PHYSICOCHEMICAL, ANTIOXIDANT AND ANTIBACTERIAL PROPERTIES OF BETEL LEAF AND KAFFIR LIME ESSENTIAL OIL NANOEMULSIONS

by

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LIST OF ABBREVIATIONS

Abbreviations	**Defination**
ºC	Degree celsius
%	Percent
µm	Micrometre
CFU	Colony forming unit
DLS	Dynamic light scattering
DPPH	1,1-diphenyl-2-picrylhydrazyl
EIP	Emulsion inversion point
EO	Essential oil
FDA	Food and Drug Administration
GRAS	Generally recognized as safe
h	hour
HLB	Hydrophilic-lipophilic balance
NA	Nutrient Agar
nm	Nanometer
MHA	Mueller-Hinton agar

MHB	Mueller-Hinton broth
min	Minute
mL	Millilitre
mm	Millimeter
mPa·S	Millipascal-second
mV	Millivolts
NA	Nutrient agar
OR	Ostwald ripening
o/w	Oil-in-water
PDI	Polydispersity index
PIC	Phase inversion composition
PIT	Phase inversion temperature
PSD	Particle size distribution
rpm	Revolutions per minute
SE	Spontaneous emulsification
TEM	Transmission electron microscopy
UV-Vis	Ultraviolet-visible spectroscopy

v/v	Volume/volume
w/o	Water-in-oil

SIFAT FIZIKOKIMIA, ANTIOKSIDAN DAN ANTIBAKTERIA NANOEMULSI BERASASKAN MINYAK PATI DAUN SIRIH DAN LIMAU PURUT

ABSTRAK

Kajian semasa fokus pada sifat fizikokimia, antioksidan dan antibakteria nanoemulsi berasaskan minyak pati (EO) daun sirih (*Piper betle L.*) dan limau purut (Citrus hystrix) yang disediakan dengan menggunakan kaedah pengemulsi spontan. Tiga nanoemulsi yang stabil, iaitu nanoemulsi EO daun sirih, nanoemulsi EO limau purut dan nanoemulsi EO sirih:limau purut disediakan. Nanoemulsi EO yang dirumuskan terdiri daripada 5% (v/v) fasa lipid (EO dan minyak jagung pada nisbah 8:2) dengan 15% (v/v) Tween 80 dan 80% (v/v) air air deionisasi. Nanoemulsi EO yang disiapkan dicirikan untuk ukuran saiz partikel indeks polidispersi (PDI), taburan saiz partikel (PSD), potensi zeta, kelikatan, kekeruhan, morfologi, aktiviti antioksidan dan aktiviti antibakteria. Semua nanoemulsi EO mempunyai ukuran saiz partikel yang kecil (20-60 nm), PDI yang rendah, PSD yang sempit, potensi zeta yang rendah dan penampilan yang telus. Nanoemulsi EO daun sirih menunjukkan kelikatan dan kekeruhan yang terendah, diikuti nanoemulsi EO sirih:limau purut dan limau purut. Imej mikroskopi elektron penghantaran (TEM) menunjukkan bahawa zarah minyak mempunyai bentuk hampir sfera, terserak secara seragam dan dalam julat nanometer (20-60 nm). Nanoemulsi EO daun sirih menunjukkan aktiviti perencatan radikal 2,2-difenil-1-pikrilhidrazil (DPPH) yang paling tinggi dengan 89.4%, diikuti dengan nanoemulsi EO sirih:limau purut (88.4%) dan limau purut (33.4%) untuk aktiviti antioksidan. Aktiviti antibakteria ditentukan oleh ujian resapan cakera dengan menggunakan nanoemulsi EO limau purut terhadap *Escherichia coli*, *Salmonella* *spp* dan *Staphylococcus aureus*. Bakteria jenis Gram-positif didapati lebih rentan daripada bakteria jenis Gram-negatif disebabkan oleh struktur dinding sel bakteria yang berlainan. Penemuan ini menunjukkan bahawa nanoemulsi EO daun sirih dan limau purut berpotensi besar untuk digunakan sebagai bahan fungsional dalam aplikasi makanan.

PHYSICOCHEMICAL, ANTIOXIDANT AND ANTIBACTERIAL PROPERTIES OF BETEL LEAF AND KAFFIR LIME ESSENTIAL OIL NANOEMULSIONS

ABSTRACT

The current study focused on the characterization of physicochemical, antioxidant and antibacterial properties of betel leaf (*Piper betle L.*) and kaffir lime (*Citrus hystrix*) essential oil (EO) nanoemulsion prepared using spontaneous emulsification method. Three stable EO nanoemulsions, namely betel leaf EO nanoemulsions, kaffir lime EO nanoemulsions and betel:kaffir EO nanoemulsions were prepared. The formulated EO nanoemulsions consisted of 5% (v/v) of oil phase (EO and corn oil at the ratio of 8:2) with 15% (v/v) of Tween 80 and 80% (v/v) of deionized water. The freshly-prepared EO nanoemulsions were then characterized for their particle size, polydispersity index (PDI), particle size distribution (PSD), zeta potential, viscosity, turbidity, morphology, antioxidant activity and antibacterial activity. All EO nanoemulsions had small particle size (20-60 nm), low PDI, narrow PSD, low zeta potential and transparent appearance. Betel leaf EO nanoemulsion exhibited the lowest viscosity and turbidity, followed by betel:kaffir and kaffir lime EO nanoemulsion. Transmission electron microscopy (TEM) images revealed that the oil particles had almost spherical shape, evenly distributed and in the range of nanometer (20-60 nm). Betel leaf EO nanoemulsion showed the highest 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity with 89.4%, followed by betel:kaffir (88.4%) and kaffir lime (33.4%) EO nanoemulsion for their antioxidant activity. Antibacterial activity was determined by disk diffusion test by using kaffir lime EO nanoemulsion against *Escherichia coli*, *Salmonella spp*, and *Staphylococcus aureus*.

