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ORIGINAL ARTICLE

Formulation, characterization, and stability of food grade oil-in-water nanoemulsions of essential oils of *Tasmannia lanceolata*, *Backhousia citriodora* and *Syzygium anisatum*

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Abstract

Oil-in-water nanoemulsions were formulated using sunflower oil mixed with each of the essential oils of *Tasmannia lanceolata* (Tasmanian pepper leaf [TPL]), *Backhousia citriodora* (lemon myrtle [LM]) and *Syzygium anisatum* (anise myrtle [AM]) and stabilized with Tween 80 using ultrasonication. An oil-surfactant ratio of 3:1 was found to produce the lowest emulsion droplet sizes of 96.6 nm for LM, 122.2 nm for AM and 131.8 nm for TPL. Increase in surfactant concentration above 10r resulted in larger droplet sizes, 165.8–2,647.2 nm for LM (radius, $r = .82$), 153.7–2,573.5 nm for AM ($r = .93$) and 157.4–2,621.6 nm for TPL ($r = .83$). Sonication for 3 min produced smaller droplet size; however, sonication for 9 min resulted in increase of droplet size by 1.48, 1.43 and 1.47 times for oils of LM ($r = .82$), AM ($r = .93$) and TPL ($r = .83$), respectively. A positive correlation was found between sonication amplitude (20–50%) and droplet size for nanoemulsions of LM ($r = .93$), AM ($r = .98$) and TPL ($r = .95$). TPL and LM nanoemulsions showed broad- spectrum antimicrobial activities against yeasts and bacteria. The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) against weak-acid resistant yeasts were between 0.001–0.003 and 0.002–0.007 mg/ml for nanoemulsion of TPL and between 0.003–0.014 mg/ml and 0.005–0.027 for nanoemulsion of LM, respectively. The stability and antimicrobial activity of TPL and LM essential oil nanoemulsions confirm their potential for application as food preservatives especially in beverage products that are commonly spoiled by weak-acid resistant yeasts.

1 | INTRODUCTION

Plant essential oils and their bioactive compounds are gaining interest among natural antimicrobial products as food additives due to their acceptability among consumers and reported antimicrobial activities (Gyawali & Ibrahim, 2014; Nazzaro, Fratianni, Coppola, & Feo, 2017; Prakash, Kedia, Mishra, & Dubey, 2015). Plant essential oils have multiple uses and have traditionally been used as food preservatives, therapeutic agents, medicine, and healing applications (Hintz, Matthews, & Di, 2015). The application of plant essential oils in food products could play a role in improving food safety and quality by inhibiting the growth of food borne or spoilage causing microorganisms (Buranasuksombat, Kwon, Turner, & Bhandari, 2011; Fratianni *et al*., 2010; Ghosh, Mukherjee, & Chandrasekaran, 2014). Currently, chemical preservatives are widely used in controlling food pathogenic bacteria and spoilage microorganisms; however, they are negatively perceived by consumers due to the adverse health effects such as cancer, acute toxicity, allergies, attention deficit hyperactivity disorder

in children and teratogenic effects on fetuses (Eigenmann & Haenggeli, 2004; Faleiro, 2011). Consumer awareness of the negative health effects associated with chemical preservatives, and the emergence of microbial resistance to chemical preservatives has shifted the focus toward the use of natural extracts such as plant essential oils as an alternative natural solution (Aneja, Dhiman, Aggarwal, & Aneja, 2014; Calo, Crandall, O'Bryan, & Ricke, 2015; Savoia, 2012; Tiwari *et al*., 2009).

Even though, essential oils are gaining much attention as safe natural antimicrobial agents, their low solubility in water (hydrophobicity) limits the total amount that can be loaded into aqueous or liquid–solid food systems which lowers their antimicrobial activity (Donsì, Annunziata, Vincensi, & Ferrari, 2012). Loading essential oil into an encapsulated nanoemulsion system would allow the oil droplets to disperse in an aqueous system, which maximizes droplet loading capacity and maintains their antimicrobial activity by increasing droplets bioavailability (larger surface area) to target the microorganisms that inhabit in the aqueous phase of the food system (Donsì, Annunziata, Sessa, & Ferrari, 2011; Donsì *et al*., 2012; Weiss, Gaysinsky, Davidson, & McClements, 2009).

Nanoemulsions are a class of emulsions with extremely small sized oil droplets in the range of 20–200 nm (Abbas, Bashari, Akhtar, Li, & Zhang, 2014; Huang, Yu, & Ru, 2010; Sugumar, Singh, Mukherjee, & Chandrasekaran, 2016). A nanoemulsion usually consists of oil, water and an emulsifier and can be formed using either high-energy or low-energy methods. The addition of an emulsifier helps to lower the interfacial tension between the water and oil phases which allows the formation of small sized oil droplets, prevents phase separation, and stabilizes the nanoemulsion (Gupta, Eral, Hatton, & Doyle, 2016). The use of ultrasonication reduces the size of oil droplets to the range of a nanoemulsion, enhancing their solubility and bioavailability with the aid of small amounts of surfactant (3–10%), in comparison to the conventional mechanical methods which require higher surfactant concentration (>20%) (Bouchemal, Briançon, Perrier, & Fessi, 2004; Huang *et al*., 2010). Production of ultrasound-assisted nanoemulsions is gaining popularity due to the low production cost, lower energy consumption, ease of operation and less surfactant requirement (Abbas *et al*., 2015). Ultrasonication generates high-intensity (low-frequency) ultrasound producing intense shear forces which disrupt the interface of oil and water phases, creating a fine and stable emulsion with the use of a low amount of surfactant (Abbas *et al*., 2014). The final characteristics of nanoemulsions in terms of small droplet size, low polydispersity index (PDI) and stability depend on pre-sonication factors such as surfactant-oil mixing ratio, sonication time, sonication amplitude and operational temperature (Hashtjin & Abbasi, 2015). Thus, optimizing the pre-sonication conditions and sonication processing settings is critical to maximize the nanoemulsion end-product qualities.

