การเตรียมและฤทธิ์ต้านจุลชีพของนาโนอิมัลชันน้ำมันมะกอก

นางสาวกุสุมา แจ่มศักดิ์

จุฬาลงกรณ์มหาวิทยาลัย
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บทคัดย่อและแฟ้มข้อมูลฉบับเต็มของวิทยานิพนธ์ตั้งแต่ปีการศึกษา 2554 ที่ให้บริการในคลังปัญญาจุฬาฯ (CUIR)
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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
สาขาวิชาอาหารเคมีและโภชนศาสตร์ทางการแพทย์ ภาควิชาอาหารและเภสัชเคมี
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
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ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

PREPARATION AND ANTIMICROBIAL ACTIVITY OF OLIVE OIL NANOEMULSIONS

Miss Kusuma Jaemsak

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy Program in Food Chemistry and Medical Nutrition
Department of Food and Pharmaceutical Chemistry
Faculty of Pharmaceutical Sciences
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กุสุมา แจ่มศักดิ์ : การเตรียมและฤทธิ์ต้านจุลชีพของนาโนอิมัลชันน้ำมันมะกอก (PREPARATION AND ANTIMICROBIAL ACTIVITY OF OLIVE OIL NANOEMULSIONS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ภญ. ดร.วรางคณา วารีสน้อยเจริญ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ภญ.อารีรัตน์ ลออปักษา, 93 หน้า.

ในปัจจุบันน้ำมันมะกอกเป็นที่นิยมใช้ทั้งในอุตสาหกรรมยา อาหาร และเครื่องสำอาง เนื่องจากประโยชน์ต่อสุขภาพ โดยมีฤทธิ์ต้านอนุมูลอิสระ ต้านการอักเสบ และต้านจุลชีพได้ การศึกษาก่อนหน้าพบว่า นาโนอิมัลชันสามารถเพิ่มการดูดซึม และชีวปริมาณออกฤทธิ์ของสารที่มีการละลายน้ำต่ำ เช่นน้ำมันมะกอกได้ อย่างไรก็ตาม การศึกษาเกี่ยวกับนาโนอิมัลชันน้ำมันมะกอกยังมีไม่มากนัก การศึกษานี้มีวัตถุประสงค์เพื่อผลิตนาโนอิมัลชันน้ำมันมะกอก โดยวิธีพลังงานต่ำและวิธีพลังงานสูง ศึกษาคุณสมบัติทางเคมีกายภาพ เช่น ขนาดอนุภาคนาโนอิมัลชัน ค่าการกระจายของอนุภาค และค่าความต่างศักย์ซีต้า หลังการเก็บไว้ที่สภาวะต่างๆเป็นเวลา 180 วัน นอกจากนั้นยังทำการประเมินฤทธิ์ในการต้านเชื้อแบคทีเรียและราอีกด้วย ผลการศึกษาพบว่า การเตรียมนาโนอิมัลชันด้วยวิธีพลังงานต่ำ โดยการใช้สารลดแรงตึงผิวคือ Tween® 80 ควรใส่สารร่วมลดแรงตึงผิวร่วม Imwitor® 308 เพื่อทำให้ได้นาโนอิมัลชันที่มีขนาดเล็กกว่า 200 นาโนเมตร การเพิ่มปริมาณน้ำมันมะกอกส่งผลให้นาโนอิมัลชันมีขนาดใหญ่ขึ้น และต้องใช้ปริมาณสารลดแรงตึงผิวมากขึ้นเพื่อลดแรงตึงผิวระหว่างของเหลวในระบบนาโนอิมัลชัน การเตรียมนาโนอิมัลชันน้ำมันมะกอกด้วยวิธีอัลทราโซนิก ซึ่งเป็นวิธีพลังงานสูง พบว่า ปัจจัยที่สำคัญที่ทำให้ได้นาโนอิมัลชันคือ แอมพลิจูด 25% เวลาที่ใช้ผลิต 5 นาที และพลังงาน 2250 จูล และนาโนอิมัลชันที่มีปริมาณน้ำมันมะกอก 5% สารลดแรงตึงผิว 30 % และสารร่วมลดแรงตึงผิวร่วม 15 % (T30 O5) มีความคงตัวมากที่สุดหลังการเก็บไว้ที่สภาวะ 25 °C ± 2 °C / 60% RH ± 5% RH, 30 °C ± 2 °C / 65% RH ± 5% RH และ 40 °C ± 2 °C / 75% RH ± 5% เป็นเวลา 180 วัน ศึกษาฤทธิ์ในการต้านจุลชีพโดยการวัดขนาดของโซนยับยั้งการเจริญของจุลชีพด้วยวิธี disc diffusion และทดสอบฤทธิ์ในการต้านจุลชีพโดยใช้ค่า MIC, MBC และ MFC ด้วยวิธี broth microdilution พบว่า นาโนอิมัลชันน้ำมันมะกอกมีผลเพิ่มฤทธิ์ต้านจุลชีพเมื่อเทียบกับน้ำมันมะกอกถึง 42-105 เท่า ในแบคทีเรีย และ 28-42 เท่าในเชื้อรา การเพิ่มปริมาณน้ำมันมะกอกในสูตรตำรับทำให้การฤทธิ์ต้านจุลชีพมีมากขึ้น โดยสูตรตำรับที่ให้ฤทธิ์ต้านจุลชีพได้ดีที่สุดคือ สูตรที่มีปริมาณน้ำมันมะกอกร้อยละ 10 (T30 O10) กล่าวโดยสรุปนาโนอิมัลชันน้ำมันมะกอกมีฤทธิ์ต้านจุลชีพได้ โดยความสามารถในการออกฤทธิ์ขึ้นกับส่วนประกอบของนาโนอิมัลชัน ทั้งนี้ควรจะมีการศึกษากลไกการออกฤทธิ์ต้านจุลชีพของนาโนอิมัลชันต่อไป เพื่อนำนาโนอิมัลชันไปประยุกต์ใช้ในด้านอาหารและยา

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ภาควิชา	อาหารและเภสัชเคมี	ลายมือชื่อนิสิต .....
สาขาวิชา	อาหารเคมีและโภชนศาสตร์ทางการแพทย์	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
ปีการศึกษา	2560	ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5776102033 : MAJOR FOOD CHEMISTRY AND MEDICAL NUTRITION
KEYWORDS: OLIVE OIL NANOEMULSION ANTIMICROBIAL ACTIVITY

KUSUMA JAEMSAK: PREPARATION AND ANTIMICROBIAL ACTIVITY OF OLIVE OIL NANOEMULSIONS. ADVISOR: ASSOC. PROF. WARANGKANA WARISNOICHAROEN, Ph.D., CO-ADVISOR: ASSOC. PROF. AREERAT LAORPAKSA, 93 pp.

Due to their health benefits including antioxidant, anti-inflammatory and also antimicrobial activities, extra virgin olive oil (EVOO) has received much attention in food, cosmetics, and pharmaceutical industries. Nanoemulsions (NEs) solubilize and increase absorption and bioavailability of lipophilic substances (i.e. olive oil). However, there is insufficient data in the formation of olive oil NEs. This study aimed to investigate the formation of oil-in-water NEs containing EVOO using low-energy approach and high-energy approach. NEs were characterized for size, polydispersity and zeta potential after storage at different conditions for up to 180 days. They were determined for antimicrobial susceptibility. From the result, NEs prepared by low energy emulsification using Tween® 80 (surfactant) indicated the requirement of co-surfactant (Imwitor® 308) to form NEs with smaller droplet sizes (< 200 nm). An increase in oil amount resulted in larger NEs droplets and more surfactant needed to form an interfacial film of droplet. For NEs prepared by high energy ultrasonication, parameters to generate the optimal NEs were ultrasound amplitude 25%, treatment time 5 min and input energy 2250 J. NEs containing 5% EVOO, 30% surfactant and 15% co-surfactant (T30 O5) was the most stable after storage at 25 °C ± 2 °C / 60% RH ± 5% RH, 30 °C ± 2 °C / 65% RH ± 5% RH and 40 °C ± 2 °C / 75% RH ± 5% for 180 days. The antimicrobial activity of NEs was measured as a zone of inhibition by disc diffusion, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) by broth microdilution method. The results showed that encapsulation of EVOO into NEs increased antimicrobial activity about 42-105 times against bacteria and 28-42 times against fungi compared to corresponding sole EVOO. Furthermore, adding more olive oil in preparation resulted in more antimicrobial activity. T30 O10 was the most effective formulation in antimicrobials against tested organisms. Conclusively, EVOO NEs showed antimicrobial activity which depends on the compositions of EVOO NEs. Further investigation should be the mechanism of NEs as antimicrobials in order to apply the NEs employed in food and pharmaceutical aspects.

Department:	Food and Pharmaceutical Chemistry	Student's Signature	.....
		Advisor's Signature	.....
Field of Study:	Food Chemistry and Medical Nutrition	Co-Advisor's Signature	.....
Academic Year:	2017		

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CHAPTER I

INTRODUCTION

1.1 Background and Rationale

In recent years, nanotechnology-based products have been developed to employ in many applications including food, beverage and pharmaceuticals. Encapsulation of functional substances especially those with low water solubility and poor chemical stability into a nano carrier system can increase the solubility, stability and bioavailability of the components (Sugumar et al., 2013). Moreover, the encapsulation of the lipophilic substances such as essential oils and beta-carotene into a colloidal delivery system can help disperse them into aqueous-based products (Saberi, Fang, and McClements, 2013). Nanoemulsions consist of at least two immiscible liquid mixed by emulsifiers such as surfactants and co-surfactants. Due to small droplet size typically less than 100 nm and high surface area of nanoemulsions, they can enhance the functionality of substances and also improve appearance and stability (Gupta, Eral, Hatton, and Doyle, 2016). Nanoemulsions are considered to be more stable to gravitational separation and aggregation than conventional emulsions (McClements, 2011).

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Natural antimicrobial agents are one of the functional substances which have been attractive for use in wide applications. An ideal antimicrobial should be

non-toxic, economical and effective for a wide range of microorganisms (Davidson, Cekmer, Monu, and Techathuvanan, 2015). Natural antimicrobials from plant origins include organic acids, phytoalexins, oil components and phenolic compounds. Oils that have antimicrobial activity against food-borne pathogens such as clove oil, thyme oil, lemongrass oil, and olive oil may have a potential to be applied in many food products.

Olive oil has received much attention due to its health benefits. The presence of high monounsaturated fatty acids, polyunsaturated fatty acids and minor components such as tocopherol, phenolic compound, chlorophyll, hydrocarbon and carotenoids are contributed to antioxidant and antiinflamatory properties. It can decrease LDL cholesterol and elevate HDL cholesterol (Cicerale, 2011). Moreover, in some studies have shown that olive oil also has antimicrobial activity against bacteria, fungi and mould. Phenolic compounds in olive oil are susceptible for a potent antibacterial activity Morerecently, there have been reports on the inhibitory effect of olive oil against foodborne microorganism such as *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium, Bacillus cereus*, *Listeria monocytogenes* and fungi such as *Aspergillus* and *Candida* species. The antimicrobial mechanisms of olive oil against microorganism are proposed to be denaturating the proteins, affecting cell membrane permeability (Gokmen, Kara, Akkaya, Torlak, and Onen, 2014) and disrupting the cell wall peptidoglycan, which finally lead to leakage of intracellular cytoplasmic constituents and cell death (Janakat, Al-Nabulsi, Allehdan,

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Olaimat, and Holley, 2015). Olive oil extracted by a cold press method and without using heat or chemicals is called the extra virgin olive oil (EVOO) (Limbo, Peri, and Piergiovanni, 2014). It has the high antimicrobial activity due to its higher amount of phenolic compounds.

It has been reported that encapsulating the antimicrobial compound into the nano-sized formulations could increase its antimicrobial activity. For example, incorporation of oils such as basil oil into nanoemulsions enhanced its antibacterial activity against food-borne pathogens (Ghosh, Srivastava, Nath, and Celis, 2013). Nanoemulsions could possibly alter the phospholipid bilayer integrity, thus allowing lipophilic molecules within the oils to penetrate into the cell membrane of the microorganisms (Moghimi, Ghaderi, Rafati, Aliahmadi, and McClements, 2016).

However, the information about the antimicrobial activity of nanoemulsions incorporating olive oil is still limited. The formation of olive oil nanoemulsions and determination of antimicrobial activity of olive oil nanoemulsions by using disc diffusion and broth microdilution method was conducted in this study.

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1.2 Objectives of the study

1. To investigate factors on preparation of nanoemulsions incorporating olive oil
2. To determine physicochemical properties of olive oil nanoemulsions
3. To evaluate antimicrobial activity of olive oil nanoemulsions

1.3 Benefits of the study

This study provides information on the compositions of oil, surfactant and co-surfactant, in order to optimize the formation of such nanoemulsions. The results of this study may be beneficial for further use of olive oil nanoemulsions with antibacterial activity for food and pharmaceutical products.

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CHAPTER II

LITERATURE REVIEW

2.1 Extra virgin olive oil (EVOO)

Olive oil is classified in 6 categories namely extra virgin olive oil, virgin olive oil, refined olive oil, refined olive pomace oil, olive oil composed of refined and virgin olive oils and olive-pomace oil, due to their quality and milling process (Figure 1). Extra virgin olive oil and virgin olive oil are suitable for human consumption. Lampante which refined by a physical-chemical process is called refined olive oil and becomes edible oil after the process. The solid residue from the milling process is called the pomace, which extracted with solvent becomes refined olive pomace oil.

Olive oil is an edible vegetable oil which has high contents in beneficial substances such as fatty acids, phenolic compounds, tocopherol, and carotenoids. The compositions of the fatty acid in olive oil are monounsaturated fatty acids, oleic acid (65-83%); saturated fatty acids (8-14%); polyunsaturated fatty acids, linoleic acid (omega-6) (6-15%), and alpha-linolenic acid (omega-3), (0.2-1.5%) (Limbo et al., 2014). The main components in olive oil include oleic acid, phenolics, and squalene.

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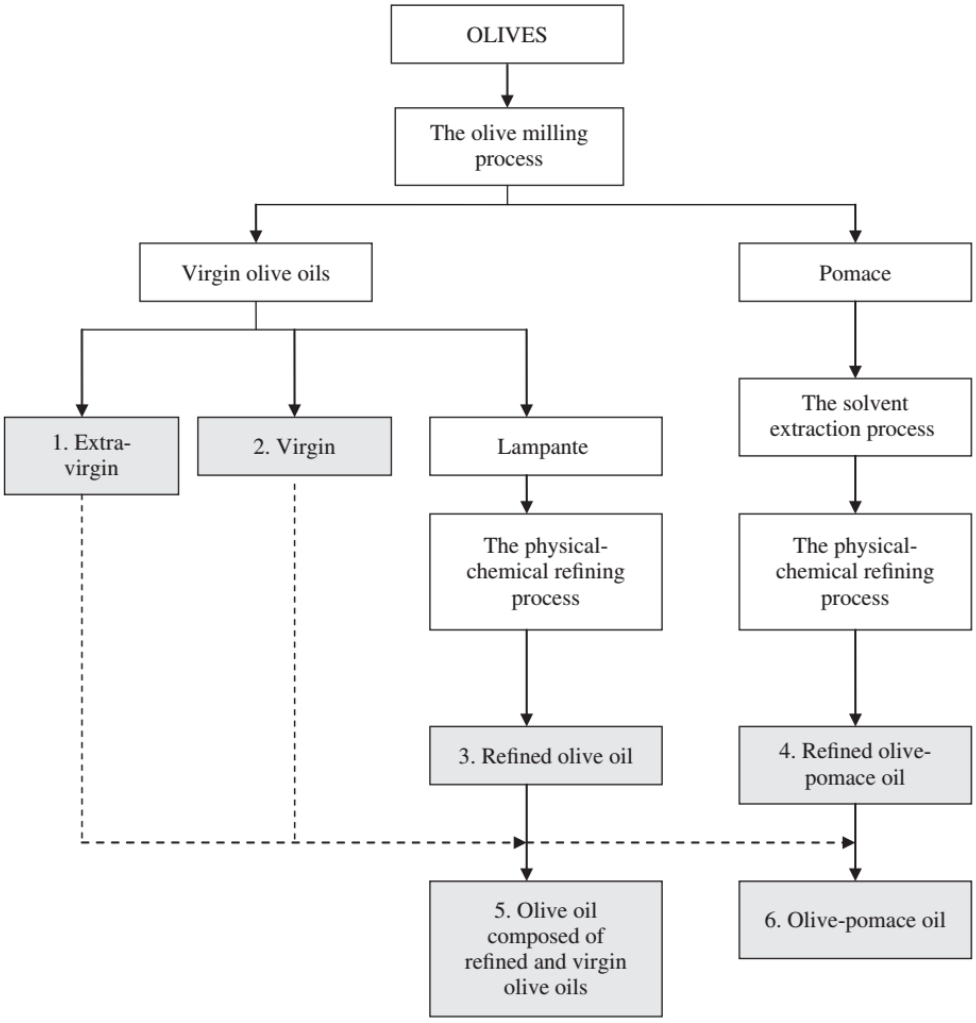

Figure 1 The olive oil milling process (Peri, 2014)

Oleic acid is found in meat such as beef and poultry (30-45 % oleic acid), palm, peanut, soybean, and sunflower (25-49 % oleic acid). Several studies have shown health benefits of an oleic acid such as cancer prevention, antioxidant, antiatherogenic, anti-inflammation, reducing LDL-cholesterol and antimicrobial activity (Omar, 2010).

