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Original Article

Anti-inflammatory activity of phytosomal ethanolic extract of propolis (*Tetragonula* sp.) in a rat model of carrageenan-induced edema

[Actividad antiinflamatoria del extracto etanólico fitosomal de propóleo (*Tetragonula* sp.) en un modelo de edema inducido por carragenina en ratas]

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Abstract

Context: Propolis from *Tetragonula* sp. has demonstrated anti-inflammatory effects in studies. However, the limited absorption and permeability of flavonoids reduce their bioavailability and therapeutic use. Phytosomes, as nanocarriers, offer a promising way to improve the bioavailability of flavonoids.

Aims: To develop a phytosomal gel incorporating ethanolic extract of *Tetragonula* sp. propolis (EEP) and to assess its anti-inflammatory efficacy utilizing a rat model of carrageenan-induced paw inflammation edema.

Methods: Propolis was extracted via maceration utilizing 70% ethanol and subsequently evaluated for total flavonoid content. Phytosomes of EEP were prepared using the antisolvent precipitation method with varying propolis-to-phospholipid weight ratios (1:1, 1:2, 1:3, and 2:1). The optimal formulation, determined by entrapment efficiency, was characterized using FTIR, TEM, and PSA techniques. The phytosomal formulation was then incorporated into a gel matrix. *In vitro* permeation assessments were performed utilizing a Franz diffusion cell. The anti-inflammatory effectiveness was assessed in a rat model of carrageenan-induced paw edema, with six rats per treatment group (n = 6). Data were statistically analyzed using One-way ANOVA followed by Tukey's post-hoc test ($p<0.05$) via SPSS 19.0 software.

Results: The highest entrapment efficiency (89.62 ± 0.79%) was observed at a 1:3 extract-phospholipid ratio. The optimized phytosomes demonstrated a particle size of 161.90 ± 2.00 nm, with a polydispersity index of 0.389 ± 0.023, and a spherical shape. The phytosomal gel achieved a high cumulative permeation of 90.751 ± 1.000% within 180 minutes. Furthermore, *in vivo* studies revealed that the phytosomal gel produced a significantly greater inhibition of paw edema ($p<0.05$) than both the non-phytosomal propolis gel and the diclofenac sodium gel.

Conclusions: The phytosomal gel of ethanolic propolis extract significantly enhanced anti-inflammatory activity, demonstrating its potential as an effective topical delivery system for natural flavonoid-based therapies.

Keywords: anti-inflammatory activity; phytosome; propolis; *Tetragonula* sp.; flavonoids; carrageenan-induced edema.

Resumen

Contexto: Propóleo de *Tetragonula* sp. ha demostrado efectos antiinflamatorios. Sin embargo, la limitada absorción y permeabilidad de los flavonoides reducen su biodisponibilidad y su uso terapéutico. Los fitosomas, como nanoportadores, ofrecen una forma prometedora de mejorar la biodisponibilidad de los flavonoides.

Objetivos: Desarrollar un gel fitosomal que incorpore extracto etanólico de propóleo de *Tetragonula* sp. (EEP) y evaluar su eficacia antiinflamatoria mediante un modelo de edema de la pata inducido por carragenina en rata.

Métodos: El propóleo se extrajo mediante maceración con etanol al 70% y, posteriormente, se evaluó el contenido total de flavonoides. Los fitosomas de EEP se prepararon empleando el método de precipitación con antisolvente, con diferentes proporciones en peso de propóleo a fosfolípidos (1:1, 1:2, 1:3 y 2:1). La formulación óptima, determinada en función de la eficiencia de atrapamiento, se caracterizó mediante técnicas de FTIR, TEM y PSA. Luego se incorporó la formulación fitosomal a una matriz de gel. Las evaluaciones de permeación *in vitro* se realizaron en una celda de difusión de Franz. La eficacia antiinflamatoria se evaluó en un modelo de rata con edema de pata inducido por carragenina, con seis ratas por grupo de tratamiento (n = 6). Los datos se analizaron estadísticamente mediante ANOVA unidireccional, seguida de la prueba post hoc de Tukey ($p<0,05$), con el software SPSS 19.0.

