International Journal of Applied Pharmaceutics

ISSN- 0975-7058 Vol 15, Special Issue 1, 2023

Original Article

OPTIMIZATION AND CHARACTERIZATION OF QUERCETIN VITAMIN C NANO-PHYTOSOME FORMULATION

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Received: 15 Nov 2022, Revised and Accepted: 05 Jan 2023

ABSTRACT

Objective: To design an optimal formulation for quercetin and vitamin C nano-phytosome.

Methods: Nano-phytosomes are prepared by the thin layer hydration technique using a 2-level-5-factor design experimental. A total of 32 experimental formulas were used for data analysis. The ratio of quercetin: soy lecithin (X_1), the ratio of quercetin: cholesterol (X_2), stirring speed (X_3), stirring temperature (X_4), and stirring time (X_5) were used as independent factors, while globule size as a dependent factor. Data analysis was carried out by Design Expert12® application. Characterization of the optimal formula included physicochemical evaluation, globule size analysis, zeta potential, polydispersity index, entrapment efficiency, Transition Electron Microscopy (TEM) analysis, and FTIR analysis.

Results: The optimal formula consisted of quercetin: vitamin C: lecithin: cholesterol ratio of 1: 1: 1.046: 0.105 mol; stirring speed 763.986 rpm; stirring time of 59 min, at temperature 51.73 °C which produced 59.26 nm average globule size, PDI value 0.66; zeta potential value-35.93±0.95 mV and average SPAN value 0.61. This formulation showed entrapment efficiency of quercetin 91.69±0.18 % and vitamin C 90.82±0.13 %. The TEM and FITR analysis showed the morphological of the globules and interactions between the drugs, soy lecithin, and cholesterol to form nano-phytosomes.

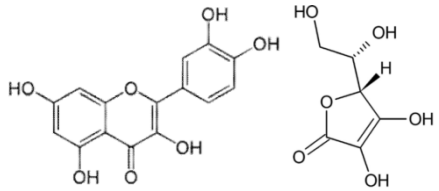
Conclusion: The conditions to obtain the optimal formula for quercetin vitamin C nano-phytosome consisted of quercetin: vitamin C: lecithin: cholesterol ratio of 1: 1: 1.046: 0.105 mol; stirring speed 763.986 rpm; stirring time of 59 min, and at temperature 51.73 °C

Keywords: Nano-phytosome, Quercetin, Vitamin C, Optimization, Factorial design

DOI: https://dx.doi.org/10.22159/ijap.2023.v15s1.47507 Journal homepage: https://innovareacademics.in/journals/index.php/ijap

INTRODUCTION

Quercetin (fig. 1) is one of the most widely consumed flavonoids. This compound is commonly found in vegetables, fruits, and tea [1, 2]. Quercetin has many pharmacological effects, including anti-inflammatory, anti-apoptotic, psychostimulant, preventing platelet aggregation, increasing capillary permeability, and enhancing mitochondrial biogenesis [2–4]. Ansar (2016) reported that quercetin can protect the liver from damage caused by hepatotoxins. In addition, the latest research by Zhang (2020) revealed that quercetin had activity against the SARS-Cov-2 virus, particularly showed significant inhibition against 3CLpro and PLpro of the virus [5–9].



(A) **(B)**

Fig. 1: Quercetin (A) and vitamin C (B) chemical structure [10, 11]

Ascorbic acid or as known as vitamin C (fig. 1) is a water-soluble vitamin, a strong reducing agent or antioxidant that improves an immune system to fight against bacterial infections, viruses, and other pathogenic agents. There is also evidence that vitamin C co-administered with quercetin provides synergistic antiviral action [12, 13].

However, despite its great potential, quercetin like other flavonoids, has limitations in its bioavailability and absorption. From the Biopharmaceutics Classification System (BCS) data, quercetin is included in BCS class II, which means low solubility and high permeability drug. The solubility of quercetin is about 1 µg/ml in water; 5.5 µg/ml in gastric juice and 28.9 µg/ml in intestinal fluid. This has limited its dissolution in the gastric after oral administration thus resulted in low bioavailability [14].

