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A new stable and bioactive formulation of *Geniotrigona thoracia* propolis microemulsion for oral delivery

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ABSTRACT

Propolis, particularly those produced by stingless bees, is known to have a broad range of medicinal benefits due to its antioxidant properties. However, its strong and unpleasant flavour and sticky texture make it less palatable. This study aimed to improve the oral administration of stingless bee propolis extract for nutraceutical use by formulating it into a microemulsion. The microemulsion was created using a water titration method and a pseudo-ternary phase diagram to determine the optimal surfactant: co-surfactant ratio. The formulation was then characterized for its globule size, pH, viscosity, and phenolic content, and subjected to stability tests under ambient and accelerated conditions. Anti-microbial and antioxidant activity were evaluated through the disk diffusion method and the ABTS assay. The microemulsion had an average droplet size of 229.2 nm, a pH of 4.78 $\pm$ 0.18, and a viscosity of 0.149 Pa, which falls within the typical range for oral formulations. The microemulsion successfully preserved the antioxidant and antibacterial properties of the propolis extract. Long-term and accelerated stability studies indicated that the microemulsion's pH, appearance, and viscosity remained stable for up to 12 months, although there was a 24 % loss in phenolic activity over the same period. Overall, a stable and potent propolis microemulsion was successfully developed using pharmaceutical and food-grade ingredients, providing a safe and effective way to orally deliver propolis.

1. Introduction

Propolis is a resinous substance that bees collect from the leaf buds and bark of poplar trees or other species (Maroof & Gan, 2021). Folk medicines in many regions worldwide have used propolis for its biological and pharmacological properties, including hepatoprotective effects, antitumor, antioxidative, antimicrobial, and anti-inflammatory effects (Silva-Carvalho et al., 2015; Maroof et al., 2020). Due to these properties, propolis is commonly applied in food, beverages, cosmetics, and medicine for improving health and preventing diseases (Alam et al., 2023; Irigoiti et al., 2021).

Stingless bees are widespread and well-adapted to tropical and subtropical regions of the world, such as Central and South America, Africa, Northern Australia, and Southeast Asia (Maroof et al., 2023; Ivorra et al., 2020). In Malaysia, approximately 40 different indigenous species of stingless bees, locally known as "lebah kelulut", exist, but stingless bee culture in Asia are mostly from two domesticated native species: *Geniotrigona thoracica (G. thoracica)* and *Heterotrigona itama (H. itama)* (Ivorra et al., 2020).

Although more than 800 constituents have been identified in propolis (Kasote et al., 2022), the functional properties are mainly attributed to the polyphenols of flavonoids, phenolic acids, and their esters (Maroof & Gan, 2022; Galeotti et al., 2018). However, raw propolis is difficult to use due to its low water solubility, susceptibility to oxidation, low bioavailability, and short half-life, limiting its use in humans unless suitably formulated (Fan et al., 2014). To be utilized for human consumption, raw propolis (which contains wax and other insoluble materials) must first undergo extraction to yield an extract containing the

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biologically active constituents. However, upon extraction, the bitter taste,low water solubility and low thermal and oxidative stability of the extracts raise some challenges for food applications and consumer acceptance (Maroof et al., 2023).

Microemulsions (MEs) have generated considerable interest over the years as a good drug delivery system (Pavoni et al., 2020). MEs are colloidal, optically isotropic, transparent or slightly opalescent formulations of low viscosity consisting of surfactant, co-surfactant, oil and water (Fan et al., 2014). They have advantages such as thermodynamic stability, ease of preparation, good solubilization capacity of lipophilic, hydrophilic, and amphiphilic solutes, making them very versatile (Tartaro et al., 2020). MEs have emerged as novel vehicles for drug delivery via multiple routes, including transdermal, topical, oral, and parenteral, allowing for more targeted, sustained, or controlled release formulations that can improve therapeutic outcomes and reduce drug toxicity (Pavoni et al., 2020). For natural products developed into nutraceuticals, MEs can aid in taste masking and enhance the antimicrobial activity of formulations (Seibert et al., 2019).

Emulsions loaded with propolis extract (PE) have been developed for several applications. Žilius et al. (2016) created a ME using PEG-8 caprylic/capric glycerides (labrasol) and ethanolic PEs, with the goal of delivering phenolic components present in propolis *ex vivo* into the skin. In this oil in water ME, the phenolic compounds were mostly present in the ME lipid phase, thus limiting their availability at the skin's surface. Nevertheless, the formulation is viewed as a good source of antioxidant with reduced potential to cause oxidative stress on biological systems.

