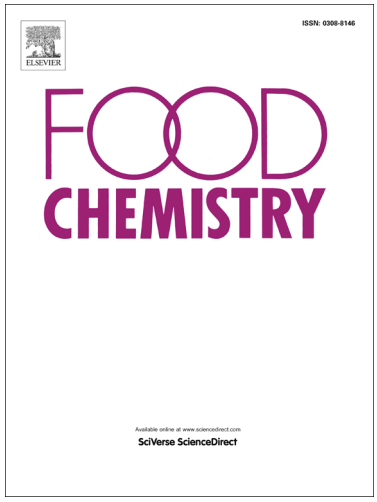


Formulation, characterisation and antibacterial activity of lemon myrtle and anise myrtle essential oil in water nanoemulsion

Nilesh Prakash Nirmal, Ram Mereddy, Li Li, Yasmina Sultanbawa

PII: S0308-8146(18)30188-2
DOI: https://doi.org/10.1016/j.foodchem.2018.01.173
Reference: FOCH 22362

To appear in: *Food Chemistry*

Received Date: 8 September 2017
Revised Date: 11 January 2018
Accepted Date: 29 January 2018

Please cite this article as: Nirmal, N.P., Mereddy, R., Li, L., Sultanbawa, Y., Formulation, characterisation and antibacterial activity of lemon myrtle and anise myrtle essential oil in water nanoemulsion, *Food Chemistry* (2018), doi: https://doi.org/10.1016/j.foodchem.2018.01.173

Formulation, characterisation and antibacterial activity of lemon myrtle and anise myrtle essential oil in water nanoemulsion

Running title: Nanoemulsion formulation, characterisation and antibacterial activity

Nilesh Prakash Nirmal[1*], Ram Mereddy[2], Li Li[3] and Yasmina Sultanbawa[1]

[1]Centre for Nutrition and Food Sciences, Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, [2]Department of Agriculture and Fisheries, Health and Food Sciences Precinct, Block 10, 39 Kessels Road, Coopers Plains, Brisbane, QLD-4108

[3]Australian Institute for Bioengineering and Nanotechnology, The University of Queensland, St. Lucia, Queensland 4072, Australia

* Corresponding author. Tel.: +61 7 344 32474; Fax: +61 7 32169591

E-mail address: n.nirmal@uq.edu.au

Abstract:

This study focussed on the formulation, characterisation of lemon myrtle (LM) and anise myrtle (AM) essential oil (EO) in water nanoemulsion and their antibacterial activity. The required hydrophilic lipophilic balance (rHLB) value of LM EO and AM EO was 14 and 12, respectively. The Central Composite Rotatable Design (CCRD) model produces the smallest droplet size and polydispersity index (PDI) for LMEO ($d \approx 16.07$ nm; PDI ≈ 0.209) and AMEO ($d \approx 30.23$ nm; PDI ≈ 0.216) at 1% EO and 10% surfactant mixture (Smix) ratio using ultrasonication for 5 min. Whereas, increased in EO, decrease in Smix concentrations and ultrasonication time produces higher droplet size of nanoemsulions. LMEO (LM-15, LM-17) nanoemuslions was clear and transparent compared to AMEO (AM-15, AM-17). All the selected nanoemuslions showed good stability at 4, 25 and 40^{o}C during storage, except LM-15 at 40^{o}C. LMEO nanoemulsion showed enhanced antibacterial activity compared to LMEO alone ($P<0.05$).

Keywords: Lemon myrtle, Anise myrtle, Essential oil, HLB value, Nanoemulsion, Droplet size, Stability

1. Introduction

Lemon myrtle (*Backhousia citriodora*) and anise myrtle (*Syzygium anisatum*) are native to Australia and belong to the family Myrtaceae. The fresh or dried leaves of LM and AM have been used widely as ingredients in food flavourings, perfumes, personal care products and pharmaceutical preparations (Clarke, 2012). The major functional properties of LM and AM are attributed to the EO. The yield of EO from fresh leaves of LM varied from 1.1-3.2 % and EO contained 95% of citral compound, which is an isomeric mixture of geranial (E-isomer)

and neral (Z-isomer) (Wilkinson, Hipwell, Ryan, & Cavanagh, 2003). The typical phytochemical profile of LMEO is β- myrcene (0.1-0.7%), 6-methyl-5-hepten-2-one (0.1-2.5%), linalool (0.3-1.0%), citronellal (0.1-0.9%), *cis*-isocitral (0.6-2.7%), *trans*-isocitral (1.0-4.2%), *exo*-isocitral (0.1-2.0%), neral (32.0-40.9 %), geranial (46.1-60.7 %) and trans-geraniol (0.4-0.7%) (Sultanbawa, 2016b). Brophy and Boland (1991) reported that the yield of EO from AM varies from 1.3 -2.0 % and AM EO had two different chemotypes depending upon the content of anethole and methyl chavicol. AM EO (anethole type) comprised of 71.2-93.7 % of (E)-anethole and 5.0-15.3% of methyl chavicol, whereas AM EO (methyl chavicol type) contained 22.1-42.8 % of (E)-anethole and 55.8-75% % of methyl chavicol (Sultanbawa, 2016a).

These compounds are known as antimicrobial agents with activity against both bacteria and fungi (Hayes & Markovic, 2002; Senatore, Oliviero, Scandolera, Taglialatela-Scafati, Roscigno, Zaccardelli, et al., 2013). EOs act as natural antimicrobials and some EOs have been classified as GRAS by the US Food and Drug Administration (Weiss, Gaysinsky, Davidson, & McClements, 2009). The application of EO in a food product could inhibit the growth of food borne bacteria and other pathogenic microorganisms (Buranasuksombat, Kwon, Turner, & Bhandari, 2011). Even though EOs are natural and safe their application in food is limited by technological hurdles related to their hydrophobic nature, preservation of their activity and their interaction with other food ingredients (Donsi, Annunziata, Vincensi, & Ferrari, 2012). Therefore, to overcome these limitations EOs need to be protected from the interaction with other food ingredients and harsh conditions of food manufacturing and storage (Davidov-Pardo & McClements, 2015). The oil in water nanoemulsion of EO provides an efficient approach to increasing the physical stability of the active compounds and increases their bioactivity. The EO's in the emulsion can target the microorganisms

located in the water rich phase of the food system (Donsì, Annunziata, Sessa, & Ferrari, 2011).

Nanoemulsion can be prepared by different processing methods, such as low or high energy required methods (Solans & Solé, 2012). The energy required for the system to increase interfacial area between the two phases can be provided by mechanical stirring, high-pressure homogenization or ultra-sonication (Rebolleda, Sanz, Benito, Beltran, Escudero, & Gonzalez San-Jose, 2015). Ultrasonic homogenisation is considered as a 'green technology' due to its high efficiency, economic performance and low instrumental requirements (Abbas, Karangwa, Bashari, Hayat, Hong, Sharif, et al., 2015). Ultrasonication uses low energy consumption, low surfactant and produces smallest droplet size homogenous emulsion than conventional mechanical processes (Delams, Piraux, Couffin, Texier, Vinet, Poulin, et al., 2011). The physicochemical properties of nanoemulsions are influenced by type and concentration of oil and surfactant, hydrophilic-lipophilic balance (HLB) of oil and processing condition (Solans, Izquierdo, Nolla, Azemar, & Garciacelma, 2005). Optimal stability is achieved when HLB value of surfactant mixture is close to that required of the oil, which produces the smallest mean droplet size with narrow distribution (Fernandes, Mascarenhas, Zibetti, Lima, Oliveira, Rocha, et al., 2013; Rodrigues, Costa, Almeida, Cruz, Ferreira, Vilhena, et al., 2015). Therefore formulation of an efficient delivery system should consider the required HLB value of oil, concentration of oil and surfactant, and mechanical energy applied. To date the literature reports the formulation and antimicrobial activity of a LMEO nanoemulsion (Buranasuksombat, Kwon, Turner, & Bhandari, 2011) and anise oil nanoemulsion (Topuz, Ozvural, Zhao, Huang, Chikindas, & Golukcu, 2016) using a microfluidizer and high pressure homogenizer techniques, respectively. However, these studies did not report any information about HLB values and storage stability at different temperatures

The objective of the present study was to determine the required HLB value of LMEO and AMEO for the development of a nanoemulsion. Central Composite Rotatable Design (CCRD) study was applied to generate a range of formulations with different EO and surfactant mixture concentrations and ultrasonication times to assess the mean droplet size and polydispersity index of the developed nanoemulsion. The selected smallest mean droplet size nanoemulsion was characterized for turbidity and density. Storage stability of nanoemulsion were determined at different temperature conditions (4, 25 and 40^{o}C) for 4 weeks. Additionally, antibacterial activity of EOs and selected nanoemuslions was evaluated.

