PHYSICOCHEMICAL AND ANTIBACTERIAL PROPERTIES OF CALAMANSI LIME AND PANDAN LEAF ESSENTIAL OIL NANOEMULSIONS

by

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This dissertation is composed of my original work and contains no material previously published or written by other person except where due reference has been made in the text. The content of my dissertation is the result of work I have carried out since the commencement of my research project and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution.

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LIST OF ABBREVIATIONS

Abbreviation	Caption
µL	Microlitre
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
CFU/ml	Colony-forming unit/millilitre
CL	Calamansi lime
CL:PL	Mixture of calamansi lime and pandan leaf at 1:1 ratio
DAG	Diacylglycerols
DLS	Dynamic light scattering
EO	Essential oil
FDA	Food and Drug Administration
FFA	Free fatty acids
GC	Gas chromatography
GRAS	Generally recognized as safe
h	Hour
HLB	Hydrophilic-lipophilic balance
HPLC	High-performance liquid chromatography
HSD	Honestly significant difference
kV	Kilovolt
LCT	Long-chain triglycerides
LPS	Lipopolysaccharides
MAG	Monoacylglycerols
MCT	Medium-chain triglyceride
MHA	Mueller-Hinton agar

MHB	Mueller-Hinton broth
min	Minute
mL	Millilitre
mm	Millimetre
mPa·s	Millipascal-second
mV	Millivolts
nm	nanometre
o/w	Oil-in-water
PCS	Photon correlation spectroscopy
PDI	Polydispersity index
pH	Power of hydrogen
PIT	Phase inversion temperature
PL	Pandan leaf
PSD	Particle size distribution
PTA	Phosphotungstic acid
QLS	Quasi-elastic light scattering
rpm	Revolutions per minute
SEM	Scanning electron microscopy
T%	Percentage of transmittance
TAG	Triacylglycerols
TEM	Transmission electron microscopy
v/v	Volume/volume
w/v	Weight/volume

LIST OF SYMBOLS

Symbol	**Caption**
α	Alpha
β	Beta
°	Degree
℃	Degree Celsius
=	Equal
<	Less than
–	Minus
%	Percent
+	Plus
±	Plus-minus
:	Ratio

SIFAT FIZIKOKIMIA DAN ANTIBAKTERIAL NANOEMULSI MINYAK PATI LIMAU KASTURI DAN DAUN PANDAN

ABSTRAK

Sejak kebelakangan ini, terdapat minat yang tinggi dalam penggunaan minyak pati sebagai agen antimikrobial semula jadi dalam pengawetan makanan atas sebab penolakan pengguna terhadap penggunaan pengawet sintetik dalam makanan. Nanoemulsi telah dikenali sebagai sistem penyampaian minyak pati yang amat baik dengan peningkatan kelarutan air dan perlindungan terhadap degradasi. Dalam kajian ini, sifat fisikokimia termasuk ukuran saiz partikel, nilai polidispersi (PDI), taburan saiz partikel, potensi zeta, kekeruhan, kelikatan dan morfologi, serta aktiviti antibakterial nanoemulsi minyak pati limau kasturi (*Citrofortunella microcarpa*) dan daun pandan (*Pandanus amaryllifolius*) yang disediakan menggunakan kaedah pengemulsian spontan telah disiasat. Dalam penyediaan nanoemulsi, 5% (v/v) minyak (minyak pati dengan minyak jagung pada nisbah 8: 2) dicampurkan dengan 15% (v/v) Tween 80 dahulu, diikuti dengan penambahan ke dalam 80% (v/v) air suling dengan menggunakan pengaduk magnet pada 750 rpm dan 25 ℃. Nanoemulsi minyak pati daun pandan menunjukkan saiz partikel (19 nm) dan nilai PDI yang terkecil (0.250), manakala nanoemulsi mintak pati limau kasturi mempunyai saiz partikel (41 nm) dan nilai PDI (0.655) yang terbesar. Nanoemulsi minyak pati daun pandan lebih homogen, sementara nanoemulsi minyak pati limau kasturi menunjukkan sebaran saiz yang lebih luas. Semua sampel menunjukkan nilai potensi zeta negatif dan kelikatan yang rendah dengan penampilan yang telus. Partikel minyak berbentuk sfera dengan pelbagai saiz telah didedahkan oleh mikrograf mikroskop electron penghantaran. Semua sampel mengekalkan kestabilan fizikal selepas tempoh penyimpanan selama 1 bulan pada 4 ℃ dan 25 ℃. Aktiviti

antibakterial yang signifikan tidak ditunjukkan oleh semua sampel terhadap *Staphylococcus aureus*, *Escherichia coli* and *Salmonella spp.* Nanoemulsi minyak pati menunjukkan potensi yang tinggi untuk dimasukkan ke dalam produk makanan berasaskan air, namun penyelidikan yang lebih lanjut mengenai formulasi nanoemulsi diperlukan untuk meningkatkan keberkesanan antimikrobial mereka bagi aplikasi pengawetan makanan.

