Development of nanostructured lipid carrier clove oil as anesthetic agent in white shrimp (*Litopenaeus vannamei*)

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การพัฒนาน้ำมันกานพลูในรูปอนุภาคนาโนชนิดที่มีโครงสร้างไขมันเป็นตัวพาเพื่อใช้เป็นยาสลบ
สำหรับกุ้งขาวแวนนาไม

น.ส.สมฤดี แก้วมาลุน

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สมฤดี แก้วมาลุน : การพัฒนาน้ำมันกานพลูในรูปอนุภาคนาโนชนิดที่มีโครงสร้างไขมันเป็นตัวพาเพื่อใช้เป็นยาสลบ สำหรับกุ้งขาวแวนนาไม. ( Development of nanostructured lipid carrier clove oil as anesthetic agent in white shrimp (*Litopenaeus vannamei*)) อ.ที่ปรึกษาหลัก : รศ.น.สพ. ดร.นพดล พิหารัตน์, อ.ที่ปรึกษาร่วม : อ. ดร.ธีรพงศ์ ยะทา

น้ำมันกานพลูเป็นน้ำมันหอมระเหยที่สกัดได้จากดอกตูมแห้งและใบของต้นกานพลู (*Syzygium aromaticum*) น้ำมัน กานพลูมีส่วนประกอบหลักที่สำคัญคืออยูจีนอล ฤทธิ์ทางชีวภาพของน้ำมันกานพลูทำให้สัตว์น้ำมีอาการเชื่องซึม สงบ จนถึงทำให้สัตว์ ซึมลึกและสลบซึ่งเป็นประโยชน์ในการจัดการสัตว์น้ำ เช่น ลดการเคลื่อนไหว, ลดความเครียด, ลดอัตราการตายและลดพฤติกรรม การกินกันเองของสิ่งมีชีวิตในระหว่างการจัดการและขนส่งสัตว์น้ำ อย่างไรก็ตามการใช้น้ำมันกานพลูต้องนำไปละลายในเอทานอล ก่อนทุกครั้งเนื่องจากน้ำมันกานพลูไม่ละลายในน้ำ ดังนั้นการใช้เอทานอลในการละลายน้ำมันกานพลูก่อให้เกิดการระคายเคืองและ ความเป็นพิษต่อสัตว์น้ำและผู้ใช้ ในการศึกษาครั้งนี้เพื่อหลีกเลี่ยงผลข้างเคียงดังกล่าว เราได้พัฒนาน้ำมันกานพลูในรูปอนุภาคนาโน ชนิดที่มีโครงสร้างไขมันเป็นตัวพาเพื่อใช้เป็นยาสลบ โดยไม่ใช้เอทานอลในกระบวนการเตรียมเพื่อเพิ่มความสามารถในการละลายน้ำ , เพิ่มประสิทธิภาพในการออกฤทธิ์ที่ดีขึ้น, เพิ่มความคงตัวและลดความเป็นพิษของน้ำมันกานพลูเมื่อเทียบกับสารมาตรฐานน้ำมัน กานพลู โดยในการศึกษาครั้งนี้เราได้ทำการศึกษาลักษณะรูปร่าง คุณสมบัติทางเคมีกายภาพ ประสิทธิภาพการออกฤทธิ์ ความเป็น พิษและการกระจายตัวของน้ำมันกานพลูในรูปอนุภาคนาโนไขมัน ผลการศึกษาพบว่า clove oil NLCs มีขนาดอนุภาค 175.07±0.72 nm ค่าประจุ -48.37±.38 ค่าดัชนีการกระจายตัวของอนุภาคอยู่ที่ 0.115±0.230 อนุภาคมีรูปร่างกลม ประสิทธิภาพ การกักเก็บร้อยละ 88.55 มีความสามารถในการควบคุมการปลดปล่อยสารสำคัญของน้ำมันกานพลู และมีความคงตัวที่ดี หลังจาก นั้นนำ clove oil NLCs ที่เตรียมได้ทำการทดลองประสิทธิภาพการออกฤทธิ์การสลบในกุ้งขนาด 3 กรัมที่ความเข้มข้น 10, 20, 30, 40, 50, 60, 70, 100, 120, 140, 160, 180 and 200 ppm เปรียบเทียบกับกลุ่มสารมาตรฐานน้ำมันกานพลู ผลพบว่าที่ความ เข้มข้น 10 ppm ทั้งในกลุ่ม clove oil NLCs และ STD clove oil ไม่มีฤทธิ์ในการสลบ กุ้งเริ่มเข้าสู่การสลบในระยะที่ 2 (Complete loss of equilibrium) ในความเข้มข้นที่ 30 ppm โดยที่ความเข้มข้น 30 ppm เหมาะสมที่ใช้ในการสลบกุ้ง ซึ่ง สามารถทำให้กุ้งสลบในระยะที่ 2 ภายในเวลา 5 นาที หลังจากสัมผัสกับน้ำมันกานพลูในกลุ่ม clove oil NLCs แต่กุ้งในกลุ่ม STD clove oil เริ่มเข้าสู่การสลบในระยะที่ 2 ภายในเวลา 5 นาทีที่ความเข้มข้น 50 ppm การศึกษาความปลอดภัยของน้ำมันกานพลูใน กลุ่ม clove oil NLCs และ STD clove oil ต่อสัตว์น้ำโดยการประเมินความเป็นพิษของน้ำมันกานพลูในกุ้งที่ความเข้มข้นต่างๆ เป็นเวลา 48 ชั่วโมง พบว่าระดับความเข้มข้นของน้ำมันกานพลูที่ทำให้กุ้งตายครึ่งหนึ่งของจำนวนกุ้งที่ใช้ทดสอบ ที่ความเข้มข้น 140 ppm ในกลุ่มของ STD clove oil ในขณะที่กลุ่ม clove oil NLCs ที่ความเข้มข้น 140 ppm ไม่มีอัตราการตายและไม่มีความเป็น พิษเกิดขึ้น และผลการศึกษาการกระจายตัวของอนุภาคในตัวกุ้งและระยะเวลาการขับออก พบว่ากุ้งในกลุ่ม clove oil NLCs สามารถขับน้ำมันกานพลูออกจากร่างกายได้ภายในเวลา 30 นาที ในขณะเดียวกันกุ้งในกลุ่ม STD clove oil ยังสามารถตรวจพบ น้ำมันกานพลูในตัวกุ้ง ซึ่งจากผลการทดลองจะแสดงให้เห็นว่า clove oil NLCsใช้เป็นยาสลบสำหรับกุ้งขาวแวนนาไมได้อย่างมี ประสิทธิภาพ สามารถเหนี่ยวนำกุ้งเข้าสู่การสลบในระยะที่ 2 ได้เร็วกว่าและยังใช้ในปริมาณความเข้มข้นของสารที่น้อยกว่า ไม่มี ความเป็นพิษต่อสัตว์น้ำและสามารถขับออกได้เร็วกว่าเมื่อเปรียบเทียบกับ STD clove oil.

| สาขาวิชา | วิทยาศาสตร์ทางการสัตวแพทย์และเทคโนโลยี | ลายมือชื่อนิสิต ............................................... |
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| ปีการศึกษา | 2563 | ลายมือชื่อ อ.ที่ปรึกษาหลัก ............................. |
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Somrudee Kaewmalun : Development of nanostructured lipid carrier clove oil as anesthetic agent in white shrimp (*Litopenaeus vannamei*). Advisor: Assoc. Prof. Dr. NAPADON PIRARAT Co-advisor: Instructor Dr. TEERAPONG YATA

Clove oil is an essential oil extracted from the dried buds and leaves of the clove plant (Syzygium aromaticum). Clove oil contains eugenol as the main active ingredient. Clove oil used as an anesthesia functions as calming effect, loss of equilibrium, non-reactivity and consciousness in lead to reduce activity, physiological stress, mortality rate, and preventing cannibalism method during handling and transportation. However, the component of clove oil is insoluble in water and must be dissolved in ethanol. So, clove oil ethanolic solution leading to toxic and irritation in animal and user. In this study, to avoid this side effect, the anesthetic preparation of clove oil without ethanol is an attempt to be developed. We have prepared a new generation of clove oil in the form of NLCs to enhance water miscibility, high efficacy, better physical stability, and low toxicity compared with STD clove oil. The clove oil NLCs obtained were characterized in terms of physicochemical, efficacy, toxicity and biodistribution of clove oil NLCs. The results showed that clove oil NLCs had particle size of 175.07 ± 0.72 nm, zeta potential of -48.37 ± .38, PDI of 0.115 ± 0.230. Clove oil NLCs have spherical particle shape, %encapsulation efficiency of clove oil NPs was 88.55% and demonstrated sustained release of active ingredients. The anesthetic effects of clove oil were studied in white shrimp (average 3 g). Clove oil concentration at 10, 20, 30, 40, 50, 60, 70, 100, 120, 140, 160, 180 and 200 ppm of clove oil NLCs and STD clove oil were used in study. The results showed no effect of clove oil to white shrimp at 10 ppm of both groups. After exposure to clove oil NLCs at 30 ppm, showed the shrimp can be started to stage 2 (Complete loss of equilibrium) while shrimp of STD clove oil group be started to stage 2 within 5 minutes at concentrations of 50 ppm. The optimum concentration of anesthetic was 30 ppm in shrimp which induced shrimp to stage 2 within 5 minutes after exposure to Clove oil NLCs. The toxicity of clove oil to different concentration of clove oil was studied and median lethal concentration (LC50) at 48 h were report in toxicity study. The result show that the STD clove oil concentration of 140 ppm resulted in median lethal concentration (LC50 48h), whereas the concentration of 140 ppm of clove NLCs led to a 0% mortality over a 48-h period. And the result of biodistribution study of clove, it was found that shrimp in clove oil NLCs group was able to excrete clove oil from the body within 30 minutes but shrimp in STD clove oil group was still detected clove oil in shrimp. In conclusion, the clove oil NLCs has been successfully developed with enhanced water miscibility, increased high anesthetic efficacy with a sustained release, reduction of clove oil toxicity and clove oil NLCs do not accumulate on the shrimp body.