Nano-phytosome is a novel drug delivery system in the form of a nanometer-sized lipophilic molecular complex which improve the stability and bioavailability of a phytoconstituent, especially polyphenolic compounds [15]. Nano-phytosomes are products obtained from stoichiometric chemical interactions between phospholipids and plant extracts or phytoactive constituents in aprotic solvents resulting spherical shape vesicle encapsulating the drugs [16, 17]. Application of nano-phytosomes shows physical stability enhancement and increased phytoconstituent uptake by the cells to give better therapeutic benefits [18–20].

This study aims to design an optimal formula for the formulation of vitamin C quercetin nano-phytosomes. The optimization is focused on the comparison of the molar composition of the active substance with lecithin and cholesterol, as well as conditions in the formulation such as temperature, time, and stirring speed. The desired optimal condition is based on the globule size, zeta potential, PDI, entrapment efficiency, and the stability of the product formed.

MATERIALS AND METHODS

Materials

Quercetin were purchased from Sigma Aldrich®, (Germany), ascorbic acid were bought from Merck® (USA), soya lecithin were obtained from Lansida group (Indonesia), cholesterol were from PT. CRODA Indonesia, distilled water, *phosphate buffer saline*, methanol and dichloromethane (pro analytical grade) were purchased from Merck®, (USA).

Methods

Experimental design of quercetin vitamin C nano-phytosome formulation

The optimization of the quercetin vitamin C nano-phytosome formula was carried out with a 2^5 factorial design, with 5 variables and 2 levels of treatments. A total of 32 experimental formulas were used for data

analysis. The ratio of quercetin composition: soy lecithin (X_1), the ratio of quercetin: cholesterol (X_2), stirring speed (X_3), stirring temperature (X_4), and stirring time (X_5) were used as independent factors, while globule size was used as the dependent factor.

Manufacturing process of quercetin vitamin C nano-phytosome

Nano-pyrosomes were made using the thin layer hydration method with different molar ratios of quercetin, vitamin C, phosphatidylcholine, and cholesterol. Quercetin, vitamin C, were dissolved in methanol, while soy lecithin and cholesterol were dissolved in dichloromethane. The mixture was then evaporated by using a rotary evaporator (BUCHI®, Rotavapor R-100) at a temperature of 45 °C to produce a thin film. Then vacuum drying was carried out to completely evaporate the organic solvent. The film was hydrated using phosphate buffer saline at 45 °C. The sonication process is carried out after hydration by using a bath sonicator (Elma™, Elmasonic S-10 H) for the purpose of reducing the size of the globules [20].

Evaluation of quercetin vitamin C nano-phytosome

Physicochemical evaluation

The physicochemical evaluation of the nano-phytosomes includes organoleptic observation, evaluation of the pH value, and physical stability using freeze and thaw methods [21, 22].

Morphological and size evaluation of the globules

Observation of the morphological structure of nano-phytosome was determined by Transition Electron Microscope (TEM). The globule size, zeta potential and size distribution was determined by *Dynamic Light Scattering* (DLS) methods using a particle size analyzer (PSA) (Malvern Panalytical–Zetasizer Ver.7.12, UK) [20]. The size distribution was expressed by the SPAN value. SPAN is a measure of the range of the size distribution that can be determined with the equation below.

$$\text{SPAN} = \frac{\text{D(v90\%)} - \text{D(v10\%)}}{\text{D(v50\%)}}$$

Where D(v90%), D(v50%), and D(v10%) are the equivalent volume diameters at 90, 50, and 10% cumulative volume, respectively.

Determination of entrapment efficiency

Nano-phytosomes were taken and transferred to test tubes. The solution was centrifuged for 5 min at 15,000 rpm. After centrifugation, the supernatant was collected and the amount of free drugs was determined spectrophotometrically.

The concentration of free drugs (quercetin and vitamin C) in the sample was measured by multi-component measurement using Vierordt's method. This method was carried out because quercetin and vitamin C showed different maximum wavelengths (λ_1 dan λ_2) in one sample analysis [23].