Gram-positive bacteria were more susceptible than Gram-negative bacteria due to the different cell wall structure of the bacteria. These findings demonstrated that betel leaf and kaffir lime EO nanoemulsion had a great potential to be used as functional ingredients in food application.

CHAPTER 1 INTRODUCTION

1.1 Research background

Essential oil (EO) is a complex mixture of volatile and non-volatile compounds typically lipoophilic which naturally produced by plants as secondary metabolites. Betel leaf EO contains bioactive compounds including monoterpenes, sesquiterpenes, phenylpropanoids and aldehydes (Nayaka et al, 2021). It has been reported that phenylpropanoid groups such as acetyl eugenol, eugenol, chavicol and safrole were the major compound found in betel leaf EO through gas chromatography-mass spectrometry analysis (Karak, 2018). Kaffir lime EO contains sabinene, α-pinene, D-limonene, myrcene, citronellal and terpinen-4-o as major compounds (Automatik and Minyak, 2013). These active compounds contribute to the antioxidant and antibacterial activities of EO.

Although essential oils including betel and kaffir lime EOs have numerous health-related benefits but their application in food is still limited. The reason might be due to their hydrophobic properties, susceptibility to degradation and strong smell that make their incorporation in food challenging. The reaction between EOs and food components may also minimize their antibacterial activity. These factors impose difficulty to the manufacturers on the incorporation of EOs into food and thus the development of appropriate delivery system for EO to be utilized in food is necessary.

To overcome the above problems, EO can be formulated into nanoemulsion. (Buranasuksombat, 2011). Nanoemulsions are colloidal dispersion consisting of droplets at the nanometer range (20-200 nm), dispersed in an aqueous media (Tadros et al., 2004). The reduced droplets size makes EO nanoemulsion to be more stable towards destabilization phenomena such as coalescence, gravitational separation and particle aggregation (Mason et al., 2006). EO nanoemulsions appear clear and transparent because they barely scatter light, therefore appropriate to be added into clear beverages and food (McClements, 2002). It is revealed that the functionality of the small particles of EOs is enhanced due to their high surface area. EO nanoemulsion can diffuse into bacteria cell to reduce and kill the food borne pathogens (Salvia-Trujillo et al., 2015) and can be incorporated into food products to minimize lipid oxidation (Li et al., 2015). In addition, EOs are classified as generally recognized as safe (GRAS) by the US Food and Drug Administration (FDA) (Weiss et al., 2009) therefore their incorporation into food product is permitted. For instance, EO nanoemulsions can be applied in food like savory meat, fish, cheese, soups and sauces (Seow et al., 2014). Hence, formulating betel leaf and kaffir lime EOs into nanoemulsion is expected to improve their feasibility as functional ingredient in the food system.

1.1 Rationale of the study

Essential oil (EO) exerts various health benefits and has antibacterial and antioxidant properties. However, the application of EOs in the food and beverages are limited due to their hydophobicity, sensitivity to degradation and strong aroma. Being highly hydrophobic, EOs are insoluble in water and hence difficult to be dispersed in water-based food systems. The high volatility and strong aroma of EOs may negatively affect the organoleptic properties of food products (Noori et al., 2018). In addition, food components such as lipids, proteins and carbohydrates may interact with active EO components and reduce their antibacterial activity in food (Gutierrez et al., 2008). The susceptibility of EOs to degradation may also minimize their antioxidant and antibacterial activities (Turek and Stintzing, 2013). As a consequence, high concentration of EO is required to be incorporated in order to achieve the desired effect (Zhaveh et al., 2015).

To mitigate the drawbacks, encapsulation of EOs in nanoemulsion system can be a good solution in food and beverages system (Gharenaghadeh et al., 2017) whereby the dispersibility of EOs in water will be improved and their susceptibility to degradation and influence on the organoleptic properties of food will be reduced. Moreover, the small particles size and high surface to volume ratio of EO nanoemulsions may enhance the antibacterial activity of EO by improving their diffusion into the bacteria cell and inhibit their growth. The production of EO nanoemulsion particularly from betel leaf and kaffir lime EO may also help to maximize the utilization of underutilized herbal plants in Malaysia.

1.2 Objectives

1.2.1 General and specific objectives

The present research aims to produce stable betel leaf and kaffir lime essential oil nanoemulsions as functional ingredients in food applications. The specific objectives of the research are:

1. To investigate the physicochemical properties of betel leaf and kaffir lime essential oil nanoemulsions produced using spontaneous emulsification method.
2. To study the antioxidant and antibacterial properties of betel leaf and kaffir lime essential oil nanoemulsions.