The rich Australian indigenous flora contains a variety of plants that are abundant in essential oils and phytochemicals with promising biological activities. For that reason, attention has focused in recent years on these native plants to investigate their potential in medicine, pharmaceutical industry, cosmetics, aromatherapy, foods, and beverages as spices, flavoring and preservative agents. Tasmanian pepper, Anise myrtle and lemon myrtle are three Australian native plants whose essential oils contain dominant components with high antimicrobial and antioxidant properties. Tasmanian pepper (*Tasmannia lanceolata)* belongs to the Winteraceae family and is found in forested regions in Tasmania, Victoria and north to the Blue Mountains of Australia (Pengelly, 2002). Tasmanian pepper is a medium sized bushy woody plant that grows up to five meters in height. It has dark green leaves, red-purple-colored stems, and dark berry fruit about 6–7 mm in diameter (Dragar, Garland, & Menary, 1998). It is characterized by a strong heat and pungent flavor, high content of sesquiterpene and monoterpene essential oil and antioxidants (Menary & Menary, 2003; Netzel, Netzel, Tian, Schwartz, & Konczak, 2006; Pengelly, 2002; Smyth, Sanderson, & Sultanbawa, 2012). Leaves are used as herb while berries are used as a spice. Both parts owe their sharp pungency to the presence of a sesquiterpene dialdehyde called polygodial (Chaliha, Cusack, Currie, Sultanbawa, & Smyth, 2013; Dragar *et al*., 1998; Konczak, Zabaras, Dunstan, & Aguas, 2010; Pengelly, 2002). The major components in Tasmanian pepper leaves extracts include Linalool 1.81%, Bicyclogermacrene 1.51%, Myristicin 1.00%, Calamenene 3.42%, Cadina-1,4-diene 1.58%, Spathulenol 1.94%, Guaiol 4.46%, Drimenol 1.91%, 5-Hydroxycalamenene 1.47%, Polygodial 36.74%, n-Pentacosane 1.54%, hexacosanal 2.71% (Sultanbawa, 2016c). Anise myrtle (*Syzygium anisatum)* belongs to the Myrtaceae family. It is a rare Australian native plant found in the moist rainforest areas located in the Bellingen Valley of northeast New South Wales and some parts of Queensland (Blewitt & Southwell, 2000; I. Southwell, Russell, & Smith, 2001). Its leaves are used as a herb either fresh or as ground powder to provide aniseed flavors in sweet and savory Australian cuisines and cosmetic products (Konczak *et al*., 2010; Nirmal, Webber, Mereddy, & Sultanbawa, 2018b). Chemical composition of Anise myrtle includes (E)-anethole (71.2–93.7%) and methyl chavicol (5.0–15.3%) in the *Syzygium anisata* essential oil (anethole type) and (E)-anethole (22.1–42.8%) and methyl chavicol (55.8–75.0%) in the *Syzygium anisata* essential oil methyl chavicol type (Sultanbawa, 2016a). Lemon myrtle (*Backhousia citriodora*) is a member of the Myrtaceae family and is found in the subtropical rainforests of central and south-east regions of Queensland, Australia spreading along the coastal regions from Brisbane to Cairns (Buchaillot, Caffin, & Bhandari, 2009). Lemon myrtle leaves have one of the strongest citral aromas, that can compete with the entire members of the citrus family (I. Southwell *et al*., 2001). Chemical analysis of the lemon myrtle essential oil reveals that it is dominated (82–91%) by citral ($C_{10}H_{16}O$), a monoterpene aldehyde which is responsible for the lemon scent in the leaves. This terpene aldehyde was found to be a mixture of the two geometric isomers neral 9 ([2E]-3,7-dimethylocta-2,6- dienal), and geranial 10 ([2Z]-3,7-dimethylocta-2,6-dienal) also known as citral a and citral b, respectively in the ratio of 1.2–1.5 (Archer, 2004). The key constituents of the essential oil of the citral chemotype of Lemon myrtle essential oil are β- Myrcene, 2.3-Dehydro-1.8 cineole, 6-Methyl-5-hepten-2-one, Citronellal, *exo*-Isocitral, Z-Isocitral, Linalool, *E*-Isocitral, Neral, Geranial, Nerol and Geraniol (Southwell, 2021; Sultanbawa, 2016b).

Numerous reports have been published on the antimicrobial and antifungal potential of Lemon myrtle essential oil against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, methicillin-resistant *S. aureus* (MRSA), *Aspergillus niger*, *Clostridium perfringens*, *Klebsiella pneumoniae* and *Propionibacterium acnes* (Hayes & Markovic, 2002; Wilkinson, Hipwell, Ryan, & Cavanagh, 2003; Zouhir, Jridi, Nefzi, Ben Hamida, & Sebei, 2016). Lemon myrtle essential oils have also been reported to be effective against food pathogenic bacteria and food spoilage yeasts (Alderees, Mereddy, Webber, Nirmal, & Sultanbawa, 2018), against food-borne pathogens (Thielmann, Muranyi, & Kazman, 2019), and against the plant postharvest pathogen *Monilinia fructicola* (Lazar-Baker, Hetherington, Ku, & Newman, 2011). Antiviral, antioxidant and antiinflammatory properties of the Lemon myrtle essential oils have also been reported (Southwell, 2021). Lemon myrtle essential oil in Australia is used as a lemon flavor in the food and beverage industry and is especially attractive as a flavoring agent in dairy products as it does not contribute to curdling (Sultanbawa, 2016b). However, to the best of our knowledge, there is no published literature on the formulation of a stable nanoemulsion formulated with Tasmanian pepper leaf, lemon myrtle and anise myrtle essential oils. In addition, the antimicrobial activity of the nanoemulsions formulated with Tasmanian pepper leaf, lemon myrtle and anise myrtle essential oils against weak-acid resistant yeasts have not been studied.