To measure the concentration with this method, the information required is:

- Absorbance (A) of substance X at λ_1 and λ_2 as well as values of a_{x1} and a_{x2} respectively
- The absorbance of substance Y at λ_1 and λ_2, as well as the values of a_{y1} and a_{y2} respectively
- Sample absorbance at λ_1 and λ_2, (A_1 and A_2) respectively.

From the Lambert-Beer equation where A= εlC, the equation to calculate the concentration of 2 different substances in one sample can be derived as follows [23]:

$$A_1 = a_{x1}bC_x + a_{y1}bC_y \ldots\ldots\ldots\ldots (1)$$

$$A_2 = a_{x2}bC_x + a_{y2}bC_y \ldots\ldots\ldots\ldots (2)$$

Where A is the absorbance, a is the molar absorptivities from each compound, b is the width of the cell used (1 cm) and C is the concentration of the compound.

The encapsulation efficiency has been determined according to the equation

$$\text{EE (\%)} = \frac{W_{(1)} - W_{(2)}}{W_{(1)}} \text{x } 100$$

$W_{(1)}$ is the amount of the drugs added during the manufacture of nano-phytosomes, and $W_{(2)}$ is the amount of the free drugs remaining in the supernatant [20].

Fourier transform infrared spectroscopy analysis

FTIR spectra of quercetin, vitamin C, soy lecithin, cholesterol and the nano-phytosomes were obtained on a Perkin Elmer Spectrum 1000 FT-IR Spectrometer. Scanning was done at the wave number range of 4000–600 cm^{-1}.

Data analysis

Data are shown as mean±standard deviation. The response parameter values were analyzed using Design Expert 12® application.

RESULTS

Optimization of 5 factors 2 levels as shown in table 1 was carried out in 32 test formulas. Analysis of the test formulas by Design Expert 12® application obtained a polynomial equation. The Y response is the globule size and X_1–X_5 is the optimization factor. The polynomial equation giving the sequential model in terms of coded factors is given as follows:

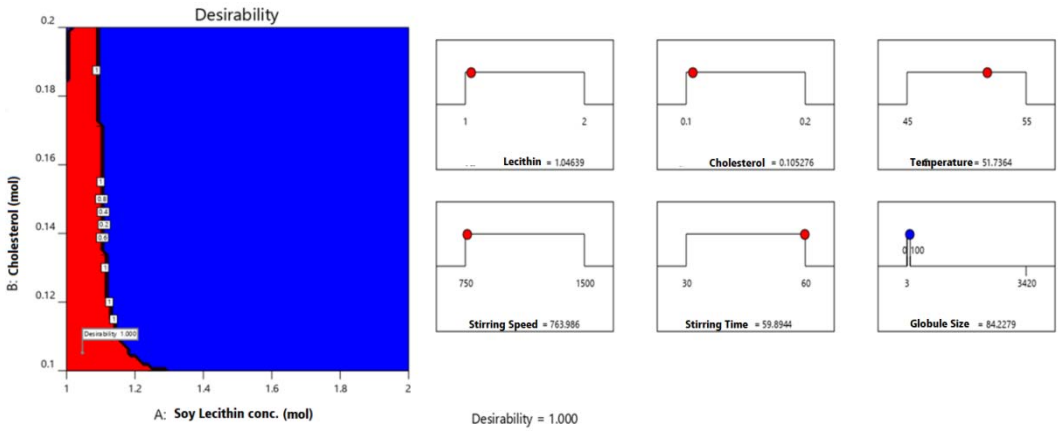
$$Y = 560.44+181.31X_1+65.56X_2-140.87X_3-290.06X_4-407.19X_5-245.19X_1X_2+152.38X_1X_3-216.81X_1X_4-268.06X_1X_5-170.37X_2X_3-41.06X_2X_4+22.81X_2X_5+143.25X_3X_4+82.88X_3X_5+224.81X_4X_5+120.25X_1X_2X_3+72.31X_1X_2X_4+173.81X_1X_2X_5+53.62X_1X_3X_4-77.38X_1X_3X_5+285.06X_1X_4X_5-18.87X_2X_3X_4+103.25X_2X_3X_5-74.81X_2X_4X_5-81.50X_3X_4X_5-100.87X_1X_2X_3X_4-79.13X_1X_2X_3X_5+11.56X_1X_2X_4X_5-107.87X_1X_3X_4X_5+78.25X_2X_3X_4X_5+11.50X_1X_2X_3X_4X_5$$

