Type Culture Collection (ATCC TIB-71). This cell line was cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, penicillin and streptomycin. The cells were incubated at 37^{O}C in a 5% CO_2 incubator and sub passaged every 4-5 days.

Preparation of Benjakul extract: The 95% ethanol extract was dissolved in sterile dimethyl sulfoxide (DMSO) to a final concentration of 50 mg/ml. The extract was diluted with RPMI to obtain final concentrations of between 1-100 µg/ml.

Table 3.3 List of chemicals and reagents of assay for NO inhibitory effect

Name	Source
Dimethyl sulfoxide (DMSO)	RCL Labscan, Thailand
Distilled water	Milford, USA
Fetal bovine serum (FBS)	Biochem, Germany
Hydrochloric acid (HCl)	Univar, Australia
Isopropanol	RCI labscan, Thailand
Lipopolysaccharide from *E.coli* O55:B5 (LPS)	Sigma, USA
N-(1-Naphthyl)ethylenediamine dihydrochloride	Sigma, USA
Penicillin-Streptomycin (P/S)	Sigma, USA
Phosphate buffered saline (PBS)	Amresco, USA
Phosphoric acid 85% (H_3PO_4)	Sigma, USA
Prednisolone ≥ 90 %	Sigma, USA
RPMI medium 1640	Gibco, USA
Sodium bicarbonate ($NaHCO_3$)	BHD, England
Sodium hydroxide (NaOH)	Univar, Australia
Sulfanilamide ($H_2NC_6H_4SO_2NH_2$)	Sigma, USA
Thiazolyl blue tetrazolium bromide (MTT)	Sigma, USA
Trypan blue 0.4%	Gibco, USA
Trypsin-EDTA	Gibco, USA

Assay for NO inhibitory effects in RAW 264.7 cells was modified from Tewtrakul and Itharat, 2007. The assay was used to evaluate the inhibition of release of Nitric Oxide (NO) that is produced by mouse macrophage leukemia-like (RAW 264.7) in inflammatory conditions. The cells (RAW 264.7) were cultured in a flask with RPMI 1640 medium containing 10% FBS, penicillin (100 units/ml) and streptomycin (100 units/ml), RAW 264.7 cells were washed by phosphate buffer saline (PBS) and suspended by 0.25% trypsin-EDTA. The cells were cultured in 96-well sterile plate ($1x10^5$cells/well) with 100 µl complete RPMI and incubated in 5%

CO_2, 37°C overnight. Complete RPMI (100µl/well) containing 10ng/ml of Lipopolysaccharide (LPS) was replaced in control and only complete RPMI replaced in normal. Next, 100 µl/well of each sample was added following the concentration, 100 µl /well of complete RPMI was added in control medium, 100 µl/well of 0.2% DMSO added in control solvent, then incubated overnight. Supernatant 100 µl was transferred to another 96-well sterile plate, followed by 100 µl of Griess reagent. The NO production was determined by measuring the accumulation of nitrite which interacted with Griess reagent. The absorbance was measured by spectrophotometer at wavelength 570 nm. This method was carried out in triplicate. The inhibition (%) was calculated using the following equation and IC_{50} value calculated using Prism program.

$$\% \text{ inhibition} = \frac{C - S}{C} \times 100$$

Control(C) : [LPS (+), sample (-)] - [LPS (-), sample (-)]

Sample (S) : [LPS (+), sample (+)] - [LPS (-), sample (+)]

MTT assay was determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromine(MTT) colorimetric method. This method was continued from NO assay and used to determine cytotoxicity of extracts. The plates were incubated at 37°C in 5% CO_2 incubator for 24 hours. MTT solution (10 µl, 5mg/ml in PBS) was added in each well and incubated 2 hours. Supernatant was removed and 100 µl of isopropanol containing 0.04 M HCl added to dissolve the formazan production by cells. The density of formazan solution was measured by micro spectrophotometer at wavelength 570 nm. If the density of cell viability was more than 70% it should be considered as survival.

$$\%\text{survival} = \left(\frac{S}{C}\right) \times 100$$

Control (C) : LPS (-), sample (-)

Sample (S) : LPS (-), sample (+)

3.4 Forced Degradation study (Pre-formulation or Stress test)

Forced degradation was performed. It is very important to know when to perform forced degradation studies for the development of new drug substance and new drug product (Charde, 2013). The typical tests are heat, humidity, acid hydrolysis, alkaline hydrolysis, and oxidation.

Table 3.4 List of chemicals and reagents of forced degradation study

Name	Source
Deionized water	Milford, USA
Hydrochloric acid	Univar, Australia
Hydrogen peroxide	Univar, Australia
Sodium hydroxide	Univar, Australia

3.4.1 Temperature forced degradation

Prepare BKE by weighing up to 50 mg in a test tube, then heat at 80°C for 3 hours. The sample was tested for anti-inflammatory activity.

3.4.2 Moisture hydrolysis

Prepare BKE by weighing up to 50 mg in a test tube. Add 3 drops of distilled water and heat at 80°C for 3 hours. The sample was tested for anti-inflammatory activity.

3.4.3 Acid hydrolysis

Prepare BKE by weighing up to 50 mg in a test tube. Add 3 drops of 3N hydrochloric acid and heat at 80°C for 3 hours. Then add 3 drops 3N sodium hydroxide. The sample was tested for anti-inflammatory activity.

3.4.4 Alkaline hydrolysis

Prepare BKE by weighing up to 50 mg in a test tube. Add 3 drops of 3N sodium hydroxide and heat at 80°C for 3 hours. Then add 3 drops of 3N hydrochloric acid. The sample was tested for anti-inflammatory activity.

3.4.5 Oxidation

Prepare BKE by weighing up to 50 mg in a test tube. Add 3 drops of 30% hydrogen peroxide and heat at 80 °C for 3 hours. The sample was tested for anti-inflammatory activity.

3.5 Solubility study of Benjakul extract in oils

The solubility of Benjakul in various oils was determined by adding BKE and oils (oleic acid, isopropyl myristate, caprylic capric triglyceride and tween80) in the same ratio (1g: 1g) in a test tube. The mixture vials were then kept at 37 ℃ in an isothermal shaker for 24 hours to achieve equilibrium. The equilibrated samples were removed from the shaker (100 rpm) and centrifuged at 3,000 rpm for 15 min (Sajida li, 2013). 10 mg of supernatant was taken and diluted with acetonitrile (concentration 1 mg/ml) and filtered through a 0.22-μm membrane filter. Piperine content was determined by HPLC at a wavelength of 256 nm.

3.6 Preparation of nanoemulsion formulation

Table 3.5 List of chemicals and reagents of formulation

Name	Source
Distilled water	Milford, USA
Oleic acid	Panreac, EU
Myritol (Caprylic capric triglyceride)	Chemipan, Thailand
Isopropyl myristate (IPM)	Merck, France
Polysorbate 80	Chemipan, Thailand
Propylene Glycol	PC.Drug center, Thailand
Xanthan Gum	China

Nanoemulsion was prepared by ultrasonication method using an ultra-sonicate homogenizer (Priya, 2015). First, the oil phase and aqueous phase were prepared. The oil phase contains BKE and oil mixed (oleic acid: myritol: isopropyl myristate), and aqueous phase (propylene glycol, paraben cocn.) contains the surfactant (tween80), water. Sonicate the oil-water phase mixture by probe sonication at 20% amplitude for 5 minutes. Then added 2.5% xanthan gum with continuous stirring to get nanoemulsion. Then apply centrifugation test at 5,000 rpm for 30 min and observed for phase separation (Azeem, 2009). Compositions of Benjakul nanoemulsion were shown in Table3.6.

Table3.6 Composition of Benjakul nanoemulsion

Formulation (code)	Contents of ingredients (% w/w)						
	Oil Mix	Extract	Xanthan gum	Tween80	Propylene glycol	Paraben Conc.	Distilled water
NE-base-T8	10	0	0	8	5	1	76
BKE0.5-NE-T8	10	0.5	0	8	5	1	75.5
BKE1-NE-T8	10	1	0	8	5	1	75
BKE2-NE-T8	10	2	0	8	5	1	74
BKE3-NE-T8	10	3	0	8	5	1	73
BKE3-NE-T8-x0.2	10	3	0.2	8	5	1	65
BKE3-NE-T8-x0.3	10	3	0.3	8	5	1	61

BKE: Extract: NE: Nanoemulsion, T: Tween80, x: Xanthan gum

3.6.1 Study of the physio-chemical properties of nanoemulsion of Benjakul extract.

3.6.1.1 Determination of Particle size, Polydispersity index (PDI), and Zeta potential

In preparation of Benjakul extract nanoemulsion, the particle size was recorded as mean droplet diameter. The mean of the Benjakul nanoemulsion was measured using a nano-size analyzer (Zetasizer Nano ZS, Malvern Instruments, Malvern, U.K.) at 25 ºC (Dal ma, 2016). Size measurements of fresh and stored emulsions were carried out by selecting 12-13 numbers of runs and expressing in nm, and the PDI was calculated by cumulate analysis. Each sample was measured at least three times, and the average values were used (Kim, 2014). The zeta potential of nanoemulsion was determined by using Zetasizer in the same way as particle size and PDI. The zeta potential was calculated from the electrophoretic mobility using the Smoluchowski equation. All measurements were made in triplicate and values are expressed in mV. Results of measurement were reported as mean±standard deviation (SD)

3.6.1.2 Rheological properties

The rheological measurement was conducted by using a controlled-stress rheometer (Rotational Rheometer, ARES G2, TA Instruments,USA). The steady shear rate sweep test was performed the shear rate range of 0.01-1000 s-1 by using the 50 mm-parallel plates geometry with a gap width of 1.00 mm.

3.6.1.3Morphology by using transmission electron microscopy (TEM)

Transmission Electron Microscopy (TEM) Morphology is the study and structure of the nanoemulsion using an electron microscope. It consists of an electron source, which produces the electrons to feed into the system. The electrons are then squeezed into beams. An electromagnetic Lens, which acts as a condenser lens, can adjust the electron beam to a small size as needed. A very thin specimen (60-90 nm) can be penetrated by the electrons. Through the passage of electrons, the sample is projected by the objective lens. This adjusts the focus and

creates the intermediate image, which is amplified by a projector lens on to a fluorescent screen. It is a 2D image. Objects with low atomic numbers, produce a white image and objects with higher atomic numbers produce a black image. (Ric newsletter, 2014)

Morphology of the representative Benjakul nanoemulsion was observed by using the transmission electron microscopy technique. Benjakul nanoemulsion was stained with 0.5% w/v uranyl acetate solution and observed under a JEM-1220 transmission electron microscope (Japan).

3.6.1.4 Determination of piperine of Benjakul nanoemulsion

The piperine content in nanoemulsion of BKE was determined by using a high performance liquid chromatography (HPLC). Benjakul nanoemulsion was dissolved in acetonitrile at concentrations 100 mg/ml and filtered through a 0.45 μm membrane filter. These solutions were analyzed by HPLC. The standard curve of piperine was constructed and used to calculate piperine content of Benjakul nanoemulsion.

3.6.1.5 *In vitro* Release Study by Franz diffusion

Table 3.7 List of chemicals and reagents of *in vitro* Release Study by Franz diffusion cell

Name	Source
Absolute ethanol	QRëC, New Zealand
Distilled water	Milford, USA
Sodium hydroxide	Univar, Australia
Monosodium dihydrogenortho-phosphate ($NaH_2PO_42H_2O$)	Univar, Australia
Disodium hydrogenortho-phosphate dihydrate ($NaHPO_42H_2O$)	Univar, Australia

Release study of Benjakul nanoemulsion was performed according to the method described in previous research revealed with some modification**.** BKE was released from nanoemulsion by using modified Franz diffusion cells in triplicate. About 3 mg Benjakul nanoemulsion was prepared and placed on the cellulose dialysis membrane for release. The membrane with Benjakul nanoemulsion on one side was placed between the donor and receptor units, which were filled with 10% Absolute EtOH in pH 7.4 phosphate buffer solution to increase solubility of BKE. Throughout the period of the diffusion study, the receiving solution was kept well stirred with a magnetic stirrer, and the temperature was maintained at 37±1°C. A precise amount of the receiving solution was withdrawn by sampling 5ml at predetermined intervals for 480 mins for analysis of released piperine content by using HPLC technique**.** The diode array detector was set at a wavelength of 320 nm.