CHAPTER 2 LITERATURE REVIEW

2.1 Essential oils

Essential oil (EO) is a concentrated hydrophobic aroma compound containing major active secondary metabolites including terpenes and oxygenated compounds which associated to many therapeutic activities (Aggarwal, 2011). EOs can be obtained from different parts of plants such as fresh leaves, flowers, fruits and roots through different extraction process of expression, extraction, and steam distillation (Cassel et al, 2009)

Many plants naturally produce bioactive chemicals that can act as defence agents to protect themselves from pathogenic attack (Murugan, 2015). EOs which are systhesized as secondary metabolites hereby play an important role in plant defense due to the strong antibacterial, antiviral and antifungal properties. Thus, the potential of EOs to be applied in food and beverage system as natural antibacterial s is enhanced (Burt, 2004). Apart from food industry, EOs are also widely used in other industries that manufacture perfumes, toiletries, soaps (São Pedro, 2013) and even insect repellents (Isman, 2000). There are only about 300 out of over 3000 varieties of known EOs are currently of commercial interest (Dima and Dima, 2015). Lavender, tea tree, peppermint and jasmine are among popular and common EOs found in the markets. However, there are more EOs from various flowers and spices to be discovered and utilized in different fields. The application of EOs as preservatives in the food and beverages industry has gotten a lot of attention in recent years, owing to the consumer concerns on the negative effects of synthetic preservatives (Seow et al., 2014).

2.1.1 Betel leaf (*Piper betle L.*)

Betel leaf (Figure 2.1) with glossy heart shaped leaf and white catkin which belongs to the *Piperaceae* family is one of the precious herbal plants found in central and eastern Malaysia (Muruganandam et al., 2017). The betel leaf, which is also known as ‘Paan' in India and ‘Sirih’ in Malaysia and Indonesia is an evergreen and perennial creeper (Varier, 1997). According to Guha (2006), the plantation of betel plant is vital for people's livelihoods and economics in South Asia.

Since ancient times, fresh betel leaves wrapped with the areca nut, mineral slaked lime, catechu, flavouring substances and spices are chewed in some Asian countries (Guha, 2006). Besides the socio-cultural uses, betel leaf is also a crucial medicinal plant in the traditional treatment in Southeast Asian countries because of its therapeutic properties and nutritional values. It is used to treat bad breath, constipation, headache, itches, swelling of gum, and injuries (Agarwal, 2012). The freshly-squeezed juice of betel leaves is used for the treatment of skin ailments in Sri Lanka (Arambewela et al., 2010). The traditional application of betel leaves is mainly related to their antibacterial and antifungal properties.

Figure 2.1 Betel leaf (*Piper betle L.*) (Source: Rekha et al., 2014)

In terms of chemical constituents, betel leaf contains water, proteins, carbohydrates, minerals, fat, fibre, EO, tannin, alkaloid, vitamins and minerals (Farid, 2017). The presence of phenols and terpenes in EO gives betel leaf its strong pungent aromatic scent (Bajpai, 2010). The components like saponins, flavonoids and polyphenols are known to impart strong antifungal and antibacterial properties to betel leaf (Maisuthisakul et al., 2007). According to recent studies in Sri Lanka, betel leaf extract inhibits the growth of microorganism such as *Escherichia coli*, *Streptococcus pyogenes* and *Staphylococcus aureus* at lower activity (Rekha et al., 2014).

However, betel leaf is very perishable due to dehydration, fungal infection and dechlorophyllation. Hence, there is a huge wastage of the betel leaves during storage and transportation. To reduce such wastage, it can be processed into EO to be used as raw ingredients in various fields. Beside being used for herbal therapeutic products, betel leaf

EO has a great potential to be used as natural food preservative and flavouring agent in the food industry (Mitali et al., 2019).

2.1.2 Kaffir lime (*Citrus hystrix*)

Kaffir lime (Figure 2.2) or “limau purut,” in Malay, with scientific name *Citrus* is a herb that botanically belongs to the *Rutaceae* family. The plant is commonly grown in some Asian countries such as Thailand, Malaysia and Indonesia. It is a small, thorny and bushy tree with aromatic leaves that grows to be around 3-5 m tall. This underutilized tropical fruit is a bumpy, green citrus fruit with a very acidic flavour that matures to yellow skin (Abirami et al, 2014).

Figure 2.2 Kaffir lime (*Citrus hystrix*) (Source: Anuchapreeda et al., 2020)

Kaffir lime is also used in traditional medicine to cure many diseases such as heart disease, dizziness, indigestion and as physical nourishment (Hutadilok-Towatana, 2006). The fruit peel was traditionally used by Malaysians to wash their hairs and body whereas the fruit juice is used on skin for softening purpose or mixed with bath water to control body odour (Nor, 1999). Kaffir lime leaves and fruits are commonly used as aromatics and spices for flavouring purpose due to their unique and strong fragrance. It is also reported that kaffir lime leaves extract exhibit antioxidant, anticancer and anti-inflammatory properties (Abirami et al., 2014). D-limonene is the most prominent bioactive compound, accounting for highest percentages of 19.08% in kaffir lime (Sreepian et al., 2019).

