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Nano Phytosomes of Quercetin: A Promising Formulation for Fortification of Food Products with Antioxidants

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ABSTRACT

Background: Phytosomes are recently introduced drug delivery system and novel botanical formulation to produce lipophilic molecular complex to improve absorption and bioavailability of phytoconstituent. Quercetin is a well-known flavonoid with different biological effects and contributed in food preserving by free radical scavenging activity. However, bioavailability of Quercetin is an important limiting factor for its antioxidant activities. **Purpose:** To overcome this limitation, in the present study we aimed to produce Quercetin-loaded nano phytosomes to improve its physicochemical stability and bioavailability. ***Methods:*** Quercetin-loaded nano phytosome was prepared by using phosphatidylcholine (PC) and cholesterol (CH). Quercetin nano phytosomes system was characterized by particle size analyzer and differential scanning calorimetry (DSC). ***Results:*** Results showed that formulation with the Quercetin: PC: CH molar ratio of 1: 2: 0.2 had lower particle size (80 nm) and higher encapsulation efficiency percent (98%). Results also indicated that incorporation of cholesterol improved the physical stability of nano phytosome for over three weeks. The DSC data showed that incorporation of Quercetin in the phospholipid bilayer reduced the phase transition temperature of bilayer in the nano phytosome structure resulting higher release and bioavailability. ***Conclusion:*** Nano phytosomal formulation of Quercetin showed promising potential in fortification of food products with water insoluble antioxidants.

Introduction

Free radicals are reactive molecules with unpaired electrons which can oxidize other molecules to obtain electrons and stabilize themselves. They can oxidize macromolecules, such as DNA, proteins, carbohydrates and lipids.[1-3] Several plant-derived compounds have shown many pharmaceutical properties in the treatment of cancer, prevention of the occurrence of many chronic diseases and have been used as supplement for formation of different types of para pharmaceutical productions.[4,5] On the other hands, these compounds due to the antioxidant activity may be more effective than many synthetic antioxidants. They were used as efficient antioxidant in vegetable oils, milk powder, animal fat and other production in food industrial.[6-8] Antioxidants prevent the formation of peroxides and therefore slow down the process of the food oxidation. Other techniques such as air-tight packaging, using inert gases like nitrogen, vacuum packing and refrigeration can be used to delay the oxidation process in foodstuffs. However, these can still be inefficient and addition of antioxidants can be an effective way to prolong the shelf life of the foodstuffs. It was found that both the concentration and absorption of natural antioxidants are important in producing the maximum beneficial effect.[9-11]

Synthetic antioxidants have shown evidences of toxicity in animal models. Flavonoids, the most common group of polyphenolic compounds, have many properties such as antioxidant, antimicrobial, anticancer and anti-inflammatory effects.[12] Quercetin is a natural polyphenolic flavonoid and is distributed widely in fruits, vegetables and herbs which is being investigated for its widespread health benefits including antioxidant and antimicrobial activities.[13,14] Quercetin also can be used in cardiovascular protection by reducing oxidative damage caused by LDL cholesterol through scavenging free radicals and chelating transition metal ions. Quercetin inhibits an enzyme that has been related to nerve, eye and kidney damage in diabetic patients. However, despite of this wide range of therapeutic activity, Quercetin like other flavonoids has a major limitations in bioavailability and absorption.[15-17] Its poor absorption is likely as result of three factors including poor lipid solubility, due to the sugar moieties which elevate the molecules hydrophilicity, its

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large size molecules which cannot be absorbed by passive diffusion from intestine into bloodstream, and finally degradation of phenol moiety of Quercetin by gastrointestinal bacteria's which destroyed Quercetin in gastric environment.[18-22] Therefore, because of the advantage of Quercetin as a therapeutically active agent and its limited absorption, it is necessary to develop a new formulation for Quercetin.[23]

The improved drug bioavailability of nanoparticles is attributed to the fact that particles in the nano-size range are efficient in crossing permeability barriers.[24] Thus, the absorption and oral bioavailability of dutasteride, salmon calcitonin, olanzapine, and ibuprofen were demonstrated to be significantly enhanced when administered as nanoparticles, in comparison with oral-free drugs.[25-27] It has been indicated that the transport of nanoparticles is mediated by Peyer's patches (M cells) in the small intestine, and they enter the systemic circulation through lymphatic transport which enhances oral drug absorption. Moreover, bypassing the liver through lymphatic transport leads to a reduced first-pass metabolism and consequently enhances oral drug bioavailability.[28]