Resultados: La mayor eficiencia de atrapamiento (89,62 ± 0,79%) se observó con una relación extracto-fosfolípido de 1:3. Los fitosomas optimizados presentaron un tamaño de partícula de 161,90 ± 2,00 nm, un índice de polidispersidad de 0,389 ± 0,023 y una forma esférica. El gel fitosomal logró una alta permeación acumulada de 90,751 ± 1,000% en 180 minutos. Además, los estudios in vivo revelaron que el gel fitosomal produjo una inhibición significativamente mayor del edema de la pata ($p<0,05$) que el gel de propóleo no fitosomal y el gel de diclofenaco sódico.

Conclusiones: El gel fitosomal de extracto etanólico de propóleo mejoró significativamente la actividad antiinflamatoria, lo que demuestra su potencial como sistema de administración tópica eficaz para terapias naturales basadas en flavonoides.

Palabras Clave: actividad antiinflamatoria; edema inducido por carragenina; fitosoma; flavonoides; propóleos; *Tetragónula* sp.

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INTRODUCTION

Propolis is a bioactive resin-like substance produced by bees, including stingless species such as *Tetragonula* sp., which are widely distributed in Southeast Asia, and has been used in traditional medicine for its antioxidant, antimicrobial, and anti-inflammatory activities (Paula et al., 2023; Przybyłek & Karpiński, 2019; Sahlan et al., 2021).

In Indonesia, stingless bees such as *T. sapiens*, *T. clipearis*, and *T. fuscobalteata* are known to produce propolis with distinct physical and chemical characteristics compared with those of the genus *Apis* (Trianto et al., 2023). The propolis obtained from these species tends to be more viscous and requires specialized extraction techniques. Among its active constituents, flavonoids are particularly significant due to their anti-inflammatory potential. However, flavonoids exhibit limited stability and bioavailability, primarily due to their poor solubility and inadequate permeability across biological membranes (Boufadi et al., 2021; Chittasupho et al., 2023; Costa et al., 2021).

To address these limitations, drug delivery systems such as phytosomes have been investigated. Phytosomes are composed of phospholipid complexes designed to enhance the solubility, permeability, and stability of flavonoid compounds. While various techniques for phytosome production exist—including solvent evaporation and thin-film hydration— antisolvent precipitation has been identified as a method that improves absorption efficiency (Alharbi et al., 2021; Barani et al., 2021; Varadkar & Gadgoli, 2022). However, the antisolvent precipitation method has demonstrated superior entrapment and stability but remains underutilized in propolis formulations (Dewi et al., 2024; Permana et al., 2020; Ridwan et al., 2024).

Previous studies demonstrated that propolis has anti-inflammatory effects (Abduh et al., 2024; Kowalczyk et al., 2025; Zulhendri et al., 2022). Nevertheless, limited research has focused on propolis derived specifically from *Tetragonula* species and delivered via phytosomal systems. Therefore, this study aimed to develop a phytosomal gel formulation of *Tetragonula* propolis extract and assess its anti-inflammatory effects *in vivo* using a rat model of carrageenan-induced paw edema.

MATERIAL AND METHODS

Plant material and chemicals

Propolis from *Tetragonula* sp. was obtained from Indonesian stingless bee breeders. Chemicals used included ethanol (70% and 96%), dichloromethane, n-hexane, aluminum chloride, sodium acetate, quercetin (Merck®), aquadest, phospholipid (Phospholipon® 90), carbopol 940, sodium carboxymethylcellulose (Na CMC), triethanolamine (TEA), methylparaben, glycerin, fatty acids (palmitic, oleic, stearic), virgin coconut oil, squalene, paraffin wax, cholesterol, vaseline album, Whatman paper, pH 7.4 phosphate buffer (Merck®), and carrageenan (Sigma-Aldrich®). All excipients in the gel were pharmaceutical-grade; other solvents and reagents were analytical-grade.

