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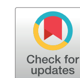
Research article

Physicochemical characteristics of liposome encapsulation of stingless bees' propolis



N.A. Ramli [a], N. Ali [b,c,*], S. Hamzah [b], N.I. Yatim [c]

[a] Faculty of Chemical & Process Engineering Technology, Universiti Malaysia Pahang, 26300, Kuantan, Pahang, Malaysia
[b] Faculty of Ocean Engineering Technology and Informatics, Universiti Malaysia Terengganu, 21030, Kuala Nerus, Terengganu, Malaysia
[c] Higher Institution Centre of Excellence (HICoE), Institute of Tropical Aquaculture and Fisheries, Universiti Malaysia Terengganu, 21030, Kuala Nerus, Terengganu, Malaysia

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ABSTRACT

Nutraceuticals from natural sources have shown potential new leads in functional food products. Despite a broad range of health-promoting effects, these compounds are easily oxidized and unstable, making their utilization as nutraceutical ingredients limited. In this study, the encapsulated stingless bees' propolis in liposome was prepared using soy phosphatidylcholine and cholesterol by thin-film hydration technique. Three different formulations of phosphatidylcholine composition and cholesterol prepared by weight ratio was conducted to extract high propolis encapsulation. Physicochemical changes in the result of the encapsulation process are briefly discussed using scanning electron microscopy and Fourier Transform Infrared Spectroscopy. A dynamic light-scattering instrument was used to measure the hydrodynamic diameter, polydispersity index, and zeta potential. The increment of the liposomal size was observed when the concentration of extract loaded increased. In comparing three formulations, F2 (8:1 w/w) presented the best formulation as it yielded small nanoparticles of 275.9 nm with high encapsulation efficiency (66.9%). F1 (6:1 w/w) formed large particles of liposomes with 422.8 nm, while F3 (10:1 w/w) showed low encapsulation efficiency with (by) 38.7%. The liposome encapsulation will provide an effective nanocarrier system to protect and deliver the flavonoids extracted from stingless bees' propolis.

1. Introduction

Propolis is a natural product from bees' hives that has a resinous consistency and is highly variable in terms of physical appearance and color, depending on the botanical, geographical origin, and time of its collection [1, 2]. In general, propolis is composed of 50–60% resins, 30–40% waxes and fatty acids, 5–10% essential oils, 5% pollens and microelements [3]. Propolis contains minerals and phenolic compounds such as flavonoids aglycones, phenolic acids and their esters, phenolic aldehydes, alcohols, ketones, steroids, terpenes, sugars, and amino acids [4, 5]. Among them, flavonoid, together with phenolic compounds, are reported to contribute greatly to biological and pharmacological activities compared to the other propolis constituents [6], and it is proposed as a good marker of propolis quality [7]. The nutritionist has highlighted dietary flavonoids as a novel bioactive medicinal agent with various therapeutic properties for human well-being, such as antioxidant, anti-inflammatory, anticancer, and antimicrobial activities [8, 9].

However, in Malaysia, there is a lack of experimental research on the nutraceutical of stingless bees propolis, locally known as 'lebah kelulut' [10]. Untapped propolis produced by stingless bees are usually regarded as waste by traditional beekeepers in Malaysia. Previous nutraceutical experiments are more likely to rely on the propolis of honey bees than on stingless bees. A substantial gap in research publications on stingless bee products has been identified relative to the common honey bee products that are well-studied. In our previous study, it was found that sticky propolis produced by *Heterotrigona itama* (*H. Itama*) stingless bees' species showed a higher composition of TFC (91%) and TPC (49%) compared to the hard propolis, which resulted in the highest antioxidant activities compared with the other nine species of stingless bees in the same origin which was 85% of DPPH degradation [11]. Based on this finding, propolis from stingless bees of *H. Itama* species is chosen to be extracted for liposome encapsulation.

Flavonoid is a ubiquitously dispersed group of bioactive compounds in the plant kingdom, including the propolis. The flavonoid, one of the most potent compounds, has limited its stability and bioavailability [12].

* Corresponding author.
E-mail address: noraaini@umt.edu.my (N. Ali).