In another study, Fan et al. (2014) explored if the immune-enhancing properties of propolis flavonoids (PF's) might be improved by converting it to a propolis ME. When compared to PF's alone, their findings demonstrated that propolis ME (at medium and high doses of PFs) dramatically boosts immunological organ indices, overcomes immunosuppression, accelerates lymphocyte proliferation, and improves serum interleukin concentrations. Consequently, ME may be a useful formulation that can enhance PF's bioavailability. Dantas et al. (2010), used ethanolic extracts of Brazilian propolis incorporated into MEs for topical application, and optimized the formulation by varying compositions of Tween 20, Tween 80, ethanol, and water. The MEs exhibited antimicrobial activity against gram positive bacteria.

However, studies on the development of oral propolis MEs are limited. Such formulations are important since the oral route is the most common delivery route for natural product supplements. We have already conducted and published a thorough evaluation of the propolis extract in terms of the individual compounds present, total phenolics and flavonoid content (Maroof et al., 2023). In this study, we aim to formulate a ME of the characterized stingless bees *G. thoracica* propolis for oral administration as a health supplement using food and pharmaceutical grade components. We present a systematic screening of ME components in the formulation to guide selection of the final formulation combinations. The step is followed by an extensive physical, chemical and biological characterization of the formulation as well as long term stability studies to guide its commercial potential.

2. Materials and methods

2.1. Materials

Folin-Ciocalteu reagent, sodium carbonate, gallic acid were purchased from Sigma-Aldrich (Germany). Ethanol was obtained from Merck (Germany). Food grade glycerol, propylene glycol, Tween 20 and Tween 80 were purchased from Sigma-Aldrich (Germany). Oral pharmaceutical grade polyoxyl 35 castor oil (Kolliphor EL) and polyoxyl 40 hydrogenated castor oil (Kolliphor RH40) were purchased from BASF (Ludwigshafen, Germany). Pharmaceutical grade Labrasol was purchased from Gattefossé (Saint-Priest, France).

2.2. Extraction of raw propolis samples

G. thoracica propolis produced by bees cultured in the Gemas district of Negeri Sembilan, Malaysia was collected and stored overnight at −20 °C. The samples were then ground using a pestle and mortar and sieved into a particle size of 300–500 μm. The resulting samples were stored separately in airtight containers at −20 °C until subjected to supercritical fluid extraction (SFE). The SFE procedure involved setting the main extraction vessel at 1) 200 Bar in the first hour and subsequently 2) 150 Bar from the 2nd to 4th hour. The carbon dioxide (CO_2) flow rate was set at 100 g/min in the first three hours followed by 80 g/min in the final hour. Ethanol flow was at 15 ml/min in the first three hours followed by 10 ml/min in the final hour. Each of the three cyclones was set to a different pressure level: 60, 80, and 90 bars. The supernatant was then evaporated in a rotavapor at 40 °C, resulting in a sticky resin-like substance which was used for the formulation.

2.3. Formulation of blank microemulsion

2.3.1. Selection of oil

Propolis' equilibrium solubility was determined by employing a single medium chain triglyceride (MCT) i.e. oleic acid and a single long chain triglyceride (LCT), i.e. olive oil as described by Lin et al. (2009) with slight modifications. Propolis solubility in the oils was determined by adding 100 mg of propolis to 2 mL of oil in a test tube followed by vortexing (KMC-1300V, Vision Scientific Co., Kyunggi-do, Korea) for approximately 10 min.

The propolis in oil solution was left at room temperature for 24 hours to observe any signs of instability, such as changes in color, creaming, or sedimentation. If 100 mg of propolis could be dissolved in the solvent without any signs of instability, successive amounts of 100 mg of propolis were added until the mixture showed a persistent cloudy appearance or visible grains of sedimented propolis were deposited at the bottom of the test tube.

2.3.2. Screening of surfactants

Five surfactants (Kolliphor RH, Labrasol, Kolliphor EL, Tween 20 and Tween 80) were screened for ME formulation as described by Azeem et al. (2009) with slight modifications. Briefly, 10 mL of surfactant solution was prepared at a 1:10 ratio of surfactant to water. Subsequently, 4 μL of oil selected in Section 2.3.1 was added with vigorous vortexing (KMC-1300V, Vision Scientific Co., Kyunggi-do, Korea). If a one phase clear solution was obtained, the addition of the oil was repeated until the solution became cloudy or turbid.

2.3.3. Selection of co-surfactants

Glycerol and propylene glycol (PG) were screened as potential co-surfactants. The surfactant selected in Section 2.3.2 was mixed at a 1:1 ratio of surfactant: co-surfactant and was left to stand at room temperature for 24 hours. Mixtures that showed no signs of instability or immiscibility were brought over to the next step.