2. Material and methods

2.1. Essential oil and surfactant

Lemon myrtle essential oil (LMEO) 100 % pure extracted from *Backhousia citriodora* and anise myrtle essential oil (AMEO) 100% pure extracted from *Syzygium anisatum* were procured from Auroma, Hallam, Victoria, Australia. Non-ionic surfactants Tween 80 (HLB= 15) and Span 80 (HLB 4.5) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Double distilled water purified with Milli-Q system fitted with 0.2 µm filters (Millipore Co., Bedford, MA, USA) was used in all the experiments.

2.2. Formulation of nanoemulsion

To prepare nanoemulsion, firstly EO (10%) and surfactant (10%) were mixed together by vortex (Ratek Instruments Pty Ltd, Victoria, Australia) and constituted as organic phase. The aqueous phase consists of only double distilled water (80%). The quantities of each constituent in the emulsion were measured using an analytical balance (Sartorius, Gottingen, Germany). A coarse emulsion was prepared by mixing the organic and aqueous phases, at 2000 rpm for 2 minutes. A fine emulsion was prepared by sonifying the coarse emulsion using a high intensity ultrasonic processor (Branson SFX 550, Shanghai, PRC) with up to 550 watts of output power at 20 kHz. The Sonifier was equipped with titanium alloy microtip probe of 3 mm diameter and operated at 50% amplitude with pulses of 5 s (5 s ON and 7 s OFF) for 2 min to avoid heating of the sample (Abbas, et al., 2015).

To determine the required hydrophilic-lyphophilic balance (rHLB) of the EO, emulsions were prepared using different surfactant HLB values. The surfactant HLB value ranging from 8 to 15 were prepared using different combinations of Span 80 (HLB 4.3) and Tween 80 (HLB 15) according to Rodrigues, et al. (2015) as follows: 65.4:34.6 (HLB 8), 57:43 (HLB 9), 46.7:53.3 (HLB 10), 37.4:62.6 (HLB 11), 28:72 (HLB 12), 18.7:81.3 (HLB 13), 9.3:90.7 (HLB 14), 0:100 (HLB 15). The emulsions were prepared as mentioned above.

2.3. Experimental design

After determining the rHLB value for each EO concentration, a CCRD model was used to study the effects of EO concentration (1-10 % w/w), surfactant mixture (Smix) concentration (1-10% w/w) and ultra-sonication time (1-5 min) on the droplet size of the nanoemulsions. The CCRD model generated seventeen combinations with six replicates as the central point (Supplementary material).

2.4. Characterisation of nanoemulsion

2.4.1. Particle size measurement

The particle size distribution, mean droplet size and polydispersity index (PDI) of nanoemulsion were determined by a dynamic light scattering device (Nano-ZS Malvern Instrument, UK). Nanoemulsion was diluted using double distilled water (1:20) to avoid multiple scattering effects. Mean particle diameter was reported as z-diameter. All measurements were performed in triplicate

2.4.2. Turbidity measurement

The turbidity of selected nanoemulsion was measured using a UV-visible spectrophotometer (Genesys-20, Thermo-Scientific, Madison, Wisconsin, USA) according to the method of Qian and McClements (2011). The emulsion was diluted with distilled water to a range of different oil droplet concentrations (0- 10%) and the turbidity was measured at 600 nm.

2.4.3. Density measurement

The densities of selected nanoemulsions were calculated from the mass to volume ratio of each emulsion. An analytical balance and calibrated glass cylinder were used to measure the mass and volume of samples, respectively.

2.5. Storage stability of selected nanoemulsions

The selected nanoemulsions were transferred into airtight glass bottles. The stability of nanoemulsion was observed at 4, 25 and 40^{o}C temperatures. The stability was measured every week for a storage period of 4 weeks and assessed for mean particle diameter (Abbas, et al., 2015). The creaming and phase separation were observed visually.

2.6. Antibacterial analysis

The minimum inhibitory concentration (MIC) of selected nanoemulsions, essential oil and Smix against two gram positive (*Staphylococcus aureus - ATCC 33591, Listeria monocytogenes – ATCC 19111*) and two gram-negative (*Escherichia coli – ATCC 11775, Pseudomonas aeruginosa – ATCC 9626*) bacteria were determined by the broth microdilution assay as described by NCCLS (2008). Briefly, bacterial culture was streaked on plate count agar (PCA) and incubated for 24 hr at 37^{o}C for further use. A culture spore suspension (10^{8} CFU/mL) was prepared by transferring a loop of culture from PCA plate in saline water to achieve 0.1-0.15 OD at 600 nm. Cell suspension was diluted in sterile nutrient broth (NB) to produce 10^{6} CFU/mL cell counts. The MIC of nanoemulsion, essential oil and Smix were determined by two fold serial dilution method in 96 well plates. Initially, 100 µL of sterile NB was added in each well and then each assay solution (100 µL) was mixed with NB in the second column of the plate. Thereafter, 100 µL of mixed solution from the second column was transferred to third column of the plate and so on, to produce the desired concentration ranges of nanoemulsion (0.5-0.003%), pure essential oil (2.5-0.009 %) and Smix (15-0.46 %). Each assay solution repeated in three rows. Then 100 µL of bacterial suspension (10^{6} CFU/mL) were added in each well. The first column of the plate serve as negative control. Plates were incubated for 24 h at 37^{o}C. The MIC for bacteria was determined as the lowest

concentration of assay solution inhibiting the visual growth of the test culture on the microplate.

2.7. Statistical analysis

All analyses were performed in triplicate and results were expressed as the mean ± standard deviation (SD). Analyses of variance (ANOVA) were performed and mean comparisons were done by post hoc Tukey's (HSD) and Duncan's range tests by using a XLSTAT package (Microsoft Excel). *P* values less than 0.05 were considered statistically significant.