Therefore, the objective of this study was to formulate stable nanoemulsions with Tasmanian pepper leaf, lemon myrtle and anise myrtle essential oils. The current study also aimed to determine the maximum oil concentration that can be loaded into a stable nanoemulsion system while maintaining the size of oil droplets in the nano range by optimizing mixing ratio of a food-grade surfactant (Tween 80) with the oil phase, sonication time and sonication amplitude. Stable nanoemulsions with no sign of phase separation or creaming and having the lowest droplet size and PDI value were evaluated for their storage stability at 5°C and 25°C for a period of 28 days. In addition, the antimicrobial activity (MIC, MBC and MFC) of the selected essential oil nanoemulsions were evaluated against 10 weak-acid resistant yeasts and two food-borne bacteria.

2 | MATERIALS AND METHODS

2.1 | Essential oils and surfactant

Essential oils of lemon myrtle and anise myrtle were supplied from Australian Rainforest Products Pty Ltd. (New South Wales, Australia) and essential oil of Tasmanian pepper leaf was supplied by Essential oils of Tasmania Pty Ltd. (Tasmania, Australia). Essential oils kept in their original bottles protected from light exposure and stored at 4°C until further use.

Sunflower oil (100% pure, Crisco, Australia) was purchased from a retail supermarket and stored at room temperature. Non-ionic surfactant Tween 80 was purchased from Sigma- Aldrich Co. (St. Louis, MO, USA). Double distilled water purified in Milli-Q system (Millipore Co., Bedford, MA, USA) was used in all experiments. Nanoemulsion components were measured based on volume/volume percentage (v/v%).

2.2 | Formulation of nanoemulsion

The nanoemulsion formulas were prepared based on a mixture of four components, essential oil, sunflower oil, Tween 80 and Milli-Q water in different ratios (v/v %). Concentrations of essential oil (20%) and sunflower oil (10%) were kept fixed for all the emulsion formulas since any increase beyond these concentrations resulted in a viscous emulsion due to lower water phase to oil phase ratio. The concentration of Tween 80 was increased in increments of 5% (from 5 to 30%) whereas the Milli-Q water concentration was decreased by the same percentage. Tween 80 and Milli-Q water were added at these ratios 5–65%, 10–60%, 15–55%, 20–50% and 25–45% respectively. The nanoemulsion was prepared according to Abbas, Karangwa, Bashari, Hayat, Hong, Sharif and Zhang (Abbas *et al*., 2015) with few modifications. The organic phase was prepared first by mixing essential oil, sunflower oil and Tween 80 together and vortexed (Ratek Instruments Pty Ltd, Victoria, Australia) for 30 s. The aqueous phase representing the Milli-Q water was added to the organic phase and vortexed for 60 s at 2000 rpm creating a coarse emulsion. An encapsulated nanoemulsion was prepared by ultrasonicating the coarse emulsion in a high intensity ultrasonic processor (Branson SFX 550, Shanghai, PRC) with 550 watts maximum power output of at 20 kHz frequency. The sonifier is equipped with 1/2 "diameter stepped disruptor horn fitted with a 1/8" diameter titanium alloy tapered microtip. The microtip probe was immersed in the course emulsion and sonicated for different times (10 s pulse-ON and10 s pulse-OFF) at 10, 20, 30, 40, and 50% amplitude.

2.3 | Characterization of nanoemulsion

Measurement of PDI, droplet size distribution and mean droplet size of the nanoemulsion formula were determined using a dynamic light scattering instrument (ZEN3600 Malvern Instruments Limited, UK). The nanoemulsion was diluted with Milli-Q double distilled water (1:40) prior to the measurement process to reduce multiple scattering effects and eliminate the effect of viscosity. Mean droplet diameter was expressed as z-diameter. All measurements were taken at 25°C, in triplicate.

2.4 | Storage stability of selected nanoemulsions at different temperatures

Intrinsic stability of the nanoemulsion was observed during storage at 5°C and 25°C for a period of 28 days. Change in droplet size was measured weekly, also nanoemulsion was observed for the formation of phase separation and creaming.

2.5 | Microorganisms

The antimicrobial activity of Tasmanian pepper leaf, lemon myrtle and anise myrtle essential oil nanoemulsions was evaluated against two bacteria, Gram-positive *Staphylococcus aureus* (ATCC 9144) and Gram-negative *Escherichia coli* (ATCC 11775) and ten yeasts, *Candida albicans* (ATCC 10231), *Candida krusei* (ATCC 6258), *Dekkera anomala* (ATCC 58985), *Dekkera bruxellensis* (ATCC 56866), *Rhodotorula mucilaginosa* (ATCC 20129), *Rhodotorula glutinis* (ATCC 96365), *Saccharomyces cerevisiae* (ATCC 38555), *Schizosaccharomyces pombe* (ATCC 26189), *Zygosaccharomyces bailii* (ATCC 38923) and *Zygosaccharomyces rouxii* (ATCC 10682). Microorganisms purchased from *In Vitro* Technologies, VIC, Australia.

