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Original Article

Preparation and physicochemical evaluation of hydrogel containing quercetin phytosomes

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ABSTRACT

The objective of this study was to prepare and evaluate some physiochemical properties of the hydrogel containing quercetin phytosomes. Quercetin phytosome suspension was prepared by anti-solvent precipitation method followed by evaluation of the drug entrapment efficiency, mean particle size, and particle distribution index (PDI). The particle size of quercetin phytosomes was in the narrow range of 400 - 500 nm with PDI < 0.4, the absolute value of zeta potential was higher than 30 mV. Analysis of Fourier-Transform Infrared Spectroscopy and proton nuclear magnetic resonance spectroscopy proved the presence of physical and chemical interactions between quercetin and phospholipid. The quercetin–hydrogenated soybean phosphatidylcholine complex was formed by hydrogen and ion-dipole bonding between the -OH group of the phenyl rings of quercetin and the -P=O, $-N(CH_3)_3$ group of the phospholipids. In scanning electron microscope (SEM) images, the quercetin phospholipid complex was found to be irregular and disc-shaped with a rough surface. Later, quercetin phytosomes were incorporated into the hydrogel system using Carbopol 934 or sodium carboxymethyl cellulose. The optimal formula of quercetin phytosome hydrogel containing 0.1 % active ingredient had the particle size in the range of 400 - 500 nm with PDI < 0.4 as they were in the phytosome suspension. The release rate and deposition of quercetin from the phytosome quercetin hydrogel were increased significantly by more than 3 times and 6 times, respectively in comparison with the conventional hydrogel.

1. INTRODUCTION

Quercetin is a natural flavonoid that has been proven with therapeutic potential for the prevention and treatment of diseases such as cancer, allergy, cardiovascular. Particularly, quercetin has been shown superior antioxidant and anti-inflammatory capacity than other flavonoids[1, 2]. Various studies have implemented on the antioxidant properties of quercetin[3, 4, 5]. However, the complicated molecular structure, low solubility and low permeability[6] are the main hurdles that limit the effectiveness of quercetin when given in dosage forms. Thus, the challenge is to find ways to overcome the above-limited properties of the active ingredient. There have been various approaches that were implemented,

namely quercetin nanoparticles[7, 8], a new non-aqueous self-double-emulsifying drug delivery system[9], nanoemulsion[10], liposomes[11] and phytosomes[12].

Phytosomes, also named as phyto-phospholipid complexes, is formed by interactions between the polar head of phospholipids and phytoconstituents that are usually polyphenols[13]. In the phytoconstituents phospholipid complexes, the active constituents are bound up with phospholipids forming a dipole-shaped spherical particle structure, thereby improving the solubility, partition coefficient and transportation of active substances through the lipid membrane, as a result, overcome the obstacle of nature active constituents[14].

Various studies showed that the percutaneous absorption of phytoconstituents was improved by the application of phytosomes[15, 16]. Thus, phyto-phospholipid complexes were widely employed in transdermal dosage forms[17, 18, 14]. On the basis of references, phytosome technology was applied to prepare a topical semi-solid dosage form containing quercetin phytosomes.

Currently, the hydrogel is a dosage form that widely applied in skincare, drugs, and cosmetics for prolonged release of the active ingredient, being non-invasive and having advantages in cutaneous and percutaneous drug delivery. The phytosomes are highly biocompatible vesicles with bio-membrane like structure which helps them easily transported into the intracellular[15]. Thanks to the advantages of hydrogel products and phytosome complexes, we decided to prepare and integrate quercetin phytosomes into the hydrogel dosage form, a skincare product, in order to exploit the antioxidant capacity and the anti-aging effect by enhancing the retention in the skin. This study was implemented on the preparation and evaluation of hydrogel containing quercetin phytosomes.

2. MATERIALS AND METHODS

2.1. Materials

Quercetin dihydrate was obtained from Sigma-Aldrich (U.S.A). Soybean hydrogenated phosphatidylcholine (HSPC) was supported by Lipoid (Germany). HPLC-grade dichloromethane and methanol were purchased from Merck (U.S.A). Cholesterol (CH) and sodium carboxymethyl cellulose medium viscosity (NaCMC) were purchased from Sigma-Aldrich (U.S.A). Water was purified by reverse osmosis in-house systems. Other reagents were analytical grade commercial products purchased from Beijing Chemical Reagent Factory (China).

2.2. Animals

Adult male Wistar rats were used, each weighed between 130 and 165 g (the National Institute of Drug Quality Control (Vietnam)). The rats were housed in a 12 h light/dark cycle in a temperature (25±2°C) and humidity (55±15%) controlled room with food and water ad-libitum. The animals were allowed to acclimatize for at least seven days before the start of the experiment. The protocol of the animal experiment (protocol number 598/QD-DHH) was approved by the Animal Care and Use Committee of the Hanoi University of Pharmacy, Vietnam.

2.3. Methods

2.3.1. Preparation of quercetin phytosomes

Quercetin phytosomes were prepared by the anti-solvent precipitation method as the following steps: quercetin, soybean hydrogenated phosphatidylcholine and cholesterol were dissolved in ethanol. The achieved solution was stirred by the IKA magnetic machine at 80°C at a speed of 500 rpm for 16 h in the reflux condition. Quercetin phytosomes were precipitated by pumping the ethanol phase into either distilled water or 0.2 mg/ml NaCMC solution in the condition of temperature was 60 °C and stirring speed was 850 rpm. The size of phytosome particles in the achieved suspension was reduced by ultrasound for 15 minutes at 50 Hz, power of 50 W.

2.3.2. Characterization of quercetin phytosome suspension

High-Performance Liquid Chromatography

Quercetin was assayed by validated method using HPLC system (Shimadzu, Japan, Nexera XR) in the condition of Apollo C18 column, size 4.6 × 250 mm, particle size 5µm; mobile phase: a mixture of 0.2% phosphoric acid solution and methanol with a volume ratio of 40:60; flow speed: 1 ml/min; injection sample volume: 50 µl; UV detector wavelength at 370 nm.

Particle size, particle size distribution (PDI) and zeta potential

The particle size, PDI and zeta potential were determined by the dynamic diffraction method (Malvern Panalytical, UK, Zetasizer ZS90). The quercetin phytosome suspension was diluted by double distilled water that was filtered through a 0.2 μm cellulose acetate membrane to verify the count rate in the range of 200 and 400 kcps.

Morphology

The morphology of the phytosome particles was examined by scanning electron microscopy FESEM S-4800 (Hitachi, Japan, S-4800) in the condition a magnification of M = 20x - 800,000x; resolution δ = 1.0 nm; acceleration voltage U = 0.5 - 30 kV. The quercetin phytosomes were coated with gold prior to measurement.

Fourier transform infrared spectroscopy (FTIR)

FTIR spectra were obtained by a Fourier Transform Infrared Spectroscopy (Bruker Optics, Germany, IFS-66/S) using the potassium bromide (KBr) disk method. Five to ten milligrams of dried samples of quercetin phytosomes, quercetin, HSPC, and physical mixture were grounded and mixed with 150 mg of spectra-grade KBr then pressed into a 12 mm diameter disk using a Carver hydraulic press (Carver, U.S.A., Model 3912). Samples were analyzed in the 400-4,000 cm^{-1} spectrum with an instrument resolution of 0.1 cm^{-1}.

Preparation of physical mixture: quercetin, hydrogenated soybean phosphatidylcholine and cholesterol (in the ratio as the same that of phytosome complex) were grounded and mixed to form a homogeneous powder.

