https://doi.org/10.31925/farmacia.2023.1.10 **ORIGINAL ARTICLE**

ACUTE TOXICITY ASSESSMENT OF *GRAPTOPHYLLUM PICTUM* (L.) GRIFF. LEAVES ETHANOLIC EXTRACT AND ITS NANOFORMULATIONS: COMPARATIVE STUDY OF PHYTOSOME AND CYCLODEXTRIN INCLUSION COMPLEX

IDHA KUSUMAWATI [1,2]*, SUBHAN RULLYANSYAH [1], MEGA FERDINA WARSITO [3], MUHAMMAD ZDULQORNAIN [1], FIRDAUSYAH NURAINI [1], AISYAH FARAH RIZKA [1], YUSUF ALIF PRATAMA [1], RETNO WIDYOWATI [1], EKA PRAMYRTHA HESTIANAH [4], KATSUYOSHI MATSUNAMI [5]

[1]*Department of Pharmaceutical Science, Faculty of Pharmacy, Universitas Airlangga, Nanizar Zaman Joenoes Building, Jl. Mulyorejo, Surabaya, 60115, East Java, Indonesia*
[2]*Natural Product Drug Discovery and Development Research Group, Faculty of Pharmacy, Universitas Airlangga, Nanizar Zaman Joenoes Building, Jl. Mulyorejo, Surabaya, 60115, East Java, Indonesia*
[3]*Research Center for Applied Microbiology, National Research and Innovation Agency (BRIN), Komplek CSC-LIPI, Jl. Raya Bogor Km 46, Cibinong 16911, West Java, Indonesia*
[4]*Veterinary Anatomy Department, Faculty of Veterinary, Universitas Airlangga, Jl. Mulyorejo, Surabaya 60115, East Java, Indonesia*
[5]*Department of Pharmacognosy, Graduate School of Biomedical & Health Sciences, Hiroshima University 1-2-3, Kasumi, Minami-ku, Hiroshima, Japan*

**corresponding author: idha-k@ff.unair.ac.id*

Manuscript received: December 2022

Abstract

Graptophyllum pictum (L.) Griff has been used in traditional medicine to treat various diseases and shows great potential for clinical use, but is often limited by poor oral bioavailability and lack of information regarding its safety. In this study, the herbal extract was developed into two forms *e.g.*, phytosome and cyclodextrin complex in order to enhance its bioavailability. This study also examines the toxicity of *G. pictum* leaves ethanolic extract (GPLE) and its nanoformulations. The GPLE phytosome (GPLE-P) was formed using hydration method. The GPLE cyclodextrin inclusion complex (GPLE-CDIC) was produced through co-precipitation methods. Both formulations produced vesicle in nanometer size. The formation of the vesicles was confirmed through FT-IR and differential scanning calorimetry (DSC) analysis. The *in vitro* toxicity test showed that GPLE and GPLE-P caused erythrocyte morphological changes at 500 μg/mL, while GPLE-CDIC at 250 μg/mL. Oral administration of all formulations did not cause any erythrocyte morphological changes. The complete blood cell analysis showed no significant difference in the number of blood components from the untreated group compared to the treated group. Both formulations can be safely administered orally without any immediate unwanted effect.

Rezumat

Graptophyllum pictum (L.) Griff a fost folosită în medicina tradițională și prezintă un potențial clinic important, dar utilizarea sa este adesea limitată de biodisponibilitatea orală slabă și lipsa de informații privind siguranța ei. În acest studiu, extractul etanolic de frunze a fost folosit pentru obținerea a două forme farmaceutice diferite, fitozom și complex cu ciclodextrina, pentru a crește biodisponibilitatea. Acest studiu prezintă, de asemenea, toxicitatea extractului etanolic de frunze de *G. pictum* (GPLE) și nanoformulările sale. Fitozomul GPLE (GPLE-P) a fost obținut prin metoda hidratării. Complexul de incluziune al ciclodextrinei cu GPLE (GPLE-CDIC) a fost obținut prin metode de co-precipitare. Ambele formulări au condus la obținerea de vezicule cu dimensiune nanometrică, fiind confirmate prin analiza FT-IR și calorimetria cu scanare diferențială (DSC). Testul de toxicitate *in vitro* a arătat că GPLE și GPLE-P au cauzat modificări morfologice ale eritrocitelor la 500 μg/mL, în timp ce GPLE-CDIC la 250 μg/mL. Administrarea orală a tuturor formulărilor nu a provocat modificări morfologice ale eritrocitelor. Analiza completă a celulelor sanguine nu a arătat nicio diferență semnificativă în numărul de componente sanguine din grupul netratat comparativ cu grupul tratat. Ambele formulări pot fi administrate în siguranță pe cale orală, fără efecte imediat nedorite.

Keywords: *Graptophyllum pictum* (L.) Griff, acute toxicity, phytosome, cyclodextrin inclusion complex

Introduction

Medicinal plants have been used worldwide to prevent and treat various diseases, but unfortunately, there is a limited scientific foundation to prove their quality, efficacy and safety. Despite their widespread use and their medicinal importance, they are not utterly safe

as there is a substance with a healing property that can also induce adverse effects if used irresponsibly or in overdose. Additionally, the lack of proper standardization increases the probability of adverse effects.

Likewise of other pharmaceutical products, herbal medicine must go through a series of toxicology studies to postulate data concerning the product's safety before the commencement of the clinical trials [1]. The multi-component nature of herbal remedies means that some may have therapeutic value while others may provoke toxicity [2]. These interactions can occur with other medications or chemical compounds as well. In general, toxicology studies give information regarding the adverse effect of herbal preparation or product on living organisms, *i.e.*, symptoms, mechanisms and treatments. Toxicity studies can be categorized as acute, sub-chronic and chronic differing in the quantity and duration of product treatment to the object [3]. It helps determine the proper dosage for long-term use and its impact on the organs after therapy [4]. Therefore, it is important to assess plant safety through well-controlled and validated scientific toxicity studies.

Graptophyllum pictum (L.) Griff has been used as traditional medicine as contraceptive, delivery aid; to treat wound swelling, ulcers, abscesses, haemorrhoids [5, 6], renal impairments [7], rheumatism, urinary infection, scabies, hepatomegaly, ear diseases and as fertility enhancer [8]. In Indonesia, *G. pictum* has been used to treat tonsilitis, abscess, rheumatism, breast engorgement, breast abscess [9] and haemorrhoid [10-12]. The biological properties of this plant such as anti-haemorrhoid [11, 12], analgesic anti-inflammation [13], immunomodulator [14], antioxidant [15, 16], anti-plaque [17], oxytocic and anti-implantation [5], antidiabetic activities [18] and nephroprotective effects [6, 7] have been scientifically proven. In addition, these plant leaves contain various chemical components such as tannin, alkaloid, steroid and glycoside [8], phenol (including flavonoid, anthraquinone) [8, 12, 13, 16], saponin, coumarin and sugar [12, 13].

G. pictum leaves extract (GPLE) had been reported for its anti-haemorrhoid activity, and it was assumed that the activity is due to the flavonoid compound which has anti-inflammatory and antioxidant properties [12, 13, 16]. In addition, micronized purified flavonoid fraction was also reported to reduce rectal discomfort, pain and secondary haemorrhage following the haemorrhoidectomy [19]. Therefore, the dose of the preparations was arranged based on the flavonoid concentrations. Poor solubility, critical absorption conditions which lead to erratic bioavailability, non-uniform bio-distribution in the body fluids, and fluctuating pharmacokinetic and pharmacodynamic responses are unfortunate obstacles to the oral administration of flavonoids.

