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RUTIN ETHOSOMES BY SOLE SOLVENT INJECTION: PREPARATION METHOD, PHYSICAL PROPERTIES, DRUG LOADING, DEFORMATION, AND *IN-VITRO* MEMBRANE PERMEABILITY

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Summary

Rutin is a significantly familiar Vietnamese herbal compound, from *Sophora japonica L*. However, its relatively large molecule, low solubility in water, and poor skin permeability hinder its topical permeability and absorption. Nanovesicles, especially ethosomes, are considered potential skin delivery vehicles with deformable ability. In this research, only solvent injection was used to fabricate rutin ethosomes. Various polyols, like propylene glycol, polyethylene glycol 200, Transcutol, and glycerol and mixtures of them, at various ratios, were investigated in rutin binary ethosome preparation. Then, rutin ethosomes were characterized by physical properties, loading efficiency, and deformability. The optimum binary ethosomes were made from ethanol, glycerol, and propylene glycol with a size below 120 nm and uniform, with a PDI of approximately 0.1. After deformation, rutin ethosomes had smaller sizes, narrower distributions, and retained entrapment efficiencies of nearly 90%, indicating the flexibility of rutin ethosome structure. Drug release after 24 hours in an *in vitro* dermal model was improved the highest in binary ethosomes - nearly 5-fold, followed by classical ethosomes with approximately a 3-fold increase, compared with pure rutin. Therefore, these rutin nanovesicles were successfully prepared with favourable physical properties, high entrapment efficiency, an elastic structure, and significantly higher release for skin delivery. Facile solvent injection, with controlled temperature and duration, followed by simple filtration through an appropriate pore size, which is a potential method for larger-scale production of binary ethosomes.

Keywords: *Ethosomes; Solvent injection; Binary Ethosomes; Rutin; Deformation.*

1. Introduction

For centuries, herbal medicines have played a crucial role in treating various illnesses. In recent pharmaceutical advancements, natural bioactive compounds sourced from plants have gained attention as alternative therapeutic agents and dietary supplements aimed at promoting health, curing diseases, and preventing pathological conditions [1]. Rutin is a significantly familiar Vietnamese herbal compound from *Sophora japonica* L., and is traditionally used in daily life, functional food, and even pharmaceuticals. Rutin exhibits diverse pharmacological effects, such as protecting the kidneys and liver, supporting brain function, reducing inflammation, managing diabetes, combating cancer, and lowering blood pressure. In dermatology, rutin is an antioxidant, a free radical scavenger, an oxidative - stress preventer, and a collagen synthesis modulator, which enables its usage for skin anti-aging and skin protection [2]. Moreover, rutin is antibacterial [3], wound-healing [4], skin-whitening [5], and UV-protective, also strengthens the UV-blocking ability of sun protection factor (SPF) agents [6], which has been widely applied in skin products. Despite its many advantages, rutin, like many plant-based actives, faces limitations in practical use. Its relatively large molecule, low solubility in water, and poor skin permeability hinder its ability to be effectively absorbed when applied topically.

Nanotechnology is one powerful technique to enhance the bioavailability of herbal bioactive compounds, enable targeted delivery to specific biological sites, and regulate the rate of drug release [1]. Among nanocarriers, ethosomes are considered one of the most outstanding vehicles for dermal delivery [7]. Ethosomes are advanced lipid-based carriers specifically designed to enhance skin drug delivery. Their unique composition, particularly the high ethanol content, imparts remarkable deformability, allowing the vesicles to squeeze through the narrow intercellular spaces of the stratum corneum. Additionally, ethanol can disrupt skin lipid membrane structure, significantly improving transdermal and dermal penetration, making ethosomes highly effective for delivering both hydrophilic and lipophilic drugs into deeper skin layers. Moreover, ethosomes can encapsulate large molecules such as peptides, offer sustained drug release, and protect sensitive compounds from degradation. These properties make them a promising and convenient system for topical and transdermal therapies [8],[9].