3.6.1.6 *In vitro* anti-inflammatory activity of Benjakul nanoemulsion by inhibition of nitric oxide production from RAW 264.7 cells

(1) Preparation of Benjakul nanoemulsion

Benjakul nanoemulsion was dissolved in sterile RPMI making stock at a concentration of 100 mg/ml. Sample were prepared at various concentration of 30, 10, 1, and 0.1 µg/ml, and tested as described on topic 3.3.2

3.6.1.7 *In vitro* Cytotoxicity activity of Benjakul nanoemulsion

Animal cell lines: The HaCaT cell line is a well-known immortalised human keratinocyte cell line. This cell line was cultured in DMEM medium containing 10% heat-inactivated fetal bovine serum, penicillin and streptomycin. The cells were incubated at 37°C in 5% CO_2 and sub passaged every 4-5 days.

Table 3.8 List of chemicals and reagents of cytotoxicity activity by MTT assay

Name	Source
Dimethyl sulfoxide (DMSO)	RCL Labscan, Thailand
Distilled water	Milford, USA
Fetal bovine serum (FBS)	Biochem, Germany
Hydrochloric acid (HCl)	Univar, Australia
Penicillin-Streptomycin (P/S)	Sigma, USA
Phosphate buffered saline (PBS)	Amresco, USA
DMEM medium	Gibco, USA
Sodium bicarbonate ($NaHCO_3$)	BHD, England
Thiazolyl blue tetrazolium bromide (MTT)	Sigma, USA
Trypan blue 0.4%	Gibco, USA
Trypsin-EDTA	Gibco, USA

Preparation of Benjakul nanoemulsion: BKE and Benjakul nanoemulsion was dissolved in sterile DMEM medium to a final concentration of 1 mg/ml. It was diluted with DMEM to obtain final concentration of 0.1-50 µg/ml.

Assay for MTT effects in HaCaT cells: MTT assay was determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromine(MTT) colorimetric method. The plates were incubated at 37°C in 5% CO_2 for 24 hours. MTT solution (10 µl, 5mg/ml in PBS) was added in each well and incubated 2 hours. Supernatant was removed and 100 µl of DMSO added to dissolve the formazan production by cells. The density of formazan solution was measured by micro spectrophotometer at wavelength 570 nm. If density of cell viability was more than 70% it was considered as survival.

$$\%\text{survival} = \left(\frac{S}{C}\right) \times 100$$

Control (C) : Medium

Sample (S) : Medium, sample

3.7 Stability test of Benjakul extract nanoemulsion.

3.7.1 Heating cooling cycle (Charoenjittichai, 2016)

Nanoemulsions were kept in the refrigerator at 4 ℃ for 48 hours and in the hot air oven at 45 ℃ for 48 hours per one cycle. The test was carried out for six cycles. Measurements of particle size, size distribution, and zeta potential were performed in triplicate and nanoemulsions were optically evaluated in terms of phase separation and creaming.

3.7.2 Accelerated stability study (U.S. Department of Health and Human Services Food and Drug Administration, 2003)

Stability testing was done using transparent vials. The product was put in these vials and exposed to 40 ± 2°C with 75 ± 5% RH as accelerated testing for 6 months period. The product was tested for anti-inflammatory activity and cytotoxicity activity of samples on days 0, 15, 30, 60, 90, 120, 150, and 180.

3.8 Statistical analysis

All data are the mean of three replications. Values for BKE parameters are expressed as the mean ± standard error of mean, those for Benjakul nanoemulsion are expressed as mean ± standard deviation. Data were analyzed by using one-way ANOVA and Dunnett's multiple comparison tests.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Quality control of 95%Ethanol Benjakul extract

4.1.1 Study on chemical fingerprint of Benjakul preparation

4.1.1.1 Determination pf piperine content

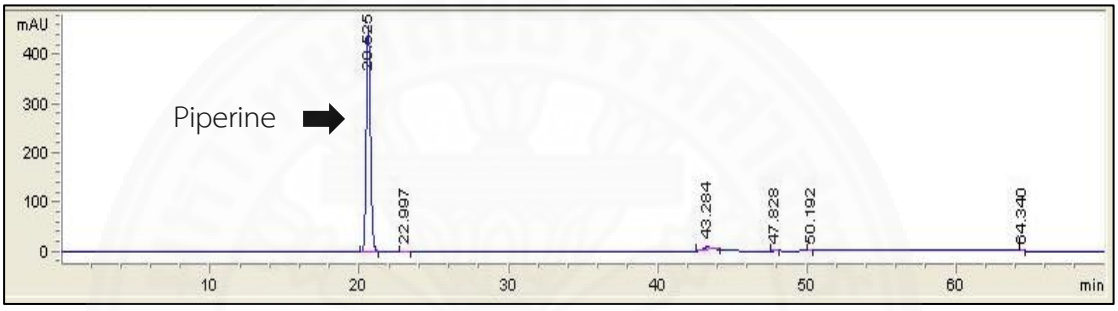

Figure 4.1 Chromatogram of standard piperine at wavelength 256 nm by High Performance Liquid Chromatography

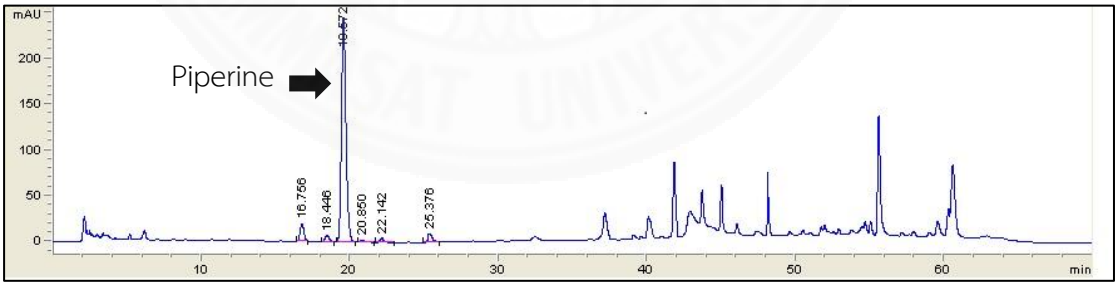

Figure 4.2 Chromatogram of piperine in BKE at wavelength 256 nm by High Performance Liquid Chromatography

BKE major constituent is piperine which is stable and found in *Piper retrofractum* Vahl.. Therefore, it was used as a chemical marker for quality control of extract. The standard of piperine obtained from HPLC analysis is shown by chromatogram in Figure 4.1, which shows the peak of standard piperine at 20 minutes consistent with chromatogram of BKE in Figure 4.2.

This study, the standard curve of piperine shown in Figure 4.3, which has the following regression equation 4.1 (R^2 = 0.9998) for calculated the piperine content in BKE.

y = 22.425x + 4.0467 (Eq.4.1)

From calculated found BKE had piperine content 88.89 mg/g

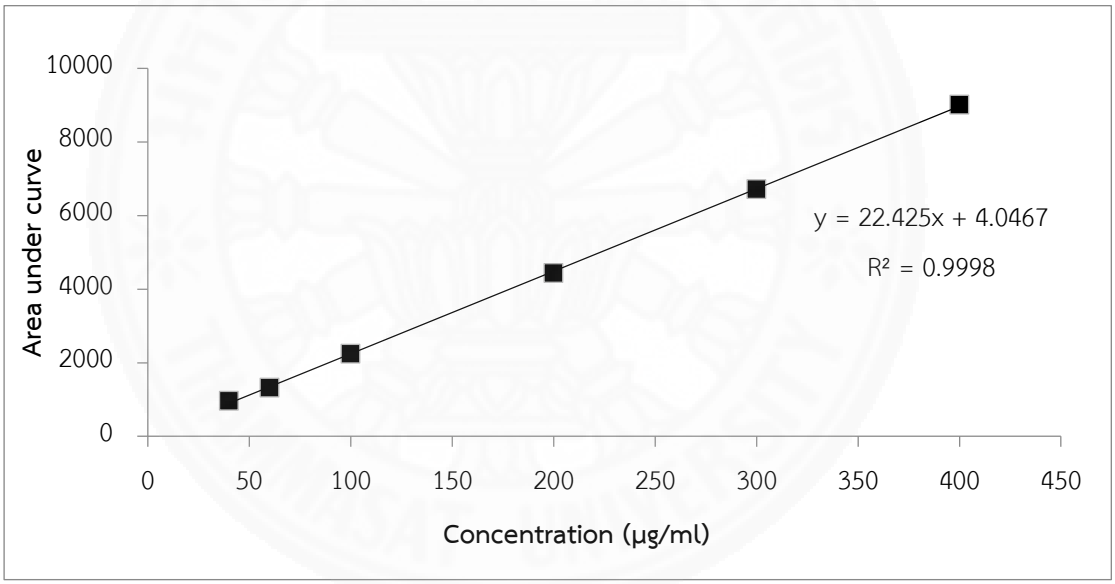

Figure 4.3 Calibration curve of standard piperine (concentration range from 40-400 µg/ml)

4.1.2 Determination of anti-inflammatory activity of Benjakul extract

The Anti-inflammatory activity of BKE, on LPS induced nitric oxide production release from RAW264.7 cells by the extract showed potent inhibition with IC_{50} value of 19.72 ± 0.72 µg/ml.

Piperine is a potent anti-inflammatory agent. It could inhibit the inflammation processes in the injured tissues by reduction of NO released from macrophages, reduction of PGE2 production and inhibition of IL-6 expression. (Bang,*et.al.*,2009 and Umar,*et.al.*,2013) Therefore, it has been currently used for treatment of inflammation-associated diseases.

4.1.3 Anti-inflammatory activity and piperine content of Benjakul extract treated under forced degradation conditions

BKE was treated under thermolysis, hydrolysis, acid hydrolysis, base hydrolysis and oxidation reaction on anti-inflammatory activity and determination of piperine in BKE before the formulation of the product.

The results found BKE treated under various stress conditions i.e. thermolysis, hydrolysis, acid hydrolysis, base hydrolysis and oxidation showed anti-inflammatory activity with IC_{50} values of 21.17±2.09, 15.97±1.4, 19.47±3.2, 20.87±3.2, 15.35±1.8 µg/ml respectively. Table 4.1 statistical analyses showed that anti-inflammatory properties of BKE was not significantly different from an untreated BKE.

In addition, piperine content of BKE treated under various stress conditions showed an increase from BKE (not tested) shown in Figure 4.4. Statistical analysis showed that piperine content under thermolysis, moisture, and alkaline hydrolysis was significantly different from an untreated BKE.

The previous study found that piperine as the main compound in *Piper retrofractum* Vahl., *Piper nigrum* Linn. was increased because of derivative of piperine as methyl piperate. (Rattarom, 2013) It may be converted to piperine when it was kept at a high temperature in long period time. Thus, Benjakul extract which composed of *Piper chaba* Hunt., *Piper sarmentosum* Roxb., *Piper interruptum* Opiz. can found methyl piperate. Therfore, piperine was increased.

This study found that BKE treated under forced degradation condition had an increase of piperine content but anti-inflammation activity of BKE treated under thermolysis, hydrolysis, acid hydrolysis, alkaline hydrolysis and oxidation were not significantly different. These results indicated that formulation of BKE products was feasible.