Phenolic compounds in olive oil can be divided into three groups: simple phenol, secoiridoids and lignans, which possess antimicrobial, antioxidant and anti-inflammatory properties (Waterman and Lockwood, 2007). Extra virgin olive oil (EVOO) has a higher amount of phenolic compounds than the refined virgin olive oil. EVOO contains about at least 36 phenolic compounds. The concentrations of phenolic compounds in olive oil depend on growth, agricultural techniques, maturity of olive oil at harvest, processing, and storage (Cicerale, 2011). Major phenolic compounds of olive oil consist of hydroxytyrosol, tyrosol, ligstroside, and oleuropein. Oleuropein is a type of phenolic compounds which is mostly found in olive oil and possesses potent antimicrobial (gram negative and gram positive bacteria) and antiviral properties (Tamendjari et al., 2014). The structure of oleuropein is shown in Figure 2.

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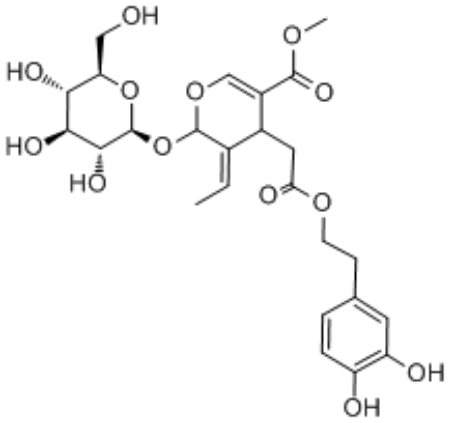

Figure 2 Chemical structure of oleuropein (Waterman and Lockwood, 2007)

Squalene is a hydrocarbon and a triterpene which is a precursor for the synthesis of plant and animal sterols such as cholesterol and steroid hormones. Squalene is found mostly in olive oil, shark oil and a lesser amount of wheat germ and rice bran. Health benefits of squalene are anti-inflammatory, antioxidant and antimicrobial properties (Limbo et al., 2014). A chemical structure of squalene is shown in Figure 3.

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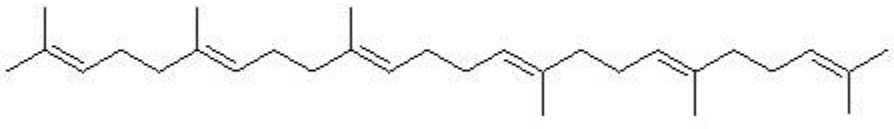

Figure 3 Chemical structure of squalene (Shah, Bhalodia, and, Shelat, 2010)

2.2 Surfactant

Emulsions are the thermodynamically unstable system. Surfactant or emulsifier is necessary to maintain the interface interaction between liquids from separation in emulsion system by decreasing interfacial tension. Adding a surfactant in emulsion tends to lower interfacial energy and improves the stability of the emulsion. The surfactant is an amphiphilic compound in which a structure consists of lipophilic and hydrophilic parts. The solubility properties of surfactant are called the HLB (Hydrophilic-Lipophilic Balance) value. The HLB number is in range of 0-20. The surfactant with high HLB value (above 11) has hydrophilic property and is used for preparation of oil in water (O/W) emulsion (oil droplets dispersed in a continuous phase). In other words, a surfactant with low HLB value (below 9) has oil-soluble property and is used as an emulsifier for water in oil (W/O) emulsion preparation (ICI Americans INC, 1980). The surfactant is classified in 4 classes according to ionization properties: anionic surfactant (i.e. sodium stearate, calcium oleate, and sodium lauryl sulfate), cationic surfactant (i.e. benzalkonium chloride, cetyl trimethylammonium), amphoteric surfactants (i.e. lecithin, amphoacetate, and betaine) and non-ioinic surfactant (i.e. Span® and Tween®).

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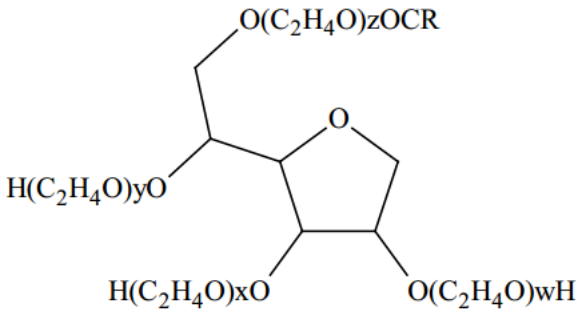
Tween® 80 or polyoxyethylene (20) sorbitan monooleate is a food grade non-ionic surfactant classified as GRAS (Generally recognized as safe), and is derived from polyethoxylated sorbitan and oleic acid. Tween® 80 is a non-ionic and strongly hydrophilic surface active agent. The HLB value of Tween 80 is 15.0 which is suitable for making O/W emulsion. An acceptable daily intake (ADI) of Tween® 80 is in the range 0-25 mg/kg body weight (BW). The application of Tween® 80 is permitted in food, pharmaceuticals, cosmetics and industrial applications (European Food Emulsifier Manufacturer's Association, EFEMA, 2013). The structure of Tween® 80 is shown in Figure 4



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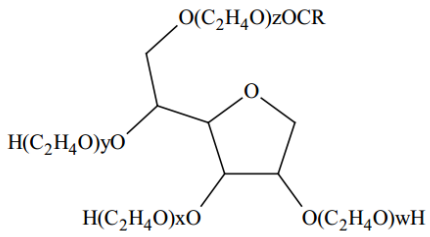
where w + x + y + z = approximately 20 and RCO- is the oleic acid moiety

Figure 4 Chemical structure of Tween® 80 (EFEMA, 2013)

Tween® 20 or polyoxyethylene sorbitol monolaurate is derived from the partial esters of sorbitol and dianhydrides with lauric acid. An acceptable daily intake of Tween® 20 is also in the range 0-25 mg/kg BW. Due to the high HLB value of 16.7. Tween® 20 is used as an emulsifier in O/W emulsion. It usually employs in food, pharmaceutical, cosmetic and other industrial applications. A formula of Tween® 20 is shown in Figure 5.



where w + x + y + z = approximately 20 and RCO- is the lauric acid moiety

Figure 5 Chemical structure of Tween® 20 (EFEMA, 2013)

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2.3 Co-surfactant

Co-surfactant can be used to enhance the solubilizing capacity of a surfactant and to improve emulsion formation by reducing the interfacial tension of immiscible liquid. Addition of a co-surfactant brings to a synergistic effect with a surfactant (Setya, Talegaonkar, and Razdan, 2013). Co-surfactant leads to higher packing densities of the emulsifier film at the interface, and increases the rigidity of the emulsifier film at interface layer of the emulsion. The model of using co-surfactant in nanoemulsion system is shown in Figure 6.

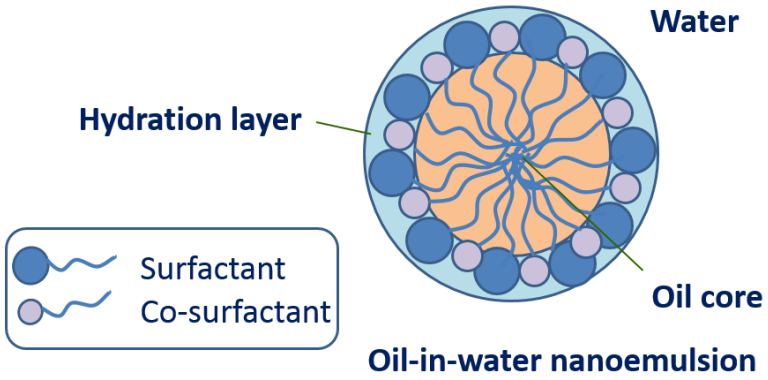

Figure 6 O/W nanoemulsion system (Pachioni-Vasconcelos et al., 2016)

Imwitor® 308 is glycerol esters of caprylic acid derived from vegetable sources. Imwitor® 308 is used in wide application due to its solubility both in oil and water. It can act as a co-emulsifier, solubilizer, lubricant, absorption promotor. It also has bacteriostatic properties (Cremer, 2012). Other co-surfactants which can be used for nanoemulsions are propylene glycol, polyethylene glycol, lauroglycol 90, ethanol and carbitol (Setya et al., 2013). A formula for Imwitor® 308 is shown in Figure 7.

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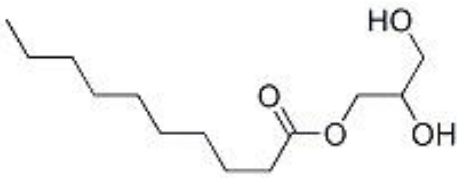

Figure 7 A formula of Imwitor® 308

2.4 Nanoemulsion

Nanoemulsions consist of at least two immiscible liquid stabilized by emulsifiers (with or without co-surfactants). The emulsifier can stabilize nanoemulsions by reducing the interfacial tension and preventing droplet deformation and coalescence(Leong, Wooster, Kentish, and Ashokkumar, 2009). Due to small droplet size typically less than 100 nm and high surface area of nanoemulsions (NEs), they can enhance the functionality of substances and also improve appearance and stability (Solans and Solé, 2012).

NEs can be formed in three types; (A) oil in water emulsion (O/W) contains oil droplets dispersed in continuous aqueous phase, (B) water droplets are dispersed in oil phase is called water in oil emulsion (W/O) and multiple emulsions are primary emulsion dispersed in another liquid phase (C and D) (Figure 8).

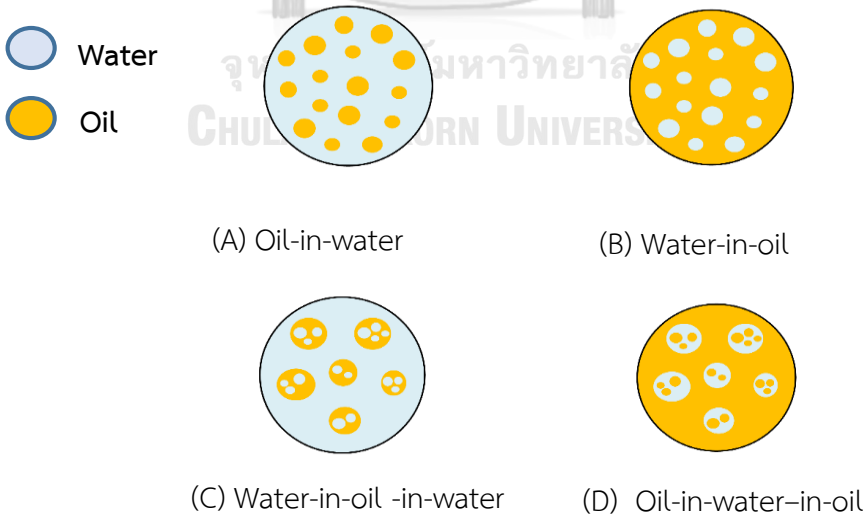

Figure 8 Types of nanoemulsions (Gupta et al., 2016)

The instability of nanoemulsion contributes to occurences of creaming, Ostwald ripening, gravitational separation, flocculation, coalescence, and sedimentation (Tadros, Izquierdo, Esquena, and Solans, 2004). The instability mechanisms in food emulsion are shown in Figure 9.

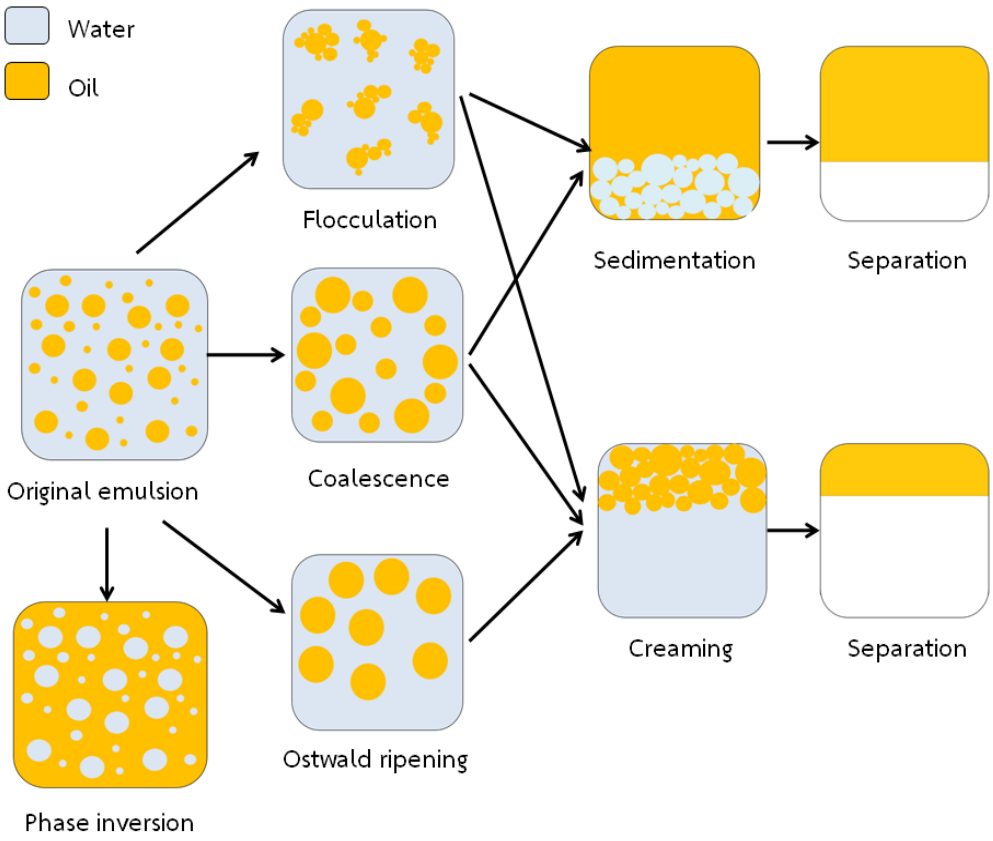

Figure 9 Schematic diagram of instability mechanisms in nanoemulsions. (Adapted from McClement, 2011)

Flocculation is a mechanism in which the globules are forming floccules due to attractive interaction which tend to rise up or settle down of dispersed globules in the emulsion more rapidly than usual. In coalescence, the droplets become a bigger drop. Phase inversion is the physical mechanism that cause changes in types of emulsion between O/W and W/O. This process happens by varying in the phase volume, increasing amount of electrolytes and temperature changes (Jaiswal, Dudhe, and Sharma, 2015). Sedimentation and creaming are the gravitational separation form of instability in an emulsion. Sedimentation is the downward movement of droplets in which the droplets have a higher density than the surrounding continuous phase. This mechanism usually found in W/O emulsion. Creaming is an upward movement of the droplet in which the droplets have a lower density than the continuous phase. This mechanism has found in O/W emulsion and can be evaluated by creaming index of the emulsion (McClements, 2011). In Oswald ripening, the oil droplets diffuse through the aqueous phase which finally form too large droplets (McClements, 2011).

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NEs are considered to be more stable to gravitational separation and aggregation than conventional emulsions (McClements, 2011). NEs have a wide variety of potential applications in food, cosmetics, and pharmaceutical industries. NEs can increase bioavailability and reduce chemical degradation of encapsulated lipophilic substances such as vitamins, flavor, colors, and drugs (Herrera, 2012). In food industries, NEs are used to incorporate low water solubility substance such as ß-carotene or essential oil such as basil oil, thyme oil in order to increase bioaccessibility of lipophilic

substances. The bioaccessibility of ß-carotene after encapsulated in NEs was increased (Liang et al., 2013). Rebolleda et al. (2015) have found that incorporating of wheat bran oil into NEs could improve the bioaccessibility of the wheat bran oil into the water system. Yi et al. (2015) studied the encapsulation of ß-carotene and results indicated that encapsulated labile bioactive compounds (such as ß-carotene) into NEs could improve its antioxidant properties. Furthermore, Majeed et al. (2016) determined the antimicrobial activity of clove oil NEs compared to clove oil and canola oil mixture. The result was indicated that clove oil NEs enhanced antimicrobial activity of the mixture of clove and canola oil. In pharmaceutical applications, NEs are used for delivery of drugs and biological agents which are susceptible to hydrolysis and oxidation. Furthermore, NEs are used for targeted delivery of lipophilic drugs (Jaiswal et al., 2015).

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2.4.1 Preparation of nanoemulsion

2.4.1.1 Low energy emulsification

The low energy methods for NEs preparation include phase inversion temperature (PIT), phase inversion composition (PIC), emulsion inversion point (EIP) and spontaneous emulsification (Wooster et al., 2016). Low energy method is based on the spontaneous formation of emulsions under specific compositions or environmental conditions which cause changes in interfacial properties (McClements, 2011). The emulsification process is carried out at constant parameter either their

environmental conditions or compositions. The **phase inversion temperature** (PIT) method relies on the changes in solubility and physicochemical properties of surfactant with changing temperature initiate the transformation in a type of emulsion (such as O/W turns to W/O) (McClements, 2011). The **phase inversion composition** (PIC) is the method which alters the composition of NEs with constant temperature. **Emulsion inversion point** (EIP) method or catastrophic phase inversion is the method which alters the type of emulsion by increasing the volume fraction of disperse phase, adding a surfactant or changing the temperature. **Spontaneous emulsification** is the method that two immiscible phases spontaneously form to NEs at a proper temperature. This method is produced by varying the compositions of oil and aqueous phases, environmental conditions such as temperature, pH, ionic strength, and mixing conditions such as stirring speed, a rate of addition and order of addition (McClements, 2011). Advantages of spontaneous emulsification method are economical and low energy use. The limitation of spontaneous emulsification is requiring a large amount of surfactant and co-surfactant to form NEs and are not suitable for large-scale industrial productions (Gupta et al., 2016). The schematic diagram of a mechanism for spontaneous emulsification is shown in Figure 10.