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| Field of Study: | Veterinary Science and technology | Student's Signature ............................... |
| Academic Year: | 2020 | Advisor's Signature ............................. |
| | | Co-advisor's Signature ........................ |

## ACKNOWLEDGEMENTS

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จุฬาลงกรณ์มหาวิทยาลัย

CHULALONGKORN UNIVERSITY

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# CHAPTER I

# INTRODUCTION

## Importance and rationale

The whiteleg shrimp *Penaeus vannamei* is considered as one of commercially important species in aquaculture. A key factor in improving the quality of products is animal welfare in shrimp farming. Improved animal welfare also plays an important role in determining the social acceptability of an animal production system. In fact, several procedures in shrimp farming such as capture, handling, and transportation can cause stress in animals. Following behavioural physiological changes, shrimp production may be compromised as a result from physical damage.

Alternatively, the effect of stress in animals during these procedures could be reduced by using anaesthetics. Clove oil has been exploited as an appropriate anaesthetic for aquatic animals because of its natural agents obtained from the clove tree *Syzygium aromaticum*, low costs, high efficacy, no mortality nor adverse side effects (Javahery et al., 2012). In work published from our group (Yostawonkul et al., 2019), we have successfully developed and evaluated an efficient, cost-effective, safe, and easy-to-use version of clove essential oil by converting the poorly water-soluble clove oil into nanoemulsions used for safe anaesthesia in tilapia.

In fact, there are three major types of lipid nanoparticles; nanoemulsion, solid lipid nanoparticles (SLNs), and nanostructured lipid carriers (NLCs). Nanoemulsion is the first version of lipid-based nanoparticles. SLNs and NLCs are the modified versions of nanoemulsions. Of which, NLCs have proven to be superior over other lipid-based carrier systems (Amandeep et al., 2019).

Recently, our group have proposed a new generation of clove oil in the form of NLCs. Our preliminary study showed that this newly generated formulation is superior to the previous version of clove oil nanoparticles in the form of nanoemulsions. Interestingly, this new version is more satisfied by users and offers better physical stability and more efficient dispersion in water. (Personal communication) In our *in vivo* pilot study, clove oil in the form of NLCs induced

anaesthesia in tilapia more rapidly at lower concentrations than clove oil nanoemulsions. However, this new version has not been physico-chemically characterized and evaluated for use in shrimp. Additional research is necessary to conduct in order to determine optimal time and dose responses as well as the in vivo toxicity. These fundamental knowledges are required to be evaluated and monitored before moving forward to the practical implementation.

In this study, we were primarily focused on the physico-chemically characterization as well as *in vitro* and *in vivo* biological characterization of our improved version of clove oil nanoparticles 2 in the form of NLCs. The whiteleg shrimp was selected as a target animal species. Specifically, we studied for concentration optimization and time evaluation for induction and recovery of clove oil nanoparticles-treated animals. We also to investigate the toxicity and the biodistribution of clove oil nanoparticles in shrimp body.
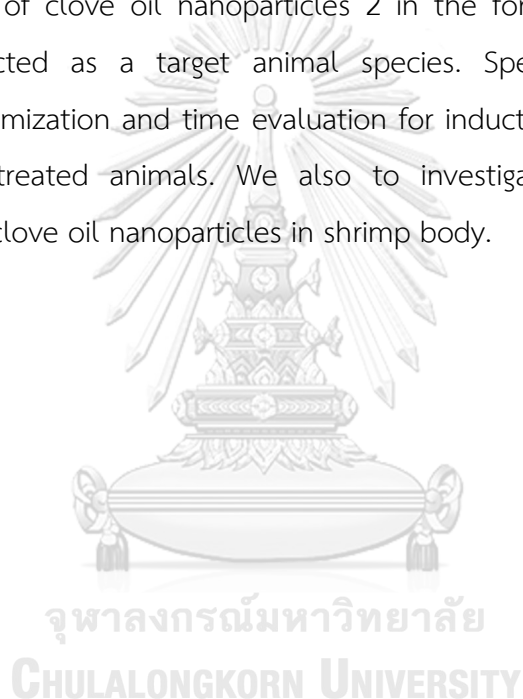
## Objectives

1. To formulate and physico-chemically characterize the newly generated version of clove oil in the form of NLCs.

2. To evaluate the appropriate concentration of clove oil NLCs for induction and recovery Time.

3. To investigate the biodistribution of the newly generated formulation of clove oil NLCs in shrimps' body.

4. To assess the toxicity of the newly generated version clove oil NLCs.

## Hypotheses

1. A newly generated formulation of clove oil in the form of NLCs can be developed and used as an aquatic anaesthesia agent in shrimps.

2. Clove oil NLCs can be absorbed, distributed and metabolized in shrimps' body after immersion.

3. Clove oil NLCs has lower toxicity in comparison with standard clove oil.

## Advantages of Study

1. This study will be an improved version of clove oil in the form of NLCs can be developed and used as an aquatic anaesthesia agent in shrimps.

2. This study showed clove oil NLCs can be absorbed, distributed and metabolized in shrimps' body after immersion.

3. This study showed Nanodelivery system to improve the efficacy of clove oil NLCs for anaesthesia and has low toxicity.
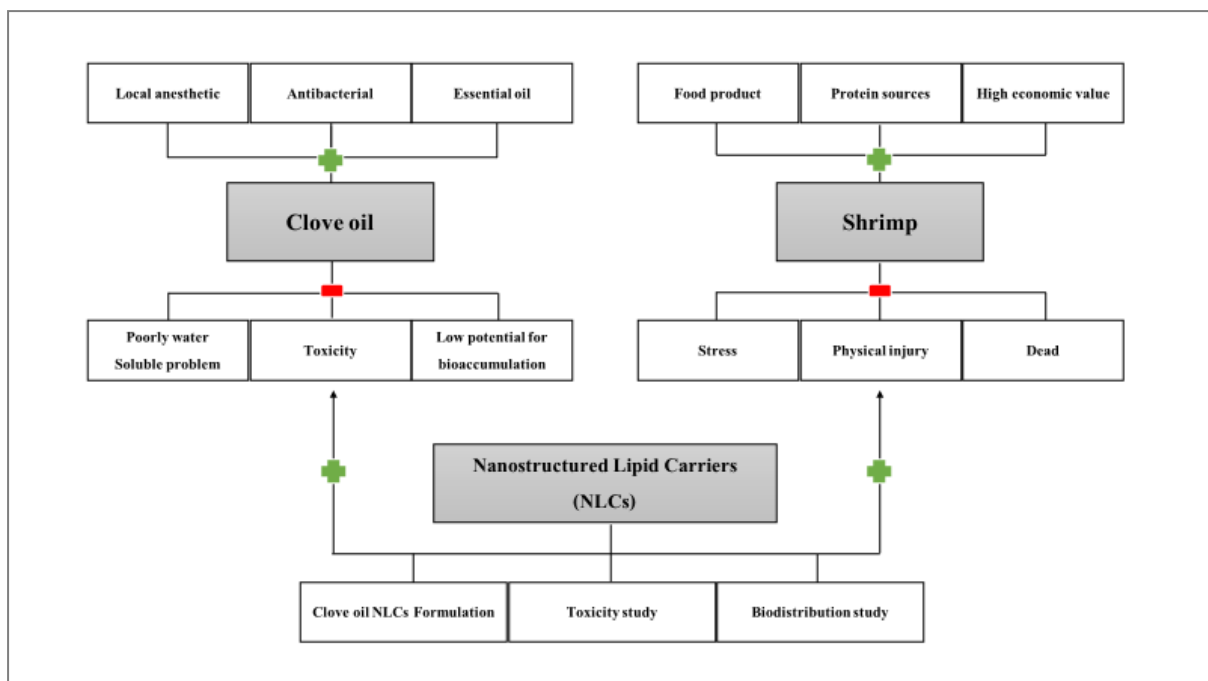
**Figure 1 :** Conceptual framework

## CHAPTER II

## LITERATURE REVIEW

### 2.1 Anaesthetics in Aquaculture

Anaesthesia functions as a pain killer in aquatic animals, resulting in a calming effect, loss of equilibrium, non-reactivity and consciousness, all of which are depended on dose of administrations (Summerfelt, 1990). In aquaculture, anaesthetics are used in several procedures in order to decrease metabolism and prevent physical injury. Ideal anaesthetics should be easy to handle, affordable and exhibits the effects immediately after being soluble in water. Fast anaesthesia should be occurred after 1-5 minutes and fast recovery should be less than 5 minutes. Another important characteristic of anaesthetic is quick excretion or metabolization without or low residue as well as zero or short withdrawal period. More importantly, anaesthetic agents should be non-toxic to animal, users and environment (Coyle et al., 2004). Anaesthetics can be derived from by various agents, namely quinaldine sulphate, tricaine methanesulphonate (also known as MS-222), phenoxyethanol and benzocaine. Unfortunately, these chemical anaesthetic agents are relatively toxic to animals and environment and more costly than natural agents. A specific withdrawal time is required for these synthetic anaesthetic agents before the animal can be consumed or released into the environment (FDA, 2007), such as MS-222 approved for use in fish in the United States, but there must still be a period of discontinuation of the drug for at least 21 days before releasing the fish to natural water sources or consuming due to chemicals causing accumulation in aquatic animals and consumers (Bowker et al., 2011). Interestingly, clove oil derived from the clove tree *Syzygium aromaticum* has long been proposed to be another best of choice as safe anaesthetic agent for aquatic animals (Woody et al., 2002).