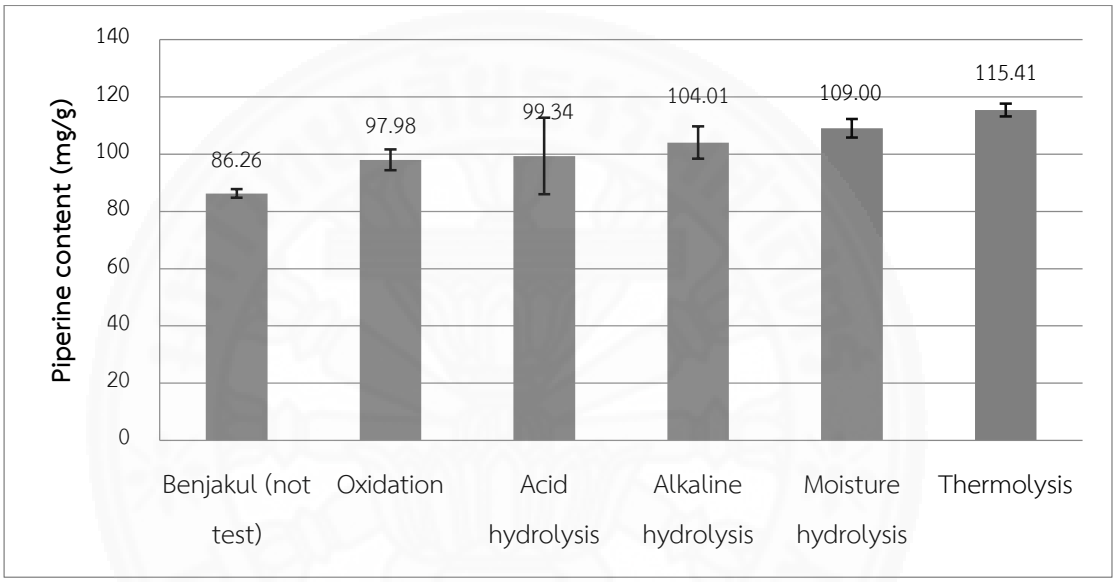

Figure 4.4 Determination of piperine in BKE treated under forced degradation conditions

Table 4.1 The percentage of inhibitory effect on NO production and cytotoxicity of BKE under stress conditions (Mean ± SEM), (N=3)

Stress conditions type	%Inhibition of Nitric Oxide Production and (%survival)					IC_{50} (µg/ml)
	0.1 µg/ml	1 µg/ml	10 µg/ml	30 µg/ml	50 µg/ml	
BKE (NT)	NT	-19.83±2.9 (99.48±21.25)	18.52±2.4 (98.21 ± 4.81)	71.42±1.0 (102.11 ± 12.83)	90.69±2.3 (88.34 ± 17.68)	19.72±0.72
Thermolysis	-4.70±3.7 (101.52±0.8)	-0.83±2.1 (107.70±3.4)	22.58±5.2 (89.16±3.8)	73.10±1.9 (102±8.6)	NT	21.17±2.09
Moisture hydrolysis	-1.36±1.4 (89.74±5.7)	-2.62±0.9 (80.56±3.13)	28.55±2.4 (77.50±3.8)	71.04±2.9 (88.76±8.4)	NT	15.97±1.4
Acid hydrolysis	-7.75±3.8 (102.36±6.7)	-2.62±1.1 (95.46±4.0)	25.78±2.9 (84.18±3.6)	67.40±5.3 (90.53±6.5)	NT	19.47±3.2
Base hydrolysis	-4.31±5.1 (92.24±3.3)	-2.28±4.5 (91.25±2.3)	25.68±4.3 (81.55±5.9)	56.91±2.5 (94.91±4.1)	NT	20.87±3.2
Oxidation	-9.10±3.8 (108.45±11.4)	-5.10±1.3 (98.39±5.4)	33.23±4.4 (95.01±14.6)	84.44±0.6 (110.95±5.5)	NT	15.35±1.8

*indicates no significant difference at *p* value > 0.05 when compared to BKE (NT), NT = Not test

4.1.4 Determination of solubility of piperine content in Benjakul extract in oils

The solubility of BKE in each oil and surfactant was determined by using piperine as a marker. This study of the piperine solubility in each oil showed BKE can be dissolved in the oil according to Figure 4.5. Therefore, the solubility can be used as the data for the nanoemulsion formulation of BKE.

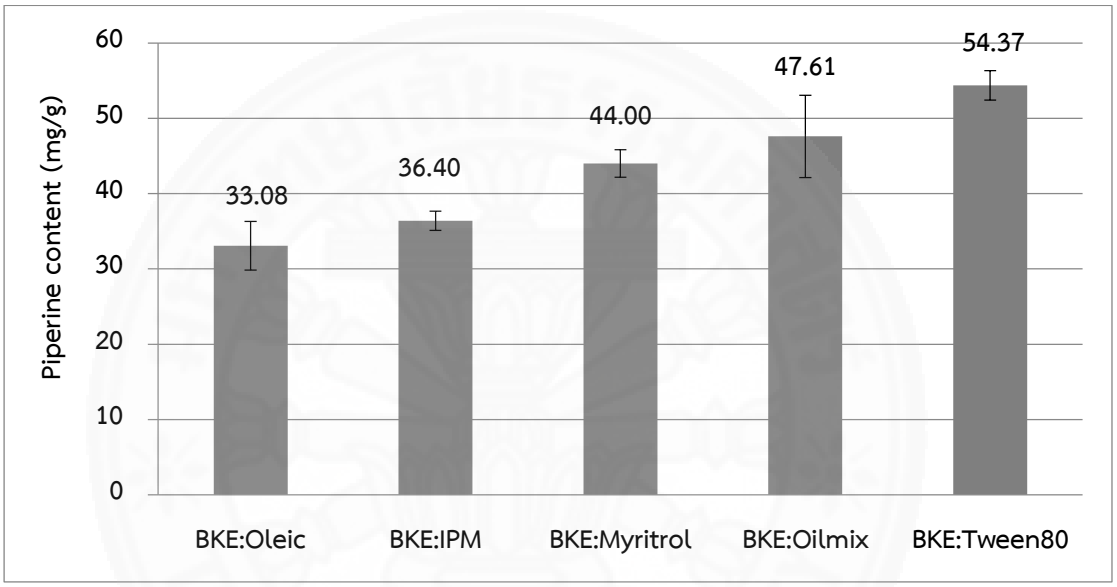

Figure 4.5 Piperine content in BKE in oils and surfactant

4.2 Preparation of nanoemulsion formulation

4.2.1 Experimental design

This study performed by using the variables of the formulation i.e. oil mix (oleic acid: myritol: isopropyl myristate), propylene glycol and tween80. Each formulation consisted of the 3 factors (oilmix, propylene glycol and tween80) in varying proportion as shown in Table 4.2

Table 4.2 The variables of base nanoemulsion formulation

Number	Code.	Formula (%)		
		O:I:M (Oleic acid: Myritol: Isopropyl myristate) (1:1:1)	PG (Propylene glycol)	T (Tween80)
1	1O1I1M5-T5	5	0	5
2	1O1I1M10-T5	10	0	5
3	1O1I1M15-T5	15	0	5
4	1O1I1M5-PG5-T5	5	5	5
5	1O1I1M10-PG5-T5	10	5	5
6	1O1I1M15-PG5-T5	15	5	5
7	1O1I1M5-T8	5	0	8
8	1O1I1M10-T8	10	0	8
9	1O1I1M15-T8	15	0	8
10	1O1I1M5-PG5-T8	5	5	8
11	1O1I1M10-PG5-T8	10	5	8
12	1O1I1M15-PG5-T8	15	5	8
13	1O1I1M5-T12	5	0	12
14	1O1I1M10-T12	10	0	12
15	1O1I1M15-T12	15	0	12
16	1O1I1M5-PG5-T12	5	5	12
17	1O1I1M10-PG5-T12	10	5	12
18	1O1I1M15-PG5-T12	15	5	12

4.2.2 Determination of physical properties of nanoemulsion formulation

The nanoemulsion was prepared first by testing physical properties of 18 different mixtures of the basic formulation, as shown in Table 4.3 and Table 4.4, and found that the formula 11 was not separated in the new prepared condition, at 24 hours and at 7 days and after centrifugation at 5,000 rpm for 30 minutes. It has a particle size of 150.9 nm with a PDI of 0.2 and zeta potential of -3.8 mV. Particle size of nanoemulsion varies from 10 to 1,000 nm and its low PDI value and it reduced the chance of separation. Although, another formula has similar properties but found separated at 24 hours and 7 days. Therefore, formula 11 was selected for a basic formulation with BKE.

Formulations of nanoemulsions containing Benjakul remedy were developed and characterized their physicochemical properties. The results showed that increase in the Tween 80 content led to the larger droplet size, the higher value of PDI and the less zeta potential. This finding related with the previous study (Asasutjarit *et al.,* 2007). They found that the excess emulsifier molecules could deposit on surface and shielded the charge of the solid lipid nanoparticles. However, these parameters of nanoparticles were acceptable when using the optimum concentrations of emulsifiers.

Table 4.3 Physical properties of base nanoemulsion formulation (Mean ±SD), (N=3)

Formulation number	Samples	Physical properties						Physical properties after centrifugation at 5,000 rpm for 30 min	
		Turbid	Slightly turbid	Slightly clear	Clear	Not separate	Separate	Not separate	Separate/ %Creaming
1	1O1I1M5-T5			✓		✓			✓/(3.8%)
2	1O1I1M10-T5	✓				✓		✓	
3	1O1I1M15-T5	✓				✓		✓	
4	1O1I1M5-PG5-T5				✓	✓		✓	
5	1O1I1M10-PG5-T5		✓			✓		✓	
6	1O1I1M15-PG5-T5	✓				✓		✓	
7	1O1I1M5-T8				✓	✓		✓	
8	1O1I1M10-T8		✓			✓		✓	
9	1O1I1M15-T8	✓				✓		✓	

Table 4.3 Physical properties of base nanoemulsion formulation (Mean ±SD), (N=3) (Continued)

Formulation number	Samples	Physical properties						Physical properties after centrifugation at 5,000 rpm for 30 min	
		Turbid	Slightly turbid	Slightly clear	Clear	Not separate	Separate	Not separate	Separate/ %Creaming
10	1O1I1M5-PG5-T8				✓	✓		✓	
11	1O1I1M10-PG5-T8		✓			✓		✓	
12	1O1I1M15-PG5-T8		✓			✓		✓	
13	1O1I1M5-T12			✓		✓		✓	
14	1O1I1M10-T12			✓		✓		✓	
15	1O1I1M15-T12		✓			✓		✓	
16	1O1I1M5-PG5-T12				✓	✓		✓	
17	1O1I1M10-PG5-T12			✓		✓		✓	
18	1O1I1M15-PG5-T12	✓				✓		✓	

Table 4.4 Physical properties (particle size, polydispersity index (PDI), and zeta potential of base nanoemulsion formulation (Mean ±SD), (N=3)

Formulation number	Samples	Particle size (nm)	PDI	Zeta-potential (mV)
1	1O1I1M5-T5	66.6±1.6	0.5±0.1	-10.7±1.0
2	1O1I1M10-T5	139±1.2	0.3±0.0	-6.2±0.4
3	1O1I1M15-T5	158.4±1.0	0.4±0.0	-4.1±0.2
4	1O1I1M5-PG5-T5	70.8±0.9	0.3±0.0	-7.2±0.4
5	1O1I1M10-PG5-T5	137.6±0.7	0.3±0.0	-4.6±0.7
6	1O1I1M15-PG5-T5	198.1±6.2	0.4±0.0	-3.8±0.3
7	1O1I1M5-T8	42.4±1.0	0.9±0.0	-8.8±2.0
8	1O1I1M10-T8	123.3±0.6	0.2±0.0	-4.6±0.7
9	1O1I1M15-T8	186.3±3.8	0.2±0.0	-3.6±0.5
10	1O1I1M5-PG5-T8	40.9±0.7	0.8±0.0	-6.0±1.1
11	**1O1I1M10-PG5-T8**	**150.9±1.8**	**0.2±0.0**	**-3.8±0.1**
12	1O1I1M15-PG5-T8	226.3±6.6	0.4±0.1	-4.7±0.4
13	1O1I1M5-T12	104.2±8.3	1±0.0	-6.3±0.6

Table 4.4 Physical properties (particle size, polydispersity index (PDI), and zeta potential of base nanoemulsion formulation (Mean ±SD), (N=3) (Continued)

Formulation number	Samples	Particle size (nm)	PDI	Zeta-potential (mV)
14	1O1I1M10-T12	165.5±2.5	0.6±0.0	-5.7±0.2
15	1O1I1M15-T12	262.8±26.8	0.2±0.0	-4.0±0.8
16	1O1I1M5-PG5-T12	25.8±4.3	0.4±0.1	-4.8±0.7
17	1O1I1M10-PG5-T12	147.3±0.7	0.5±0.0	-3.8±0.3
18	1O1I1M15-PG5-T12	911.5±16.0	0.2±0.0	-0.8±0.6

4.3 Preparation of Benjakul nanoemulsion

4.3.1 Determination of Particle size, Polydispersity index (PDI), and Zeta Potential

The formula 11 was selected as the basic formulation to prepare the nanoemulsion of BKE. Thus, preparing the nanoemulsion of BKE. BKE was added in different ratios as shown in Table 4.5 and physical properties of each formulation were tested. This BKE has a small amount of piperine per 1 g, and a previous study used 0.5% BKE in the preparation, which may contain less piperine. Thus, this study used 1%, 2% and 3% of BKE respectively.