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Flavonoid stabilization is a crucial parameter for effective human administration, as it is susceptible to oxygen, sun, heat, and water [13, 14]. As a result, these factors reduce the shelf life of food items. Besides, the flavonoid is vulnerable to low bioavailability, which requires accelerated body clearing, low aqueous solubility, and slow oral absorption [15]. These disadvantages would result in a lack of potency of bioactive compounds and decreased medicinal benefits. Therefore, attempts to increase bioactive compounds' durability and bioavailability have continuously been pursued to address these constraints.

Nanotechnology is an adequate tool to obtain low bioavailability of nutraceutical delivery system [16, 17]. One of the new technologies designed to avoid bioactive compounds' degradation while retaining its functional characteristics, is encapsulation. Encapsulation allows the defence of a wide variety of bioactive substances by embedding them in a protective carrier matrix. Not just for safety, nanotechnology developments will increase the bioavailability of bioactive substances by reducing the scale of nano-size particles to prevent the risk of accelerated clearance in the body [10]. In this respect, liposomes could be a viable encapsulation mechanism that could solve the issues on bioactive flavonoids and phenolic compounds as mentioned above. The liposome is a colloidal, vesicular structure dependent on bilayer phospholipids [18], and can entrap bioactive compounds in its protective matrix.

This research describes an approach used to protect flavonoids present in propolis, through its encapsulation in liposomes. A carefully engineered nanocarrier may significantly accelerate nutraceutical distribution, and expand the spectrum of potential therapeutic and medicinal applications. By integrating the characteristics of liposomes, a high encapsulation performance and loading capability with enhanced physical stability can be achieved in the manufacturing of nutraceutical carriers. The actual challenge lies in the formulation and preparation condition to obtain the required physicochemical characteristics, for a high encapsulation efficiency of the compound's desired target. However, methods for synthesizing liposomes are still under research development to enhance the collective interrelated effects of liposomes' physicochemical stabilities. In this context, soy phosphatidylcholine's effect on cholesterol ratio and concentration of propolis extract loaded, was studied to obtain the best liposome formulation for a high propolis extract capture. This study found the characterization and assessment of three liposomal formulations, particularly liposomes' encapsulation performance. The liposome formulations produced are compared to their performance with average scale reference values, polydispersity index, zeta potential, and encapsulation efficiency.

2. Material and methods

2.1. Material

Soy lecithin L-α-phosphatidylcholine from soybean with ≥99% (TLC) in lyophilized powder and phosphate buffer saline (PBS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Aluminium chloride, acetone, chloroform, and methanol from Merck (Darmstadt, Germany). The quercetin used is HPLC grade (purity ≥95%), and cholesterol obtained from sheep's wool (purity ≥99%). All the solvents used are analytical grade.

2.2. Preparation of stingless bees' propolis extract

The propolis sample was collected from stingless bees' colonies of *Heterotrigona itama* (*H. Itama*) stingless bees' species located in a rubber smallholding plantation, in Kuala Nerus District, Terengganu, Malaysia. The dry sample of propolis was ground and soaked in acetone overnight at room temperature. The sample was percolated gradually for its filtrate and the remaining residue was extracted with 70% methanol. This step was repeated until the filtrate became colorless. The methanol was removed in a rotary evaporator at 45 °C, providing the dry methanolic extract of propolis. The extract was kept in a desiccator for further drying before being preserved at 4 °C before usage.

2.3. Preparation of propolis extract-loaded liposomes (PEL)

A thin-film hydration technique was used to synthesize PEL [19]. The compositions of phosphatidylcholine and cholesterol were prepared by weight ratio (8:1, 6:1, and 10:1 w/w). These formulations were prepared as in Table 1. The phosphatidylcholine, cholesterol, and propolis extract were mixed in 10 ml of methanol: chloroform (1:1 v/v) solvent. The solvent was then evaporated by a rotary evaporator with a temperature setting condition at 45 °C. Finally, a dry lipid film formed in the round bottom flask will be dissolved using 10 ml PBS solution. This liposomal suspension was stirred continuously at 40 °C, above its phase transition temperature (T_c) for 30 min. Subsequently, the liposome suspension was sonicated using a QSonica (Q700, USA) ultrasonicator, with an amplitude of 40% and a comprehensive treatment of 25 min. The flask was plunged into a cold-water bath to prevent the raising of temperature of samples. A pulsed duty cycle was fixed for all treatments with 8s on and 2s off. After preparation, the liposome suspension was centrifuged (15000 rpm, 4 °C) for 30 min. The solvent containing the free unentrapped extract in the suspended stage was separated. Then, the extracted liposomes were cleaned using 2 ml PBS solution, recentrifuged and resuspended, to obtain 2 ml of liposome suspension before being homogenized [20].