3. Results and discussion

3.1. The required HLB value for LMEO and AMEO

The effect of different HLB surfactant mixtures (Smix) on droplet size and PDI of LMEO and AMEO is presented in Table 1. Different HLB value surfactant combinations ranging from 8 to 15 were prepared by mixing Span 80 (HLB 4.3) and Tween 80 (HLB 15) in various proportions. The required HLB of an oil can be determined by using a set formulation with a wide range of HLB and surfactant, which can be prepared by combining low and high HLB values (Rodrigues, et al., 2015). One day after nanoemulsions preparation HLB 8, 9, 10, 11 and 15 produced mean droplet size above 100 nm, whereas HLB 12, 13 and 14 produced mean droplet size below 100 nm, for both LMEO and AMEO. On day one, the lowest mean droplet size and PDI for LMEO and AMEO were obtained at HLB 14 (84.7 nm, 0.223 PDI) and 12 (82.1 nm, 0.256 PDI), respectively. After one week, mean droplet size and PDI of nanoemulsions were decreased. The decrease in mean droplet size of nanaoemulsion is the

phenomenon of micellization where the formation of a colloidal cluster of individual surfactant molecules occurs. These micelles are in dynamic equilibrium with constantly disintegrating and reforming, until reaching kinetic stability (Patist, Kanicky, Shukla, & Shah, 2002). The smaller the interfacial tension between the oil phase and water, the more stable will be the emulsion (Rodríguez-Rojo, Varona, Núñez, & Cocero, 2012). In another study, the wheat bran oil in water nanoemulsion with minimum droplet size (84.6 nm) and the narrow PDI (0.257) was obtained when mixture of span 80 (37.4%) and tween 80 (62.6%), with HLB value 11 was used (Rebolleda, Sanz, Benito, Beltran, Escudero, & Gonzalez San-Jose, 2015). The required HLB value for evening primrose seed oil was determined and reported to be 12 when mixture of span 80 (28%) and tween 80 (72%) was used (Rodrigues, et al., 2015). Rodríguez-Rojo, Varona, Núñez, and Cocero (2012) determined the required HLB for rosemary EO was 15. The HLB value reported for the above studied oil's and EO was varying from 11-15, similarly we found HLB 14 and 12 for LMEO and AMEO, respectively. The composition, structure and charge of the interfacial layer surrounding the oil droplets can be affected by the different types of surfactant (different HLB value) to prepare the emulsion (McClements, Saliva-Trujillo, Zhang, Zhang, Zou, Yao, et al., 2016). The minimum mean droplet size indicates the optimal stability, which determines the HLB of the system (Orafidiya & Oladimeji, 2002; Rodríguez-Rojo, Varona, Núñez, & Cocero, 2012). Therefore, the determination and development of the required HLB value of EO was an important step to formulate stable minimum droplet size nanoemulsion.

The determined surfactant mixture ratio at required HLB value for LM (HLB 14) and AM (HLB 12) were used to conduct further experiments.

3.2. Effect of EO concentration, surfactant concentration and ultra-sonication time on mean droplet size and distribution

The effect of EO and surfactant mixture concentration and ultrasonication time on the droplet size of nanoemulsion is shown in Figure 1A (LMEO) and Figure 2A (AMEO). The concentration of EO and Smix was selected from 1 to 10 % and the CCRD was carried out for randomised selection of different ratio of EO and Smix with different sonication times (1-5 min). The mean droplet size and PDI decreased when EO to Smix ratio was 1: 10 with 5 min of sonication, the smallest droplet size and PDI for LMEO (d ≈ 16.07 nm; PDI ≈ 0.209) and AMEO (d ≈ 30.23 nm; PDI ≈ 0.216) was obtained, respectively. When EO to Smix ratio was changed to 5.5 :13.1 with 3 min of sonication, the droplets size and PDI increased slightly for LMEO (d ≈ 28.81 nm; PDI ≈ 0.209) and AMEO (d ≈ 61.37 nm; PDI ≈ 0.169), respectively. Smallest droplets form at the highest surfactant concentrations and the system becomes optically transparent at the highest surfactant levels (Chang & McClements, 2014). Higher surfactant concentration largely reduces the interfacial tension at the oil-water interface, which produces smaller droplets (Davidov-Pardo & McClements, 2015). When the EO concentration exceeded the Smix (e.g. run order 4, 9, 13) the mean droplet size of LMEO (d ≈ 275.8 to 365.4 nm) and AMEO (d ≈ 186.8 to 630.6 nm) increased. The large particle size observed at the high EO content can be attributed to the Ostwald ripening and / or coalescence effect that brings about changes in phase behaviour of the oil-surfactant-water system at a certain composition (Chang & McClements, 2014). The wide range of mean particle size may suggest different nanocarrier system such as micelles or nano oil droplets due to the different range of surfactant and oil concentrations. This suggests that the concentration of EO and surfactant play a critical role in the preparation of stable emulsions (Davidov-Pardo & McClements, 2015). The concentration of surfactant in the emulsion should be enough to cover all EO droplets and keep them in a dispersed phase.

Figure 1B and 2B represent the particle size distribution of smallest mean droplet size nanoemulsion for LMEO and AMEO, respectively. LMEO nanoemulsion at both EO to Smix ratios of 1:10 and 5.5:13.1 showed monomodal particle size distribution. AMEO nanoemulsion at EO to Smix ratio of 1:10 showed monomodal particle size distribution, whereas at 5.5:13.1 showed bimodal size distribution. Biomodal size distribution of AMEO indicate the presence of major and minor unimodal distributions. Flavoured oils e.g. citrus oil reported to be more suitable for forming nanoemuslions than triacylglycerol oils (Ostertag, Weiss, & McClements, 2012). In this study, EO's are directly used as the oil phase without any addition of medium or long chain triglycerides. On the other hand, Chang and McClements (2014) reported that emulsion containing flavour oils are more susceptible to Ostwald ripening due to their high water solubility and suggested to add minimum amount of medium chain triglyceride (MCT) to emulsion to prevent rapid coalescence. However, in this study the selected nanoemulsion (LM-17, LM-15, AM-17, and AM-15) were stable during long term storage at different conditions (see below). LMEO and AMEO nanoemulsions were prepared by high energy approaches, whereas Chang and McClements (2014) used spontaneous emulsification procedure to prepare nanoemulsions.

Ultra-sonication process formulates nanoemulsion by cavitation phenomenon where formation and collapse of vapour cavities in the liquid medium occurred due to high intensity ultrasound (Mahdi Jafari, He, & Bhandari, 2006). Ultra-sonication time from 1 to 5 min were used for CCRD study to determine the suitable process time for emulsification. CCRD study produced seventeen combinations of EO and Smix with different ultrasonication times varying from 1 to 6.3 min. The ultra-sonication time required for the formation of smallest droplet size nanoemulsion depended on the EO:Smix ratio. Ultra-sonication time and intensity affect the adsorption rate of the surfactant to the droplet surface and the droplet size distribution (Rebolleda, Sanz, Benito, Beltran, Escudero, & Gonzalez San-Jose, 2015). In

other studies, Li and Chiang (2012) found that the increase in ultra-sonication time and power after certain value increases the droplet coalescence due to the over processing of the emulsion. Therefore, the optimization of emulsification parameters and processing method is not only important to save energy and chemicals but also to produce smallest fine droplet size and particle distribution.

The selected smallest fine droplet size nanoemulsions LMEO (LM-17, d ≈ 16.07 nm; LM-15, d ≈ 28.81 nm) and AMEO (AM-17, d ≈ 30.23 nm; AM-15, d ≈ 61.37 nm) were further characterised and used for stability study at different temperatures.

3.3. Characterization of selected nanoemulsion

Nanoemulsion with smallest mean droplet size was further characterised for optical properties. The optical properties of emulsion are important for its application in different food systems such as turbid or optically transparent delivery systems (Qian & McClements, 2011). The optical properties of selected nanoemulsion in terms of turbidity were studied and presented in Figure 3. Nanoemulsions were diluted by double distilled water to different droplet concentrations to determine the variation in turbidity at 600 nm. As the droplet concentration of emulsion increase the turbidity of the emulsion increased. Nanoemulsion prepared with 5.5 % EO + 13.1% Smix (sonication time 3 min) had higher turbidity as compared to nanoemulsion prepared by 1 % EO + 10% Smix (sonication time 5 min) irrespective of either EO. LMEO nanoemulsion at LM-15 (0.0047 $cm^{-1}\%^{-1}$) produced slightly turbid emulsion than the LM-17 (0.0003 $cm^{-1}\%^{-1}$) which was a clear and transparent emulsion. AMEO nanoemulsion showed 0.002 $cm^{-1}\%^{-1}$ turbidity at AM-17 and 0.010 $cm^{-1}\%^{-1}$ turbidity when prepared by AM-15. When nanoemulsion was prepared by mixing 1 % EO and 10% Smix (LM/AM-17, sonication time -5 min), LMEO produced optically transparent

nanoemulsion than AMEO owing to smallest mean droplet size of LMEO nanoemulsion compared to AMEO. The smallest mean droplet size nanoemulsion are known to scatter light less effectively than the larger particle size, which account for the lower turbidity of emulsion containing smaller droplet size (McClements, 2002).