Proton nuclear magnetic resonance spectroscopy (^{1}H-NMR)

^{1}H-NMR experiments were conducted to investigate the possibility of the intermolecular interaction between quercetin and HSPC. The ^{1}H-NMR spectra of the samples were taken at 25°C on a Bruker Avance 500 MHz spectrometer equipped with 5 mm TCI HCN Z gradient cryoprobe. Spectra were processed by Bruker Topspin 2.1 software and analyzed by CARA 1.8.4 software. Quercetin phytosomes were dissolved in deuterated and deaerated dimethyl sulfoxide (DMSO-d_6) at a concentration of 20 mg/ml. HSPC and quercetin were dissolved in DMSO-d_6 at concentrations, equivalent to the concentration of HSPC and quercetin in quercetin phytosome solution. The solution was added to a suitable glass tube at the liquid level is about 4.5 to 5 cm and then covered the cap. The glass tube was then placed into the test chamber, scanning the ^{1}H nuclear magnetic resonance spectrum at 500 MHz.

Quercetin entrapped determination

In principle, solubility of quercetin and quercetin phytosomes in ethyl acetate at 25°C were respectively 14.500 ± 0.062 mg/ml and 0.164 ± 0.002 mg/ml. Our result proved that ethyl acetate solvent could dissolve selectively quercetin to separate from the phytosome mixture (solubility of quercetin phytosomes was only at 1.13 % compared to free quercetin). Thus quercetin entrapped in the complexion efficiency was determined on the assay of the total amount of quercetin in the phytosome mixture and free quercetin separated by dissolving in ethyl acetate. Equation calculated quercetin entrapped in the complexion efficiency:

$$EE = 1\frac{Mq}{Mt} \times 100\ (\%) \quad (1)$$

Where: EE: Quercetin entrapment efficiency; Mq: the amount of free quercetin; Mt: the total amount of quercetin content in the phytosome mixture.

Implementation: Quercetin phytosome powder was dried in a vacuum oven at 50 °C for 48 hours. Exactly amount of phytosome sample was weighed and placed in a 20 ml volumetric flask. Ethyl acetate was added to get the sufficient volume, well shaken and then sonicated for 10 minutes. The sample was cool down to 25 °C and filtered through a 0.20 μm PTFE membrane. Exactly 1 ml of filtrated solution was withdrawn then evaporating completely ethyl acetate by drying in a vacuum oven at 40 °C for 1 h. The residue was dissolved in ethanol and diluted to the appropriate concentration for quantification by HPLC method.

2.3.3. Preparation of quercetin phytosome hydrogel

The formula of hydrogel containing quercetin phytosome was as following:

Phytosome quercetin suspension	50,0 g
NaCMC or Carbopol 934	investigation
Triethanolamine	qs. pH = 6.0 - 7.5
Glycerin	5.0 g
Nipagin	0.1 g
Purified water	qs. 100.0 g

Hydrogel excipients (NaCMC or Carbopol 934) were immersed into water for absolutely swelling and then neutralized by triethanolamine in case of using Carbopol 934. The preservative was dissolved into the polyol solvent before combination into the quercetin phytosome suspension. Then, the phytosome suspension was dispersed into swelled hydrogel excipients to get the final concentration of 0.1% quercetin in the hydrogel. Stirring gently to form a smooth, homogeneous hydrogel mixture. The phytosome hydrogel was evaluated by various physical characteristics and determined the amount of quercetin.

2.3.4. Characterization of quercetin phytosome hydrogel

In vitro release of quercetin from hydrogel

The 0.2 µm cellulose acetate membrane and excised rat skin were used in the *in vitro* release study. The male white rats were sacrificed by excess chloroform inhalation. In this study, we only took the rat abdominal skin to ensure the thickness uniformity. The abdominal hairs were shaved, subcutaneous fat was carefully removed to obtain a full-thickness abdominal skin (2x2 cm). The skin tissue was washed with distilled water; blotted dry and visually inspected for any damage. The integrity of the skin tissue was confirmed under an optical microscope before the *in vitro* release study to verify that there was no scratch or injury on the stratum corneum–epidermis layer which could influence the result of this study. The processed skin tissue was stored at -20 °C until the further experiment. The *in vitro* release study was carried out in the Franz diffusion test system (Hanson Research, Germany, 6-Cell Drive System) consisting of 7 ml receptor chambers and donor chambers with a diffusion area of 1.767 cm^2. The amount of 0.5 g of the hydrogel or the phytosomes was put on and spread evenly over the surface of membranes. The rat skin tissue was freeze-thawed in phosphate buffer of pH 6.8 at room temperature for 1 h and mounted in the donor chamber keeping the stratum corneum side in intimate contact with the sample. The donor chamber was inserted into the receptor chamber keeping the dermis in close contact with the receptor solution (phosphate buffer of pH 6.8) maintained at 32 ± 0.5 °C. The magnetic stirrer was kept at a speed of 400 rpm. Aliquots (1 ml) were withdrawn from the receptor chamber at predetermined time intervals up to 20 h and immediately replenished with the same volume of pre-warmed receiver solution to maintain sink condition. Aliquots were filtered through a 0.2 µm membrane filter and analyzed for drug content by the mentioned HPLC method above. The cumulative amount released at each time interval was calculated and plotted against time.

The cumulative amount of quercetin permeated through excised rat skins were calculated by Eq (2):

$$Qs = \frac{Vo \times Cn + \sum_{n-i}^{n-1} Ci \times Vi}{s} \quad (2)$$

Where C_n was quercetin concentration of the receiver solution at each sampling time, C_i was the active ingredient concentration of the sample, and V_0 and V_i were the volumes of the receiver solution and the sample, respectively. S was the effective diffusion area (S = 1.767 cm^2).

Deposition of quercetin in rat skin

The remains of quercetin in rat skin after 20 hours were determined by washing out the rat skin after the release study 3 times with pH 6.8 phosphate buffer solution and blotting dry. The treated skin area was cut into small pieces and homogenized in a rotor-stator homogenizer (ProSientific, USA). Quercetin was extracted two times with methanol in ultrasonic condition for 30 minutes. Methanol was supplemented to the ultrasonic mixtures to have sufficient 10 ml mixtures were centrifugated at 6,000 rpm for 10 minutes and taken a clear solution then filtered through 0.45 µm PTFE membrane. The active ingredient retained in the rat skin was determined by the HPLC method described above.

Viscosity

The viscosity of the hydrogel was determined

by means of oscillatory measurement with a rheometer (TA Instrument, United States, Discovery Hybrid Rheometer) equipped with a cone-plate sensor (with a diameter of 4 cm and a cone angle of 4°); The volume used for each measurement is approximately 1.4 ml. The samples were first thermoregulated in 180 s before each run in 120 s at 0.5 Pa, 32°C ± 1°C and a frequency of 1 Hz.

2.3.5. Stability of quercetin phytosome suspension and hydrogel

Quercetin phytosome suspension and hydrogel was filled in wide-mouth glass bottles, with tight lids and stored under laboratory conditions (30 ± 5 °C, RH 65 - 85%). Stability of the quercetin phytosome suspension samples at initial, after one week and three weeks, was assessed on the criteria including particle size, PDI, Zeta potential value, EE%. The stability of the quercetin phytosome hydrogel samples at initial and after a month was assessed on the criteria including Quercetin content, particle size, PDI, Zeta potential value, pH.