2.3.4. Pseudo-ternary phase diagram formation

To obtain the optimal concentration range of surfactant (selected in 2.3.2) and co-surfactant (selected in 2.3.3) for the formation of ME, pseudo-ternary phase diagrams were constructed. The surfactant and co-surfactant were mixed in three different ratios (1:1, 2:1 and 3:1), labeled as S_{mix}. Nine different combinations of oil and S_{mix} in different weight ratios (1:9, 1:8, 1:7, 1:6, 1:5, 1:4, 1:3, 1:2, 1:1) were then tested. The aqueous titration method was used to construct the pseudo-ternary phase diagrams, which involved the stepwise addition of water to each weight ratio of oil and S_{mix}, followed by a mixing stage with a vortex mixer at 25 °C. The pseudo-ternary diagrams were constructed with an axis representing the aqueous phase, another for the oil phase, and the final axis for a mixture of surfactant and co-surfactant at a fixed weight ratio (S_{mix}) (Tang et al., 2019).

2.3.5. Addition of propolis to blank ME formulation to produce the propolis filled ME formulation

To prevent potential toxicity caused by high surfactant concentration, an area with S_{mix} concentration < 50 % was identified from the pseudo-ternary phase diagrams. PE was then added to the blank ME to yield a final formulation of 300 mg PE/ml of ME, which was mixed overnight at 600 rpm.

2.4. Characterization of propolis filled ME

2.4.1. Thermodynamic stability testing

The propolis filled ME formulation was subjected to different thermodynamic stability tests to assess its physical stability.

2.4.1.1. Heating-cooling cycle. The selected formulation was subjected to six cycles of heating and cooling (4-45 °C) with storage at each temperature extreme lasting for at least 48 hours. The occurrence of phase separation, creaming, precipitation, and breaking were then visually observed and recorded.

2.4.1.2. Centrifugation test. The formulations were centrifuged at 3500 rpm for 30 min and were visually inspected for phase separation and precipitation.

2.4.1.3. Freeze-thaw cycle. Three freeze-thaw cycles were performed by storing the formulation at temperatures ranging between -20 °C and +25 °C, for a minimum of 48 hours at each temperature. The presence of phase separation, creaming, precipitation, and breaking was visually observed.

2.5. Physicochemical characterization

2.5.1. Droplet size

The droplet size of the ME was determined in triplicate by photon correlation spectroscopy using a Zetasizer 1000 HS (Malvern Instruments, Worcestershire, UK). Light scattering was monitored at 25 °C at a 90° angle.

2.5.2. Viscosity

The viscosity of the MEs was determined in triplicate by utilising a Brookfield R/S plus rheometer (Brookfield Engineering Laboratories Inc., Middleboro, MA) using a C50-1 spindle in triplicate at 25 °C.

2.5.3. pH

The pH of the formulations was measured in triplicate using a pH meter (Mettler Toledo MP 220, Greifensee, Switzerland) at 25 °C.

2.5.4. Total phenolic content

The total phenolic content (TPC) of propolis filled ME was determined using the Folin-Ciocalteau assay as adapted from Maroof et al. (2023) with some modifications. Briefly, gallic acid was used as a standard and total phenolics were expressed as mg/g gallic acid equivalents (GAE). Standard solutions of gallic acid (0.1-1.0 mg/ml) was prepared in ethanol. ME (1-10 mg/ml) was also prepared in ethanol. Subsequently, 50 µl of sample was added into a 96 well plate and 50 µl of Folin-Ciocalteu reagent (dissolved in water at 1:10). Then, 50 µl of 7.5 % sodium carbonate was added into the same well and mixed. Absorbance was read at 760 nm after 2 hours of incubation in the dark at room temperature.

2.5.5. Long-term and accelerated stability studies

The stability study was conducted following the International Council for Harmonisation (ICH) guidelines Q1A (R2), as described by Dongala et al. (2019). Samples of the optimized formulation were placed in a programmable environmental chamber for 12 months at 30 °C and 60 % relative humidity (RH) for ambient stability testing, and for 6 months at elevated temperatures [40 °C ± 2 °C and 75 % ± 5 % RH for accelerated stability studies]. Samples were withdrawn at 3-month intervals and monitored for changes in organoleptic properties, viscosity, and loss of phenolic content. To maintain humidity, saturated salt solutions of sodium bromide (for 60 % RH) and sodium chloride (for 75 % RH) were used. The percentage reduction in phenolic content was calculated by subtracting the phenolic content at each time point from the total phenolic content at the start of the trial (baseline).

2.5.6. Antioxidant activity by ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) radical scavenging assay

The ABTS assay procedure was based on Arnao et al. (2001), with some modifications. Stock solutions of ABTS (7 mM) and potassium persulfate solution (2.4 mM) were prepared. To prepare the working solution, the two stock solutions were mixed in equal quantities and allowed to react for 14 hours at room temperature in the dark. The resulting solution was then diluted with methanol to obtain an absorbance of 0.70 ± 0.01 units at 734 nm using a SpectraMax iD3 Multi-Mode microplate reader. A fresh ABTS solution was prepared each time the assay was run.