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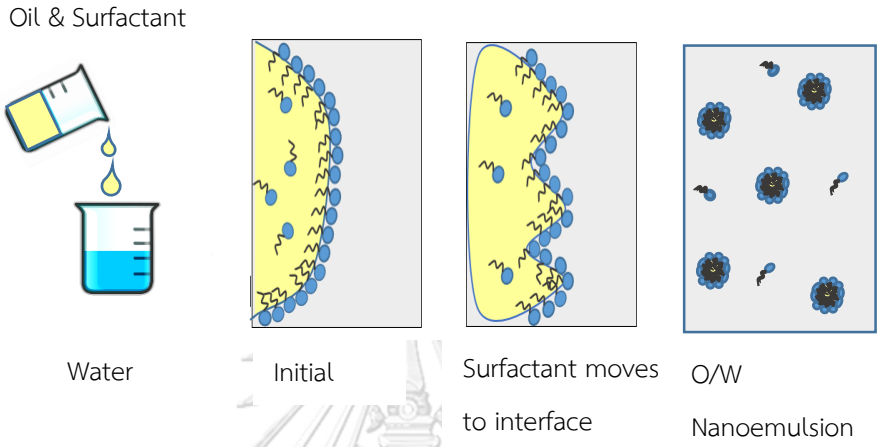

Figure 10 Schematic diagram of mechanism for spontaneous emulsification of nanoemulsion formation (McClements, 2011)

2.4.1.2 High energy emulsification

High energy approaches use mechanical devices to generate NEs by mechanical force to break up droplets into a smaller size. The mechanical device used to generate high energy are known as a homogenizer, a microfluidizer, and an ultrasonicator. The limitation of this method is unsuitable for hot labile or unstable molecules such as protein and peptide.

Ultrasonication is one of the high energy technique used for NEs preparation. In this technique, the energy is provided through sonicator probe, which contains piezoelectric quartz crystal. Ultrasonicator converts the electricity into the acoustic wave and produces mechanical vibration via the tip and formation of cavities

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in liquid and NEs can be obtained (Figure 11) (Cole-Parmer, 2017). The breaking up step of the droplet is shown in Figure 12. The first step, the dispersed phase droplet is disrupted into continuous phase by interfacial acoustic waves. The next step, the droplets are breaking up through cavitation. The small droplets can be produced. The input parameters of ultrasonication are amplitude, energy and sonication time.

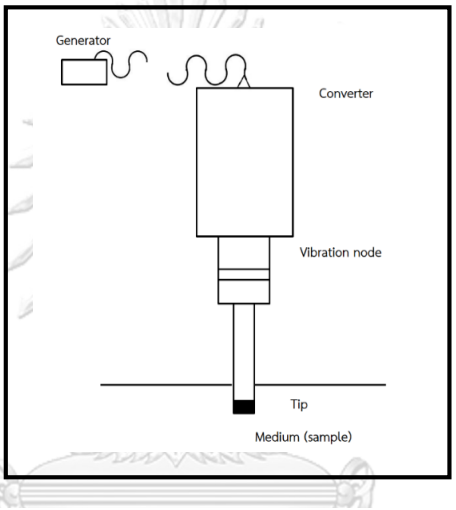

Figure 12 Experimental set-up for ultrasonication (Adapted from Cole-Parmer, 2017)

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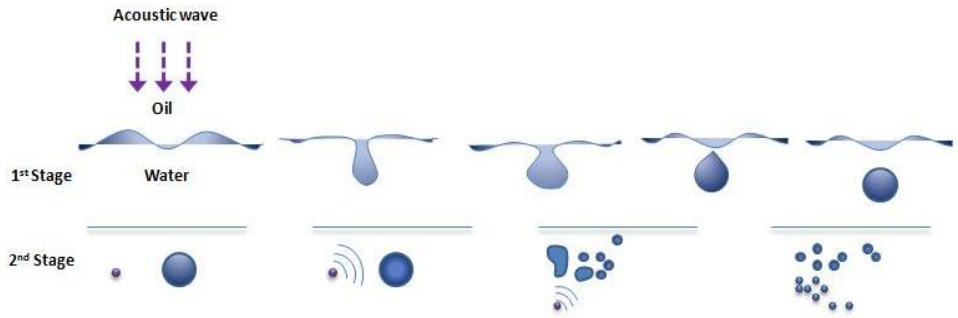

Figure 11 Schematic diagram of the formation of NEs by ultrasonication method (Chalothorn, 2011)

2.5 Nanoemulsions and antimicrobial activity

Based on the several studies, encapsulation of functional lipophilic substances in NEs can improve their efficacy, bioaccessibility, stability and also antimicrobial activity (Shah et al., 2010). In 2016 Moghimi et al. were investigated the antimicrobial activity of *Thymus daenensis* NEs and pure *Thymus daenensis* essential oil. The result found that the *T. daenensis* NEs enhanced antimicrobial activity against *E. coli* than pure *T. daenensis* essential oil about 10 times. The mechanisms can be explained by the incorporation of *T. daenensis* essential oil into nanoemulsions brings the functional oil droplets close to the cell membrane, leading to disrupt the cell membrane by altering the phospholipid bilayer integrity and loss of cell internal constituents such as protein, nucleic acid, and potassium. Furthermore, due to their reduced particle sizes, the surface areas exposed to the microbial membrane are increased (Topuz et al., 2016). Formulation of oregano oil NEs can increase solubility and enhance the antimicrobial activity (Bhargava, Conti, da Rocha, and Zhang, 2015). The mechanisms of NEs in antimicrobial activity is as below (Figure 13).

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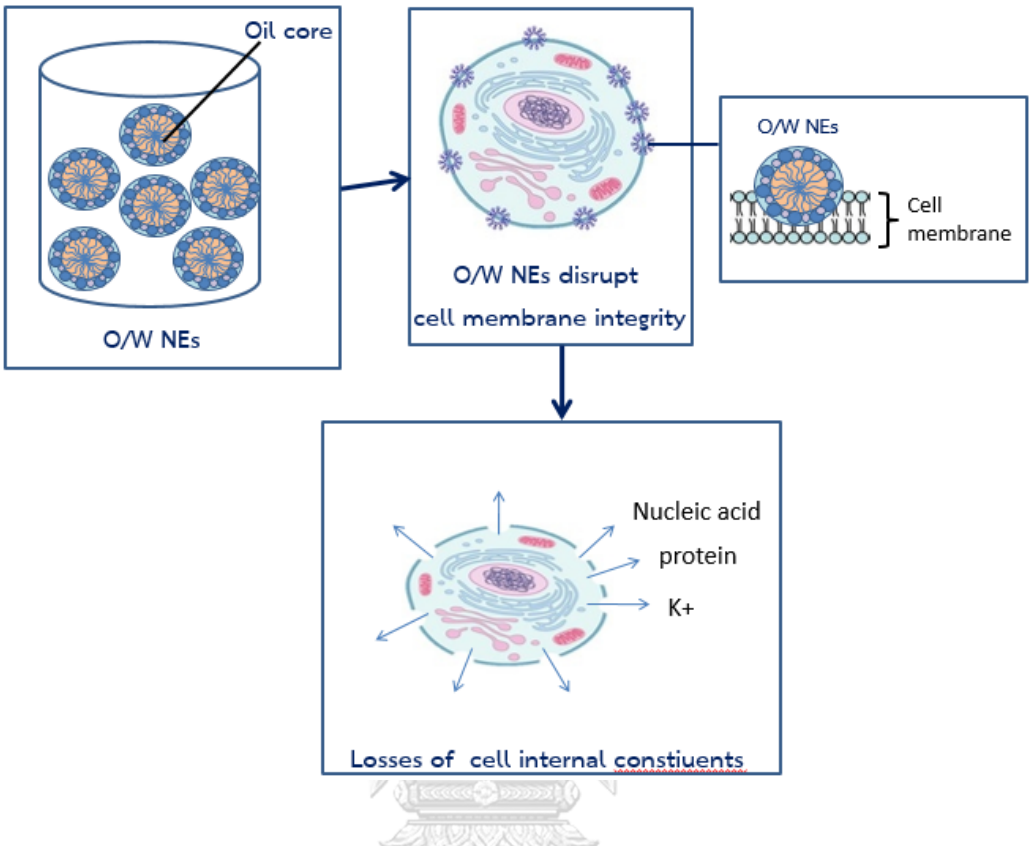

Figure 13 The mechanisms of nanoemulsions in antimicrobial activity. (Adapted from Moghimi et al., 2016)

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For the study of antimicrobial of NEs, food-borne pathogen may be concerned. A food-borne pathogens enter the body through consumption of contaminated food and water. Most symptoms of foodborne diseases are diarrhea, nausea, and vomiting. Life-threatening illness include infected bacterium in the bloodstream, paralytic illness (found in *Clostridium botulinum* toxin), kidney damage and kidney failure (found in Shiga toxin-producing *E. coli* (STEC) which most commonly identified as *E. coli* O_{157}). Most food-borne illness results from inadequate cooking,

cross-contamination, and poor personal hygiene (United State Department of Food and Drug Administration (US FDA), 2017). Information about the most common food-borne pathogens is shown in Table 1.

In this study, the preparation of nanoemulsions incorpotating olive oil, surfactant (Tween® 80) and co-surfactant and determination of antimicrobial activity of olive oil nanoemulsions will be conducted.

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Table 1 Information about the most common food-borne pathogens (US FDA*, 2017)*

Pathogens	Basics	Common sources	Symptoms
Pathogenic *Escherichia coli (E. coli)*	A group of bacteria that can produce toxic toxins.	Raw milk, untreated water, raw and undercooked meat, poultry, or shellfish	Severe stomach cramps, bloody diarrhea, and nausea. *E. coli* O_{157}:H_7 can cause permanent kidney damage which leads to death.
Staphylococcus aureus (S. aureus)	The bacteria are transferred to food by a human with poor hygiene. It is carried on the skin and the nasal passages of humans	Dairy products, salad, raw meat and poultry and human skin, infected cuts, pimples, noses and throats	Nausea, stomach cramps, vomiting, and diarrhea Watery diarrhea and cramps, or nausea and vomiting
Bacillus cereus (B. cereus)	This is a food-borne intoxication caused by consumption of enterotoxins	Uncovered milk, meats, vegetables, fish, rice, and starchy foods	

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Table 1 (cont.)

Pathogens	Basics	Common sources	Symptoms
Pseudomonas aeruginosa (P. aeruginosa)	An opportunistic pathogen	Can be isolated from soil and water and commonly associated with spoilage of food.	Alleged to cause gastroenteritis in human if ingested in large number
Candida albicans (C. albicans)	An opportunistic pathogenic yeast that is commonly found in the human gut flora	Poor dry storage of grains, oilseeds, fruits and vegetable leads to mould growth and production of mycotoxins	Hemorrhagic syndromes, liver tumors (Alflatoxins)
Aspergillus niger (A. niger)	An opportunistic pathogen	Have been found in meat, vegetable, dairy and grain products. Mycotoxins are produced at warm temperature (13-35 °C) and humidity	Mycotoxicosis and carcinogen

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CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Reagents

1. Extra virgin olive oil (Bertolli®, Italy)
2. Polyoxyethylene (20) sorbitan monolaurate or Tween® 20 (Acros, Belgium)
3. Polyoxyethylene (20) sorbitan monooleate or Tween® 80
   (Croda Inc., United Kingdom)
4. Dimethyl sulfoxide (AnalaR®, England)
5. Glyceryl monocaprylate or Imwitor® 308 (Sasol, Germany)
6. Mueller-Hinton agar (Difco™, USA)
7. Mueller-Hinton broth (Difco™, USA)
8. Sabouraud dextrose agar (Difco™, USA)
9. Sabouraud dextrose broth (Difco™, USA)
10. Gentamicin disc (Oxoid™, UK)
11. Amphotericin B for injection (Biolab, Thailand) Lot. J 169230

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3.1.2 Microbial culture

For antimicrobial study, the microbial culture were obtained from Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University. *Bacillus cereus* ATCC 11778 was obtained from Betagro Co. Ltd, Thailand.

Gram positive bacteria: *Staphylococcus aureus* ATCC 6538

Bacillus cereus ATCC 11778

Gram negative bacteria: *Escherichia coli* ATCC 25922

Pseudomonas aeruginosa ATCC 27853

Yeast: *Candida albicans* ATCC 10231

Mould: *Aspergillus niger* ATCC 16404

3.1.3 Instruments and Equipment

1. RM40 refractometer (Mettler Toledo, Switzerland)
2. High intensity ultrasonic processor VC/VCX 750 (Sonic, USA)
3. SV-10 sine-wave vibro viscometer (A&D, Japan)
4. Zetasizer Nano ZS (Malvern Instruments, UK)
5. Spectrophotometer 22RS (Labomed Inc, USA)
6. Microplate reader (Clariostar®, Germany)
7. Mixed cellulose esters membrane 0.22 μM (Merck Millipore Ltd., Ireland)
8. 96-well plate (Thermo Fisher Scientific, China)
9. Temperature controlled incubator (Memmert, Germany)

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3.2 Experimental design

The experiment was conceptually designed as shown in Figure 14. The NEs were prepared by low and high energy emulsification by which the concentrations of olive oil were in the range of 1% to 10% w/w. The NEs were determined for their size, size distribution, and zeta potential. The stability of NEs was tested after being stored in an air-tight container and protect from light for 180 days at three different temperatures, at (i) 25 °C ± 2 °C / 60% RH ± 5% RH, (ii) 30 °C ± 2 °C / 65% RH ± 5% RH and (iii) 40 °C ± 2 °C / 75% RH ± 5% RH, according to the International Conference on Harmonization (ICH) guideline (ICH, 2003). The NEs formulations containing high amount of oil phase and the droplet size of less than 100 nm were carried out for antimicrobial test. Disc diffusion and broth dilution methods were used for determination of minimum inhibitory concentration (MIC).

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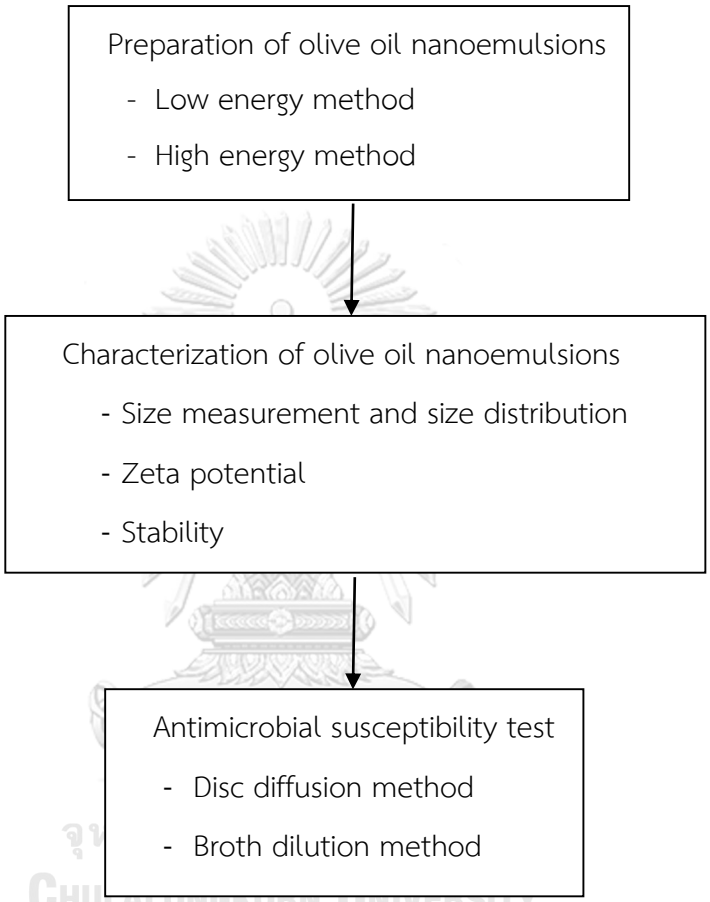

Figure 14 Experimental design of the study

3.3 Methods

3.3.1 Preparation of nanoemulsions containing olive oil

Oil-in-water nanoemulsions (NEs) were formulated using extra virgin olive oil (EVOO) as an oil phase, polyoxyethylene sorbitan monolaurate (Tween® 20) as a surfactant and glyceryl mono caprylate (Imwitor® 308) as a co-surfactant.

In order to obtain NEs, the preparation methods used were low-energy emulsification and high-energy emulsification. For the low-energy emulsification, oil-in-water nanoemulsions were formulated at varied concentrations of oil (EVOO), Tween® 20 and Imwitor® 308. NEs formulation in the absence of a co-surfactant was also prepared. For the formulation containing a co-surfactant, a weight ratio of surfactant to co-surfactant at 2:1 was used (Table 2). NEs were prepared by weighing desired amounts of the ingredients and adding water to adjust the final weight. The mixture was heated up to 70-75 °C for approximately 10 min. Then the samples were cooled down to the ambient temperature with stirring throughout. The samples were kept in airtight containers. For the experiment on characterization of NEs, the samples were freshly prepared and used within 24 h.

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Table 2 Compositions of the NEs formulations

NEs code	Compositions (%w/w)		
	EVOO	Surfactant	Co-surfactant
• **Low energy method**			
Tw5 O1	1	5	-
Tw10 O1	1	10	-
Tw20 O1	1	20	-
Tw30 O1	1	30	-
T5 O1	1	5	2.5
T5 O2	2	5	2.5
T10 O1	1	10	5
T10 O2	2	10	5
T20 O1	1	20	10
T20 O2	2	20	10
T30 O1	1	30	15
T30 O2	2	30	15
• **High energy method**			
T20 O5	5	20	10
T30 O5	5	30	15
T30 O10	10	30	15

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For high-energy emulsification, the coarse emulsions were prepared by weighing the desired amounts of the ingredients and stirring. Then water was added to adjust the final weight. The coarse emulsions were subjected to emulsify by ultrasonication. The diameter of the ultrasonic probe tip is 3 mm. The parameters used in the ultrasonic treatment are ultrasound amplitude (20-35%), treatment time (5-20 min) and input energy (1250-2500 J) (Salvia-Trujillo, 2014). The samples obtained from both methods were kept in airtight containers at different storage conditions as in ICH guideline for stability test (ICH, 2003). The NEs were further characterized by the following experiments.