The nanotechnology-based formulation is a promising technology to enhance the activity and specificity of herbal medicine and improve the mean residence time and biodistribution. It can increase the bio-availability and active surface energy that leads to the potentiation of receptor binding selectivity, thereby enhancing the effectiveness and safety profile [20]. In addition, the flavonoid micronization to less than 2 μm will improve its solubility and absorption and shorten the onset of action [21].

Phytosomes incorporate natural active phytochemicals into phospholipids to produce lipid-compatible complexes [22, 23]. This vesicular system has been proven to enhance absorption and bioavailability without causing pharmacological or structural changes of the phyto-constituents [23]. Furthermore, the small-sized particles of the phytosome had a pronounced ability to traverse from a water-soluble condition into the lipid-soluble state of the enterocyte cell membrane into the cell, arriving the blood and protecting the active ingredients of the herbal drug from the enzyme and bacteria in the gastrointestinal tract [24, 25]. It also has high encapsulation efficiency with a better release profile [23], which further leads to a lower dosage of active constituents for exerting a biological effect.

β-cyclodextrin (β-CD) is a cyclic oligosaccharide containing seven glucopyranose units. These units could form inclusion complexes with diverse molecules by encapsulating the non-polar part of the guest into its hydrophobic cavity and stabilizing the polar position by the polar rims, which make it suitable for the controlled delivery agent of organic, inorganic, biological and pharmaceutical molecules [26]. For example, cyclodextrin complex inclusion of myricetin reported an increased solubility and improved oral bioavailability by 9.4 times [27].

Despite its various medicinal effect and usage, there is no study about the oral toxicity of the extract and its formulation, which provide safety information. This study will evaluate the safety profile of the extract and its formulation through *in vitro* and *in vivo* toxicology studies, especially in terms of the erythrocyte morphology and complete blood count.

Materials and Methods

Plant material

G. pictum (L.) Griff leaves (GPL) were harvested when the branch diameter of the plant reached 1 - 2 cm, and the leaves had reddish-purple colour. The samples were acquired from a tea plantation at Lawang, Malang, East Java, Indonesia, in October 2017. The specimen (RM GP102017) was identified and stored at the Herbarium of the Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Universitas Airlangga, Indonesia.

Chemicals

Technical grade ethanol for the extraction process was purchased from PT Brataco, Indonesia. Chemicals that were used in the formulation were phosphatidylcholine

(Sigma), CMC-Na (Sigma), β-CD (Sigma) and Cab-O-Sil (Sigma). Other substances that were used during this study were *Sapindus rarak* extract (as positive control), analytical grade solvents and chemicals (Merck).

Extraction

This research is a continuation of previous research, so the same extract (GPLE) was used in this study. *G. pictum* leaves were dried in the oven at 40°C until the water content was less than 10% and then grounded. Next, the extraction process was carried out using MAE (microwave accelerated extraction) method. The dry powder (100 g) was extracted in a microwave (30% generator power) for one minute using 70% ethanol (plant: solvent, 1:10, w/v). After the solvent was evaporated under low pressure using a rotary evaporator (Buchi, Japan), the extracts were freeze-dried to finish drying.

Total flavonoid concentration

Total flavonoid content was determined according to Christ and Müller's method [28]. In this method, the aglycone of flavonoid was released by acid hydrolysis of the GPLE. Next, $AlCl_3$ in a methanol-ethyl acetate-acetic acid solvent was added to form the complex. The absorbance of flavonoid complex was measured using a spectrophotometer at 425 nm. The experiments were conducted in quintuplicate, and the activity was expressed as hyperoside equivalent (HE, mg/100 g samples) [29, 30].

Preparation of GPLE phytosome (GPLE-P) and β-cyclodextrin inclusion complex formulation (GPLE-CDIC)

GPLE-P was prepared by using the hydration method. The ratio of 1:2 between phosphatidylcholine (PC) is depicted in Table I. PC was dissolved in 96% ethanol and added with the extract solution equivalent to 20 mg of flavonoids. The aging process is carried out using a rotary evaporator without heating for 30 minutes to an hour, and to decrease phytosomes size by using homogenization by Ultraturax at 8600 rpm. The solution was dried at room temperature by adding Cab-O-Sil [31].

Table I

Composition of GPLE-P and GPLE-CDIC formulas

	GPLE-P	**GPLE-CDIC**
GPLE	1.12 g	1.12 g
Phosphatidylcholine	0.56 g	-
Ethanol	50.0 mL	-
Cab-O-Sil	1.20 g	-
β-cyclodextrin	-	5.60 g
Water	-	25.0 mL

The production of cyclodextrin inclusion complexes was carried out through the co-precipitation method. GPLE was mixed with soluble cyclodextrin in the water at a ratio of 5:1. The mixture was then dried at room temperature while stirring.

Characterization of the formulation

Encapsulation efficacy

The encapsulation efficacy (EE) was determined using the dialysis method. Total flavonoid content was analysed according to the method described in 2.4 by using spectroscopic method.

Differential scanning calorimetry

The DSC analysis was conducted using HP DSC 2+ (Mettler, Toledo) to study the thermal behaviour of the extract (GPLE), phosphatidylcholine (PC), Cab-o-Sil, physical mixture of phytosome (PMP), phytosome (GPLE-P), β-cyclodextrin (β-CD), physical mixture of cyclodextrin inclusion complex (PM-CDIC), and cyclodextrin inclusion complex (GPLE-CDIC). The analysis was performed from 30ºC to 300ºC at a scanning rate of 10ºC/min, under a constant nitrogen stream.

Particle size, polydispersity index and zeta potential

Particle size, polydispersity index (PDI) and zeta potential (ZP) were determined with Malvern Zetasizer ZS 90 (Malvern Instruments Inc., UK). ZP predicted the physical stability of the colloidal systems.

Scanning Electron Microscopy

The morphological examination of the vesicles was performed using scanning electron microscope (Hitachi SU3500, Hitachi High-Technologies Europe GmbH).

Infrared Spectroscopy

The IR absorption spectrum of the GPLE, phosphatidyl-choline, Cab-o-Sil, PMP, GPLE-P, β-CD, PM-CDIC and GPLE-CDIC were analysed by FTIR (UATR Spectrum Two, Perkin Elmer). The samples were dried in a desiccator, blended with potassium bromide, and pelleted in the sample holder for further analysis. The IR spectrum for each sample was recorded at 4000 - 450 cm^{-1}. The compatibility of the GPLE with the excipients was investigated based on the spectrum.

Animals

Three months old male *Rattus novergicus* (ICR) weighing 200 - 250 g from Animal Centre of Faculty of Pharmacy, Universitas Airlangga, Indonesia, were used in this study. The animals were acclimatized a week before the experiment started. Animal experiment was conducted according to the ethical standards and approved by the Animal Experimentation Ethical Committee of Universitas Airlangga (protocol number 2.KE.88.05.2018).