Where is Y = Response of globul**e** size; X_1= soya lecithin; X_2= cholesterol; X_3= stirring speed; X_4= temperature; X_5= stirring time; X_1X_2= 2 factor interaction; $X_1X_2X_3$= 3 factor interaction; $X_1X_2X_3X_4$= 4 factor interaction; $X_1X_2X_3X_4X_5$= 5 factor interaction.

Table 1: Factorial design 2^5 [16, 20, 24]

Code	Factor (X)	Level	
		Low	High
X_1.	Quercetin: Soya Lecithin ratio (mol)	1:1	1: 2
X_2.	Quercetin: cholesterol ratio (mol)	1: 0.1	1: 0.2
X_3.	Stirring speed (rpm)	750	1500
X_4.	Stirring temperature (°C)	45	55
X_5.	Stirring time (minute)	30	60

The predicted optimal formula for the quercetin vitamin C nano-phytosome is described in fig. 2B. These conditions are predicted to produce a mean globule size of 84.228 nm with a desirability value of 1.00.



(A) **(B)**

Fig. 2: Prediction of the optimal nano-phytosome formula using design expert 12®application; (A) Desirability value of the results; (B) Prediction of the optimal formula

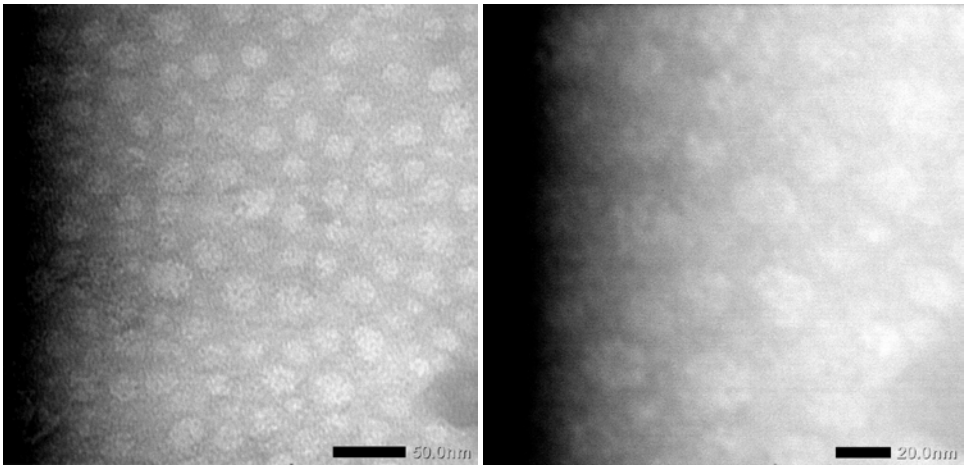
Based on organoleptic observations, it was found that the optimum nano-phytosome preparations formed had a liquid consistency, greenish-yellow in color, with characteristic odor of quercetin (fig. 3). Evaluation of the pH obtained a value of 7.57±0.20. There was no sight of visible precipitations and changes in physical appearances during the freeze and thaw stability test.

Evaluation of the predicted optimal formula showed that the mean globules size of the optimal formula is 59.26±34.94 nm (table 2), with zeta potential, PDI, and SPAN values listed. as was shown in table 2, the entrapment efficiency for quercetin and vitamin C in the nano-phytosome is 91.69±0.18 % and 90.82±0.13 % respectively. The results of globule morphology observations using TEM (fig. 4) showed spherical globules in shape with clear boundaries between globule particles.