Table 4.5 Physical properties (particle size, polydispersity index (PDI), and zeta potential of Benjakul nanoemulsion formulation (Mean ±SD), (N=3)

Formulation number	Sample	Particle size (nm)	PDI	Zeta-potential (mV)
1	NE-base-T8	289.6±3.3	0.3±0.0	-6.1±0.4
2	BKE0.5-NE-T8	265.4±1.6	0.3±0.0	-3.3±0.3
3	BKE1-NE-T8	275.9±4.4	0.3±0.0	-2.0±0.3
4	BKE2-NE-T8	241.5±3.6	0.2±0.0	-0.9±0.0
5	BKE3-NE-T8	276.2±5.3	0.3±0.0	-1.3±0.2
6	**BKE3-NE-T8-x0.2**	**587.6±25.1**	**0.6±0.1**	**-5.8±0.2**
7	BKE3-NE-T8-x0.3	909.0±16.7	0.6±0.0	-4.0±0.5

The preparation of the nanoemulsion of BKE showed that the formula 6 (BKE3-NE-T8-x0.2) was physically in the new prepared condition at 24 hours, and at 7 days the appearance was rather turbid. It was not separated after centrifugation at 5,000 rpm for 30 minutes, the particle size was 587.6 ± 25.0 nm, and the PDI was 0.6 ± 0.09 and a potential electric potential (Zeta potential) -5.7 ± 0.2 mV. BKE3-NE-T8-x0.2 was liquid and may not have stuck to the skin. Therefore, xanthan gum was used as a viscosity additive, so the particle size was increased after xanthan gum was added.

The main compound of Benjakul is piperine so it was the marker of this study. Piperine in Benjakul extract has low content. Thus, a concentration of extract in the product must be increased as high concentration for study on drug release. However, the preparation of extract could be prepared dilution of concentration with another base solution. Therefore, the formula 6 which had the highest concentration of BKE was selected.

4.3.2 Rheological properties

The flow curve and viscosity profile results for NE-base-T8 were shown in Figure 4.6 and Figure 4.7. It was found that there is a linear relationship between shear stress and shear rate. The viscosity profile of NE-base-T8 had Newtonian flow characteristics which was fluid. However, BKE3-NE-T8-x0.2 has xanthan gum which it can make the viscosity increase. The flow curve, and viscosity profile of BKE3-NE-T8-x0.2 were different from NE-base-T8. The viscosity profile of BKE3-NE-T8-x0.2 had non-Newtonian flow characteristics (pseudoplastic and has a constant viscosity. The shear viscosity of BKE3-NE-T8-x0.2 decrease when the shear rate of BKE3-NE-T8-x0.2 increases as shown in Figure 4.8 and Figure 4.9.

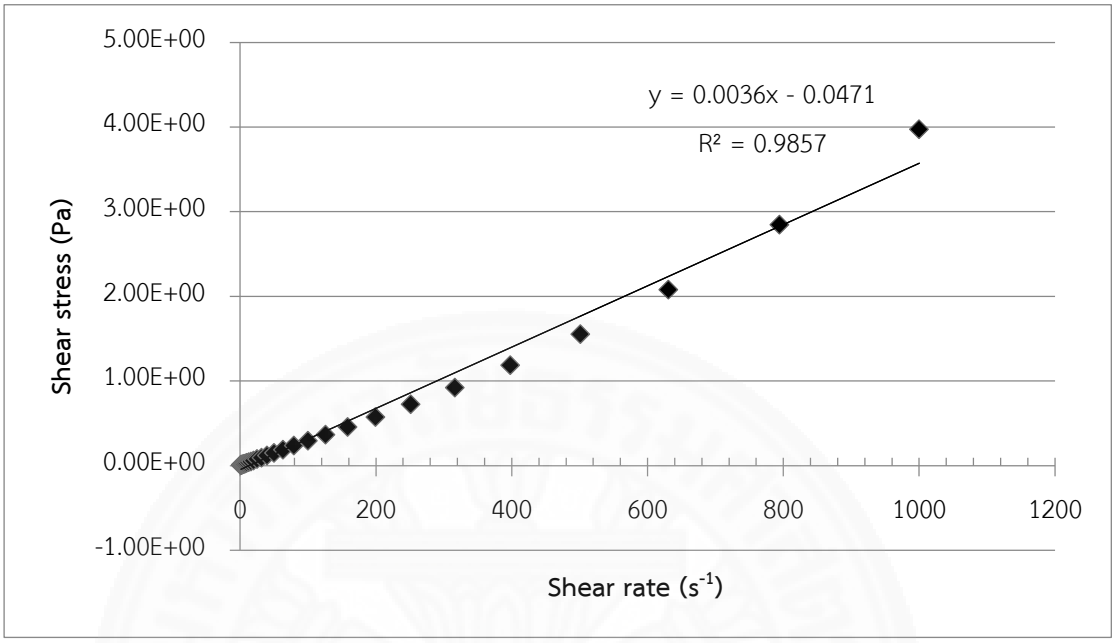

Figure 4.6 Flow curve of NE-base-T8

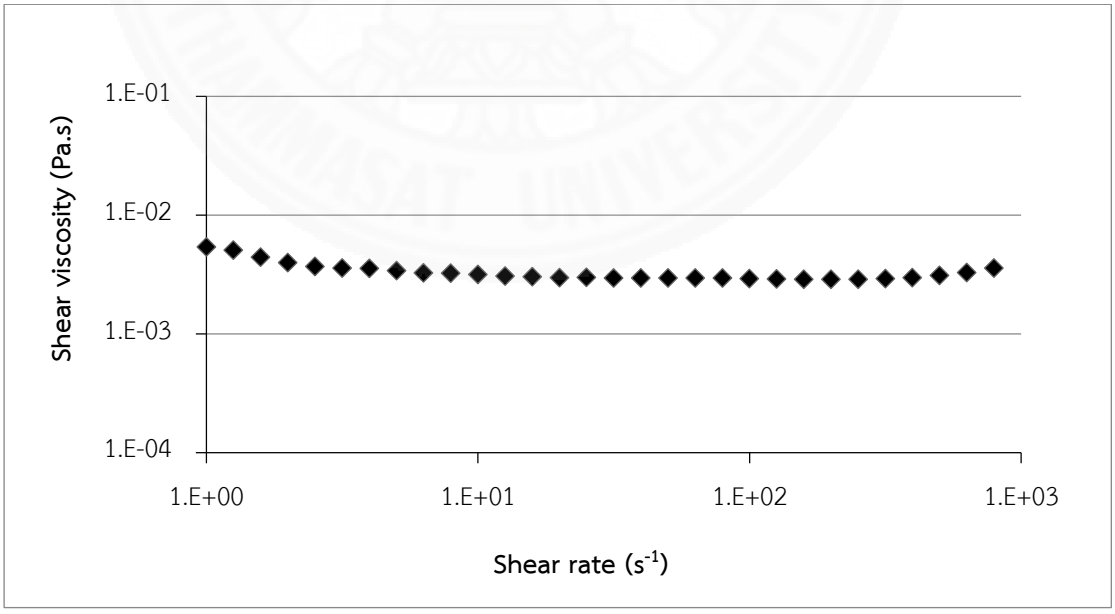

Figure 4.7 Logarithmic plots of viscosity against shear rates of NE-base-T8

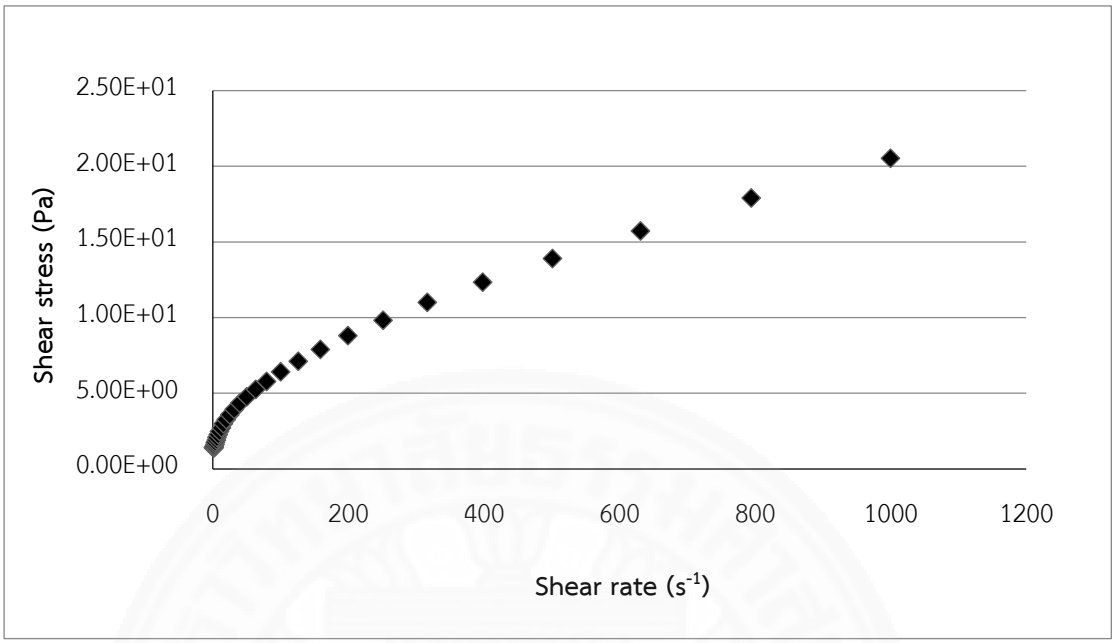

Figure 4.8 Flow curve of BKE3-NE-T8-x0.2

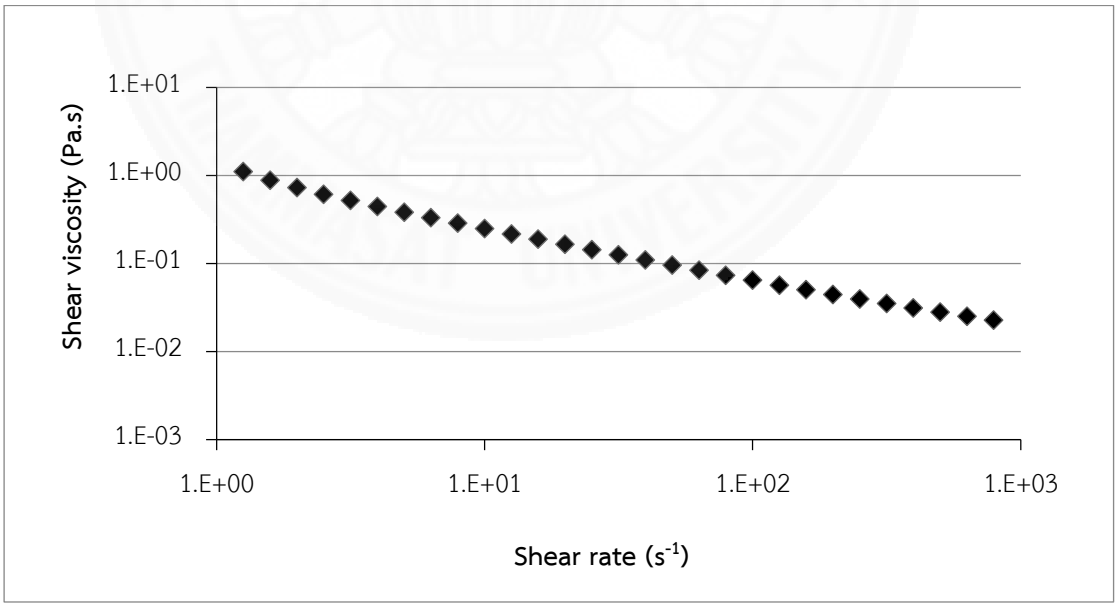

Figure 4.9 Logarithmic plots of viscosity against shear rates of BKE3-NE-T8-x0.2

4.3.3 Morphology by using transmission electron microscopy (TEM)

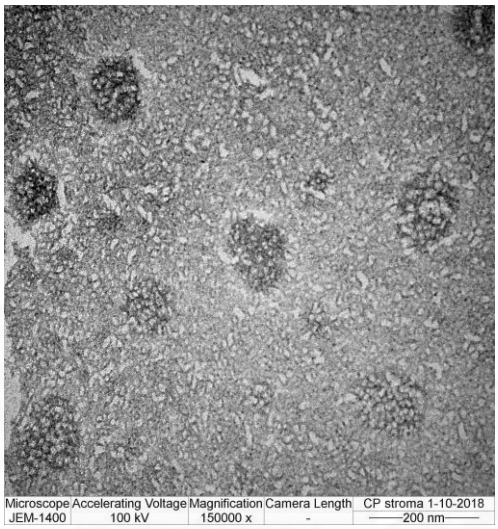

Figure 4.10 Transmission electron microscopy of NE-base-T8

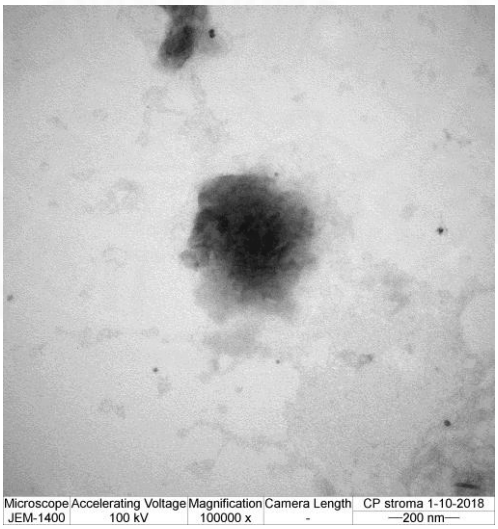

Figure 4.11 Transmission electron microscopy of BKE3-NE-T8-x0.2

Using transmission electron microscopy (TEM) indicated the morphology of NE-base-T8 and BKE3-NE-T8-x0.2 as shown in Figure 4.10 and Figure

4.11 respectively. The black area which was dyed with 0.5% uranyl acetate is a particle of nanoemulsion clearly showing its spherical form.