PHYSICOCHEMICAL AND ANTIBACTERIAL PROPERTIES OF CALAMANSI LIME AND PANDAN LEAF ESSENTIAL OIL NANOEMULSIONS

ABSTRACT

In recent years, there is considerable interest in using essential oils (EOs) as natural antimicrobial agents in food preservation due to consumer's rejection on the utilization of synthetic preservatives in food. Nanoemulsion has been known to be an excellent delivery system of EOs with the improvement of water solubility and protection against degradation. In this study, the physicochemical properties including particle size, polydispersity index (PDI), particle size distribution, zeta potential, turbidity, viscosity and morphology, as well as antibacterial activity of calamansi lime (*Citrofortunella microcarpa*) and pandan leaf (*Pandanus amaryllifolius*) EO nanoemulsions prepared using spontaneous emulsification method were investigated. In the preparation of nanoemulsions, 5% (v/v) of oil (EOs and corn oil at the ratio of 8:2) was first mixed with 15% (v/v) of Tween 80, followed by addition into 80% (v/v) of distilled water using magnetic stirrer at 750 rpm and 25 °C. Pandan leaf (PL) EO nanoemulsion showed the smallest particle size (19 nm) and lowest PDI value (0.250), whereas calamansi lime (CL) EO nanoemulsion had the largest particle size (41 nm) and highest PDI value (0.655). PL EO nanoemulsion was more homogeneous, while CL EO nanoemulsion had broader size distribution. All samples exhibited low negative zeta potential values and viscosity with transparent appearance. Different sizes of spherical oil particles were shown by transmission electron microscopy micrographs. All samples remained physically stable after 1 month of storage at 4 °C and 25 °C. There was no significant antibacterial activity shown by all samples against *Staphylococcus aureus*,

Escherichia coli and *Salmonella spp*. The EO nanoemulsions showed high potential to be incorporated into water-based food products, however further investigation on the formulation of nanoemulsions is required in order to improve their antibacterial efficacy for food preservation.

CHAPTER 1

INTRODUCTION

1.1 Research background

Novel preservation technologies have gained much interest from consumers and food industries due to the damage of food quality caused by thermal processing and the harmful effects exerted by chemical preservatives on health (Singh et al., 2010). Natural antimicrobials have exhibited notable potential among various novel alternatives, where one of the examples involved the use of natural food grade antimicrobial compounds such as essential oils (EOs) (Saeed et al., 2019).

EOs are hydrophobic natural aromatic substances obtained from different plant parts by hydrodistillation, expression, steam distillation or solvent extraction techniques (Burt, 2004). EOs are known to possess antimicrobial and antioxidant properties due to the presence of bioactive components such as terpenes, terpenoids, esters, phenols and oxides (Yazgan et al., 2019). Lime EOs which can be extracted from the peels and leaves of lime are mainly contained limonene and other terpenes. These compounds are responsible for the strong antibacterial activity of lime EOs against *Staphylococcus aureus*, *Escherichia coli* and *Salmonella* Typhimurium (Matan et al., 2014). EOs extracted from pandan leaves, a tropical plant that is commonly found in Malaysia, also have been found to exhibit antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*. It is due to the great amount of antibacterial compounds such as phytol and α-thujaplicin (Mar et al., 2019).

Despite their strong antimicrobial properties, there are limitations on the utilization of EOs in foods and beverages. EOs which are hydrophobic with relatively low water solubility have limited application in real food systems especially in water-based food products due to their non-uniform distribution in the food matrices (Ryu et al., 2018). Meanwhile, their strong flavour properties can easily affect the sensory properties of food products and reduce consumer's acceptability. The volatile EOs are also highly susceptible to degradation in the presence of light, oxygen and temperature (Sotelo-Boyás et al., 2017). On top of that, direct incorporation of EOs into complex food matrices may reduce their antimicrobial activity due to the interaction of EOs with other food components (Ryu et al., 2018). Nevertheless, many studies have shown that these problems can be overcome by formulating EOs into nanoemulsions (Bento et al., 2020; Liew et al., 2020; Liu et al., 2019).

Nanoemulsions are described as oil-in-water (o/w) or water-in-oil (w/o) emulsion systems with mean droplet diameters ranging from 20 to 200 nm. Due to the small droplet size, they have some benefits over conventional emulsions. Nanoemulsions are kinetically more stable than conventional emulsions and have long-term stability against droplet aggregation and gravitational separation (Wooster et al., 2008). Moreover, nanoemulsions tend to be transparent that makes them suitable to be incorporated into transparent beverages. Greater antimicrobial activity of EOs also could be achieved since the extremely small droplet size in nanoemulsions can effectively increase the distribution of antimicrobial agents in food matrices and allow more interaction with microorganism with its larger surface area (Zhang et al., 2017). Hence, incorporation of EOs into nanoemulsions not only can reduce their impact on organoleptic properties of food products, but also can improve their dispersibility in water, stability, and bioavailability.

There is limited information available on the use of lime and pandan leaf EO nanoemulsions in the preservation of food products. Therefore, this study is designed to produce stable lime and pandan leaf EO nanoemulsions as natural preservatives for food applications. In this study, spontaneous emulsification method was used to fabricate nanoemulsions from calamansi lime (*Citrofortunella microcarpa*), pandan leaf (*Pandanus amaryllifolius*) and mixture of calamansi lime and pandan leaf EOs at a 1:1 ratio. Then, the physicochemical properties of EO nanoemulsions including particle size, polydispersity index (PDI), particle size distribution (PSD), zeta potential, turbidity, viscosity and morphology as well as their antibacterial activity against common foodborne pathogens namely *Staphylococcus aureus*, *Escherichia coli* and *Salmonella spp*. were investigated.