The photograph of LM (17 and 15) and AM (17 and 15) nanoemulsions is shown in Figure 3. In general, nanoemulsion prepared by using AMEO produced slightly turbid emulsion as compared to LMEO. Optical properties of nanoemuslions make them suitable for their use in different systems without altering visual quality (Rebolleda, Sanz, Benito, Beltran, Escudero, & Gonzalez San-Jose, 2015). The nanoemuslion prepared by 1% wheat bran oil, 7.3% surfactant and 50s ultrasoncation produced 0.36 $cm^{-1}\%^{-1}$ turbidly emulsion (Rebolleda, Sanz, Benito, Beltran, Escudero, & Gonzalez San-Jose, 2015). The turbidity observed in the present study ranged from 0.0003 to 0.010 $cm^{-1}\%^{-1}$ which is much lower than the reported value of wheat bran oil nanoemulsion. The density of nanoemulsion was determined to evaluate the degree of denseness of the emulsion. The densities of LM-17, LM-15, AM-17 and AM-15 were 1006.6, 1003.6, 1008.9 and 1006.2 kg m^{-3}, respectively. AM nanoemulsion shows slightly higher density than LM nanoemulsion. When the EO concentration in the emulsion increase, the density of emulsion decreased. Essential oils or flavour oils are low viscosity oils than hydrocarbons (Qian & McClements, 2011). In this study, we did not use any carrier oils (medium chain or long chain triglycerides) hence the effect of the decrease in density with the increase in EO occurred due to the viscosity of EO. All the characterised parameters of nanoemulsion are well correlated with each other for each nanoemulsion.

3.4. Stability of selected nanoemulsions during storage

The stability of selected nanoemulsions LM-17, LM-15, AM-17 and AM-15 during four weeks of storage at 4, 25 and 40^{o}C temperature is shown in Figure 4. The storage stability of prepared nanoemulsions is an important parameter for industrial applications to observe the changes in mean particle size of emulsion at different temperatures for long term physical stability. Nanoemulsions from LM and AM were stable at 4^{o}C with a minor increase in the droplet size of LM nanoemulsion during storage. However, nanoemulsion AM-15 showed decrease ($P< 0.05$) in the size after one week of storage followed by a minor increase towards the end of storage. When emulsions were stored at 25^{o}C, a steady increase in mean droplet size was observed for LM-15 ($d \approx 28.81$ to 102.33 nm). While nanoemulsions LM-17, AM-17 and AM-15 showed a minor increase in mean droplet size at the end of storage. AM-15 nanoemulsion mean droplet size decreased after one week of storage at 25^{o}C. The decrease in mean droplet size of nanoemulsion after a week could be due to the continuous re-constructing behaviour of the micelle to achieve kinetic stability. At the ambient temperature, there is a kinetic energy barrier which prevent oil, water and surfactant emulsion system from reaching the highest kinetic equilibrium (Hashtjin & Abbasi, 2015). Therefore, the reduction in this barrier over the storage time probably enhances the kinetic equilibrium of the system. However, at higher storage temperature, Ostwald ripening occurred faster causing increase in droplet size. At higher storage temperature (40^{o}C) LM-15 showed drastic increase ($d \approx 28.81$ to 230.37 nm) in mean droplet size after first week of storage. Thereafter, the droplet size of LM-15 doubled after every week of storage and phase separation occurred at the end of storage time at 40^{o}C. At the higher temperature, the movement of dispersed droplets in continuous phase of LM-15 nanoemulsion could have raised the opportunities of droplet collisions. This increases droplet coalescence or Ostwald ripening of LM-15, ultimately make phase separation at the end of storage time. Ostwald ripening rate is directly proportional to

the temperature and indirectly affected by temperature through the diffusion coefficient, solubility and interfacial tension (Li & Chiang, 2012).

Although a slight increase in mean droplet size was observed in the LM-17 ($d \approx 16.07$ to 80.52 nm), AM-17 ($d \approx 30.23$ to 41.98 nm) and AM-15 ($d \approx 61.37$ to 96.29 nm) during storage at 40°C, still showed the size below 100 nm. AMEO nanoemulsion was stable at 40°C for four weeks compared to LMEO nanoemulsion. LM-17 was also stable at 40°C as compared to LM-15. The excessive oil and surfactant content and less sonication time of LM-15 nanoemulsion might affect the interfacial tension between oil and aqueous phases. However, lower oil and surfactant concentrations and longer sonication time were used for LM-17 nanoemulsion formation, which probably encapsulated all the insoluble EO in the core. Ostwald ripening can be eliminated by encapsulating insoluble species in the core of emulsion which increases free energy of the trapped component (Delams, et al., 2011). Rebolleda, Sanz, Benito, Beltran, Escudero, and Gonzalez San-Jose (2015) reported that a nanoemulsion prepared using 1% wheat bran oil, 7.3% surfactant (Span80: Tween 80, 37.4:62.6) and 50s of ultrasound and stored at 25°C for 60 days showed droplet size increase by 2 fold and sedimentation occurred on the last day of storage. When nanoemuslion was stored at different temperatures ranging from room temperature to 70°C, the Ostwald ripening was observed to increase with temperature and time of storage (Delams, et al., 2011). The mean droplet size of nanoemulsion prepared by starch stabilized curcumin and stored at 40°C increased steadily, whereas the sample stored at 4 and 25°C showed minor increase in the size (Abbas, et al., 2015).

Therefore, this result indicates that 4 and 25°C temperatures are good for storage of LM and AM nanoemulsion and specifically for long term of storage and stability, 4°C temperature storage is recommended.

3.5. Antibacterial activity of selected nanoemulsions

Essential oils are hydrophobic, reactive, volatile in nature and they rapidly evaporate from surface (Varona, Martín, & Cocero, 2009). Therefore, to retain their biological activities and minimize the impact on organoleptic properties of foods, these essential oils need to be protected from environmental stress and food ingredient where they are incorporated (Donsi, Annunziata, Vincensi, & Ferrari, 2012). The LM-17 and AM-17 nanoemulsions were studied for antibacterial activity against two gram positive and two gram negative bacteria (Table 2). AMEO as it is and nanoemulsion did not show any inhibitory activity against the tested bacteria. LMEO and its nanoemulsion showed inhibitory activity against, *S. aureus*, *L.monocytogenes*, and *E.coli*. The Smix (HLB-12) at the LMEO concentration of 15% showed inhibitory activity against *S. aureus*, *L.monocytogenes*, and *E.coli.* Whereas, Smix (HLB -14) showed minimum inhibitory activity against *E. coli* at 15%. Therefore, the Smix concentration used in the nanoemulsion formulation did not contribute towards antibacterial efficacy of nanoemulsion. A lowered minimum inhibitory concentration was observed for the LMEO nanoemulion as compared to LMEO alone. Encapsulation of EO at the nanoscale increases the physical stability of bioactive compounds and increases their bioactivity through activation of cell absorption mechanism (Weiss, Gaysinsky, Davidson, & McClements, 2009). The MIC and MBC values of terpenes nanoemulsion against *E.coli*, *L. delbrueckii* and *S. cerevisiae* showed lower or equal to the values of unencapsulated mixture (Donsì, Annunziata, Sessa, & Ferrari, 2011). On the whole, the lower MIC or EC_{50} value of tested material against the microorganism indicate the higher efficacy. Therefore, encapsulation of essential oil into nanoscale increases the bioactivity of LMEO.