## 2.2 Anaesthesia of aquatic invertebrates

Less is known about invertebrate anaesthetics. Procedures in crustacean culture are generally performed without anaesthesia despite the fact that fast and uncontrollable movement of shrimp can cause handling problems as well as cannibalistic behaviour during holding and transporting. Therefore, there has been numerous investigations searching for safe and effective anaesthetics for crustacean in particular for transportation. Response to anaesthesia are different between than crustaceans and fish. One possible explanation is their certain types of synaptic receptor. For example, many crustaceans do not respond to MS-222. As compared to fish, significantly higher concentrations are required to induce anaesthesia in crustaceans. For most crustaceans, carbon dioxide is an effective anaesthetic. Another approach to immobilize crustaceans is cooling despite the high mortality rate (Coyle et al., 2004). Another previously reported anaesthetic agents for freshwater prawns (*Macrobrachium osenbergii*) is Aqui-S. However, higher concentrations (100 to 200 mg /L) are required as compared to those used on finfish (20 mg / L) (Coyle et al., 2005).

## 2.3 Clove oil



**Figure 2 :** Extraction of eugenol from clove is a pale-yellow liquid with warm.

Clove oil obtained from the *Eugenia caryophyllata* tree stem, leaves, flowers and buds. That is considered a potential anaesthetic for fish (Woody et al., 2002). The utilization of clove oil is ideas as characterized by fast induction, sustained recovery, and low mortality rate as compared to many chemical anaesthetics. Clove oil is mainly composed of eugenol, methyleugenol and eugenyl acetate. The active ingredient is eugenol which is poorly water soluble. It is therefore necessary to dilute the product with alcohols (Grush et al., 2004). Therefore, clove oil in ethanol leading to toxic and irritation in animal and user. After oral administration, absorption and metabolization of eugenol occurs rapidly while excretion can be achieved by urine within 24 h, without negative side effects in fish (Fischer et al., 1990). According to (Akbulut et al., 2011), clove oil and eugenol have advantages over other anaesthetics due to their high effectiveness, cost-effective and low toxicity to fish and some invertebrates. Eugenol has there also proposed as a safe aesthetic agent for laboratory use (Liu and Gibson, 1977).
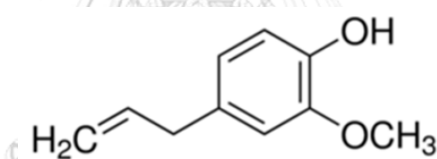


**Figure  3 :** Structures of eugenol, major components of clove oil

The US Food and Drug Administration (FDA) also approves clove oil as a mild topical anaesthetic or analgesic as well as a food additive for human use (Alqareer et al., 2006; FDA, 2007). However, some negative side effects associated with the toxicity of eugenol could be observed such as ventilator failure and medullary collapse (Sladky et al., 2001). A number of previous studies suggested that the harmful effects of clove oil on aquatic animals is associated with high concentration and the length of anaesthesia time. It is also suggested a reduction in coral growth and bleaching were also observed after repeated administration of the clove oil (Boyer et al., 2009).

Interestingly, clove oil could serve as an important natural antibacterial agent used in various applications, including dentistry, pharmaceuticals, and aromatherapy.

It has been also used as an analgesic, antiseptic, warming, disinfectant, antibacterial, and local anaesthetic (Akbari et al., 2010; Li et al., 2018; Norton et al., 1996) has suggested that clove oil is an efficient anaesthetic agent for Grass shrimp (*Palaemonetes sinensis*) (Wycoff et al., 2018) demonstrated the feasibility of clove oil as a transient anaesthetic agent for crustaceans.

## 2.4 Toxicity and pharmacokinetics of clove oil

Clove oil is generally recognized as a safe substance when consumed in lower concentrations, not over 1,500 mg/kg. On the other hand, the World Health Organization (WHO) has determined that the daily amount in clove oil per day acceptable is 2.5 mg/kg in weight in humans (Gülçin et al., 2012). Clove oil can also function as anaesthesia for a variety of fish. However, long exposures can lead to mortality and sub-acute morbidity (Javahery et al., 2012).

When administered orally, eugenol is readily absorbed and rapidly reaches plasma and blood with a mean half-life of 14.0 h and 18.3 h, respectively. After repeated daily administration, a cumulative effect was hypothesized and associated with relief of neuropathic pain (Guénette et al., 2007).

## 2.5 Nanotechnology-based drug delivery system

The use of nanotechnology-based drug delivery system has been proposed as a promising strategy for controlled and prolonged release as well as precise delivery of active ingredients including drugs, vaccines, and biopharmaceuticals. Typically, there are two basic types of nanoparticles based on their chemical nature: organic and inorganic (Degli Esposti et al., 2018). Organic nanoparticles are prepared from various biomolecules and biomaterials. In contrast, inorganic nanoparticles are prepared with inorganic elements. Nanoparticles of gold, mesoporous silica, magnetic iron oxide, and quantum dots are examples of inorganic nanoparticles. Of the organic nanoparticles, lipid nanoparticles and polymeric nanoparticles are the most widely used. Both organic and inorganic nanoparticles are of interest in life sciences in particular as biocompatible and biodegradable materials. The characteristics of organic and inorganic nanoparticles are different, and these characteristics determine

how the nanoparticles are exploited. Organic nanoparticles have been exploited as safe drug delivery systems due to their well-established biodegradability and biocompatibility. In contrast, inorganic nanoparticles are being actively developed for nanotechnology-based biosensors and diagnostics due to their optical and magnetic properties (Vashist et al., 2012).

## 2.6 Lipid nanoparticles

Lipid nanoparticles have the ability to encapsulate water-insoluble substances and facilitate them well-dispersed in an aqueous solution. Therefore, lipid nanoparticles have attracted special interest as an effective strategy for the delivery of poorly water soluble or insoluble active ingredients. Three major types of lipid nanoparticles are nanoemulsion, solid lipid nanoparticles (SLNs), and nanostructured lipid carriers (NLCs). Nanoemulsion is the first generation of lipid nanoparticles comprised of a liquid lipid and considered to be less effective than the latter two systems. SLNs (the second generation of lipid nanoparticles) were developed to circumvent the disadvantages of nanoemulsions because they have improved release kinetics of encapsulated drug with good physical stability. In contrast to nanoemulsions, SLNs are synthesized from a solid lipid, whereas the NLCs are formulated by using both solid and liquid lipids. NLC are the modified version of nanoemulsions and SLNs and thus regarded as the third generation of lipid-based nanoparticles. NLC are a nanoparticulate colloidal system in which partial-crystallized particles are stably formed and dispersed in an aqueous matrix (Tamjidi et al., 2013). Due to their high drug loading, entrapment efficiency and stability, NLC have recently proposed to be superior to other lipid-based carrier systems in delivering pharmaceutical agents. Bioavailability and stability of bioactive compounds-encapsulated NLCs can be enhanced, providing controlled release of encapsulated drugs (Pardeike et al., 2009; Teeranachaideekul et al., 2007).

## 2.7 Advantages of Nanoparticles.

Nanotechnology-based drug delivery system represents a new and promising strategy for improving multi-performance characteristics of conventional drugs with

several improved pharmaceutical actions. To date, numerous publications have reported various aspects of nanoparticle-mediated drug delivery systems. Nanoscale drugs are superior to free drugs, including encapsulation, sustained, controlled or triggered release, and selective targeting to diseased sites, thus improving the pharmacokinetic behavior and target ability of drugs. Encapsulation is defined as "the creation of a structure built around the drug" so that they are completely covered to prevent exposure of the encapsulated drugs to off-target tissues (Kumari et al., 2014).

This offer a particular advantage for toxic substances such as chemotherapeutic drugs, where off-target toxicity is a key limitation. The controlled release system is referred to as "a constant supply of the active ingredient that exhibits zero-order kinetics" (Zhao et al., 2017). An amount of the drug is continuously released for a specific period of time and equivalent to the eliminated by the body. Furthermore, drugs can be delivered in a sustained or triggered manner by choosing appropriate materials and design features. Most interestingly, targeting nanoparticles directly to a certain biological location (referred to as site specific targeting) is a key issue in drug delivery. Specific binding forces that promote cellular contact and particle uptake primarily are resulted from interactions between certain ligands and complementary molecules or receptors on the cell membrane (Gao et al., 2005). Ligands can be either of biological origin (i.e., aptamer protein, peptide, and antibody) or abiotic ligands such as chemical moieties or surface functionalities (Leroueil et al., 2008). These systems have potential to reduce off-target effects and enhance accumulation at the diseased organ or tissue.