4.3.4 Determination of piperine content of BKE3-NE-T8-x0.2

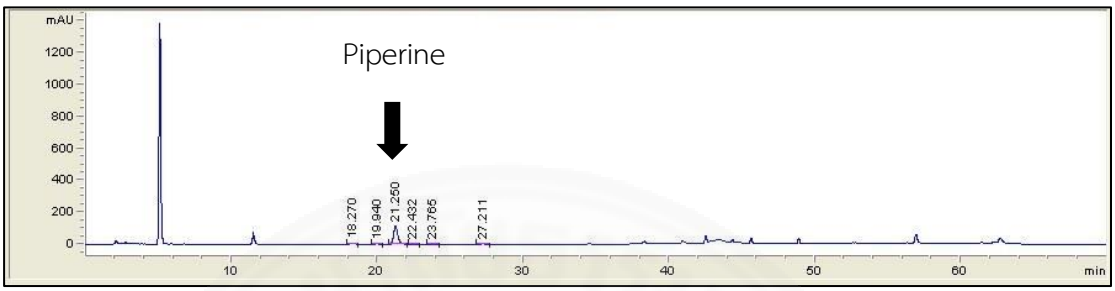

Figure 4.12 Chromatograms of piperine in BKE3-NE-T8-x0.2 at wavelength 256 nm by High Performance Liquid Chromatography

Analysis of the content uniformity of BKE3-NE-T8-x0.2 by HPLC analysis using piperine as a chemical marker, found that 100 g BKE3-NE-T8-x0.2 has piperine content 0.269 g.

4.3.5 *In vitro* Release Study

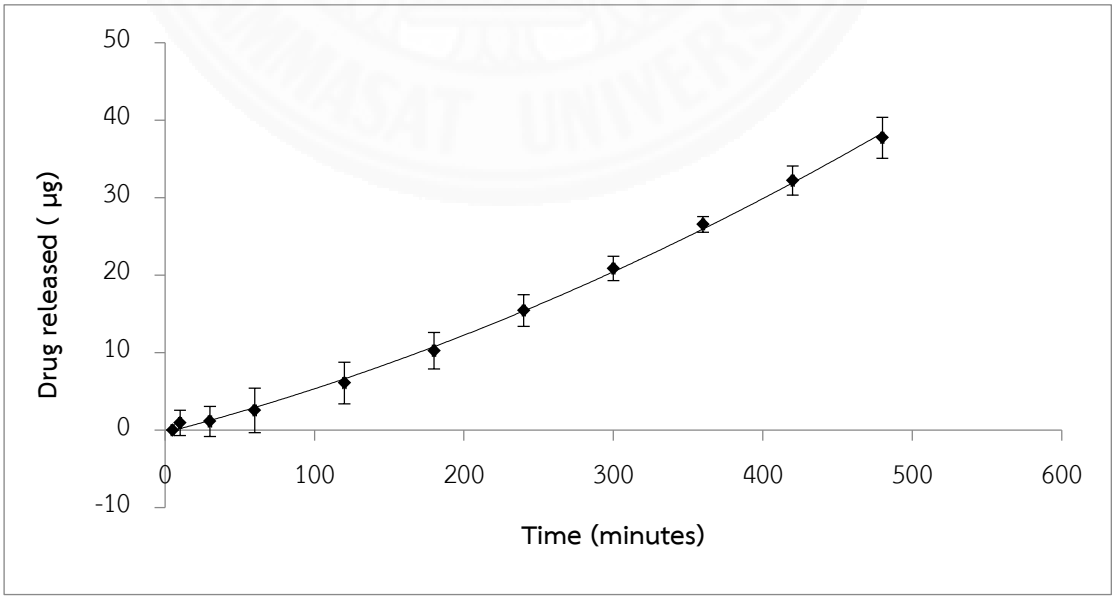

Figure 4.13 Drug release through cellulose membranes of BKE3-NE-T8-x0.2

The results study of the release of BKE from BKE3-NE-T8-x0.2 by Franz diffusion cell using a cellulose membrane, produced a release profile as shown in Figure 4.3. It was found that BKE is continuously released from the nanoemulsion at a rate of 0.0855 μg/min

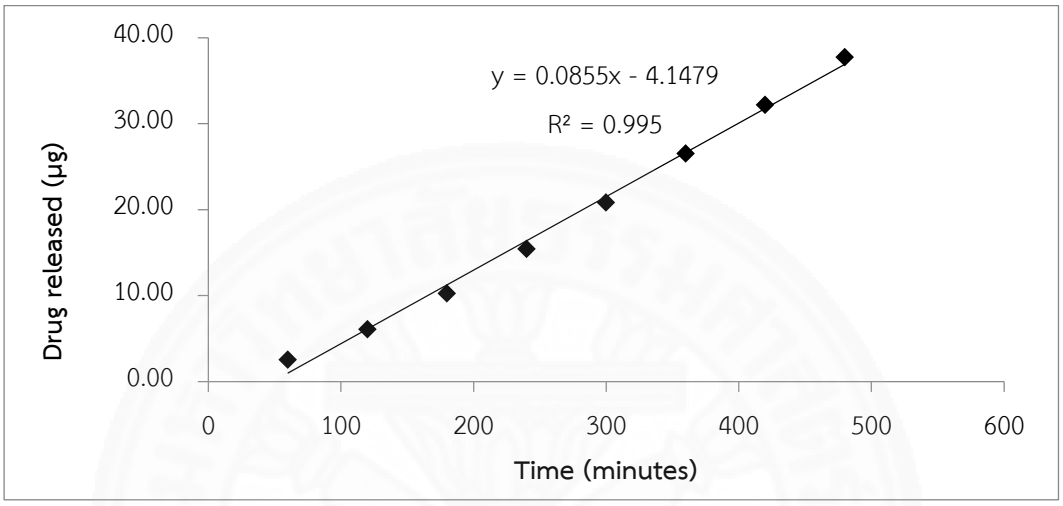

Figure 4.14 The release profile of BKE from BKE3-NE-T8-x0.2: Plot of cumulative release amount of BKE against time

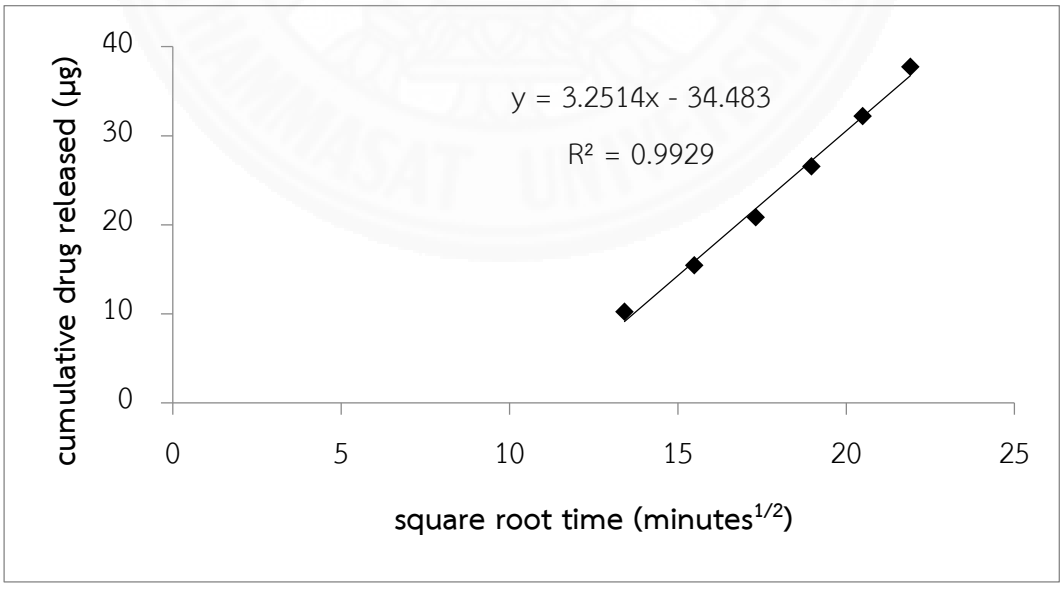

Figure 4.15 Plot of cumulative release amount of BKE against square root of time

Figure 4.14 shows a trend profile of release of BKE found that release of BKE shows a good linear correlation with time (R^2 >0.98), while the plot between release of BKE with square root of time showed R^2 <0.98 in Figure 4.15

These results indicated that release profile obeyed zero order models with R^2=0.9950 in Figure 4.14. Therefore, confirmed that BKE was molecularly dispersed in the nanoemulsion. However, it should be studied permeation properties and clinical trial study when the nanoemulsion is in the human skin.

Xanthan gum was used as a viscosity enhancer. Furthermore, it could be control drug release rate from the nanoemulsion. In this study, concentrations xanthan gum were fixed, but, the concentration of BKE was varied. It was found that piperine in the nanoemulsion containing the high concentration of piperine was released faster than the piperine in the nanoemulsion containing the low concentration. This result could be explained by the effect of concentration gradients of piperine in the donor- and the receptor compartment of the Franz diffusion cells.

4.3.6 Anti-inflammatory activity of BKE3-NE-T8-x0.2

Table 4.6 The percentage of inhibition of BKE3-NE-T8-x0.2 on LPS induced NO production from RAW264.7 cells and cytotoxicity (Mean±SEM), (N=3)

Samples	%Inhibition of Nitric Oxide Production and (%cytotoxicity)					IC_{50} (µg/ml)
	0.1 µg/ml	1 µg/ml	10 µg/ml	30 µg/ml	50 µg/ml	
NE-base-T8	3.95±2.0 (131.35±15.6)	9.65±3.0 (120.22±12.2)	37.59±4.9 (116.09±16.6)	73.64±3.4 (122.21±7.9)	NT	17.28±2.7
BKE3-NE-T8-x0.2	14.43±3.2 (99.15±8.4)	18.54±2.7 (99.03±15.1)	56.31±5.0 (101.90±15.0)	86.14±1.9 (113.74±20.4)	NT	8.36±1.0[(a),(b)]
BKE	NT	-19.83±2.9 (99.48±21.2)	18.52±2.4 (98.21±4.8)	71.42±1.0 (102.11±12.8)	90.69±2.3 (88.34±17.6)	19.72±0.7
Prednisolone	25.19±5.2 (120.94±17.1)	45.20±9.2 (100.98±4.1)	50.36±8.7 (92.31±3.1)	NT	70.63±7.8 (89.81±5.7)	1.72±0.7

[(a)] indicates significant difference at p value < 0.05, compared to BKE

[(b)] indicates no significant difference at p value > 0.05, compared to Prednisolone

NT = Not test

Anti-inflammatory activity of BKE3-NE-T8-x0.2 shown in Table 4.6 had IC_{50} value 8.36±1.06 µg/ml and BKE IC_{50} = 19.72±0.72 µg/ml and their values were also significantly different. In addition, BKE3-NE-T8-x0.2 was significantly different when compared to NE-base-T8. These results suggested that NE-base-T8 containing BKE could inhibit NO released from macrophages properly. Moreover, the synergist effects of NE-base-T8 and BKE could lead to the more potent NE-base-T8 for inhibition of inflammatory processes in the injured tissue.

NE-base-T8 could inhibit inflammatory effect on NO production. The ingredient of this base which effect on anti-inflammation may be oleic acid because oleic acid was reported on the inflammatory response of the skin. A topical application of oleic acid was studied on ear-edema test and the results showed that oleic had the anti-inflammatory activity. (Morais *et al.,* 2017)

4.3.7 Cytotoxicity activity of BKE3-NE-T8-x0.2

This test was performed on human skin. Skin irritation test was conducted to evaluate the potential of BKE, NE-base-T8 and BKE3-NE-T8-x0.2 by using MTT assay and is reported as the %cell viability of HaCaT cells. The results shown in Table 4.7 indicate that the percentage of survival of all samples was higher than 70%. BKE3-NE-T8-x0.2 was also not significantly different from the base nanoemulsion. Therefore, it may be used for further *in vivo* and clinical trials, since it has been shown to be safe.

