Formulation and evaluation of encapsulated *Graptophyllum pictum* (L.) Griff. ethanolic extract in lipid vesicles for hemorrhoid treatment

[Formulación y evaluación de extracto etanólico encapsulado de *Graptophyllum pictum* (L.) Griff. en vesículas lipídicas para el tratamiento de hemorroides]

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Abstract

Context: *Graptophyllum pictum* (L.) Griff. leaves (GPL) are traditionally used in Indonesia to treat hemorrhoids, and the anti-hemorrhoid activity of the ethanolic extract of *G. pictum* leaves (GPLE) has been proven scientifically. However, since it contains a variety of compounds, both hydrophilic and lipophilic, which has diverse solubility, absorption, and penetration characteristic, there is a need to be formulated to improve its effectiveness. Lipid nanocarrier formulations, such as liposome and ethosome, can be developed as the delivery system for GPLE to enhance its effectiveness.

Aims: To evaluate the anti-hemorrhoid activity of the liposomal and ethosomal formulation of GPLE.

Methods: The hydration method was used to prepare GPL liposomal (GL) and ethosomal (GE) formulations. An in vivo study was conducted to evaluate the penetration and effectiveness of both formulations. The in vivo test was evaluated based on the recto-anal tissue histological observation after treatment of the formulation to croton oil-induced hemorrhoid mice. The number of inflammatory cells, goblet cells, hemorrhage area, and mucosal thickness were compared between both formulations and the extract.

Results: GL had a smaller particle size, 145.7 ± 11.32 nm, with a PDI value of 0.389 ± 0.019. The zeta potential (ZP) of GL was -2.0200 ± 0.0513 mV and -0.0109 ± 0.0257 mV for GE. GE exhibited a better penetration and activity profile than GL, which was assumed to be correlated to its smaller particle size and component of the formulation. GE was able to penetrate up to the submucosa layer; meanwhile, GL only reached the muscularis mucosa. GE revealed better anti-hemorrhoid activity as it showed a lesser number of inflammatory cells, goblet cells, and necrosis cells. Additionally, it exhibited a smaller hemorrhage area and muscular mucose cell thickness compared to GL. However, there was no significant difference statistically.

Conclusions: These findings suggest that lipid vesicles are a suitable delivery system for GPLE in the anti-hemorrhoid treatment.

Keywords: encapsulation; hemorrhoid; herbal; lipid carrier; medicine.

Resumen

Contexto: Las hojas de *Graptophyllum pictum* (L.) Griff. (GPL) se utilizan tradicionalmente en Indonesia para tratar las hemorroides, y la actividad antihemorroidal del extracto etanoílico de hojas de *G. pictum* (GPLE) se ha demostrado científicamente. Sin embargo, dado que contiene una variedad de compuestos, tanto hidrófilos como lipofílicos, que presentan diversas características de solubilidad, absorción y penetración, es necesario formularlo para mejorar su eficacia. Las formulaciones de nanotransportadores lipídicos, como el liposoma y el etosoma, pueden desarrollarse como sistema de administración de GPLE para mejorar su eficacia.

Objetivos: Evaluar la actividad antihemorroidal de la formulación liposomal y etosomal de GPLE.

Métodos: Se utilizó el método de hidratación para preparar las formulaciones liposomal (GL) y etosomal (GE) de GPLE. Se realizó un estudio in vivo para evaluar la penetración y eficacia de ambas formulaciones. La prueba in vivo se realizó a partir de la observación histológica del tejido recto-anal tras el tratamiento de la formulación a ratones con hemorroides inducidas por aceite de croton. Se comparó el número de células inflamatorias, células caliciformes, área de hemorragia y grosor de la mucosa entre ambas formulaciones y el extracto.

Resultados: GE presentó un tamaño de partícula menor, 145.7 ± 11.32 nm, con un valor de índice de polidispersidad (PDI) de 0.376 ± 0.031. Mientras tanto, GL mostró un tamaño de partícula de 407.80 ± 11.32 nm, con un valor de PDI de 0.389 ± 0.019. El potencial zeta (ZP) de GL fue de -2.0200 ± 0.0513 mV y de -0.0109 ± 0.0257 mV para GE. GE mostró un mejor perfil de penetración y actividad que la GL, lo que se supuso correlacionado con su menor tamaño de partícula y componente de la formulación. GE fue capaz de penetrar hasta la capa submucosa, mientras que la GL sólo alcanzó la muscularis mucosa. GE reveló mejor actividad antihemorródica, ya que mostró un menor número de células inflamatorias, células caliciformes y células de necrosis. Además, mostró un área de hemorragia y un grosor de las células de la muscularis mucosa menores en comparación con GL. Sin embargo, no hubo diferencias significativas desde el punto de vista estadístico.