In vitro Toxicity Studies

The *in vitro* toxicity was conducted using rat blood. The formulations were prepared with final working extract concentration 1000 ppm and serially diluted in 96 well microtiter plates until it reaches the lowest concentration of 62.5 ppm. *Sapindus rarak* infusion was used as positive control and DMSO 0.5% as the negative control. The morphology of the erythrocytes was observed under a microscope with 1000× magnification.

In vivo Toxicity Studies

Rats were randomly separated into 13 groups (6 animals each). The normal (N) group was healthy animals. Other groups were treated daily for 14 days, as shown in Table II. The sample suspension was given to rats once daily with oral gavage at the same time without fasting. The blood of each animal was drawn after 14 days of treatment. The toxicity of each group was observed according to the erythrocyte morphology and complete blood count.

Table II

Treatment group

Group	Treatment
Group-1	Negative Control (CMC-Na 0.5% suspension)
Group-2	Positive Control (*S. rarak* extract)
Group-3	GPLE 10 mg/kgBW
Group-4	GPLE 20 mg/kgBW
Group-5	GPLE 40 mg/kgBW
Group-6	GPLE-P 10 mg/kgBW
Group-7	GPLE-P 20 mg/kgBW
Group-8	GPLE-P 40 mg/kgBW
Group-9	GPLE-CDIC 10 mg/kgBW
Group-10	GPLE-CDIC 20 mg/kgBW
Group-11	GPLE-CDIC 40 mg/kgBW
Group-12	Blank phytosome (P)
Group-13	Blank cyclodextrine inclusion complex (CDIC)

The sample (GPLE, GPLE-P, GPLE-CDIC) dose of 10 mg/kg means the weight of the sample, which is equivalent to 10 mg of total flavonoids

Erythrocytes numbers determination

Blood was drawn from rat using Na-EDTA pre-washed Thoma-erythrocytes pipette until it reached 0.5. The blood was then diluted using 0.9% normal saline using the same pipette until it reached 101. Next, the aspirator was removed, the diluted blood was homogenized manually in the pipette, then put into the counting chamber at a 30° angle and left for 2 - 3 minutes. Erythrocytes total was counted under a microscope with 10× magnification.

Erythrocytes morphology observation

The morphological analysis of the erythrocytes was done using the method described by Cyprotex (literatur cyprotex). Each row of the microplate well was filled with 20 μL of the sample. The first row contained 1000 μg/mL extract solution, followed by double dilution using saline. Next, 180 μL of blood was added to each well. The microplate was then incubated at 37°C for 45 minutes. The morphology of the erythrocytes was observed under a microscope with 1000× magnification. It was grouped into several types, *i.e.*, echinocyte and stomatocyte.

Statistical analysis

All experiments were performed with five replications and the results are expressed as means ± SD (standard deviation). Statistical differences between groups were estimated using two-ways analysis of variance (ANOVA) for erythrocyte morphological changes test while the blood cell count data was analysed using one-way ANOVA. Both statistical analyses followed with LSD test and were considered statistically significant at $p < 0.05$. Statistical analyses were carried out using SPSS 25.0 software (SPSS Inc., Chicago, IL, USA).

Results and Discussion

G. pictum has been used traditionally to treat haemorrhoids [10-12]. The GPLE has been proven for its anti-haemorrhoid activity [11, 12], and flavonoid compound was presumed to be the active ingredients due to its anti-inflammatory and anti-oxidant properties [12, 13, 16]. Flavonoids work as phlebotonic agent [32, 33], that improve haemorrhoid symptoms [34-37]. Unfortunately, flavonoids are difficult to absorb in the small intestine [38]. The micronized flavonoid was reported to improve solubility and absorption, and shortened the onset of action [21]. Thus, developing the microparticle or nanoparticle delivery system will improve the bioavailability profile [19]. The safety of the GPLE-P and GPLE-CDIC was assessed according to the changes in the erythrocyte morphology and complete blood count analysis.

Phytosomes, one of the lipid-based nanovesicles, had an important function in improving the pharmacokinetic and pharmacodynamic profile of natural active ingredients [22]. It incorporates plant extract, fraction, or phyto-chemicals into hydrophilic polar head groups of phospholipid (mainly phosphatidylcholine) to form a lipid-compatible molecular complex in an aprotic solvent [22, 23, 39]. The ratio between plant material and phosphatidylcholine is usually about 1:1 or 2:1 [22, 40]. The polyphenolic ring of the phytochemicals interacts with hydrophilic moiety, phosphate group, of phospholipids (*i.e.*, choline) in aprotic solvents (*i.e.*, acetone,1,4-dioxane, hexane, metyhlenechloride and ethyl acetate) to form the body of phytosomes. Mean-while, the phosphatidyl lipophilic moiety forms a tail to incorporate the water-soluble choline-bound phytochemicals [22]. Phytosomes can protect the encapsulated phytoconstituents against gastric fluids

and gut microorganisms. They also enhance the transition of the enterocyte cell membrane from a water-soluble to a lipid-soluble state and then penetrate inside the cell to reach the bloodstream [40]. The ratio of GPLE and phosphatidylcholine used to form the molecular complex in this study was 2:1, where the GPLE was enclosed by the phosphatidylcholine molecules.

Cyclodextrins inclusion complexes improve the chemical and biological properties of the complex active substances. It also improves water solubility, dissolution and bioavailability; improves stability and shelf life of the product [41]; enhances specificity and release profile of the phytochemicals [41, 42]; improves organoleptic properties of the substances [41, 42]; and prevents interaction between active compounds and drug substance with excipients [41]. Cyclodextrins are categorized as "generally recognized as safe" (GRAS) material by US FDA, with an acceptable daily intake (ADI) of 5 mg/kg bw *per* day [43]. It is regarded as non or only partly digestible by the enzymes of the human gastrointestinal tract, and the absorption is negligible because of its high molecular weight and hydrophilic nature. Therefore, it is practically nontoxic. However, a high cyclodextrin concentration can be harmful to the kidney [44, 45]. Thus, cyclodextrin is non-toxic if used in safe concentration ranges and regarded as safe for drug complexation.

Characterization of the extract

This research is a continuation of previous research [12], so the same extract (GPLE) was used in this study. The yield of the GPLE obtained from the extraction process was 4.52% w/w, with a total flavonoid content of 1.79 ± 0.02% w/w (n = 6). The flavonoid content in the formulation was used to calculate each dose for the treatment group, which deem responsible for the anti-inflammatory and anti-haemorrhoid activity.

Characterization of the formulations

The particle size of the GPLE-P was 743.3 ± 33.3 nm, while the GPLE-CDIC was 340.1 ± 89.9 nm (Table III), thus both formulations can be categorized as nanoparticles formulation. The particle size homogeneity was determined based on PDI value. The particles in both formulations were shown in nanometer size.