## 2.8 Methods for construction of nanoparticles

Nanoparticles can be synthesized by various methods, which involve mechanical, chemical and other pathways (Koczkur et al., 2015). Two general approaches to the synthesis of organic nanoparticles are "top-down" and "bottom-up" approaches. In the "bottom-up" techniques, nanoparticles are synthesized from atoms or molecules via synthetic chemistry and self-organization to generate a wide range of nanoscale systems such as lipid-based nanoparticles, polymeric

nanoparticles, liposomes, and niosomes. In the "top-down" techniques, larger (macroscopic) particle size of initial materials are reduced by mechanical milling and other more complex ones such as microfluidics and lithography. Small molecules can be loaded into nanoparticles either by physical encapsulation, or by conjugation on the surface or in the core. Synthesis methods used to produce these organic nanoparticles are sometimes sophisticated and poorly controllable. In contrast, the production and secretion of biological vectors involve a simple, powerful and precise mechanism of biosynthesis and self-assembly by cells (Hung and Leonard, 2015). For example, assembly and release of bacteriophage or exosome particles occur in living cells.

## 2.9 Physicochemical characterization of nanoparticles

### 2.9.1 Particle size (Dynamic Light Scattering, Scanning and Transmission Electron Microscopy)

A fundamental and important investigation in nanoscience and nanotechnology is nanoscale characterizations. Current research demands the rapid method of determining characteristics and properties of newly developed nanoparticles. Physicochemical properties of nanoparticles are primarily analyzed and characterized by the particle size (average diameter), charge, and morphology. Size distribution, degree of aggregation, and surface area can be also measured and evaluated (Mourdikoudis et al., 2018). Of which, Dynamic Light Scattering (DLS) is the most frequently used technique in order to determine of the particle size and size distribution (de Assis et al., 2008). Moreover, direct visualization of the nanoparticles can be achieved by two electron microscopy techniques; scanning electron microscopy (SEM) and transmission electron microscopy (TEM), two of which can be exploited in order to more accurately determine the size, shape and surface morphology. Another microscopy technique known as scanning force microscopy or AFM offers the most accurate characterization of size, size distribution and actual description (Polakovič et al., 1999).

### 2.9.2 Surface charge

Surface charge of nanoparticles is another important parameter to be considered for directly evaluating its storage stability. Surface charge plays an important role in the electrostatic interaction with bioactive compounds as well as their interaction between nanoparticles and the biological environment. Zeta potential is a parameter that indirectly measures the surface charge. This value can be derived by evaluating the potential difference between the outer Helmholtz plane and the surface of shear. In order to ensure stability and avoid aggregation of the particles, either high positive or negative zeta potential values are recommended (Otsuka et al., 2003).

### 2.9.3 Encapsulation efficiency

The amount of drug trapped in the formulation can be calculated and expressed as the entrapment efficiency (EE) and loading efficiency (LE). These parameters can be measured by using the following Eqns (Bhagav et al., 2011).

1. The entrapment efficiency (EE)= active ingredient content in the product obtained (mg)/total drug content (mg)×100

2. loading efficiency (LE)= active ingredient content in the product obtained (mg)/total product weight (mg)×100.

Active ingredient content can be measured by using reverse phase high-performance liquid chromatography (HPLC) techniques (Singh and Vingkar, 2008).

### 2.9.4 Stability testing

The stability testing is necessarily conducted in order to support the information regarding the presence of active ingredients in produces under the various environmental factors such as temperature, humidity, and light over the period of time or specific time interval. This information is crucial to establish a retest period for the active ingredients and thus determine a shelf life for the product. Storage conditions can be therefore recommended (Gurpreet and Singh, 2018).

# CHAPTER III

# MATERIALS AND METHODS

## 3.1 Anaesthetic agents

Pure essential clove oil was purchased from Thai-China flavours and fragrances industry Co., Ltd. (Phra Nakhon Si Ayutthaya, Thailand). Sorbitan Oleate (80), Polysorbate 20 and Glycerol were from Croda Thailand. Cetearyl Alcohol and coco-glucoside was supplied from Chemico Inter Corporation Thailand.

## 3.2 Experimental animals

Whiteleg shrimp (*Litopenaeus vannamei*) with an average weight of 3 g were purchased from a healthy farm and then transported to the aquaculture laboratory at Chulalongkorn University. Shrimp were distributed into tanks with continuously aerated clean water pH 8.2, 20 ppt salinity, at 28°C.

## 3.3 Preparation of clove oil NLCs

The formulation of clove oil NLCs composed of 20% clove oil. Clove oil (20 g) was warmed in a water bath at 70 °C. Then, Sorbitan Oleate 80 (3 g) and Cetearyl alcohol and coco-glucoside (2 g) was added into warmed clove oil and used as an oil phase. Mixing of Polysorbate 20 (3 g), Glycerol (2 g) and DI water (70 g) were warmed in water bath at 70 °C as an aqueous phase. A pre-emulsion was mixture by adding the warmed aqueous phase into the oil phase and stirred at 300 rpm for 10 min. Finally, clove oil was emulsified by high-speed homogenizer at 10,000 rpm for 10 min. Clove oil NLCs was successfully fabricated via high-speed homogenization.
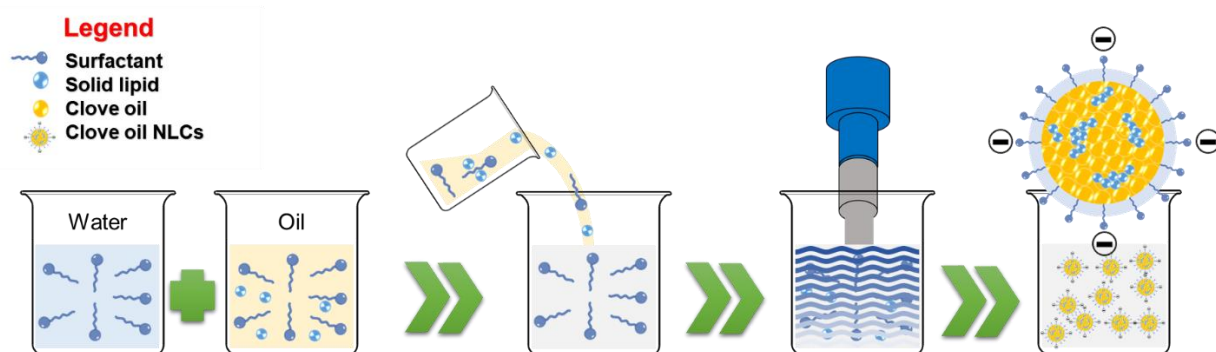
**Figure 4 :** Schematic diagram of the preparation of Clove oil-encapsulated nanoparticles. Clove oil-encapsulated NLCs are fabricated.

## 3.4 Physical properties and characterization of clove oil NLCs

The clove oil NLCs formulation were evaluated for particle size, polydispersity index (PDI) and zeta potential by the Dynamic light scattering (Nanosizer, Malvern, UK). The measurements were performed in triplicate with particle suspensions diluted 1000 time in distilled water at room temperature.

## 3.5 Morphological study of clove oil NLCs

The morphology of clove oil NLCs was examined by transmission electron microscopy (TEM) (JEM-2100 plus, JEOL, Japan). Before analysis, Clove oil NLCs was dispersed in distilled water. A drop of nanoparticle suspension was placed on a carbon-coated copper grid followed by drying. This grid was mounted in the instrument. Photographs were taken at appropriate magnifications of 200.0kX.

## 3.6 Clove oil NLCs stability testing

For determining shelf life of a clove oil NLCs, accelerated stability studied are performed. The samples were kept in glass bottles and tightly sealed. Samples are stored at different temperatures and ambient humidity conditions (30°C/60% RH and 40°C/75%) for up to 3 months. After specified time intervals (0, 1, 2 and 3 months) samples were collected and analysed for particle size and zeta potential using Dynamic light scattering (DLS).

### 3.7 *In vitro* drug release study of clove oil NLCs

HPLC technique was used to determine the encapsulation efficiency (EE) and release profile of eugenol. The amount of drug trapped in the formulation can be calculated and expressed as the entrapment efficiency (EE). These parameters can be measured by using the following Equation:

$$\%EE = \frac{C_i - C_F}{C_i} \times 100$$

Where Ci represents initial concentration of eugenol in NLCs; Cf represents the concentration of unencapsulated eugenol.

The controlled drug release study was performed to evaluate eugenol release from clove oil. The release of STD clove oil dissolved in ethanol was also studied under the same conditions as the clove oil NLCs for comparison. All measurements were performed in triplicate.

### 3.8 Anaesthetic concentration and recovery time

Individuals of white shrimp were held in thirteen separates plastic tanks (10 shrimps/tank) containing different concentrations of STD clove oil and clove oil NLCs (10, 20, 30, 40, 50, 60, 70, 100, 120, 140, 160, 180 and 200 ppm). The time of induction and recovery to reach each stage were recorded by one observer per stage. After shrimps showed a complete loss of equilibrium (induction time stage 2), the treatment was stopped and shrimps were transferred to the recovery tank (no anaesthesia present) and observed until it fully recovery in stage 2. All experiment were performed in triplicate.

The stages of induction and recovery from anaesthesia under identical experimental conditions were as described in Table 1-2.