2.6 | Antimicrobial activity

The MIC was defined as the lowest oil concentration to inhibit a visible microorganism growth, performed by a broth serial microdilution method using 96-well plates according to Ahmad and Viljoen (2015) with few modifications (Ahmad & Viljoen, 2015). Cultures were grown for 24 hr at 35°C in nutrient broth (Oxoid, England) for bacteria and for 48 hr at 25°C in Sabouraud dextrose broth (Oxoid, England) for yeasts. Cultures were measured at $OD_{540} = 0.5$ McFarland, diluted using their designated double strength broth to 1×10^5 colony forming unit (CFU) per ml and 100 μL were dispensed in each well of the 96-well plate. A stock solution of essential oil nanoemulsion was serially diluted two-fold in 25 ml centrifuge tubes from 1 to 0.002% v/v and 100 μL of each concentration dispensed in triplicate from highest to lowest in the plate. Growth control (culture + broth + tween 80 + sunflower oil) and sterility control (broth + tween-80 + essential oil + sunflower oil) were included in every test in the same 96-well plate. After incubation for 48 hr for yeasts and 24 hr for bacteria, the absence of a white growth at the bottom of the well is an indication for the MIC. The MBC and MFC were determined by inoculating 20 μL from wells with no observed growth into a new 96-well plate containing 100 μL broth (normal strength) and incubated under the same conditions mentioned above. Wells without visible growth with the least essential oil concentration were selected as the MBC or MFC. The experiment was repeated three times and the average of MIC, MBC and MFC was expressed in mg/ml.

2.7 | Statistical analysis

Statistical analyses were performed using GraphPad Prism version 7.00 (GraphPad 2016) and figures were generated in Microsoft Excel (Office 2016). Statistical significance of differences among treatment groups was done using one-way analysis of variance (ANOVA) followed by Tukey's test as a *post hoc* comparison and $p < .05$ was considered significant.

3 | RESULTS

3.1 | Effect of oil-surfactant mixing ratio

The addition of surfactant at the appropriate ratio is critical to stabilize an emulsion through lowering its interfacial tension at oil–water interface. The mixing ratio of oil-surfactant has a significant impact on the stability and droplet size of emulsion. In this experiment, the oil phase was maintained at the maximum loading capacity of 30% v/v (20% essential oil +10% sunflower oil). The effect of mixing oil at different concentrations (5–25% v/v) of nonionic surfactant Tween 80 on the emulsion droplet size and PDI is presented in Table 1. Oil- surfactant ratio of 1.2:1 (30:25% v/v) produced the largest droplet size and PDI values were as follows, lemon myrtle essential oil ($d = 2{,}647.2$ nm and PDI = 0.62), anise myrtle essential oil ($d = 2{,}573.5$ nm and PDI = 0.61) and Tasmanian pepper leaf ($d = 2{,}621.6$ nm and PDI = 0.63), respectively. At oil-surfactant ratio of 1.5:1 (30:20% v/v), droplet size and PDI value decreased to $d = 683.7$–722.7 nm and PDI = 0.51–0.58 for the three essential oils. Phase separation was observed when emulsion was prepared at oil-surfactant ratio of 6:1 (30:5% v/v). The smallest droplet size and PDI value were obtained with oil-surfactant ratio of 3:1 (30:10% v/v) for lemon myrtle essential oil ($d = 96.6$ nm; PDI = 0.17), anise myrtle essential oil ($d = 122.2$ nm; PDI = 0.17) and Tasmanian pepper leaf essential oil ($d = 131.8$ nm; PDI = 0.17), respectively.

3.2 | Effect of sonication time

Emulsions with the lowest droplet size which contained 20% v/v essential oil, 10% v/v sunflower oil, 10% v/v Tween 80 and 60% water were further evaluated for the effect of sonication time on droplet size and PDI. Impact of sonication time of 3, 6 and 9 min (sonication setting: 10 s pulse-ON and 10 s pulse-OFF) on droplet size, PDI and heat production is presented in Table 2. Nanoemulsions with largest droplet size and PDI of 194.2 nm; 0.27, 174.3 nm; 0.25 and 142.6 nm; 0.26 for Tasmanian pepper leaf, anise myrtle and lemon myrtle essential oil, respectively, were obtained with 9 min of sonication time. A decrease in droplet size, DPI values and temperature were observed with a decrease in sonication time. As sonication time decreased from 9 to 6 min, a significant ($p < .05$) decrease in droplet size was observed for Tasmanian pepper leaf (187.5 nm), anise myrtle (161.1 nm) and lemon myrtle (138.2 nm) essential oil. A further significant ($p < .05$) decrease in droplet size was found when sonication time was decreased to 3 min for Tasmanian pepper leaf (131.8 nm), anise myrtle (122.2 nm) and lemon myrtle (96.6 nm) essential oil. A strong positive correlation was found between sonication time and droplet size for Tasmanian pepper leaf ($r = .83$), anise myrtle ($r = .93$) and lemon myrtle ($r = .82$) essential oil. In addition, heat generated by sonication increased as sonication time increased. This effect could be due to the exothermic nature of the sonication

TABLE 1 The effect of oil, surfactant and water concentrations on droplet size and polydispersity index of oil-in-water nanoemulsions