Ethosomes, with the involvement of other polyols, such as propylene glycol (PG) [7],[10],

isopropanol (IPA) [11], and glycerol (G) [12], are called binary ethosomes. Binary ethosomes have greater stability and drug permeability than classical ethosomes [10],[13]. Specifically, binary ethosomes increased *ex vivo* permeability by sevenfold, and 1.5 folds in *in vivo* anti-inflammatory [10]. Also, blending PG in phloretin binary ethosomes raised the percutaneous permeability 1.06 folds more than classical ethosomes, and 2.4 folds than PG solution [14]. The major methods for ethosome preparation are thin film hydration [13] and solvent - injection [7]. Solvent - injection is a more advantageous method for ethosome fabrication due to the simple process and scale-up [15].

However, there have been a few studies on dermal nanovesicle loading rutin, including the ethosomal system. Classical rutin ethosome had a size of 369 nm, and zeta potential (ZP) of -19.6 mV [16]. In another study, the size of the optimized rutin ethosome was 112 nm and an entrapment efficiency (EE) of 67.5% [17]. Rutin ethosome was fabricated by the cold method, with perphospholipid and only butylated hydroxytoluene, and had a size of around 650 nm and an entrapment efficiency of 76.9% [18]. The previous rutin binary ethosomes were investigated with polyols such as separated G, PG, and IPA, by solvent injection, followed by probe sonication for size reduction [19].

Hence, this study aims to broadly and closely investigate the effect of polyols on the fabrication of rutin binary ethosome by sole solvent injection method. Different polyols were used, including PG, G, polyethylene glycol 200 (PEG200), and Transcutol (T) and the mixture of them, at various ratios in combination with ethanol (Fig. 1.) to improve rutin ethosome physical properties and membrane permeability. Moreover, this study also focused on the modification of the size, homogeneity, drug loading after facile deformation, and *in vitro* membrane delivery.

2. Materials and methods

2.1. Materials

Rutin was an active pharmaceutical ingredient (CAS 153-18-4). Rutin, used as a standard for high performance liquid chromatography, was obtained from Ark Pharm with a purity of 98^{+} %. Ethanol was from Xilong Scientific Co., Ltd (China). Soybean lecithin (SPC) (>90% Phosphatidylcholine) was gifted from Lipoid, Germany. Transcutol was gifted from Gattefosse. Propylene glycol (PG), and glycerol (G), polyethylene glycol 200 (PEG 200) were from Xilong Scientific Co. Ltd (China).

2.2. Methods

2.2.1. Ethosome preparation:

Rutin ethosome samples were prepared following the solvent injection method with mild modifications [19]. Firstly, rutin was dissolved in ethanol/polyols using a water bath (IKA C-MAG HS 4). At 40°C, lecithin was added and dissolved completely to form a homogeneous organic phase. Distilled water (aqueous phase) was kept at the same temperature and added to the organic phase at the speed of 0.5 mL/min under magnetic agitation (IKA C-MAG HS 4) at 45°C for 30 mins. Finally, the temperature was reduced to 30°C and the ethosomal samples were continuously stirred for 16 hours. Finally, the products were stored in closed glass vials for further studies (Fig. 1). Rutin was fixed at the ratio of 2.5 mg/g, various lecithin ratios (w/v), ethanol ratios (w/w), and different polyols, including PG/Glycerol/PEG 2000/Transcutol were investigated to prepare ethosomes.

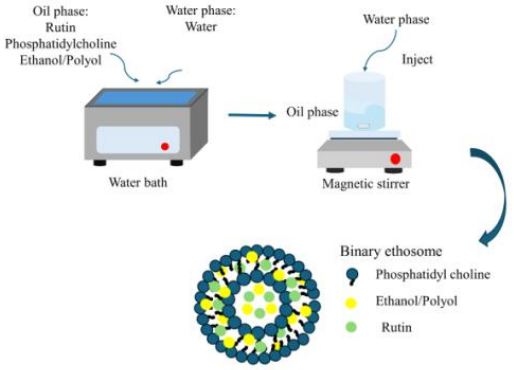

Fig. 1. Binary rutin ethosome fabrication

2.2.2. Size, polydispersity index, and zeta potential:

Size, polydispersity index (PDI), and zeta potential (ZP) of ethosome vesicles were measured by a nanoparticle analyzer (HORIBA nanoPartica SZ-100). Ethosome samples were diluted with water to ensure precise measurement when measuring size, PDI, and ZP.