**Table 1.** Stages of anaesthesia in shrimps according to (Li et al., 2018)

| Stage | Behaviour of shrimp |
|:---:|:---|
| 1 | Reaction only to strong tactile and vibration stimuli |
| 2 | Non-reactivity to stimuli |

**Table 2.** Stages of recovery in shrimps according to (Li et al., 2018)

| Stage | Behaviour of shrimp |
|:---:|:---|
| 1 | Start of erratic swimming without reestablishment of equilibrium |
| 2 | Attained an upright position on the bottoms of the aquaria |
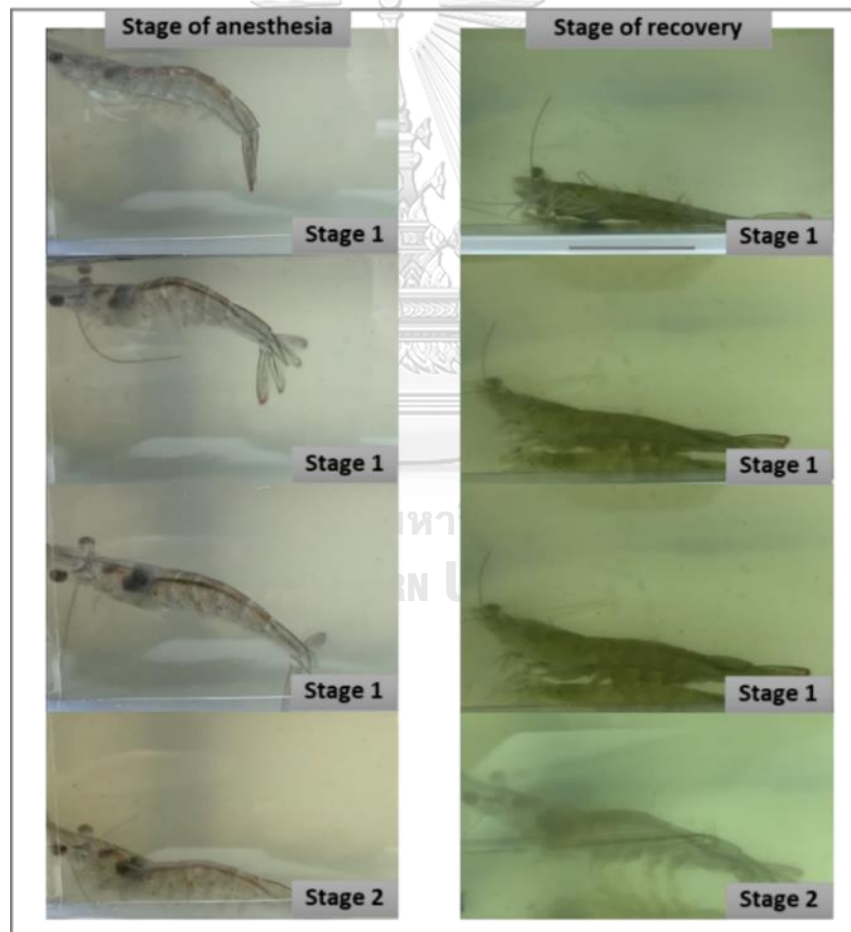


**Figure 5 :** Behavioral characteristics of shrimp in two stages of anaesthesia and recovery.

## 3.9 Toxicity study of clove oil in shrimps

The acute toxicity test consisted of two groups: STD clove oil and clove oil NLCs. Thirteen concentrations consist of 10, 20, 30, 40, 50, 60, 70, 100, 120, 140, 160, 180 and 200 ppm and a control were used to determine mortality as the basic test. Ten shrimps in three replicates were used for every concentration and also in the control. The mortality rate for each group was measured after 48 hrs.

## 3.10 Biodistribution study of clove oil on shrimps

For this experiment to investigate the biodistribution of fluorescent labelled clove oil in shrimp body, which was visualized by Bioluminescence imaging. Shrimps were divided into 3 groups; control (untreated), STD clove oil and clove oil NLCs. Ten shrimps were immersed into 50 ppm each treatment. An individual shrimp was harvested from the tank when reaching the onset of stage 2. The shrimps were immediately transferred to a well oxygenated recovery tank. After full recovery, the shrimps were collected every 2 minutes for detected excretion time, which shrimp eliminated clove oil particles. Fluorescent signal of Nile Red stained nanoparticles was examined using Fluorescence imaging and the images were analysed by IMAGE J software. All experiments were performed in triplicate.

## 3.11 Statistical analysis

GraphPad Prism software (Version 8.0) was used to generate graphs and perform statistical analyses. One-way and two-way analysis of variance, or repeated measures analysis of variance followed by Bonferroni's multiple comparison test were used for multiple comparisons. A value of $p < 0.05$ was considered statistically significant.

## CHAPTER IV

## RESULTS

### 4.1 Physical properties and characterization of clove oil NLCs

As shown in **Figure 6,** after preparation the clove oil NLCs appeared as a milky translucent dispersion with no phase separation and were well-dispersed and water-soluble. **Table 3** presents the average particle size (nm), PDI, and zeta potential (mV) of the clove oil NLCs formulation and encapsulation efficiency and drug loading of clove oil-loaded NLCs immediately after preparation.
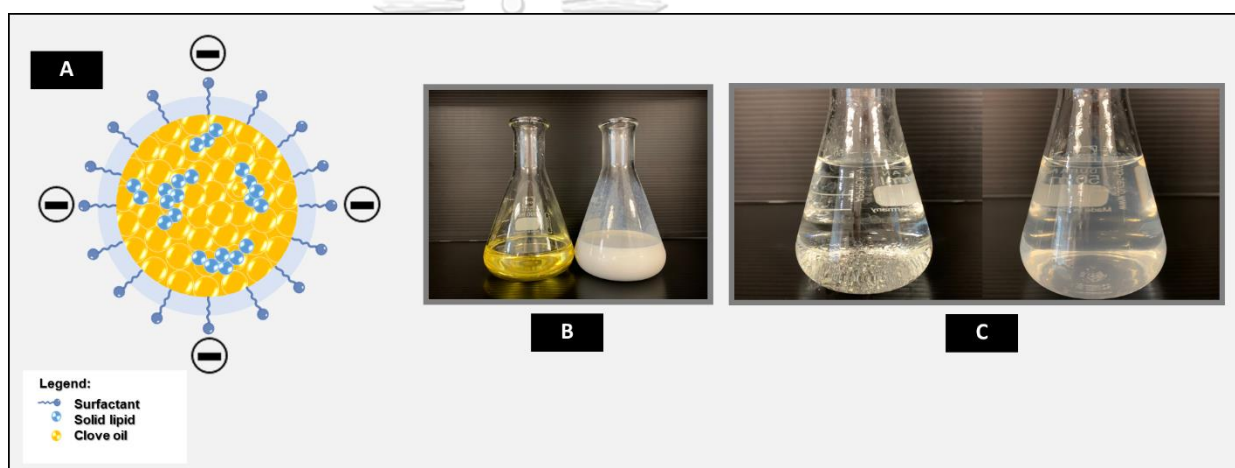


**Figure 6 :.** A: Graphical representation of clove oil-loaded in NLCs. B: Appearance of STD clove oil and clove oil NLCs from left to right. C: Comparison of STD clove oil and clove oil NLCs with water miscibility. Dispersions of NLC loaded with clove oil indicated enhanced solubility that was better than STD clove oil. The results indicate that NLCs dispersions have good potential for use as an aquatic anaesthetic.

The average particle size, PDI, and zeta potential value of the clove oil NLCs were 175.07±0.72 nm, 0.115±0.230, and -48.37±.38 mV, respectively, indicating a narrow size distribution of nanoparticles. The encapsulation efficiency was 88.55%. The mean particle was below 200 nm, their PDI values were lower than 0.3, and their zeta potential was higher, indicating their relatively narrow size distribution (Tamjidi et al., 2013).

**Table 3.** Physical properties characterisation of the clove oil NLCs, including particle size, zeta potential, and polydispersity index.

| Sample | Average Diameter (nm)±SD | Average Zeta potential (mV) ±SD | Average Polydispersity Index±SD | Appearance |
|--------|--------------------------|----------------------------------|----------------------------------|------------|
| STD clove oil | N/A | N/A | N/A | Yellow transparent |
| Clove oil NLCs | 175.07±0.72 | -48.37±.38 | 0.115±0.230 | Milky translucent |

Abbreviations: N/A, not applicable.