2.4. Physicochemical characterization

The surface characteristic of liposomes observed using scanning electron microscopy (SEM) (JEOL 6360 LA, Tokyo, Japan). The freeze-dried PEL samples were mounted onto adhesive-taped stubs and sputter coated with gold film by an automated coater (JFC 11600), to prevent any charging up to the surface by the electron beam. A dynamic light-scattering instrument (Zetasizer ZS, Malvern Instruments, Worcestershire, UK) was used to quantify the hydrodynamic diameter (Z-average), polydispersity index (PDI), and zeta potential. The liposome suspensions were diluted with distilled water to a 3.0% v/v to avoid multiple scattering effects. The samples were then transferred into an electrophoresis cuvette and repeated for triplicate. The spectra of PEL were analyzed using a spectrophotometer (UV-18000 Shimadzu, Kyoto, Japan). Samples were measured in a rectangular quartz cuvette with 1 cm path length at 25 °C. Fourier Transform Infrared Spectroscopy (FT-IR) analysis was used for the chemical composition analysis using an FT-IR spectrometer (Spectrum 100 PerkinElmer, Boston, USA). The sample was deposited in spectroscopic grade potassium bromide (KBr) disks.

2.5. Performance assessment of propolis extract-loaded liposome

The evaluation of the encapsulation and loading efficiency of the liposomes was done using ultracentrifugation method [21]. The encapsulation efficiency is evaluated by taking the centrifuged liposome's supernatant, and measured using UV-vis at 420 nm of wavelength. The

Table 1. Formulation for liposome suspension at different weight ratio.

Formulation	Weight ratio (w/w)	Weight of Phosphatidylcholine (mg)	Weight of Cholesterol (mg)
F1	6:1	60	10
F2	8:1	80	10
F3	10:1	100	10

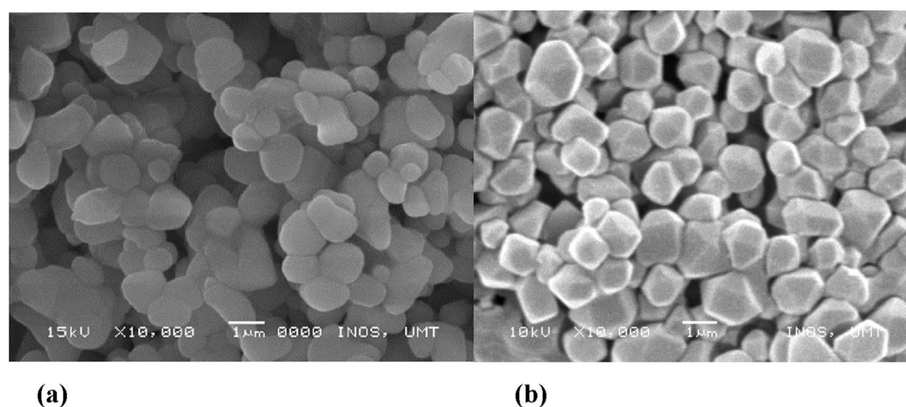

Figure 1. Scanning electron microscopy images of free liposomes (FL) (a) and propolis extract-loaded liposomes (PEL) (b) with magnification of x 10,000 (scale 1 μm).

extract was assayed by total flavonoid content (TFC), and was expressed as quercetin equivalence (QE) in propolis extract weight. A calibration curve of Absorbance (420 nm) *versus* Quercetin (μg/mL) in methanol was constructed and used to determine the total flavonoids content. The encapsulation efficiency and loading were measured as a percentage of flavonoids entrapped in the liposomes by means of Eqs. (1) and (2) used by Wallace et al. [21]:

$$\text{Encapsulation efficiency } (\%) = (W_{total} - W_{free}) \text{ x } 100/W_{total} \quad (1)$$

$$\text{Loading capacity } (\%) = (W_{total} - W_{free}) \text{ x } 100/W_{np} \quad (2)$$

Where W_{total} was the total weight of flavonoids in liposome suspension (mg), W_{free} was the weight of free flavonoids in liposome suspension (mg), and W_{np} represented the liposome weight (mg).