Table 4.7 The percentage viability of HaCaT cells exposed to BKE, NE-base-T8, and BKE3-NE-T8-x0.2 (Mean±SEM), (N=3)

Samples	%Cell viability				
	0.1 µg/ml	1 µg/ml	10 µg/ml	30 µg/ml	50 µg/ml
BKE	102.27±3.5	95.16±10.5	101.88±2.7	106.16±9.0	109.78±5.3
NE-base-T8	98.46±2.2	95.38±0.9	92.80±2.7	92.71±1.9	91.98±2.1
BKE3-NE-T8-x0.2	94.88±3.0	104.96±2.6	100.19±2.4	100.90±0.6	105.90±1.4[(a)]

[(a)] indicates no significant difference at p value > 0.05, compared to NE-base-T8 and BKE

4.4 Stability study

4.4.1 Heating cooling cycle

4.4.1.1 Physical test

(1) Appearance (N=3)

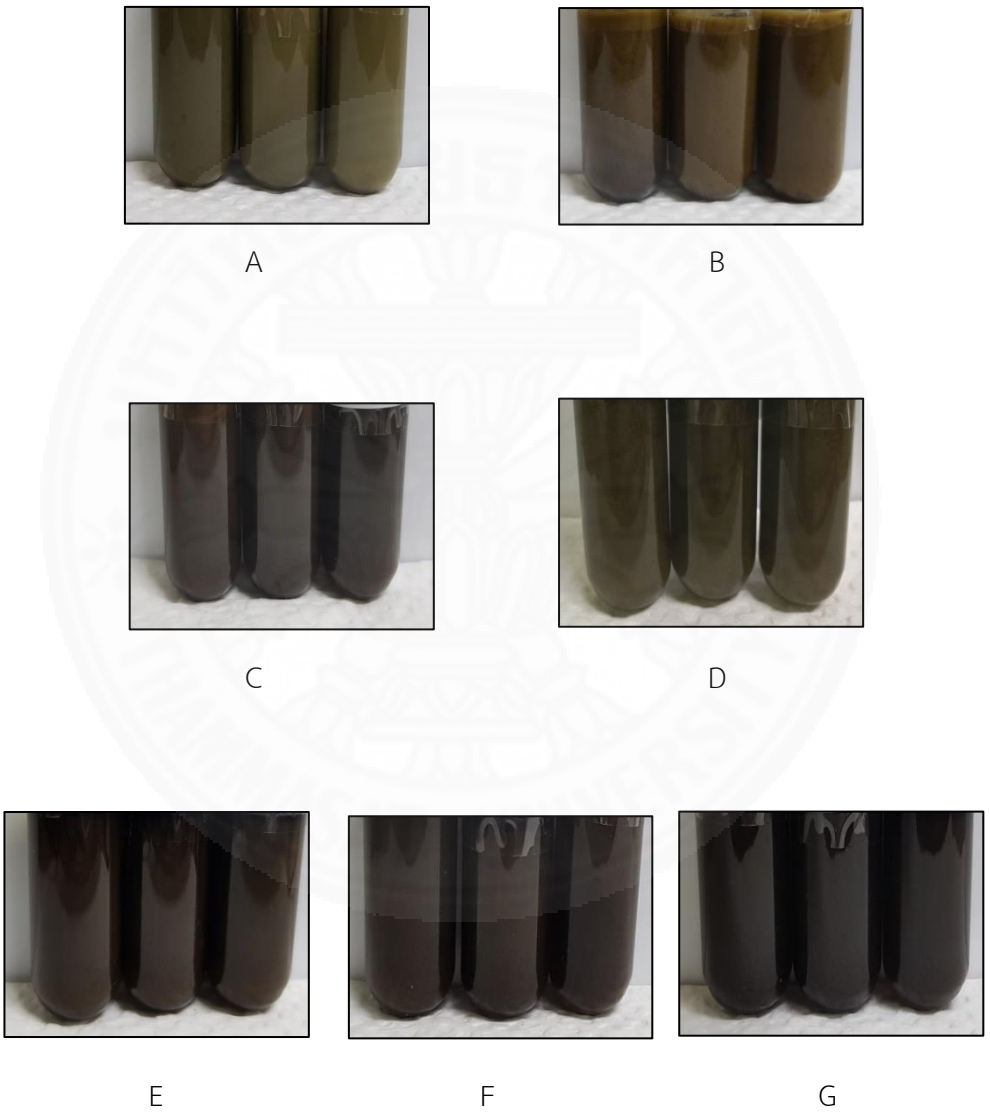

Figure 4.16 Appearance of BKE3-NE-T8-x0.2 after heating-cooling cycle test
A: Control, B: 1st cycle, C: 2nd cycle, D: 3rd cycle,
E: 4th cycle, F: 5th cycle, G: 6th cycle

Physical tests of BKE3-NE-T8-x0.2 after heating cooling cycle test included appearance (including phase separation). Appearance (including phase separation) found that there was no phase separation.

(2) Particle size, PDI, Zeta potential

Table 4.8 The properties of BKE3-NE-T8-x0.2 after heating cooling cycle test included particle size, PDI, zeta potential compared to control (Mean±SD),(N=3)

Cycle	Particle size	PDI	Zeta potential
Control	975.1±80.4	0.7±0.1	-3.4±1.0
1st Cycle	1262.0±21.7	0.8±0.0	-1.7±0.7
2nd Cycle	800.0±11.0	0.7±0.1	-2.1±0.7
3rd Cycle	800.8±72.2	0.7±0.1	-4.2±0.7
4th Cycle	967.5±14.8	0.7±0.0	-2.2±0.6
5th Cycle	848.7±5.7	0.7±0.0	-1.9±0.4
6th Cycle	817.2±21.6	0.6±0.1	-2.5±0.4

Physical tests of BKE3-NE-T8-x0.2 after heating cooling cycle test included particle size, PDI, zeta potential. From the heating-cooling cycle test, 1st cycle of BKE3-NE-T8-x0.2 had increased particle size and 2nd- 6th cycles had decreased when compared to control. The percentage of PDI found that (1st-5th cycles) had increased but the 6th cycle had decreased and zeta potential at the 3rd cycle had increased other cycle had decreased when it was compared with control.

The first measurement of control in start experiment (cycle 0), the particle size showed larger than the other cycle because xanthan gum may not dissolve. When it was kept in heat and cool condition, the particle size of BKE3-NE-T8-x0.2 of other cycle had decreased from control. However, in the study of stability, it is considered to be unstable. Although the size is reduced to small particles.

4.4.2 Accelerated stability study (Exposure to under 40±2°C with 75±5%RH)

4.4.2.1 Chemical test (Piperine content)

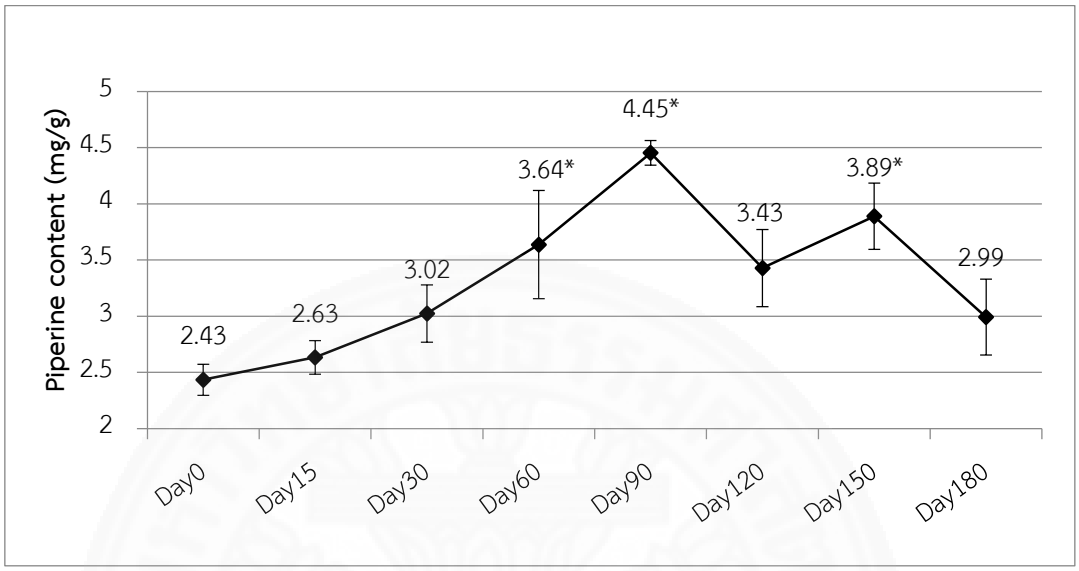

Figure 4.17 Piperine content of BKE3-NE-T8-x0.2 from stability study (Mean±SD), (N=3) by using HPLC

BKE3-NE-T8-x0.2 stability was tested by accelerated conditions (40 °C 75%RH for 6 months). The piperine content in BKE3-NE-T8-x0.2 at day 0 to day 180 were determined by HPLC. This study found that during the 180 days three samples were also a significant difference when compared with Day 0. This result due to the decomposition of the piperine derivative resulting increase in piperine content from Day0 shown in Figure 4.25. The results of the study showed that the product of BKE3-NE-T8-x0.2 may be unstable. However, piperine content of this product increased so. It is a good point of this study.

4.4.2.2 *In vitro* anti-inflammatory activity by inhibition of nitric oxide production from RAW 264.7 cell lines of BKE3-NE-T8-x0.2 from stability test

Table 4.9 The percentage of inhibition on LPS induced NO production from RAW264.7 cells and cytotoxicity from stability test of BKE3-NE-T8-x0.2 (Mean±SEM), (N=3)

Samples	%Inhibition of nitric oxide production and (%survival)					IC_{50}
	0.01 µg/ml	0.1 µg/ml	1 µg/ml	10 µg/ml	30 µg/ml	(µg/ml)
Day 0	NT	5.62±2.1 (86.49±9.9)	7.80±2.1 (82.47±3.4)	29.93±3.5 (85.66±3.3)	74.27±4.1 (88.00±8.8)	18.46±0.9
Day 15	NT	-1.48±2.2 (91.23±1.9)	10.24±0.5 (91.53±3.1)	37.04±2.6 (88.25±1.9)	75.52±1.9 (92.24±3.9)	21.36±0.9
Day 30	NT	-2.52±1.4 (100.73±5.1)	6.36±1.9 (94.33±7.1)	37.95±2.18 (86.28±4.5)	84.23±1.6 (107.14±8.3)	17.38±1.9
Day 60	NT	-2.99±1.6 (87.50±12.6)	3.40±0.5 (88.62±2.2)	38.17±0.1 (90.20±0.9)	88.83±1.8 (108.84±4.6)	14.82±0.3
Day 90	NT	-1.36±0.69 (102.49±6.7)	6.97±0.97 (95.39±4.5)	47.77±4.3 (91.54±5.7)	94.79±1.7 (91.85±8.7)	11.27±1.7[(a)]
Day 120	NT	-1.00±2.4 (94.53±7.6)	7.10±1.5 (89.16±5.7)	49.60±1.6 (90.12±9.1)	91.85±3.1 (99.78±5.8)	10.34±0.5[(a)]
Day 150	NT	-4.96±2.1 (89.75±6.2)	5.59±0.8 (93.23±10.8)	43.49±4.2 (84.15±7.9)	91.51±1.1 (90.55±7.5)	13.59±1.7
Day 180	-7.04±3.1 (91.94±9.4)	-0.17±1.4 (100.26±0.8)	16.00±6.2 (97.67±7.6)	63.44±5.8 (99.31±10.2)	NT	8.61±0.8[(a)]

[(a)] indicates significant difference at p value < 0.05, compared to Day 0

The stability test of BKE3-NE-T8-x0.2 is shown in Table 4.9 for anti-inflammatory activity by inhibitory activity on nitric oxide release. The statistical analysis found that Day90, 120, and 180 were also significantly different when compared with Day 0 ($p<0.05$). The results of the study showed that the product BKE3-NE-T8-x0.2 may be unstable. However, the anti-inflammatory activity of BKE3-

NE-T8-x0.2 at Day90, 120, and 180 IC_{50} were 11.27±1.7, 10.34±0.5 and 8.61 µg/ml, respectively. BKE3-NE-T8-x0.2 was kept in high temperature in a long period time may be increased content of piperine which was reported as high antiinflammatory compound. Benjakul extract which composed of *Piper chaba* Hunt., *Piper sarmentosum* Roxb., *Piper interruptum* Opiz. can found methyl piperate, this compound was changed to piperine when it was kept in high temperature and kept it a long period time. In addition, oleic acid in the product has anti-inflammatory activity. Therefore, further study should be investigated whether this methodology affects the structure of the components on decomposition or evaporation of the substance in the formulation.