Fig. 3: Quercetin vitamin C nano-phytosome

Table 2: Mean globule size, polydispersity index, zeta potential and SPAN value of prepared quercetin vitamin C nano-phytosome optimum formula

Sample	Mean particle size (nm)	PDI	% EE		Zeta potential (mV)	SPAN
			Quercetin	Vitamin C		
Sample 1	43.34	0.719	91.60	90.94	-35.9	0.697
Sample 2	35.12	0.527	91.59	90.85	-36.9	0.635
Sample 3	99.32	0.740	91.90	90.68	-35.0	0.493
Average	59.26±34.94	0.66±0.12	91.69±0.18	90.82±0.13	-35.93±0.95	0.61±0.1



(A) **(B)**

Fig. 4: The results of the globule morphology examination using a transmission electron microscope (TEM); (A) 50 nm scale bar, (B) 20 nm scale bar

FTIR analysis was carried out to observe the interaction formed between the active substance and the lipid component of the nano-phytosome. As shown in fig. 5, the spectra of quercetin showed a characteristic peak at 3000–3500 cm^{-1} (3268.43) due to the presence of O–H bonds [25]. In vitamin C, there is a specific spectral point at (1653.02) which indicates the presence of a double carbon group (C = C), and at the point (1752.36), which indicates the presence of a carbonyl group (C = O) [25]. Meanwhile, in the nano-phytosome spectrum, the typical peak of quercetin is still visible, but there are additional of 2 new peaks in the range of 2840-3000 cm^{-1} (2924.13; 2853.73) region, which indicates the presence of C-H stretching, originating from soy lecithin and cholesterol [26]. There is also a slight shift in the carbonyl bond and carbon double bond markers (1741.75; 1646.27), which indicates that vitamin C is also involved in the nano-phytosome.

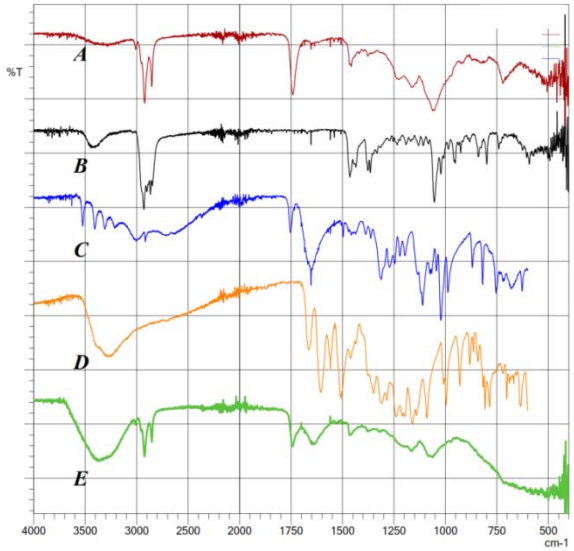


Fig. 5: FTIR spectra of soy lecithin (A), cholesterol (B), vitamin C (C), quercetin (D), and nano-phytosome sample (E)

DISCUSSION

Several studies related to the formulation of natural ingredients into phytosomes have been carried out previously by other researchers such as Rasaie (2014), Maryana (2018), and Saputra (2020) [20, 21, 27]. In the previous studies, the researchers used phospholipids from animal sources or pure form as carriers in the quercetin formulation. In this study, we used soy lecithin as a carrier. In this study, the quercetin nano-phytosome formulation was formulated with vitamin C, aiming to get a stabilizing effect of vitamin C on quercetin. Vitamin C is theoretically an anti-oxidant that can inhibit the oxidation process of quercetin in the formulation, which could increase the efficacy of quercetin [12, 13].

There are 5 factors (X) that affect the final size of the nano-phytosome globules formation. They are the ratio of quercetin composition: soy lecithin (X_1), the ratio of quercetin: cholesterol (X_2), stirring speed (X_3), stirring temperature (X_4), and stirring time (X_5) which were used as independent factors, and globule size was used as a dependent factor [16, 20, 24].