1.2 Problem Statement

Natural antimicrobial agents such as essential oils (EOs) have gained great attention from consumers due to their rejection on the incorporation of synthetic preservatives in food. EOs from calamansi lime and pandan leaf that are readily available in Malaysia and have been shown to exhibit antibacterial and antioxidant properties are highly potential in controlling the microbial growth in food products. However, direct incorporation of EOs into food systems particularly water-based food products is challenging due to their poor water solubility, high susceptibility to thermal degradation, and high sensitivity to light and oxidation. These issues appear to affect their dispersibility, stability, and antimicrobial activity in food systems, thereby minimizing their functionality. Therefore, formulating EOs into nanoemulsions not only able to improve the functional properties of EOs as natural antimicrobial agents but also expands its application in water-based food products particularly.

1.3 Objectives

General objective:

1. To produce stable calamansi lime and pandan leaf essential oil nanoemulsions as natural food preservatives.

Specific objectives:

1. To investigate the physicochemical properties of calamansi lime (*Citrofortunella microcarpa*) and pandan leaf (*Pandanus amaryllifolius*) essential oils nanoemulsions prepared using spontaneous emulsification method.

2. To study the antibacterial properties of nanoemulsions prepared from calamansi lime (*Citrofortunella microcarpa*) and pandan leaf (*Pandanus amaryllifolius*) essential oils.

CHAPTER 2

LITERATURE REVIEW

2.1 Essential oils

Essential oils (EOs) are hydrophobic natural aromatic substances which can be obtained by several techniques including steam distillation, hydrodistillation, expression, or solvent extraction from different parts of the plants (Burt, 2004). In order to provide protection from external agents such as insects, pathogens, herbivores and sunlight, EOs are usually produced as secondary metabolites in different plant organs (Asbahani et al., 2015). EOs are very complex natural mixtures consisting of 20-60 components which are unsaturated hydrocarbons oxygenated. They are usually constituted by up to 85% major components and a variety of other trace components (Bakkali et al., 2008). According to Pavela (2015), the biological properties of the EOs are generally determined by these major components.

The main groups of EOs are divided into two groups, which are terpenes (monoterpenes and sesquiterpenes) and terpenoids (phenols, alcohols, esters, ketones, aldehydes, ethers) and aromatic and aliphatic constituents (Burt, 2004). The bioactive components presented in the EOs contributed to their main antibacterial and antioxidant properties (Yazgan et al., 2019). In nature, many terpenoids in plants have been used to avoid bacterial infections in living tissues as it can cause cell death by interacting directly with the bacterial cell wall or membrane and alter the permeability of adenosine triphosphate (ATPs) or similar essential molecules (Arendt et al., 2016; Nazzaro et al., 2013). Hence, EOs have been widely used in pharmaceutical and health industries, cosmetics and perfume industries and preservation of food as antiseptic, antibacterial and preserving agents. On top of that, novel preservation technologies have gained great attention from consumers and food industries because of the damage of food quality resulting from thermal processing and the rejection of the use of synthetic preservatives in food preservation. Therefore, EOs which exhibited notable potential as natural food grade antibacterial preservatives have drawn considerable interest in recent years.

2.1.1 Calamansi lime essential oils

Lime is one of the most important citrus fruits for EO extraction due to the effectiveness in food preservation applications and fresh citrusy aroma. According to Ruberto (2002), lime EOs could be defined as aromatic oily liquids that are obtained from the flowers, twigs, leaves, and peel of the lime or as by-products of the juice industry. The calamansi lime (*Citrofortunella microcarpa*) from the *Rutaceae* family, which is widely grown in tropical and subtropical areas, is one of the examples of limes that is commonly found in Malaysia alongside with kaffir and key lime (Othman et al., 2016). Calamansi lime is a shrub with an abundant of long spine on the branches, twigs and stem with green and round or oblong-shaped leaves. The EO can be extracted from the peels and leaves by hydrodistillation (Othman et al., 2016).

Figure 2.1: Calamansi lime (*Citrofortunella microcarpa*) (Source: Baker, 2020).

Major bioactive compound of EO from the peels of calamansi lime is monoterpene hydrocarbon, limonene (94.0%) with minor components including β-myrcene (1.8%), linalool (0.4%) and α-terpineol (0.3%), whereas sesquiterpene hydrocarbons are the most abundant components in the EO obtained from the leaves including hedycaryol (19.0%), α-sesquiphellandrene (18.3%), α-eudesmol (14.4%) and β-eudesmol (8.6%) (Othman et al., 2016; Palma et al., 2019). The EO with the high concentration of limonene have been proven to show its antibacterial properties against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella* Typhimurium, *Bacillus subtilis* and *Streptococcus spp*. (Liew et al., 2020; Sotelo-Boyás et al., 2017). In addition, calamansi lime EO also exhibits high antioxidant activity with the presence of sesquiterpene hydrocarbons, β-myrcene, linalool and α-terpineol (Assaeed et al., 2020; Wang et al., 2019; Xanthis et al., 2021). However, calamansi lime EO with lipid soluble limonene can easily lose its bactericide and antioxidant activity due to its susceptibility to both physical instability and oxidative degradation (Djordjevic et al., 2008; Lu et al., 2018).

2.1.2 Pandan leaf essential oils

Pandan leaf (*Pandanus amaryllifolius*) is a tropical plant in the screw pine (*Pandanus*) genus that is easily available in Malaysia and often being used to enhance the flavour of dishes due to their nutty and botanical fragrance contributed by the key aroma compound (2-acetyl-1pyrroline) (Jimtaisong & Krisdaphong, 2013). It is an upright bright green plant with fan-shaded spray of long, slender, spiky leaves and woody aerial roots. As the plant is sterile, it bears no fruit and is to be propagated by vegetative parts. Methods including carbon dioxide extraction, cold pressing, phytol extraction and hydrodistillation can be used to extract the EO from pandan leaves (Mar et al., 2019).