Kaffir lime EO is often used in aromatherapy to treat anxiety and depression due to its pleasant and soothing aromatic smell (Sarkar et al., 2015). According to Nanasombat and Lohasupthawee (2005), kaffir lime EO exhibited antibacterial property against several types of *Salmonella* and *Enterobacteria*. Previous research had also reported that the EO of kaffir lime exhibited antibacterial activity against *Bacillus cereus, Salmonella typhi and Staphylococcus aureus* (Chaisawadi et al. 2005),

2.2 Challenges associated with the application of essential oils in food

Essential oil exerts various health benefits and have antibacterial and antioxidant properties. Both betel leaf and kaffir lime EOs have high potential to be incorporated into food and beverages as functional ingredients. However, the direct application of EOs is often limited and creates a formidable challenge to food industry. This is due to the hydrophobic properties of the EO that makes it insoluble in water and therefore difficult to be dispersed in water-based food system. EO is also unstable compounds with high sensitivity to factors like light, heat and oxidation (Troncoso-Rojas et al., 2013). For these reasons, the direct incorporation of EO into food matrix is inappropriate because majority of food preparation methods require heat treatment and exposure to air and light which might lead to degradation of EO. The oxidation degradation causes deterioration of EO compounds generating free radicals and development of unpleasant taste and off-odour (Aguilar-Veloz, 2020). The high volatility and pungent taste of the EOs may adversely affect the organoleptic properties of the foods (Noori et al., 2018) which will reduce the consumer acceptability on the food and beverage products. In addition, the susceptibility to degradation of EO may reduce the antioxidant and antibacterial activities and therefore (Turek and Stintzing, 2013) higher concentration of EO is required to be introduced in the food system in order to achieve the desirable effect (Zhaveh et al., 2015).

2.3 Nanoemulsion technology

Formulating EOs into nanoemulsions has provided a potentially advantageous alternative for the use of EOs in food matrix (Donsi et al., 2011). In recent years, nanoemulsions have been used as a delivery system for lipid compounds including EOs. Nanoemulsion is a colloidal dispersion containing nano-sized droplets, in the range of 20-200 nm suspended in an aqueous phase (Moghimi et al., 2017). Nanoemulsions are commonly used to encapsulate, protect and improve the delivery of lipid bioactive compounds. Nanoemulsions can be classified as water-in-oil (W/O) and oil-in-water (O/W) emulsions. W/O nanoemulsion represents the small water droplets distributed in an oil medium, while O/W nanoemulsion such as EO denotes the dispersion of small oil droplets in an aqueous medium (McClements and Jafari, 2018).

The development of EO into nano-sized particles provides a lot of advantages. Firstly, nanoemulsions are optically transparent or translucent because of its particle size is much smaller than the light wavelength which enabling it to be incorporated into transparent beverages (McClements et al., 2011). The small particle size and high surface to volume ratio of nanoemulsions contributes to the droplet deformation-derived phenomena are typically higher in the case of nanoemulsions (Helgeson, 2016). The physical stability and bioavailability of encapsulated active compounds are greatly enhanced in nanoemulsions and hence avoiding conventional destabilization phenomena, namely creaming, sedimentation, coalescence, and flocculation and gravitational separation (Singh et al., 2017).

In addition, the reduced droplet size greatly increases the antibacterial activity of antimcrobials as it can be dispersed easily in food area where the microorganisms grow (Donsi et al., 2011) and enhances the transport of active ingredients through biological membranes, thus intensifying the bioavailability of bioactive compounds (Acosta, 2009). Meanwhile, the encapsulation of the essential oil in nanoemulsion also helps to minimise the impact to the organoleptic properties of the foods (Donsi et al., 2011).

There was previous work on the utilization of oregano EO nanoemulsion to minimize the foodborne pathogen on fresh lettuce. (Bhargava et al, 2015). Landry et al (2015) also applied carvacrol EO nanoemulsion on food such as broccoli, radish seed, mung bean and alfalfa seeds. These work indicated the potential utilization of nanoemulsion technology in food products.

2.3.1 Important components of essential oil nanoemulsions

Emulsifier or surfactant and ripening inhibitor are two crucial components in nanoemulsion formulation. Emulsifier is a surface-active agent that facilitate the formation of nanoemulsion and keep two immiscible liquids mixed whereas ripening inhibitor helps in improving the physical stability of nanoemulsion.

2.3.1.1 Emulsifier

Emulsifier is a surface-active amphiphilic molecule that facilitate formation of emulsion by adsorbing onto the oil-water interface during homogenization. Molecular emulsifiers have amphiphilic property due to the presence of both hydrophilic and hydrophobic groups (Chakraborty and Dhar, 2017). The polar heads are oriented to aqueous phase while the non-polar tails face the oil phase. It helps in reducing the interfacial tension between immiscible liquids thus facilitating droplet disruption and creating a protective interfacial layer that prevent droplets from aggregating (McClements and Gumus, 2016).

Hydrophilic-lipophilic balance (HLB) is a good indicator to measure the affinity of surfactant to the oil phase. Surfactants that have high HLB numbers (>10) are more attracted to water (hydrophilic) hence it is used to prepare O/W emulsions because the concentration of water is much higher than oil. On the contrary, the surfactants that have low HLB numbers (<10) have affinity for oil (lipophilic) (Hasenhuettl and Hartel, 2008) so it is suitable to be utilized in the formation of W/O emulsions (Arancibia et al., 2017).

Plant-based emulsifiers such as proteins (soy or pea protein), polysaccharides (gum arabic or modified starch), phospholipids (soy or sunflower lecithin), surfactants (quillaja saponin) are often used as emulsifier or stabilizing agent in food industry. Synthetic surfactants such as Tween and Spans are frequently employed alone or in combination with these ingredients. According to Komaiko and McClements (2014), Brij, Span and Tween are synthetic surfactants that commonly used to prepare nanoemulsions by spontaneous emulsification. Since the surfactant types and surfactant to oil ratio influence the size of the emulsion droplets and also their stability, only surfactants with small molecular mass able to formulate nanoemulsions through spontaneous emulsification. This is because the small surfactant molecules tend to diffuse faster from the oil phase to the aqueous phase. Komaiko and McClements (2015) reported that medium-chain triglyceride nanoemulsions made with Tween 80 exhibited the smallest droplet size in comparison to other sucfactants using spontaneous emulsification. Tween 80 has optimum surfactant curvature when it attaches to the interface between the oil phase and aqueous phases. This is because Tween 80 has one unsaturated tail whereas other Tweens have saturated linear tails or more unsaturated tails (Komaiko and McClements, 2016).