Conclusiones: Estos hallazgos sugieren que las vesículas lipídicas son un sistema de administración adecuado para GPLE en el tratamiento antihemorroidal.

Palabras Clave: encapsulación; hemorroides; hierbas; portador lipídico; medicamento.
INTRODUCTION

Hemorrhoids are defined as symptomatic enlargement and distortion of vascular channels in the anorectal region, often coupled with the deterioration of supporting tissues in the anal cushions (Lohsiriwat, 2012; Sardiñas et al., 2016; Sun and Migaly, 2016). It has manifestations such as inflammatory reactions (Shrivastava et al., 2018), vascular hyperplasia (Aigner et al., 2009; Chung et al., 2004), vascular thrombosis, degeneration of the collagen fibers and fibroelastic tissues, in-addition to distortion and rupture of the anal sub-epithelial muscle (Lohsiriwat, 2012; Sun and Migaly, 2016). In severe cases, a prominent inflammatory reaction involving the vascular wall and surrounding connective tissue associated with mucosal ulceration, ischemia, and thrombosis is shown (Lohsiriwat, 2012; Margetis, 2019; Sun and Migaly, 2016). Although it has a low prevalence and morbidity, hemorrhoids lower the patient’s quality of life (Kaidar-Person et al., 2007; Lee et al., 2014).

Hemorrhoids can be managed by surgical or nonsurgical treatment (Brown, 2017; Sun and Migaly, 2016). These options have their own positive and negative effects. Unfortunately, there is a possibility of relapse for both treatments (Čuk et al., 2015). Hemorrhoid medicament by nonsurgical option is intended to relieve symptoms and can be delivered orally and topically, but unfortunately, it is often unsuccessful in treating hemorrhoids. Hemorrhoids medicines usually contain local anesthetics, corticosteroids, antibiotics, and anti-inflammatory drugs (Lohsiriwat, 2012). It provides temporary relief to acute symptoms such as bleeding and pain during defecation. Oral medicament containing flavonoids and calcium dobesilate is the alternative option. Topical corticosteroids in cream or suppository formulations are commonly prescribed, but their efficacy remains unproven. As the main goals of hemorrhoid treatments are to decrease vascularity, reduce redundant tissue, and increase hemorrhoidal rectal wall fixation to minimize prolapse. Therefore, a combination of oral and topical medications hopefully will increase the probability of success in hemorrhoid treatment and avoid surgical options (Misra and Imlitemsu, 2005).

Graptophyllum pictum (L.) Griff. leaves (GPL), belonging to the family Acanthaceae, have been used as traditional medicine to treat hemorrhoids in Indonesia. The pharmacological activities of these plant extracts are related to their anti-hemorrhoidal activity that has been studied extensively, such as their analgesic and anti-inflammatory activities (Ozaki et al., 1989), phagocytosis activity and immunoglobulin formation (Kusumawati et al., 2002), and their ability to activate the classical pathway of complement and chemotactic activity (Kusumawati et al., 1997).

The previous study by Kusumawati et al. (2022a) showed that the ethanolic extract of G. pictum leaves (GPLE) contains a total flavonoid content (TFC) of 16.3 ± 0.79 mg HE/g (hyperoside equivalent) and total phenolic content (TPC) of 428.3 ± 18.01 mg GAE/g (gallic acid equivalent). GPLE also shows the ability to stop bleeding and shows astringent and strong antioxidant properties. The oral and topical anti-hemorrhoid activity of GPLE was determined using a hemorrhoid rat model induced by croton oil. At the dose of oral (166.4 mg/kg) and topical (250 mg/kg) application, GPLE was shown to have a therapeutic effect on hemorrhoids through its antioxidant, anti-inflammatory, and hemostatic properties.