Oil	EO: SO: T80: W 20:10: 05:65% 20:10: 10:60%	Droplet size (nm) 172.8 ± 0.92[a] 131.8 ± 0.25[b]	PDI 0.23 ± 0.04[a] 0.17 ± 0.03[a]	Notes Phase separation Emulsion
TPL	20:10: 15:55%	157.4 ± 0.72[c]	0.19 ± 0.04[a]	Emulsion
	20:10: 20:50%	683.7 ± 1.04[d]	0.54 ± 0.02[b]	Emulsion
	20:10: 25:45%	2,621.6 ± 0.84[e]	0.63 ± 0.05[b]	Viscous
	20:10: 05:65%	172.3 ± 0.98[a]	0.21 ± 0.02[a]	Phase separation
	20:10: 10:60%	96.6 ± 0.73[b]	0.17 ± 0.01[a]	Emulsion
LM	20:10: 15:55%	165.8 ± 0.60[c]	0.35 ± 0.01[b]	Emulsion
	20:10: 20:50%	722.7 ± 1.55[d]	0.51 ± 0.03[c]	Emulsion
	20:10: 25:45%	2,647.2 ± 1.01[e]	0.62 ± 0.04[d]	Viscous
	20:10: 05:65%	148.2 ± 0.53[a]	0.20 ± 0.02[a]	Phase separation
	20:10: 10:60%	122.2 ± 0.36[b]	0.17 ± 0.01[a]	Emulsion
AM	20:10: 15:55%	153.7 ± 0.58[c]	0.23 ± 0.03[a]	Emulsion
	20:10: 20:50%	691.3 ± 1.16[d]	0.58 ± 0.04[b]	Emulsion
	20:10: 25:45%	2,573.5 ± 0.76[e]	0.61 ± 0.02[b]	Viscous

Abbreviations: AM, anise myrtle; EO, essential oil; LM, lemon myrtle; T80, Tween 80;TPL, Tasmanian pepper leaf; SO, sunflower oil; W, Milli-Q water. Means with different letters within the same columns are significantly different at $p < .05$.

TABLE 2 Effect of sonication time on emulsion temperature, droplet size and polydispersity index of Tasmanian pepper leaf, lemon myrtle and anise myrtle essential oils

Sonication amplitude	Duration (min)	Temperature (°C)	LM		TPL		AM	
			DS (nm)	PDI	DS (nm)	PDI	DS (nm)	PDI
20%	3	36.53 ± 0.70[a]	96.6 ± 0.73[a]	0.17 ± 0.01[a]	131.8 ± 0.25[a]	0.17 ± 0.03[a]	122.2 ± 0.36[a]	0.17 ± 0.01[a]
	6	43.68 ± 0.41[b]	138.2 ± 0.32[b]	0.22 ± 0.01[b]	187.5 ± 0.71[b]	0.22 ± 0.02[ab]	161.1 ± 0.68[b]	0.23 ± 0.03[b]
	9	46.73 ± 0.56[c]	142.6 ± 0.73[c]	0.26 ± 0.02[c]	194.2 ± 0.43[c]	0.27 ± 0.02[b]	174.3 ± 0.53[c]	0.25 ± 0.02[b]

Note: Sonication set for 10 s ON and 10 s OFF.
Abbreviations: AM, anise myrtle; DS, droplet size; LM, lemon myrtle; PDI, polydispersity index; TPL, Tasmanian pepper leaf. Means with different letters within the same columns are significantly different at $p < .05$.

TABLE 3 Effect of sonication amplitudes on emulsion temperature, droplet size and polydispersity index of Tasmanian pepper leaf, lemon myrtle and anise myrtle essential oils

Sonication amplitudes (%)	Temperature (°C)	LM		TPL		AM	
		DS (nm)	PDI	DS (nm)	PDI	DS (nm)	PDI
10	28.75 ± 0.35[a]	217.9 ± 0.63[a]	0.38 ± 0.01[a]	221.5 ± 0.72[a]	0.37 ± 0.02[a]	215.4 ± 0.56[a]	0.35 ± 0.01[a]
20	36.53 ± 0.57[b]	96.6 ± 0.73[b]	0.17 ± 0.03[b]	131.8 ± 0.25[b]	0.17 ± 0.03[b]	122.2 ± 0.36[b]	0.17 ± 0.02[b]
30	41.65 ± 0.63[c]	131.7 ± 0.21[c]	0.27 ± 0.01[c]	148.6 ± 0.53[c]	0.24 ± 0.01[ce]	136.4 ± 0.81[c]	0.26 ± 0.01[c]
40	44.10 ± 0.42[c]	143.0 ± 0.57[d]	0.22 ± 0.02[d]	153.5 ± 0.84[d]	0.20 ± 0.02[bc]	143.6 ± 1.03[d]	0.21 ± 0.03[bd]
50	59.75 ± 0.26[d]	158.5 ± 0.41[e]	0.29 ± 0.01[c]	164.7 ± 0.31[e]	0.27 ± 0.01[e]	152.7 ± 0.70[e]	0.24 ± 0.01[cd]

Abbreviations: AM, anise myrtle; DS, droplet size; LM, lemon myrtle; PDI, polydispersity index; TPL, Tasmanian pepper leaf. Means with different letters within the same columns are significantly different at $p < .05$.

process. The temperature of the system may rise up to tens of degrees Celsius depending on the period and intensity of the sonication process (Hashtjin & Abbasi, 2015).