There are different types of emulsifiers available in the market. However, the selection of suitable emulsifier for each application needs to be concerned because their functions vary considerably and emulsifiers can affect both fabrication methods and performances of nanoemulsion.

2.3.1.2 Ripening inhibitor

Ripening inhibitor is a substance that is introduced to the nanoemulsion's dispersed phase to prevent droplet development caused by Ostwald ripening (Wooster et al., 2008). Ostwald ripening is an unfavourable phenomenon that causes the growth of the oil droplets during storage period. Droplet growth happens as a consequence of oil molecules diffusing from smaller droplets to bigger droplets through the intervening aqueous phase due to the variations in the chemical potential of the oil inside droplets with different dimensions (Taylor, 1998; McClements, 2014)

EO nanoemulsions are particularly susceptible to Ostwald ripening even though EO is constituted with hydrophobic compounds because many compounds are relatively soluble in aqueous phase when they are encapsulated in nanoemulsion (Ryu, 2017). Therefore, ripening inhibitors are usually employed in O/W nanoemulsion that are prepared from oil phases with a relatively high solubility in water such as EO and flavour oils (Chang et al, 2012; McClement and Rao, 2011). Without the presence of ripening inhibitor, the creaming and phase separation in EO nanoemulsion will be accelerated.

Ripening inhibitors are typically water-insoluble oil, such as long chain triglycerides that are added prior to nanaoemulsion formation. Sunflower oil, corn oil and palm oil are some of the examples of ripening inhibitors. Ripening inhibitor helps to retard droplet growth through an entropy of mixing effect. As the water-soluble oil molecules like EOs diffuse from the small droplets to the large droplets, the concentration of the ripening inhibitor that remains in the smaller droplets increases. Therefore, the water-soluble oil molecules move from large to small droplets, caused by the development of concentration gradient and thereby counterbalancing the droplet growth through Ostwald ripening (McClement and Jafari, 2018). Nonetheless, the incorporation of the ripening inhibitor can decrease the efficiency of nanoemulsion formation and the antibacterial activity of the EOs, therefore EO nanoemulsions preparation must be carefully conducted.

2.3.2 Essential oil nanoemulsions formation

EO nanoemulsions can be prepared by using two different formation methodologies, namely high energy method and low energy method. High energy method, which requires specific mechanical instruments to create strong disruptive forces to overcome the kinetic barriers have been widely employed to produce large industrial scale of nanoemulsions (McClements and Jafari, 2018). Low energy methods on the other hand, rely on internal energy of the components to produce nanoemulsion spontaneously in response to alteration of the compositions or environmental conditions. In this research, low energy method, namely spontaneous emulsification is used for preparing EO nanoemulsions.

2.3.2.1 High energy method

High energy methods such as high-pressure homogenization, microfluidization and sonication are commonly used to prepare nanoemulsions for industrial applications (Salvia-Trujillo et al., 2016). In this case, the mechanical devices play an important role to provide intense disruptive forces. In high-pressure homogenization, a pump functions by forcing a coarse emulsion through a tiny valve and hence creating high shear and turbulent forces to break down the large droplets (Modarres-Gheisari et al., 2019). For microfluidization, a coarse emulsion is driven by a pump into two separate channels, which are then forced to impact on each other at high velocity, thereby causing the large oil droplets to be disrupted. An ultrasonic probe is placed in a mixture containing oil, water and emulsifier to generate strong disruptive forces. Advantages of high energy methods include lower energy and emulsifier consumption, smaller droplets size and higher stability of the nanoemulsions (Hashtjin and Abbasi, 2014). However, the rise of temperature that result in the heat sensitive bioactive compounds in the EO cannot be encapsulated well is one of the disadvantages of using high energy method (Liew et al., 2020).

2.3.2.2 Low energy method

Low-energy methods such as phase inversion composition (PIC), phase inversion temperature (PIT), emulsion inversion point (EIP) and spontaneous emulsification (SE) are common low energy methods used to produce nanoemulsions. It relies on the spontaneous formation of tiny oil droplets when the system composition or environmental conditions are changed in a certain way (Anton et al., 2008).

PIC is carried out by altering the composition at constant temperature during emulsification phase. Unlike PIT method which can only be utilised with thermoresponsive surfactants systems like alkyl ethoxylates, PIC can be used with all types of surfactants and thermolabile molecules (Feng et al, 2020). When the ratio of water to oil was kept constant, various dispersion phases were observed when the temperature was changed, which was linked to changes in the solubility of non-ionic surfactants (Shinoda and Saito, 1968). The intermediate temperature at which the phase inversion occurs is recognized as PIT or HLB temperature (Sloans and Sole, 2012). In EIP, the composition of the system is changed at a constant temperature whereby the nanoemulsion is produced by gradual dilution with oil or water (Sadtler et al., 2010).