Table III

Physical characteristics of GPLE nano-vesicles formulation

Sample	Particle Size (nm)	Zeta Potential (mV)	Polydispersity Index	Entrapment Efficiency (%)
GPLE-P	743.3 ± 33.3	-34.6 ± 2.47	0.455 ± 0.028	94.30 ± 1.91
GPLE-CDIC	340.1 ± 89.9	-31.3 ± 0.68	0.344 ± 0.002	97.7 ± 0.57

The PDI of the both formulations were in range of 0.1 - 0.4, 0.399 ± 0.028 for GPLE-P and 0.344 ± 0.002 for GPLE-CDIC (Table III); thus, they were grouped as moderately polydisperse [46]. Zeta potential is the surface charge of the particles used to determine the stability of the particles in the suspension. It showed the tendency of the particles to aggregate and predicted their biological performance. The phytosome and cyclodextrin complex particles were negatively charged; thus, they will attract to the inflammation site and had haemo-compatible characteristic. The zeta potential value of both formulations was above ± 30 mV, *i.e.*, -34.6 ± 2.47 mV and -31.3 ± 0.68 mV, for GPLE-P and GPLE-CDIC (Table III), respectively. It indicated that the particles had a good stability profile because they had good repulsion force [47, 48]. Entrapment efficiency (EE) was used to determine the amount of the extract encapsulated in the phytosome and cyclodextrin inclusion complex. The entrapment efficiency of the GPLE-P and GPLE-CDIC were 94.3 ± 1.91% and 97.7 ± 0.57% (Table III), respectively. This result showed that the formulations efficiently entrap or carry the herbal extract with EE more than 90%.

There were no identical peaks found in the end product (GPLE-P) and each of the components used in the formulation of the phytosomes (GPLE, phosphatidylcholine, Cab-o-Sil and PMP), which indicated that there were interactions between GPLE and excipients in the formulation (Figures 1a - 1c and 1e). However, the physical mixture of the phytosome still showed some characteristic of the individual excipients that was part of the formulation, such as peaks at 111.77°C and 254.65°C (Figure 1d), which is similar to the peaks shown in the phosphatidylcholine and Cab-o-Sil thermogram with slightly shifted temperature, 115.31°C (Figure 1b) and 247.99°C (Figure 1c) respectively. At the same time, it did not show any peak related to GPLE, which had peaks at 146.33°C and 196.16°C (Figure 1a). It was different compared to a physical mixture of cyclodextrin inclusion complex (PM-CDIC), which still had the characteristics of the extract, as it showed slightly shifted peaks at 151.70°C and 197.86°C (Figure 1g). The phytosome (GPLE-P) and cyclodextrin inclusion (GPLE-CDIC) thermogram showed that both had one peak at 86.03°C (Figure 1e) and 138.70°C (Figure 1h), respectively, which means that there was the interaction between the excipients and the extract.

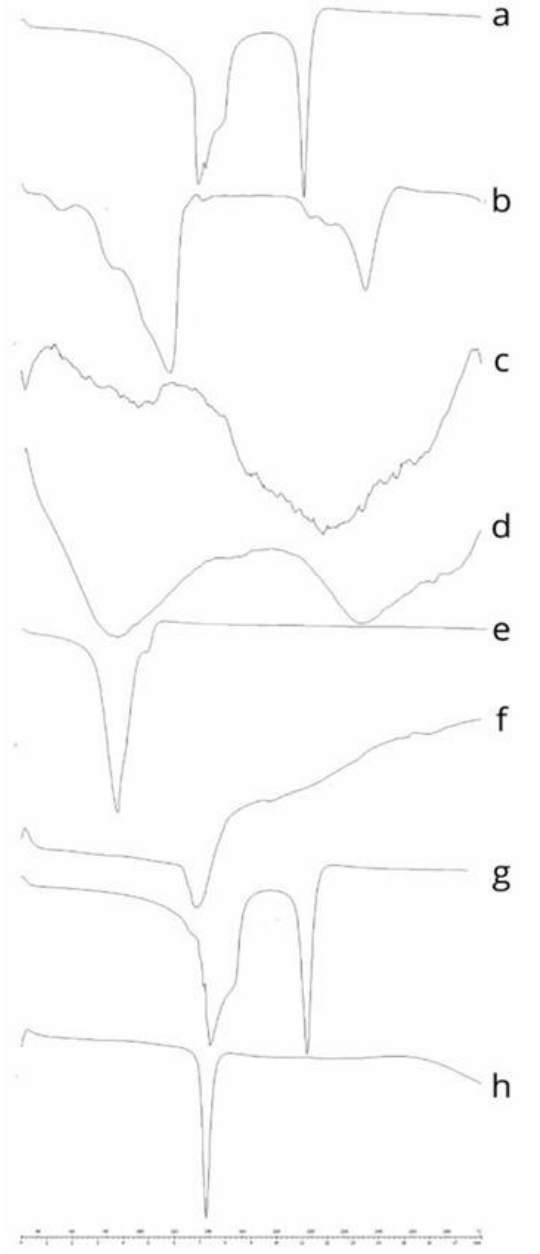

Figure 1.
Thermogram of (a) GPLE, (b) phosphatidylcholine, (c) Cab-o-Sil, (d) PM-P, (e) GPLE-P, (f) β-CD, (g) PM-CDIC and (h) GPLE-CDIC

The observation of the particles through SEM showed that both vesicular systems were spherical and had rigid structures (Figure 2). Particle sizes in both formulations were at the nanometer level. Smaller particle size leads to higher interfacial surface area for drug absorption.

The vesicular systems formation was confirmed using FTIR spectroscopy by comparing the spectrum of the vesicle with the individual components. Figure 3 shows the FTIR spectra of GPLE, β-CD, GPLE-CDIC, phosphatidylcholine, Cab-o-Sil and GPLE-P. The GPLE spectrum shows frequencies at 3302 cm^{-1} which indicate the presence of the stretching vibration of ν[O-H] from the phenolic compound, 2922 cm^{-1} stretching vibrations of aliphatic ν_{as}[C-H], aromatic stretching of ν[C-H] at 1416 cm^{-1} from a flavonoid, asymmetric stretching of ν_{a}[C-O] at 1148 cm^{-1} for esters compounds and out-of-plane bending of δ[C-H] at 775 cm^{-1}. Meanwhile, the phosphatidylcholine spectrum had a [C=O] stretching band of unsaturated aldehydes and ketones at 1662 cm^{-1}, the [C-H_2] scissoring band at 1401 cm^{-1}, and the stretching vibration of [P=O] at 1181 cm^{-1}. The characteristic Cab-O-Sil is a large peak at 3465 cm^{-1} representing [O-H] stretching from silanol groups and asymmetric stretching vibrations of a broad, strong peak at 1212 - 1076 cm^{-1} from [Si-O-Si]. The spectrum of phytosomal formulation in Figure 3 showed that it still had the characteristic of phosphatidylcholine at 709 cm^{-1}, 873 cm^{-1}, 963 cm^{-1} and 1403 cm^{-1}, while the frequency that represents GPLE and Cab-o-Sil were disappeared. It was observed that β-CD had frequencies at 3326 cm^{-1}, 2936 cm^{-1}, 1608 cm^{-1}, 1172 cm^{-1}, 1108 cm^{-1} and 979 cm^{-1} which corresponds to stretching vibration of ν[O-H] from β-CD, stretching vibration of ν_{as}[C-H], bending vibration of δ[O-H], glucosidic stretching vibration of ν_{S}[C–O-C], stretching vibration of ν[C-C] and bending vibration of ν[C-O] respectively (Figure 3B). However, all absorption peaks of β-CD and GPLE disappeared, and only a faint out-of-plane bending of δ[C-H] at 713 cm^{-1} was observed (Figure 3C), which provides an important indication of the production of GPLE-CDIC.