2.3.6. Data analysis and statistic

A minimum of three experiments was performed. The results are expressed as mean ± standard deviation (SD). Differences were determined using a paired, one-tailed Student's t-test and ANOVA multiple comparisons. A P-value<0.05 was considered significant. Data were analyzed by using Excel (Microsoft, U.S.A)

3. RESULTS

3.1. Preparation and evaluation of quercetin phytosome suspension

Quercetin phytosome samples containing the different molar ratios of CH:HSPC, precipitating in the different medium (distilled water and 0.2 mg/ml NaCMC solution) were prepared and evaluated the complexion efficiency and physicochemical properties. The results were shown in Table 1.

Table 1. Properties of quercetin phytosome suspension prepared with the various formulas (n = 3, Mean ± SD).

Sample (mol)	CH:HSPC	Precipitating medium (d.nm)	Particle size	PDI	Zeta potential (mV)	EE (%)
F1	0:1	Distilled water	423.2 ± 2.5	0.235 ± 0.026	-21.3 ± 1.2	80.1± 0.2
F2	0.1:1		430.5 ± 1.2	0.243 ± 0.022	-20.5 ± 1.8	83.2 ± 0.2
F3	0.2:1		439.6 ± 0.7	0.242 ± 0.031	-20.4 ± 2.1	86.0 ± 0.3
F4	0.5:1		478.6 ± 4.2	0.327 ± 0.019	-21.5 ± 2.9	60.9 ± 0.2
F1'	0:1	0.2 mg/ml NaCMC solution	398.2 ± 5.7	0.238 ± 0.021	-32.1 ± 2.3	86.8 ± 0.3
F2'	0.1:1		402.0 ± 5.0	0.257 ± 0.016	-31.0 ± 1.7	85.7 ± 0.3
F3'	0.2:1		408.2 ± 2.9	0.257 ± 0.018	-31.2 ± 1.2	87.1 ± 0.4

In the case of precipitating in distilled water, when cholesterol was added with the molar ratio of CH:HSPC by 0.1:1 and 0.2:1, the phytosome particle size and PDI were similar to those of the sample without CH (CH:HSPC by 0:1). However, the quercetin phytosomerization efficiency increased proportionally to the amount of cholesterol. This could be explained by the interaction between cholesterol and phospholipid helped phospholipid molecules more tightly arranged, resulted in increasing the hardness and thickness of the complex membrane. As a result, the phytosomes could be protected from the leaking of quercetin, the main factor caused recrystallization and physical instability of the system. When the molar ratio of CH:HSPC increased to 0.5:1 (sample F4), the phytosome particle size and PDI also increased (478.6 nm and 0.327 respectively). The complexion

efficiency decreased significantly compared to that of other samples (60.9%). It could be explained that the elevated cholesterol concentration competed for the position of quercetin in the bi-phospholipid membrane[19, 20], as a result, the substance was pushed out of the complex.

When the solution containing 0.2 mg/ml NaCMC was used as the precipitating medium, the particle size of the quercetin phytosome suspension was smaller meanwhile absolute Zeta value was higher (~ 30 mV) than those of samples that precipitated in distilled water. Instead of using organic solvents as several published studies[21, 22], in this study, the solution containing 0.2 mg/ml NaCMC was used not only to protect the environment but also to increase the homogeneity of the samples. The physical stability of the system could be enhanced when Zeta potential elevated to the absolute value higher than 30 mV. In addition, when phytosome precipitated in 0.2 mg/ml NaCMC solution, the quercetin phytosomerization efficiency was higher than that in the distilled water. This result may be due to the adsorption of NaCMC polymer on the surface of the phytosome particles. NaCMC is a synthetic polymer that can behave as a complex surfactant. In the suspension, NaCMC can adsorb on solid surfaces. NaCMC also is an ionizable polymeric surface that contains carboxylic groups in the molecule. So, the mechanism of increasing surface charge is the direct ionization of surface groups. As a result, the zeta potential value of phytosomes suspension using 0.2 mg/mL NaCMC solution as the precipitating medium was higher than that of using distilled water as a precipitating medium. The level of NaCMC adsorption on the solid surface could proportional to the % NaCMC in the hydrogel formula.

Based on the obtained results, the F3' formula with the optimal zeta potential and highest complexion efficiency was chosen to prepare three samples of quercetin phytosome suspension (M1, M2, M3). Results of particle size, PDI, Zeta potential and quercetin entrapment efficiency of the achieved phytosome suspension was as follows:

The particle size of quercetin phytosomes was in the range of 400 – 450 nm with the PDI < 0.4. The absolute value of zeta potential was approximate 30 mV. In those three phytosome samples, the quercetin entrapment efficiency was all higher than 80 %.

After 3 weeks of storage in the laboratory condition, there was no significant difference in particle size, PDI, Zeta potential in the suspension samples Table 2. The quercetin entrapment efficiency was still higher than 80 %.

In Figure 1, it could be found that quercetin existed in the polygonal crystalline form, but after complexion with soybean hydrogenated phosphatidylcholine and cholesterol, it transformed into spherical microparticle form. This could be one of the factors that increase the contact area of the phytosomes with the medium, thereby increasing the dissolution rate and improving the permeability of the active ingredient through biomembrane. This could be beneficial to quercetin bioavailability, a natural active ingredient with low solubility and poor permeability properties[6].

Table 2. Stability of quercetin phytosome suspension (n = 3, Mean ± SD).

Samples	particle size (nm)	PDI	Zeta potential (mV)	EE%
Initial				
M1	410.0 ± 2.4	0.345 ± 0.052	-30.7 ± 2.3	87.2 ± 0.5
M2	404.1 ± 2.9	0.349 ± 0.063	-31.6 ± 1.4	86.5 ± 0.3
M3	410.3 ± 2.7	0.341 ± 0.049	-31.6 ± 1.1	86.7 ± 0.5
After one week				
M1	410.3 ± 2.6	0.342 ± 0.049	-31.0 ± 1.2	85.2 ± 0.5
M2	411.5 ± 1.3	0.365 ± 0.064	-32.7 ± 1.2	86.3 ± 0.4
M3	412.3 ± 2.0	0.350 ± 0.034	-32.1 ± 2.0	85.7 ± 0.6
After three weeks				
M1	423.1 ± 1.3	0.354 ± 0.043	-31.5 ± 2.3	84.7 ± 0.3
M2	420.4 ± 1.5	0.375 ± 0.016	-32.8 ± 1.5	85.4 ± 0.4
M3	419.5 ± 2.0	0.368 ± 0.031	-32.4 ± 1.3	84.8 ± 0.3