Based on the polynomial equation given by the application, the concentration of lecithin (X_1) and cholesterol (X_2) showed a positive number which means the globule size will increase with an increase in the concentration of these compounds. Meanwhile, the temperature (X_3), speed (X_4), and stirring time (X_5) show negative numbers, which means the size of the globules will decrease as the input increases. The search for the optimal formula is limited to the region which produces the "desirability" value of 1 with an assumption that the results are not significantly different from the application predictions [28].

The results of globule morphology observations using TEM showed that the globules were spherical in shape and had clear boundaries between globule particles. This firm spherical shape is affected by the addition of cholesterol in the formula. Cholesterol as a constituent of the hydrophobic walls of nano-phytosomes provides strength and rigidity to nano-phytosome particles/globules and increases particle stability so they are not easily damaged [16]. However, excessive addition of cholesterol in the formulation can affect the permeability of phytosome molecules and increase the size of their globules [26].

The optimal formula for quercetin vitamin C nano-phytosome produced average globule size 59.26±34.94 nm; with a SPAN value of 0.61±0.1. This number indicates the narrow distribution of globular size in the dispersion. In contrast to the predicted value by the application calculations, the average globule size of the nano-phytosome preparations obtained was smaller than the application predictions. However, it has a wide standard deviation that may occur because the unpredicted variable depends on the measuring conditions and also by the extremely low or high level used in the formula design.

The average PDI value obtained is 0.66±0.12. The PDI values in the range of 0.2-0.5 show the homogenous distribution of nanosized globules in a dispersion system. The particle size distribution is determined by the structure of lipid carriers and the nature of the ingredients used during nano-phytosome preparations [29].

The zeta potential of the nano-phytosome optimal formula showed a value of-35.93±0.95mV. This value indicates that the preparation has good physical stability against agglomeration [30]. The physical stability of the nano-phytosome is also confirmed by the freeze and thaw stability test. Evaluation of the entrapment efficiency showed the quercetin entrapment is 91.69±0.18% and vitamin C at 90.82±0.13%.

FTIR analysis was carried out to observe the interaction formed between the active substance and the lipid component of the nano-phytosome. The existence of a shift or formation of a spectrum peak on the nano-phytosome when compared to each of its constituent components, can be a reference for the formation of this complex [31]. In the FTIR spectra of the nano-phytosome samples, the specific peaks of each component of the nano-phytosome were observed with a slight shift in the peak value. However, no new peak formation was seen. This shows that the bonds between the nano-phytosome components formed are a type of weak bond such as hydrogen bonds [32].

This study is preliminary research on the formulation of quercetin and vitamin C into more applicable preparations for diseases with specific

receptor targets, such as COVID-19. Research conducted by Zhang (2020) revealed that quercetin has activity against the SARS-Cov-2 virus [9]. Moreover, the study demonstrated significant inhibition by quercetin against 3CLpro and PLpro of the SARS Cov-2 virus. The role of vitamin C in the formulation is not only expected to have an immunomodulatory effect to accelerate disease therapy but also the effect of vitamin C in recycling quercetin so as to increase its efficacy [12].

CONCLUSION

The optimization showed that the optimal formula for quercetin vitamin C nano-phytosome consists of quercetin: vitamin C: lecithin: cholesterol ratio of 1: 1: 1.046: 0.105 mol; stirring speed 763.986 rpm; stirring time of 59 min, and at temperature 51.73 °C produces small globule size (59.26±34.94 nm) and good stability (zeta potential value at-35.93±0.95 mV). For future prospects of this research, it was expected that nano-phytosome preparations will be developed into preparations that are more applicable in targeted delivery for diseases with specific receptors, such as COVID-19, cancer, and other diseases.

ACKNOWLEDGMENT

The authors acknowledge the financial support by the Faculty of Pharmacy, Andalas University through a research scheme year 2021 (number of contract 15/UN16.10. D/PJ.01./2021). We also acknowledged PT.DKSH Indonesia for the particle/globule size analysis.

AUTHORS CONTRIBUTIONS

All authors are equally contributed.

CONFLICT OF INTERESTS

The authors declared no conflicts of interest.

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