The stability test results (heating-cooling test and exposure to under 40±2°C with 75±5%RH) of BKE3-NE-T8-x0.2 indicated that the product can be stable on affecting of piperine content and anti-inflammatory activity when it stored at room temperature

CHAPTER 5
CONCLUSIONS AND RECOMMENDATIONS

Benjakul is a Thai traditional medicine which is composed of five plants. It is used to relieve muscle and bone pain and may be used as a substite for NSAIDs. Benjakul tablets must be taken daily. Occasionally, it can cause adverse reactions to the patient, such as heat, abdominal cramps, nausea, and rash. Therefore, there is a case for using topical applications for medicinal products containing Benjakul extracts. This study would help to reduce the problem of such adverse reactions because the drug is absorbed through the skin into the inflammatory site without going through the gastrointestinal route. Currently, the dosage forms of topical products for muscle pain already in the market include patches, creams, gels, and lotions but there is no report of the nanoemulsion. It is a suspension of the ultra-small particle of active ingredient and one of the most effective forms delivering a transdermal drug into the skin layers.

A major constituent of BKE is piperine which was used as a chemical marker for quality control of extract. BKE 1 g. has piperine content 88.89 mg. and for its anti-inflammatory effect was IC_{50} value of 19.72±0.72 µg/ml. BKE is soluble in oils and surfactant and after the stress test, BKE loss of anti-inflammatory activity.

The inflammatory process occurs in injured cells by release of nitric oxide, and BKE has proved that it can inhibit NO production. Consequently, it could relieve muscle and bone pain effectively because these symptoms are affected by the inflammatory process. BKE is suitable for using further as an active ingredient in developing a topical product.

Development of a topical product of BKE as a nanoemulsion involves emulsions which contain oil, water, and surfactant phases. Therefore, preparation of a formula involves selecting the completed formula from base formulation of 18 formulas. The best base nanoemulsion formula was the 1O1I1M10-PG5-T8, composite as an oleic acid:myritol:isopropyl myristate 10%, propylene glycol 5%, Tween80 8% which determined low surfactant than 20% for decrease irritation skin.

The physical properties of this base formulation were tested, and separation of nanoemulsion did not occur. It has a particle size of 150.9 nm which is in the range of nanoemulsion from 10 to 1,000 nm and has low PDI to lower the chance of separation. The zeta potential value may cause the nanoemulsion to be not stable because zeta potential must have a value less than -30 or more than +30. The base nanoemulsion was a liquid and may not have stuck to the skin. Therefore, xanthan gum was used as a viscosity additive.

Preparation of Benjakul nanoemulsion used 3% of BKE because of piperine content in this BKE less which a previous study found that 0.5% BKE was used in the preparation for the irritation test on skin. However, the percentage of BKE increase may have no effect on skin because it does not touch the skin directly, it is entrapped in an oil phase which is confirmed by cytotoxicity activity. Physio-chemical properties of BKE3-NE-T8-x0.2 was also investigated. The particle size increased from base nanoemulsion but it was within still properties range of nanoemulsion and had flow curve with non-Newtonian (pseudoplastic). BKE is continuously released from the nanoemulsion at a rate of 0.855 µg/min. This result indicated that BKE was dispersed in the nanoemulsion.

Anti-inflammatory activity of BKE3-NE-T8-x0.2 by inhibition of nitric oxide production induced by LPS in RAW264.7 cells showed IC_{50} value of 8.36±1.06 µg/ml. and found that the percentage of survival of all samples was higher than 70% at every concentration for cytotoxicity activity in HaCaT cells. Therefore, BKE3-NE-T8-x0.2 was not toxic to HaCaT cells and showed no potential for being a skin irritant. In addition, it could be used for further *in vivo* and clinical trials, since it has been shown to be safe for applied skin.

Stability test of BKE3-NE-T8-x0.2 showed that it is unstable because the components of BJK was decomposted or evaporated in the formulated method. The stability test result revealed surprisingly BKE3-NE-T8-x0.2 showed increasing anti-inflammatory activity indicating that it can be stored more than two year. Therefore, the study of the structure of ingredients in formulation before preparation of formulation would be studied because its results can help evaluated stability testing.

The product is stable when it was stored at room temperature and stored a long time without the loss of piperine content and anti-inflammation activity

These results can be concluded that BKE can be developed to BKE3-NE-T8-x0.2 because it has an anti-inflammatory effect and was not toxic to skin cells although its stability was doubtful. Further research could be included biological activities in other components, permeation of the formulation and irritation test in the animal model and normal volunteers should be studied. In addition, these results provide the knowledge base and could be used to be ingredient for further developing other products.

REFERENCES

Books and Book Articles

Itharat, A.(2010).Benjakul development project for use as a health supplement in cancer patients.

Cheng, Y., Xia, N. and Gilbert, M.G.. (1999). Piperaceae. In Z. Wu & P.H. Raven (Ed.) Flora of China Vol.4. Missouri Botanical Garden, St.Louis, Missouri.

Chuakul, W., Soonthornchareonnon, N. & Saralamp, P., (1999). Plumbago indica L. Plant Resources of South-East Asia No. 12(1): Medicinal and poisonous plants 1. Backhuys Publisher, Leiden, Netherlands.

Kakatum, N. (2011). "Anti-inflammatory Activity of Thai Traditional Remedy Extract for Muscle Pain Treatment Called Sahastara and Its Plant Ingredients". Master degree of science (Applied Thai Traditional Program) Thesis Faculty of Medicine Thammasat university.

Sookdee, P. (2017). Development of film-forming spray containing Sahasthara remedy extract for anti-inflammation. Master degree of science (Applied Thai Traditional Program) Thesis Faculty of Medicine Thammasat university.

Pichiensunthon, C. and Jeerawongs, V. (2004). Treditional Pharmacy Handbook Vol.5. Ammarin publisher, Bangkok.

Rattarom, R. (2013). Regulation of apoptosis on lung cancer cells through caspase-dependent mechanism by benjakul extract and its ingredients. Doctor of Philosophy (Medical Sciences). Thammast University.

Saralamp, P., Chuakul, W., Temsiririrlkul, R. & Clayton, T. (1996). Medicinal Plant in Thailand Volume I. Department of Pharmaceutical Botany, Faculty of Pharmacy, Mahidol University, Thailand.

Varangkhana Triyasut.(2015).The clinical safety on skin of Benjakul recipe and each lant component extract in healthy volunteers (clinical trial phase I). Master degree of science (Applied Thai Traditional Program) Thesis Faculty of Medicine Thammasat university.

WHO. (1999). Rhizoma Zingiberis. In WHO monographs on selected medicinal plants. (Vol.1, pp. 277-287). Geneva, World Health Organization.

Articles

Azeem, A., Rizwan, M., Ahmad, F. J., Iqbal, Z., Khar, R. K., Aqil, M., & Talegaonkar, S. (2009). Nanoemulsion components screening and selection: a technical note. Aaps Pharmscitech, 10(1), 69-76.

Gupta, A., Eral, H. B., Hatton, T. A., & Doyle, P. S. (2016). Nanoemulsions: formation, properties and applications. Soft matter, 12(11), 2826-2841.

Barel, A. O., Paye, M., & Maibach, H. I. (2014). Handbook of cosmetic science and technology. CRC Press.

Chaveerach, A., Mokkamul, P., Sudmoon, R., & Tanee, T. (2006). Ethnobotany of the genus Piper (Piperaceae) in Thailand.

Panapisal, V. (2016). Effects of surfactant mixture ratio and concentration on nanoemulsion physical stability. Thai Journal of Pharmaceutical Sciences (TJPS), 40.

Frondoza, C. G., Sohrabi, A., Polotsky, A., Phan, P. V., Hungerford, D. S., & Lindmark, L. (2004). An in vitro screening assay for inhibitors of proinflammatory mediators in herbal extracts using human synoviocyte cultures. In Vitro Cellular & Developmental Biology-Animal, 40(3-4), 95-101.

Sharif, H. R., Sharif, M. K., & Zhong, F. (2017). Preparation, characterization and rheological properties of vitamin E enriched nanoemulsion. Pakistan Journal of Food Sciences, 27(1), 7-14.

ItharatPhD, A. (2010). Determination of cytotoxic compounds of Thai traditional medicine called Benjakul using HPLC. Journal of Medical Association Thailand, 93(7), S198-S203.

Kim, J. H., Ko, J. A., Kim, J. T., Cha, D. S., Cho, J. H., Park, H. J., & Shin, G. H. (2014). Preparation of a capsaicin-loaded nanoemulsion for improving skin penetration. Journal of agricultural and food chemistry, 62(3), 725-732.

Bang, J. S., Choi, H. M., Sur, B. J., Lim, S. J., Kim, J. Y., Yang, H. I., ... & Kim, K. S. (2009). Anti-inflammatory and antiarthritic effects of piperine in human interleukin 1β-stimulated fibroblast-like synoviocytes and in rat arthritis models. Arthritis research & therapy, 11(2), R49.

Dal Mas, J., Zermiani, T., Thiesen, L. C., Silveira, J. L., da Silva, K. A., de Souza, M. M., ... & Lucinda-Silva, R. M. (2016). Nanoemulsion as a carrier to improve the topical anti-inflammatory activity of stem bark extract of Rapanea ferruginea. International journal of nanomedicine, 11, 4495.

Zhang, J. M., & An, J. (2007). Cytokines, inflammation and pain. International anesthesiology clinics, 45(2), 27.

Kumar, S., Singhal, V., Roshan, R., Sharma, A., Rembhotkar, G. W., & Ghosh, B. (2007). Piperine inhibits TNF-α induced adhesion of neutrophils to endothelial monolayer through suppression of NF-κB and IκB kinase activation. European journal of pharmacology, 575(1-3), 177-186.

Vasavirama, K., & Upender, M. (2014). Piperine: a valuable alkaloid from piper species. International Journal of Pharmacy and Pharmaceutical Sciences, 6(4), 34-8.

Gorgani, L., Mohammadi, M., Najafpour, G. D., & Nikzad, M. (2017). Piperine the bioactive compound of black pepper: from isolation to medicinal formulations. Comprehensive Reviews in Food Science and Food Safety, 16(1), 124-140.

Jaiswal, M., Dudhe, R., & Sharma, P. K. (2015). Nanoemulsion: an advanced mode of drug delivery system. 3 Biotech, 5(2), 123-127.

Antosova, M., Plevkova, J., Strapkova, A., & Buday, T. (2012). Nitric oxide important messenger in human body. Open Journal of Molecular and Integrative Physiology, 2(03), 98.

Acid, L. Final Report on the Safety Assessment of Oleic Acid, Laurie Acid, Palmitic Acid, Myristic Acid, and Stearic Acid.

Ali, M. S., Alam, M. S., Alam, N., & Siddiqui, M. R. (2014). Preparation, characterization and stability study of dutasteride loaded nanoemulsion for treatment of benign prostatic hypertrophy. Iranian journal of pharmaceutical research: IJPR, 13(4), 1125.

Charde, M. S., Kumar, J., Welankiwar, A. S., & Chakole, R. D. (2013). Review: Development of forced degradation studies of drugs. International Journal of Advances in Pharmaceutics, 2(3), 34-39.

Rachawat, P., Pinsornsak, P., Kanokkangsadal, P., & Itharat, A. (2017). Clinical Efficacy and Safety of Benjakul Remedy Extract for Treating Primary Osteoarthritis of Knee Compared with Diclofenac: Double Blind, Randomized Controlled Trial. Evidence-Based Complementary and Alternative Medicine, 2017.

Prow, T. W., Grice, J. E., Lin, L. L., Faye, R., Butler, M., Becker, W., ... & Roberts, M. S. (2011). Nanoparticles and microparticles for skin drug delivery. Advanced drug delivery reviews, 63(6), 470-491.

Raju, R., Sunny, A., Thomas, J. K., Abraham, L., & Thankapan, T. D. C. (2014). Isolation, characterization and in-vitro anti-inflammatory activity of *Plumbago indica* Linn. Advances in Pharmacology and Toxicology, 15(1), 13.

Asasutjarit, R., Lorenzen, S. I., Sirivichayakul, S., Ruxrungtham, K., Ruktanonchai, U., & Ritthidej, G. C. (2007). Effect of solid lipid nanoparticles formulation compositions on their size, zeta potential and potential for in vitro pHIS-HIV-hugag transfection. Pharmaceutical research, 24(6), 1098-1107.