Figure 2.2: Pandan leaves (*Pandanus amaryllifolius*) (Source: Jills, 2019).

The EO of pandan leaves has been reported to contain large amount of phytol (21.35%), α-thujaplicin (18.64%), dodecanol (12.55%), tetradecanol (8.93%), benzyl acetate (8.08%) and linalool (2.45%), which may play important roles as antimicrobial compounds (Mar et al., 2019). In addition, other minor constituents such as *n*-heptadecane, eugenol, benzyl benzoate, indole and benzyl alcohol found in the EO may also contribute to the antimicrobial properties. It has been reported to exhibit antimicrobial activity against *Escherichia coli*, *Micrococcus luteus*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Mar et al., 2019). On top of that, squalene and phenolic compound (tocopherol) that are highly present in the EO are efficient antioxidants due to their capability to scavenge free radicals and reduce free radical formation (Brunetti et al., 2013; Zakaria et al., 2020). According to Nor et al. (2008), pandan leaf EO has high potential to be used as natural food antioxidants due to its capability to retard oxidation effectively as well as its excellent heat-stable antioxidant property.

2.1.3 Synergistic effect of essential oil

EOs are the most widely used natural products in food preservation, but the requirement of high concentrations to achieve adequate antimicrobial and antioxidant effects can result in negative organoleptic impacts on food (Pietrasik et al., 2013). The antimicrobial efficacy of EOs may also be improved by combining different EOs (Gadisa et al., 2019), thereby minimize their sensory impacts by increasing their effectiveness even at lower concentrations (Ouedrhiri et al., 2017). One of the four possible effects including synergistic, partial synergistic, additive and antagonism can occur contributed by the interaction of two or more agents in the combination in different manners (Kasrati et al., 2014). According to Burt (2004), additive effect is observed when combined effect of antimicrobials is equal to the sum of the individual effects, whereas antagonism is known when the mixture of antimicrobials has less inhibitory effect than when individually used. Synergistic interaction which represents the observation of greater effect of the combined antimicrobials compared to the sum of the individual effects, are the most significant as they are able to enhance the antimicrobial and antioxidant properties by making use of the efficiencies of the combined agents in the best possible manner and lead to several fold reduction in the required concentrations of the combined agents (Adrar et al., 2016; Shojaee-Aliabadi et al., 2017).

The multi-component nature of the combination plays an important role in the mechanism of the underlying synergistic effects. Different types of components or compounds in the combination of EOs may work synergistically to influence multiple biochemical processes in the bacteria and reinforcing the effect of each other, thereby improving the functional properties of the combined agents (Sharma et al., 2020).

Several studies have shown that the antimicrobial properties of combination of two or more EOs could increase compared to individual EO. Doi et al. (2019) have reported the synergism between cinnamon and oregano EOs when used for inactivation of food-borne pathogens, *Staphylococcus aureus*. Moreover, a combination of EOs from cinnamon and clove managed to exhibit a synergistic effect in controlling the growth of *Listeria monocytogenes*, *Bacillus cereus* and *Yersinia enterocolitica* (Goñi et al., 2009). In order to achieve maximum synergistic effect, appropriate combination and concentration of EOs are important factors to be optimized (Sharma et al., 2020).

2.1.4 Challenges of EOs incorporation in foods

Although the EOs are recognized as generally recognized as safe (GRAS) by the US Food and Drug Administration (FDA), the direct incorporation of EOs in foods and beverages still exhibited some limitations and several drawbacks (Salvia-Trujillo et al., 2015). Production of organoleptic properties such as strong odour and flavour and the arise of technological limitations due to the hydrophobicity, volatility and reactivity of the EOs have made it difficult for food application (Sugumar et al., 2016). Gutierrez et al. (2009) reported that high levels of EOs can influence the sensory properties of foods including taste, aroma and flavour and cause them to be unacceptable. EOs which are hydrophobic with relatively low solubility in water can result in non-uniform distribution in food matrices, thus they have to be incorporated into foods using suitable delivery systems such as in emulsions or organic solvents (Ryu et al., 2018). According to Sotelo-Boyás et al. (2017), EOs which are highly volatile also susceptible to degradation when exposed to oxygen, light and temperature. Moreover, Ryu et al. (2018) reported that EOs tend to interact with other components such as proteins and lipids with hydrophobic binding in the food systems when they are introduced into complex food matrices and thus their antimicrobial activity is often affected and reduced. Since proteins are the main component of the food matrix, their hydrophobic nature and the presence of a three-dimensional matrix layer act as a barrier that can prevent the EOs to penetrate into the inner structures of the bacterial cells (García-Díez et al., 2017; Sugumar et al., 2015). Hence, incorporation of EOs into nanoemulsion is a promising strategy to improve the functional properties and physicochemical stability of EOs in order to achieve high antimicrobial efficiency and expand its application in foods.