Flavonoids are known for their ability to increase vascular tone, reduce venous capacity, decrease capillary permeability, facilitate lymphatic drainage, and have an anti-inflammatory effect (Lyseng-Williamson and Perry, 2003; Pérez-Cano and Castell, 2016; Serafini et al., 2010). Flavonoids from Ginkgo biloba have been shown to increase venous tone and lymphatic drainage, decrease capillary hyperpermeability from inflammatory processes, and have been successfully used in clinical trials in hemorrhoid patients (Misra and Imlitemsu, 2005; Zaman et al., 2015). Micronized purified flavonoid fraction (MPFF), consisting of 90% diosmin and 10% hesperidin, is the most common flavonoid used in clinical treatment (Alonso-Coello et al., 2006). The micronization of the drug into particles of less than 2 μm improved its solubility and absorption and shortened the onset of action. In addition, MPFF reported reducing rectal discomfort, pain, and secondary hemorrhage following hemorrhoidectomy (La Torre and Nicolai, 2004).

This study aimed to develop an effective topical formulation of GPLE that is able to promote the delivery of the extract through the skin. Apart from the flavonoids, which are suggested to be responsible for the activity, GPLE contains many unknown compounds. Hence, problems arising from its use include poor solubility, low bioavailability, and difficulty reaching target organs with sufficient doses. Phospholipid-based delivery systems, such as liposomes and ethosomes, were used to overcome these problems and limitations of herbal extract. Liposomes and ethosomes were characterized by polar head and nonpolar tail groups. Selected formulations were characterized by particle size, zeta potential, polydispersity index, and encapsulation efficiency. The best formulations were subjected to in vivo hemorrhoid activity and penetration studies.

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**MATERIAL AND METHODS**

**Plant extract and chemicals**

The ethanolic extract of *G. pictum* leaves (GPLE) from a previous study that had a total flavonoid content of 16.3 ± 0.79 mg HE/g and a total phenolic content of 428.3 ± 18.01 mg GAE/g were used in this study (Kusumawati et al., 2022a). Folin-Ciocalteu and DPWH were purchased from Sigma, betamethasone from PT. Kimia Farma, and phospholipon H 90 (hydrogenated phosphatidylcholine) from Lipoid GmbH. Other reagents and solvents were in analytical grade and purchased from Merck.

**Preparation of liposomal and ethosomal systems**

Hydration methods were used to prepare the lipid-based formulations, liposome (GL) and ethosome (GE), with composition as shown in Table 1. GL was prepared by dissolving 12 mg phospholipon H 90 in 4 mL ethanol, then the solution was added with 1.23 g GPLE (equivalent to 20 mg TFC). It was mixed with 6 mL propylene glycol (PG), and the ethanolic content was evaporated using a rotary evaporator (Buchi Rotary Evaporator, Laboratory 4000G1, Schwabach, Germany). Hydration was done through the addition of 4 mL phosphate buffer (pH 6.4) while homogenized using a homogenizer (T 25 digital ULTRA-TURRAX®-IKA Disperser, China) at 8600 rpm for five minutes.

GE was prepared through 12 mg phospholipon H 90 solubilization in 4 mL ethanol. The GPLE containing 20 mg TFC was added to the solution and stirred using Ultraturax at 8600 rpm for five minutes. Two milliliters of phosphate buffer and 6 mL PG were added dropwise during homogenization.

<table>
<thead>
<tr>
<th>Composition</th>
<th>Ethosome</th>
<th>Liposome</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPLE (g)</td>
<td>1.23</td>
<td>1.23</td>
</tr>
<tr>
<td>Phospholipon H 90 (mg)</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Propylene glycol (mL)</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Phosphate buffer 0.18 M pH 6.4 (mL)</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Ethanol (mL)</td>
<td>4</td>
<td>-</td>
</tr>
</tbody>
</table>

**Liposome and ethosome characterization**

**Differential scanning calorimetry (DSC) study**

Thermal analysis of the samples, i.e., GPLE, phospholipon H 90 (Lipoid), physical mixture of liposome (PGL), physical mixture of ethosome (PGE), loaded liposome (GL) and loaded ethosome (GE), were done using differential scanning calorimeter (Mettler Toledo type HP DSC 2+, Switzerland) at a temperature range between 0 and 250°C with the heating rate at 10°C/min, under a constant nitrogen stream.