Phytosome is an advanced delivery system and a novel botanical formulation to produce lipophilic molecular complex which improves absorption and bioavailability of phytoconstituent specially polyophenolics.[29] The commonly used lipid phase substances for producing phytosomes are phospholipids from soy, and mainly phosphatidylcholine (PC).[30] Phosphatidylcholine is miscible in both water and lipid environment and well absorbed when taken orally. It has two polar and non-polar portions where, the polar head of PC, choline, interacts with phytoconstituents by creation of H-band between PC's phosphate group and hydroxyl group of phytoconstituents. On the other hand, two fatty acid chains, as non-polar portion of PC, produce microsphere with lipophilic membrane around phytoconstituents.[31-33] Due to the chemical bond between H-band of PC and phytoconstituents, phytosomes show better physical stability which enhances absorption of hydrophilic polar phytoconstituents resulted in enhanced bioavailability and greater therapeutic benefits. [34] In principle, both liposome and phytosome systems are vesicular structures prepared by phospholipids and the fundamental difference between them is the way of drug incorporation in their structure. In liposomes the active ingredient is dissolved in the medium contained in the cavity or in the layers of the membrane, whereas in the phytosome it is an integral part of the membrane, being the molecules anchored through chemical bonds to the polar head of the phospholipid.

The aim of the present study was to prepare a physically stable phytosomal formulation of Quercetin with higher encapsulation efficiency and physical stability to improve its efficacy in intestinal absorption and its preservation from oxidation in foodstuffs.

Materials and Methods

Materials

Soybean phosphatidylcholine, cholesterol, methanol and dichloromethane were purchased from Merck Company (Darmstadt, Germany). Quercetin was obtained from Sigma Aldrich Company (Steinheim, Germany). Distilled water was obtained from a Milli-Q Plus purification system and all other used chemicals and solvents were of analytical grade.

Preparation of Quercetin nano phytosome

Phytosomes were prepared by using thin layer hydration method with different molar ratio of Quercetin, PC and cholesterol. Quercetin and PC was dissolved in methanol, while cholesterol was dissolved in dichloromethane. The mixture was taken in a round bottom flask and evaporated in a rotary evaporator (Heidolph, Germany) at 45 °C until evaporation of all solvents and producing thin dry film in the round bottom flask. The vacuum drying evaporate the organic solvents completely. Moreover, the prepared lipid thin layer had been exposed to nitrogen gas flow and kept an overnight in the room temperature before hydration to ensure the complete removal of the organic solvents. The film was hydrated with distilled water in a rotary at 45 °C. Three methods were used to decrease phytosomes size, including bath sonication (Model 8852, cole- Palmer Instrument, Chicago, IL) at 45 °C, homogenization (Heidolph, Germany) with 20,000 rpm and probe sonication method (Sonix, Vibracell).

Particle size analysis

The particle size and particle size distribution of prepared nano phytosomes was measured by particle size analyzer (Wing SALD 2101, Japan). Phytosomal suspensions were diluted with double distilled water and stirred continuously during the particle size analysis. The size distribution was expressed by the volume median diameter (VMD) and SPAN value. SPAN is a measure of the width of the size distribution and smaller SPAN values are obtained when narrower distribution exists.

$$\text{SPAN} = \frac{D(v90\%) - D(v10\%)}{D(v50\%)} \qquad \text{Eq.(1)}$$

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

Determination of encapsulation efficiency

The encapsulation efficiency of Quercetin was determined by calculating the amount of entrapped Quercetin in the phytosomes. To determine the encapsulation efficiency of Quercetin in phytosome, an appropriate amount of dispersion was transferred in Millipore Amicon® Ultra filtration tube (Ultracel, cut off 30 kDa). The dispersion was centrifuged (Sigma-3k-30, Germany) for 5 min at 5000 rpm. After centrifugation the supernatant was collected and

amount of free Quercetin was determined spectrophotometrically (ʎmax= 210 nm). [35] The encapsulation efficiency has been determined according to the following equation:

$$EE\ (\%) = \frac{W_{(Added\ \ drug\ )} - W_{(free\ \ drug\ )}}{W_{(Added\ \ drug\ )}} \times 100 \qquad \text{Eq.(2)}$$

Where, $W_{(added\ drug)}$ is the amount of drug added during the preparation of phytosomes, $W_{(free\ drug)}$ is the amount of free drug measured in the lower chamber of the Millipore Amicon® after centrifugation.