2.2 Nanoemulsions

Nanoemulsions are described as oil-in-water (o/w) or water-in-oil (w/o) emulsion systems with mean droplet diameters ranging from 20 to 200 nm (Sagalowicz & Leser, 2010). Nanoemulsions generally consist of an oil phase (organic phase), aqueous phase and emulsifier. The oil phase can be formulated from a variety of nonpolar components such as monoacylglycerols (MAG), diacylglycerols (DAG), triacylglycerols (TAG), free fatty acids (FFA), mineral oils, waxes, oil-soluble vitamins, flavour oils, fat substitutes, EOs or various lipophilic nutraceuticals (McClements & Rao, 2011). TAG oils extracted from corn, soybean and sunflower which are low cost and nutritious are the most commonly used oils in nanoemulsions (McClements & Rao, 2011). Physical and chemical characteristics of the oil phase such as viscosity, density, refractive index, polarity, interfacial tension and water-solubility greatly influence the formation, stability and properties of nanoemulsions (McClements, 2011). On the other hand, the aqueous phase can be composed of water with a variety of polar molecules, proteins, minerals, carbohydrates, acids, bases and alcoholic co-solvents, whereby the composition of the aqueous phase can directly affect the physicochemical properties of the fabricated nanoemulsion (McClements & Rao, 2011). Different types of emulsifiers such as phospholipids, polysaccharides, small molecule surfactants and proteins may be added to facilitates the formation of emulsions by reducing the interfacial tension between the oil and aqueous phases and enhance the stability of nanoemulsions (Gupta et al., 2016; Mason et al., 2006).

Due to the small droplet size, nanoemulsions are kinetically more stable than conventional emulsions and have long-term stability against droplet aggregation and gravitational separation as the strength of the net attractive forces acting between the droplets reduces (Karthik et al., 2017; Wooster et al., 2008). However, they are thermodynamically unstable resulting from the free energy of the colloidal dispersion which is higher than that of the separate phases (oil and water) (Simonazzi et al., 2018). Moreover, nanoemulsions with the droplet size smaller than the wavelength of visible light ranging from 300-700 nm tend to be transparent due to no or a weak light scattering effect (Gupta et al., 2016) , which makes them suitable to be incorporated into foods and beverages. Greater antimicrobial activity of EOs could also be achieved since the extremely small droplet size in nanoemulsions can effectively increase the distribution of antimicrobial agents in food matrices, thereby reducing the amount of the EOs required (Zhang et al., 2017). The biological activity of the EOs incorporated in nanoemulsions can be improved due to the enhanced transport of active molecules through biological membranes and increased reactivity contributed by a higher surface area/volume ratio (Salvia-Trujillo et al., 2015).

2.3 Fabrication of essential oil nanoemulsion

External or internal energy source is needed to fabricate nanoemulsion which is a nonequilibrium system. Fabrication of nanoemulsion can be performed by either high-energy or low-energy emulsification methods that involve mechanical and chemical processes, respectively. The selection of methods for the formation of nanoemulsion has a great impact on the droplet size and stability mechanisms of the emulsion system through operating conditions and composition.

2.3.1 High-energy emulsification methods

High-energy emulsification methods involve the use of mechanical energy to produce intense disruptive forces to mix and disrupt oil and water phases that lead to the formation of tiny oil droplets (Wooster et al., 2008). Equipment such as high-pressure valve homogenizer, microfluidizer and ultrasonic homogenizer are used for this method. Generally, the methods utilize a two-step emulsification process that involves initial production of coarse emulsion with a large droplet size using a high-shear mixer followed by high-shear homogenization to obtain nanoemulsion (Lee et al., 2018). The droplet size produced is usually affected by a balance between two opposing processes, which are the droplet disruption and droplet coalescence that occur within the homogenizer under high-energy emulsification (Jafari et al., 2008). Proper viscosity ratio, higher homogenization intensity or duration and emulsifier concentration can result in the formation of smaller droplets (Wooster et al., 2008).

In high-pressure valve homogenization, the droplet size of coarse emulsion can be reduced effectively to nano size by subjecting to very high pressures and pumped through a restrictive valve. The mechanism of homogenizer involves a pump that pulls the coarse emulsion into a chamber on its backstroke followed by forcing through a narrow valve at the end of the chamber on its forward stroke, thereby breaking down the larger droplets into smaller ones due to the production of strong disruption forces during collision (McClements & Rao, 2011). The higher the homogenization pressure and/or the number of passes, the smaller the droplet size produced, which can leads to a lower interfacial tension and increase in the emulsifier adsorption on the droplet surface to prevent re-coalescence (Jafari et al., 2008). In addition, an emulsifier that is able to adsorb on the droplet surfaces rapidly is needed in sufficient amounts to cover the surfaces of the newly formed droplet during homogenization to prevent coalescence (Jafari et al., 2008).

Microfluidizer is similar in design to high-pressure valve homogenizer as it also employs high pressure to force the premix of coarse emulsion through a narrow orifice for the production of intense disruptive forces. Nonetheless, the channels in which the emulsion flows are different in microfluidizer. The emulsion flowing is divided through a channel into two streams and redirected into an interaction chamber, in which they collide with each other at very high speed and pressure thus leading to the production of intense disruption forces which form small droplets (McClements, 2011). The droplet size of nanoemulsion formed by microfluidizer generally decreases with increasing energy input, except in the case of energy input which is higher above certain optimal conditions, in which it can contribute to the formation of larger droplet size due to 'overprocessing' (Jafari et al., 2007).

Emulsification can also be done in an ultrasonic system by using a sonicator probe. When a high-intensity electric field is applied, the probe contains piezoelectric quartz crystals oscillate to generate ultrasonic waves producing intense mechanical vibration and cavitation forces. As a result, the formation of liquid jets at high speed and the collapse of the vapor cavities creates intense disruptive forces in the liquid surrounding the probe that lead to droplet disruption and formation of nano-sized droplets (McClements & Rao, 2011; Salem & Ezzat, 2019). Both emulsion composition and the power intensity of sonification influence the droplet size of nanoemulsion produced (Anton et al., 2008). The droplet size decreases when there is an increase in energy density or duration at optimal conditions.