**Scanning electron microscope (SEM)**

The morphological characterization of particles was observed using SEM (Hitachi FlexSEM 100). Samples were coated into stainless steel 316L using the dip coating method. The observations were done in 30,000× magnification.

**Particle size, PDI, ZP analysis and encapsulation efficacy (EE)**

The particle size analyzer (Zetasizer ZS ZEN3500, Malvern Instruments Inc., UK) was used to determine particle size, PDI, and ZP of the nanoparticle in each formulation. PDI was used to determine the particle size homogeneity, and ZP to predict the stability of the colloidal system. The analysis was done in five replicates.

The encapsulation efficacy (EE) was determined using the dialysis method (Maestrelli et al., 2006), calculated based on flavonoid content, and analyzed using thin-layer chromatography (TLC). Briefly, the acetic cellulose membrane (MWCO 12,000-14,000) was soaked in ethanol 30% for 1 hour. The dialysis bag was added with 2 mL samples and 0.1% Triton X, then put into 30 mL ethanol 30% while stirred at 300 rpm for 5 hours. The analysis was done in six replications, and the diffused flavonoid was calculated for the EE.

\[
EE(\%) = \frac{[\text{total drug}] - [\text{diffused drug}]}{[\text{total drug}]} \times 100 \tag{1}
\]

**FTIR**

The IR absorption spectrum of the phosphatidylcholine, GPLE, PGL, GL, PGE, GE, PG, and buffer was analyzed by FTIR (UATR Spectrum Two, Perkin Elmer). The IR spectrum for each sample was recorded at 4000 – 450 cm⁻¹. The compatibility of the GPLE with the excipients investigated based on the spectrum.
Animal

Three months BALB/c male mice weighing 20-25 g from the Animal Center of the Faculty of Pharmacy, Universitas Airlangga, were used in this study. Before treatment, the animals were acclimatized for a week in ample cages in a well-ventilated room at a temperature set at 23 ± 2°C and humidity 45 ± 5%. They had access to water and food ad libitum. Animal experiments were conducted according to ethical standards and approved by the Animal Experimentation Ethical Committee of Universitas Airlangga (protocol number 2.KE.88.05.2018).

In vivo activity study

Experiments were carried out using a croton oil-induced hemorrhoid mice model based on the method described by Kusumawati et al. (2022a). Our previous research studied the effect of different doses of GPLE in the range of 100-500 mg/kg BW on its anti-hemorrhoid activity in mice. It was found that the anti-hemorrhoid activity was dose-dependent, and at 250 mg GPLE/kg BW, there was already a substantial improvement in hemorrhoid condition in the mice. Therefore, in this study, 250 mg/kg of GPLE was used as the treated animal dose, and the GL and GE groups were treated with a dose equivalent to GPLE. All samples were applied intrarectally on mice once daily at the same time.

Mice were randomly divided into six groups, i.e., normal (N), basis/control (C), betamethasone (BTM), GPLE, GL, and GE, with eight mice in each group. The normal (N) group consisted of healthy and untreated animals; the control (C) group of animals received only basis gel (Natrium CMC 0.05%). The reference group was treated with 0.25 mg/kg betamethasone (BTM). Hemorrhoids were induced in all the groups, except the normal group, by inserting into the anus a cotton ball impregnated with 6.1 mL of a mixture of croton oil (deionized water, pyridine, diethyl ether, and 6% croton oil in diethyl ether in a ratio of 1:4.5:10) for 10 seconds under mild ether anesthesia. Five days after induction, all the animals were subjected to respective treatment as assigned to the groups once daily for 14 days. On the 14th day, 1 h after the treatment, animals sacrificed using anesthetic injection (40 mg/kg ketamin/5 mg/kg xylazine).

On the fifteenth day, an hour after the treatment, rat anorectal histology samples were obtained by fixing rat anorectal biopsies into 10% formalin solution followed by paraffin method fixation and stained using hematoxylin-eosin (HE). All sample histology slides were observed by one pathologist using an optical microscope (Olympus BX50, USA), and were captured by Software Cell D (Olympus, USA). Histological observation of the anorectal tissue was made to note the appearance of inflammatory cells, tissue thickness, hemorrhage, and degrees of necrosis (Azeemuddin et al., 2014; Nishiki et al., 1988).