4. Conclusions

This study reported the required hydrophilic-liphophilic balance for LMEO (Span 80: Tween 80, 9.3:90.7, HLB 14) and AMEO (Span 80: Tween 80, 28:72, HLB 12). LMEO and AMEO were successfully incorporated into a water system by the formulation of nanoemulsion using ultrasonication. The smallest mean droplet size and stable nanoemulsion were achieved by the combinations of 1% EO + 10% Smix and ultrasnocation = 5 min and 5.5% EO + 13.1% Smix and ultrasonication = 3 min. The minimum mean droplet size achieved for LMEO was 16.07 ± 0.13 nm (LM-17) and 28.81± 0.13nm (LM-15). Similarly for AMEO was 30.23 ± 0.23nm (AM-17) and 61.37 ± 2.30 nm (AM-15). Nanoemulsions LM-17 and AM-17 were clear and optically transparent compared to LM-15 and AM-15. Nanoemulsion prepared from AMEO (17&15) had good storage stability at 4, 25 and 40^{o}C over four week storage period. Although LM-17 showed good storage stability at different temperatures, LM-15 showed coalescence and phase separation at 40^{o}C during storage. LMEO nanoemulsion showed higher antibacterial activity than LMEO alone. AMEO as it is and nanoemulison did not show any inhibitory activity against the tested bacteria. For long term storage and stability of nanoemulsions, 4 and 25^{o}C storage temperatures are recommended.

Acknowledgment

NPN would like to thank Queensland Alliance for Agriculture and Food Innovation (QAAFI) and The University of Queensland (UQ) for the financial support of this project and UQ-postdoctoral fellowship.

Conflicts of interest: none

References

Abbas, S., Karangwa, E., Bashari, M., Hayat, K., Hong, X., Sharif, H. R., & Zhang, X. (2015). Fabrication of polymeric nanocapsules from curcumin-loaded nanoemulsion templates by self-assembly. *Ultrasonics Sonochemistry, 23*, 81-92.

Brophy, J. J., & Boland, D. J. (1991). The leaf essential oil of two chemotypes of *Backhousia anisata* Vickery. *Flavour and Fragrance Journal, 6*, 187-188.

Buranasuksombat, U., Kwon, Y. J., Turner, M., & Bhandari, B. (2011). Influence of emulsion droplet size on antimicrobial properties. *Food Science and Biotechnology, 20*(3), 793-800.

Chang, Y., & McClements, D. J. (2014). Optimization of orange oil nanoemulsion formation by isothermal low-energy methods: influence of the oil phase, surfactant, and temperature. *Journal of Agricultural and Food Chemistry, 62*(10), 2306-2312.

Clarke, M. (2012). Australian native food industry stocktake. In *RIRDC Publication*, vol. 12/066). Canberra, Australia: Union Offset Printing.

Davidov-Pardo, G., & McClements, D. J. (2015). Nutraceutical delivery systems: resveratrol encapsulation in grape seed oil nanoemulsions formed by spontaneous emulsification. *Food Chemistry, 167*, 205-212.

Delams, T., Piraux, H., Couffin, A. C., Texier, I., Vinet, F., Poulin, P., Cates, M. E., & Bibette, J. (2011). How to prepare and satbilize very small nanoemulsion. *Langmuir, 27*(5), 1683-1692.

Donsì, F., Annunziata, M., Sessa, M., & Ferrari, G. (2011). Nanoencapsulation of essential oils to enhance their antimicrobial activity in foods. *LWT - Food Science and Technology, 44*(9), 1908-1914.

Donsi, F., Annunziata, M., Vincensi, M., & Ferrari, G. (2012). Design of nanoemulsion-based delivery systems of natural antimicrobials: effect of the emulsifier. *Journal of Biotechnology, 159*(4), 342-350.

Fernandes, C. P., Mascarenhas, M. P., Zibetti, F. M., Lima, B. G., Oliveira, R. P. R. F., Rocha, L., & Falcão, D. Q. (2013). HLB value, an important parameter for the development of essential oil phytopharmaceuticals. *Revista Brasileira de Farmacognosia, 23*(1), 108-114.

Hashtjin, A. M., & Abbasi, S. (2015). Optimization of ultrasonic emulsification conditions for the production of orange peel essential oil nanoemulsions. *Journal of Food Science and Technology, 52*(5), 2679-2689.

Hayes, A. J., & Markovic, B. (2002). Toxicity of Australian essential oil *Backhousia citriodora* (Lemon myrtle). Part 1. Antimicrobial activity and in vitro cytotoxicity. *Food and Chemical Toxicology, 40*(4), 535-543.

Li, P.-H., & Chiang, B.-H. (2012). Process optimization and stability of d-limonene-in-water nanoemulsions prepared by ultrasonic emulsification using response surface methodology. *Ultrasonics Sonochemistry, 19*(1), 192-197.

Mahdi Jafari, S., He, Y., & Bhandari, B. (2006). Nano-Emulsion Production by Sonication and Microfluidization—A Comparison. *International Journal of Food Properties, 9*(3), 475-485.

McClements, D. J. (2002). Theoretical prediction of emulsion color. *Advances in Colloid and Interface Science, 97*(1), 63-89.

McClements, D. J., Saliva-Trujillo, L., Zhang, R., Zhang, Z., Zou, L., Yao, M., & Xiao, H. (2016). Boosting the bioavailability of hydrophobic nutrients, vitamins, and nutraceuticals in natural products using excipient emulsions. *Food Research International, 88*, 140-152.

NCCLS. (2008). Performance standards for antimicrobial susceptibility testing: ninth informational supplement. In *NCCLS documents*, (pp. 120-126). Wayne: National Committee for Clinical Laboratory Standard.

Orafidiya, L. O., & Oladimeji, F. A. (2002). Determination of the required HLB values of some essential oils. *International Journal of Pharmaceutics, 237*(1), 241-249.

Ostertag, F., Weiss, J., & McClements, D. J. (2012). Low-energy formation of edible nanoemulsions: Factors influencing droplet size produced by emulsion phase inversion. *Journal of Colloid and Interface Science, 388*(1), 95-102.

Patist, A., Kanicky, J. R., Shukla, P. K., & Shah, D. O. (2002). Importance of Micellar Kinetics in Relation to Technological Processes. *Journal of Colloid and Interface Science, 245*(1), 1-15.

Qian, C., & McClements, D. J. (2011). Formation of nanoemulsions stabilized by model food-grade emulsifiers using high-pressure homogenization: Factors affecting particle size. *Food Hydrocolloids, 25*(5), 1000-1008.

Rebolleda, S., Sanz, M. T., Benito, J. M., Beltran, S., Escudero, I., & Gonzalez San-Jose, M. L. (2015). Formulation and characterisation of wheat bran oil-in-water nanoemulsions. *Food Chemistry, 167*, 16-23.

Rodrigues, R. F., Costa, I. C., Almeida, F. B., Cruz, R. A. S., Ferreira, A. M., Vilhena, J. C. E., Florentino, A. C., Carvalho, J. C. T., & Fernandes, C. P. (2015). Development and characterization of evening primrose (*Oenothera biennis*) oil nanoemulsions. *Revista Brasileira de Farmacognosia, 25*(4), 422-425.

Rodríguez-Rojo, S., Varona, S., Núñez, M., & Cocero, M. J. (2012). Characterization of rosemary essential oil for biodegradable emulsions. *Industrial Crops and Products, 37*(1), 137-140.

Senatore, F., Oliviero, F., Scandolera, E., Taglialatela-Scafati, O., Roscigno, G., Zaccardelli, M., & De Falco, E. (2013). Chemical composition, antimicrobial and antioxidant activities of anethole-rich oil from leaves of selected varieties of fennel [Foeniculum vulgare Mill. ssp. vulgare var. azoricum (Mill.) Thell]. *Fitoterapia, 90*, 214-219.