3. Results and discussion

3.1. Morphology

The suspensions of free liposomes (FL) and PEL were freeze-dried before observation under SEM to examine these samples' morphology in solid-state. The microscopy images of nanosized liposomes with an almost spherical shape are presented in Figure 1. The particles were well-distributed with homogenous size. These images prove that both liposomes were successfully isolated. From these images, the particle surface of FL appears smoother than PEL, due to the propolis extract incorporated at the outer surface of the liposomes.

The UV-Vis spectrum of studied propolis extract presented an intense absorption band at 280 nm (Figure 2.). This absorption band was attributed to the presence of an A-ring of flavonoids [22]. The FL spectrum displayed no apparent peak of absorption. A similar absorption peak was observed after the encapsulation of extract, and it exhibited Einstein's shift softly, due to the existence of other materials composed in the suspension, confirming the entrapment of the propolis extract in the liposomes.

The potential chemical interaction between propolis extract and liposomes was evidenced by the FTIR spectral data as in Figure 3. The absorption at 3404 cm^{-1} correspondents to hydroxyl groups (O–H), was attributed to the presence of flavonoid and phenolic compounds in the propolis extract [23, 24]. The bands at 2927 cm^{-1} and 2864 cm^{-1} were corresponded to the symmetrical stretches of CH_3 and asymmetrical stretches of CH_2, respectively. These absorptions bands were correlated - or associated - with waxes residues of the methanolic extract [25]. The absorptions bands at 1701 cm^{-1} (C=O), 1635 cm^{-1} (C=C), 1454 cm^{-1} (aromatic C–H) and at 1377 cm^{-1} (aromatic C–O), were associated with the presence of flavonoids. The profile of infrared spectrum obtained for the propolis extract studied, was similar to the previous data published for propolis from other origins [26, 27].

Compared to PEL, the functional group of O–H found at 3323 cm^{-1}, and C=O stretching at 1735 cm^{-1} became broader and almost vanished after the addition of propolis extract. This phenomenon has shown that propolis extract is entrapped within the liposome bilayer, possibly through hydrophobic interactions and hydrogen bonding [28, 29]. The propolis extract's trapping into the liposome phospholipid bilayer was due to hydrophobic interactions between the aromatic rings of propolis flavonoid with phospholipid acyl chains [28]. In addition, hydrogen

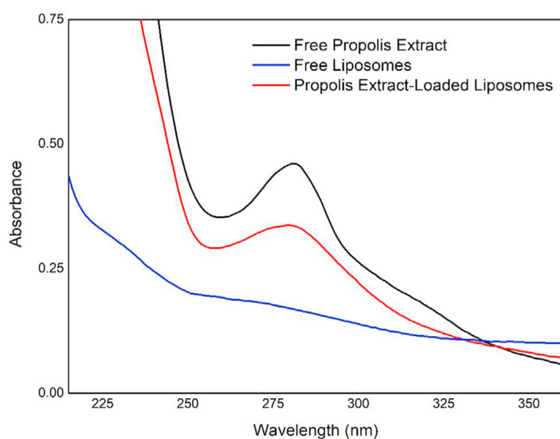

Figure 2. UV-Vis spectra of free propolis extract, free liposomes and propolis extract-loaded liposomes.

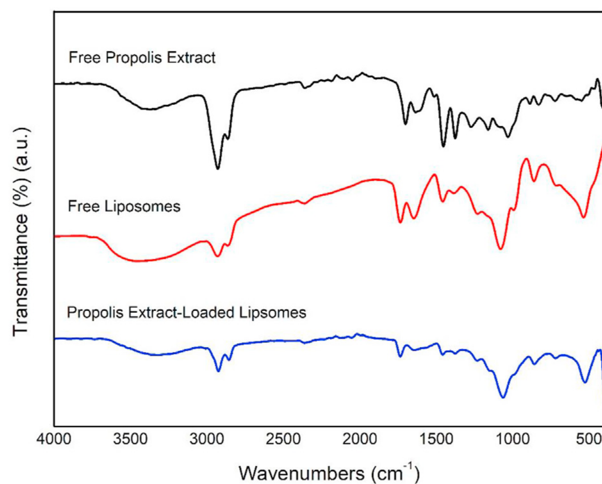

Figure 3. FTIR spectra of free propolis extract, free liposomes and propolis extract-loaded liposomes.