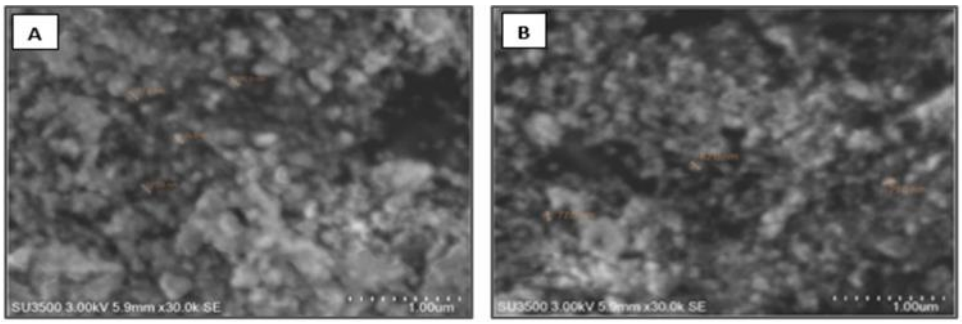

Figure 2.
SEM of (A) GPLE-P dan (B) GPLE-CDIC

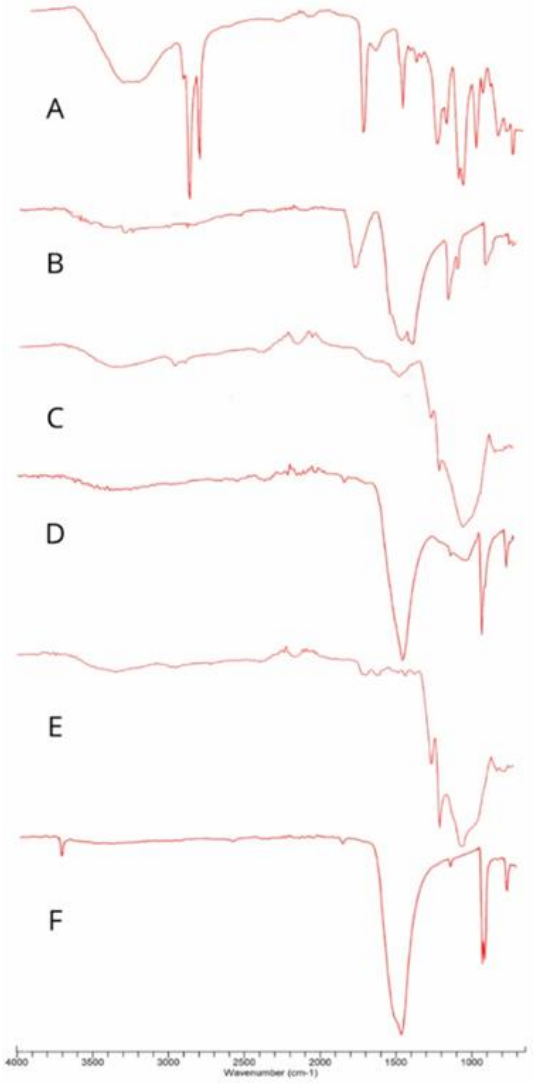

Figure 3.
FTIR spectra of (A) GPLE, (B) β-CD, (C) GPLE-CDIC, (D) phosphatidylcholine, (E) Cab-o-Sil and (F) GPLE-P

The formation of the final products was also confirmed using FTIR spectroscopy. It showed that in the GPLE-P, the characteristic of phosphatidylcholine at 709 cm^{-1}, 873 cm^{-1}, 963 cm^{-1} and 1403 cm^{-1} was still observed, while the frequency that represents GPLE and Cab-o-Sil were disappeared as there were no peaks in the same wavelength. Meanwhile, in GPLE-CDIC, all absorption peaks of β-CD and GPLE disappeared, only δ[C-H] at 713 cm^{-1} was observed (Figure 3C), which provides an important indication of the interaction between all ingredients in the formation of the cyclodextrin inclusion complex, GPLE-CDIC.

Toxicity Studies

Erythrocyte has an important role as a gas carrier in the body. It has deformable and elastic characteristics to sustain its passage through narrow capillaries of the microvasculature [49]. Normal erythrocyte has a flexible biconcave shape, and the cell morphology can be altered into stomatocytes or echinocytes when exposed to the addition of amphiphiles or various agents, variation of the electrolyte concentration, the medium's pH increase and temperature changes [50, 51]. Previous studies reported that amphipathic molecules and extract could interact with outer phospholipids monolayer of erythrocyte membrane and induce morphological changes [51, 52]. It is assumed that echinocyte spicules on the outer membrane form due to the agent inducing erythrocytes' tendency to form convex structures. Conversely, the expansion of the inner membrane favours the formation of stomatocytes [50].

The erythrocyte morphology can be of several types: (A) normal, (B) echinocyte, and (C) stomatocyte, which can be acknowledged in Figure 4.

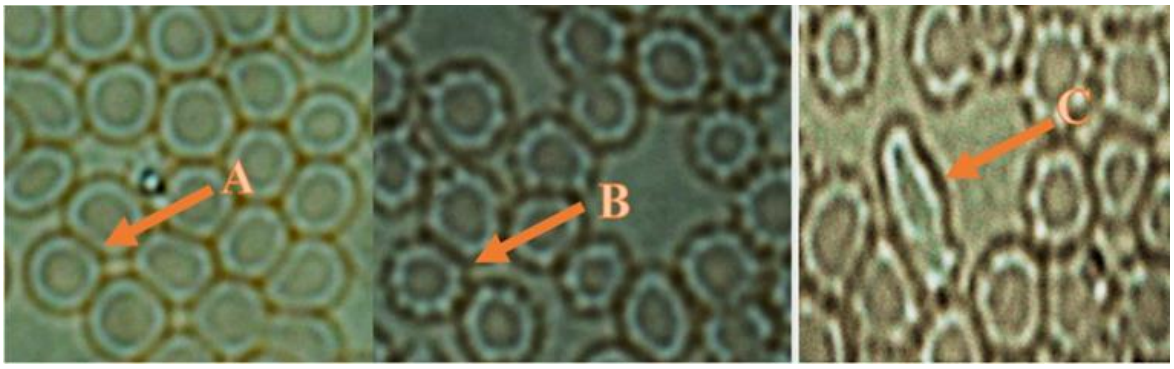

Figure 4.
Erythrocyte morphology: (A) normal, (B) echinocyte, and (C) stomatocyte

This study shows that GPLE and its nanovesicles formulation can induced an erythrocyte morphological change in a dose-response manner (Table IV). The erythrocyte morphology changes counted as the percentage of echinocyte or stomatocyte *per* normal erythrocyte. *In vitro* toxicity test showed morphology changes of the erythrocyte when treated with *S. rarak* extract at 125 μg/mL and higher concentrations. The erythrocyte morphology was changed into echinocyte when blood was treated with GPLE and GPLE-P at

a concentration of 500 µg/mL and higher, while in GPLE-CDIC, it showed a concentration of 250 µg/mL and more increased. There was a significant difference between GPLE, GPLE-P and GPLE-CDIC at 500 µg/mL. GPLE-CDIC and *S. rarak* showed no significant difference at 500 µg/mL in terms of the percentage of echinocyte, while there was no stomatocyte in the GPLE-CDIC compared to *S. rarak* treated group. At 1000 µg/mL, the echinocyte of *S. rarak* was significantly higher than in other treated groups, but the stomatocyte of *S. rarak* was the only one that showed the changes.