To determine the ABTS scavenging capacity of PEs and MEs (0.1 ml) (0.01-0.10 mg/ml), they were allowed to react with the ABTS solution (0.1 ml). After 7 minutes, the absorbance was measured at 734 nm using a SpectraMax iD3 Multi-Mode microplate reader. The ABTS scavenging capacity of the extract was compared with that of Trolox. Finally, the percentage inhibition was calculated using the following formula:

$$\text{The percentage ABTS of scavenging rate} = \frac{A0 - (A1 - A2)}{A0} \times 100$$

…where A0 is the absorbance of the blank (ABTS working solution + methanol), A1 is the absorbance of sample with ABTS working solution and A2 is the absorbance of sample in the absence of ABTS working solution. All determinations were performed in triplicate (n = 3).

2.6. Biological activity by testing antibacterial activity

Gram-positive *Staphylococcus aureus (S. aureus)* (ATCC 6538-P) and two gram-negative bacteria, *Escherichia coli (E. coli)* (ATCC 8739) and *Pseudomonas aeruginosa (P. aeruginosa)* (ATCC 9027), were selected based on their clinical and pharmacological importance. The antibacterial activity of PE and ME was investigated using the disk diffusion method. The bacterial stock cultures were incubated on Luria-bertani agar at 37 °C for 24 h before use. The cell suspension was adjusted with sterile saline solution to obtain turbidity values comparable to that of McFarland no. 0.5 standard (10^{10} cells/mL). The bacterial inoculum was uniformly spread on plates using a sterile L-shaped spreader. Samples were then dissolved in 70 % ethanol (EtOH) to obtain 100 mg/mL of test samples for antimicrobial analysis. Subsequently, 6-mm filter paper discs were impregnated with 20 µl of the test samples. The discs were allowed to remain at room temperature until complete diluent evaporation and kept under refrigeration (4 °C) until ready to be used. A commercial gentamicin (10 µg disc) for gram-negative bacteria and erythromycin (15 µg disc) for gram-positive bacteria were used as positive controls. For the negative controls, sterile commercial paper discs (6 mm diameter) were impregnated with 20 µL diluent (70 % EtOH), which was used to dilute microparticles and allowed to remain at room temperature until complete diluent evaporation and kept under refrigeration (4 °C) until ready to be used. The discs were loaded onto the surface of the agar plates. The zones of growth inhibition around the disks were measured after 24 hours of incubation at 37 °C. The assays were run in triplicate (Seibert et al., 2019).

2.7. Data analysis

The data were presented as the mean ± standard deviation of three independent tests and were analyzed using Microsoft Excel 2013. Statistical differences between groups were determined using either the t-test or analysis of variance (ANOVA) followed by the Bonferroni post-hoc multiple comparison test. A p-value of less than 0.05 was considered statistically significant for all tests.

3. Results

3.1. Formulation development of blank ME

3.1.1. Screening of oil

In comparison to olive oil, oleic acid has a greater solubility for propolis. Oleic acid was chosen as the oil phase of ME because it did not sediment even at concentrations greater than 0.5 g/ml, whereas PE's saturated solubility in olive oil was found to be 0.33 ±0.02 g/ml after which further addition caused PE sedimentation.

3.1.2. Screening of surfactants

Tween 20 and Kollihor RH exhibited the lowest solubilization capacity, with solubilization of 40 ± 8.00 µl and 24 ± 4.00 µl of oleic acid, respectively. In comparison, Tween 80, Kolliphor EL, and Labrasol demonstrated significantly higher solubilization capacities, reaching 100 ± 8.00 µl, 96 ± 4.00 µl, and 300 ± 4.00 µl, respectively.

3.1.3. Screening of co-surfactants

Propylene glycol was chosen as a co-surfactant since glycerol was immiscible with labrasol when mixed. The solution was left standing for 24 hours at room temperature.