Extraction of propolis

Propolis was frozen, ground, and extracted with 70% ethanol at room temperature, with the extraction repeated 3 times over 24 hours. Extracts were filtered, concentrated via rotary evaporation, and the yield (%) was calculated using the formula [1] (Nurfatimah et al., 2024).

$$\text{Yield (\%)} = \frac{\text{Extract weight}}{\text{Sample weight}} \times 100\% \quad [1]$$

Total flavonoid content

The content was quantified using a colorimetric aluminum chloride assay with quercetin as the standard, measured at 440 nm with a UV-Vis spectrophotometer (Analytic Jena Specord 200 plus, Hauser Company, Germany), and reported as milligrams of quercetin equivalents (QE) per gram of sample extract (Nicolescu et al., 2025; Shraim et al., 2021).

Preparation of EEP phytosomes

Phytosomes were prepared via antisolvent precipitation using propolis extract at phospholipid-to-propolis extract ratios of 1:1, 1:2, 1:3, and 2:1. Ethanolic extract (96%) and phospholipid (dissolved in dichloromethane) were refluxed at 60°C for 2 hours. The mixture was then treated with n-hexane to precipitate, filtered, and dried (Damle & Mallya, 2016; Ridwan et al., 2024).

Entrapment efficiency

One milliliter of phytosome dispersion was centrifuged at 12,000 rpm, 4°C for 45 min, and the supernatant was analyzed for unencapsulated flavonoids using UV-Vis spectrophotometry at 440 nm (Analytic Jena Specord 200 plus, Hauser Company, Germany). The entrapment efficiency (%) was determined using the formula [2].

$$\text{Entrapment efficiency (\%)} = \frac{(T - S)}{T} \times 100\% \quad [2]$$

where T is the content in the propolis extract, and S is the flavonoid amount in the supernatant (Dewi et al, 2024).

Characterization

Physical test of phytosome surface morphology

Phytosomes were dissolved in water at a 1:10 ratio, placed on a carbon-coated copper grid for 1 minute, and subsequently analyzed using a Transmission Electron Microscope (TEM) (Jeol JEM 1400, Jeol Ltd., Japan) (Deleanu et al., 2023).

Spectroscopic test using Fourier transform infrared (FTIR)

In the FTIR analysis (ParkirElmer Inc., USA), samples comprising EEP, phospholipid, a physical mixture, and EEP phytosome were combined with KBr at a ratio of 1:100, ground thoroughly, and subsequently pressed into a mold for examination across the spectral range of 4000-400 cm^{-1} (Damle & Mallya, 2016).

Particle size test and polydispersity index

These tests were assessed using dynamic light scattering facilitated by a particle size analyzer (PSA) (Nano Particle Analyzer Horiba SZ-100, Horiba, L.td., Japan). The suspension was diluted 1:100 with distilled water at 25°C and observed at 90° scattering (Jain et al., 2023).

Optimum EEP phytosome preparation

The optimized phytosome was prepared via anti-solvent evaporation, using the ideal EEP-to-phospholipid ratio and an effective sonication time (0-5 min) for size reduction (Elmasonic, Elma Schmidbauer GmbH, Germany).

Optimum EEP phytosome characterization

Characterization involved measuring particle size, polydispersity index (PSA), TEM morphology, and FTIR analysis to confirm complex formation, as in previous studies (Ridwan et al., 2024).

Gel formulation

The enhanced EEP phytosome was incorporated into a gel matrix comprising Carbopol 940, Na CMC, TEA, methyl paraben, and glycerin, with the pH adjusted to 4.5–6.5. Details are given in Table 1.

Permeation study

In vitro permeation was measured using a Franz cell with a cellulose membrane, with 1 mg of gel (containing EEP and phytosome) from the receptor medium, filled with phosphate buffer (pH 7.4) (Chaerunisaa et al., 2021). The receptor was used at 37 ± 0.5°C with stirring. Aliquots of 2 mL were taken at set intervals (0-180 mins) and replaced with fresh medium. Flavonoid levels in each aliquot were measured via a UV-Vis spectrophotometer at 440 nm (Chittasuppo et al., 2023; Dewi et al., 2018; Ridwan et al., 2023).