Table IV

Erythrocyte morphology changes of rat blood *in vitro* toxicity test of the GPLE and its nanovesicles formulation

Samples	1000 µg/mL		500 µg/mL		250 µg/mL		125 µg/mL		62.5 µg/mL	
	Percentage per normal erythrocyte (%)									
	E	S	E	S	E	S	E	S	E	S
C (+): *S. rarak*	10.41 ± 0.14^{b}	13.25 ± 0.22^{a}	7.27 ± 0.34^{a}	6.06 ± 0.25^{a}	8.87 ± 0.15^{a}	0	10.78 ± 0.36^{a}	0	0	0
C (-): DMSO 0.5%	0^{e}	0^{b}	0^{d}	0^{b}	0^{c}	0	0^{b}	0	0	0
GPLE	2.02 ± 0.12^{d}	0^{b}	1.63 ± 0.25^{c}	0^{b}	0^{c}	0	0^{b}	0	0	0
GPLE-P	6.69 ± 0.15^{c}	0^{b}	3.41 ± 0.15^{b}	0^{b}	0^{c}	0	0^{b}	0	0	0
GPLE-CDIC	11.76 ± 0.14^{a}	0^{b}	7.36 ± 0.17^{a}	0^{b}	3.65 ± 0.12^{b}	0	0^{b}	0	0	0

E = Echinocyte; S = Stomatocyte; The number in each sample followed by the same letter are not significantly different at the 0.05 level of LSD's multiple comparison tests. The sample (GPLE, GPLE-P, GPLE-CDIC) dose of 1000 µg/L means the weight of the sample, which is equivalent to 1000 µg/L of total flavonoids

The morphological changes were more obvious in the higher concentration of treated groups. GPLE-CDIC had a significantly higher echinocyte percentage than the *S. rarak*, although there was no stomatocyte in the GPLE-CDIC group at a concentration of 1000 µg/mL. Meanwhile, the GPLE-P group had significantly lower toxicity than the positive control group, although it was still significantly higher than GPLE at 500 and 1000 µg/mL. It was assumed that β-cyclodextrin causes erythrocytes' haemolysis as the membrane disruption effect, which elicited the removal of membrane components from erythrocytes [53].

The *in vivo* toxicity test showed no toxicity, as there were no erythrocyte morphology changes (Table V), either in GPLE, GPLE-P, or GPLE-CDIC formulation, at a concentration up to 40 mg/kg bw. The positive control showed toxicity at the concentration of 10 mg/kg bw.

Table V

Erythrocyte morphology changes of rat blood in acute toxicity test of the GPLE and its nanovesicles formulation

Samples	10 mg/kg bw		20 mg/kg bw		40 mg/kg bw	
	Percentage per normal erythrocyte (%)					
	E	S	E	S	E	S
C (+): *S. rarak*	43.18 ± 0.35^{a}	0	56.17 ± 0.61^{a}	0	*	*
C (-): DMSO 0.5%	0^{b}	0	0^{b}	0	0	0
GPLE	0^{b}	0	0^{b}	0	0	0
GPLE-P	0^{b}	0	0^{b}	0	0	0
GPLE-CDIC	0^{b}	0	0^{b}	0	0	0

E = Echinocyte; S = Stomatocyte; * = not tested; The number in each sample followed by the same letter are not significantly different at the 0.05 level of LSD's multiple comparison tests. The sample (GPLE, GPLE-P, GPLE-CDIC) dose of 10 mg/kg means the weight of the sample, which is equivalent to 10 mg of total flavonoids

Table VI

Differential blood cell count values of rats in acute toxicity test of the GPLE and its nanovesicles formulation

Sample	Blood Cell Counts				
	Erythrocyte (10^{6}/µL)	Haemoglobin (g/dL)	Haematocrit (%)	Leucocyte (10/µL)	Thrombocyte (10/µL)
Untreated group	4.42 ± 0.12^{a}	10.68 ± 0.36^{b}	43.84 ± 0.93^{b}	7.72 ± 0.72^{a}	113.80 ± 0.76^{c}
GPLE 40 mg/kg bw	4.60 ± 0.10^{a}	8.43 ± 0.07^{c}	39.49 ± 0.16^{c}	8.29 ± 0.11^{a}	139.36 ± 0.17^{b}
GPLE-P 40 mg/kg bw	4.40 ± 0.08^{a}	10.98 ± 0.36^{ab}	48.94 ± 0.60^{a}	7.74 ± 0.65^{a}	139.06 ± 0.68^{b}
GPLE-CDIC 40 mg/kg bw	4.54 ± 0.12^{a}	11.74 ± 0.15^{a}	48.52 ± 0.22^{a}	7.44 ± 0.72^{a}	141.96 ± 0.88^{a}

The number in each sample followed by the same letter are not significantly different at the 0.05 level of LSD's multiple comparison tests

The result of blood cell count analysis (Table VI), in terms of erythrocyte and leucocyte number, showed no significant differences ($p > 0.05$) between the GPLE, GPLE-P and GPLE-CDIC groups compared to the untreated group. It was shown that the haemoglobin in the GPLE-P treated group showed no significant difference from the untreated group. While the GPLE-CDIC was comparable to GPLE-P, it was statistically higher than the untreated group. Meanwhile, the GPLE group showed a significant

decrease in haemoglobin concentration compared to the other group. Additionally, the percentage of haematocrit in the GPLE group was the lowest. Meanwhile, both formulations were significantly higher than the untreated group. In terms of thrombocyte concentration, all treated groups showed a significant increase compared to the control, and the highest concentration was in the GPLE-CDIC group.

The GPLE-P and GPLE-CDIC showed improvement in toxicity profile compared to GPLE group in terms of haemoglobin, haematocrit and leucocyte concentration. This result was following a study by Kubota *et al*. that reported the non-toxic effect of orally administered β-cyclodextrin to rats when they were fed for 52 weeks. Orally administered β-cyclodextrin had an absorption rate of 0.6%, and within 10 hours, about 90% will be excreted *via* urine without undergoing relevant metabolism in rats [54]. The lipid-based vesicle, phytosome, consists of phospholipids, mainly phosphatidylcholine. It has excellent biocompatibility and a similar structure to the cellular membrane [55]. Therefore, this vesicular system has good tissue compatibility and can reduce drug toxicity due to the decreasing dose of the active compounds.

Conclusions

This study showed that the phytosomal and cyclodextrin inclusion complex formulation of GPLE had a comparable safety profile compared to the untreated group and a slightly better toxicity profile compared to GPLE. The nanovesicles were prepared and characterized for various physicochemical parameters. The characterization showed that GPLE formed a complex with either lipid vesicle or β-cyclodextrin. GPLE had a slightly better safety profile than GPLE-CDIC and GPLE-P when tested *in vitro* using rat blood. GPLE showed a lower number of erythrocyte morphological changes at the concentration of 500 μg/mL compared to GPLE-P. Still, the erythrocyte was affected by GPLE-CDIC at a lower concentration of 250 μg/mL. However, no significant differences were observed when the nanoformulations were tested *in vivo* in rats. Henceforth, further study is essential to examine the effect of GPLE formulation on rat blood, *in vivo* release profile, and pharmacokinetics of the phytoconstituent.