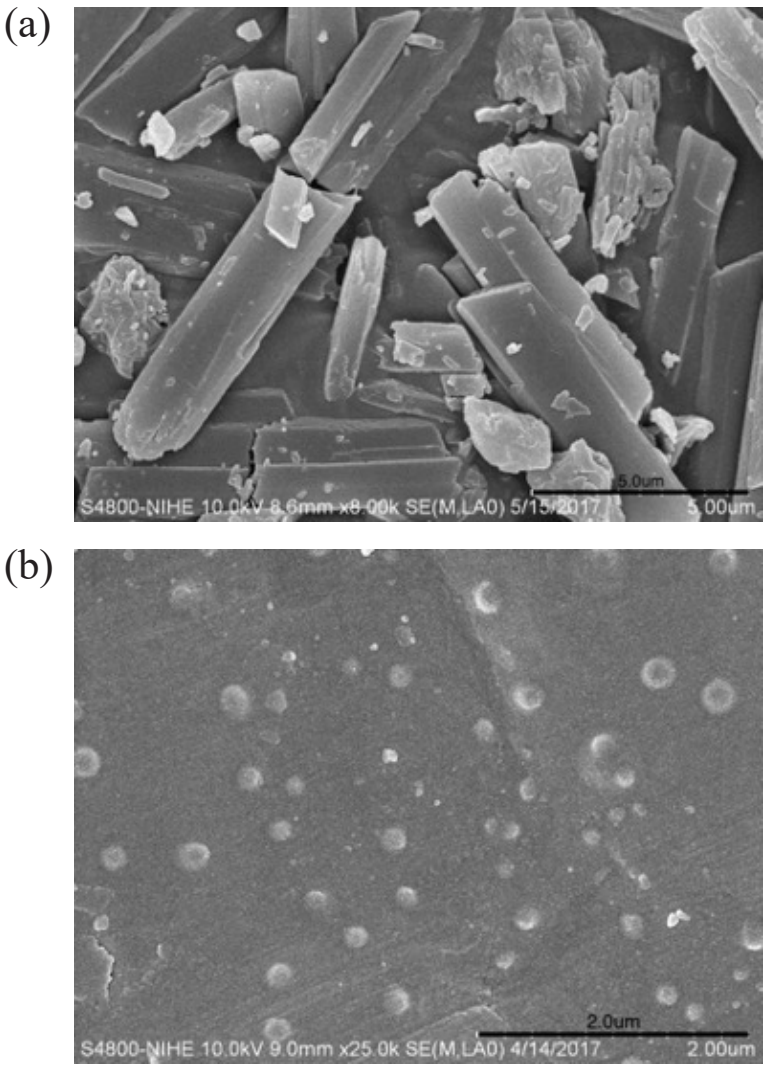


Figure 1. Images of (a) quercetin crystalline and (b) quercetin phytosomes taken by scanning electron microscope.

The quercetin phytosome suspension was evaporated at 60 °C in vacuum conditions to obtain the powder of the phytosome complex. The interaction between quercetin, soybean hydrogenated phosphatidylcholine, and cholesterol in the phytosome complex was evaluated by analysis of the FTIR and ^{1}H-NMR spectrum.

The interaction between quercetin and HSPC in the complex could be evaluated by the analysis of the IR spectra Figure 2. The -O-H phenolic group stretching vibration of quercetin molecular were observed as the bands at the range from 1168.6 cm^{-1} to 1380.7 cm^{-1}. In the IR spectrum of HSPC, -P=O and P-O- stretching vibrations were observed as strong bands at 1239.0 cm^{-1} and 1090.5 cm^{-1} respectively, $-N^{+}(CH_3)_3$ stretching vibration (C-N stretching) was observed as a weak band at 1173.4 cm^{-1} [23]. There was a significant difference between phytosome complex IR spectra and the one from the physical mixture containing quercetin, HSPC, and cholesterol. In the IR spectrum of the quercetin phytosomes, the complexion between quercetin and HSPC could be observed by the interaction between O-H groups in quercetin molecular and -P=O, P-O-C, $-N^{+}(CH_3)_3$ groups in HSPC molecular. The specific absorption peaks of quercetin and HSPC mentioned above were shifted to 1063.5 cm^{-1} on the phytosome spectra and the peak intensity was changed. The IR spectrum of the physical mixture showed an additive effect of both quercetin and HSPC, the specific absorption peaks of HSPC was also presented all. Moreover, there was no new peak observed in the physical mixture and phytosome complex. These results also pointed out that cholesterol was not involved in complex bonds.

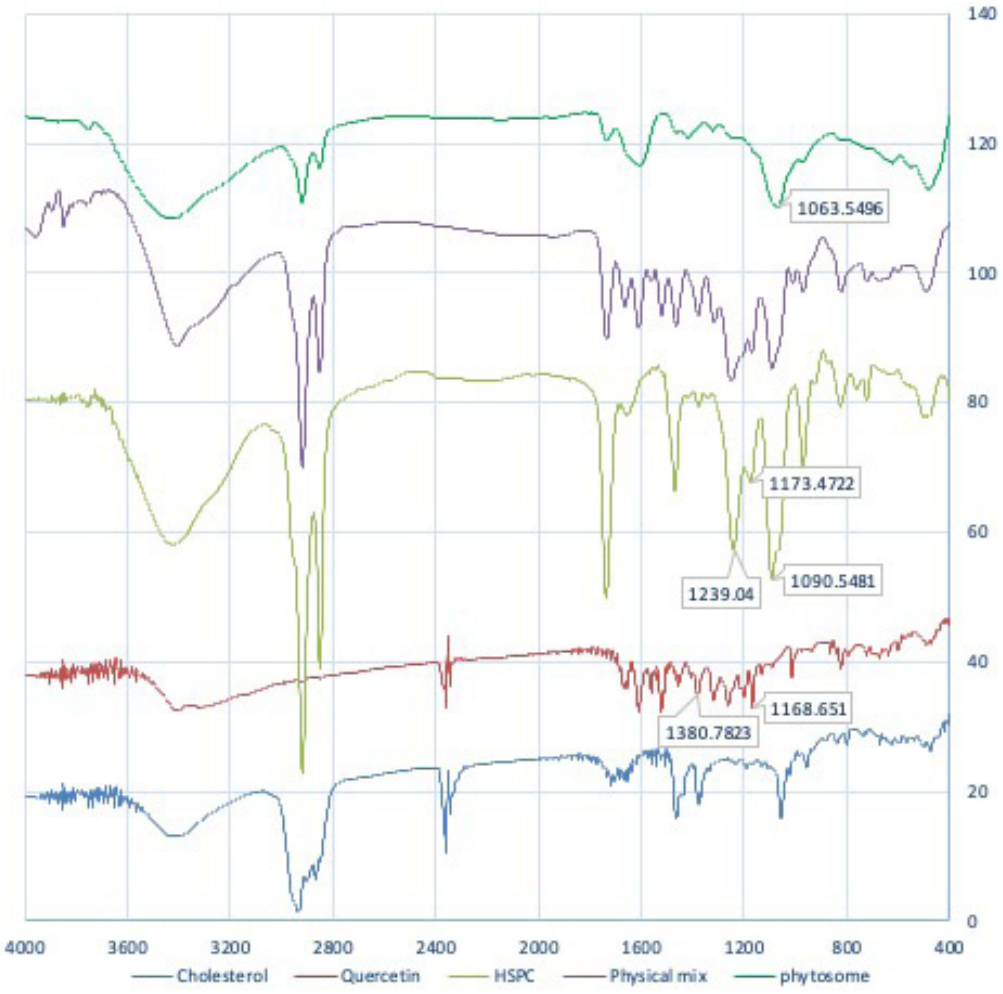
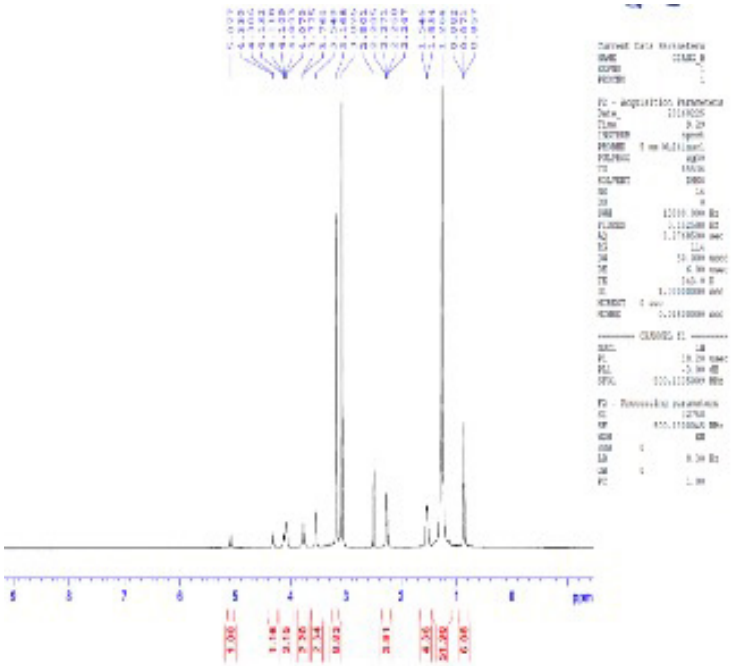


Figure 2. FTIR diagram of quercetin phytosomes, quercetin, HSPC and physical mixture of quercetin, soybean hydrogenated phosphatidylcholine and cholesterol.