Anti-inflammatory evaluation

The use of male Wistar rats in this study were approved by the University of Mataram Ethics Committee, approval number 101/UN.18.F8/ETIK/2024. The rats were randomly assigned to four groups (n = 6): Group 1: 0.1 mL carrageenan + gel (negative control). Group 2: 0.1 mL carrageenan + gel with EEP (5%w/v). Group 3: 0.1 mL carrageenan + gel with EEP phytosome (5%w/v). Group 4: 0.1 mL carrageenan + gel with diclofenac diethyl amine (1%) (positive control). 0.1 mL of 1% carrageenan was injected subplantarly to induce inflammation, and paw volumes were measured at 30, 60, 90, 120, and 150 minutes. Inhibition percentages were calculated (Ismail et al., 2016; Xiang et al., 2023).

Table 1. The formula of the gel containing EEP phytosome.

Ingredients	Percentage of amount (%)	Function
EEP in phytosome	5	Active ingredient
Carbopol 940	0.59	Gelling agent
Glycerine	1.91	Emollient
Triethanolamine	0.2	Alkalizing agent
Methyl paraben	0.2	Preservative
Na CMC	2	Gelling agent
Aquadest	Ad 100	

Statistical analysis

Data are shown as mean ± SD. Statistical analysis was performed using SPSS 19.0 (IBM Corp., USA). Prior to the main analysis, data normality was verified using the Shapiro-Wilk test, and homogeneity of variance was confirmed. Subsequently, a One-Way Analysis of Variance (ANOVA) was conducted, followed by post-hoc Tukey tests for multiple comparisons between groups. A value of $p<0.05$ was deemed statistically significant.

RESULTS

Phytochemical analysis

The ethanolic extraction of propolis yielded 43.54%. Phytochemical screening confirmed the existence of flavonoids, phenols, tannins, saponins, and alkaloids. The total flavonoid content was 1.05 ± 0.0274 mg QE/g.

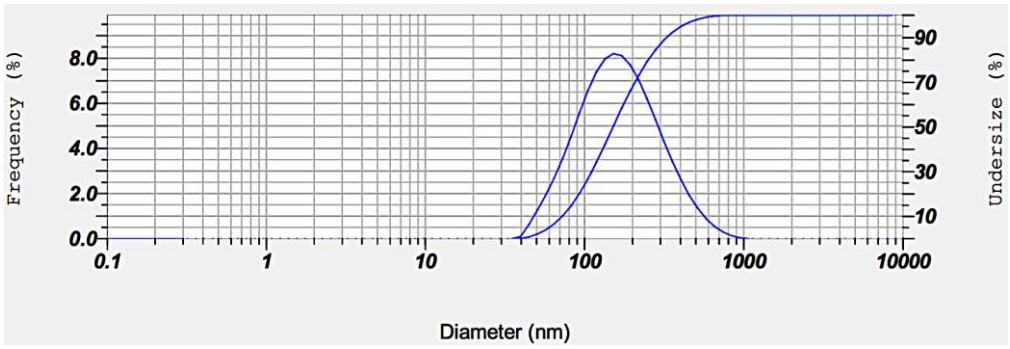

Figure 1. Representative PSA spectrums showing the frequency and cumulative undersize distribution of the optimized phytosomes.

Phytosome of EEP preparation and characterization

Optimization of weight ratio

For the preparation and characterization of the EEP phytosomes, an antisolvent method was employed, utilizing varying EEP-to-phospholipid weight ratios (Dewi et al., 2024; Ridwan et al., 2024). The study determined that the optimal weight ratio was 1:3, achieving an entrapment efficiency of 89.624 ± 0.79% (refer to Table 2). This ratio was selected for subsequent analysis aimed at optimizing particle size and polydispersity index.

Table 2. Entrapment efficiency (%) of EEP phytosome.

EEP: Phospholipid	Entrapment efficiency (%)
1: 1	78.866 ± 0.70[a]
1: 2	86.546 ± 1.34[b]
1: 3	89.624 ± 0.79[c]
2: 1	86.541 ± 0.03[b]

Results are expressed as mean ± SD from three experiments. Different letters indicate statistically significant differences ($p<0.05$).

Optimization of particle size and polydispersity index (PDI)

The 1:3 EEP to phospholipid ratio showed the highest entrapment efficiency (89.62 ± 0.79%). Sonication for 3 minutes optimized particle size (161.9 ± 2.0 nm) and PDI (0.389 ± 0.023). The optimized particle size and polydispersity index are illustrated in Fig. 1.