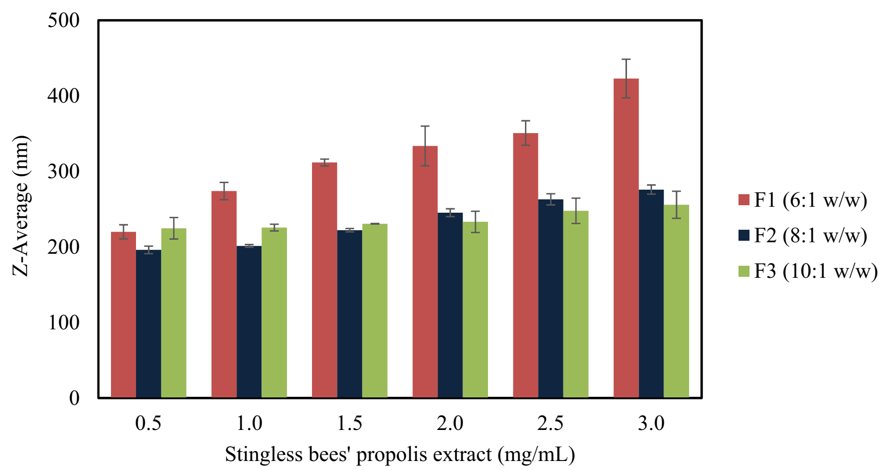

Figure 4. Effect of Stingless bees' extract concentration and liposome formulation on the particle size of propolis extract-loaded liposomes. Soy phosphatidylcholine:cholesterol (6:1 w/w) = F1, (8:1 w/w) = F2, (10:1 w/w) = F3.

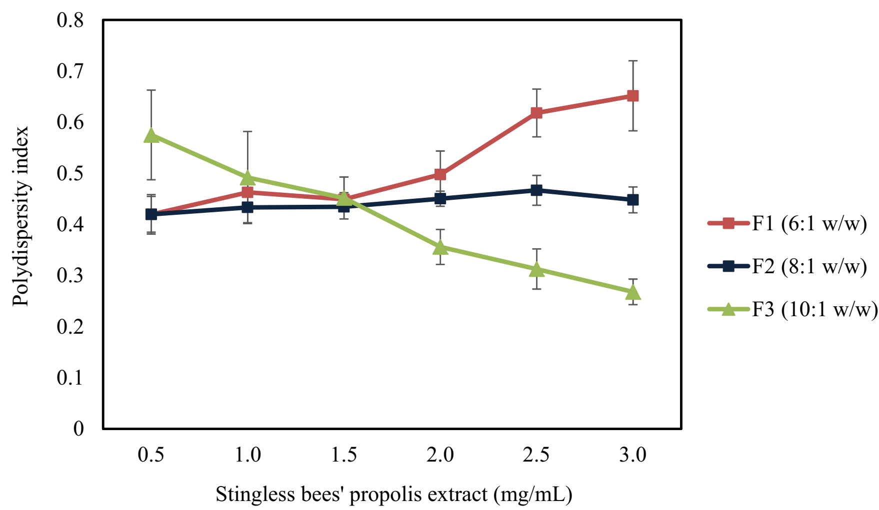

Figure 5. Effect of Stingless bees' extract concentration and liposome formulation on the polydispersity index of propolis extract-loaded liposomes. Soy phosphatidylcholine:cholesterol (6:1 w/w) = F1, (8:1 w/w) = F2, (10:1 w/w) = F3.