## 4.2 Morphological study of clove oil NLCs

**Figure 7** shows the TEM morphology of the clove oil NLCs preparation. The particles were spherically shaped, had a smooth morphology, were well dispersed, and had a narrow size distribution. From the TEM image, no aggregation or agglomeration were observed. The spherical clove oil NLCs were in the nanometre size range, which was in good agreement with the dynamic light scattering measurement results.
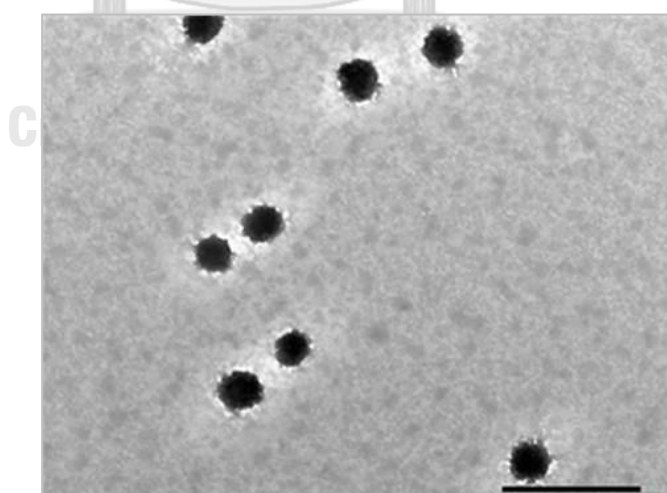


**Figure 7 :** Clove oil morphology visualised by Transmission Electron Microscopy (TEM).

## 4.3 Clove oil NLCs stability testing

**Figure 8** presents the physical stability of clove oil NLCs formulation for 0, 1, 2, and 3 months at 30℃ and 40℃ based on particle size and zeta potential measurement. At the end of this period, the clove oil NLCs formulation showed no macroscopic phase separation in 30℃ and 40℃, so the remaining formulation was analysed for size and zeta potential. It is observed that particle size and zeta potential for clove oil NLCs stored in both temperatures were considered stable. Consequently, incorporation of clove oil load in NLCs successfully developed a stable and uniform particle.
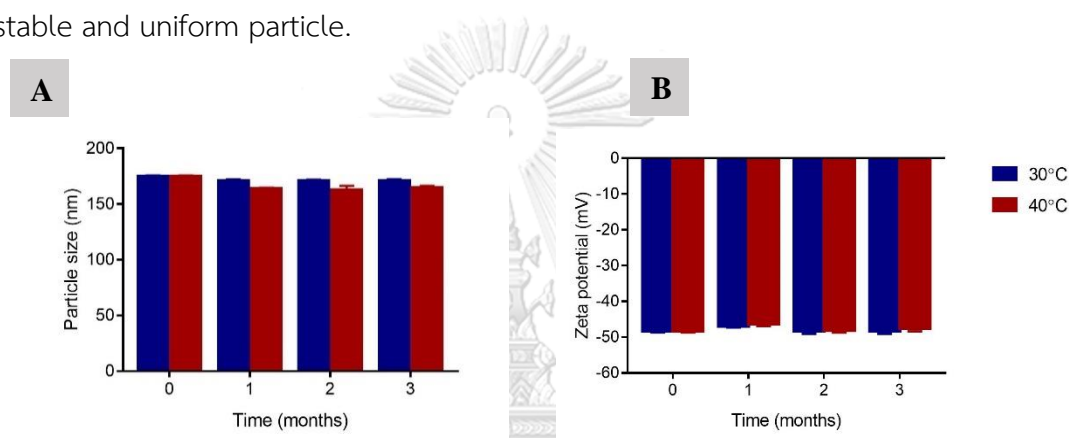


**Figure 8 :** Particle size (A) and zeta potential (B) of clove oil NLCs generated following storage at 30℃/60% RH and 40℃/75% RH for up to 3 months. No statistically significant difference of particle size and zeta potential (p<0.05) were found when stored at 30℃ compared to 40℃. Data is expressed as mean ± standard deviation (n=3).

## 4.4 *In vitro* drug release study of clove oil NLCs

The releasing ability of STD clove oil and clove oil NLCs was demonstrated in **Figure 9**. The result indicates that the release profiles of STD clove oil exhibited a burst release. Eugenol was immediately released in a few minutes and reached to 30 % level within 1 hour. In contrast, the Clove oil NLCs were capable to control the release of eugenol. Only 5% of eugenol were gradually released from the clove oil NLCs in the first hour. The level of eugenol was released less than 20% when measured at 2 hours. The linear regression trendline of drug release was found between 2 to 8 hours duration.
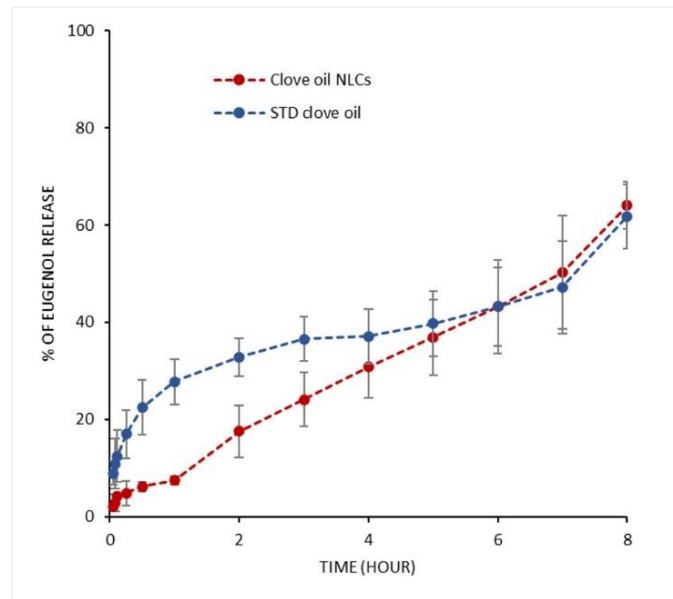
**Figure 9 :** Eugenol release profile compared to the release profile from STD clove oil and clove oil NLCs at different times.

## 4.5 Anaesthetic concentration and recovery time
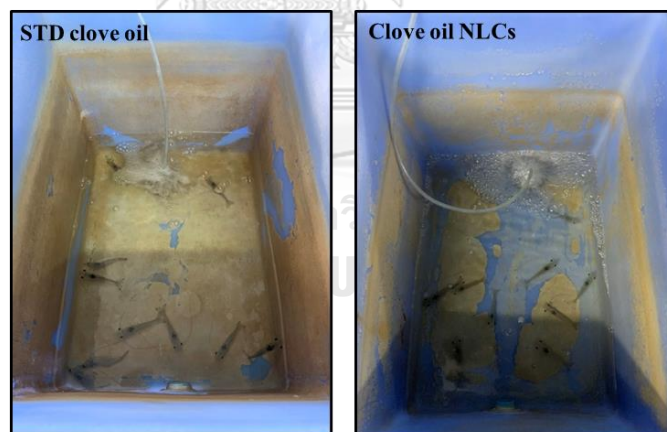


**Figure 10 :** Anaesthetic experiment of STD clove oil and clove oil NLCs on shrimp at different concentrations.

Most shrimp exposed to high concentrations of clove oil encountered with a risk of ventilatory failure. The affected shrimp showed a neural paralytic syndrome, cramped body movements, loss of coordination and orientation in water, reduced

respiratory rate and oxygen consumption. The result shown in **Figure 11A-11B** indicate a significant difference in induction and recovery times between STD clove oil and clove oil NLCs. Both STD clove oil and clove oil NLCs could induce the shrimp entered to stage 2 without complications. The lowest concentration of STD-CO and CO-NLCs which allowed shrimp to reach stage 2 was 20 ppm. Interestingly, the induction time was such different between 2 groups. STD clove oil needed 31 minutes while clove oil NLCs needed only 6.30 minutes to let the shrimp turn into stage 2 of general anaesthesia. However, the induction time from STD clove oil treatment decreased to 12 and 5 minutes at 30 and 40 ppm concentration, respectively. In addition, increasing the clove oil NLCs concentration to medium concentration (range 30-70 ppm of clove oil compound) could reduce the induction time for few minutes ($p > 0.05$). High concentrations (range 100-200 ppm) of STD clove oil and clove oil NLCs were demonstrated rapidly action on anaesthesia. The shrimps were unconscious within 0.85-3 minutes after immersed them into the clove oil contained tanks. With increasing concentrations of clove oil, the duration required to reach sedation and anaesthesia decreased, while the recovery times increased. The shortest duration to sedation (0.85 minute of clove oil NLCs and 1.10 minutes of STD clove oil) were obtained at higher concentrations of STD clove oil and clove oil NLCs (200 ppm). Moreover, the longest duration to sedation (6.30 minutes of clove oil NLCs and 31 minutes of STD clove oil) were achieved at a lower concentration of 20 ppm. Furthermore, clove oil at a concentration of 10 ppm was ineffective within 5 minutes of exposure. The shrimps were recovered from the complete loss of equilibrium stage at 20 ppm treated with STD clove oil and clove oil NLCs within 3.50 and 3 minutes, respectively. The medium concentration (30-70 ppm clove oil) did not show significant difference in recovery time between STD clove oil and clove oil NLCs ($p > 0.05$). The longer duration of recovery time was recorded in high concentration clove oil, especially in STD clove oil treatment. The highest recovery time was 20 minutes. Nonetheless, the recovery time of high concentration clove oil NLCs group was not longer than 8 minutes.