3.3.2 Characterization of olive oil nanoemulsions

3.3.2.1 Measurement of size and size distribution

Droplet sizes of NEs were measured by dynamic light scattering (DLS) using non-invasive back scattering technology. The experiment was performed at 25 °C on Zetasizer Nano ZS equipped with a helium-neon laser operating at 633 nm. The NEs samples were diluted to surfactant concentrations at 1% w/w so as to avoid the interparticulate interaction and multiple scattering (Warisnoicharoen, Lansley, and Lawrence, 2000). Prior to size measurement, all sample solutions were clarified by ultrafiltration through a 0.22 μm cellulose acetate Millipore membrane. The width of size distribution is referred as a polydispersity index (PDI) which is in range of 0 to 1.

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PDI value greater than 1 indicates that the distribution of nanoemulsion is polydispersity and DLS is unsuitable for the sample.

3.3.2.2 Determination of refractive index

The value of the refractive index (RI) was used as a parameter for a light scattering experiment. The principle of the refractometer is based on the physical of light refraction. In optically dense medium, light passes slow down and speed up when it passes into less optically dense medium. The change in speed is accompanied by a change in direction, and angle of incidence. This angle which occurs that the refractometer measures. The RI measurement was performed by RM40 refractometer with a built-in solid state thermostat. The NEs samples were diluted to 5% w/w surfactant before test. Minimal volume of a test sample was dropped on a measuring cell and the value was read at a controlled temperature of 25 °C.

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3.3.2.3 Determination of viscosity

The NEs prepared at different concentrations of surfactant were studied for viscosity change. The viscosity of NEs was determined using SV-10 sine-wave vibro viscometer at room temperature. The SV series has 2 thin sensor plates that are driven with electromagnetic force at the same frequency by vibrating at constant sine-wave vibration in reverse phase like a tuning fork. The electromagnetic drive controls the vibration of the sensor plates to keep in constant amplitude. The

driving electric current, which is exciting force, will be detected as the magnitude of viscidity produced between the sensor plates and the sample fluid. The coefficient of viscosity is obtained by the correlation between the driving electric current and the magnitude of viscidity.

3.3.2.4 Zeta potential measurement

The electrophoretic mobility of oil droplets, reported as a zeta potential, was measured by laser droppler electrophoresis using a Zetasizer Nano ZS. This device performs the surface electrical charge of the nanodroplets and the zeta potential was then calculated using Henry's equation (Malvern, 2016).

3.3.3 Stability

Since, low energy method was the method which incorporated low amount of olive oil (1-2%) into NEs. High energy method was selected to study the stability test due to it can incorporate more oil (5-10%). Stability of olive oil NEs was evaluated by any changes in particle size, PDI and zeta potential after storage for 14, 28, 90 and 180 days at (i) 25 °C ± 2 °C / 60% RH ± 5% RH, (ii) 30 °C ± 2 °C / 65% RH ± 5% RH and (iii) 40 °C ± 2 °C / 75% RH ± 5% RH in the temperature-controlled incubator.

From above experiments, the NEs systems that contained the maximum capacity of oil encapsulation with a lesser amount of surfactant phase and maintained better stability were selected to test for antimicrobial susceptibility.

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3.4 Antimicrobial susceptibility test

The antimicrobial activity of olive oil NEs was evaluated by *in vitro* growth inhibition of microorganism by disc diffusion method and broth dilution method. For comparison, the antimicrobial activity of solely olive oil was also studied. All antibacterial susceptibility assays were carried out under aseptic technique and performed at least in triplicate.

The bacteria were cultured at 37 ℃ on a Mueller-Hinton agar. Before experimental use, the cultures were sub-cultured in slant agar medium, incubated for 24 h. The fungi were cultured at room temperature in a Sabouraud dextrose agar and sub-cultured in slant agar medium for 48 h before the experiment.

3.4.1 Disc diffusion method

Disc diffusion method is an antimicrobial susceptibility test using Kirby-Bauer technique (Gokmen et al., 2014). This method determines antimicrobial activities by measuring zone of inhibition (mm) against the test organisms. The test media were Mueller-Hinton agar (MHA) for bacteria and Sabouraud dextrose agar (SDA) for fungi.

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After bacterial culture, each of the organism was standardized and matched a 0.5 McFarland (approximately 1.5×10^8 CFU/mL). The test organisms were inoculated with steriled swab on the surface of the agar plate. The steriled discs (13 mm diameter) were impregnated with 20 µL of samples and then placed on the inoculated agar and incubated at 37 ℃ for 24 h for bacteria (Laincer et al., 2014), 30 ℃ for 24 h for yeast

and 30 °C for 48 h for mould (Lorian, 1996). For a negative control disc, the samples were replaced by sterile water. A positive control is a disc (6 mm diameter) containing an antibiotic drug (gentamicin 10 µg for bacteria, amphotericin B 5 µg for fungi) (Lass-Florl, Perkhofer, and Mayr, 2010). The solution of surfactant and co-surfactant or micelle solution (no oil added) at the same concentration of NEs was also tested for comparison.

For the antimicrobial test of EVOO, EVOO at the same amount used in the NEs formulation was dissolved in 1% w/v dimethyl sulfoxide (DMSO) (i.e. 5% EVOO in 1% w/v DMSO and 10% EVOO in 1% w/v DMSO) before the experiment. It was noted that DMSO (1% w/v) had no antimicrobial activity (Sookprasert and Suptang, 2012).

3.4.2 Broth dilution method

In order to determine minimum inhibitory concentration (MIC) of olive oil and NEs containing olive oil, the broth dilution method was used. The MICs value (mg/mL) is determined by the lowest concentration of samples that shows no visible microbial growth (Lorian, 1996). The concentration that was the MIC of the tested samples was further determined for the minimum bactericidal concentration (MBC) and the minimum fungicidal concentration (MFC). MBC and MFC are the lowest concentrations of an antimicrobial that kill the microbial growth of 99.9% (Xue, Michael Davidson, and Zhong, 2015).

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3.4.2.1 Antimicrobial test of NEs (Martinez-Gutierrez et al., 2010)

Not only EVOO NEs formula were selected to determine MIC but also the corresponding micellar solution. Briefly, the bacteria were inoculated for 18 h before susceptibility test and then was suspended with 0.9% normal saline to standardize and was adjusted to match a 0.5 McFarland turbidity (approximately $1.5x10^8$ cells/mL). The samples were sequentially diluted in a 96-well plate containing Mueller-Hinton Broth (MHB) medium for bacteria and Sabouraud dextrose broth (SDB) medium for fungi. In a 96-well plate, each well contained 100 µL of a serial dilution of the sample and 100 µL of bacterial suspension. The dilutions of EVOO NEs and micelle were serially made for each consecutive well from 80% to 2% (Table 3). The final volume of each well was 200 µL. The final concentration of oil in EVOO NEs or co-surfactant (CoSAA) are shown in Table 4. The plate was covered and incubated at 37 °C for 24 h for bacteria, 30 °C for 24 h for yeast and 30 °C for 48 h for mould. The well containing 100 µL of bacterial suspensions and 100 µL of ultrapure water was used as a negative control. Blank control well contained 100 µL of media (without culture) and 100 µL of various concentrations of NEs (for sample) or water (for negative control). The results were determined by absorbance measurement after cell incubation using the microtiter plate reader at 620 nm for bacteria and 490 nm for fungi. The cell growth inhibition was calculated as in equation (1). The absorbances of the sample (A_s) or the negative control (A_c) were substracted from the blank control (A_{bk}). The MIC value was defined as the NEs concentration that showed 100% cell inhibition (Figure 15).

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$$\% \text{ cell inhibition} = \frac{(A_c - A_{bk}) - (A_s - A_{bk})}{(A_c - A_{bk})} \times 100 \qquad (1)$$

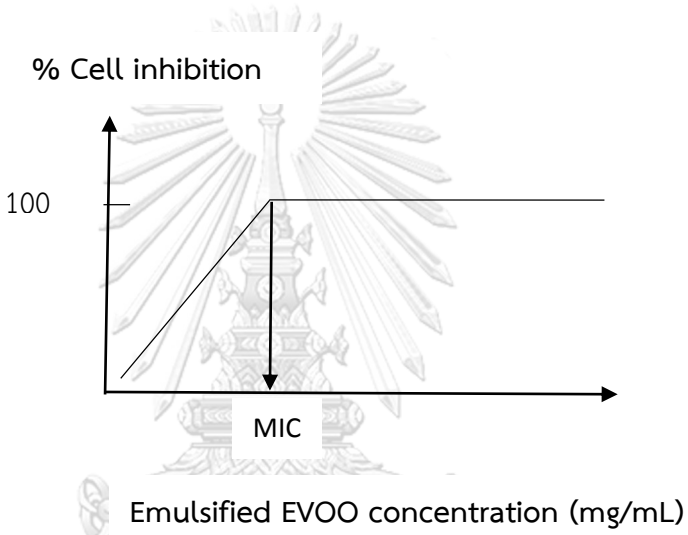

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Figure 15 A plot of graph for determination of MIC of EVOO NEs

Table 3 Compositions of test samples in each well of a 96-well plate

Volume (µl)	Well no. 1	2	3	4	5	6	7	8	9	10	11	12
EVOO NEs	80	70	60	50	40	30	20	10	8	4	2	-
Ultrapure water	20	30	40	50	60	70	80	90	92	96	98	100
Culture suspension	100	100	100	100	100	100	100	100	100	100	100	100
Final conc. of EVOO NEs or micelle (mg/mL)	400	350	300	250	200	150	100	50	40	20	10	0

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Table 4 Final concentrations of EVOO (in NEs) and co-surfactant (in micelle) in 96-well plate.

	Final conc. of EVOO (mg/mL)		Final conc. of CoSAA (mg/mL)	
Well No.	**5 % EVOO NEs**	**10% EVOO NEs**	**Micelle T20**	**Micelle T30**
1	20.0	40.0	40.0	60.0
2	17.5	35.0	35.0	52.5
3	15.0	30.0	30.0	45.0
4	12.5	25.0	25.0	37.5
5	10.0	20.0	20.0	30.0
6	7.5	15.0	15.0	22.5
7	5.0	10.0	10.0	15.0
8	2.5	5.0	5.0	7.5
9	2.0	4.0	4.0	6.0
10	1.0	2.0	2.0	3.0
11	0.5	1.0	1.0	1.5
12	0.0	0.0	0.0	0.0

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. To determine the MBC and MFC of EVOO NEs, the minimal concentration of EVOO NEs concentration that showed no growth of microbial (MIC) was further spread on MHA plates for bacteria or SDA plates for fungi. Colonial growth was determined after 24-h incubation of agar plate at 37 °C for bacteria, 30 °C for 24 h for yeast and 30 °C for 48 h for mould.

3.4.2.2 Antimicrobial test of extra virgin olive oil

Antimicrobial test of olive oil was performed with the same method of NEs, except that the olive oil was dissolved in 1% w/v DMSO before the experiment. The experiment was done in a test tube instead of a 96-well plate. The composition of the test sample (a final volume of 2 mL) is shown in Table 5. A final volume was adjusted to 2 mL by using Tween® 80 since Tween® 80 can help solubilize oil prior to test without antimicrobial activity (Mann and Markham, 1998).

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Table 5 Compositions of test samples of EVOO (final volume of 2 mL).

EVOO (mL)	Tween® 80 (mL)	Culture suspension (mL)	EVOO (%)
1.50	-	0.50	**75**
1.40	0.10	0.50	**70**
1.20	0.30	0.50	**60**
1.00	0.50	0.50	**50**
0.94	0.56	0.50	**47**
0.92	0.58	0.50	**46**
0.91	0.59	0.50	**45.5**
0.90	0.60	0.50	**45**
0.86	0.64	0.50	**43**
0.80	0.70	0.50	**40**

3.5 Statistical analysis

Each experiment was performed at least in triplicate and all values were reported as means ± SD. The relationship between droplet sizes and surfactant concentrations were analyzed using a linear regression and the square of Pearson's correlation coefficient (r^2) was observed. A linear regression equation obtained was used to calculate the particle size of NEs at an infinite dilution. Statistical Analysis Software version 23.0 (IBM® SPSS Statistics) was used to determine differences between groups by one-way analysis of variance (ANOVA), followed by Bonferroni's post hoc test at a significant level of $p < 0.05$.

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CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 Characterization of olive oil nanoemulsions

4.1.1 Low energy method

4.1.1.1 Size measurement and analysis

The hydrodynamic diameters of the dilute EVOO NEs and the corresponding PDI were determined by dynamic light scattering (DLS). The DLS results are illustrated in Figures 16 - 17. The preparation of NEs was spontaneous emulsification method which depended upon the compositions of the system to spontaneously form nanoemulsion. This study, oil-in-water NEs were prepared using a food-grade surfactant, Tween® 20, and co-surfactant, Imwitor® 308. The long chain triglyceride, extra virgin olive oil (EVOO), was incorporated in the NEs. NEs formation in the presence of Imwitor® 308 clearly showed the smaller particle size than the systems without the co-surfactant i.e.Tw10 O1 (301.80 ± 33.80 nm) compared to T10 O1 (127.77 ± 2.25 nm) at 1% W/W surfactant. NEs prepared using Imwitor® 308 and incorporated EVOO at 1% w/w had the droplet sizes in a range of 50-150 nm (Figure 16). Upon increasing the percentage of oil from 1% to 2% w/w, particle sizes of EVOO NEs were slightly different from those containing the lower amount of oil except for EVOO NEs formulated using 5% w/w surfactant which showed a dramatic increase in size. Most of the PDI values of EVOO NEs did not exceed 0.7, suggesting the rather narrow size distribution of NEs (Figure 17) (Malvern, 2016).

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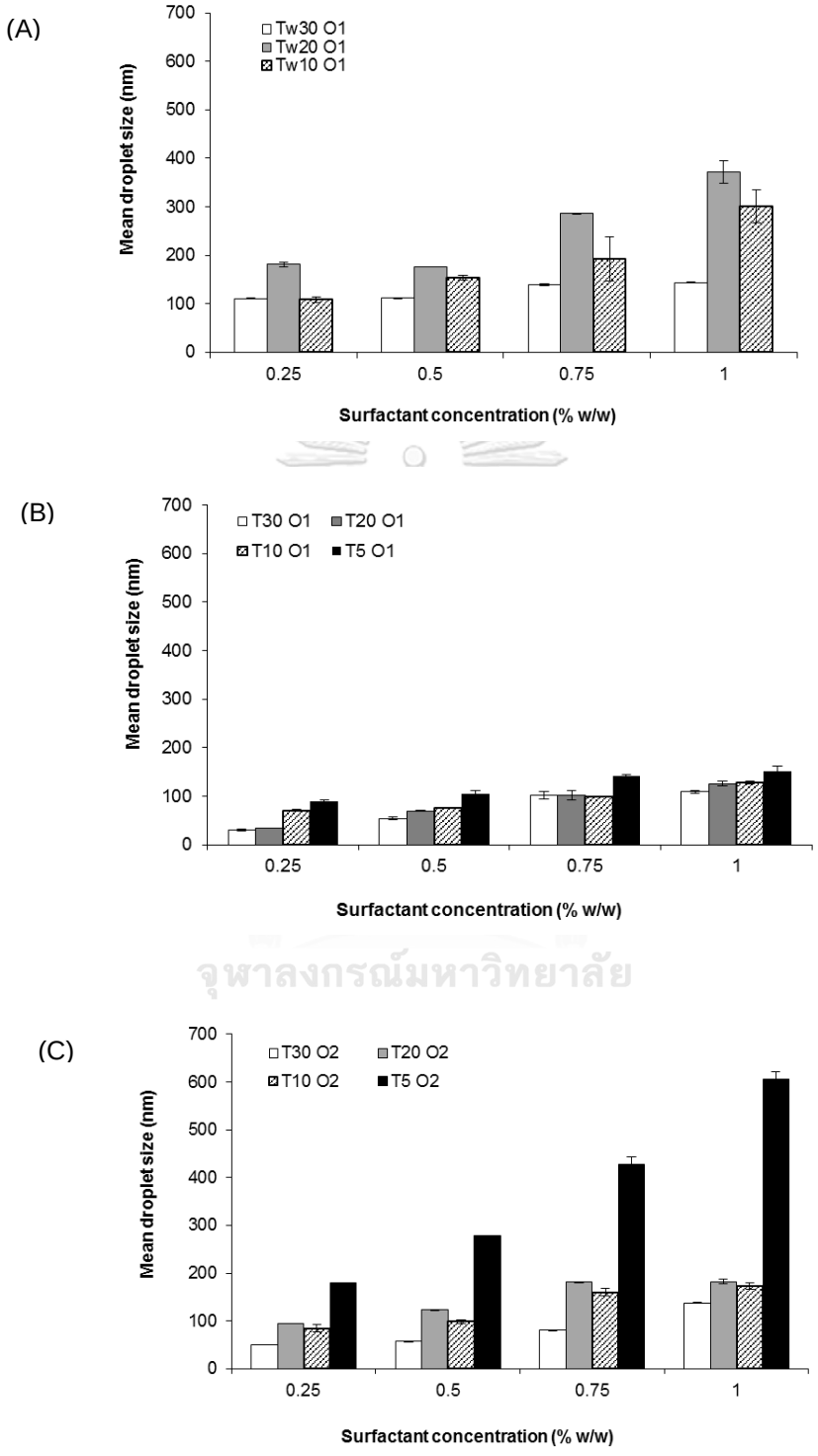

Figure 16 Mean diameter (nm) of EVOO NEs droplet after dilution to different surfactant concentrations.