2.2.3. Morphology:

The visualization of ethosome samples was investigated by transmission electron microscopy (TEM) (JEOL Inc, Jem - 1400 flash).

2.2.4. Rutin quantification:

The rutin quantification was evaluated by high performance liquid chromatography (HPLC) [16]. The stationary phase was a C_{18} reverse-phase column (150 x 4.6 mm, 5 µm), while the mobile phase was the mixture of acetic acid 1% and

acetonitrile (80:20) with an isocratic flow rate at 1 mL/min. Rutin was detected and quantified by DAD at 355 nm. Samples were measured at 355 nm (Thermo Scientific™, UltiMate™ 3000 Standard (SD) HPLC Systems). The standard concentrations were in the range of 0.1-100 µg/mL, prepared by absolute ethanol dissolution and water dilution. The collected samples were directly quantified or diluted with water if necessary.

2.2.5. Drug entrapment efficiency (EE)

EE was a value showing the content of rutin which was encapsulated in ethosomal vesicle according to the equation below:

$$EE = \frac{R_e}{R_t}.100\% = \frac{R_t - R_f}{R_t}.100\%$$

Where Re is encapsulated rutin, R_f is free rutin, and R_t is the total content of rutin.

To determine free rutin content, the ultrafiltration method was applied with a 10000 Da cutoff at the speed of 10.000 rpm in 15 min. The rutin in the filtrate was quantified by HPLC.

2.2.6. Deformation test:

The deformation of binary ethosomes was tested following previous methods [20, 21] with a slight modification. Briefly, the freshly - prepared ethosomes were deformed by going through a 0.22 µm syringe filter triple times. The deformable ethosomes were evaluated for the change in size, homogeneity, and drug loading.

2.2.7. *In vitro* membrane permeability:

The penetration ability of rutin ethosome formulations was investigated by an experiment using Franz vertical diffusion cells. Dialysis membranes with 14KDa cut-off were placed in the joint to separate donor and receptor chambers. Rutin ethosomes and rutin were added into a donor compartment at the fixed quantity of 500 µg rutin. In the receptor compartment, pH 7.4 phosphate buffer was stirred at a fixed rate. The temperature of the receptor compartments was kept at 37°±0.5°C during the experiments. At time intervals, a fixed volume of medium was collected to determine rutin concentration by the HPLC method. An equal volume of buffer was added to replace this amount of the medium.

2.2.8. Data analysis:

T-test and one-way ANOVA test to evaluate statistical significance (setting at $p < 0.05$) were performed with Microsoft Excel. Data was reported as average ± standard deviation (SD). A value of $p < 0.05$ was considered statistically significant (*). Each experiment was carried out in triplicate.

3. Results and discussion

3.1. Effect of preparation condition on rutin ethosome physical properties

3.1.1. Effect of temperature and incubation duration in oil phase preparation on rutin ethosome physical properties:

The temperature of the oil phase preparation was evaluated in the range of 35°C to 50°C. The water phase was kept at the same temperature before the injection steps. The modification of size, homogeneity, and zeta potential of rutin ethosome based on the temperature change was presented in Fig 2. The formulation of the rutin ethosome used for this assessment was 30% ethanol (w/w), with rutin and phosphatidylcholine at the molar ratio of 1:1.

As can be seen from Fig. 2A, the size of rutin ethosome decreased from 430.37±15.16 nm to 335.87±10.83 nm when the temperature went up from 35°C to 40°C, while from 40°C to 50°C, the size continuously increased up to 389.43±11.29 nm. The same trend was observed in the homogeneity of these nanovesicles, as the PDI was lowest at 40°C, 0.29±0.01. The absolute value of zeta potential may be higher at higher temperatures, gaining from above 20 mV to around 30 mV. The entrapment efficiency could be considered the same for all temperatures. Therefore, 40°C was the chosen temperature for phase preparation.

Additionally, the incubation duration of the oil phase at 40°C played an important role in rutin ethosome preparation (Fig 2B). The incubation step was necessary for the formation of a homogenous mixture for the oil phase, but also exposed to the risk of degradation and modification of phosphatidylcholine structure. Hence, the phase preparation duration strongly affected the physical properties of rutin, especially its uniformity. At 30 min, the size and PDI of rutin ethosomes were lowest, 326.07±46.54 nm and 0.27±0.03, respectively.