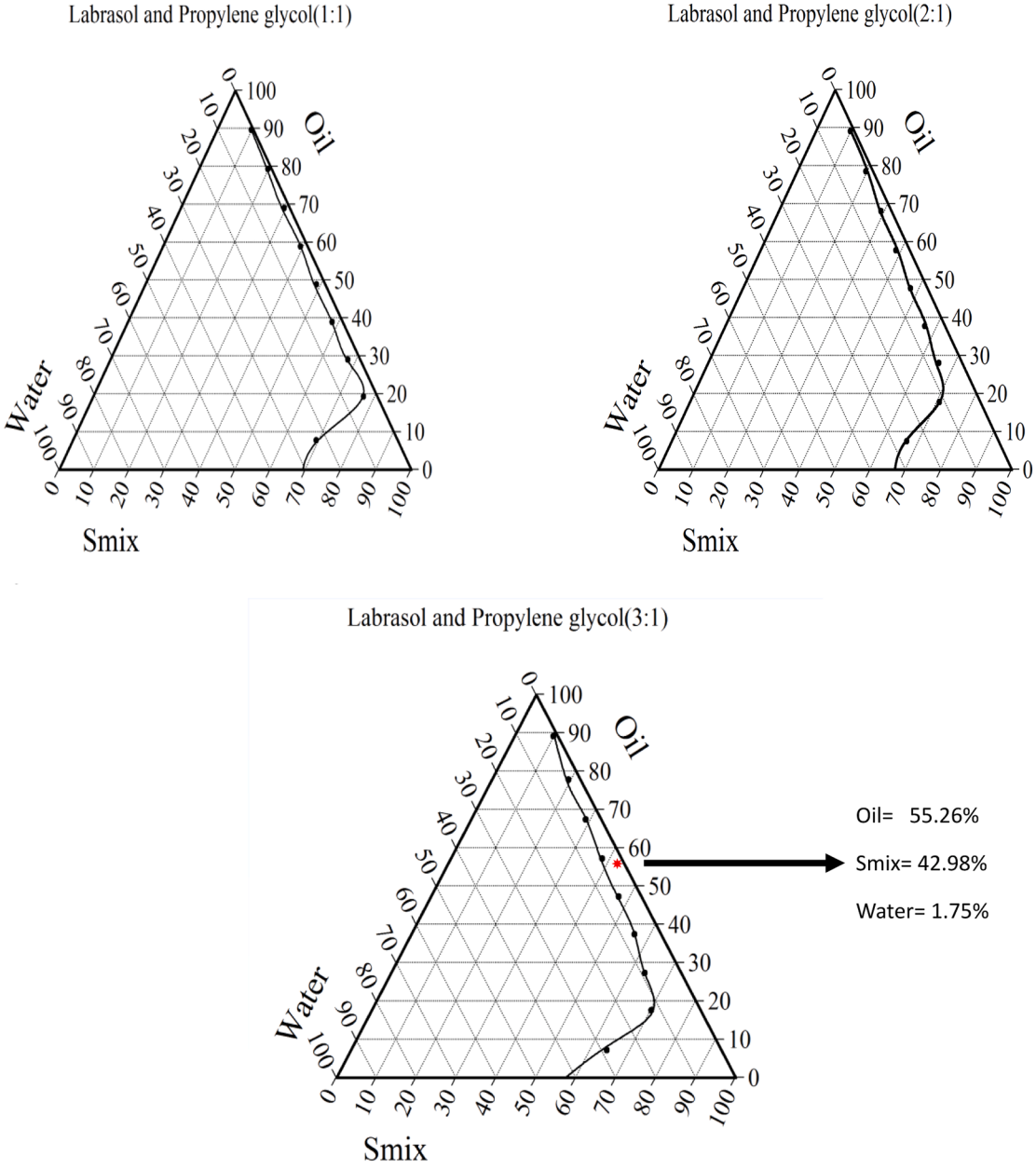

Fig. 1. Pseudo ternary phase diagrams of blank ME formed by different surfactant to cosurfactant ratio (Smix). The red dot located within the ME area on the diagram represents the chosen blank ME formulation. ME = microemulsion.

3.1.4. Pseudoternary phase diagrams and choice of formulation ingredient ratio for propolis-filled ME

Oleic acid served as the oil phase, labrasol and propylene glycol as S_{mix}, and water as the aqueous phase for constructing the ternary phase diagrams (Fig. 1).

Fig. 1 displays pseudo-ternary phase diagrams of the quaternary systems under investigation at 1:1, 2:1, and 3:1 S_{mix} values. The ratio of surfactant to co-surfactant that yielded the maximum area for ME was 3:1, hence deemed the optimal ratio. A region within this area was selected for generating the blank ME.

3.2. Formulation development and characterization of propolis filled ME

To prevent potential toxicity caused by high surfactant concentration, an area with S_{mix} concentration < 50 % was identified from the pseudo-ternary phase diagrams. The blank ME was composed of 55.26 % oil, 42.98 % S_{mix}, and 1.75 % water. Propolis was then added to the blank ME to yield a final formulation of 300 mg propolis/ml of ME, which was mixed overnight at 600 rpm.

3.3. Physicochemical characterization of propolis filled ME

3.3.1. Thermodynamic stability studies

The propolis-filled ME formulation exhibited good physical stability during thermodynamic stability testing, returning to its original state upon removal of stress. There was no indication of phase separation, turbidity, creaming, or cracking, suggesting that MEs possess good stability.

3.3.2. Droplet size, pH, viscosity and TPC

Blank ME displayed a clear and transparent appearance, while propolis-filled ME retained the golden brownish hue of the free PE, as observed through organoleptic assessment. Propolis filled ME's were found to have a droplet size of 239.2 ± 4.12, pH of 4.78 ± 0.18, viscosity of 0.149 ± 0.00, and TPC of 15.06 ± 1.86 mg/g GAE.