Figure 3 showed ^{1}H-NMR spectra of HSPC, quercetin and the phytosomes. ^{1}H chemical shifts are reported in parts per million. The spectrum of quercetin exhibited characteristics H-shift described below: ^{1}H-NMR (500 MHz, DMSO-d6), δ10.77 (s, 1H, 7-OH); δ9.57 (s, 1H, 3-OH); δ9.32 (s, 1H, 3'-OH); δ9.28 (s, 1H, 4'-OH). The spectrum of HSPC exhibited characteristics H-shift ^{1}H-NMR (500 MHz, DMSO-d6), δ3.17 (s, 9H). Upon comparison of the chemical shifts of quercetin alongside the phytosome complex, the key differences emerged in both the downfield aromatic region (δ > 7) and the upfield region (δ < 4). The N-methyl groups of quercetin phytosomes were seen at δ 3.32 whereas this group of HSPC was seen at δ3.17. The formation of molecular aggregated with quercetin was clearly seen by the change in proton signals in the aromatic region. The charged head of the phospholipid molecule was involved in weak intermolecular interaction such as hydrogen bond and ion-dipole interaction with the phenolic (7-OH, 3-OH, 3'-OH, 4'-OH). This was supported by the lowered intensity of these proton signals and their distinct downfield chemical shifts. This indicated the embedding of quercetin in the phospholipid and thus confirmed the formation of the phytosome complex.

(a)



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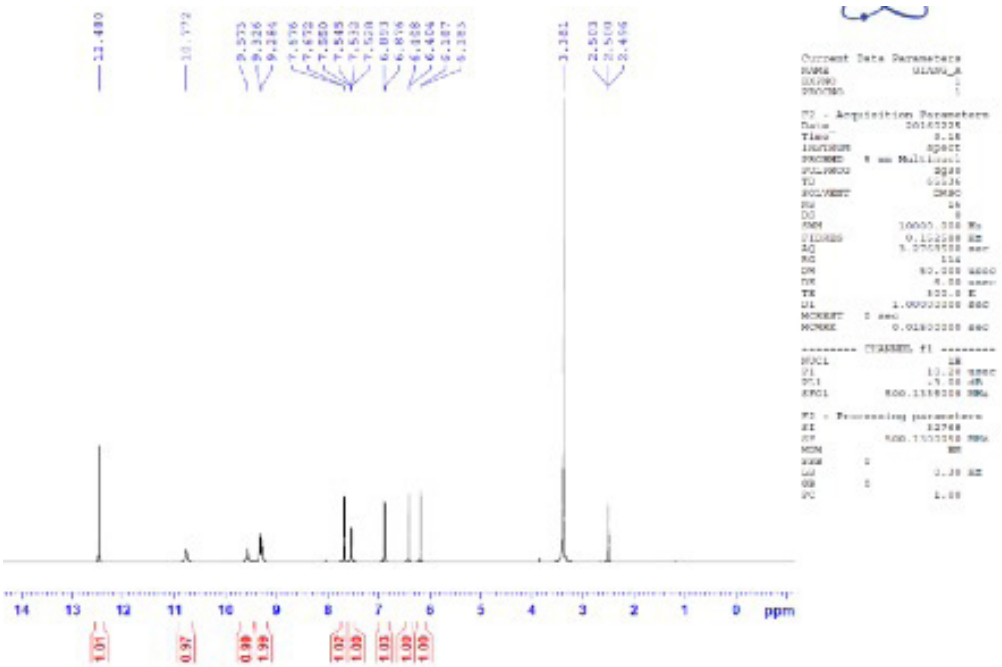

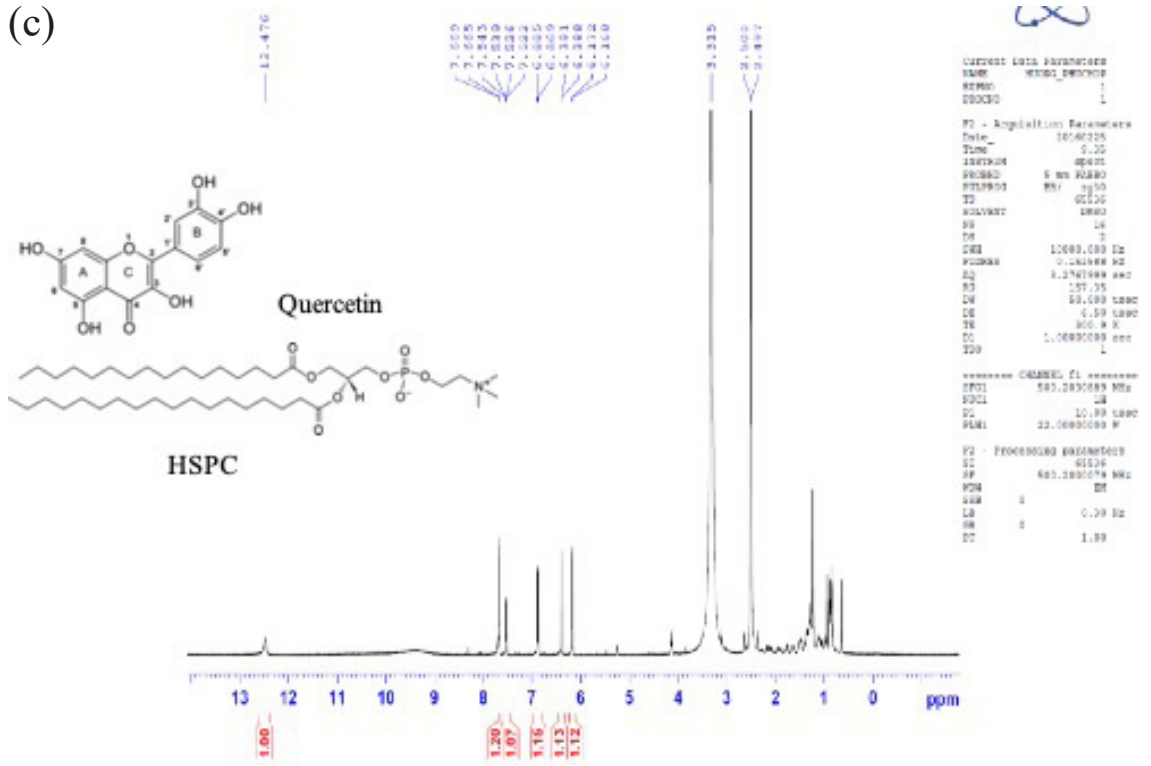

Figure 3. [1]H-NMR spectra of HSPC (a), quercetin (b) and phytosomes (c).