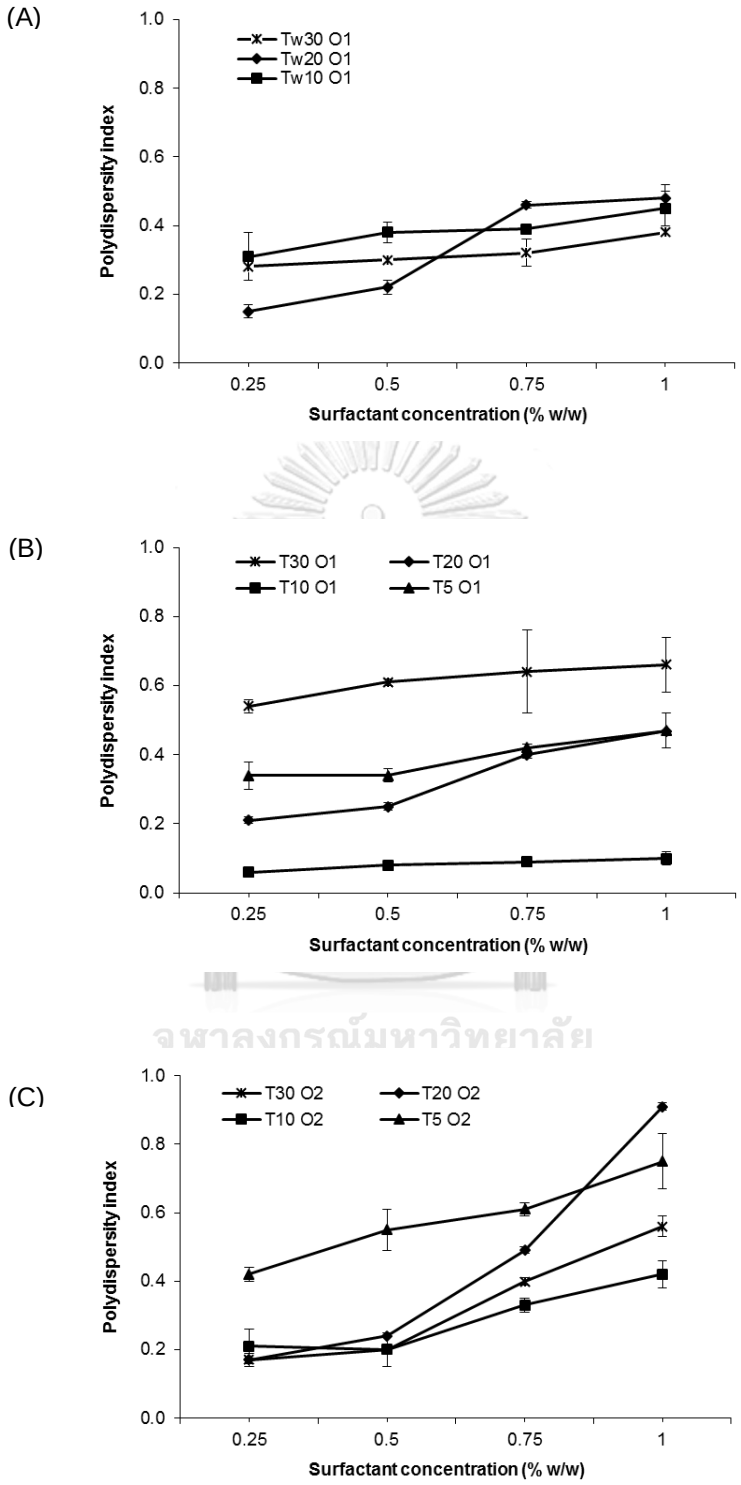

Figure 17 Polydispersity index of EVOO NEs droplet after dilution to different surfactant concentrations.

The results indicated the requirement of any co-surfactants especially at a lower amount of surfactant in order to form NEs with smaller droplet sizes. The co-surfactant can help lower an interfacial tension and increase the fluidity of the interfacial layer, thus allowing greater penetration of the oil (Wooster et al., 2016). A two-fold increase in oil amount resulted in larger NEs droplets. An increase in oil molecules inside the droplet core brings about more surfactant molecules needed to form an interfacial film around the droplet (Yi, Zhang, Liang, Zhong, and Ma, 2015).

The size measurement was normally make done upon dilution the NEs to 1% surfactant to prevent interparticle interaction. However, in order to test whether NEs were definitely in nano-scale size, determination of NEs at the infinite dilution was carried out.

The data from DLS results were further analyzed to obtain a size of NEs at infinite dilution using a linear regression equation; $Y = a + bX$, where Y, X and b are droplet sizes, surfactant concentration, and slope, respectively. The y-intercept (a) was considered as a size of NEs at infinite dilution. The parameter for regression analysis is shown in Table 6 and the sample of the regression line is shown in Figure 18.

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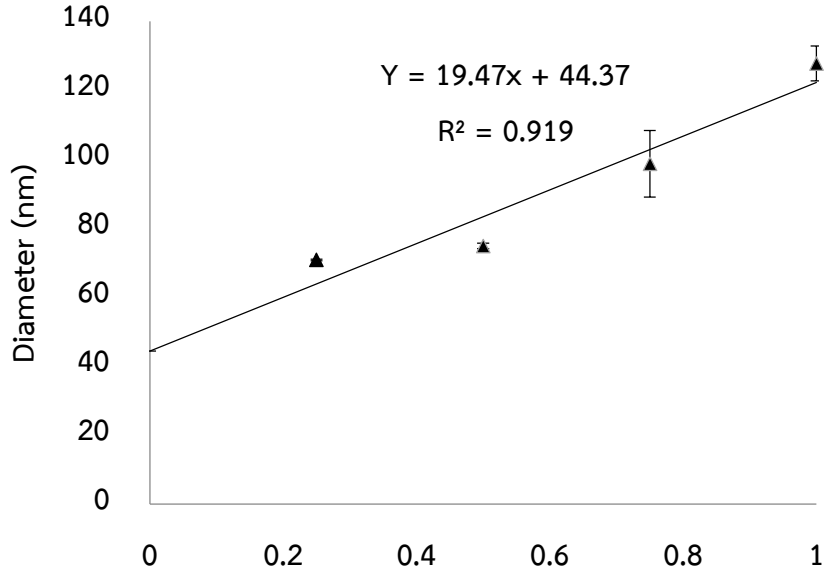

Figure 18 A sample of the regression line in T10 O1 system.

Table 6 Droplet size of nanoemulsions derived from a linear regression equation. The r^2 is a coefficient of determination.

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NEs	Slope	r^2	size (nm)	NEs	Slope	r^2	size (nm)
Tw10 O1	61.86	0.9338	34.23	T30 O1	28.68	0.9364	2.02
Tw20 O1	68.32	0.8856	83.37	T5 O2	142.93	0.9848	15.70
Tw30 O1	12.46	0.8644	95.20	T10 O2	32.46	0.9187	47.76
T5 O1	22.43	0.9474	66.03	T20 O2	32.14	0.9020	64.78
T10 O1	19.47	0.9190	44.37	T30 O2	28.78	0.8663	8.98
T20 O1	30.85	0.9929	5.52				

The calculated particle size at infinite dilution of dilute EVOO NEs from a regression analysis indicated that the particle diameters of EVOO NEs were less than 100 nm. A decline in regression line with surfactant concentrations indicated the presence of some extent of attractive interaction among particles (Malvern, 2016).

In this study, the particle size was characterized from Zetasizer Nano ZS. However, the size analysis of DLS results relies on the assumption that particles are spherical or near-spherical in shape and monodisperse (Warisnoicharoen et al., 2000).

4.1.1.2 Refractive index and viscosity

The values of refractive indices of NEs at varying concentrations of Tween® 20 (surfactant), Imwitor® 308 (co-surfactant) and extra virgin olive oil (EVOO) are shown in Table 7. The refractive indices of NEs fell in the range of 1.3-1.4. For the viscosity study, the dilute NEs containing 1% w/w EVOO stabilized by Tween® 20 and Imwitor® 308 were measured and the result is tabulated in Table 7.

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From the result, it has been found that the higher values of RI were obtained with an increment of oil concentration. Increasing amount of surfactant and co-surfactant also caused a higher number of RI. For the viscosity, NEs were found to be slightly more viscous than the dispersed aqueous phase (1.08 mPa·s).

Table 7 Values of refractive index and viscosity of extra virgin olive oil nanoemulsions. The surfactant (SAA), cosurfactant (CoSAA) and oil are Tween® 20, Imwitor® 308 and extra virgin olive oil (EVOO), respectively.

Weight ratio of EVOO: SAA: CoSAA	NEs coding	Refractive index [a]	Viscosity (mPa·s)
1:5:0	Tw5 O1	1.340 ± 0.001	ND
1:10:0	Tw10 O1	1.346 ± 0.001	ND
1:20:0	Tw20 O1	1.360 ± 0.001	ND
1:30:0	Tw30 O1	1.375 ± 0.001	ND
1:5:2.5	T5 O1	1.343 ± 0.001	1.24
1:10:5	T10 O1	1.352 ± 0.002	1.27
1:20:10	T20 O1	1.373 ± 0.001	1.32
1:30:15	T30 O1	1.393 ± 0.001	1.41
2:5:2.5	T5 O2	1.343 ± 0.001	ND
2:10:5	T10 O2	1.354 ± 0.001	ND
2:20:10	T20 O2	1.375 ± 0.001	ND
2:30:15	T30 O2	1.395 ± 0.001	ND

[a] mean ± S.D. (n=3). ND = not determined.

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The use of low-energy method could incorporate less amount of olive oil (1-2% olive oil). In order to incorporate more oil, more energy and amount of surfactant are required to generate the NEs. Hence, the NEs were further prepared by high energy method. Moreover, in high energy method Tween® 80 was used as surfactant instead of Tween® 20 due to it conducted the smaller size of droplets of EVOO NEs (APPENDIX A).

4.1.2 High energy method

4.1.2.1 Measurement of size and size distribution

The coarse emulsion (T20 O5) was freshly prepared prior to ultrasonication. The parameters, namely ultrasound amplitude, treatment time and input energy were varied to obtain the NEs. The effect of each parameter on NEs formation was determined at constant value of other parameters.

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For study of input energy parameter, ultrasonic amplitude was fixed at 20% and treatment time of 15 min while input energy was varied in the range 1250-2500 J. The result is tabulated in Table 8.

Table 8 Size, PDI and zeta potential of NEs with varying input energy (J)

Input energy (J)	Size (nm)	PDI	Zeta potential (mV)
1250	43.18 ± 7.75	0.35 ± 0.01	-3.35 ± 0.34
1500	34.66 ± 0.41	0.40 ± 0.02	-4.35 ± 0.83
1750	51.53 ± 0.75	0.27 ± 0.01	-1.97 ± 0.23
2000	41.90 ± 0.15	0.28 ± 0.00	-1.61 ± 0.06
2250	33.99 ± 0.46	0.31 ± 0.00	-2.42 ± 0.26
2500	46.29 ± 0.61	0.71 ± 0.03	-5.15 ± 2.01

Values of mean ± S.D., (n=3)

From the result (table 8), the input energy 2250 J was conducted the smallest droplet size of EVOO NEs and then use as an input energy parameter in the next study of treatment time parameter, the 25% amplitude and input energy of 2250 J were constant while the treatment time was varied from 5-20 min to obtain the NEs. The result is shown in Table 9. At 5 min of treatment time, the smallest EVOO NEs were obtained and then use as a time parameter for amplitude study. For the study of amplitude (%), the time and input energy were fixed constant at 5 min and 2250 J, respectively. The amplitude was varied from 20-35%. The result is shown in Table 10. In all NEs, the zeta potential values were approximately very low in charges (slightly the negative value). Since the surfactant and co-surfactant used were non-ionic, the

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majority of charges obtained for zeta-potential was possibly from the oil molecule (Majeed et al., 2016).

Table 9 Size, PDI and zeta potential of NEs with varying treatment (min)

Treatment time (min)	Size (nm)	PDI	Zeta potential (mV)
5	26.90 ± 0.46	0.28 ± 0.02	-2.61 ± 0.55
10	29.17 ± 0.25	0.24 ± 0.01	-2.42 ± 0.26
15	29.92 ± 0.36	0.31 ± 0.04	-2.48 ± 0.58
20	32.03 ± 0.51	0.33 ± 0.00	-1.94 ± 0.20

Values of mean ± S.D., (n=3)

Table 10 Size, PDI and zeta potential of NEs by varies amplitude (%)

Amplitude (%)	Size (nm)	PDI	Zeta potential (mV)
20	46.07 ± 2.15	0.38 ± 0.06	-3.62 ±0.24
25	20.04 ± 0.08	0.37 ± 0.01	-2.08 ± 0.20
30	30.27 ± 0.48	0.24 ± 0.00	-2.32 ± 0.49
35	28.77 ± 0.35	0.24 ± 0.00	-2.03 ± 0.17

Values of mean ± S.D., (n=3)

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From the data above (Tables 8-10; Appendix B), the optimized ultrasonic parameters for NEs preparation which yielded the smaller size, low PDI and short treatment time were amplitude 25%, input energy 2250 J and treatment time of 5 min. The optimized condition obtained was then used to prepare NEs at high amount of oil in high energy method. The EVOO NEs composition which yielded the smallest droplet size (29.53 ± 0.10 nm) contained 30% Tween® 80, 15% Imwitor® 308 and 5% olive oil. The average size of T20 O5, T30 O5, and T30 O10 were 32.32, 29.53 and 78.99 nm, respectively (Table 11).

Table 11 Size, PDI and zeta potential of NEs prepared by ultrasonication

NEs system	Size (nm)	PDI	Zeta potential (mV)
T20 O5	32.32 ± 0.44	0.37 ± 0.02	-3.62 ± 0.34
T30 O5	29.53 ± 0.10	0.22 ± 0.01	-1.59 ± 0.17
T30 O10	78.99 ± 2.91	0.63 ± 0.10	-3.19 ± 0.36

Values of mean ± S.D., (n=3)

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4.2 Nanoemulsion stability

NEs systems, T20 O5, T30 O5 and T30 O10, were selected for stability test since this three formulas contain the maximum capacity of oil with the lesser amount of surfactant phase. Stability evaluation of NEs was analyzed from a change in size, PDI, and zeta potential after storage at three conditions for 180 days according to ICH guideline. The results are shown in Figures 19-21 and appendix C. From the results,

NEs which were stored at 25 °C ± 2 °C / 60% RH ± 5% RH for 6 months still had the droplet diameters in nano-scale size of less than 200 nm. Droplets sizes of NEs were growing over storage time. Droplet diameter of NEs were increased about 20 - 25 nm during 90 days of storage for all formulations. Concentrations of surfactant and oil seemed to be factors to maintain the stability of NEs. From the result, NEs containing higher concentration of surfactant (T30 O5) has a smaller size than T20 O5 upon storage. T30 O10 had a bigger size than T30 O5 due to containing higher amount of oil but equal concentration of surfactant phase. In this study, T30 O5 was the most stable formula because the compositions of surfactant phase were enough to maintain the interfacial force between immisible liquids. The optimum amount of surfactants play a role in lowering the interfacial tension between the layers, thus preventing coalescence, gravitational separation and flocculation (McClements, 2011). The PDI values of three formulas were approximately less than 1 indicating that the size distribution of NEs was monodisperse. However, upon storage, the NEs system showed an increase in PDI indicating the instability of the system. In general, zeta potential values of stable NEs are among ± 30 mV due to electrostatic stabilization (Evans and Napper, 1973). In this study, the NEs were generated from non-ionic surfactant and co-surfactant which caused less in zeta potential value. The stabilize mechanism of non-ionic NEs system is steric stabilization (Tuntarawongsa and Phaechamud, 2013 (APPENDIX D)). The NEs, T20 O5, T30 O5 and T30 O10, were further studied for antimicrobial activity.

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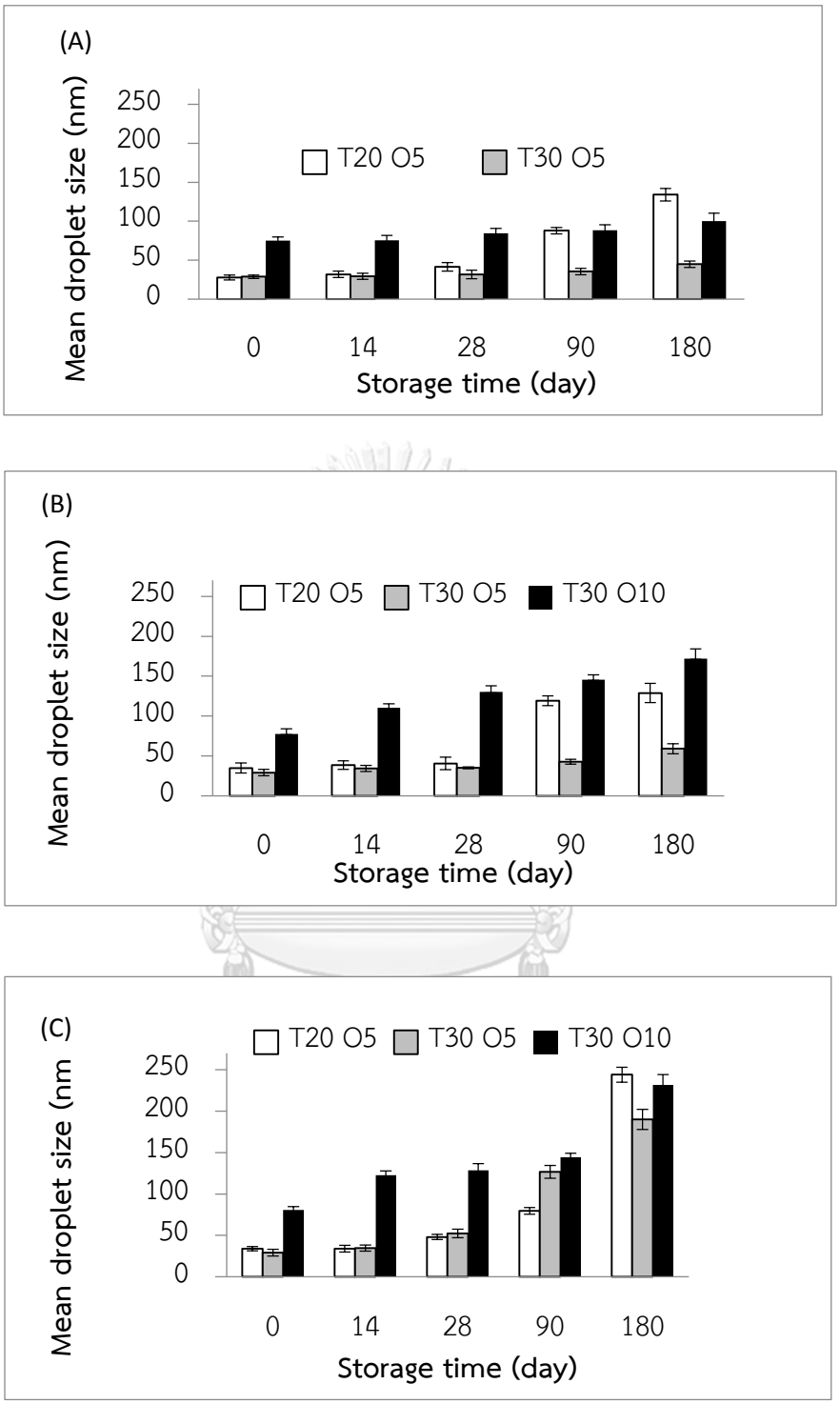

Figure 19 Mean droplet size of EVOO NEs after storage for 0, 14, 28, 90 and 180 days at (A) 25 °C (B) 30 °C and (C) 40 °C.