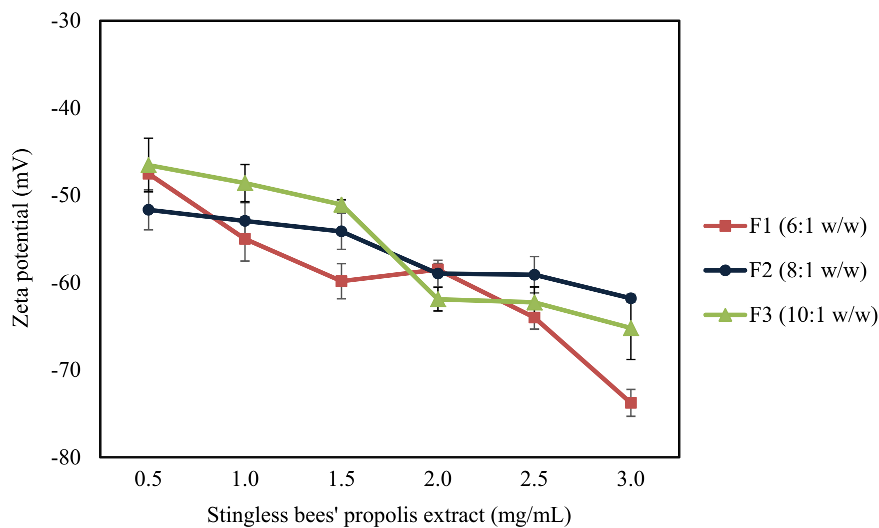

Figure 6. Effect of extract concentration and liposome formulation on zeta potential of propolis extract-loaded liposomes. Soy phosphatidylcholine:cholesterol (6:1 w/w) = F1, (8:1 w/w) = F2, (10:1 w/w) = F3.

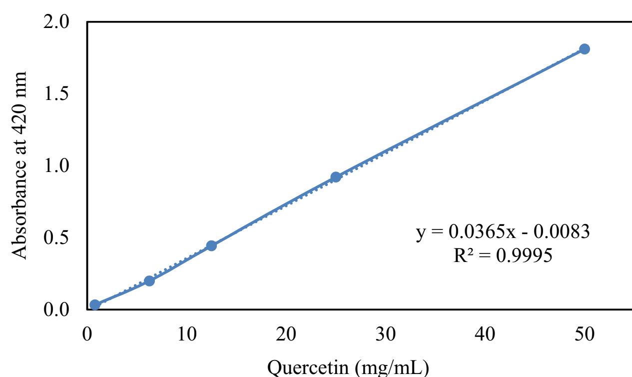

Figure 7. Calibration curve of absorbance at 420 nm *versus* quercetin (mg/mL) in methanol.

bonding also occurred between the phospholipid bilayer's polar head groups and the propolis extract's flavonoids, making it possible to introduce propolis extract on the liposomes' outer surface. This finding is also related to the image of SEM depicting the rougher surface of PEL relative to the unloaded liposomes.

3.2. Effect of extract concentration and liposome formulation on PEL

In this study, three formulations were analyzed to evaluate the ratio of phosphatidylcholine to cholesterol. Incorporating cholesterol as an essential component in the lipid membrane is a well-established strategy to impart better liposome stability. It also aims to increase the rigidity of the phospholipid bilayer and control its permeability [30]. This approach leads to higher vesicle integrity for the fluid formulation, thus will increase the efficiency of liposomes on encapsulation [31].

An increasing trend of average particle size for all formulations was observed after being encapsulated with increasing concentration of stingless bees' extract (Figure 4). The figure demonstrates that size increases as the concentration of the extract increased. An increasing amount of hydrophobic propolis molecules entrapped in the phospholipid bilayer gave rise to the liposome size, significantly for F1. A previous study reported the increase in particle size of liposomes loaded with green tea extract, which is higher than the unloaded liposomes [18]. The maximum concentration of extract that could be incorporated was 3.0 mg/ml for all those formulations. The instability of liposome suspension was observed when the concentration of the extract was added in excess. Large particles' aggregation was formed right after the preparation, showing that excess extract could not be entrapped more in the liposomes.