Physical stability studies

Phytosomal Quercetin suspensions were stored at 4 °C and samples were regularly withdrawn and physical stability of prepared phytosomes was evaluated at 7, 14, 21 and 28 days.

Differential scanning calorimetry (DSC)

A differential scanning calorimeter (DSC 60, Shimadzu, Japan) was used to measure enthalpy and melting point of all substance used in the study. The equipment was calibrated using indium and zinc. Samples were heated in the range of 25-300 °C at a scanning rate of 20 °C /min in aluminum pans (40 µL) under nitrogen gas. The melting points and enthalpies of fusion were calculated using the Mettler STARe software (version 8.01).

Results and Discussion

Particle size and encapsulation efficiency

The particle size of nano phytosomes is extremely important as it can be affect the stability and bioavailability of phytoconstituent encapsulated systems. Smaller particles possess a large surface area and have faster release as well as higher stability.[36] Table 1 shows the effects of changing molar ratio of phosphatidylcholine to cholesterol on the characteristics of the phytosome, including particle sizes, SPAN value and encapsulation efficiency of Quercetin. Average particles size of Quercetin phytosomes when prepared by molar ratios of 1: 2: 0 and 1: 2: 0.2 of Quercetin: PC: CH, were 79 nm and 82 nm, respectively. Considering the mean particle size and size distribution, the optimum ratio of PC to CH was found 2: 0.2 (Figure 1). It can be seen from the data in Table.1 the phytosomes size was enhanced with increasing cholesterol content. This might be due to the interaction between cholesterol and phosphatidylcholine which induces a tighter packing of PC in membrane resulting increase in the mechanical stiffness of the membranes.[37-39] Cholesterol also increases the thickness of phospholipid bilayer.[40] Quercetin exhibits a high affinity for phytosomes which is due to the its planar configuration, that can easily located into the organized structure of the phospholipids within the phytosomes membranes. [41] As it was shown in Table 1, encapsulation efficiency of quercetin in the phytosomes was in the range of 96-98% which did not change by different molar ratios of Quercetin: PC: CH.

Table 1. Mean particle size, SPAN value and encapsulation efficiency of prepared Quercetin phytosomes with different molar ratio of Quercetin (QU), phosphatidylcholine (PC) and cholesterol (CH). Data were expressed as mean ± SD of three experiments.

Formulation	QU: PC: CH	Mean particle size (nm)	SPAN	Encapsulation efficiency (%)
F_1	1: 1: 0	383.33 ± 1.53	1.36 ± 0.16	97.00 ± 1.00
F_2	1: 2: 0	79.67 ± 1.53	0.84 ± 0.03	97.67 ± 0.58
F_3	1: 2: 0.2	83.33 ± 2.89	0.88 ± 0.04	96.67 ± 0.58
F_4	1: 2: 0.4	200.67 ± 6.51	1.14 ± 0.03	96.67 ± 1.15
F_5	1: 2: 0.8	393.67 ± 2.89	1.15 ± 0.37	97.33 ± 0.58

Physical stability study

Phytosomes must be stable during the storage period and remain at the appropriate size range before reaching their targeted tissues when used as a drug delivery system. Physical stability of optimum formulation without cholesterol, has been studied for seven days and results indicated to instability due to the size increasing (Table 2). Results showed that, particle size of nano phytosomes was increased up to 6 folds after 7 days. However, addition of cholesterol into the nano phytosomes formulation resulted in physical stability of particle size over a 21 days period. Previous studies also showed that the physical stability of liposome can be enhanced by cholesterol addition. [42] The formation of the lipid bilayer and its fluidity is influenced by the amount of cholesterol introduced between the phosphatidylcholine molecules. Presence of cholesterol is advantageous as it makes the bilayer sufficiently flexible. The molecular structure of cholesterol includes a tetracyclic hydrocarbons rings, a single hydroxyl group at carbon 3 and an isooctyl hydrocarbon side chain at carbon 17.[43] Where cholesterol is incorporated into phospholipid bilayers, hydroxyl polar group of cholesterol is placed next to the phosphatidylcholine carbonyl groups by formation hydrogen bond. Therefore, this bonding between cholesterol and phosphatidylcholine can enhance electrostatic repulsion between phospholipid bilayer and finally increase its stability by limiting the

movement of acyl chains of phosphatidylcholine.[37]