SPC essentially went through a glassy state (solid) to the liquid-crystalline phase (liquid), and then reached the liquid-disordered point to ensure the molecular interaction with other ingredients in rutin ethosomal formulas. The melting points of various phosphatidylcholines were often in the range of 40°C and 55°C, with several below this range [22]. Thus, approximately and above these temperatures, SPC could move freely in the solvent to unite with rutin and ethanol, as well as water, to form ethosomal structures. The phase transition of SPC, and the bonding formation

between vesicle materials, required a period of time at a certain temperature around and higher the melting point of SPC. Additionally, heat was essential in the dissolving of rutin in ethanol at the desired concentrations. Therefore, phase preparation conditions, especially for the oil phase, were at 40°C in 30 min - incubation.

3.1.2. Effect of injection temperature on rutin ethosome physical properties:

Different temperatures of injection step for rutin ethosome fabrication were evaluated in the range of 40°C to 50°C (Fig. 2C). Among them, 45°C delivered the best size and PDI, as ethosome size was 186.47±4.71 nm and PDI was below 0.3.

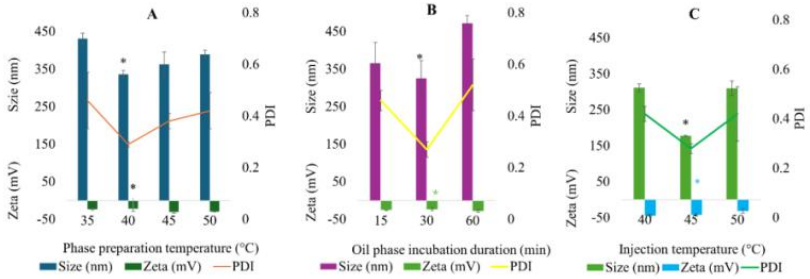

Fig. 2. Physical properties of rutin ethosomes at different phase preparation conditions: Phase preparation temperature (A), oil phase incubation duration (B), injection temperature (C)

Therefore, rutin ethosome could be fabricated by the solvent injection method with reasonable physical properties. Specifically, the oil phase was mixed and incubated at 40°C for 30 mins, and injection was set at 45°C with the injection rate of 0.5 mL/min of water phase into the oil phase.

3.2. Effect of formulation ingredients on rutin ethosome properties

3.2.1. Effect of the phosphatidylcholine ratios on rutin ethosome physical properties:

Soybean phosphatidylcholine (SPC) ratios controlled the physical properties of rutin ethosome following sole solvent injection (Fig. 3.). At the ratio SPC: rutin of 1:1, the size of rutin ethosome was smallest, 177.28±2.03 nm and more homogenous, as PDI was 0.28±0.02. Increasing SPC quantity led to larger rutin nanovesicles and less uniform systems. Based on the physical properties, the molar ratio SPC: rutin of 1:1 was chosen for further experiments.

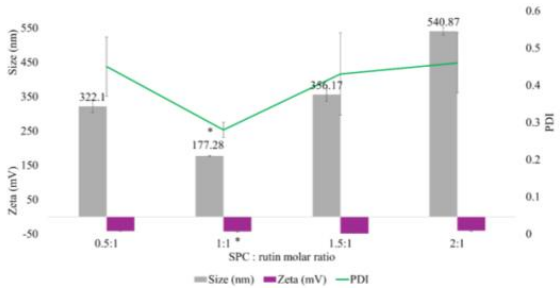

Fig. 3. Rutin ethosome physical properties at various SPC:rutin molar ratios

3.2.2. Effect of ethanol ratios on rutin ethosome physical properties:

Formulations of ethosomes were evaluated with the different ratios in the range from 27.5% to 40% (w/w) (Fig. 4). The ethanol ratios were within the range of 20 and 45 % (w/v) in ethosomal formulations [23]. The higher the ethanol ratios from 30% to 40%, the larger the size of rutin ethosomes. While with ethanol lower than 30%, these values were even worse than ethanol ratios in the range of 32.5% to 37.5%. Another rutin-loaded ethosomes, by the thin-film hydration method, had the size of 247 ± 7 nm or 369.5 ± 4.2 nm [24]. In other research, the size of the ethosomal vesicles was 634.9 nm without sonication [18], showing a significantly larger size than the resulting rutin ethosome in this study. Hence, 30% ethanol (w/w) was chosen for rutin ethosome fabrication, with a size of 177.28 ± 2.03 nm, PDI at 0.28 ± 0.02, and zeta potential of -42.4 ± 3.25 mV.