2.3.2 Low-energy emulsification methods

Low-energy emulsification methods which do not require a high mechanical input can also be used to produce nanoemulsions, whereby the two most commonly utilized methods are phase inversion temperature (PIT) and spontaneous emulsification (McClements & Rao, 2011; Solans & Solé, 2012). The low-energy methods involve a chemical process that is dependent on the internal chemical energy of the system to produce small droplets by gentle stirring (Solè et al., 2006). The nanoemulsions are fabricated by manipulating the intrinsic physicochemical properties and phase behaviour of the system components under certain optimal conditions (Komaiko & McClements, 2016). Droplet size of nanoemulsion produced by low-energy emulsification methods is affected by the environmental temperature, emulsion composition such as type of surfactant, surfactant-oil-water ratio and ionic strength as well as the stirring speeds and duration (Anton et al., 2008). Low-energy approaches are able to produce smaller droplet sizes more efficiently than high-energy approaches, but the types of oil and emulsifier that can be utilized are limited (Salem & Ezzat, 2019).

In PIT method, changes in temperature induced alteration in the curvature of non-ionic surfactants which modify the solubility of surfactants leading to the inversion of an o/w emulsion to a w/o emulsion or vice versa (Walker et al., 2015). A kinetically stable nanoemulsion can be produced by quick cooling or heating so that the temperature is close to the phase inversion temperature (PIT) (Jintapattanakit, 2018). The surfactant exhibits both hydrophilic and lipophilic properties and is equally soluble in the oil and aqueous phase at the PIT, which is also known as hydrophilic-lipophilic balance (HLB) temperature (García-Celma et al., 2016; Jintapattanakit, 2018). At this temperature, extremely small sizes of droplets can be produced due to an ultra-low interfacial tension, but coalescence of droplets can occur easily thus it has a low stability (McClements & Rao, 2011; Solans & Solé, 2012). Hence, the use of the PIT method in the preparation of nanoemulsion has a major disadvantage, which is the oil droplets prone to coalescence if the temperature increases (Lee et al., 2018).

The process of spontaneous emulsification is simpler compared to the PIT method. In spontaneous emulsification method, nanoemulsions can be fabricated spontaneously by adding organic phase (oil and hydrophilic/amphiphilic surfactant) slowly into aqueous phase (water) at a particular temperature (Komaiko & McClements, 2016; Lee et al., 2018). Nanometric oil droplets are formed as a result of the noticeable increase in interfacial area and interfacial turbulence caused by the rapid movement of the amphiphilic surfactant from the oil phase into the aqueous phase when the two phases are mixed together with gentle stirring (Anton & Vandamme, 2009; McClements & Rao, 2011). There are several limitations and drawbacks for this approach including the requirement of relatively high amounts of surfactants and the utilization of only low-viscosity hydrocarbon-based oil and synthetic small molecule non-ionic surfactants (Lee et al., 2018).

2.4 Formulation of essential oil nanoemulsion

The selection of a suitable group of ingredients is very important to formulate a nanoemulsion successfully (McClements, 2015). Formulation plays a crucial role not only in determining several nanoemulsion properties which are related to their surface and biological activity but also in achieving minimum droplet size (Donsì & Ferrari, 2016). According to McClements (2015), nanoemulsions which are simply formed by homogenizing an oil with an aqueous phase would quickly separate into its component phases. Hence, additional ingredients such as emulsifiers and ripening inhibitors are required to ease the formation and improve stability of nanoemulsions.

2.4.1 Emulsifiers/surfactants

Emulsifiers are surface-active amphiphilic molecules consisting of polar and nonpolar regions that are able to reduce the interfacial tension when adsorbing onto the oil-water interface during homogenization (Bai et al., 2016). As a result, the droplet disruption and a protective interfacial layer are facilitated and created thus inhibiting droplet aggregation (McClements & Gumus, 2016). Several factors should be considered in selecting a suitable emulsifier and of its concentration with respect to the oil phase, including the surface coverage needed for stable emulsions, the dynamics of surface adsorption, as well as the rearrangement at o/w interfaces and the hydrophilic-lipophilic balance (HLB) of the surfactant molecule (Donsì et al., 2012). These factors usually affect the final mean droplet size compared to the emulsification process efficiency itself (Donsì & Ferrari, 2016).

According to Chang and McClements (2014), surfactants with too high or too low HLB numbers are not suitable in the formation of nanoemulsions as the surfactant monolayer curvature is too high to form nano-sized oil droplets and the surfactant prone to remain in the oil phase. Generally, monolayers with low interfacial tensions can be formed using surfactants with HLB numbers between 8 and 18 by promoting the conditions required for the formation of very fine droplets at the o/w boundary, but there is no clear prediction can be derived for the mean droplet size which can be achieved upon emulsification (Donsì & Ferrari, 2016). In addition, surfactant concentration also plays a crucial role on the o/w interfacial tension and the droplet surface coverage, whereby it can influence the droplet break-up efficiency, the absorption kinetics and the rate of droplet coalescence (McClements & Jafari, 2018). The emulsification efficiency also depends on the viscosity of the dispersed phase with respect to the viscosity of the continuous phase, whereby a smaller mean droplet size can be formed for a given energy input when the ratio is close to unity (Donsì, 2018; Walstra, 1993). In short, an adequate selection of the emulsifier layer is able to control the long-term stability of nanoemulsions by promoting the steric hindrance and electrostatic repulsion between the droplets (Donsì, 2018).