3.3.3. Long-term and accelerated stability studies

Throughout both long-term and accelerated stability studies, there was no significant variation in pH or viscosity (Table 1). The formulation retained uniformity during the entire study period. While the TPC count significantly decreased at the 12-month mark of long-term stability tests, no significant changes were observed during other trial periods and conditions.

3.3.4. Antioxidant activity

The ABTS antioxidant assay revealed that free PE exhibited significantly higher antioxidant activity in comparison to ME. The antioxidant activity decreased in the following order Trolox(0.039 ± 0.003^{c})> Free PE(0.048 ± 0.00^{b})> Propolis loaded ME(0.06 ± 0.00^{a}).

3.4. Biological characterization by antibacterial activity testing

Both free PE and ME showed no activity (inhibition zone = 0.0 ± 0.0 mm) against the tested gram-negative bacteria (Table 2). The antibacterial efficacy of the samples against *S. aureus* decreased in the following order: PE (20.00 ± 00) > ME (14 ± 1.00).

Table 2
Antibacterial activities of propolis extract and microemulsion formulation of propolis against *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa.*

Agent	Zone of inhibition (mm)		
	Staphylococcus aureus	*Escherichia coli*	*Pseudomonas aeruginosa*
Gentamicin (10 µg disc) for gram-negative bacteria and erythromycin (15 µg disc) for gram-positive bacteria	24.8 ± 0.72^{a}	24.2 ± 0.11^{a}	23.5 ± 0.98^{a}
Free PE (52.80 ± 4.29 mg GA /g of propolis sample)	20.00 ± 00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}
ME (15.06 ± 1.86 mg GA/g of ME sample)	13.96 ± 1.30^{c}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}

The data obtained were analysed statistically using one-way ANOVA followed by the Bonferroni post hoc multiple comparison test. For all tests, a value of <0.05 was considered significant. Different letters indicate significant differences between groups. ($n = 3$). PE= Propolis extract, ME= microemulsion.

4. Discussion

Food-grade MEs have emerged as a promising approach for delivering bioactive compounds. In this study, we first characterized and then developed a ME for the oral delivery of *Geniotrigona thoracica* propolis and assessed its physicochemical properties, antioxidant and antibacterial activity, and long-term stability. We previously characterized the extracted sample for its chemical composition, total phenols, flavonoids, antibacterial, and antioxidant activities in another study (Maroof et al., 2023).

4.1. Formulation development of blank ME

In formulating the ME, we screened a range of oils, surfactants, and co-surfactants to identify the optimal ingredients and their ideal ratio. Given the lipophilic nature of propolis, a high concentration of surfactant may be necessary for the ME, which could potentially lead to toxicity and irritancy issues in the body (Azeem et al., 2009). Therefore, careful selection of the surfactant type and optimization of its concentration are crucial.

The ability of a ME to maintain medication solubility is also influenced by the drug's solubility in the oil phase, particularly in oral administration. If a drug's solubilization capacity at the interface is enhanced by surfactants, dilution with the aqueous phase can lead to surfactant migration away from the interface, significantly reducing drug loading capacity and potentially causing precipitation in the gastrointestinal tract (Azeem et al., 2009). In our study, the solubility of propolis was much higher in oleic acid (a MCT) compared to olive oil (a LCT), consistent with previously published data on other lipophilic compounds like ezetimibe (Bandyopadhyay et al., 2012) and probucol

Table 1
Long term and accelerated stability test for propolis-loaded microemulsion.

Parameter	Time point (Months)						
	0	3		6		9	12
		Long-term	Accelerated	Long-term	Accelerated	Long-term	Long-term
Appearance	Uniform	Uniform	Uniform	Uniform	Uniform	Uniform	Uniform
pH	4.78 ± 0.18	4.58 ± 0.05	4.46 ± 0.19	4.53 ± 0.24	4.59 ± 0.31	4.63 ± 0.11	4.62 ± 0.00
Viscosity	0.149 ± 0.00	0.144 ± 0.00	0.143 ± 0.00	0.153 ± 0.00	0.145 ± 0.00	0.148 ± 0.00	0.148 ± 0.00
Phenolic retention (%)	-	95.28 ± 7.13	91.88 ± 9.77	88.83 ± 3.45	80.10 ± 9.32	79.98 ± 8.04	75.57 ± 6.76*

* Difference when compared to the value at baseline is significant at $p < 0.05$ ($n = 3$). ME= microemulsion.

(Christensen et al., 2004). This phenomenon is likely due to the shorter chain length and improved fluidity of MCTs (Bandyopadhyay et al., 2012).

After selecting oleic acid as the oil phase, the objective was to screen for surfactants with high solubilization capacity for the oil. In this study, five non-ionic surfactants (Labrasol, Cremophor EL, Kolliphor RH, Tween 20, and Tween 80) were screened, and their oil solubilization capacity was determined. Non-ionic surfactants were preferred as they are less affected by pH and ionic strength fluctuations, are generally safe, and are biocompatible. A surfactant solution diluted in water (1:10) was used as it was more selective in terms of oil solubilization (Azeem et al., 2009).