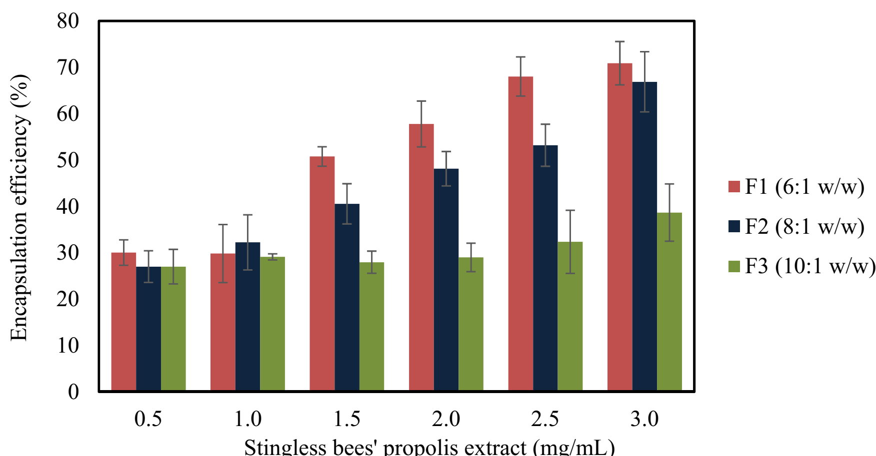

Figure 8. Effect of extract concentration and liposome formulation on encapsulation efficiency of propolis extract-loaded liposomes. Soy phosphatidylcholine:cholesterol (6:1 w/w) = F1, (8:1 w/w) = F2, (10:1 w/w) = F3.

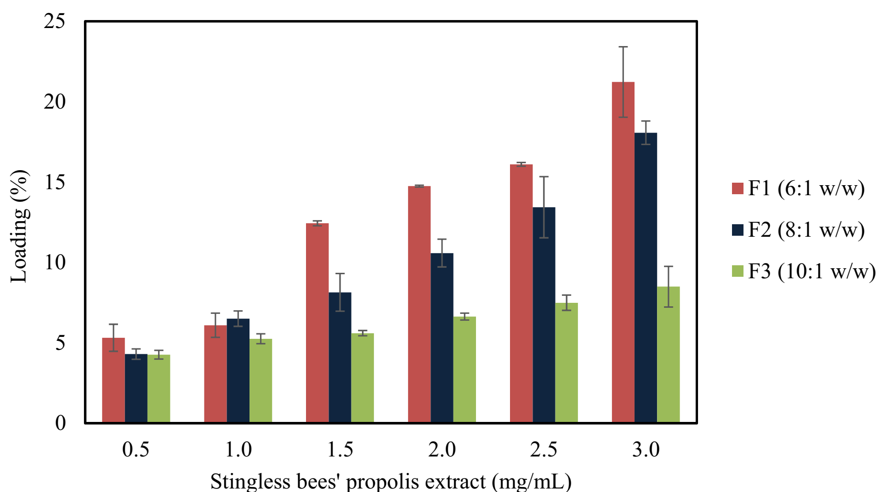

Figure 9. Effect of extract concentration and liposome formulation on the loading capacity of propolis extract-loaded liposomes. Soy phosphatidylcholine:cholesterol (6:1 w/w) = F1, (8:1 w/w) = F2, (10:1 w/w) = F3.

The most significant increment was in F1, with a vesicle size change rate of 92%. The possible reason for this result might be due to the loosening effect of the phospholipid membrane. A low amount of phosphatidylcholine in the formulation led to an increment in the bilayer's permeability, resulting in the increment of particle size after loaded with propolis extract. In general, nanocarriers should be controlled in the small particle size range to prevent mononuclear phagocyte recognition and clearance by the reticuloendothelial system [30]. According to Li et al. (2016), liposomes with particle size of 100 nm–300 nm have excellent potency to transport the bioactive agents between and into cell membranes [32]. Conversely, particles larger than 500 nm are reported to be more likely excreted before reaching the bloodstream [33]. Thus, this formulation was discarded since it showed unfavorable characteristics of nanocarrier in terms of particle size.

On the other hand, the size for F2 and F3 at the highest concentration of the extract was slightly smaller than F1, with 275.9 nm and 255.8 nm, respectively. Particles of small size ranges have a comparatively high cell uptake with faster drug release and a longer half-life in the blood than larger particles. It also recorded that the penetration of smaller sized nanoparticles through the rat intestinal loop's submucosal layers was more effective than the larger particles that were primarily localized in the epithelial lining [34]. In this regard, F2 and F3 will be further assessed.