Fourier transform infrared (FT-IR)

FTIR analysis confirmed complexation between EEP and phospholipids. TEM images revealed spherical particles of uniform size, consistent with PSA data (Fig. 2).

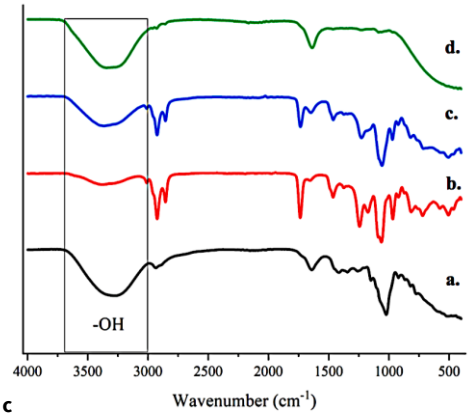

Figure 2. FT-IR spectrum of **(a)** EEP; **(b)** phospholipid; **(c)** phytosome of EEP; **(d)** physical mixture of EEP and phospholipid.

Transmission electron microscope(TEM)

The transmission electron microscope was used to characterize the morphology of the optimal EEP phytosome. As shown in Fig. 3, the EEP phytosome was made up of spherical vesicles, around 50 nm in size. The verified particle size of the EEP phytosome was 161.900 ± 2.000 nm (see Table 3).

Evaluation of gel containing EEP phytosome

The EEP phytosome gel was formulated according to the parameters in Table **3**, using a combination of two gelling agents. The physical characteristics of the EEP phytosome-filled gel, as detailed in Table 4, indicate that it has met the requirements for gel preparation.

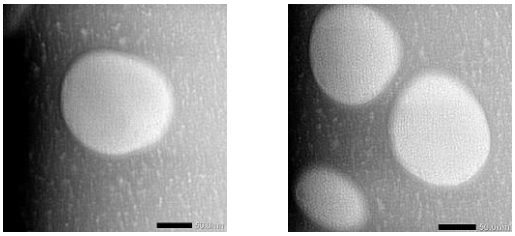

Figure 3. The morphology of the optimal EEP phytosome analyzed by transmission electron microscopy.

Table 3. Phytosome particle size and PDI of EEP particles at different sonication times.

Sonication time (min)	Particle size (nm)	PDI
0	384.867 ± 28.382[a]	0.608 ± 0.008[a]
1	263.400 ± 1.442[b]	0.578 ± 0.036[b]
3	161.900 ± 2.000[c]	0.389 ± 0.023[c]
5	443.500 ± 37.455[d]	0.607 ± 0.055[a]

Data are mean ± SD of triplicates. Different letters indicate statistically significant differences ($p<0.05$).

In vitro permeation assay

In vitro studies comparing the efficacy of the EEP phytosomal gel with the conventional EEP gel show the permeation profile of the phytosomal formulation in Fig. 4. Notably, the permeation of the conventional EEP gel could not be precisely quantified, as the flavonoid concentration remained below the detection limit (LOD) of the UV-Vis spectrophotometric method. Conversely, the phytosomal gel exhibited superior performance, with the cumulative percentage of total flavonoid content released reaching 90.751 ± 1.000% within 180 minutes.

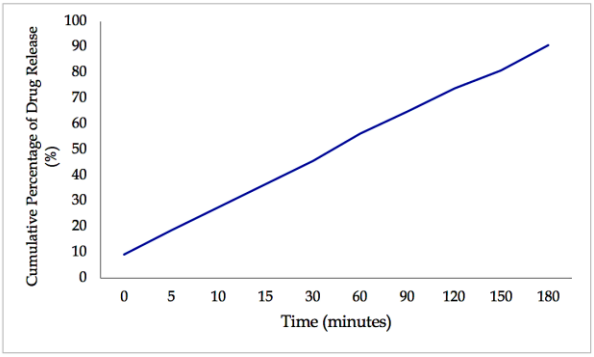

Figure 4. Cumulative percentage of TFC content release of gel containing EEP phytosome.