Types of emulsifiers that are commonly used in the food industry are small molecule surfactants, polysaccharides, phospholipids and proteins (McClements & Gumus, 2016). In the fabrication of nanoemulsions, Tween 80 which is a non-ionic surfactant derived from polyoxyethylene sorbitan and oleic acid are the most commonly used emulsifier due to their low cost and good emulsifying properties (Raikos et al., 2017). According to McClements (2015), the non-ionic emulsifier with HLB number equal to 15.0 is able to stabilize the o/w nanoemulsions effectively and has a low toxicity compared to other synthetic emulsifiers.

2.4.2 Ripening inhibitor

It is easier for EOs which have low viscosity, low interfacial tension and high polarity to form nanoemulsions with smaller droplets by either high-energy or low-energy methods (Zhang & McClements, 2018). However, one of the major challenges during the development of EOs nanoemulsions is the high sensitivity to Ostwald ripening, which causes them to be less physically stable, resulting in faster droplet coalescence (Jang et al., 2019). This is because it still has significant solubility in the aqueous phase despite it is an oil phase which is predominantly hydrophobic (Ryu et al., 2018). Ostwald ripening is induced by the curvature differences of the particles involving the diffusion of dispersed phase molecules through the continuous phase leading to the expansion and shrinkage of larger droplets and smaller droplets respectively (Liu et al., 2019). This phenomenon involving the droplet growth happens when the solubility of dispersed phase in larger droplets is lower than in small droplets due to its concentration gradient (Thompson et al., 2018). In accordance, ripening inhibitor is required to be incorporated into the oil phase to retard droplet growth due to Ostwald ripening prior to o/w nanoemulsion formation containing EOs with highly water-soluble oil phase (Zhang & McClements, 2018).

Long-chain triglycerides (LCT) oils such as sunflower oil, corn oil, canola oil and soybean oil which are highly hydrophobic and exhibit negligible water solubility are some of the examples of ripening inhibitors which are used for fabrication of food-grade EO nanoemulsion containing EOs (Hidajat et al., 2020; Liew et al., 2020; Majeed et al., 2016; Moraes-Lovison et al., 2017). By mixing EOs with LCT oils, the partitioning of EOs between the oil droplets and the aqueous phase can be positively altered, thereby reducing the rate of Ostwald ripening (Donsì et al., 2014). According to Ryu et al. (2018) who had performed study on the effect of ripening inhibitor types including canola oil, coconut oil, corn oil and palm oil on thyme oil formation, stability and antimicrobial activity, an optimum concentration of ripening inhibitor was determined to be around 40% of the oil phase in order to achieve the formation of nanoemulsions that were stable during storage while maintaining its antimicrobial activity. The amount of ripening inhibitor needed to retard Ostwald ripening is different, depending on the concentration and the structural characteristics of the emulsifiers present (Han et al., 2018). Ziani et al. (2011) had mixed thyme EOs and corn oil at the ratio of 1:3 in the presence of 0.5% Tween 80, whereas the lime EOs were mixed with corn oil at the ratio of 8:2 with 15% Tween 80 by Liew et al. (2020) for the formation of stable nanoemulsions with small droplet size.

2.5 Characterization of essential oil nanoemulsion

The suitability of nanoemulsions for particular applications depending on the bulk physicochemical characteristics of nanoemulsions such as droplet size, rheology, optical transparency and stability against droplet aggregation and coalescence (McClements & Jafari, 2018a). In order to characterize the properties of nanoemulsions formed, instrumental techniques that enable the determination of particle size, zeta potential, optical properties and morphology are required.

2.5.1 Particle size, polydispersity index (PDI) and particle size distribution (PSD)

Dynamic light scattering (DLS) analysis, which is also known as photon correlation spectroscopy (PCS) or quasi-elastic light scattering (QLS) is the most commonly employed technique in determining the size distribution profile of small particles (Bahuguna et al., 2020). Malvern Zetasizer is used to conduct the analysis for the monitoring of the fluctuations of light scattering intensity due to the Brownian motion of particles and relates this to the particle size, which is expressed as Z-average diameters (nm) (Gurpreet & Singh, 2018). DLS not only allows a rapid and adequate evaluation of particle size of nanoemulsion, but also is often used to determine the particle size distribution and their stability through storage (Silva et al., 2012).

Particle size, polydispersity index (PDI) and particle size distribution (PSD) that are highly correlated with each other are good parameters in describing the stability, uniformity, dispersibility, as well as the quality of nanoemulsions (Mason et al., 2006). PDI which represents the uniformity of the particle size within a nanoemulsion and stability of the formed nanoemulsion is expressed as a dimensionless number (Bahuguna et al., 2020). The numeric value of PDI ranges from 0 to 1, whereby a lower PDI value indicates a monodispersed distribution, while a broad size distribution is shown by a higher PDI value (Bahuguna et al., 2020). PSD exhibiting the relationship between the particle size and concentration is a key predictor for droplet homogeneity of nanoemulsion formed (Kaddumukasa et al., 2017). According to Zhang et al. (2017), nanoemulsion with high homogeneity is indicated by unimodal particle size distribution with a narrow peak.