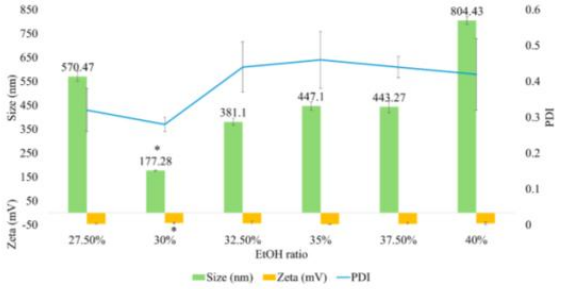

Fig. 4. Rutin ethosome physical properties at various ethanol ratios

3.2.3. Effect of polyol types on rutin ethosome properties:

Incorporation with other polyols could improve the flexibility and drug loading of ethosome. Additionally, other polyols could be considered permeability enhancers [25]. The combinations of 30% ethanol (w/w) with other polyols: G, PG, PEG200, and Transcutol at 5% (w/w) changed rutin physicochemical properties (Table 3). While PEG200 and Transcutol increased the size of rutin ethosome, G and PG considerably reduced the size of these vehicles, in agreement with the previous study [25], clearly seen in Fig. 2. Thus, G and PG were chosen for further investigation of rutin ethosome formation.

Table 3. Binary rutin ethosome properties with various polyol types

Polyol	EtOH 30%	G 5%	PG 5%	PEG200 5%	Trancutol 5%
Size (nm)	177.28 ± 2.03	171.43 ± 6.21	141.13 ± 7.65	455.10 ± 68.13	350.80 ± 43.82
PDI	0.28 ± 0.02	0.29 ± 0.07	0.25 ± 0.05	0.27 ± 0.14	0.39 ± 0.13
Zeta (mV)	-42.4 ± 3.25	-46.90 ± 4.67	-37.55 ± 0.78	-39.25 ± 0.64	-41.90 ± 1.13
EE (%)	89.67 ± 0.68	89.84 ± 0.44	90.69 ± 0.26	91.28 ± 0.26	92.13 ± 0.44

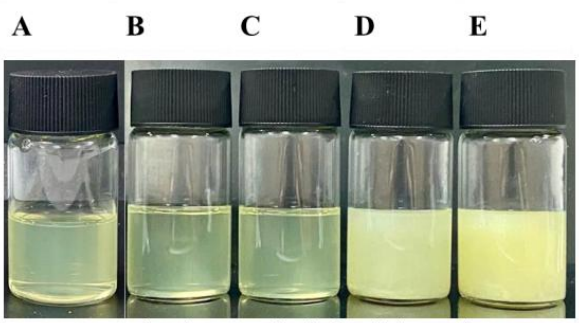

Fig. 3. Rutin ethosome: classical and binary ethosomes A. EtOH30%, B. EtOH30% + Glycerol5%, C. EtOH30% + PG5%, D. EtOH30% + PEG200 5%, E. EtOH30% + Trancutol5%