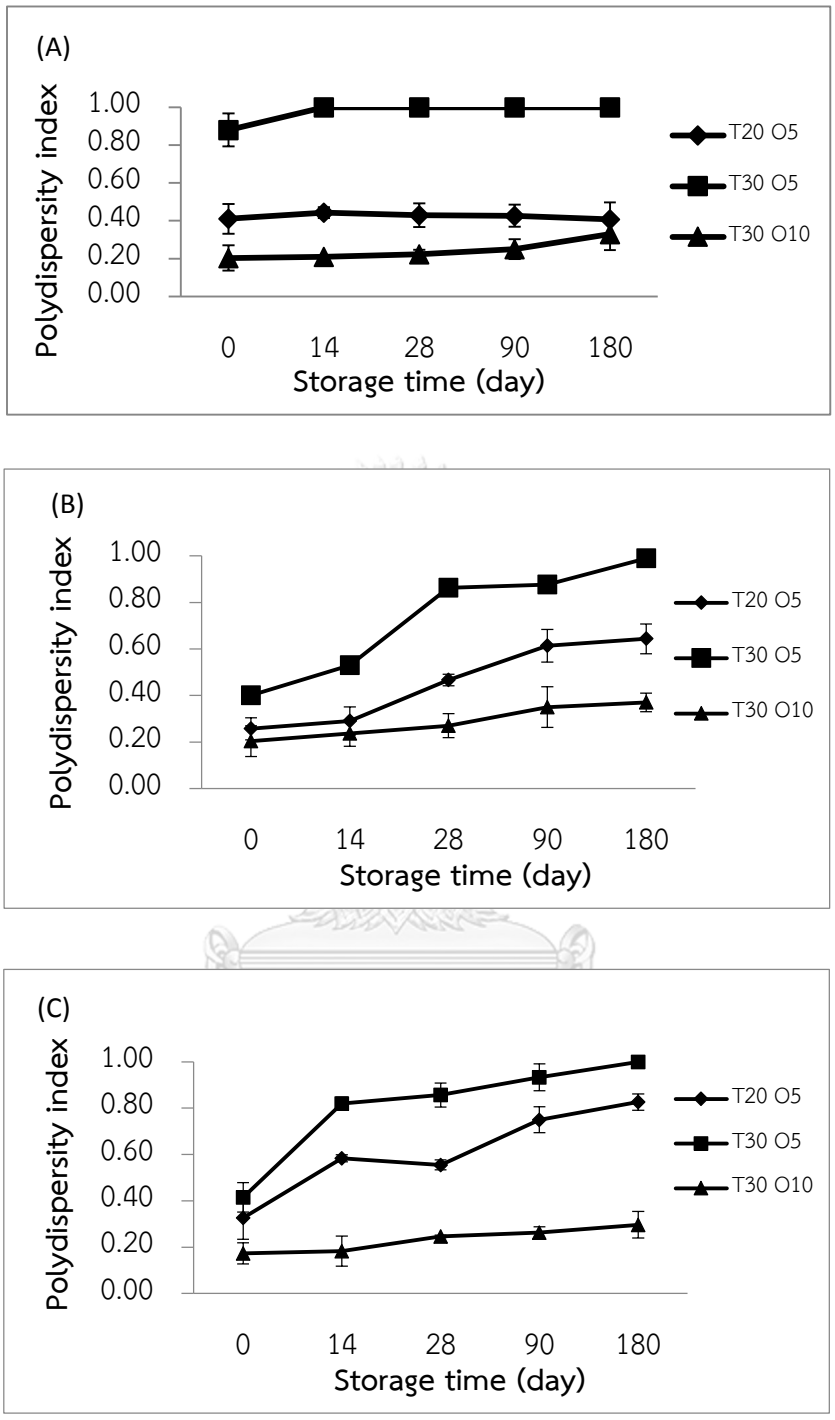

Figure 20 Polydispersity index of EVOO NEs after storage for 0, 14, 28, 90 and 180 days at (A) 25 °C (B) 30 °C and (C) 40 °C

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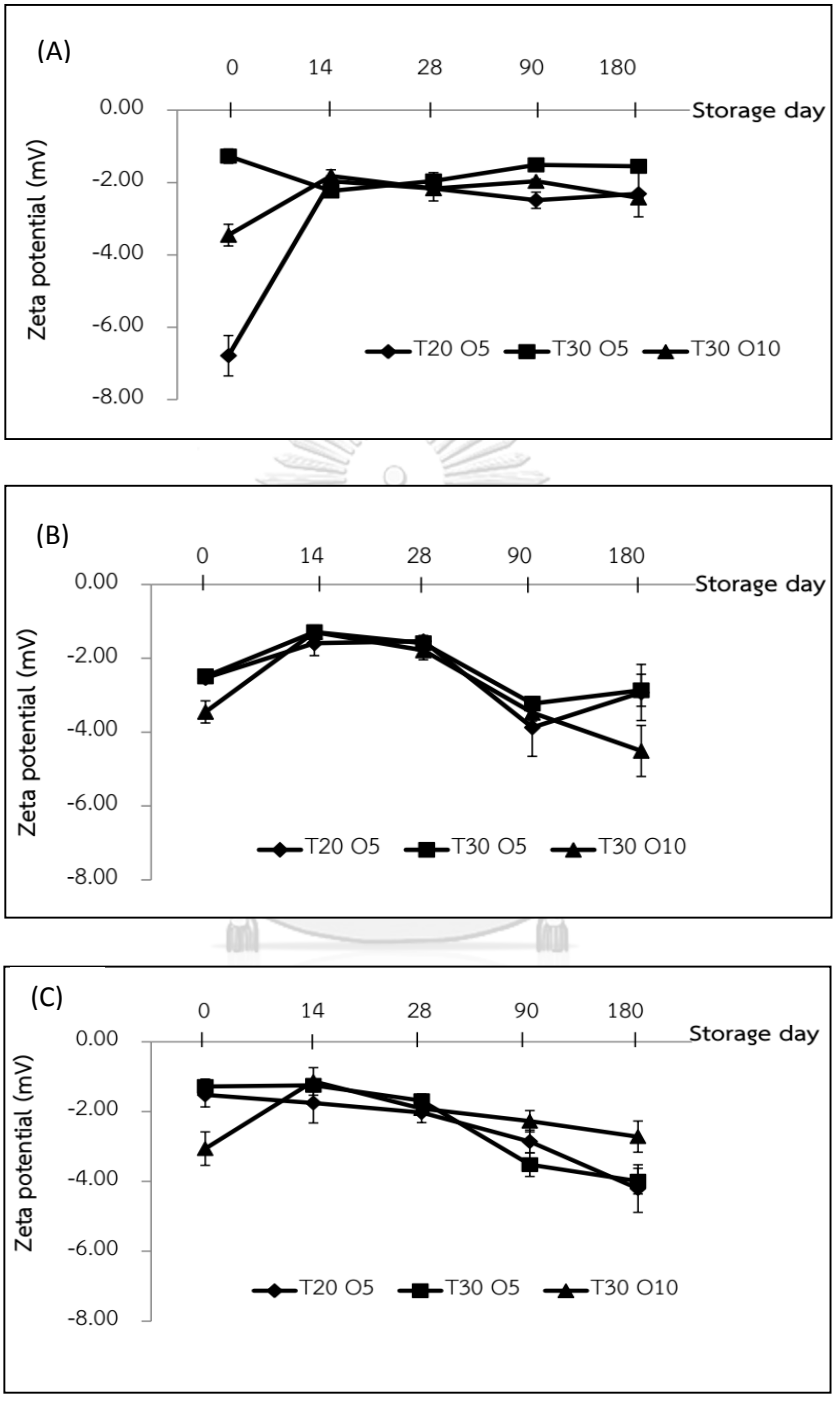

Figure 21 Zeta potential of NEs after storage for 0, 14, 28, 90 and 180 days at (A) 25 ℃ (B) 30 ℃ and (C) 40 ℃

The effect of zeta potential on different temperature was observed. In general, NEs had an electrostatic repulsion for stabilized the system. In non-ioinic system this electrostatic repulsion is less than steric stabilization. There is the slightly change of the zeta potential. An increase in coalescence rate and a decrease in kinetic equilibrium at high temperature cause an incidence of droplet aggregation and polydispersity (Hashtjin and Abbasi, 2015).

4.3 Antimicrobial activity

This study determined antimicrobial activity of olive oil NEs using disc diffusion and broth dilution methods. Inoculation contained approximately 2.24 - 5.96 x 10^7 CFU/mL of *P. aeruginosa* ATCC 27853, 2.46 – 3.4 x 10^6 CFU/mL of *S. aureus* ATCC 6538, 2.46 – 3.37 x 10^6 CFU/mL of *B. cereus* ATCC 11778, 2.20 - 3.37 x 10^6 CFU/mL of *E. coli* ATCC 25922, 2.56 - 3.26 x 10^6 CFU/mL of *A. niger* ATCC 16404 and 1.8 – 2.53 x 10^6 CFU/mL of *C. albicans* ATCC 10231.

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In the first part of the antimicrobial test, disc diffusion method was used to evaluate the antimicrobial activity of the compositions in EVOO NEs. After that, broth dilution method was used to determine the MIC, MBC and MFC values of EVOO NEs and their corresponding to micelles.

4.3.1 Disc diffusion method

4.3.1.1 Antimicrobial test of olive oil and other ingredients

Antimicrobial test of EVOO, Tween® 80 and Imwitor® 308 was done against *S. aureus* ATCC 6538 and *C. albicans* ATCC 10231 to represent bacteria and fungi. The result were EVOO, 20% Tween® 80 and 30% Tween® 80 showed no zone of inhibition except the disc containing co-surfactant (disc 2 and 3) and a mixture of co-surfactant and surfactant (disc 5 and 8) (Figure 22). Since the EVOO was insoluble in the media, so it showed no clear zone (disc 6). It was in a necessity for adding an emulsifier or solvent into the medium to ensure that the test sample was exposed to the microorganism (Mann and Markham, 1998). In this study, the 1% DMSO was used to dissolve olive oil and the maximum concentration of the oil dissolved was 10% (disc 10), however it showed no antimicrobial activity against *S. aureus* ATCC 6538 and *C. albicans* ATCC 10231. Since the bacteriostatic properties of Imwitor® 308, the zones of inhibition were observed in disc containing Imwitor® 308 (disc 2, 3, 5 and 8) (Cremer, 2012).

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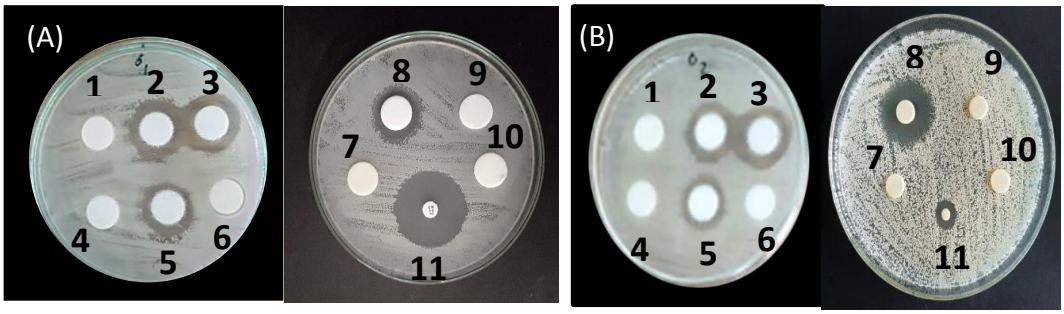

Figure 22 Antimicrobial test on (A) *S. aureus* ATCC 6538 and (B) *C. albicans* ATCC 10231 using disc diffusion method

1 = 20 % Tween® 80, 2 = 10 % Imwitor® 308, 3 = 15 % Imwitor® 308,

4 = 30% Tween® 80, 5 = 30% Tween® 80 and 15% Imwitor® 308, 6 = pure olive oil

7 = 1% DMSO, 8 = 20% Tween® 80 and 10% Imwitor® 308, 9 = 5% oil in 1 %DMSO

10 = 10% oil in 1% DMSO, 11 = gentamicin 10 µg (A) or amphotericin B 5 µg (B)

(A) *S. aureus* ATCC 6538 (B) *C. albicans* ATCC 10231

Disc diameter of 13 mm for disc 1-10 and 6 mm for disc 11

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4.3.1.2 Antimicrobial test of EVOO NEs

The antimicrobial activity of NEs was determined by measuring zone of inhibition. As shown in Table 12 and Figure 23, the range of inhibition zones of NEs were 13.13 - 18.33 mm for bacteria and 23.63 - 27.17 mm for fungi. The result showed that all EVOO NEs exhibited antimicrobial activity against gram positive, gram negative bacteria and fungi. For bacteria, It was possibly due to cell wall of gram positive bacteria contain only a layer of peptidoglycans attached to the inner membrane but gram negative bacteria is surrounded by a thin peptidoglycan, which is covered by an

outer membrane containing lipopolysaccharide and protecting toxic chemical to reach the inner space between the double membrane. Moreover, gram negative consists of porin channels which can protect the entry of harmful chemicals into the cell (Silhavy, Kahne, and Walker, 2010) (See structure of bacterial cell wall in Appendix E). From the results, T30 O10 exhibited inhibitory effect on microorganism significantly different (p<0.05) from the corresponding T30 micelle in gram negative bacteria and fungi (Table 12). T30 O5 has shown inhibitory effect on microorganism significantly different from the corresponding T30 micelle in tested gram negative bacteria and fungi. T20 O5 has showed inhibitory effect significantly different (p<0.05) from the corresponding T20 micelle only in *C. albicans* ATCC 10231. From the experiment, EVOO NEs showed however a limited zone of inhibition. It was possibly due to the insolubility of oil components which was unable to penetrate in agar media (Mann and Markham, 1998). Hence, the antimicrobial assay was further studied using broth dilution technique and used Tween as an emulsified O/W emulsions.

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Table 12 Zone of inhibition (mm) of nanoemulsions containing 5% and 10% EVOO and corresponding micelles.