2.5.2 Zeta potential

The zeta potential is used as a method to measure the surface charge of oil droplets in emulsions (McClements & Rao, 2011). Zeta potential is closely related to the stability of nanoemulsion, indicating the degree of repulsion between adjacent particles with similar charge within the suspension (Bahuguna et al., 2020). Its value highly depends on the pH of the solution, nature of the surfactant, particle size and morphology, as well as electrolyte concentration (Simunkova et al., 2009). The zeta potential value, which is estimated from the electrophoretic mobility of oil droplets, is determined by using Malvern Zetasizer (Gurpreet & Singh, 2018). Formation of a stable nanoemulsion is indicated by droplets with zeta potential values higher than ± 30 mV (Silva et al., 2012). Droplet aggregation and coalescence may occur when the zeta potential value is low, as a result of the increasing attraction force between particles (Bahuguna et al., 2020).

2.5.3 Turbidity

It is important to determine the turbidity of nanoemulsion, especially when they are incorporated in beverages or food products which have high transparency (Piorkowski & McClements, 2014). According to McClements and Jafari (2018a), the nanoemulsion is dependent on the light scattering profile, which relies on the size, concentration and refractive index contrast between dispersed droplets and the continuous phase. The percentage of transmittance (T%) of formulated nanoemulsion can be measured using UV-Vis spectrophotometer, whereby the decrease in T% indicates the increased turbidity of the nanoemulsion (Gurpreet & Singh, 2018). The turbidity of nanoemulsion is directly associated with the particle size (Zhang et al., 2015). Nanoemulsion tends to be turbid or optically opaque when the particle size is much larger than the wavelength of light, resulting in the higher intensity of light scattering (McClements & Jafari, 2018a).

2.5.4 Viscosity

Viscosity is one of the important parameters for the evaluation of nanoemulsion stability and releasing of the active components (Bahuguna et al., 2020). Nanoemulsion can be characterized using a tuning-fork vibration viscometer (Liew et al., 2020). The viscosity of nanoemulsion is influenced by the nature and amount of the components of nanoemulsion including oil, water and surfactants (Borthakur et al., 2016). The higher the amount of water, the lower the viscosity of nanoemulsion. The increase in the amount of surfactants also resulted in nanoemulsion with lower viscosity due to the reduction of interfacial tension between the aqueous and organic phases (Borthakur et al., 2016).

2.5.5 Morphology

Electron microscopy, such as scanning electron microscopy (SEM) and transmission electron microscopy (TEM) is used to examine the microstructure of nanoparticles (Lee et al., 2018). According to Borthakur et al. (2016), TEM is widely employed to carry out the study of morphology and the size of droplets in nanoemulsion. The result of particle size obtained by dynamic light scattering (DLS) can have better support by performing TEM analysis (Silva et al., 2012). Liew et al. (2020) examined the microstructure and the particle size in the lime essential oil nanoemulsions concluded that the nanoparticles exhibited spherical morphology with sizes within the nanometre range (20-200 nm), confirming the results obtained by DLS.

2.6 Antimicrobial activity of essential oil nanoemulsions

EOs established antimicrobial actions based on their hydrophobic nature, whereby they can interact with the lipids of the cell membrane strongly and increase the permeability of the membrane, leading to the disruption of bacterial structure, denaturation of cellular proteins, leakage of cytoplasmic contents such as ions, nucleic acids, ATP and amino acids and finally cell death (Seow et al., 2014; Yildirim et al., 2017). The antimicrobial activity for nanoemulsion incorporated with EOs is often found to be more effective than for free EOs. Topuz et al. (2016) and de Meneses et al. (2019) have proved that both anise oil nanoemulsion and clove oil nanoemulsion exhibited higher antimicrobial properties compared to bulk anise oil and free clove oil respectively by showing lower minimum inhibitory concentration (MIC) and greater bacterial population reduction. This is because the nanoemulsions with reduced particle size are able to improve the biological activity of EOs by increasing the surface area and affinity with bacterial cell, as well as enhancing the stability and solubility of the antimicrobial agents (Acevedo-Fani et al., 2017).

Many studies and reviews have been done on the antimicrobial activities of nanoemulsions containing EOs or their bioactive components. EO nanoemulsions had been reported to be highly effective against a wide-range of Gram-positive and Gram-negative bacteria including some major food-borne pathogens. According to Artiga-Artigas et al. (2017), the shelf life of low-fat cut cheese had been successfully extended with its surface coating with oregano EO nanoemulsion that showed antimicrobial activity against *Staphylococcus aureus*. Nanoemulsion containing star anise EO with polylysine and nisin as composite antimicrobial agents was reported to significantly inhibit *Escherichia coli* growth in ready-to-eat Yao meat products (Liu et al., 2020). Besides, in a study reported by Mendes et al. (2018), *Eugenia brejoensis* EO nanoemulsion produced by using high-speed homogenization had showed in vitro antimicrobial activity against *Pseudomonas fluorescens*. Sotelo-Boyás et al. (2017) also reported that the growth of *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Shigella dysenteriae* could be inhibited by chitosan nanoparticles and nano capsules incorporated with lime EO.

However, Gram-positive bacteria are found to be slightly more sensitive towards EOs compared to Gram-negative bacteria due to their respective cell walls with different structures (Seow et al., 2014). Major part of the cell wall of Gram-positive bacteria consists of peptidoglycans and small amount of proteins, whereas Gram-negative bacteria possess a more complex structure with a thinner lipid-based peptidoglycan layer and an outer membrane composed of double layer of phospholipids, as well as higher amount of proteins (Costa et al., 2014). Hence,