3.2.4. Effect of polyol ratios on rutin ethosome properties:

As PG and G could improve rutin ethosome physical properties, these polyols were used in higher quantities in rutin binary ethosome formulations, from 5% to 20%, in combination with 30% ethanol (w/w). The higher the ratio of PG, the larger the rutin ethosome size, and the smaller their PDI. Specifically, at 20% of PG, the size of rutin ethosomes was 192.30 ± 5.00 nm, PDI only 0.15 ± 0.08, while at 5%, the size and the PDI were 141.13 ± 7.65 nm, and 0.25 ± 0.05, respectively. In the case of G, the size significantly reduced when the ratio was up to 10%, as the size was 133.27 ± 2.97 nm at 10%, compared to 171.43 ± 6.21 nm at 5%. Thus, the combination of G and PG may improve both the size and PDI of rutin ethosomes. Table 4 shows the size and PDI of rutin consist G and PG at 5% and 10% had considerably smaller size and PDI than rutin ethosomes only fabricated from 30% ethanol. Notably, with G5%+PG 5%, the size was only 126.33±1.70 nm and PDI of 0.16±0.02, which were the best resulting binary rutin ethosomes. All rutin binary ethosomes had high entrapment efficiency, around 90%. In comparison with meloxicam ethosomes, 169 nm, 0.2 PDI, and 83.1 EE%, the rutin binary ethosome could be considered successfully prepared and had better physical properties and drug loading [10]. Therefore, rutin with 30% ethanol, glycerol 5%, and PG 5% (w/w) was chosen for the preparation of rutin binary ethosome.

Table 4. Rutin ethosome properties of various polyol ratios

Polyol	30%	G 5%	PG 5%	G 10%	PG 10%	G5%+PG 5%	G 20%	PG20%	PG10% + G10%
Size (nm)	177.28 ± 2.03	171.43 ± 6.21	141.13 ± 7.65	133.27 ± 2.97	150.70 ± 4.48	126.33 ± 1.70	159.13 ± 9.36	192.30 ± 5.00	140.87 ± 3.91
PDI	0.28 ± 0.02	0.29 ± 0.07	0.25 ± 0.05	0.30 ± 0.04	0.22 ± 0.02	0.16 ± 0.02	0.24 ± 0.04	0.15 ± 0.08	0.19 ± 0.08
Zeta (mV)	-42.4 ± 3.25	-46.90 ± 4.67	-37.55 ± 0.78	-28.65 ± 1.20	-33.60 ± 4.95	-28.75 ± 0.78	-41.15 ± 0.49	-40.65 ± 0.07	-38.55 ± 1.34
EE (%)	89.33 ± 0.44	89.42 ± 0.68	90.86 ± 0.44	88.91 ± 0.68	83.32 ± 0.26	88.74 ± 0.26	84.54 ± 0.44	90.09 ± 0.44	82.39 ± 0.26

3.3. Deformation of rutin ethosome

The flexibility of rutin ethosome membranes was briefly tested (Table 5). Both the size and PDI of rutin ethosome after deformation were smaller, with a slight decrease in entrapment efficiency of rutin in these nanovesicles, from 89.59 ± 0.44% down to 88.32 ± 0.44%.

Table 5. Rutin ethosome properties after deformation

	Fresh preparation		After deformation	
	EtOH30%	**EtOH30%+PG5&Gly5%**	**EtOH30%**	**EtOH30%+PG5&Gly5%**
Size (nm)	177.28 ± 2.03	126.33 ± 0.60	153.77 ± 8.26	117.6 ± 2.49
PDI	0.28 ± 0.02	0.16 ± 0.02	0.25 ± 0.09	0.07 ± 0.02
Zeta (mV)	-42.4 ± 3.25	-28.75 ± 0.78	-44.40 ± 0.99	-29.75 ± 0.64
EE (%)	89.59 ± 0.44	89.16 ± 0.68	88.32 ± 0.44	86.29 ± 0.44

The morphology and distribution of rutin ethosome, before and after deformation were shown clearly in Fig. 4. Rutin ethosome had membrane-core structures (Fig. 1). In cases of rutin ethosomes with only ethanol, there were collisions of small vesicles into larger vesicles, and the distribution was significantly broader than rutin binary ethosomes. After deformation, the vesicles were separated and shape-modified, in both types of rutin ethosomes, which led to a considerable reduction in PDI, from 0.16 ± 0.02 to 0.07 ± 0.02 for binary ethosome. The uniformity of binary ethosomes, consisting of PG and G after deformation, confirmed the elasticity of vesicle membranes. Thus, PG and G improved the deformable ability of ethosomes, which resulted in higher permeability for skin delivery. Additionally, a simple intrusion may be applied to obtain rutin ethosomes with better physical properties, as deformable binary rutin ethosomes had a size below 120 nm and PDI below 0.1.