For SE, the oil phase which contains oil, surfactant, ripening inhibitor is mixed with aqueous phase that contain only water. The rapid diffusion of surfactant from the oil phase to the aqueous phase occurs when they come into contact and not in equilibrium state (Anton and Vandamme, 2009). The spontaneous emulsification method is useful for applications that require incorporation of tiny quantities of lipophilic components into clear aqueous-based beverages such as flavours, nutraceuticals, vitamins or antimicrobials (Komaiko and McClement, 2015). Figure 2.3 shows the schematic representation of spontaneous emulsification method where oil phase (oil + surfactant) is added dropwise and mixed with an aqueous phase under constant magnetic stirring. The molecular view is also included where the surfactant moves from the organic phase to the water phase (red arrows), causing interfacial turbulence and spontaneous oil droplet formation.

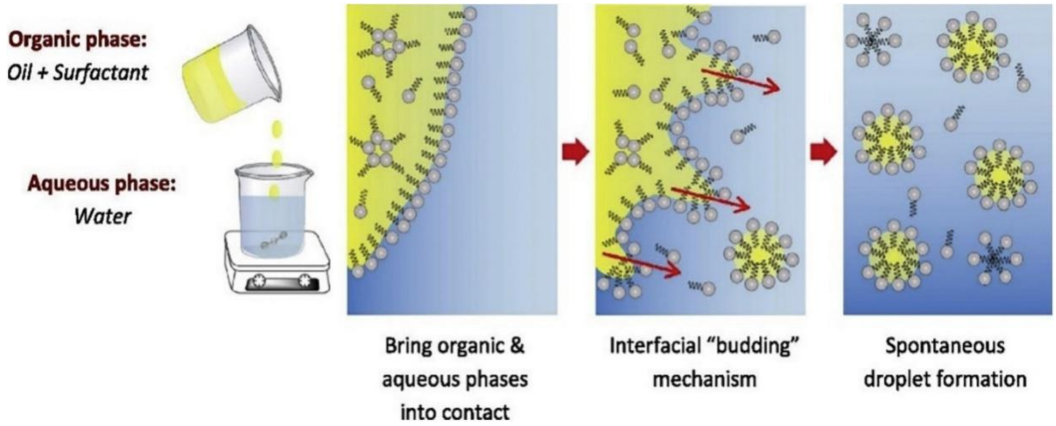

Figure 2.3 Schematic representation of spontaneous emulsification method (Source: Saberi, 2013)

One of the advantages of using low energy methods is that it does not utilize any use of the mechanical device. This method can be used to prepare EO nanoemulsion over a wide range of temperatures rather than at a fixed temperature close to the phase inversion. The low energy method is preferable due to its capacity to encapsulate heat sensitive compound in EO (Komaiko and McClements, 2016). However, this method requires large amount of surfactants with the careful selection of surfactants during the preparation of the EO nanoemulsions (Hashtjin and Abbasi, 2014).

2.4 Stability of essential oil nanoemulsions

Stability of EO nanoemulsions is usually influenced by various destabilization mechanisms because EO nanoemulsions are thermodynamically unstable system. The destabilization phenomena such as creaming, sedimentation, flocculation, coalescence and Ostwald ripening (OR) may contribute to phase separation between organic and aqueous phase in EO nanoemulsion. Among all the destabilization phenomena, nanoemulsion is particularly prone to a growth in particle size over time due to Ostwald ripening (Wooster et al., 2008).

Creaming is a destabilization process where the nanoemulsion droplets migrate upward whereas sedimentation is a phenomenon where nanoemulsion droplets move downward due to the density difference between dispersed droplets and continuous phase. EO nanoemulsion which usually O/W nanoemulsion usually faces the problem of creaming compared to sedimentation because the oil droplets are less dense than water that make the oil droplets tend to move upward. Reduced droplet sizes increased continuous phase viscosity and a smaller density difference between dispersed and continuous phases could help to minimize the gravitational separation (McClements and Jafari, 2018).

Flocculation is a phenomenon whereby two or more droplets aggregate forming a bigger cluster while maintaining their identity. It is a reversible process because the droplets aggregate without breaking of the interfacial surfactant film. It is usually a precursor for coalescence as it keeps the droplets close together for an extended period. Coalescence which is often coming after flocculation destabilizes the EO nanoemulsions system by causing the flocking together of smaller droplets to form a larger sized droplet. It is caused by the attractive interaction among the droplets.

Ostwald ripening causes the increase in droplet size due to the diffusion of oil molecules from tiny to large droplets aided by the differences in droplet curvature (McClement, 2005). The increase in water-solubility of the organic phase will lead to the increase in the rate of Ostwald ripening. OR occurs in emulsions with sub-micron droplets as well as conventional emulsions with a dispersed phase with high water solubility (Taylor, 1998). In this case, due to the difference in Laplace pressure, the chemical potential of the encapsulated molecule changes as it becomes more soluble as the droplet diameter decreases. Thus, EO nanoemulsions are still susceptible to OR in the fact that many compounds are relatively soluble in aqueous phase when they are encapsulated in nanoemulsion even though EOs are predominantly hydrophobic nature (Ryu, 2017). Hence, the ripening inhibitor such as corn oil is often introduced during the preparation of EO nanoemulsions to prevent this unfavourable destabilization phenomenon (Wooster et al., 2008).

Figure 2.4 represents common destabilization phenomena of nanoemulsions. Transmission electron microscopy (TEM) images of initial nanoemulsion are shown with a scale bar of 500 nm. Creaming and sedimentation phenomena can be seen by direct observation. Flocculation, coalescence and Ostwald ripening are shown with confocal micrographs with a scale bar of 50 μm.