Although some studies (You et al., 2019; Koli et al., 2021), select surfactants based on a drug's solubility, we propose that solubilization of oil with the surfactant is equally important since a surfactant with good solubilizing power for propolis may not have a good affinity for the oil phase. Surfactants with high hydrophilic-lipophilic balance (HLB) values, such as Tween 20 (16.7) and Kolliphor RH (16.0), had the lowest solubilization capacity for oleic acid (40 μl and 24 μl, respectively). In contrast, Tween 80 (HLB: 15.0), Kolliphor EL (HLB: 13.5), and Labrasol (HLB: 11.0) had solubilization capacities of 100 μl, 96 μl, and 300 μl, respectively. Similarly, Mahdi et al. (2011) have previously documented the declining solubility trend with an increase in HLB value to create a similar pseudo-ternary phase diagram, albeit for palm kernel oil.

Single-chain surfactants alone are usually inadequate in reducing the oil/water (o/w) interfacial tension to a point where a ME can form (Callender & Wettig, 2021). To resolve this, co-surfactants can be used to improve the interfacial film flexibility and enable the oil to penetrate the interfacial region by altering the hydrocarbon tail mobility (Azeem et al., 2009). However, the compatibility of co-surfactants with the selected surfactant is important as poor mixtures of surfactant and co-surfactant may lead to phase separation (Garti et al., 2005). Thus, we optimized blends of oil, water and surfactant to co-surfactant ratio (S_{mix}), to create stable MEs which remain stable after propolis loading. In our study, we excluded glycerol due to its incompatibility with labrasol and selected PG as a co-surfactant.

To construct the pseudo-ternary diagram, we slowly titrated water with oil and S_{mix} in different ratios and visually observed for transparency or turbidity in the system. After vortexing the mixture, a clear and transparent mixture indicated a monophasic sample. Every composition of monophasic ME was marked as a point in the phase diagram, and the ME region covered the area. The surfactant to co-surfactant mass ratio is a crucial factor that determines the phase properties, such as the size and position of the ME region. Additionally, the type and concentration of the oil used also affect the phase properties (Shinde et al., 2018). Therefore, constructing phase diagrams is crucial in optimizing ME preparations, where the greater the ME area, the higher the ME's surfactant capacity.

S_{mix} 3:1 exhibited the maximum area as compared to the other ratios. This effect was attributed to differences in the packing of surfactant and co-surfactant at the o/w interface (Azeem et al., 2009). Subsequently, we determined the optimal mix of oil, S_{mix}, and water to obtain a stable ME based on the phase diagram.

4.2. Formulation and characterization of propolis filled ME

4.2.1. Formulation

To minimize the potential toxicity of using a high surfactant concentration, we chose an area with a concentration of S_{mix} less than 50 % from the pseudo-ternary phase diagram. The formulation ratio we chose was from the center of the ME region (Fig. 1), far from the boundary to provide some tolerance towards changes (e.g. water evaporation, oxidation of oil) induced during storage. Thus, we selected a blank ME composition of 55.26 % oil, 42.98 % S_{mix}, and 1.75 % water. The ME formed spontaneously without the aid of high shear equipment or significant heat input (heat and gentle mixing are required only if it is necessary to dissolve any of the ingredients), and its microstructures are independent of the order of addition of the excipients. Finally, we formed propolis-loaded MEs by adding 300 mg/mL propolis to the blank ME and mixing overnight at 600 rpm.

4.2.2. Physicochemical characterization

Stress testing is essential to rule out the potential development of metastable formulations. In this study, we tested the thermodynamic stability of a representative propolis-filled formulation using cooling cycles, freeze-thaw cycles, and centrifugation (Shinde et al., 2018; Zafar et al., 2017). We did not observe any phase separation, turbidity, creaming, or cracking. The heating-cooling cycle tested whether the formulation would withstand exposure to cold chain temperatures and extremely high temperatures during transportation, while the freeze-thaw cycle tested whether the formulation would return to its original state if kept in a freezer and then brought to room temperature.

The blank ME was observed to have a clear and transparent appearance, whereas the propolis-filled ME retained the characteristic golden brownish colour of free PE. Droplet size plays a crucial role in the stability and performance of MEs as it affects the kinetics and amount of propolis that is released and absorbed into the body. In order to achieve optimal absorption, smaller droplet sizes are preferred as they provide a greater interfacial area in contact with biological membranes (Tang et al., 2019). Our ME demonstrated an average droplet size of approximately 240 nm, which is consistent with other studies reporting particle sizes ranging from 5-250 nm (Tang et al., 2019; Ponce Ponte et al., 2022; Butt et al., 2018; Pineda-Reyes & Olvera, 2018).