Culture	Zone of inhibition (mm)				
	Nanoemulsions			Micelles	
	T20 O5	T30 O5	T30 O10	T20	T30
B. cereus ATCC 11778	13.13 ± 0.06[a]	14.83 ± 0.29[b]	13.40 ± 0.52[a]	13.02 ± 0.25[a]	14.33 ± 0.58[b]
S. aureus ATCC 6538	16.33 ± 0.76[a]	18.33 ± 1.26[b]	18.17 ± 0.29[a]	16.15 ± 0.49[a]	18.18 ± 3.54[b]
E. coli ATCC 25922	15.70 ± 0.17[a]	17.17 ± 1.15[b]	15.67 ± 0.15[a]	13.75 ± 0.35[a]	15.50 ± 1.32[a]
P. aeruginosa ATCC 27853	13.50 ± 0.50[a]	14.67 ± 0.58[a]	15.27 ± 1. 08[a]	13.00 ± 0.00[a]	13.42 ± 0.38[a]
C. albicans ATCC 10231	26.33 ± 0.58[a]	27.17 ± 1.04[b]	26.50 ± 1.32[b]	24.50 ± 0.71[c]	25.50 ± 0.71[a]
A. niger ATCC 16404	23.63 ± 0.38[a,b]	25.15 ± 0.81[b]	24.38 ± 1.10[b]	22.63 ± 1.11[a]	22.88 ± 0.85[a]

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Value of mean ± S.D., (n=3)

The data within the same tested culture with different superscripts are significantly different ($p < 0.05$)

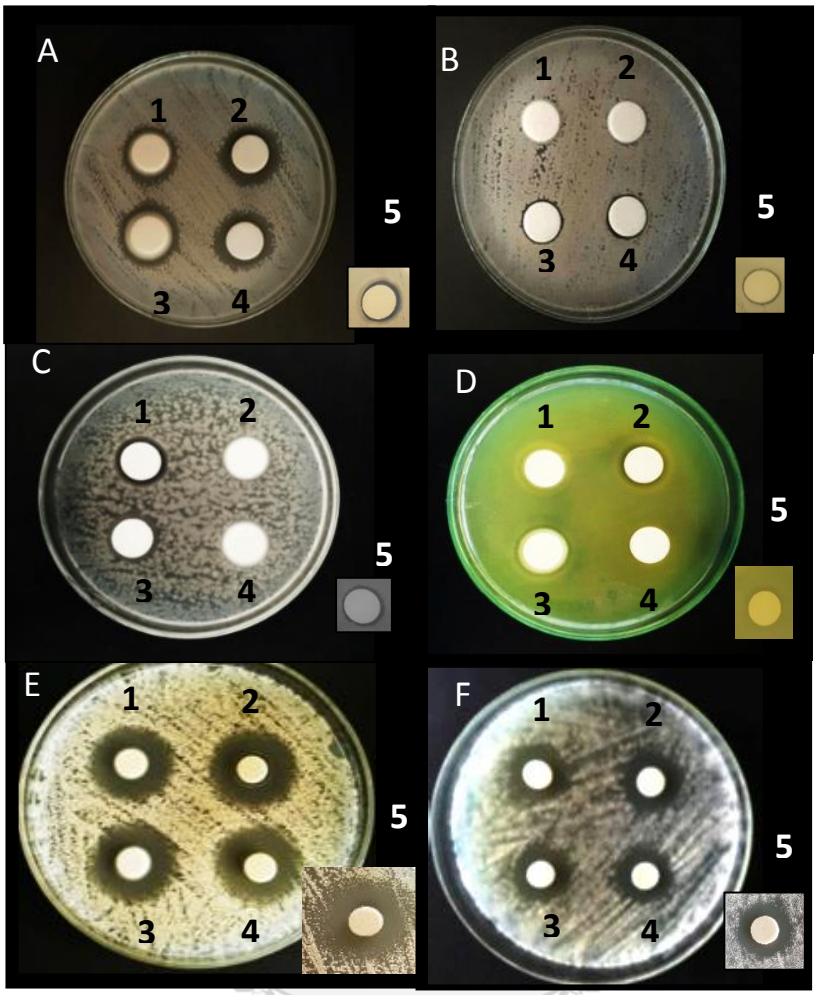

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Figure 23 Antimicrobial test of NEs and corresponding micelles using disc diffusion method against *(A) S. aureus* ATCC 6538, *(B) B. cereus* ATCC 11778, *(C) E. coli* ATCC 25922, *(D) P. aeruginosa* ATCC 27853, *(E) C. albicans* ATCC 10231, *(F) A. niger* ATCC 16404 *of (1) T20 O5, (2) T30 O5, (3) T30 O10, (4) Micelle T20, (5) Micelle T30*
Disc diameter of 13 mm.

4.3.2 Broth dilution method

The minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) were determined by broth dilution method.

4.3.2.1 Antimicrobial test of EVOO

EVOO was dissolved in 1% in DMSO before test. From the result, olive oil at a concentration as high as 75% did not show any antimicrobial activity against *B. cereus* ATCC 11778, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *C. albicans* ATCC 10231, and *A. niger* ATCC 16404 (Table 13). However, concentrations of olive oil exhibited antibacterial activity against *S. aureus* ATCC 6538 at concentrations greater than 45 %. To determine the MBC of *S. aureus* ATCC 6538, the experiment was repeated with the concentration of EVOO between 45% to 50% (Table 14). The EVOO at a concentration higher than 45% was effective in bactericidal activity against *S. aureus* ATCC 6538. The MBC of EVOO was 45.5% (420 mg/mL). Incorporating EVOO in NEs was able to increase antimicrobial activity of sole EVOO; 42-105 times for bacteria and 28-42 times for fungi.

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Table 13 Antimicrobial test of EVOO using broth dilution method

	Concentration of EVOO (%)					
	40	**45**	**50**	**60**	**70**	**75**
S. aureus ATCC 6538	+	+	0	0	0	0
B. cereus ATCC 11778	+	+	+	+	+	+
E. coli ATCC 25922	+	+	+	+	+	+
P. aeruginosa ATCC 27853	+	+	+	+	+	+
C. albicans ATCC 10231	+	+	+	+	+	+
A. niger ATCC 16404	+	+	+	+	+	+

+: growth observed, 0: no growth observed

Table 14 Antimicrobial test of EVOO against *S. aureus* ATCC 6538.

	Concentration of EVOO (%)					
	43	**45**	**45.5**	**46**	**47**	**50**
S. aureus ATCC 6538	+	+	0	0	0	0

+: growth observed, 0: no growth observed

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4.3.2.2 Antimicrobial test of EVOO NEs

The MIC values of EVOO NEs by broth dilution test were shown in Table 15 were determined by absorbance measurement after cell incubation in 96-well plate and calculated from equation (1). The percentage of cell inhibition with varied emulsified oil in EVOO NEs concentrations was determined (Figures 24-26).

Table 15 Antimicrobial test using broth dilution method to determine the MIC (mg/mL) of emulsified olive oil in EVOO NEs and corresponding micelles.

Formulas	MIC (mg/mL) of emulsified EVOO in EVOO NEs					
	S. aureus ATCC 6538	*B. cereus* ATCC 11778	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*C. albicans* ATCC 10231	*A. niger* ATCC 16404
T20 O5	7.5	7.5	10.0	G	12.5	15.0
T30 O5	5.0	5.0	5.0	G	10.0	12.5
T30 O10	4.0	4.0	5.0	G	10.0	10.0
Micelle T20	40.0	30.0	40.0	40.0	40.0	40.0
Micelle T30	30.0	22.5	30.0	30.0	30.0	30.0

G: Growth observed

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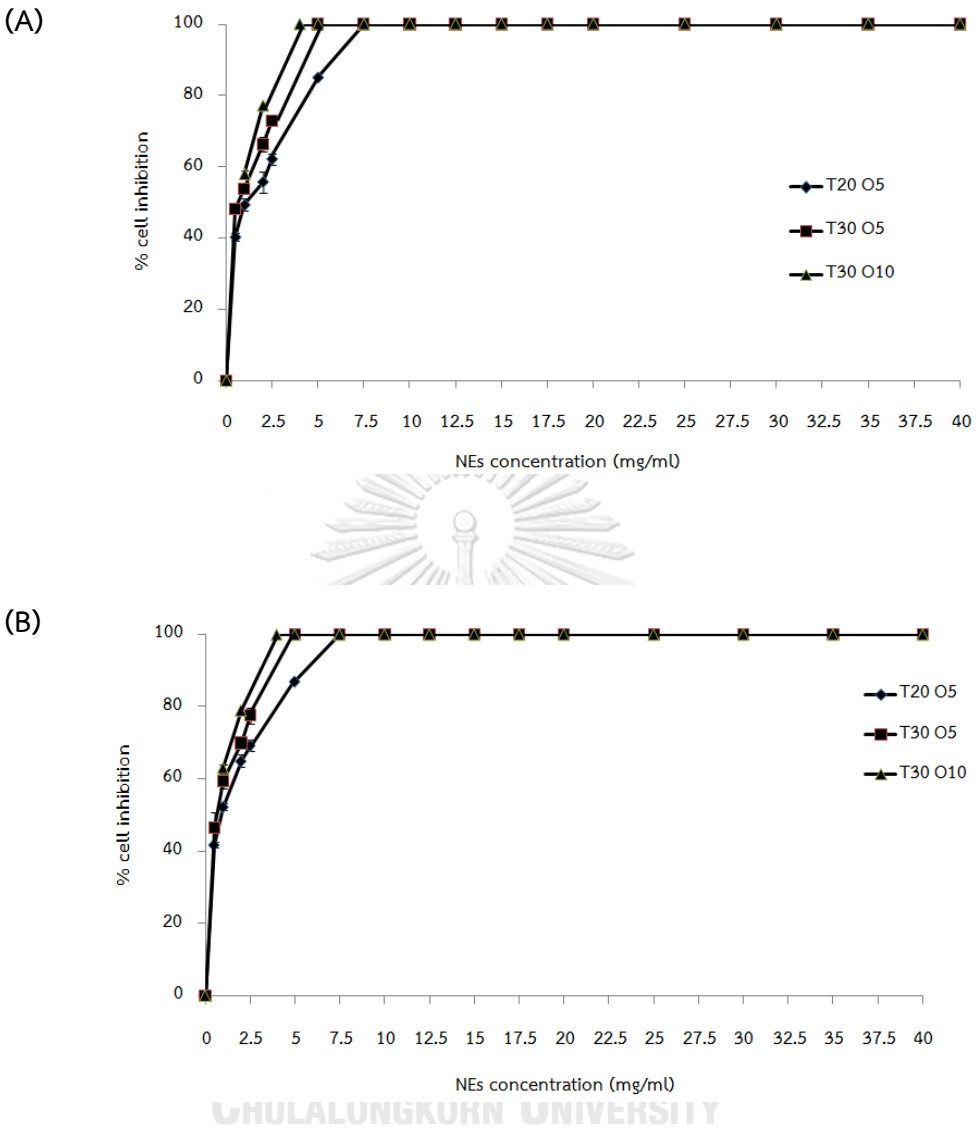

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Figure 24 % Cell inhibition of EVOO NEs against (A) *B. cereus* ATCC 11778 (B) *S. aureus* ATCC 6538

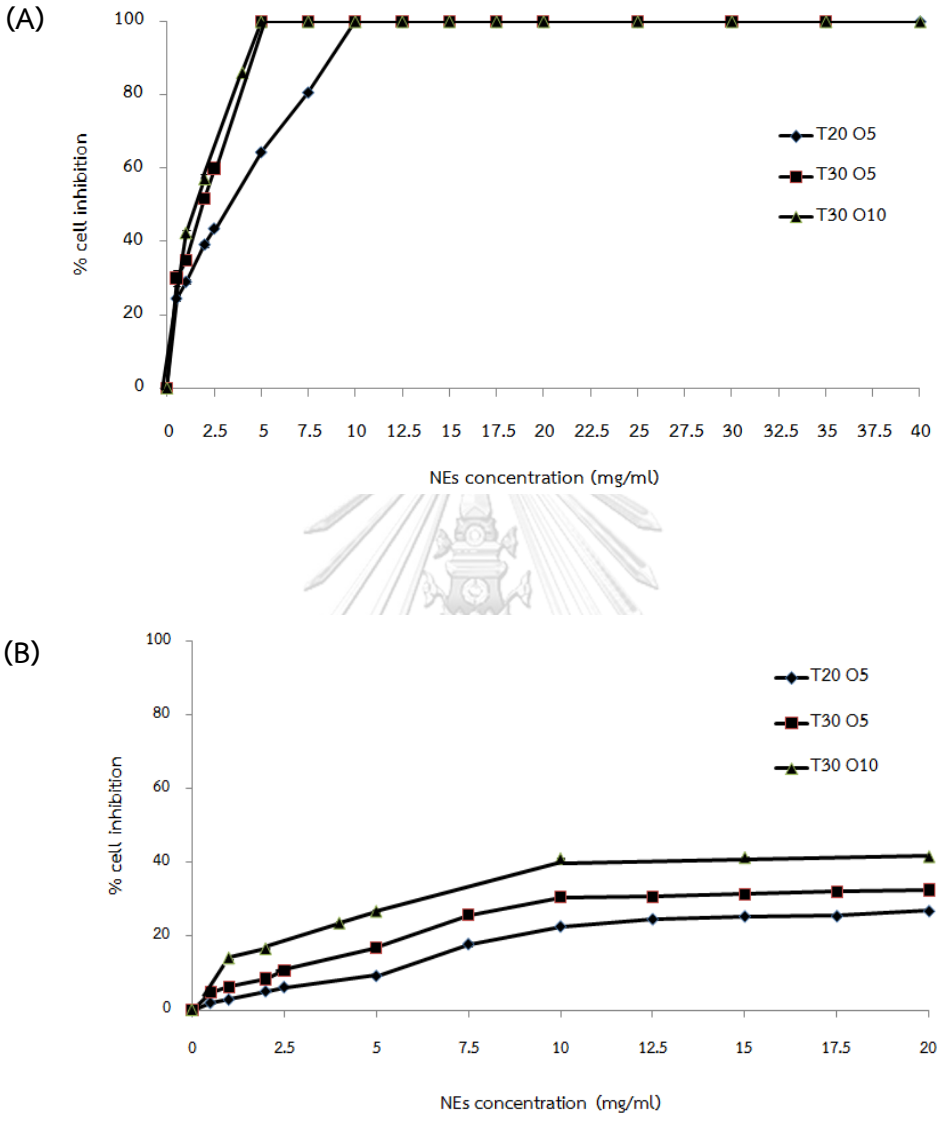
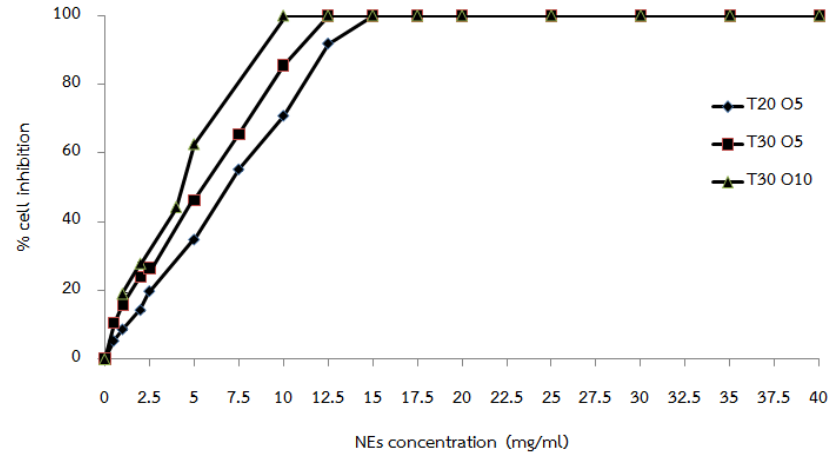


Figure 25 % Cell inhibition of EVOO NEs against *(A) E. coli* ATCC 25922 *(B) P. aeruginosa ATCC 27853*

(A)



(B)

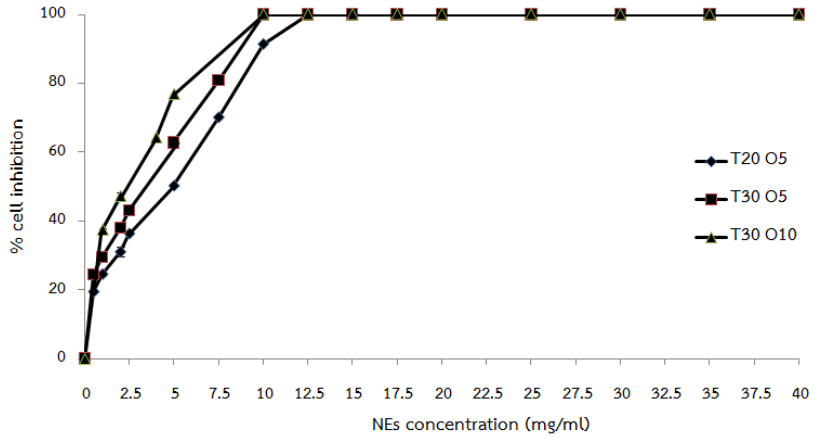


Figure 26 %Cell inhibition of EVOO NEs against (A) *A. niger* ATCC 16404 (B) *C. albicans* ATCC 10231

Table 16 Determination of MBC (mg/mL) and MFC (mg/mL) of EVOO NEs and corresponding micelles.

Formulas	MBC (mg/mL)				MFC (mg/mL)	
	S. aureus ATCC 6538	*B. cereus* ATCC 11778	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*C. Albicans* ATCC 10231	*A. niger* ATCC 16404
T20 O5	12.5	15.0	15.0	G	17.5	20.0
T30 O5	10.0	15.0	12.5	G	15.0	17.5
T30 O10	10.0	10.0	10.0	G	12.50	15.0
Micelle T20	G	G	G	G	G	G
Micelle T30	G	G	G	G	G	G

G: Growth observed

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The result of % cell inhibition and MIC determination (Table 15), the most susceptible antimicrobial activity of NEs was against *S. aureus* ATCC 6538. Furthermore, adding more olive oil in NEs formulation resulted in more antimicrobial activity for almost microorganism as seen in decreased MIC value except for *P. aeruginosa* ATCC 27853. Percentage of inhibition of *P. aeruginosa* ATCC 27853 were less than 40% inhibition in all EVOO NEs (Figure 25) and growth of culture was found, so the MIC value could not be determined from broth microdilution method at highest concentration of EVOO in this study (Table 15). The EVOO NEs had more antimicrobial

activity than micelle; 4-10 times for gram positive bacteria, 3-8 times for gram negative bacteria and 2-4 times for fungi. From the following experiment, the MBC and MFC of EVOO NEs were determined in Table 16. The MBC of *P. aeruginosa* was unable to be determined. The micelle T20 and T30 also showed the growth of all tested culture and no bactericidal and fungicidal effects were observed. The MFC values of mould (*A. niger*) and yeast (*C. albicans*) were 15-20 mg/mL and 12.5-17.5 mg/mL, respectively and MBC values were 10-15 mg/mL. The differences in fungicidal and bactericidal values were due to the structure of cell wall of microorganism. The structures of mould are multicellular filamentous fungi but yeast is unicellular microorganism. The cell wall of mould is complex and tends to resist to antimicrobial agents. Moulds are generally more resistant to antimicrobial agents than yeasts and considerably more resistant than bacteria due to the structure of cell wall which has a permeability barrier to inactivate a biocide (Bartnett, Polarine, and Lopolito, 2017).

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The result indicated that there was synergistic effect of antimicrobial activity between the composition and the nano-scale system like NEs. The nanoparticles can possibly penetrate into bacterial cell membrane leading to destabilization of the pathogen lipid membrane and finally cell death (Shah, Bhalodia, and Shelat, 2010). These results were in agreement with those reported by Moghimi et al. (2015). The previous study showed that incorporation of a *Thymus daenensis* essential oil into NEs had 10 times more antibacterial activity than pure essential oil. Moreover, the result

of Sugumar et al. (2013) showed that NEs had an effective bactericidal property and reduced the growth rate of bacteria more than surfactant used alone.