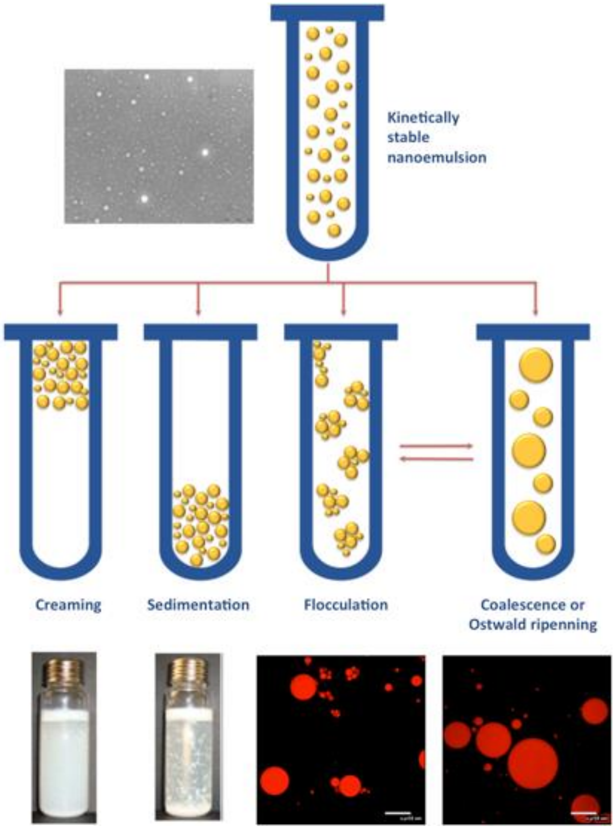

Figure 2.4 Destabilization phenomena in nanoemulsions (Source: Trujillo, 2014)

2.5 Characterization of essential oil nanoemulsions

The physicochemical properties of EO nanoemulsion have to be characterized as these properties are typically known to affect the texture, stability, taste and flavour of the foods. The physicochemical characteristics which include particle size, polydispersity index (PDI), particle size distribution (PSD), zeta potential, viscosity, turbidity and morphology of EO nanoemulsions are usually analyzed. Good stability of the EO nanoemulsion has the properties of small particle size (20-200 nm), low PDI, narrow PSD, high zeta potential and spherical shape. Particle size of EO nanoemulsion is the most crucial parameter to be measured because it has great impact on the appearance, rheology and the bioavailability of the bioactive compounds (McClements and Rao, 2011). Small particle size of EO nanoemulsion (20-200 nm) must be attained to achieve stable emulsion network and enable the incorporation of nanoemulsion in food and obtain a longer storage life. PDI is the width of the comprehensive size distribution of the nanoparticles. It is a dimensionless number ranging from 0 (monodisperse particle) to 1 (very broad dispersion) that used to determine if the average size of the nanoparticles generated has strayed from the predicted predictions (Kelmann et al., 2007). PSD is an index that is used to determine the size distribution of particles present in the given sample. The measurement of particle size, PDI and PSD of EO nanoemulsions can be determined by dynamic light scattering (DLS) (Zhang et al., 2017) in order to characterize the EO nanoemulsions.

Many characteristics of EO nanoemulsion including stability against aggregation and functional properties are determined by the type and amount of the superficial charge of droplets (McClements, 2015). Zeta potential is the potential difference in the interfacial surface boundary between two immiscible phases of EO nanoemulsion. Depending on the surfactant charge and surrounding condition, the electrical properties of nanoemulsion can range from strongly positive to neutral to strongly negative. Zeta potential which characterizes droplet superficial charge describes the electro-kinetic potential in a system. It is presented in millivolts (mV) and can be measured using zetasizer. Zeta potential values above 60 mV represent excellent electrostatic stability while values between 60 mV and 30 mV suggest good stability. The values of 5 mV to 15 mV shows a region of with limited flocculation while 3 mV to 5 mV indicate maximal flocculation (Bourbon et al., 2018). The droplets will repel each other and hence the EO nanoemulsion is stable when the zeta potential value is high. On the contrary, the low zeta potential corresponds to the destabilization phenomena such as creaming, coalescence, and Ostwald ripening (Chebil et al., 2013).

Viscosity is an important indicator to characterize the stability of EO nanoemulsion. The viscosity of a fluid medium determines its resistance to flow. The measurement of the viscosity can be achieved by using viscometer. The viscosity of nanoemulsions increases as the droplet size becomes larger and the dispersion phase becomes more concentrated. Thus, the droplets of EO nanoemulsions tend to merge and increase viscosity and viscoelastic behavior due to greater concentration of attractive forces present (Tadros, 2004).

Turbidity is defined as the rate at which liquid loses its transparency because of the presence of foreign particles suspended in the aqueous medium. It can be measured easily and precisely by using ultraviolet-visible (UV-Vis) spectrophotometer. Turbidity which measures the percentage of transmittance corresponds to the stabilization of the EO nanoemulsion and it is highly related to particle size. In the case of food and beverages especially transparent beverages, EO nanoemulsion with low turbidity is preferable because it will not affect the colour of the food and beverages itself when EO nanoemulsion is introduced (Molet-Rodríguez et al, 2018).