3.3 | Effect of sonication amplitude

Selected emulsions with the lowest droplet size were again evaluated for the effect of sonication amplitude on droplet size, PDI and

TABLE 4 Changes in droplet size of Tasmanian pepper leaf, lemon myrtle and anise myrtle essential oil nanoemulsions during storage of 28 days at 5°C and 25°C

Storage Temperature	(day)	Mean droplet size (nm)		
		TPL	LM	AM
5°C	0	131.8 ± 0.25[a]	96.6 ± 0.73[a]	122.2 ± 0.36[a]
	7	179.5 ± 3.42[b]	109.3 ± 2.41[a]	119.4 ± 0.85[a]
	14	171.2 ± 1.27[b]	98.6 ± 1.48[a]	122.8 ± 0.50[a]
	21	172.8 ± 0.85[b]	95.6 ± 1.48[a]	121.8 ± 0.52[a]
	28	169.3 ± 0.60[b]	95.8 ± 0.89[a]	125.7 ± 1.30[a]
25°C	0	131.8 ± 0.15[a]	96.6 ± 0.73[a]	122.2 ± 0.36[a]
	7	158.5 ± 3.34[b]	98.7 ± 0.29[a]	123.4 ± 2.10 [a]
	14	181.2 ± 1.97[c]	105.2 ± 3.95[a]	124.1 ± 0.35[a]
	21	184.2 ± 0.72[c]	93.7 ± 2.44[a]	127.2 ± 1.02[a]
	28	185.9 ± 1.94[c]	107.6 ± 1.45[a]	127.6 ± 0.12[a]

Abbreviations: AM, anise myrtle; LM, lemon myrtle; TPL, Tasmanian pepper leaf.

temperature. The sonication amplitude appears to have a significant impact on the emulsion droplet size, PDI and temperature produced during sonication (Table 3). Sonication at 10% amplitude did not generate enough energy to lower the droplet size which had resulted at the lowest temperature (28.75°C) producing the largest droplet size (215.4–221.5 nm) and PDI (0.35–0.38) values. However, when emulsions were sonicated at 20% amplitude, a significant ($p < .05$) decrease in droplet size (96.6–122.2 nm) and PDI (0.16–0.18) values with a temperature of 36.53°C were observed. Sonication above 20% amplitude resulted in a significant ($p < .05$) increase in droplet size, PDI values and temperature, except a non-significant increase in temperature between sonication amplitude of 30 and 40%. The overall positive correlation between sonication amplitude (20–50%, excluding 10%) and essential oil emulsion droplet size of lemon myrtle was $r = .93$, Tasmanian pepper leaf was $r = .95$ and anise myrtle was $r = .98$ while for emulsion temperature, r was $= .87$. It is worth mentioning that sonication above 20% amplitude gave a foamy appearance to the emulsion, which usually settled approximately 30 min after sonication.

3.4 | Nanoemulsion storage stability

The stability of selected nanoemulsions with the least droplet size and PDI values was evaluated during a storage period of 28 days at 5°C and 25°C which is presented in Table 4. No phase separation was observed during the storage period of 28 days. Droplet size of Tasmanian pepper leaf essential oil nanoemulsion had a significant ($p < .05$) increase from day-0 (131.8) to day-7 (169.5 nm) at 5°C and then had no signific

ant ($p > .05$) changes in droplet size from day-7 (169.5) to day-28 (169.3 nm) of storage. At 25°C, the size of Tasmanian pepper leaf nanoemulsion droplet significantly ($p < .05$) increased from day-0 (131.8) to day-14 (181.2 nm) and then had a small non-significant ($p > .05$) increase until day-28 (185.9 nm). Increase in temperature during storage, had caused an increase in Tasmanian pepper leaf nanoemulsion droplet size, however lemon myrtle and anise myrtle essential oil nanoemulsions were not affected by the increase in storage temperature. The increase in droplet size for the Tasmanian pepper leaf nanoemulsion could be due to the the movement of the dispersed droplet through the continuous phase with an increased opportunity of droplets collisions (Henry, Fryer, Frith, & Norton, 2009). In nanoemulsion systems, due to the small droplet size, the reduction of interfacial areas and free energy breakdown processes are minimal such as creaming, sedimentation, flocculation, and coalescence. However, Ostwald ripening could be a main mechanism producing instability of nanoemulsions (Li & Chiang, 2012). Both Lemon myrtle and Anise myrtle nanoemulsions were stable and showed no significant increase in droplet size throughout the storage period of 28 days at both 5°C and 25°C. The increased stability of both the Lemon myrtle and Anise myrtle nanoemulsions in the current study could be due to the use of sunflower oil which is known to be an Ostwald ripening inhibitor.

3.5 | Antimicrobial activity of nanoemulsions

The minimum inhibitory concentration (MIC), minimum fungicidal concentration (MFC) and minimum bactericidal concentration (MBC) of Tasmanian pepper leaf and lemon myrtle essential oil nanoemulsions against weak-acid resistant yeasts and food related bacteria is given in Table 5. Nanoemulsion of anise myrtle essential oil had no antimicrobial activity against all tested microorganisms at levels up to 2% v/v. Blank nanoemulsion (Tween 80 + sunflower oil + water) without Tasmanian pepper leaf or lemon myrtle essential oil was evaluated to investigate whether the antimicrobial activity was due to essential oils alone or the combination of Tween 80 and sunflower oil. The blank nanoemulsion showed no antimicrobial activity against yeasts and

TABLE 5 Antimicrobial activity of Tasmanian pepper leaf and lemon myrtle essential oil nanoemulsions against yeasts and bacteria