The difference in the PDI index exerted by the formulations is represented in Figure 5. At the highest concentration of propolis extract, F1 displayed the highest PDI index of 0.65. It indicates a polydisperse particulate system due to large particle size liposomes, as mentioned above. Any of the small-sized liposomes were partly attached to larger liposomes, due to loosening effect of the liposome membrane, which was prepared with lower phosphatidylcholine amount, resulting in a particle size non-regularity. The smaller size of liposomes produced in the other two formulations resulted in more excellent dispersion with a lower PDI index. F3 displayed the lowest PDI index among others with 0.26, suggesting that the most uniform liposomes scale was synthesized in the formulation. The dispersion of liposome was reduced to a lower index attributed to the tightly packed membrane with highest amount of phosphatidylcholine, thus the agglomeration of particles was avoided.

On top of that, zeta potential was measured to dictate the pharmacokinetic profiles and the fate of liposome stability. According to Figure 6, it can be observed that there was an inverse relation between zeta potential and propolis extract concentration. As the concentration of propolis extract increased, the zeta potential of all formulations reduced. This impact on the pattern could be related to the molecular association between the phospholipids and the entrapped propolis extract, which altered the significant charge at the liposomes' outer surface. In addition, the presence of propolis extract particles on the outer surface of liposomes, as seen in the scanning electron microscopy images, could also influence the calculation of zeta potential. However, all formulations prepared had a high magnitude of zeta potential (>30 mV), which led to a tremendous electrostatic repulsion between the particles, thus preventing agglomeration between the particles [35].

Effectiveness in encapsulation is an ultimate issue in the case of applying liposome as a nanocarrier. The calibration curve of quercetin in methanol with $R^2 = 0.997$ was constructed (Figure 7) to calculate the encapsulation efficiency and loading based on the total flavonoids content. Figure 8 presented below showed an increasing trend of encapsulation efficiency for all formulations with the increasing propolis extract concentration. This increasing trend was similar to the loading, as shown in Figure 9. At maximum concentration of propolis extract, F1 and F2 presented significantly higher encapsulation efficiency and loading than F3. The low encapsulation efficiency of F3 was probably attributed to the small size of the particle and the compact composition of hydrophobic molecules, including cholesterol in the liposomal structure. Limited space in the phospholipid bilayer, cholesterol, and propolis extract tend to compete to be aligned within the packing of the alkyl chain of the phospholipid [31]. Bozzuto and Molinari [30] also mentioned the reduced size of liposomes, leading to reduced drug storage capacity. These factors resulted in the reduction of encapsulation efficiency with the increasing amount of phosphatidylcholine.

In comparison between the other two formulations, F1 presented a slightly higher encapsulation efficiency and loading than F2. This is due to the larger size of particles yielded by the formulation. Higher fluidity of the phospholipid in F1 contributed to a greater propolis extract capacity loaded into the bilayer. However, the smaller size of liposomes is more favorable compared to the larger particles, as discussed earlier. Thus, to choose the best formulation for synthesizing propolis-extract loaded liposomes, F2 was selected as the optimum formulation compared to the others. It represented a small particle size, which was 275.9 nm, with high encapsulation efficiency and loading, 66.9% and 18.1%, respectively. Herein, a significant effect of the ratio of phosphatidylcholine to cholesterol, on the physicochemical characteristics and encapsulation efficiency of a liposomal system was proven.

4. Conclusion

The encapsulation of stingless bees' propolis extract in liposomes was proven to occur through intermolecular hydrogen bonding and hydrophobic interaction between phospholipid membrane and propolis extract, as observed in FTIR analysis. Three formulations were prepared, and the liposomes were able to incorporate propolis extract up to 3.0 mg/mL with increasing encapsulation efficiency and loading capacity, as well as the liposome size. At 3.0 mg/mL, F2 (8:1 w/w) presented high encapsulation efficiency (66.9%) and loading capacity (18.1%) while maintaining its small liposome size at 275.9 nm. This formulation is considered the best among others since F1 (6:1 w/w) formed the largest size of liposome (422.8 nm), which are likely to be excreted from the body before reaching the bloodstream. Meanwhile, F3 (10:1 w/w) showed the lowest encapsulation efficiency (38.7%) and loading capacity (8.5%), which limits the transportation of beneficial active compound into the body. Liposome encapsulation of propolis flavonoid synthesized in this study could be a suitable delivery system of functional foods for human health.

Declarations

Author contribution statement

N. A. Ramli: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

N. Ali, S. Hamzah: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

N. I. Yatim: Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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