Solans, C., Izquierdo, P., Nolla, J., Azemar, N., & Garciacelma, M. (2005). Nano-emulsions. *Current Opinion in Colloid & Interface Science, 10*(3-4), 102-110.

Solans, C., & Solé, I. (2012). Nano-emulsions: Formation by low-energy methods. *Current Opinion in Colloid & Interface Science, 17*(5), 246-254.

Sultanbawa, Y. (2016a). Anise myrtle (*Syzygium anisatum*) oils. In V. R. Preedy (Ed.), *Essential Oils in Food Preservation, Flavor and Safety*, (pp. 215-219). CA, USA: Elsevier.

Sultanbawa, Y. (2016b). Lemon myrtle (*Backhousia citriodora*)oils. In V. R. Preedy (Ed.), *Essential Oils in Food Preservation, Flavor and Safety*, (pp. 517-521). CA, USA: Elsevier.

Topuz, O. K., Ozvural, E. B., Zhao, Q., Huang, Q., Chikindas, M., & Golukcu, M. (2016). Physical and antimicrobial properties of anise oil loaded nanoemulsions on the survival of foodborne pathogens. *Food Chemistry, 203*, 117-123.

Varona, S., Martín, Á., & Cocero, M. J. (2009). Formulation of a natural biocide based on lavandin essential oil by emulsification using modified starches. *Chemical Engineering and Processing: Process Intensification, 48*(6), 1121-1128.

Weiss, J., Gaysinsky, S., Davidson, P. M., & McClements, D. J. (2009). Nanostructured encapsulation systems: food antimicrobials. In G. Barbosa-canovas, A. Mortimer, D. Lineback, W. Spiess, K. Buckle & P. Colonna (Eds.), *Global Issues in Food Scinece and technology*). New York: Academic Press.

Wilkinson, J. M., Hipwell, M., Ryan, T., & Cavanagh, H. M. A. (2003). Bioactivity of *Backhousia citriodora:* antibacterial and antifungal activity. *Journal of Agriculture and Food Chemistry, 51*(1), 76-81.

Figure Legends:

Figure 1 (A) Mean droplet size and polydispersity index of LMEO nanoemulsion. (B) Particle size distribution at CCRD run order 15 (EO/Smix/DW, 5.5/13.1/81.4, ultrasonication =3 min) and 17 (EO/Smix/DW: 1/10/89, ultrasonication =5 min). LM: lemon myrtle, EO: essential oil.

Figure 2 (A) Mean droplet size and polydispersity index of AMEO nanoemulsion. (B) Particle size distribution at CCRD run order 15 (EO/Smix/DW: 5.5/13.1/81.4, ultrasonication = 3 min) and 17 (EO/Smix/DW: 1/10/89, ultrasonication = 5 min). AM: anise myrtle, EO: essential oil

Figure 3 Turbidity of selected nanoemulsion at different oil droplet concentration. A) lemon myrtle essential oil and B) anise myrtle essential oil. LM: lemon myrtle, AM: anise myrtle, 17= EO/Smix/DW: 1/10/89, ultrasonication = 5 min; 15= EO/Smix/DW: 5.5/13.1/81.4, ultrasonication = 3 min.

Figure 4 Storage stability of LMEO and AMEO nanoemulsion at different temperature A) 4^{o}C B) 25^{o}C and C) 40^{o}C. LM: lemon myrtle, AM: anise myrtle, 17= EO/Smix/DW: 1/10/89, ultrasonication = 5 min; 15= EO/Smix/DW: 5.5/13.1/81.4, ultrasonication = 3 min.

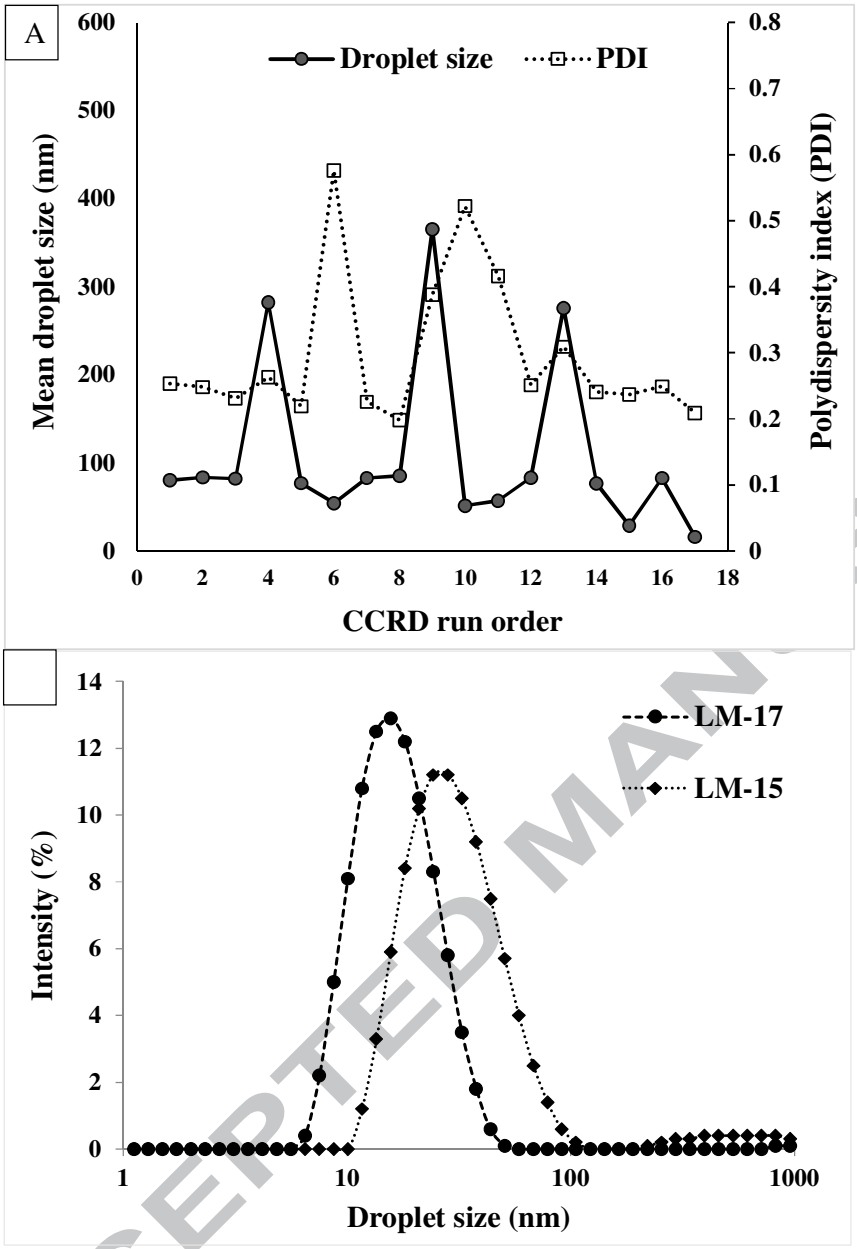
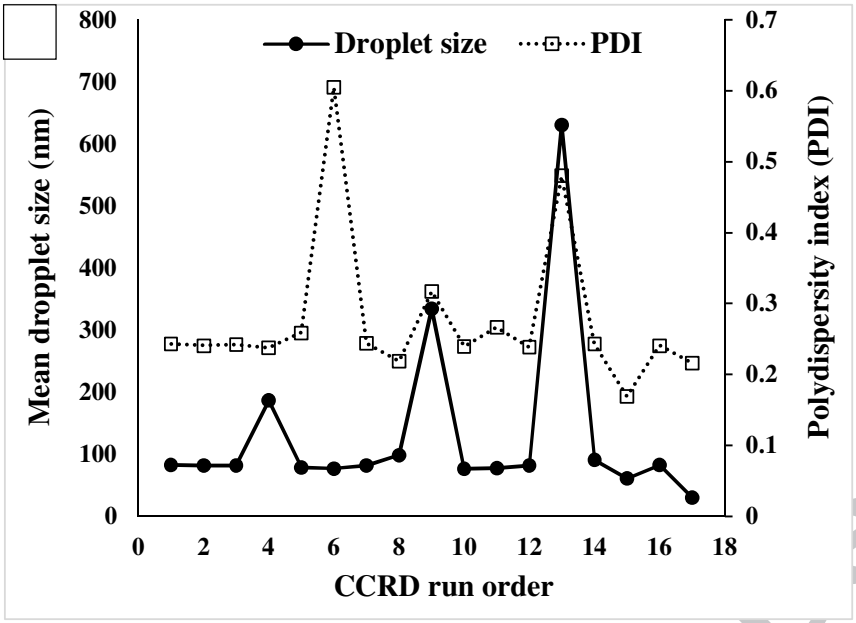