Microorganisms	Nanoemulsion Tasmanian pepper leaf MIC mg/ml	Nanoemulsion Tasmanian pepper leaf MFC/MBC mg/ml	Nanoemulsion Lemon myrtle MIC mg/ml	Nanoemulsion Lemon myrtle MFC/MBC mg/ml
E. coli	0.019 ± 0.11	0.042 ± 0.18	0.042 ± 0.18	0.083 ± 0.36
S. aureus	0.013 ± 0.05	0.027 ± 0.09	0.026 ± 0.10	0.042 ± 0.18
Z. rouxii	0.003 ± 0.01	0.005 ± 0.02	0.011 ± 0.17	0.021 ± 0.09
Z. bailii	0.003 ± 0.01	0.007 ± 0.02	0.013 ± 0.05	0.026 ± 0.09
C. albicans	0.002 ± 0.01	0.003 ± 0.01	0.013 ± 0.05	0.021 ± 0.10
S. cerevisiae	0.003 ± 0.01	0.004 ± 0.03	0.010 ± 0.04	0.018 ± 0.12
R. mucilaginosa	0.002 ± 0.02	0.003 ± 0.01	0.011 ± 0.06	0.021 ± 0.09
C. krusei	0.002 ± 0.01	0.003 ± 0.02	0.010 ± 0.05	0.018 ± 0.12
R. glutinis	0.001 ± 0.02	0.003 ± 0.01	0.010 ± 0.05	0.018 ± 0.14
S. pombe	0.003 ± 0.04	0.007 ± 0.08	0.014 ± 0.08	0.027 ± 0.09
D. anomala	0.001 ± 0.01	0.003 ± 0.01	0.003 ± 0.01	0.005 ± 0.03
D. bruxellensis	0.001 ± 0.01	0.002 ± 0.01	0.003 ± 0.02	0.005 ± 0.02

Note: Results for anise myrtle essential oil nanoemulsion is not included as there was no inhibition activity.

bacteria at the tested concentrations (data not shown). Therefore, the observed antimicrobial activity was derived from Tasmanian pepper leaf and lemon myrtle essential oils. Nanoemulsions of Tasmanian pepper leaf and lemon myrtle essential oils showed broad-spectrum antifungal and antibacterial activity. The MIC and MFC values against yeasts for nanoemulsion of Tasmanian pepper leaf essential oil were 0.001–0.003 and 0.002–0.007 mg/ml, respectively, and for nanoemulsion of lemon myrtle essential oil were 0.003–0.011 and 0.005–0.027 mg/ml, respectively. Bacteria were less susceptible to the antimicrobial activity of the essential oil nanoemulsions and required higher doses of nanoemulsion of Tasmanian pepper leaf and lemon myrtle essential oils compared to yeasts. The MIC and MBC values against bacteria were 0.013–0.019 and 0.027–0.042 mg/ml, respectively, for the nanoemulsion of Tasmanian pepper leaf and 0.026–0.042 and 0.042–0.083 mg/ml, respectively, for the nanoemulsion of lemon myrtle.

4 | DISCUSSION

Plant essential oils have shown strong antibacterial and antifungal activities (Gyawali & Ibrahim, 2014). The application of essential oils as natural preservatives in liquid and semi-liquid food products is challenging due to their hydrophobicity. Oil and water are two immiscible liquids that can be emulsified by the addition of a surfactant at the appropriate amount to reduce the interfacial tension and disperse oil droplets into water. The addition of sufficient amount of surfactant is critical to create a stable emulsion, otherwise phase separation will occur rapidly as oil molecules merge together (coalescence) to reduce their surface free energy. Surfactant molecules will attach to oil droplets creating repulsive forces on their surface (surface active) to form a stable emulsion with no phase separation (McClements, 2012). In this study, a phase separation was experienced when the emulsion was prepared at 30% oil and 5% surfactant (6:1 ratio). This is due to the low surfactant concentration required to assemble around the available oil molecules at their hydrophobic-end, thus failing to reduce the interfacial tension between oil and water causing a phase separation (Roy & Guha, 2018). However, when the surfactant concentration increased to 10%, it resulted in a stable emulsion having the lowest oil droplet size; since there were sufficient numbers of surfactant molecules (proportional to the oil droplets) to bind and completely cover the surface area of oil droplets dispersing them finely into the carrier phase (Hasani, Pezeshki, & Hamishehkar, 2015; Roy & Guha, 2018). As the surfactant concentration increased to 15, 20 and 25%, it caused an increase in oil droplet diameter. Whenever the surfactant molecules are presented in the emulsion beyond the concentration of emulsified oil molecules (critical micelle concentration), they become freely available (unattached to oil droplets) in the water phase forming micelles which lead to agglomeration responsible for the observed increase in oil droplet size (Wulff-Pérez, Torcello-Gómez, Gálvez-Ruíz, & Martín-Rodríguez, 2009).

Similar findings were observed in other reports done by Wulff-Pérez, Torcello-Gómez, Gálvez-Ruíz and Martín-Rodríguez (Wulff-Pérez *et al*., 2009) where oil-in-water nanoemulsion prepared at relatively low surfactant concentration proved to be stable against Ostwald ripening and coalescence, even when the emulsion was loaded with high oil concentration of 25%.

Another parameter that influenced the emulsion droplet size was the intensity of sonication. Increasing the power setting of the ultrasonic processor will increase the amplitude of the ultrasonic waves which in return generate intense acoustic cavitation effects that break emulsion droplets into smaller ones at a higher rate. However, there is

a limit at certain sonication amplitudes where droplet size reduction becomes ineffective due to a phenomenon called acoustic shielding which counters further reduction effect (Abbas, Hayat, Karangwa, Bashari, & Zhang, 2013; T. S. H. Leong, Manickam, Martin, Li, & Ashokkumar, 2018). The Bjerknes forces or acoustic radiation forces create a pressure forcing the cavitation bubbles in the emulsion to accumulate and cluster like clouds (coalescence), where bubbles located in the middle of these clouds receive less acoustic pressure in comparison to bubbles near the surface creating an acoustic shielding effect (T. Leong, 2016). This phenomenon was experienced in this study as the sonication amplitude was increased to 30, 40 and 50, oil droplets began to significantly increase in size in comparison to 20% amplitude which was the optimum setting producing the smallest oil droplets. Furthermore, 10% amplitude produced the largest oil droplets since it did not generate enough power to lower the size of emulsion particles. This makes 20% amplitude the critical or optimum sonication point, as any further increase or decrease in amplitude will counter the benefit of oil particle size reduction.