**In vivo skin penetration study**

The penetration study was done according to Kusumawati et al. (2022b) with slight modifications. The recto-anal area from each treatment group (C, BTM, GPLE, GL, and GE) was treated with a sample mixed with 0.01% rhodamine red as an indicator. After 1 h, the mice were decapitated, and the rat's recto-anal region was dissected and frozen in dry ice. Each specimen was sectioned with a 2 µm cryotome and observed with a fluorescence microscope (Olympus IX71-F22FL/DI inverted microscope system, DP71 camera, Cell D software). All procedures were carried out in the dark to prevent the influence of ambient light. The penetration level of the samples was determined semi-quantitatively based on a scoring system (Table 2).

**Statistical analysis**

The results are expressed as means ± SD (standard deviation of the mean). Statistical differences between groups were estimated using one-way analysis of variance (ANOVA) with Duncan's test and were considered statistically significant at p<0.05. The analysis was done using GraphPad Prism Vers. 9.0.2.

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**Table 2. The scoring criteria of the histologic specimens.**

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
<th>Figure</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>The sample has not penetrated or located in the lumen (L)</td>
<td><img src="https://jppres.com" alt="Image" /></td>
</tr>
<tr>
<td>1</td>
<td>Sample penetration reaches the stratum surface epithelium (SE)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Sample penetration reaches the muscularis mucosa (MM)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Sample penetration reaches the submucosa (SM)</td>
<td></td>
</tr>
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</table>

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RESULTS AND DISCUSSION

Liposome and ethosome characterization

Differential Scanning Calorimetry (DSC)

DSC is used for the characterization of the melting and crystallization behavior of the sample (Chen et al., 2012). The thermogram of the GPLE, phosphatidylcholine, the physical mixture of liposome (PGL) and ethosome (PGE) with GPLE, GL, and GE are illustrated in Fig. 1. DSC thermogram of the GPLE showed endotherm at 136.33°C and 196.16°C, while the phosphatidylcholine at 115.31°C and 234.88°C. PGL showed sharp endothermic peaks at 55.02°C and 185.11°C, and the board one at 87.56°C, which was assumed due to polymorphic transition as a result of decomposition during the melting process. PGE exhibited three sharp endothermic temperatures at 91.03°C, 160.05°C, and 263.47°C. GL and GE displayed one endothermic peak at 233.14°C and 198.37°C, respectively.

In the thermogram of PGL, there is a shifted temperature of the GPLE in the mixture for about 10°C lower, and decomposition of the component was observed as there is a broadening peak at 87.56°C. The PGE thermogram showed the characteristic of phosphatidylcholine, which shifted 15°C lower and 40°C higher than the raw materials. At the same time, the melting temperature of the extract was affected by another compound and appeared at 160°C. The dropped temperature observed is due to an interaction between excipients and GPLE. In the liposome and ethosome formulation, there was only one peak, assumed to be due to the integration of all material in the vesicular system, demonstrating the formation of GL and GE.

Scanning electron microscope (SEM)

The morphological analysis of the vesicles from GE and GL showed a spherical and smooth surface, with GE having elasticity in its vesicles (Fig. 2). The morphological analysis of the vesicles was done using SEM. The result showed that both ethosome and liposome vesicles had smooth spherical morphology and no pores, with GE having elastic characteristics.

Particle size, zeta potential analysis, and encapsulation efficacy (EE)

The particle sizes of GL and GE were 407.80 ± 11.32 nm and 145.70 ± 5.76 nm (Table 3), respectively. It indicated that the particle size was in the nanometer range, and the GL particle size was about three times larger than GE. The PDI of the GE and GL vesicles were 0.376 ± 0.031 and 0.389 ± 0.019 (Table 3), respectively. Both formulations showed vesicles with negative surface charge, -0.0109 ± 0.0257 mV for GE and -2.0200 ± 0.0513 for GL (Table 3). The EE value of GL was 88.64 ± 2.17% and GE 90.43 ± 1.02% (Table 4), which indicates that GL and GE had similar characteristics.