In vivo study of anti-inflammatory activities

The anti-inflammatory study demonstrated that the gel containing EEP phytosome exhibited a significantly higher level of inhibition (122.27 ± 19.08%) in comparison to the EEP gel (81.41 ± 9.69%) and diclofenac gel (92.51 ± 31.86%) at 150 minutes post-treatment ($p<0.05$) (Table 5).

DISCUSSION

The current study has demonstrated that flavonoids present in *Tetragonula* sp. propolis exhibit considerable anti-inflammatory activity (Sahlan et al., 2021). However, their lipophilic nature restricts absorption and effectiveness. Phytosomal encapsulation has significantly improved delivery, as evidenced by increased inhibition of various pharmacological activities relative to unencapsulated flavonoids (Lu et al., 2019; Nandhini & Ilango, 2021; Talebi et al., 2025).

The total flavonoid content of EEP was 1.05 ± 0.0274 mg QE/g sample. Flavonoids inhibit the secretion of arachidonic acid, which plays a role in reducing inflammatory reactions. Furthermore, flavonoid compounds—such as genistein, apigenin, quercetin, and kaempferol—regulate the cytokine expression and activation (Al-Khayri et al., 2022). However, due to the lipophilic nature of flavonoids, a novel transdermal delivery approach was developed to enhance their solubility and penetration through the stratum corneum, the skin barrier (Costa et al., 2021). Phytosomes serve as delivery systems designed to enhance the permeation of flavonoids via transdermal administration (Barani et al., 2021).

Phytosomes can be prepared using methods such as solvent evaporation, antisolvent precipitation, and thin-layer hydration, each of which affects entrapment efficiency (EE) (Damle & Mallya, 2016). EE measures the amount of flavonoid in the phytosome, influenced by the extract-to-phospholipid ratio (Dewi et al., 2024). This study used antisolvent precipitation to develop EEP phytosomes with various ratios. The 1:3 ratio achieved the highest EE of 89.624 ± 0.79%, significantly higher than those of the 1:1 and 1:2 ratios ($p<0.05$). Higher phospholipid concentrations increase EE via hydrogen bonding between flavonoid hydroxyl groups and phosphatidylcholine phosphate groups (Huang et al., 2020). FTIR analysis confirmed EEP-phytosome formation and revealed strong molecular interactions. The hydroxyl region (3600–3000 cm^{-1}) shifts indicated hydrogen bonding, while widened P–O and N–O bands (1300–1000 cm^{-1}) showed successful complexing with choline. Changes in the carbonyl (1750–1600 cm^{-1}) and C–H stretching regions (around 2900 cm^{-1}) suggest that hydrogen bonding and hydrophobic interactions stabilize the phytosome. The phytosome spectrum was notably stronger than that of pure EEP (El-Menshawe et al., 2018; Hashemzadeh et al., 2023; Lu et al., 2019).

Particle size ranged from 10 to 210 nm, which is theoretically optimal for facilitating penetration through the stratum corneum (Danaei et al., 2018). The efficacy of this system was ascribed to the utilization of phosphatidylcholine, which closely resembles the lipid bilayer architecture of human skin. By encapsulating the active flavonoids within this biomimetic structure, the phytosome enhances chemical stability and promotes superior absorption (Rajamma et al., 2022; Talebi et al., 2025).

Table 4. Physical characterization of gel containing EEP phytosome.

Organoleptic	Homogeneity	pH	Spreadability (cm)	Viscosity (cP)	Adhesiveness (sec.)
White, odorless	Homogeneous	6.23 ± 0.02	5.1 ± 0.18	7.322 ± 153.30	6.5 ± 0.93

Table 5. Percentage of inflammatory inhibition for each test group.

Test groups	Inhibition (%)				
	30 min	60 min	90 min	120 min	150 min
Control positive (gel containing diclofenac diethyl amine	37.750 ± 12.527[a]	62.026 ± 15.664[b]	77.842 ± 16.973[e]	90.230 ± 29.178[h]	92.510 ± 31.860[k]
Gel containing EEP phytosome	68.912 ± 15.744[a]	72.439 ± 13.224[c]	74.067 ± 13.674[f]	94.761 ± 12.099[i]	122.267 ± 19.081[l]
Gel containing EEP	52.062 ± 9.922[a]	63.070 ±14.163[d]	71.730 ± 13.483[g]	72.812 ± 15.780[j]	81.412 ± 9.693[m]

Data are mean ± SD. Different letters indicate significant differences ($p<0.05$).