The acoustic shielding effect is not only associated with the increase in sonication amplitude but also with sonication duration (T. S. H. Leong *et al*., 2018). As sonication duration increased from 3 to 6 and 9 min in this study, oil droplets are found to significantly increase in size due to the development of more bubbles in a form of foamy layer that block some acoustic waves to reach oil particles.

Increasing sonication time means there is more energy available to disrupt and reduce oil droplets size; however, there is a certain limit where exceeding the optimum or equilibrium (leveling-off state) of sonication time or power will lead to an insignificant reduction in oil droplet diameter (Kentish *et al*., 2008; Maali & Mosavian, 2013). Reducing oil droplets is one important step in creating a new nanoemulsion but maintaining droplet size during storage (aging process of droplets) is also a critical step since Ostwald ripening (oil droplets growing in size) is a known issue that nanoemulsion oil particles often experience during storage (Chebil, Desbrières, Nouvel, Six, & Durand, 2013). Adding a highly hydrophobic compound (ultrahydrophobe) into the dispersed oil will provide a kinetic stabilization which significantly decreases the Ostwald ripening of the nanoemulsion (Taylor, 2003).

Sunflower oil was used in making the nanoemulsion to serve as a carrier to encapsulate the essential oils and as a ripening inhibitor to retard Ostwald ripening effect during the storage period according to Doost, Dewettinck, Devlieghere and Van der Meeren (Doost, Dewettinck, Devlieghere, & Van der Meeren, 2018). The incorporation of sunflower oil as an essential oil carrier at a concentration of less than 50% has been reported to have no inference or reduction in the antimicrobial activity of the essential oil nanoemulsion. In the current study, a blank nanoemulsion (without essential oil) contained 10% sunflower oil showed no antimicrobial activity when tested at the same concentrations of the essential oil MIC values. The incorporation of sunflower oil at 10% into the nanoemulsion had no influence on the antimicrobial activity which was lower than what was reported by Doost, Dewettinck, Devlieghere and Van der Meeren (Doost *et al*., 2018) which found no antimicrobial interferences when sunflower oil was used at less than 50%. Therefore, sunflower oil was found to be a suitable hydrophobic compound that reduced the Ostwald ripening effect of the nanoemulsion during storage at two different temperatures without interfering with the essential oils' antimicrobial activity.

Anise myrtle essential oil nanoemulsion (including 10% sunflower oil) had no antimicrobial activity against all tested microorganisms even at a high concentration of 2% v/v. There are two chemotypes of anise myrtle oil which have the same chemical compounds but at different concentrations (Brophy & Boland, 1991). The first reported type of anise myrtle contains about 93- 95% anethole and 4.40–5.60% methyl chavicol, while the second type consists of 20–33% anethole and 66–77% methyl chavicol. Difference in concentration between anethole and methyl chavicol could have played a role in lack of antimicrobial activity with anise myrtle. In a previous report by Wilkinson and Cavanagh (Wilkinson & Cavanagh, 2005) which tested two types of anise myrtle essential oil, they reported that one type of the anise myrtle oil showed good antimicrobial activity against *E. coli* and *C. albicans*, but the other type had no activity. In addition, another report by Nirmal, Mereddy, Li and Sultanbawa (Nirmal, Mereddy, Li, & Sultanbawa, 2018a) found no activity of anise myrtle essential oil against *E. coli* and *S. aureus*. However, Hood, Wilkinson and Cavanagh (Hood, Wilkinson, & Cavanagh, 2003) found antimicrobial activity of anise myrtle essential oil against *E. coli* and *S. aureus*. Perhaps, more chemotypes of anise myrtle essential oil should be evaluated for their antimicrobial activity and chemical profiling in the future to draw a conclusion regarding their inhibition ability against yeasts and bacteria. The antimicrobial activity of Tasmanian pepper leaf and lemon myrtle essential oil nanoemulsions showed that they possess a strong broad-spectrum antimicrobial activity against weak-acid resistant yeasts and food spoilage bacteria. Incorporating these plant essential oils into a nanoemulsion will make them suitable as natural antimicrobial agents specifically in a beverage system.

5 | CONCLUSION

The ultrasonication technique made it possible to form stable nanoemulsions at lower ratio of surfactant to oil. Many studies have reported that lower droplet size of an emulsion is achieved by increasing ratio of surfactant to oil; However, lower droplet size was obtained in this study at low surfactant (10%) to oil (30%) ratio using ultrasonication. Results of this study have shown that nanoemulsions loaded with high oil and low surfactant at an average droplet size of <200 nm can be produced utilizing Australian native plant oils and food-grade nonionic surfactant with an energy-efficient ultrasonication within 3 min. The strong antifungal activity of the Tasmanian pepper leaf and Lemon myrtle essential oil nanoemulsions against the tested yeast strains provide useful information on the potential use of these Australian native plant essential oils in the food and beverage industry where weak acid- resistant yeasts are a significant threat to the stability of the products.

AUTHOR CONTRIBUTIONS

Fahad Alderees: Methodology, Visualization, and Writing- original draft. Saleha Akter: Writing - original draft and editing. Ram Mereddy: Conceptualization, Supervision, and Writing - review and editing. Yasmina Sultanbawa: Conceptualization, Supervision, Fund acquisition, Writing - review & editing.

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CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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