3.2. Preparation of hydrogel containing quercetin phytosomes

The influence of hydrogel excipients

Two kinds of hydrogel excipients*:* NaCMC with the concentration in the range of 3 - 5*%* and Carbopol 934 *(*Cb934*)* with a concentration of 0.2 - 0.6*%* were chosen to prepare hydrogel samples according to the above-described method. Afterward, the hydrogel was kept to stabilize for 24 hours and then investigated the characteristics. The obtained results were shown in Table 3.

In general, hydrogel samples were homogeneous with the typical yellowish-green color of phytosome quercetin, without separation. Among prepared hydrogel samples, CT1, CT2, and CT4 samples had low viscosity so they could not respond to the requirement of thickening. The hydrogel samples containing Carbopol 934 (CT4 - CT8) were more transparent and thicker than the one with NaCMC.

When incorporated into the hydrogel dosage form, the physical properties of quercetin phytosomes including zeta potential, particle size, and PDI all increased compared to the original phytosome suspension Table 3. In prepared NaCMC hydrogel samples *(*CT1, CT2, CT3*)* and Cb 0.6*%* sample *(*CT8*)*, quercetin phytosome particle size increased up to more than 500 nm. Based on the obtained results, CT5, CT6, CT7 hydrogel samples were selected to continue evaluating the *in vitro* release rate of the active ingredient compared to the free quercetin suspension and quercetin phytosome suspension at the same concentration of the active ingredient. The obtained results were shown in Figure 4.

It was easy to recognize that after 20 hours, the cumulative amount released of quercetin from phytosome suspension was significantly higher than from free quercetin suspension ($p < 0.05$), at 69.74 µg/cm^2 and 17.10 µg/cm^2, respectively. This suggested that the quercetin-HSPC complex increased the solubility of quercetin, resulting in an increase in the release rate of quercetin through 0.2 µm cellulose acetate membrane.

In the hydrogel system, Carbopol 934 helped increase the viscosity. As a result, quercetin released from hydrogel at a slower rate in comparison with that of the phytosome suspension. The cumulative amount released of the phytosome suspension was significantly higher than prepared

hydrogel samples CT5, CT6, CT7 ($p < 0.05$). The release rate of quercetin from hydrogel was inversely proportional to the concentration of Carbopol 934. It could be explained that the higher the concentration of Carbopol was used, the higher the viscosity of the hydrogel could be obtained, which slowed down the diffusion rate of quercetin across the released membrane. The CT5 hydrogel sample had the highest release rate of quercetin. Thus, the CT5 hydrogel sample prepared with Carbopol 934 excipient at a concentration of 0.3% was selected for further study.

Table 3. Properties of the hydrogel containing quercetin phytosomes prepared base on various excipients (n = 3, Mean ± SD).

Samples	Quercetin (%)	Viscos-ity (Pa.s)	pH	Quercetin phytosome suspension			Quercetin phytosome gel		
				Particle size (nm)	PDI	Zeta potential (mV)	Particle size (nm)	PDI	Zeta potential (mV)
CT1 (NaCMC 2%)	-	40.05 ± 3.76	7.09 ± 0.01	421.0 ± 3.2	0.301 ± 0.023	-30.5 ± 1.6	522.9 ± 2.2	0.438 ± 0.012	-42.3 ± 1.2
CT2 (NaCMC 3%)	-	65.00 ± 3.54	7.02 ± 0.01	411.6 ± 2.1	0.404 ± 0.013	-30.8 ± 1.3	520.2 ± 2.4	0.467 ± 0.024	-46.5 ± 0.9
CT3 (NaCMC 4%)	0.101 ± 0.012	112.34 ± 3.24	7.05 ± 0.02	405.8 ± 1.6	0.329 ± 0.045	-31.7 ± 0.9	522.2 ± 2.1	0.449 ± 0.014	-54.1 ± 1.4
CT4 (Cb 934 0.2%)	-	59.45 ± 2.31	6.65 ± 0.01	419.6 ± 2.6	0.326 ± 0.052	-33.3 ± 1.1	443.0 ± 2.1	0.424 ± 0.024	-39.8 ± 2.3
CT5 (Cb 934 0.3%)	0.102 ± 0.008	110.25 ± 2.56	6.4 ± 0.01	411.9 ± 1.7	0.343 ± 0.032	-30.1 ± 1.2	453.7 ± 3.2	0.430 ± 0.032	-34.5 ±1.5
CT6 (Cb 934 0.4%)	0.105 ± 0.010	130.00 ± 4.33	6.66 ± 0.01	417.6 ± 2.1	0.350 ± 0.028	-30.1 ± 2.1	440.5 ± 3.1	0.423 ± 0.021	-36.8 ± 2.1
CT7 (Cb 934 0.5%)	0.104 ± 0.009	150.34 ± 2.65	6.54 ± 0.02	410.1 ± 1.7	0.335 ± 0.043	-32.0 ± 1.1	450.5 ± 3.1	0.433 ± 0.034	-35.8 ± 1.2
CT8 (Cb 934 0.6%)	-	180.44 ± 3.21	6.82 ± 0.05	415.4 ±3.4	0.332 ± 0.023	-29.5 ± 1.6	528.1 ± 4.2	0.467 ± 0.056	-34.4 ± 1.1

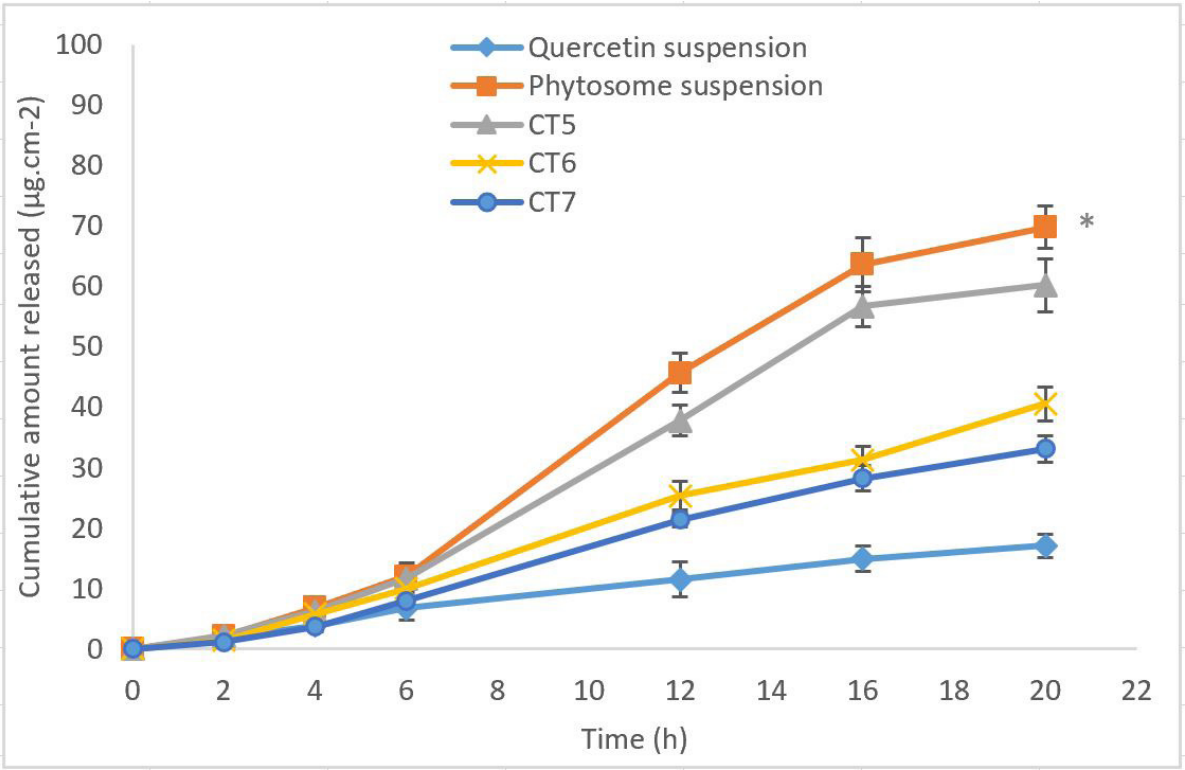

Figure 4. The cumulative amount of quercetin released through 0.2µm cellulose acetate membrane from prepared hydrogel and suspension samples (n = 3, Mean ± SD, *$p<0.05$)