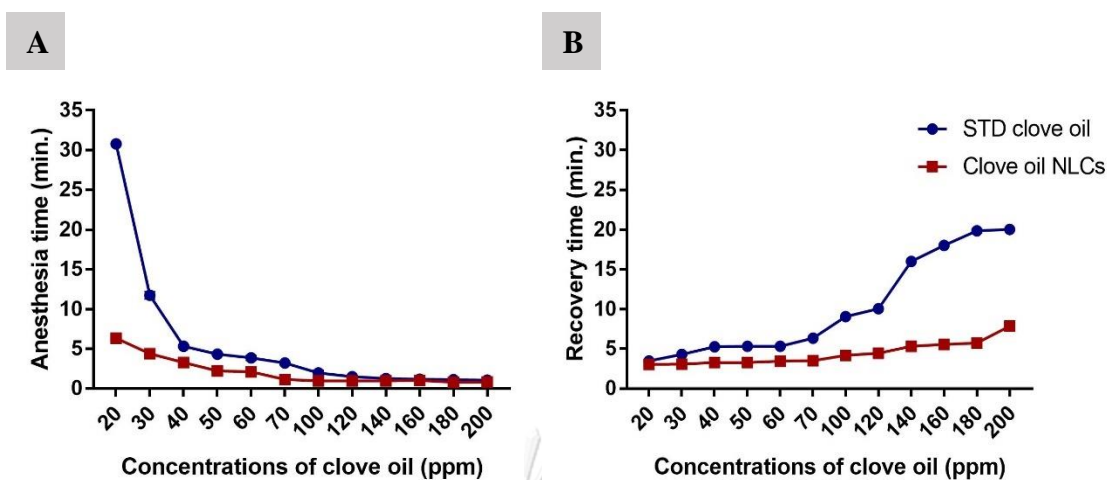**Figure 11 :** (A) Times required for induction to stage of anaesthesia in white shrimp after exposure to clove oil NLCs and (B) STD clove oil and times required for stage of recovery from anaesthesia in the anesthetized white shrimp induced by clove oil NLCs and STD clove oil.

## 4.6 Toxicity study of clove oil in shrimps
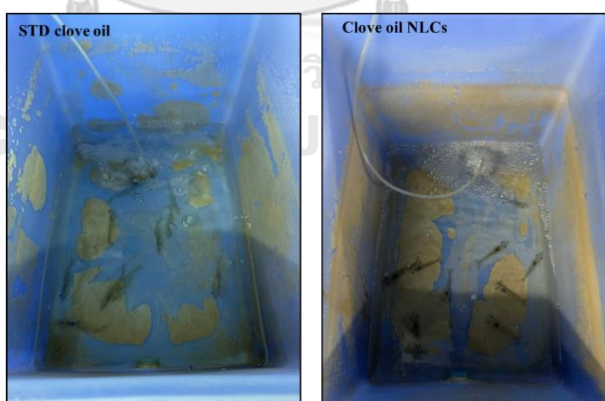


**Figure 12 :** Experimental toxicity comparison of STD clove oil and clove oil NLCs on shrimp.

The results demonstrated that there were no shrimps died after received STD clove oil and clove oil NLCs under 140 ppm and 160 ppm, respectively. The median lethal concentration of STD clove oil was 140 ppm. Half of shrimps were defuncted. The lost was raised when the concentration was increased. whereas 140 ppm of clove oil NLCs were 0% mortality over a 48-hour period. Moreover, the highest concentration of clove oil NLCs (200 ppm) was not reach to the LC50 level (**Table 4**).

Compared to clove oil in the form of NLCs, STD clove oil can be considered toxic for white shrimp. A potential explanation for this observation could be the advantage of nanoencapsulation technology, since it is well established that *nanoencapsulation can reduce* doses, and therefore reduce the potential *toxicity* of active ingredients (Ferreira and Nunes, 2019).

**Table 4.** Results of median lethal concentration (LC50 48 hours) clove oil in white shrimp at 28°C and a salinity of 20 ppt. These concentrations were selected after completing the survival and limited killing concentration tests (10 shrimp in triplicate).

| Clove concentrations (ppm) | STD clove oil | | Clove oil NLCs | |
|---|---|---|---|---|
| | Shrimp mortality (number) | Mortality (%) | Shrimp mortality (number) | Mortality (%) |
| Control | 0 | 0 | 0 | 0 |
| 10 | 0 | 0 | 0 | 0 |
| 20 | 0 | 0 | 0 | 0 |
| 30 | 0 | 0 | 0 | 0 |
| 40 | 0 | 0 | 0 | 0 |
| 50 | 0 | 0 | 0 | 0 |
| 60 | 0 | 0 | 0 | 0 |
| 70 | 0 | 0 | 0 | 0 |
| 100 | 0 | 0 | 0 | 0 |
| 120 | 0 | 0 | 0 | 0 |
| 140 | 5±0.0 | 50 | 0 | 0 |

| 160 | 6±0.71 | 60 | 1±0.0 | 10 |
| 180 | 8±1.41 | 80 | 2±0.71 | 20 |
| 200 | 9±0.71 | 90 | 2±0.0 | 20 |

## 4.7 Biodistribution study of clove oil on shrimps

The biodistribution of STD clove oil and clove oil NLCs in white shrimp was studied following the shrimp being immersed in fluorescently labelled clove oil. Nile red staining clove oil has been used as a lipid quantification technique in oil accumulation studies. Whole-shrimp body images revealed a fluorescence signal in the organ of all individuals at different times after full recovery. The fluorescence images, fluorescence intensity is represented by a multicolour range from green (low intensity of clove oil) to red (high intensity of clove oil). From whole-body imaging **(Figure 13)**, systemically-administered clove oil was found mainly in shrimp bodies. Clove oil NLCs cleared in the shrimp bodies was remove from the shrimp within 30 minutes after recovery, while clove oil was still detected in the shrimp of the STD clove oil group. These results imply that STD clove oil may result in delayed clove oil elimination. Meanwhile, clove oil NLCs may result in faster excretion.
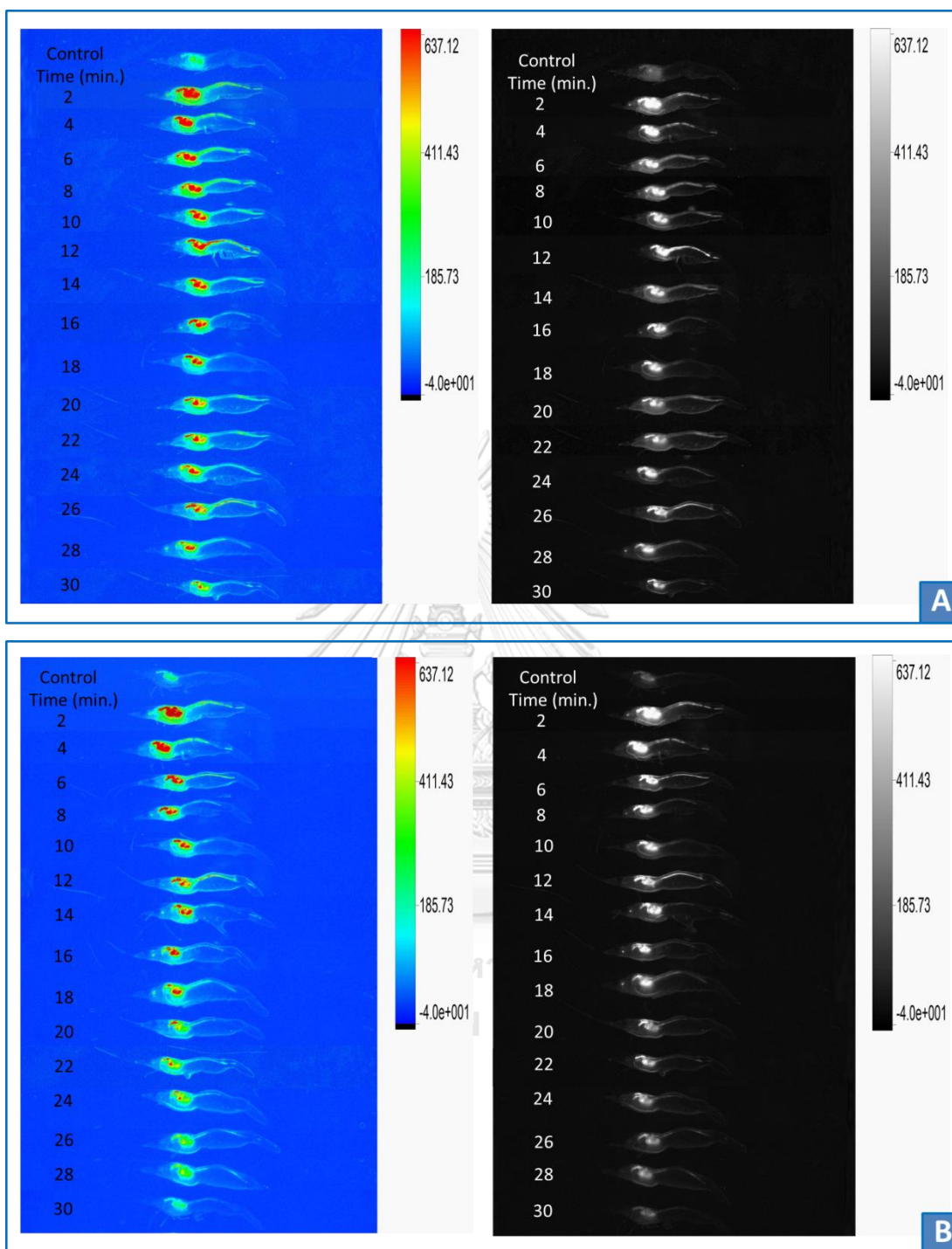
**Figure 13 :** Clove oil biodistribution after exposure.

**(A)** RGB spectrum and Gray of STD clove oil in white shrimp at 2 to 30 minutes after recovery. **(B)** RGB spectrum and Gray scale of NLCs of clove oil in white shrimp 2 to 30 minutes after recovery. Images reveal the residual and excretion of clove oil. The image representation of fluorescence measurement indicates the multicolour

distribution of images. High intensity clove oil appears as a red colour, while low intensity of clove oil appears as green. Non-immersed shrimp were used as a background subtraction control.