In fact Imwitor® 308 has only bacteriostatic effect (Cremer, 2012). Moreover, incorporating EVOO into NEs has an antimicrobial activity compared to EVOO due to their nano-scale size of droplet which increases the surface area exposed to the microbial membrane (Topuz et al., 2016).

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CHAPTER V
CONCLUSION

Nanoemulsions (NEs) have a number of advantages. Small droplet size of NEs brings about an increase in surface area of the NEs droplets which can enhance their permeation and bioavailability of encapsulated compounds and increase in shelf life of products. In this study, NEs containing olive oil were prepared using low-energy emulsification and high-energy method. The food-grade oil-in-water NEs with sufficiently small particles could be formed. The physicochemical properties and antimicrobial activity of olive oil NEs were determined.

The compositions, namely extra virgin olive oil, surfactant (Tween® 80, and co-surfactant (Imwitor® 308), were controllable in order to optimize the formation of such NEs. The preparation of NEs by spontaneous emulsification indicated the requirement of co-surfactant to form NEs with smaller droplet sizes (< 200 nm). An increase in oil amount resulted in larger NEs droplets. An increase in oil molecules inside the core of the droplet brings about more surfactant molecules needed to form an interfacial film around the droplet. The high energy ultrasonication was used to form stable NEs. Ultrasound amplitude, treatment time and input energy were parameters for NEs formulation. These parameters were varied to obtain the optimized NEs. The parameters were generated the optimized NEs are energy input 2250 J, treatment time 5 min and amplitude 25%.

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It was found that, the droplet sizes of EVOO NEs increased during storage time up to 6 months but were still in nano-scale (< 200 nm). The EVOO NEs containing 5% olive oil, 30% Tween® 80 and 15 % Imwitor® 308 (T30 O5) was the most stable. Most of the PDI values of EVOO NEs did not exceed 0.7 suggesting the rather narrow size distribution of preparation.

Incorporating extra virgin olive oil into NEs enhanced their antimicrobial activity about 42-105 times against bacteria and 28-42 times against fungi compared to sole EVOO. The small droplet size and the high surface area of NEs can enhance the functionality of olive oil by locating the oil droplets close to the microbial cell membrane. This could lead to disruption the cell membrane by altering the phospholipid bilayer integrity and loss of cell internal constituents, thus promoting cell death. The information can be used to design NEs for application in food and pharmaceutical industries. The further investigation would be mechanisms of NEs on antimicrobial activity.

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APPENDICES

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APPENDIX A

Table 1A Size of EVOO NEs (Tween® 20 used as surfactant)

	Z-average (mean±S.D.)			
Conc. (%)	**T30 O1**	**T20 O1**	**T10 O1**	**T5 O1**
0.25	110.97 ± 1.02	86.30 ± 0.99	108.43 ± 5.52	2892.67 ± 365.92
0.5	111.93 ± 0.70	176.67 ± 0.32	153.4 ± 4.81	3343.00 ± 205.42
0.75	139.00 ± 1.45	186.10 ± 1.10	191.9 ± 45.7	3530.33 ± 595.91
1	143.47 ± 0.80	372.6 ± 23.35	301.8 ± 33.8	4244.00 ± 303.16

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Table 2A Size of EVOO NEs (Tween® 80 used as surfactant)

	Z-average (mean ± S.D.)			
Conc. (%)	**T30 O1**	**T20 O1**	**T10 O1**	**T5 O1**
0.25	10.79 ± 0.14	14.71 ± 0.42	15.37 ± 0.33	368.00 ± 38.43
0.5	12.70 ± 0.16	21.06 ± 0.72	20.88 ± 0.56	392.43 ± 28.63
0.75	13.38 ± 6.11	27.49 ± 10.44	319.80 ± 49.71	587.70 ± 38.22
1	14.49 ± 0.11	29.26 ± 13.96	376.57 ± 46.39	1181.00 ± 44.26

APPENDIX B

Table 3A Effect of input energy (J) on size, PDI and zeta potential of EVOO NEs (T20 O5).

Energy (J)	Size (nm)	PDI	Zeta potential (mV)
1250	48.95	0.36	-3.37
	46.22	0.35	-3.75
	34.38	0.34	-2.93
mean	**43.18**	**0.35**	**-3.35**
S.D.	**7.75**	**0.01**	**0.34**
1500	34.93	0.41	-3.72
	34.87	0.41	-5.29
	34.19	0.39	-4.04
mean	**34.66**	**0.40**	**-4.35**
S.D.	**0.41**	**0.02**	**0.83**
1750	50.81	0.28	-1.8
	51.47	0.27	-1.87
	52.31	0.27	-2.23
mean	**51.53**	**0.27**	**-1.97**
S.D.	**0.75**	**0.01**	**0.23**
2000	41.75	0.28	-1.65
	41.89	0.28	-1.66
	42.05	0.28	-1.53
mean	**41.90**	**0.28**	**-1.61**
S.D.	**0.15**	**0.00**	**0.06**
2250	33.48	0.32	-2.72
	34.12	0.31	-2.24
	34.37	0.31	-2.29
mean	**33.99**	**0.31**	**-2.42**
S.D.	**0.46**	**0.00**	**0.26**

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Table 3A (cont.)

Energy (J)	Size (nm)	PDI	Zeta potential (mV)
2500	45.98	0.74	-7.46
	45.89	0.69	-4.23
	46.99	0.68	-3.77
mean	46.29	0.71	-5.15
S.D.	0.61	0.03	2.01

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Table 4A Effect of treatment time (min) on size, PDI and zeta potential of EVOO NEs (T20 O5)

Time (min)	Size (nm)	PDI	Zeta potential (mV)
5 min	26.40	0.30	-2.17
	26.99	0.29	-2.27
	27.31	0.26	-3.39
mean	**26.90**	**0.28**	**-2.61**
S.D.	**0.46**	**0.02**	**0.55**
10 min	28.98	0.25	-2.72
	29.45	0.24	-2.24
	29.09	0.24	-2.29
mean	**29.17**	**0.24**	**-2.42**
S.D.	**0.25**	**0.01**	**0.26**
15 min	29.68	0.30	-2.49
	29.75	0.28	-1.89
	30.34	0.35	-3.05
mean	**29.92**	**0.31**	**-2.48**
S.D.	**0.36**	**0.04**	**0.58**
20 min	31.47	0.32	-1.76
	32.16	0.33	-2.22
	32.46	0.32	-1.83
mean	**32.03**	**0.33**	**-1.94**
S.D.	**0.51**	**0.00**	**0.20**

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Table 5A Effect of amplitudes (%) on size, PDI and zeta potential of EVOO NEs (T20 O5).

Amplitude (%)	Size (nm)	PDI	Zeta potential (mV)
20%	43.69	0.45	-3.35
	46.66	0.34	-3.75
	47.87	0.36	-3.77
mean	**46.07**	**0.38**	**-3.62**
S.D.	**2.15**	**0.06**	**0.24**
25%	20.01	0.38	-2.17
	19.99	0.36	-1.86
	20.13	0.37	-2.22
mean	**20.04**	**0.37**	**-2.08**
S.D.	**0.08**	**0.01**	**0.20**
30%	29.74	0.25	-1.76
	30.42	0.24	-2.59
	30.66	0.25	-2.62
mean	**30.27**	**0.24**	**-2.32**
S.D.	**0.48**	**0.00**	**0.49**
35%	28.42	0.24	-1.92
	28.78	0.25	-1.94
	29.12	0.24	-2.22
mean	**28.77**	**0.24**	**-2.03**
S.D.	**0.35**	**0.00**	**0.17**

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APPENDIX C

Table 6A Droplet size (nm) of EVOO NEs after storage at difference conditions (mean ± S.D., n=3)

Day (s)	Size (nm)		
at 25 °C	T20 O5	T30 O5	T3 O10
0	27.69 ± 2.97	28.66 ± 2.08	75.17 ± 4.71
14	31.62 ± 4.10	29.24 ± 4.01	75.49 ± 6.36
28	41.52 ± 5.44	31.47 ± 5.42	84.30 ± 6.61
90	87.95 ± 4.10	35.45 ± 4.08	88.36 ± 7.12
180	134.01 ± 7.88	44.77 ± 4.23	100.00 ± 10.48

Day (s)	Size (nm)		
at 30 °C	T20 O5	T30 O5	T30 O10
0	34.86 ± 3.49	29.24 ± 4.01	77.70 ± 6.25
14	38.57 ± 3.66	34.34 ± 3.79	110.24 ± 5.27
28	40.70 ± 4.71	35.17 ± 1.15	130.16 ± 7.86
90	119.15 ± 3.73	42.83 ± 2.96	145.63 ± 6.23
180	128.93 ± 7.53	59.17 ± 6.30	172.04 ± 12.14

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Table 6A (cont.)

Day (s)	Size (nm)		
at 40 °C	T20 O5	T30 O5	T30 O10
0	33.92 ± 2.52	29.16 ± 3.97	80.63 ± 4.09
14	33.83 ± 4.09	34.64 ± 3.63	122.53 ± 5.17
28	48.07 ± 3.25	52.45 ± 5.15	128.75 ± 7.77
90	79.62 ± 4.12	126.67 ± 7.80	144.31 ± 4.96
180	244.22 ± 9.00	190.01 ± 12.10	231.71 ± 12.75

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Table 7A Zeta potential (mV) of olive oil NEs after storage at different conditions (mean ± S.D., n=3).

Day (s)	Zeta potential (mV)		
at 25 °C	T20 O5	T30 O5	T30 O10
0	-6.79 ± 0.56	-1.27 ± 0.20	-3.45 ± 0.30
14	-1.96 ± 0.15	-2.23 ± 0.04	-1.82 ± 0.17
28	-2.17 ± 0.13	-1.96 ± 0.23	-2.17 ± 0.34
90	-2.49 ± 0.23	-1.51 ± 0.15	-1.97 ± 0.07
180	-2.31 ± 0.64	-1.55 ± 0.15	-2.43 ± 0.02

Day (s)	Zeta potential (mV)		
at 30 °C	T20 O5	T30 O5	T30 O10
0	-2.54 ± 0.13	-2.49 ± 0.11	-3.45 ± 0.30
14	-1.59 ± 0.34	-1.29 ± 0.11	-1.29 ± 0.19
28	-1.54 ± 0.12	-1.59 ± 0.21	-1.79 ± 0.25
90	-3.87 ± 0.78	-3.23 ± 0.19	-3.48 ± 0.22
180	-2.93 ± 0.76	-2.87 ± 0.43	-4.51 ± 0.69

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Table 7A (cont.)

Day (s)	Zeta potential (mV)		
at 40 °C	T20 O5	T30 O5	T30 O10
0	-1.52 ± 0.34	-1.28 ± 0.21	-3.06 ± 0.48
14	-1.75 ± 0.57	-1.25 ± 0.18	-1.14 ± 0.40
28	-2.03 ± 0.29	-1.69 ± 0.19	-1.91 ± 0.19
90	-2.86 ± 0.33	-3.52 ± 0.34	-2.28 ± 0.31
180	-4.20 ± 0.68	-3.99 ± 0.37	-2.72 ± 0.45

Table 8A Polydispersity index of olive oil NEs after storage at different conditions (mean ± S.D., n=3).

Day (s)	PDI		
at 25 °C	T20 O5	T30 O5	T30 O10
0	0.41 ± 0.08	0.88 ± 0.09	0.20 ± 0.07
14	0.44 ± 0.03	1.00 ± 0.00	0.21 ± 0.00
28	0.43 ± 0.06	1.00 ± 0.00	0.22 ± 0.02
90	0.43 ± 0.06	1.00 ± 0.00	0.25 ± 0.05
180	0.41 ± 0.09	1.00 ± 0.00	0.33 ± 0.08

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Table 8A (cont.)

Day (s)	PDI		
at 40 °C	T20 O5	T30 O5	T30 O10
0	0.33 ± 0.09	0.42 ± 0.06	0.17 ± 0.05
14	0.58 ± 0.02	0.82 ± 0.03	0.18 ± 0.07
28	0.56 ± 0.02	0.86 ± 0.05	0.25 ± 0.01
90	0.75 ± 0.06	0.93 ± 0.06	0.26 ± 0.03
180	0.83 ± 0.04	1.00 ± 0.00	0.30 ± 0.06

Day (s)	PDI		
at 30 °C	T20 O5	T30 O5	T30 O10
0	0.26 ± 0.05	0.40 ± 0.04	0.20 ± 0.07
14	0.29 ± 0.06	0.53 ± 0.04	0.24 ± 0.06
28	0.47 ± 0.03	0.86 ± 0.04	0.27 ± 0.05
90	0.61 ± 0.07	0.88 ± 0.03	0.35 ± 0.09
180	0.64 ± 0.06	0.99 ± 0.02	0.37 ± 0.04

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APPENDIX D

The droplets in a disperse phase are Brownian movement and tend to aggregation. The colloidal interaction force between the particles are attractive force namely the van der Waals and hydrophobic interactions and repulsive force are steric and electrostatic interactions. Electrostatic components are net charge on the droplet surface. In order to stabilizing colloids, the repulsion force should be at least as strong as the attractive forces. In non-ionic system, the stability of the system depend on steric stabilization.

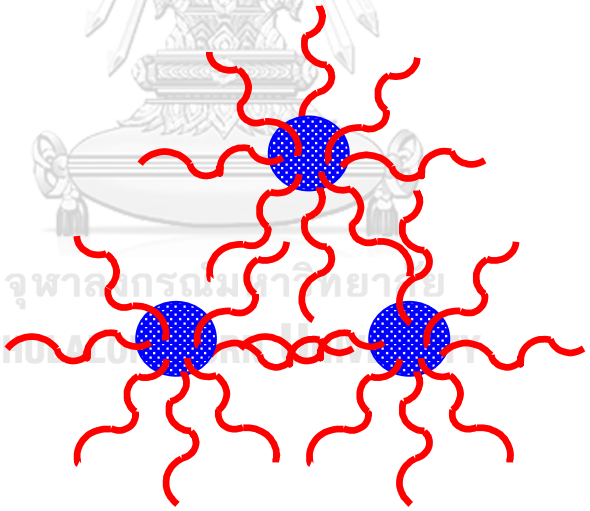

Figure 1A Steric stabilization of non-ionic NEs (Napper and Evans, 1982)

Appendix E

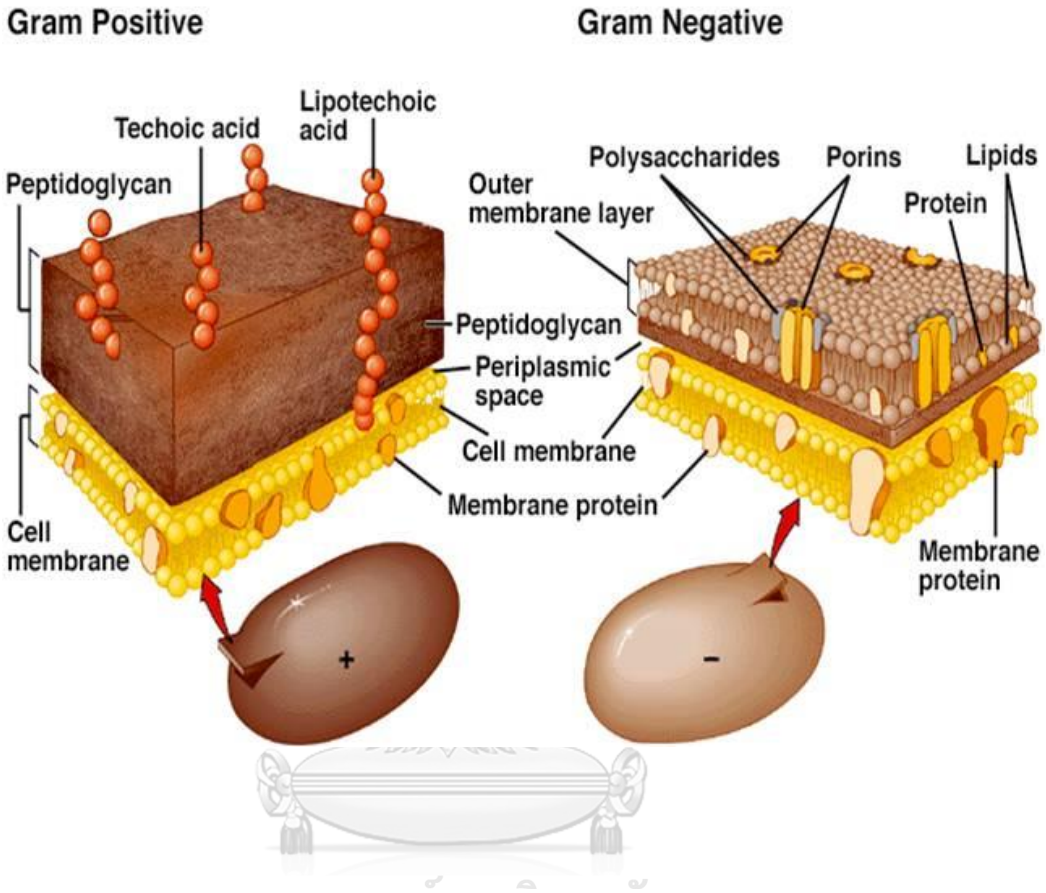

Figure 2A Compositions of cell wall of gram positive and gram negative bacteria. (Hayat, 2013)

VITA

Miss Kusuma Jaemsal was born on June 3, 1983. in Bangkok, Thailand. In 2005, she received her Bachelor from the faculty of pharmacy, Silpakorn University. After graduation, she had been work as Pharmacist hospital of Children's hospital for 6-month and then moved to Phramongkutklao hospital for one and a half year. Now she works at Prasat neurological Institue as a hospital pharmacist at inpatient unit. Her responsibilities included investigating the prescriptions, managing the stock of medicines, advising the information of medicine to a patient.

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