The influence of the preparation process

To find out the optimal process for the preparation of the hydrogel of quercetin, two different processes were also investigated. For the first preparation process, Carbopol was dispersed into quercetin phytosome suspension containing other dissolved excipients and then neutralized with 10% triethanolamine solution, under continuous stirring at 400 rpm until achieving the homogeneous hydrogel. For the second preparation process, Carbopol was homogeneously dispersed and completely swelled in purified water, neutralized with a 10% triethanolamine solution. Then quercetin phytosome suspension and other excipients were added under continuous stirring at 400 rpm. Both procedures were applied to prepare the hydrogels with the same proportion of all excipients (Carbopol 934, triethanolamine, glycerine, nipagin). The influence of the preparation process on physico-chemical characteristics and stability of the hydrogels was evaluated. The obtained results were shown in Table 4.

Table 4. Properties of the hydrogel containing quercetin phytosomes prepared by two different processes (n = 3, Mean ± S.D.).

Samples	particle size (nm)	PDI	Zeta potential (mV)
Quercetin phytosome suspension	403.4 ± 1.3	0.354 ± 0.015	-31.2 ± 1.5
1st preparation process sample	677.0 ± 23.6	0.477 ± 0.012	-35.2 ± 1.4
2nd preparation process sample	443.4 ± 1.7	0.375 ± 0.020	-35.6 ± 2.1

In the first process, the particle size in the hydrogel sample was increased significantly and not homogeneous (PDI > 0.4). So the preparation process influenced significantly the physical characteristics of the prepared hydrogels. The more suitable process was the addition of quercetin phytosome suspension to the previously prepared Carbopol hydrogel. The difference of the physical properties between two obtained hydrogel samples might belong to the swelling process of Carbopol after dispersed into phytosome suspension and the transitory lowering of the pH during the dispersion of Carbopol potentially affected the physical stability of the complex suspension.

3.3. Evaluation of phytosome quercetin hydrogel properties

Based on the selected formula, three batches of the hydrogel containing quercetin phytosomes were prepared simultaneously implemented with plain quercetin hydrogel at the same concentration of active ingredient as well as the composition of other excipients. Evaluation of properties of hydrogel samples right after preparation and 1 month of storage at room temperature. The results were shown in Table 5.

Table 5. Properties of the hydrogel containing quercetin phytosome or free quercetin (n = 3, Mean ± SD).

Properties	Quercetin gel	Quercetin phytosome gel	
		Initial	After a month
Quercetin content (%)	0.099	0.101 ± 0.002	0.101 ± 0.003
Particle size (nm)	-	458.4 ± 2.1	467.8 ± 3.1
PDI	-	0.376 ± 0.022	0.384 ± 0.031
Zeta potential (mV)	-	-35.9 ± 2.0	-37.4 ± 2.8
pH	6.41	6.39 ± 0.03	6.37 ± 0.03
Viscosity (Pa.s)	-	122.56 ± 1.21	122.12 ± 2.01

After one month of storage under laboratory conditions (30 ± 5 °C, RH 65 – 85 %), the properties of the quercetin phytosome hydrogel such as drug contain, pH and viscosity did not change significantly. The particle size, PDI values manifested slightly increasing but all in the range under 500 nm and 0.4, respectively. Zeta potential value was maintained at an absolute value higher than 30 mV that was convenient to ensure the physical stability of the system over time.

The released profile of quercetin from prepared gels through 0.2 µm cellulose acetate membrane and rat skin were shown in Figure 4 and Figure 5.

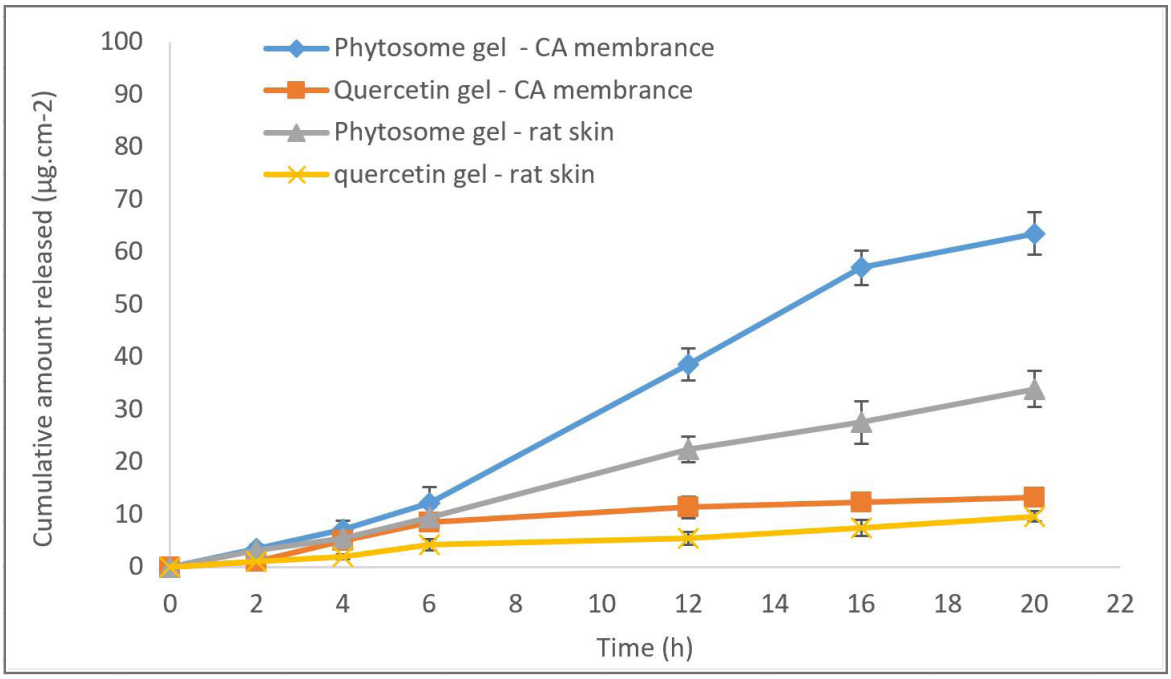

Figure 5. The cumulative amount of quercetin released through cellulose acetate membrane and rat skin (n = 3, Mean ± SD)

It can be seen that the quercetin phytosome hydrogel was capable of releasing the active substance through cellulose acetate 0.2 µm and rat skin much higher than plain quercetin hydrogel. After 20 hours, quercetin was released from prepared phytosome hydrogel through 0.2 µm cellulose acetate membrane at the level of 63.53 µg/cm^2, 4 times higher than the plain quercetin gel, at the level of 13.23 µg/cm^2. The release rate of quercetin from phytosome hydrogel through the rat skin was also at 33.89 µg/cm^2 more than 3 times higher than the plain one, at 9.66 µg/cm^2.