Acknowledgement

This work was supported by Hibah Riset Mandat grant from Universitas Airlangga, Republic of Indonesia (Grant no. 886/UN3/2018).

Conflict of interest

The authors declare no conflict of interest.

References

1. Denny KH, Stewart CW, Chapter 5 - Acute, Subacute, Subchronic, and Chronic General Toxicity Testing for Preclinical Drug Development. In: Faqi AS, editor. A Comprehensive Guide to Toxicology in Nonclinical Drug Development (2nd Edition). Boston: Academic Press; 2017; 109-127.
2. Knöss W, Toxicity of Herbal Medicines: From Past to Present to Future. In: Pelkonen O, Duez P, Vuorela PM, Vuorela H, editors. Toxicology of Herbal Products. Cham: Springer International Publishing; 2017; 1-9.
3. Mensah MLK, Komlaga G, Forkuo AD, Firempong C, Anning AK, Dickson RA, Toxicity and Safety Implications of Herbal Medicines Used in Africa. IntechOpen; 2019; 63-86.
4. Jothy SL, Zakaria Z, Chen Y, Lau YL, Latha LY, Sasidharan S, Acute Oral Toxicity of Methanolic Seed Extract of Cassia fistula in Mice. *Molecules*, 2011; 16(6): 5268-5282.
5. Olagbende-Dada SO, Ukpo GE, Coker HAB, Adesina SA, Oxytocic and anti-implantation activities of the leaf extracts of *Graptophyllum pictum* (Linn.) Griff. (*Acanthaceae*). *Afr J Biotechnol.*, 2009; 8(21): 5979-5984.
6. Srinivasan KK, Mathew JE, A.D'Silva KJ, Lobo R, Kumar N, Nephroprotective potential of *Graptophyllum pictum* against renal injury induced by gentamicin. *Iran J Basic Med Sci.*, 2015; 18(4): 412-416.
7. Srinivasan KK, Mathew JE, Joseph K, Vachala SD, Malini S, Effect of ethanol extract of *Graptophyllum pictum* (L.) Griff. on cisplatin induced nephrotoxicity in rats. *Herba Pol.*, 2011; 57(2): 15.
8. Singh P, Khosa RL, Mishra G, Pharmacognostical evaluation of aerial parts of *Graptophyllum pictum* (L.) Griff. (Syn: *Justicia picta* Linn.): A well-known folklore medicinal plant. *Anc Sci Life*, 2015; 34(4): 223-229.
9. Makkiyah F, Rahmi EP, Revina R, Susantiningsih T, Setyaningsih Y, *Graptophyllum pictum* (L.) Griff. (Syn: *Justicia picta* Linn.) and its Effectiveness: A Well-Known Indonesian Plant. *Pharmacogn J.*, 2021; 13(3): 835-838.
10. Astana PRW, Nisa U, Triyono A, Ardiyanto D, Fitriani U, Zulkarnain Z, adwaita KP, Medicinal plants used by traditional healers for hemorrhoid treatment in Borneo Island: Ethnopharmacological study. *IOP Conf Ser Earth Environ Sci.*, 2021; 913(1): 12097.
11. Hutagalung MSB, Budiono BP, Prasetyo SA, Riwanto I, Nugroho EA, Prajoko YW, Susilaningsih N, Phlebotrophic Effect of *Graptophyllum Pictum* (L.) Griff on Experimental Wistar Hemorrhoids. *J Biomed Transl Res.*, 2019; 5(1): 1-4.
12. Kusumawati I, Rullyansyah S, Rohmania, Rizka AF, Hestianah EP, Matsunami K, Histomorphometric study of ethanolic extract of *Graptophyllum pictum* (L.) Griff. leaves on croton oil-induced hemorrhoid mice: A Javanese traditional anti-hemorrhoid herb. *J Ethnopharmacol.*, 2022; 284(October 2021): 114765.
13. Azhar A, Riwanto I, Nugroho EA, Susilaningsih N, Prajoko YW, Budiono P, Prasetyo SA, Antioxidant and Anti-inflammatory effect of *Graptophyllum pictum* (L.) Griff extract Study on SOD and COX-2 serum