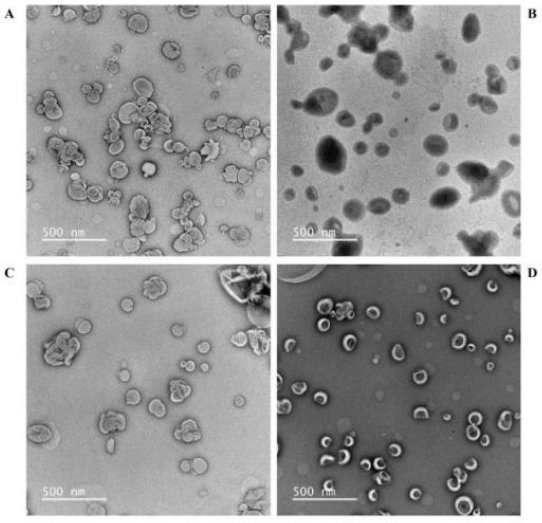

Fig. 4. TEM images of rutin ethosomes before and after deformation
A. EtOH 30%, B. EtOH30% after deformation,
C. EtOH30%+G5%+PG5%, D. EtOH30%+G5%+PG5% after deformation

3.4. In vitro membrane permeability

The rutin dissolution profiles from binary ethosomes, classical ethosomes, and pure rutin were carried out through the Franz cell model, with dialysis membranes at 14 KDa cut-off. The dialysis membranes were often used in skin penetration models [19],[26]. The pore size in the dialysis membranes allowed the diffusion of drugs across and penetration into the receptor compartment while retaining the nanocarriers in the donor one, mimicking *exvivo* animal skin as well as *in vivo* conditions. As clearly seen from Fig. 5, binary ethosomes improved the permeability of rutin the best, with nearly 32.90 ± 0.77% at the 24-hour point, followed by classical ethosomes with nearly 21% while pure rutin only released around 7% at the same time point. Thus, the optimized binary formulation further increased the release of rutin through the membrane compared to the binary ethosomes in the previous study – by 5-fold and 2.5-fold, respectively [19].

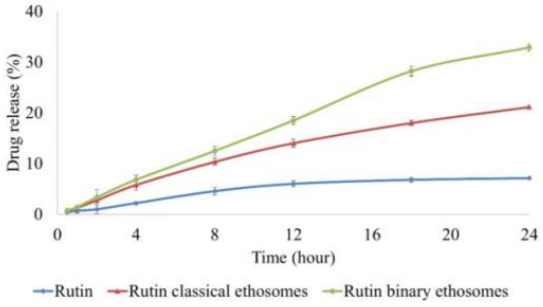

Fig. 5. *In vitro* membrane drug permeability of rutin ethosome

4. Conclusion

Rutin ethosomes and binary ethosomes were successfully fabricated by the sole solvent injection method. For rutin fabrication methods, the temperature and mixing duration of the oil phase were key factors affecting the physical properties of rutin ethosomes, with the optimized values being 40°C and 30 minutes, respectively. Moreover, the injection temperature also influenced their characteristics, with 45°C chosen for rutin

ethosome fabrication. Among various polyols, PG and G considerably reduced the size and PDI of nanovesicles compared to those made with only ethanol. The optimum binary ethosome formulation was SPC: rutin at a 1:1 molar ratio, 30% ethanol, 5% PG, and 5% G (w/w). The physical properties of the best rutin ethosome were a size below 120 nm, good uniformity, and an EE of nearly 90%. Additionally, the rutin binary ethosome exhibited excellent elasticity, as their size and uniformity even improved after deformation, with only a slight reduction in encapsulation efficiency. Moreover, in an *in vitro* membrane model, the best rutin binary ethosomes increased drug permeability through the dialysis membrane by 5-fold. Therefore, rutin binary ethosomes could be a potential nanocarrier to enhance dermal absorption. Furthermore, a sole solvent injection method, with only control of the phase preparation temperature, oil phase incubation duration, and injection temperature – followed by filtration, may be a facile approach suitable for large-scale production of ethosomes.

Declaration of Competing Interest: *The authors report no conflicts of interest.*

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