Figure: 1 (A) Mean droplet size and polydispersity index of LMEO nanoemulsion. (B) Particle size distribution at CCRD run order 15 (EO/Smix/DW, 5.5/13.1/81.4, sonication time =3 min) and 17 (EO/Smix/DW: 1/10/89, sonication time =5 min). LM: lemon myrtle, EO: essential oil.



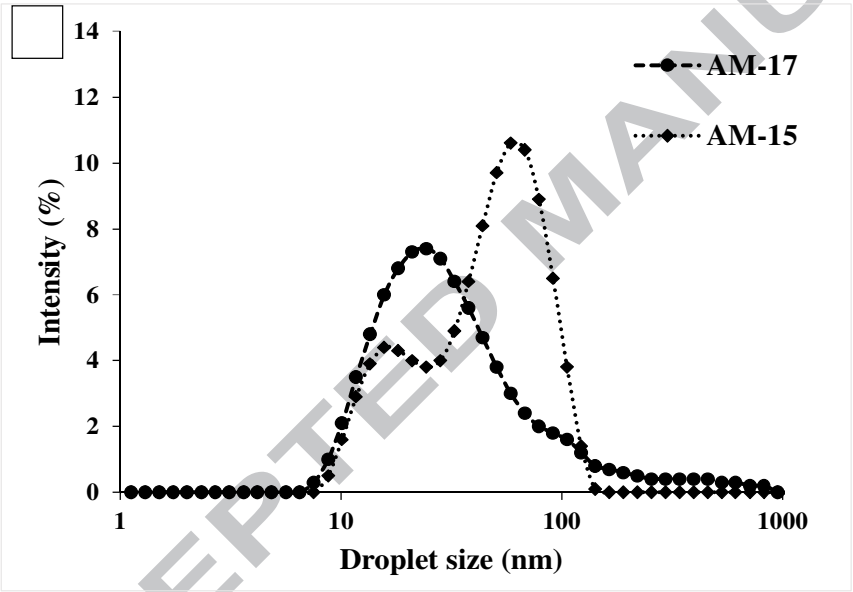


Figure: 2 (A) Mean droplet size and polydispersity index of AMEO nanoemulsion. (B) Particle size distribution at CCRD run order 15 (EO/Smix/DW: 5.5/13.1/81.4, sonication time = 3 min) and 17 (EO/Smix/DW: 1/10/89, sonication time = 5 min). AM: anise myrtle, EO: essential oil

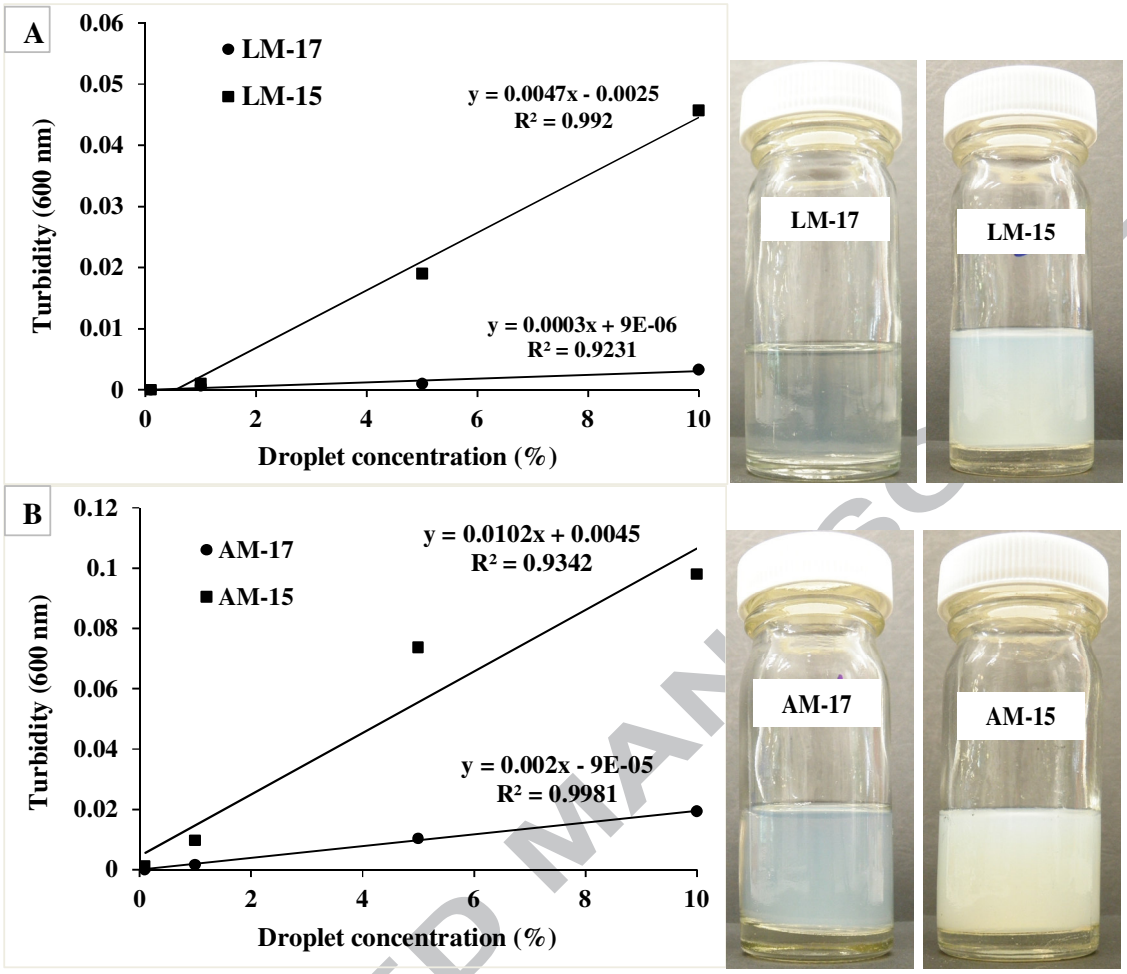


Figure 3. Turbidity of selected nanoemulsion at different essential oil droplet concentration. A) lemon myrtle essential oil and B) anise myrtle essential oil. LM: lemon myrtle, AM: anise myrtle, 17= EO/Smix/DW: 1/10/89, ultrasonication = 5 min; 15= EO/Smix/DW: 5.5/13.1/81.4, ultrasonication = 3 min.