of experimental hemorrhoids. *Medica Hosp J Clin Med.*, 2020; 7(2): 422-426.
14. Kusumawati I, Maat S, Hafid AF, The Influence of Etanolic Extract of *Graptophyllum pictum* (L.) Griff Leaves on Non Spesific Immune Responces. Surabaya, Indonesia; 1997.
15. Kusumaningsih T, Firdausi A, Diyatri I, Ridwan RD, Arundina I, Yuliati, Antioxidant Effects of *Graptophyllum pictum* Leaf Extract on Malondialdehyde (MDA) Levels of Mice Induced By a Toxic Dose of Paracetamol. *J Krishna Inst Med Sci.*, 2018; 7(3): 59-64.
16. Ozaki Y, Sekita S, Soedigdo S, Harada M, Antiinflammatory Effect of *Graptophyllum pictum* (L.) Griff. *Chem Pharm Bull.*, 1989; 37(10): 2799-2802.
17. Wahyuningtyas E, The *Graptophyllum pictum* extract effect on acrylic resin complete denture plaque growth. *Dent J (Majalah Kedokt Gigi)*, 2005; 38(4): 201-204.
18. Olagbende-Dada SO, Ogbonnia SO, Coker HAB, Ukpo GE, Blood glucose lowering effect of aqueous extract of *Graptophyllum pictum* (Linn) Griff. on alloxan-induced diabetic rats and its acute toxicity in mice. *African J Biotechnol.*, 2011; 10(6): 1039-1043.
19. La Torre F, Nicolai AP, Clinical use of micronized purified flavonoid fraction for treatment of symptoms after hemorrhoidectomy: results of a randomized, controlled, clinical trial. *Dis Colon Rectum.*, 2004; 47(5): 704-710.
20. Sandhiya V, Ubaidulla U, A review on herbal drug loaded into pharmaceutical carrier techniques and its evaluation process. *Future J Pharm Sci.*, 2020; 6(1): 51.
21. Lohsiriwat V, Hemorrhoids: From basic pathophysiology to clinical management. *World J Gastroenterol.*, 2012; 18(17): 2009-2017.
22. Alharbi WS, Almughem FA, Almehmady AM, Jarallah SJ, Alsharif WK, Alzahrani NM, Phytosomes as an Emerging Nanotechnology Platform for the Topical Delivery of Bioactive Phytochemicals. *Pharmaceutics*, 2021; 13(9): 1475.
23. Barani M, Sangiovanni E, Angarano M, Rajizadeh MA, Mehrabani M, Piazza S, Gangadharappa HV, Pardakhty A, Mehrbani M, Dell'Agli M, Nematollahi MH, Phytosomes as Innovative Delivery Systems for Phytochemicals: A Comprehensive Review of Literature. *Int J Nanomed.*, 2021; 16: 6983.
24. Onoue S, Yamada S, Chan HK, Nanodrugs: pharmacokinetics and safety. *IJN.*, 2014; 9(1): 1025-1037.
25. Rahman HS, Othman HH, Hammadi NI, Yeap SK, Amin KM, Samad NA, Alitheen NB, Novel Drug Delivery Systems for Loading of Natural Plant Extracts and Their Biomedical Applications. *Int J Nanomed.*, 2020; 15: 2439.
26. Del Valle EMM, Cyclodextrins and their uses: a review. *Process Biochem.*, 2004; 39(9): 1033-1046.
27. Yao Y, Xie Y, Hong C, Li G, Shen H, Ji G, Development of a myricetin/hydroxypropyl-β-cyclodextrin inclusion complex: preparation, characterization, and evaluation. *Carbohydr Polym.*, 2014; 110: 329-337.
28. Christ B, Muller K, For the serial determination of the content of flavonol derivatives in drugs. *Arch Pharm (Weinheim)*, 1960; 293: 1033-1042, (available in German).
29. Jafari S, Moradi A, Salaritaba A, Hadjiakhoo A, Khanavi M, Determination of Total Phenolic and Flavonoid Contents of *Leonurus cardiaca* L. in Compare with Antioxidant Activity. *Res J Biol Sci.*, 2010; 5(7): 484-487.
30. Burlec AF, Corciova A, Vlase AM, Vlase L, Mircea C, Tuchiluș C, Furnica C, Sha'at F, Robu S, Cioanca O, Hancianu M, Phytochemical composition and *in vitro* biological properties of several *Rudbeckia hirta* and *Tagetes erecta* flower extracts. *Farmacia*, 2022; 70(2): 241-247.
31. Rasaie S, Ghanbarzadeh S, Mohammadi M, Hamishehkar H, Nano phytosomes of quercetin: A promising formulation for fortification of food products with antioxidants. *Pharm Sci.*, 2014; 20(3): 96-101.
32. Martinez-Zapata MJ, Vernooij RWM, Simancas-Racines D, Uriona Tuma SM, Stein AT, Moreno RM, Vargas E, Capellà D, Cosp XB, Phlebotonics for venous insufficiency. *Cochrane Database Syst Rev.*, 2020; 2020(11): CD003229.
33. Misra MC, Imlitemsu, Drug treatment of haemorrhoids. *Drugs*, 2005; 65(11): 1481-1491.
34. Chiaretti M, Fegatelli DA, Pappalardo G, Venti MDS, Chiaretti AI, Comparison of *Centella* with Flavonoids for Treatment of Symptoms in Hemorrhoidal Disease and After Surgical Intervention: A Randomized Clinical Trial. *Sci Rep.*, 2020; 10(1): 8009.
35. Godeberge P, Sheikh P, Lohsiriwat V, Jalife A, Shelygin Y, Micronized purified flavonoid fraction in the treatment of hemorrhoidal disease. *J Comp Eff Res.*, 2021; 10(10): 801-813.
36. Sheikh P, Lohsiriwat V, Shelygin Y, Micronized Purified Flavonoid Fraction in Hemorrhoid Disease: A Systematic Review and Meta-Analysis. *Adv Ther.*, 2020; 37(6): 2792-2812.
37. Zagriadskiĭ EA, Bogomazov AM, Golovko EB, Conservative Treatment of Hemorrhoids: Results of an Observational Multicenter Study. *Adv Ther.*, 2018; 35(11): 1979-1992.
38. Oteiza PI, Fraga CG, Mills DA, Taft DH, Flavonoids and the gastrointestinal tract: Local and systemic effects. *Mol Aspects Med.*, 2018; 61: 41-49.
39. Zhang X, Xing H, Zhao Y, Ma Z, Pharmaceutical dispersion techniques for dissolution and bioavailability enhancement of poorly water-soluble drugs. *Pharmaceutics*, 2018; 10(3): 74.
40. Coc LMC, Lacatusu I, Badea N, Penes O, Cobelschi CP, Pop A, Meghea A. Curcumin co-loaded with a lipid mediator in the same nanostructured lipid delivery system *Farmacia*, 2022; 70(5): 932-943.
41. Conceicao J, Adeoye O, Cabral-Marques HM, Lobo JMS, Cyclodextrins as Drug Carriers in Pharmaceutical Technology: The State of the Art. *Curr Pharm Des.*, 2018; 24(13): 1405-1433.
42. Davis ME, Brewster ME, Cyclodextrin-based pharmaceutics: past, present and future. *Nat Rev Drug Discov.*, 2004; 3(12): 1023-1035.
43. EMA C for HMP (CHMP). Cyclodextrins used as excipients [Internet]. EMA; 2017.
44. Luke DR, Tomaszewski K, Damle B, Schlamm HT, Review of the basic and clinical pharmacology of sulfobutylether-beta-cyclodextrin (SBECD). *J Pharm Sci.*, 2010; 99(8): 3291-3301.
45. Stella VJ, He Q, Cyclodextrins. *Toxicol Pathol.*, 2008; 36(1): 30-42.

46. Bhattacharjee S, DLS and zeta potential - What they are and what they are not?. *J Control Release*, 2016; 235: 337-351.
47. Carrillo C, Sánchez-Hernández N, García-Montoya E, Pérez-Lozano P, Suñé-Negre JM, Ticó JR, Suñé C, Miñarro M, DNA delivery *via* cationic solid lipid nanoparticles (SLNs). *Eur J Pharm Sci.*, 2013; 49(2): 157-165.
48. Fathi M, Varshosaz J, Mohebbi M, Shahidi F, Hesperetin-loaded solid lipid nanoparticles and nanostructure lipid carriers for food fortification: preparation, characterization, and modeling. *Food Bioprocess Technol.*, 2013; 6(6): 1464-1475.
49. Geekiyanage NM, Balanant MA, Sauret E, Saha S, Flower R, Lim CT, Gu YT, A coarse-grained red blood cell membrane model to study stomatocyte-discocyteechinocyte morphologies. *PLoS One*, 2019; 14(4): 1-25.
50. Tachev KD, Danov KD, Kralchevsky PA, On the mechanism of stomatocyte-echinocyte transformations of red blood cells: Experiment and theoretical model. *Colloids Surfaces B Biointerfaces*, 2004; 34(2): 123-140.
51. Taib IS, Budin SB, Siti Nor Ain SM, Mohamed J, Louis SR, Das S, Sallehudin S, Rajab NF, Hidayatulfathi O, Toxic effects of *Litsea elliptica* Blume essential oil on red blood cells of Sprague-Dawley rats. *J Zhejiang Univ Sci B.*, 2009; 10(11): 813-819.
52. Suwalsky M, Vargas P, Avello M, Villena F, Sotomayor CP, Human erythrocytes are affected *in vitro* by flavonoids of *Aristotelia chilensis* (Maqui) leaves. *Int J Pharm.*, 2008; 363(1-2): 85-90.
53. Irie T, Otagiri M, Sunada M, Uekama K, Ohtani Y, Yamada Y, Sugiyama Y, Cyclodextrin-Induced Hemolysis and Shape Changes of Human Erythrocytes *in Vitro*. *J Pharmacobiodyn.*, 1982; 5(9): 741-744.
54. Kubota Y, Fukuda M, Muroguchi M, Koizumi K, Absorption, distribution and excretion of beta-cyclodextrin and glucosyl-beta-cyclodextrin in rats. *Biol Pharm Bull.*, 1996; 19(8): 1068-1072.
55. Li J, Wang X, Zhang T, Wang C, Huang Z, Luo X, Deng Y, A review on phospholipids and their main applications in drug delivery systems. *Asian J Pharm Sci.*, 2015; 10(2): 81-98.