TEM was used to observe the surface structure of the EEP phytosome, revealing a spherical shape with a smooth surface (Fig. 3). FTIR and TEM analyses confirmed the formation of stable phytosomal complexes. The improved pharmacodynamic profile is attributed to increased permeability and sustained release of flavonoids. This assertion is supported by the release study, which demonstrated that the phytosomal gel achieved a cumulative release of 90.751 ± 1.000% within 180 minutes (Fig. 4). In contrast, the conventional EEP gel remained below the limit of detection (LOD < 0.2). The synergy between the small particle size and the amphiphilic nature of the phosphatidylcholine complex effectively facilitates the transdermal delivery of flavonoids, markedly surpassing the performance of the uncomplexed extract (Chittasupho et al., 2023; Rajamma et al., 2022; Talebi et al., 2025).

The anti-inflammatory efficacy demonstrated that the EEP phytosomal gel notably suppressed edema ($p<0.05$) compared with diclofenac sodium gel and conventional EEP gel (see Table 5). This performance reflected high permeation efficiency seen in vitro (Fig. 3) (Varadkar & Gadgoli, 2022). The phytosomes' ability to deliver flavonoids across the skin ensured better delivery to the inflammatory site than the uncomplexed extract. Hydrogen bonding between flavonoids and phospholipids, as confirmed by FTIR (Fig. 2), maintained the complex stability, preventing premature degradation. Despite flavonoids' low permeation due to poor solubility (Peng et al., 2016), encapsulation in phospholipids helps them overcome stratum corneum limitations by facilitating their incorporation into cell membranes, thereby increasing absorption (Gaikwad et al., 2021; Susilawati et al., 2021). Although this study did not specifically measure biochemical markers such as TNF-α, IL-6, or PGE2, the potent anti-inflammatory activity observed in the phytosomal group is strongly supported by the known molecular mechanisms of *Tetragonula* sp. propolis. Previous studies have established that the flavonoids in this propolis exert their effects by suppressing the activation of COX-1 and COX-2 enzymes and by inhibiting the expression of genes responsible for COX-2 (Pahlaveni et al., 2020). The therapeutic success demonstrates that the phytosomal system converts favorable physicochemical traits into a powerful biological response, outperforming traditional topical treatments.

CONCLUSION

The phytosomal gel with EEP demonstrated significant anti-inflammatory activity *in vivo*, outperforming the unformulated EEP gel and commercial diclofenac gel. Its higher efficacy is due to high entrapment efficiency, optimal nanoparticle size, and greater skin permeation, whereas the unformulated EEP remained below detection. These findings confirm that the phytosomal system overcomes barriers to flavonoid bioavailability, offering a promising strategy to enhance the bioavailability of propolis bioactives as a transdermal drug delivery system for anti-inflammatory therapy in preclinical models.

CONFLICT OF INTEREST

The research was conducted entirely free from any involvement in commercial, financial, or personal matters that might be perceived as a possible conflict of interest or that could have influenced the outcomes or interpretation of the study.

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GENERATIVE ARTIFICIAL INTELLIGENCE (AI)

The authors declare that no generative artificial intelligence (AI) or AI-assisted technologies were used in the preparation, writing, or editing of this manuscript. All content was developed solely by the authors, who take full responsibility for its accuracy and integrity.

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AUTHOR CONTRIBUTION:

Contribution	Ridwan S	Pasaribu G	Putri FK	Subaidah WA	Safwan S	Wahyuni N
Concepts or ideas	x					x
Design	x		x	x		x
Definition of intellectual content	x	x	x	x	x	x
Literature search	x	x	x		x	
Experimental studies	x	x	x		x	
Data acquisition		x			x	
Data analysis	x	x			x	x
Statistical analysis		x			x	
Manuscript preparation	x	x	x	x	x	x
Manuscript editing	x				x	x
Manuscript review	x	x	x	x	x	x

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