The skin extract obtained after release study with phytosome hydrogel and plain quercetin hydrogel showed the amount of active ingredient deposited in the rat abdominal skin (Figure 6) were 14.45 ± 2.12 µg/cm^2 and 2.12 ± 1.11 µg/cm^2 respectively. These results indicated a statistically significant difference ($p < 0.05$) in quercetin deposition into the rat skin from phytosome hydrogel and plain gel during release study across rat skin. So, the phytosome hydrogel also enhanced the retention of quercetin in rat skin more than 6 times compared to that of plain quercetin gel.

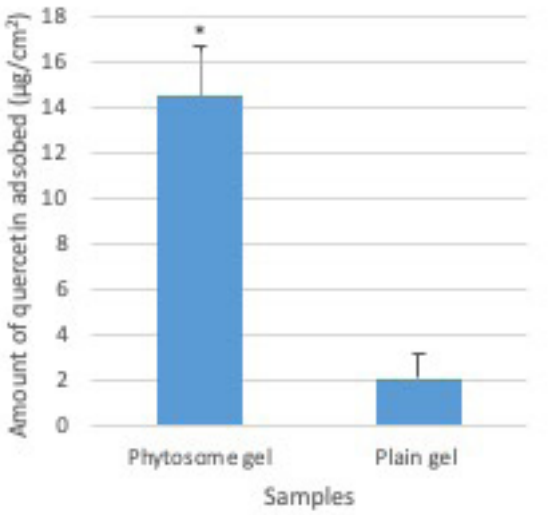

Figure 6. Amount of quercetin adsorbed in the rat skin after 20 hours (n = 3, Mean ± SD, *p<0.05)

4. DISCUSSION

Our results in preparation and evaluation of quercetin phytosome suspension were correlated to several published studies[24], the incorporation of cholesterol in the composition of phytoconstituent phospholipid complexes could enhance the physical stability of achieved phytosomes. The physical properties of prepared quercetin phytosome samples such as particle size, PDI, Zeta potential and quercetin entrapment efficiency were unchanged in three weeks stored in laboratory conditions.

In order to prove the formation of the phytosome complex, certain physical analyzing methods, such as X-Ray spectrum, differential scanning calorimetry (DSC), Fourier transform infrared spectroscopy (FTIR), proton nuclear magnetic resonance (^{1}H-NMR), powder X-ray diffractometry (PXRD) could be used to determine the interaction between the active ingredient and phospholipids by[25,24]. In this study, we selected two physical methods for analyzing the phytosome complex: infrared spectra and ^{1}H-NMR spectra. The obtained analysis results indicated the possibility of forming a bond between the proton of -OH group in the 3, 3', 4', 5, 7 position of quercetin molecular with the phosphate group and aliphatic amine in the HSPC molecular. This conclusion was based on the results of analyses of infrared spectra, nuclear magnetic resonance spectroscopy, and in accordance with other studies[26].

In our study, the optimal formula of quercetin phytosome hydrogel containing 0.1% active ingredient, had the particle size in the range of 400 - 500 nm and PDI < 0.4 as they were in the phytosome suspension. The achieved results were at a good agreement with the study of Ljiljana Djekic et al. in formulation and physicochemical characterization of hydrogels with 18β-glycyrrhetinic acid/phospholipid phytosome complex[27]. In that study, the authors also pointed out that the addition of the humectant – glycerine did not influence significantly the characteristics of the prepared hydrogels. Carbopol dispersed in water to form acidic colloidal dispersions that, when neutralized, produce highly viscous gels. In the first preparation process, Carbopol was directedly dispersed into quercetin phytosome suspension and adjusted pH after that. So, the swelling and thickening processes of Carbopol were taken place after the combination of the polymer and phytosome suspension. So the particle size of phytosome and PDI could be affected by swelling or thickening of Carbopol under the neutralizing process. So, in this study, we only concentrated on the investigation of the influence of hydrogel excipients and the preparation process on the chemical properties of quercetin phytosome hydrogel.

Compared to free quercetin, quercetin phytosomes enhanced both the rate and extent of active ingredient released through the biomembrane ($p < 0.05$). Our results were in good accordance with the published studies about phospholipid complex of rutin[14], Standardized Bacopa Extract[28], Standardized Centella Extract[29] and sinigrin[30]. The

results of the study suggested that the release of quercetin through the 0.2 μm cellulose acetate membrane is in a similar trend with the release through rat skin in the screening studies.

The achieved results proved that phytosomes enhanced not only the release rate of the active ingredient but also the deposition of quercetin into the rat skin - this is always the goal for a skincare product. The deposition of a higher quantity of quercetin into the skin layers could help slow down the passage of active ingredient through the skin and would give more antioxidant or anti-aging effects. In a similar study, Bhupen Kalita et al. reported a higher retention amount of rutin in the form of phospholipid complex in abdominal both rat and human cadaver skin[14]. That report was in association with the result of Das M. K. and Kalita B.[31]. In another study, Anisha Mazumder and co-worker[30] indicated that the administration of sinigrin via phospholipid complexes showed better skin delivery into the stratum corneum–epidermis compared to the control solution of free sinigrin. It could be explained for the excellent delivery of sinigrin by phytosomes into the stratum corneum–epidermis skin layer by the formation of lipid-compatible molecular complexes when sinigrin was bound to phospholipids.

The results of our studies indicated that the combination of phytosome suspension with Carbopol 934 could obtain phytosome hydrogel containing 0.1% quercetin that was not only able to enhance quercetin release but also improved the deposition of quercetin into rat skin in comparison with those of conventional hydrogel dosage form. Thus, the hydrogel containing phytosome quercetin could be used to overcome the limited bioavailability of quercetin and therefore increase the anti-aging effect of the compound.

5. CONCLUSIONS

In this research, the quercetin phytosome complexes were prepared by the anti-solvent precipitation method. The complex formation of quercetin phytosomes was confirmed by the FTIR and ^{1}H-NMR studies. Then, we successfully formulated prepared phytosomes into hydrogel dosage form at a concentration of 0.1% quercetin. Our results indicated that the amount of quercetin release from the phytosome hydrogel and the deposition of quercetin in rat skin was superior in comparison to conventional gel. After 20 hours, the cumulative amount of quercetin released through the rat skin and the amount of quercetin deposited in the rat skin significantly increased more than 3 times and 6 times, respectively.

6. ACKNOWLEDGMENTS

Author's contributions:

Thi-Thu-Giang Vu: performed study design and acquisition of data; analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript for important intellectual content; statistical analysis; study supervision.

Hong-Trang Nguyen: performed acquisition of data; analysis and interpretation of data; drafting of the manuscript.

Thi-Hai-Yen Tran: performed analysis and interpretation of data, performed study concept and design; critical revision of the manuscript for important intellectual content.

Bao-Tung Pham: performed acquisition of data; administrative, technical and material support; critical revision of the manuscript for important intellectual content.

Thi-Minh-Hue Pham: performed study concept and design; analysis and interpretation of data; critical revision of the manuscript for important intellectual content; obtained funding.

Conflict of interest (If any)

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