Umar, S., Sarwar, A. H. M. G., Umar, K., Ahmad, N., Sajad, M., Ahmad, S., ... & Khan, H. A. (2013). Piperine ameliorates oxidative stress, inflammation and histological outcome in collagen induced arthritis. Cellular Immunology, 284(1-2), 51-59.

Habib, S., & Ali, A. (2011). Biochemistry of nitric oxide. Indian journal of clinical biochemistry, 26(1), 3-17.

Sireeratawong, S., Itharat, A., Lerdvuthisopon, N., Piyabhan, P., Khonsung, P., Boonraeng, S., & Jaijoy, K. (2012). Anti-Inflammatory, Analgesic, and Antipyretic Activities of the Ethanol Extract of *Piper interruptum* Opiz. and *Piper chaba* Linn. International Scholarly Research Notices: Pharmacology.

de Morais, S. M., do Nascimento, J. E. T., de Sousa Silva, A. A., Junior, J. E. R. H., Pinheiro, D. C. S. N., & de Oliveira, R. V. (2017). Fatty Acid Profile and Anti-Inflammatory Activity of Fixed Plant Oils. Acta Scientiae Veterinariae, 45, 1-8.

Vannasiri, S., Sritiwong, S., & Jaijoy, K. (2010). Anti-Inflammatory, anti-nociceptive and antipyretic effects of the ethanol extract from root of *Piper sarmentosum* Roxb. Journal of Medical Association Thailand, 93(7), S1-S6.

Priya, S., Koland, M., & Kumari, S. (2015). Nanoemulsion components screening of quetiapine fumarate: effect of surfactant and co surfactant. Asian J Pharm Clin Res, 8(6), 136-40.Stöhr, J.R., Xiao, P.G. & Bauer, R. Isobutylamides and a New Methylbutylamide from *Piper sarmentosum*. Planta Medica.1999; 65(2), 175-177.

Makchuchit, S., Rattarom, R., & Itharat, A. (2017). The anti-allergic and anti-inflammatory effects of Benjakul extract (a Thai traditional medicine), its constituent plants and its some pure constituents using in vitro experiments. Biomedicine & Pharmacotherapy, 89, 1018-1026.

Tadros, T., Izquierdo, P., Esquena, J., & Solans, C. (2004). Formation and stability of nano-emulsions. Advances in colloid and interface science, 108, 303-318.

Tewtrakul, S., & Itharat, A. (2007). Nitric oxide inhibitory substances from the rhizomes of Dioscorea membranacea. Journal of ethnopharmacology, 109(3), 412-416.

Tripathi, S., Maier, K. G., Bruch, D., & Kittur, D. S. (2007). Effect of 6-gingerol on pro-inflammatory cytokine production and costimulatory molecule expression in murine peritoneal macrophages. Journal of Surgical Research, 138(2), 209-213.

U.S. Department of Health and Human Services Food and Drug Administration. (2003) Guidance for Industry Q1A(R2) Stability Testing of New Drug Substances and Products.

Arzumanian, V., Stankevicius, E., Laukeviciene, A., & Kevelaitis, E. (2003). Mechanisms of nitric oxide synthesis and action in cells. Medicina (Kaunas), 39(6), 535-41.

Qin, W., Huang, S., Li, C., Chen, S., & Peng, Z. (2010). Biological activity of the essential oil from the leaves of *Piper sarmentosum* Roxb.(Piperaceae) and its chemical constituents on Brontispa longissima (Gestro)(Coleoptera: Hispidae). Pesticide biochemistry and physiology, 96(3), 132-139.

Ridtitid, W., Ruangsang, P., Reanmongkol, W., & Wongnawa, M. (2007). Studies of the anti-inflammatory and antipyretic activities of the methanolic extract of *Piper sarmentosum* Roxb. leaves in rats. Songklanakarin Journal of Science & Technology, 29(6).

Ying, X., Yu, K., Chen, X., Chen, H., Hong, J., Cheng, S., & Peng, L. (2013). Piperine inhibits LPS induced expression of inflammatory mediators in RAW 264.7 cells. Cellular immunology, 285(1-2), 49-54.

Damanhouri, Z. A., & Ahmad, A. (2014). A review on therapeutic potential of *Piper nigrum* L. Black Pepper): The King of Spices. Medicinal and Aromatic Plants, 3(161), 2167-0412.

Schroeder, I. Z., Franke, P., Schaefer, U. F., & Lehr, C. M. (2007). Delivery of ethinylestradiol from film forming polymeric solutions across human epidermis in vitro and in vivo in pigs. Journal of controlled release, 118(2), 196-203.

Electronic Media

Acumedia Manufacturers, 2017; 7992 Tween 80, 100ml. [cited 2018 November 26]. Available from:https://foodsafety.neogen.com/pdf/acumedia_pi/7992_pi.pdf.

Malvern Panalytical Ltd, Measurement of Particle size [cited 2018 August 23]. Available from: https://www.malvernpanalytical.com/en/products/measurement-type/particle-size/?gclid=CjwKCAjw8O7bBRB0EiwAfbrTh2ENGQNe1bJceks73n0jRlVKOvUlKZMRav73Jbr7XxkAiUO9PtIBhoCHcMQAvDBwE.

Medicinal plants of Bangladesh; Traditional used of Piper retrofractum Vahl. [cited 2017 February 13]. Available from: http://www.mpbd.info/plants/piper-retrofractum.php.

MyScope training for advanced research, 2012; Transmission Electron Microscope Training module [cited 2018 August 23]. Available from: http://www.ammrf.org.au/myscope/tem/practice/

NANOCOMPOSIX, September 2012, V1.1; TRANSMISSION ELECTRON MICROSCOPY ANALYSIS OF NANOPARTICLES [cited 2018 August 23]. Available from: http://50.87.149.212/sites/default/files/nanoComposix%20Guidelines%20for%20TEM%20Analysis.pdf.

Nguyen ba trading and manufacturing co Ltd, Technical data sheet Myritol 318 (capric/caprylic Triglyceride [cited 2018 November 26]. Available from: http://nguyenbachemical.com/file/MYRITOL318.pdf.

RIC NEWSLETTER of Khon Kaen University, September 2014, NO.2; Transmission Electron Microscope [cited 2017 December 9]. Available from: http://mdresearch.kku.ac.th/files/news/filesnews/RIC_SEP_2014.pdf.

Sayunt and wirat, Physics and Engineering, 2006; Specific viscosity of fluid [cited 2018 August 23]. Available from: http://www.dss.go.th/images/st-article/pep_9_2549_viscosity.pdf.

SIDS Initial Assessment Report, 2001; OECD SIDS 1,2-DIHYDROXYPROPANE[cited 2018 November 26]. Available from: ttp://www.inchem.org/documents/sids/sids/57-55-6.pdf.

Unicorn Petroleum Industries Pvt.Ltd.; ISOPROPYL MYRISTATE (IPM) [cited 2018 November 26]. Available from: http://asia.in-osmetics.com/__novadocuments/61662?v=635464816886330000.

Thai drug control division. Thailand: MOPH; The value of medicine imports in 2012 sorted by pharmacological class [cited 2017 February 13]. Available from: http://www.fda.moph.go.th/sites/drug/download/1-2-2553.pdf.

APPENDICES

APPENDIX A

CHEMICAL REAGENTS

1. Reagents for cell cuture

1.1 RPMI 1640 (incomplete media)

RPMI 1640 with L-glutamine	10.43 g
$NaHCO_3$	2 g
Ultra-pure water	1000 ml

Adjust pH to 7.2-7.4 with 10% NaOH or 1% HCl

Filter through sterile membrane at a pore size of 0.2 micron

(Stored at 4 $^{\circ}$C)

1.2 RPMI 1640 (complete media)

RPMI 1640 (incompelete media)	1000 ml
FBS	100 ml
Penicillin-Sreptomycin	10 ml

(Stored at 4 $^{\circ}$C)

1.3 DMEM (incomplete media)

RPMI 1640 with L-glutamine	g
$NaHCO_3$	3.2 g
Ultra-pure water	1000 ml

Adjust pH to 7.2-7.4 with 10% NaOH or 1% HCl

Filter through sterile membrane at a pore size of 0.2 micron

(Stored at 4 $^{\circ}$C)

1.4 DMEM (complete media)

DMEM (incompelete media)	1000 ml
FBS	100 ml
Penicillin-Sreptomycin	10 ml

(Stored at 4 °C)

1.5 PBS (Phosphate buffer saline)

PBS	1 Tablet
Distilled water	100 ml

(Stored at 4 °C)

1.6 10% Sodium hydroxide (NaOH)

NaOH	10 g
Distilled water to	100 ml

1.7 10% Hydrochloric acid (HCl)

Conc. HCl (37%)	27 ml
Distilled water to	100 ml

1.8 FBS (Fetal bovine serum)

Slowly thaw the FBS (inactivate), heat 56 °C, 60 mins

(Aliquot, Stored at -20 °C)

1.9 P/S (Penicillin/Sreptomycin)

Slowly thaw the frozen P/S in water bath at 37 °C till completely thawed

(Aliquot, Stored at -20 °C)

1.10 Trypsin-EDTA

Slowly thaw the frozen 0.5% trypsin-EDTA, in water bath at 37 °C till completely thawed (Aliquot, Stored at -20 °C)

2. Reagents for determination Nitric Oxide

2.1 Griess reagent

Sulfanilamide	1.0 g
N-(1-Naphthyl)ethylenediamine dihydrochloride	0.1 g
Phosphoric acid	2.5 g
Adjust volume with MQ water to	100 ml

(Stored at 4 °C)

2.2 MTT solution (5 mg/ml)

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-*2H*-tetrazolium bromine or Thiazolyl blue tetrazolium bromine	200 mg
PBS	40 ml

(The reagent was protected from light wrapped in foil and stored at 4 °C)

2.3 0.04 M HCl in Isopropanol

Conc.HCl	0.83 mg
Adjust volume with Isopropanol to	250 ml

3. Reagents for determination cell viability

3.1 MTT solution (5 mg/ml)

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-*2H*-tetrazolium bromine or Thiazolyl blue tetrazolium bromine	200 mg
PBS	40 ml

(The reagent was protected from light wrapped in foil and stored at 4 °C)

3.2 DMSO (Dimethyl Sulfoxide)

Dimethyl Sulfoxide	10 ml

(The reagent was protected from light wrapped in foil and stored at 37 °C)

APPENDIX B

ยาเบญจกูล

ยาแคปซูล ยาผง ยาเม็ด ยาลูกกลอน ยาแคปซูล (รพ.) ยาชง (รพ.) ยาเม็ด (รพ.)

สูตรตำรับ

ในผงยา 100 กรัม ประกอบด้วย ดอกดปีลี รากช้าพลู เถาสะค้าน รากเจตมูลเพลิงแดง เหง้าขิงแห้ง หนักสิ่งละ 20 กรัม

คำแนะนำ บรรเทาอาการท้องอดี ท้องเฟ้อ

ขนาดและวิธีใช้

- ชนิดชง รับประทานครั้งละ 1.5–2 กรัม วันละ 3 ครั้ง หลังอาหาร
- ชนิดผง รับประทานครั้งละ 800 มิลลิกรัม–1 กรัม วันละ 3 ครั้ง หลังอาหาร
- ชนิดลูกกลอน ชนิดเม็ด และชนิดแคปซูล รับประทานครั้งละ 800 มิลลิกรัม–1 กรัม วันละ 3 ครั้ง หลังอาหาร

ข้อห้ามใช้

ห้ามใช้ในหญิงตั้งครรภ์ผู้ที่มีไข้และเด็กเล็ก

คำเตือน

- ไม่ควรใช้ยานี้ในฤดูร้อน เนื่องจากอาจทำให้ไฟธาตุกำเริบ
- ไม่ควรรับประทานติดต่อ กันนานเกิน 7 วัน

BIOGRAPHY

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Date of Birth	August09, 1990
Educational Attainment	2012: Naresuan University
	Bachelor of Applied Thai Tranditional Medicine

Publication

Thummawan C, Itharat A, Asasutjarit R. (2019). Anti-inflammatory activities of nanoemulsion containing Benjakul remedy extract. Thammasat Medical Journal, 19 (Suppl 2).

Oral Presentation

Thummawan C, Itharat A, Asasutjarit R. Stability study of Benjakul nanoemulsion product for anti-inflammation. Advancing care for our Beloved Elderly, Faculty of Medicine, Thammasat University, Thailand. July 16-18, 2018.