The nanostructure characteristic of EO nanoemulsion can be obtained by using Transmission Electron Microscope (TEM). This microscopy imaging technique provides detailed information about the sub-micron particles and the possible interaction between them (Salvia-Trujillo, 2014). It is a commonly used method to observe the morphology of EO nanoemulsion. It provides information on the size, shape and aggregation state of droplets in nanoemulsions. The imaging of EO nanoemulsion can be achieved by increasing the magnification up to few hundreds or thousands time depending on the different models of the electron microscope.

2.6 Antioxidant properties of essential oil nanoemulsions

Essential oils (EOs) which are made up of various organic molecules with conjugated carbon double bonds and hydroxyl groups exhibit antioxidant property. Antioxidant is defined as a substance that considerably reduces or suppresses the oxidation of an oxidizable substrate when present at low quantities compared to that substrate (Halliwell and Gutteridge, 1995). Antioxidant functions by donating hydrogen to free radicals, suppressing and reducing oxidative stress in order to prevent damage of cellular components result from the chemical reactions involving free radicals.

In food industry, off-flavours and rancidity result from oxidative degradation of fat-containing food products are always a challenge because the nutritional value and sensory quality are greatly influenced (Saad et al., 2007). The lipid oxidation contributes to the changes in flavour such as rancidity and colour such as carotenoids which are not favourable (Li et al., 2015). Hence, the addition of EO nanoemulsion which exhibit antioxidant property is one of the useful solutions to preserve product quality and extend the shelf life of the food products.

EO nanoemulsion which has higher solubility in water and smaller droplet size enables the active ingredients to be transported easily and the rapid penetration and hence improve the antioxidant effect. Sundararajan et al. (2018) reported that *Ocimum basilicum* EO nanoemulsion exhibited higher antioxidant activity than its EO at different concentration. From the previous study, it is shown that hydroxyl radicals scavenging activity of pure EO and EO nanoemulsion of *Citrus medica L. var. sarcodactylis* increased with the increased of concentration but nanoemulsifed EO had better performance than pure EO (Lou et al., 2017). The statement is further supported by Seibert et al. (2018) who reported that the percentage of 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) scavenging induced by *Cymbopogon densiflorus* leaf EO naoemulsion was higher than its EO.

2.7 Antibacterial properties of essential oil nanoemulsions

To maintain the quality and prolong the shelf life of food and beverages, EO nanoemulsions is useful in reducing the microbial contamination (Salvia-Trujillo et al., 2015). The reduced droplets size and surface charge enable EO nanoemulsions to be transported efficiently to the cell membrane and interact with the molecular site at the microbial cell membrane. It is reported that EO nanoemulsion is more effective to suppress the growth of Gram-positive bacteria compared to Gram-negative bacteria due to the different cell wall structure of the bacteria. Kaffir lime EO nanoemulsion also exhibited antibacterial activity against food borne pathogens such as *Escherichia coli*, *Staphylococcus aureus* and *Salmonella spp* (Liew et al., 2020). The experiment conducted by Salvia-Trujillo et al. (2015) revealed that that lemon and peppermint EO nanoemulsion showed faster inactivation of inoculated *E. coli* compared to coarse emulsion. Bhargava et al. (2015) found that oregano EO nanoemulsions potentially reduced the microorganism number like *Listeria monocytogenes*, *E. coli* and *Salmonella typhimurium*.

EO nanoemulsions can interact with microorganisms in two different ways. Firstly, nanoemulsion will contact the cell membrane and release the active compounds inside. These compounds of EOs able to move efficiently and partition into the hydrophobic part of the cell membrane due to the small nano sized particles and hydrophobic property of the encapsulated EOs (Ryu, 2017). Hence, the antibacterial activity of EO can be enhanced when the EO is formulated into the nanoemulsions. Secondly, by turning EO into nanoemulsions, EOs which have increased solubility in water allow the active components to pass through the aqueous and then diffuse into the cell membrane. The prolonged diffusion of EO in the bacteria cell membrane helps to improve the antibacterial activity of EO nanoemulsion (Ryu, 2017).

2.7.1 Selected food borne pathogens

Foods with high moisture content, nutritional levels or pH values deteriorate easily and tend to have short shelf life. Food borne pathogens such as viruses, bacteria and parasites are biological agents that lead to foodborne illnesses. *Clostridium botulinum, Salmonella spp, Staphylococcus aureus, Campylobacter jejuni, Bacillus cereus, Listeria monocytogenes*, and *Escherichia coli* O157:H7 are some of the food borne pathogens that will cause food borne illnesses and also food borne outbreak (Arshad and Batool, 2017). The food borne illnesses are the result of intake of the food which is contaminated by the pathogens or the ingestion of food that is contaminated by the toxins produced by the toxigenic pathogens (Bintsis, 2017).

In this research, three food borne pathogens, namely *Escherichia coli, Salmonella spp* and *Staphylococcus aureus* were used as test bacteria to determine the antibacterial properties the EO nanoemulsion samples. Shiga toxin-producing *E. coli* (STEC) strain O157:H7 is responsible for 63000 infections, 2100 hospitalizations and 20 deaths every year (Scallan et al., 2011). There is also multistate food borne outbreaks caused by *Salmonella spp* due to the contamination in the hot pies, peanut butter, raw tomatoes and fruit salad (Pigott, 2008). *S. aureus* lead to food borne diseases that causing estimated 241,000 illnesses per year in the United States (Scallan et al., 2011). Thus, these bacteria were selected because they are the common food borne pathogens that causes food borne illnesses and outbreak.