**Figure 14** presents quantification of fluorescence Intensity of whole-body imaging from **Figure 13** using image J software, which confirmed a sustained presence of clove oil formulation. This method determines the corrected total fluorescence by subtracting the background signal, which is useful to compare fluorescence intensity between different areas or regions. In this result, the excretion of clove oil from clove oil NLCs was faster than STD clove oil. The shrimp could excrete the clove oil within 30 minutes.



**Figure 14 :** Qualification of clove accumulation and excretion of shrimp at different durations after recovery. The comparison lines indicate a significant higher relative fluorescence intensity ($p < 0.05$) gained with STD clove oil compared to the clove oil NLCs group.

# CHAPTER V

# DISCUSSION AND CONCLUSION

The present study conducted a physicochemical characterisation of clove oil formulation. In this study, clove oil NLCs were developed as a new carrier system as an alternative formulation for anaesthetic agents. In addition, this study attempted to develop an anaesthetic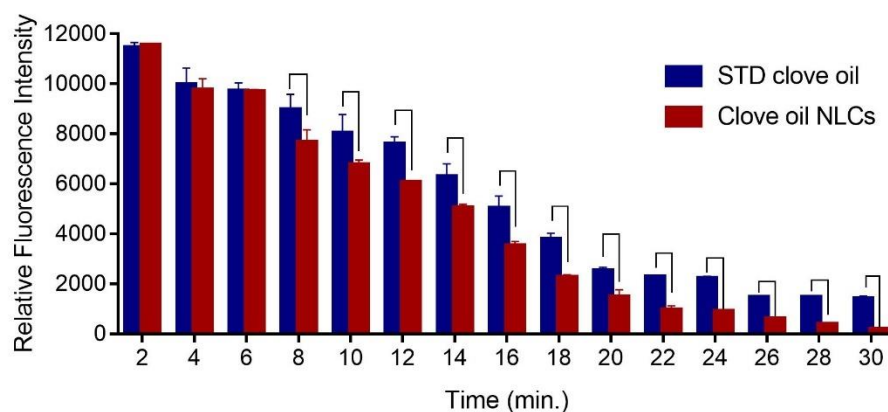 preparation of clove oil without ethanol. Clove oil NLCs have been proven to enhance the bioavailability of poorly soluble active compounds. Nonetheless, clove oil has limited solubility in water since the active ingredient is eugenol which is not entirely soluble in water and has a low to moderate bioaccumulation potential. It is therefore necessary to dilute the product with ethanol. Clove oil dissolved with ethanol has always been used for anaesthesia. Many researchers use alcohols or detergents as dissolving agents since clove oil does not easily emulsify with water without vigorous agitation (Munday and Wilson, 1997). However, ethanol causes major side effects and is toxic for animals (Akbari et al., 2010; Grush et al., 2004). The initial characterisation of the clove oil formulations showed a small droplet size, low PDI value and good zeta potential value. In addition, it also has high encapsulation efficiency that indicates the strong ability of the nanostructured lipid carrier to encapsulate the clove oil. The particles were a smooth and spherically shaped with no agglomeration or aggregation observed. The morphology was nanometre sized, which is in good agreement with the results from dynamic light scattering measurement. The stability of the clove oil formulation was evaluated according to storage at 30°C and 40°C for up to 3 months. The results showed that the average size and zeta potential maintained a fairly constant size for up to 3 months, as evidenced by measurements using DLS. Besides, the cumulative release of clove oil, the profile showed uncontrolled and irregular release in STD clove oil. The cumulative percentage release of clove oil from STD clove oil was faster than NLCs form. Clove oil adsorbed on the surface and available at the interface was responsible for the initial burst of release, while embedded clove oil was responsible for the sustained release. The result suggested that the new

generation of lipid carrier system better facilitated a controlled release of clove oil, ensuring the superior lipid-based delivery of nano-structured lipid carrier. (Rao and Naidu, 2016).

Physicochemical characterisation and stability evaluation of nanotechnology-based formulations are important for the development of these systems. In this study, clove oil formulation presented small droplet sizes and low PDI values which indicate that the system is monodispersed and can be correlated with highly stable storage for nanoparticles (Polychniatou and Tzia, 2014). PDI values of 0.3 and below are considered acceptable in drug delivery system applications using lipid-based nanoparticle formulation and indicate a homogeneous population of phospholipids nanoparticles (Mozafari, 2010; Mozafari et al., 2007; Negussie et al., 2011). The zeta potential in clove oil NLCs refers to the degree of electrostatic repulsion between particles in a dispersion. A high zeta potential value of particles offers high stability to the dispersion and prevents aggregation (Qureshi et al., 2015). The negative charges of particle can be absorbed to the emulsifier layer of oil and water interface and electric double layer similar to that of ionic form (Han et al., 2008; Keck et al., 2014). The zeta potential had a significant relationship with the long-term stability of the particle. A nanoparticle with a higher zeta potential of the nanoparticle was associated with a more stable nanoparticle. Nanoparticles with more than -30 mV showed good stability and less than or equal to -60 mV showed a very good physical stability (Uprit et al., 2013).

For the biological characteristics of clove oil as an anaesthetic upon immersion mode. According to Sladky et al.,2001, our study confirmed the increased exposure period of clove oil resulted in longer recovery time (Sladky et al., 2001). As suggested by Ross and Ross.,2009 anaesthetic efficacy is depended on its solubility in lipids, which enables the eugenol to pass through the cell wall of the gills (Ross and Ross, 2009). In fact, coating anatomic structures, especially gill epithelial cells, with oily clove oil or eugenol could lead to prolonged anaesthetic effects. The depressive action on neuromuscular transmission synapse resulted in the depression of the shrimp respiratory system and inhibited swimming performance (Fanni et al., 2021; Skår et al., 2017) as shown in crayfish (Ozeki, 1975). Eugenol, the active ingredient of

clove oil, is reported as a calcium and potassium channel blocker affected the cardiac contractile proteins and consequently caused heart rate inhibition and heart relaxation (Damiani et al., 2004; Lahlou et al., 2004; Sensch et al., 2000). In the study, the time of induction was in the same range in agreement with a study of the anaesthetic effect of clove oil in *Penaeus semisulcatus* (Soltani et al., 2004). The results clearly indicated that a shorter induction time and rapid recovery was achieved with clove oil NLCs in shrimp with a dose-dependent manner. Owing to the extremely small size with a large interfacial area of internal oil droplets-nano carrier and water miscibility of insoluble ingredients (Merisko-Liversidge et al., 2003; Subramanian et al., 2004), this might be explained a rapid absorption of clove oil through the gills.

Regarding the biodistribution of clove oil in shrimp body, the present study evaluated the *in vivo* distribution of STD clove oil and clove oil NLCs in white shrimp. The fluorescent signals measured using in vivo imaging correlated with the amount of administered clove oil which accumulated in the shrimp body. During the 30 minutes elimination kinetics experimental period, the trend of clove oil elimination began at 4 minutes after shrimp recovery. Within the 30 minutes, levels of eugenol declined quickly in shrimp body of clove oil NLCs shrimps, but the elimination rate of eugenol was quite slow in the STD clove oil shrimp. The fluorescence image indicated that the clove oil level in the shrimp body was typically higher in the absorption and distribution phases. The present study extended the evidence of preferable accumulation of STD clove oil in shrimp body by immersion administration, in which accumulation of clove oil caused toxic effects. In the study, the clove oil nanostructures were rapidly biodegraded and completely eliminated within 30 minutes as seen in the other organisms suggested by (Baati et al., 2016). Interestingly, the fluorescent signal was highly seen in the hepatopancreas and intestine of affected shrimp, suggesting the intestinal tract was also the main biodistribution and excretory route for clove oil clearance in whiteleg shrimp apart from the gill. It was noted that when encapsulated in the NLC complex, eugenol compounds showed significant lower LC50 values than those of the STD clove oil relevant with the biodistribution and excretion results. As a result, the nanoparticles

appeared to have a protective effect against the toxicity of the active compound in clove oil but maintained their anaesthesia effect (Luis et al., 2020).

In conclusion, we applied an innovative nanotechnology to develop a drug delivery system suitable for enhanced anaesthetic activity of clove oil in shrimp by bath immersion. Clove oil NLCs can therefore induce faster anaesthesia induction at a lower dosage with a rapid recovery. Interestingly, clove oil NLCs formulation are considered as high safety of margin for shrimp and for biological systems as determined by the rapid clearance in shrimp body biodistribution, as confirmed by the rapid recovery and survival rate from the toxicity study. The results from this study clearly demonstrate that clove oil is an effective anaesthesia for use with white shrimp. The effective concentration range is between 30, 40, 50, 60, 70, 100, 120, 140, 160, 180, and 200 ppm. However, use of higher clove oil concentrations require longer recovery times and induce higher shrimp mortality. Therefore, the recommended working range of concentrations is between 30, 40, 50, 60, 70, 100, 120, and 140 ppm based on anaesthesia time, recovery time, the toxicity study, and healthy behaviours of shrimp. Importantly, are the efficacy of anaesthesia and recovery, toxicity, and elimination of clove oil NLCs in relation to their physiochemical properties of nanoparticles. Our findings demonstrate that clove oil NLCs are safe for biological systems, which support the idea that lipid-based nanoparticles have improved the delivery of pharmaceutical agents which ensures that the objective that to prepare the best nanoparticles.

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