The particle sizes of both formulations were desirable for transdermal delivery systems since they were in the range of 10-600 nm (Danaei et al., 2018). The particle size of the ethosome (145.70 ± 5.76 nm) was smaller than the liposome (407.80 ± 11.32 nm) due to the higher concentration of ethanol, which causes the self-degradation properties of the vesicle bilayer with interpenetrating properties. The smaller the particle size of the vesicle, the better penetration of the encapsulated drug or extract into the deeper skin layer (Verma et al., 2003). Thus, GE, which had a smaller particle size, had better penetration ability than GL, as shown in Table 3. The ethanolic content of GE also works as a permeation enhancer (Touitou et al., 2000). These results correlate with the penetration studies, which showed that GE and GL penetrate until the submucosa and muscularis mucose layer, while the GPLE penetrates until the surface epithelium layer. It proved that lipid vesicles improved the penetrability profile of the extract, with the ethosomal formulation showing better permeability than the liposomal one.

Physicochemical characteristics of lipid nanocarriers affect the tendency of the particle to accumulate in the target cell. Therefore, the homogeneity of the nanoparticle population in the formulation was one requirement for a successful formulation (Danaei et al., 2018). The PDI is a homogeneity parameter described as the degree of non-uniformity of the particle size distribution. Lipid-based carriers are considered acceptable and homogenous when PDI is 0.3 and below (Badran, 2014; Chen et al., 2012; Putri et al., 2017). Although the FDA emphasized the importance of size and size distribution as critical quality attributes (CQAs), it does not state the acceptable criteria for PDI (Food and Drug Administration, 2019). The GL and GE formulations had PDI of 0.389 ± 0.019 and 0.376 ± 0.031, respectively, as shown in Table 3. These values fall between 0.05, considered a narrow size distribution, and 0.7, a broad size distribution (Lim et al., 2017), making them the acceptable criteria.

ZP provides information about the colloidal stability of the particle as well as the shelf life of colloidal dispersions. Generally, ZP with a value greater than ±30 mV is considered as stable since it has an electrostatic repulsion effect among the particles, thus avoiding aggregation (Mishra et al., 2018).
Figure 1. DSC thermogram profiles of (a) ethanolic extract of *G. pictum* leaves (GPLE); (b) Phosphatidylycerine; (c) physical mixture of ethosome (PGE); (d) loaded ethosome (GE); (e) physical mixture of liposome (PGL); (d) loaded liposome (GL).
The ZP values of GL and GE were -2.0200 ± 0.0513 mV and -0.0109 ± 0.0257 mV, respectively, as shown in Table 3. A zeta potential of -2.0200 ± 0.0513 mV indicates a slightly negative charge, and -0.0109 ± 0.0257 mV is considered almost neutral. The stability of liposomes with a ZP of -2 mV and -0.19 mV will depend on factors such as liposome size, shape, and composition, and the surrounding environment. In general, slightly negative and near-zero ZP can still provide some stability by creating repulsive forces between liposomes, which helps to prevent them from aggregating or clumping together. Thus, both ethosomal and liposomal formulation need to be assessed further to observe the stability of the formulation because some studies showed that although the vesicles had ZP lower than the standard value, they had good stability (Chen et al., 2012), which may cause by the steric stabilization process. Some researchers proposed that ethanol modifies the net charge of the systems into some degree of steric stabilization, which could lead to a decrease in mean vesicular size (Fang et al., 2008; Rakesh and Anoop, 2012). Ethanol can also be a negative charge provider for the surface ethosomal and stabilizer (Dubey et al., 2007; Verma and Pathak, 2012).

EE allows higher drug concentration entrapment. Thus, it will ensure the drug can be delivered more efficiently to the target organ or cell. The GL and GE exhibited no significant difference in EE (p>0.05), with EE values of 88.64 ± 2.17% w/w and 90.43 ± 1.02% w/w (Table 4), respectively. The higher value of the GE was probably due to its ethanolic content (Abdulbaqi et al., 2016).

**FTIR**

GPLE FTIR spectrum (Fig. 3g) showed major peaks at 1026.10 cm⁻¹, 1154.86 cm⁻¹, and 3290.12 cm⁻¹. FTIR spectrum of the physical mixture and final product revealed that there was no interaction between extract and excipient, as there was no major change in the spectrum in the physical mixture, either at PGL (Fig. 3f) or PGE (Fig. 3d) and the final product of either GL (Fig. 3e) or GE (Fig. 3c) formulation.