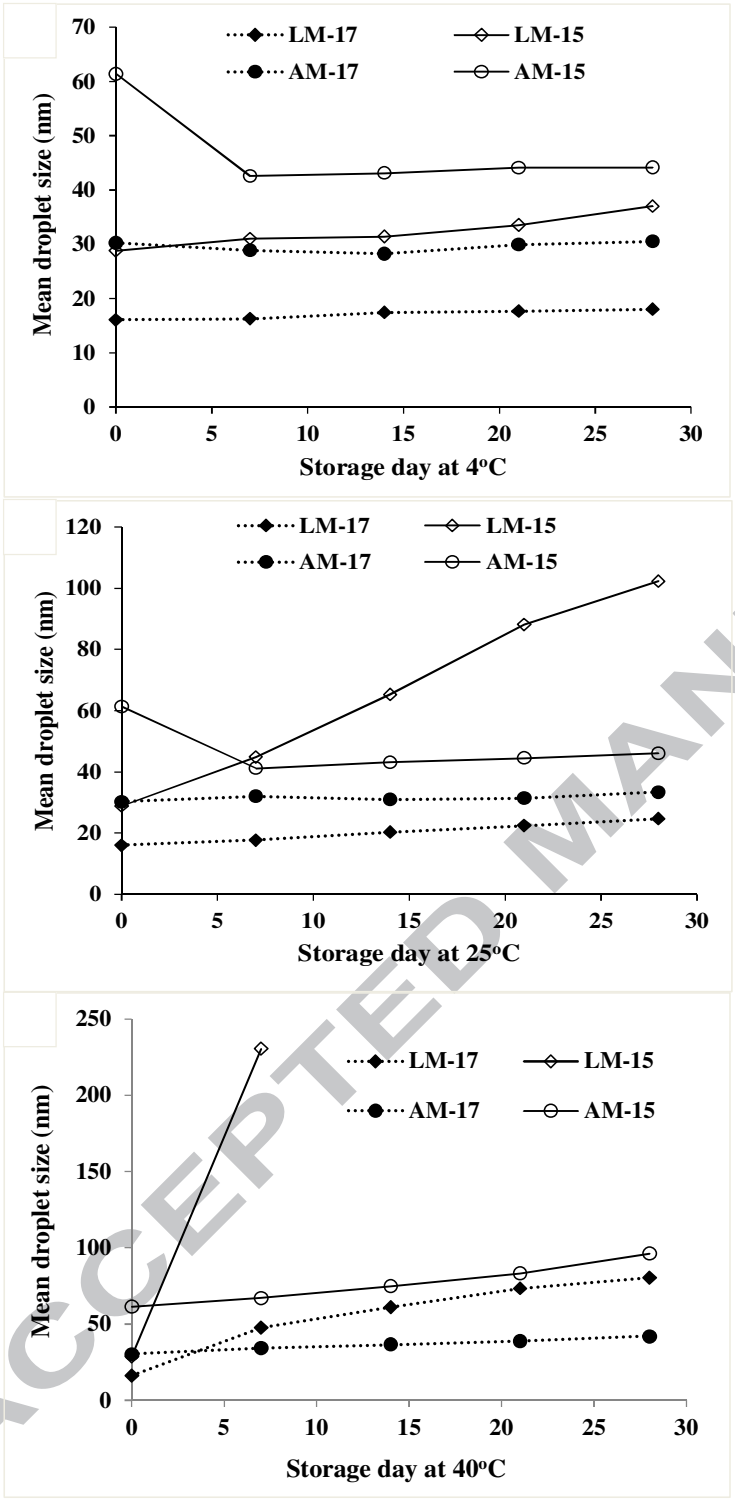


Figure 4. Storage stability of LMEO and AMEO nanoemulsion at different temperature A) 4°C B) 25°C and C) 40°C. LM: lemon myrtle, AM: anise myrtle, 17= EO/Smix/DW: 1/10/89, ultrasonication = 5 min; 15= EO/Smix/DW: 5.5/13.1/81.4, ultrasonication = 3 min.

Table 1. Effect of different HLB surfactant mixture on droplet size and polydispersity index (PDI) of LMEO and AMEO nanoemulsion

Essential Oil	Surfactant HLB value	Nanoemulsion			
		Day -1		Day-8	
		Droplet Size (nm)	PDI	Droplet Size (nm)	PDI
LM	**8**	132.4 ± 0.75	0.174 ± 0.001	143.8 ± 0.77	0.248 ± 0.012
	9	165.8 ± 1.01	0.292 ± 0.003	120.6 ± 0.55	0.196 ± 0.016
	10	158.7 ± 3.21	0.317 ± 0.047	107.1 ± 0.94	0.163 ± 0.005
	11	149.7 ± 0.98	0.397 ± 0.010	97.8 ± 0.60	0.184 ± 0.006
	12	91.4 ± 0.67	0.212 ± 0.014	86.6 ± 0.18	0.181 ± 0.006
	13	84.5 ± 0.45	0.221 ± 0.004	78.6 ± 1.24	0.212 ± 0.005
	14	**84.7 ± 1.28**	**0.223 ± 0.007**	**77.7 ± 0.55**	**0.211 ± 0.001**
	15	165.9 ± 3.90	0.288 ± 0.007	138.3 ± 0.57	0.129 ± 0.011
AM	**8**	156.6 ± 1.78	0.261 ± 0.006	133.8 ± 0.50	0.165 ± 0.009
	9	132.1 ± 0.56	0.210 ± 0.001	124.5 ± 0.06	0.182 ± 0.009
	10	110.5 ± 0.64	0.227 ± 0.005	106.7 ± 0.10	0.209 ± 0.002
	11	108.3 ± 0.21	0.271 ± 0.008	98.4 ± 0.22	0.221 ± 0.005
	12	**82.1 ± 0.16**	**0.256 ± 0.007**	**80.2 ± 1.06**	**0.234 ± 0.004**
	13	87.2 ± 0.19	0.236 ± 0.002	85.9 ± 0.60	0.219 ± 0.008
	14	83.4 ± 0.87	0.239 ± 0.007	84.1 ± 0.65	0.245 ± 0.004
	15	114.3 ± 0.99	0.336 ± 0.013	133.9 ± 0.06	0.352 ± 0.010

LM: lemon myrtle, AM: anise myrtle, HLB: hydrophilic-liphophilic balance

10% EO + 10% Smix + 80% distilled water (ultrasonication = 2 min)

Different surfactant HLB was prepared by different ration Span 80 (HLB 4.3) and Tween 80 (HLB 15) as follows : 65.4:34.6 (HLB 8), 57:43 (HLB 9), 46.7:53.3 (HLB 10), 37.4:62.6 (HLB 11), 28:72 (HLB 12), 18.7:81.3 (HLB 13), 9.3:90.7 (HLB 14), 0:100 (HLB 15).

Table 2. Minimum inhibitory concentration (%) of LMEO and AMEO nanoemulsion against gram positive and gram negative bacteria

Sample	*S. aureus*		*L. monocytogenes*		*E.coli*		*P.aeruginosa*	
	LM	AM	LM	AM	LM	AM	LM	AM
Nanoemulsion	0.062	-	0.031	-	0.25	-	-	-
EO	0.156	-	0.156	-	0.625	-	-	-
Smix	-	15	-	15	15	15	-	-

LM: lemon myrtle, AM: anise myrtle, EO: essential oil, Smix: surfactant mixture (HLB 14 = LM, HLB 12 = AM)

Nanoemulsion: 1% EO +10% Smix + 80% distilled water (ultrasonication =5 min)

Highlights:

1. The required HLB value for LM EO and AM EO are 14 and 12, respectively.
2. The smallest droplet size of 16.07 nm (LMEO) and 30.23 nm (AMEO) was obtained.
3. The selected nanoemulsions of LMEO and AMEO were transparent.
4. The selected nanoemulsion of LMEO and AMEO had good stability at 4, 25 and 40^{o}C.
5. Nanoemulsion improved antibacterial activity of LMEO.