A desirable attribute of a good pharmaceutical suspension is the ease with which it can be poured(Zafar et al., 2017). To achieve this, the flow time should be relatively short, and the corresponding apparent viscosity should be within acceptable limits (Owusu et al., 2021). Our ME exhibited a viscosity of 0.149 ± 0.00 Pa.s, which is within the acceptable viscosity range for pourable liquids (0.1-1.0 Pa.s) (Bandyopadhyay et al., 2012).

The pH of our ME formulation was found to be 4.78 ± 0.18, which falls within the normal pH range of marketed oral solutions (pH 2–9) (Attebäck et al., 2022; Ali et al., 2018).

To ensure the suitability of the developed MEs for use and determine the product's shelf life, stability tests are carried out. Unlike regular emulsions, MEs spontaneously form when the components are mixed in appropriate quantities without requiring additional mechanical energy. During stability studies, the MEs were characterized by visual inspection, determination of physicochemical properties, and phenolic content.

Throughout both accelerated and long-term stability studies, the ME's appearance remained constant. No evidence of phase separation, propolis sedimentation, cracking, or any other physical, pH, or viscosity changes were observed (Table 1). In most stability testing conditions, there was an insignificant decline in the total phenolic content, with the only exception being at the 12-month mark, indicating some phenolic activity losses at this period.

The antioxidant activity of the ME was measured using a radical scavenging assay i.e., ABTS. The ME showed lower antioxidant activity compared to free PE, which can be attributed to the lower concentration of phenolics in the ME (ME had a TPC of 15.06 ± 1.86 mg GA/g compared to PE's TPC of 52.80 ± 4.29 mg GA/g).

4.2.3. Biological characterization

In recent years, numerous studies have investigated the antibacterial activity of propolis, which can vary based on its botanical origin, geographic location, and season (Irigoiti et al., 2021). In this study, a gram-positive *S. aureus* (ATCC 6538-P) was chosen, along with gram-negative *E. coli* (ATCC 8739) and *P. aeruginosa* (ATCC 9027), to assess antibacterial activity. Both free PE and ME showed no activity (inhibition zone $= 0.0 \pm 0.0$ mm) against the gram-negative bacteria but were active against the gram-positive bacteria (Table 2). Given the

similarity in the antibacterial spectrum between propolis ME and its free form, it is plausible that the formulation successfully retained the antimicrobial activity present in propolis.

The greater antimicrobial activity of propolis against gram-positive bacteria as compared to gram-negative bacteria has been consistently observed across different regions (Przybyłek & Karpiński, 2019). This phenomenon may be attributed to the presence of the outer membrane, consisting of lipopolysaccharides in gram-negative bacteria, which can inhibit and/or retard the penetration of the antimicrobial components of propolis (Maroof et al., 2023). *S. aureus* susceptibility to propolis is significant for its use as a prophylactic health supplement and could determine the potential medical use of propolis in combination with certain antimicrobials for staphylococcal diseases (Seibert et al., 2019). Additionally, propolis is traditionally employed against throat and skin infections that are primarily caused by gram-positive bacteria.

5. Limitations and future perspectives

As reports suggest that propolis possesses beneficial antimicrobial and anticancer effects, we hope to expand our work to include biological testing for antiviral, antifungal, and anticancer activities. The matrix analysed exhibited hydrophobic characteristics, leading us to perform the ABTS assay. However, we did not complement it with a β-carotene bleaching assay or an oxygen radical absorbance capacity assay, both recognized as valid *in vitro* methods for testing antioxidant capacity. Future work should involve determining the minimum inhibitory concentration of antibacterial activity. Additionally, it is essential to investigate the improved stability and delivery of propolis during gastrointestinal digestion. This investigation should include evaluating individual compounds and comparing them against free (non-micro-encapsulated) PE. Lastly, exploring the formulation of propolis ME into other delivery methods, such as soft gel capsules and throat sprays, can facilitate ease of administration.

6. Conclusion

In this study, a novel ME of propolis has been successfully characterized and formulated for oral consumption, utilizing food and pharmaceutical grade ingredients. The newly created formulation demonstrated antioxidant and antibacterial activity and exhibited physical stability under long-term and accelerated stability conditions for up to one year. These findings suggests that this propolis ME has commercialization potential within the functional food and healthcare industry.

CRediT authorship contribution statement

Kashif Maroof: Conceptualization, Methodology, Data curation, Visualization, Investigation, Writing – original draft. **Ronald F.S. Lee:** Conceptualization, Supervision, Visualization, Investigation, Writing – review & editing. **Lee Fong Siow:** Supervision, Writing – review & editing. **Bey Hing Goh:** Conceptualization, Supervision, Writing – review & editing. **Ken Fong Chen:** Data curation, Methodology, Writing – review & editing. **Siew Hua Gan:** Conceptualization, Supervision, Visualization, Investigation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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