The drug-excipient compatibility was analyzed based on the FTIR spectrum. Two major peaks, 1026.10 cm⁻¹ and 1154.86 cm⁻¹, were observed at both the physical mixture and the final product of liposome and ethosomal formulation. The peak at 3290.12 cm⁻¹ cannot be observed in the physical mixture and final product due to the high intensity or sharp peak of buffer (Fig. 3a) and PG (Fig. 3b) at that wave-
number. This data indicates that there is compatibility of the GPLE with the excipients.

**Activity study**

The ethosomal (GE) and liposomal (GL) formulations of GPLE (250 mg/kg) demonstrated better activity in hemorrhoid treatment compared to the BTM (0.25 mg/kg) group. GL and GE groups were able to reduce the number of inflammatory cells comparable (p<0.05) to the BTM group. Additionally, GL and GE showed a lower number of inflamed cells, with GE observed to have the lowest number of inflamed cells. In terms of mucosal thickness, GPLE exhibited comparable (p>0.05) activity to the BTM group, while both GPLE formulations (GL and GE) showed better activity as they showed significantly (p<0.05) thinner mucosal compared to the BTM group. The anti-hemorrhoid activity was also observed through measurement of the hemorrhage area. GL and GE groups significantly reduced the hemorrhage area compared to the BTM group, and their activities can even bring the condition comparable to the normal group. The number of goblet cells from the BTM and GPLE indicated no significant difference (p>0.05), and the GE and GL showed significantly (p<0.05) better activity than the BTM group, as shown in Fig. 4.

GL and GE had better anti-hemorrhoid activity compared to the BTM and GPLE groups, as they showed a lower number of inflammatory cells and necrosis cells, smaller hemorrhage area, and thinner muscularis externa layer, significantly (p<0.05) as shown in Fig. 5. GL and GE activity showed no significant difference (p>0.05) in activity in all factor tested. Meanwhile, the GPLE group exhibited comparable activity to the BTM group, except in the aspect of hemorrhage area.

**Penetration study**

Penetration of substances across biological membranes depends on various chemical, physical, and biological interactions. The rectum is known to have a similar epithelium to that of the upper GI tract, and the predominant mechanism of penetration through the rectal mucosa involves trans-cellular passage across the cell membrane (Kamel et al., 2013). Fig. 6 shows the penetration ability of basis or control (C), BTM, GPLE, GL, and GE.

The penetration efficiency was analyzed semi-quantitatively and shown in Table 5. Fig. 6 and Table 5 showed that the GPLE reached the surface of the epithelium area, while the lipid nanovesicles formulation reached the deeper layer, with GL reaching muscularis mucosa and GE the submucosa. Generally, ethosomal formulation showed higher penetration, which could be attributed to the composition of ethanolic content in the formulation, which reached the submucosa layer, compared to the liposomal formulation, which only reached the muscularis mucosa.
Figure 4. Histopathology evaluation of the recto-anal mucosal area of mice. 
(A) Mucosal inflammatory cells; (B) Mucosal hemorrhage area; (C) Mucosal thickness; and 
(D) Mucosal Goblet cells. Values are expressed as mean ± SD (n = 6); different numbers of asterisks 
represent different subsets in Duncan’s test. These show significant differences at p<0.05. N: Normal; 
C: Control; BTM: Betamethasone; GPLE: ethanolic extract of G. pictum leaves; GL: loaded liposome; 
GE: loaded ethosome.

Figure 5. Histopathology evaluation of recto-anal muscularis externa portion area of mice. 
(A) Muscularis externa inflammatory cells; (B) Muscularis externa hemorrhage area; 
(C) Muscularis externa tissue thickness; and 
(D) Muscularis externa necrosis cells. Values are expressed as mean ± SD (n = 6); different numbers of asterisks represent different 
subsets in Duncan’s test. These show significant differences at p<0.05. N: Normal; 
C: Control; BTM: Betamethasone; 
GPLE: ethanolic extract of G. pictum leaves; GL: loaded liposome; GE: loaded ethosome.
**CONCLUSION**

The GE and GL groups (250 mg/kg) showed comparable or even better activity and penetration than the betamethasone (0.25 mg/kg) group in all parameters. Overall, the GE exhibited better activity and penetration than GL except in one parameter, the number of inflammatory cells in the muscularis externa. Although, there were no significant differences in all parameters from GL and GE groups. Both GL and GE have potential as active ingredient candidates for topical products used for hemorrhoid therapy. However, further studies need to be carried out to determine its stability and the long-term effects of the treatment.

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