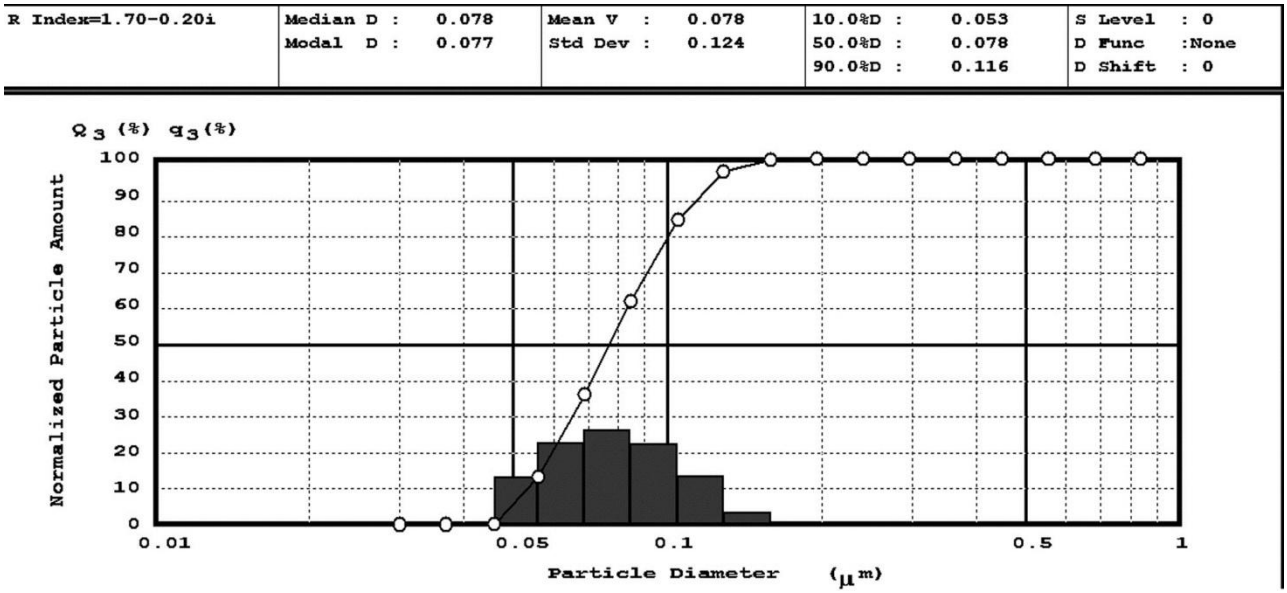

Figure 1. Particle size distribution of nano phytosome (formulation F3).

Table 2. Comparison of the prepared nano phytosomes size stability with or without cholesterol in their formulation. Results were expressed as mean ± SD of three experiments.

Formulatio	QU: PC: CH	Mean particle size (nm) ± SD						
		Day 1	Day 3	Day 5	Day 7	Day 14	Day 21	Day 28
F2	1: 2: 0	79.66 ± 1.52	162.66 ± 46.69	346.00 ± 33.42	468.33 ± 42.25	-	-	-
F3	1: 2: 0.2	78.66 ± 1.15	79.66 ± 1.52	79.66 ± 1.52	80.33 ± 0.58	80.66 ± 0.56	83.66 ± 2.30	98.33 ± 12.22

Differential scanning calorimetry

Differential scanning calorimetry thermograms of the pure Quercetin, cholesterol, phosphatidylcholine, and Quercetin-loaded nano phytosome were displayed in (Figures 2). The endothermic peak of Quercetin was observed at 321.22 ºC (Figure 2A) corresponding to its melting point. DSC thermogram of phosphatidylcholine and cholesterol also showed endothermic peaks at 205 °C and 152.45 °C, respectively (Figure 2, B and 2, C). Thermogram of Quercetin-loaded phytosome (Figure 2, D) interestingly showed disappearance of the endothermic melting peak of Quercetin and significant shift of the endothermic melting peak of cholesterol to the lower melting points. These observations indicated that Quercetin was molecularly distributed on the surface and inside the matrix of nano phytosome and lost its crystalline structure. Quercetin and phosphatidylcholine interact by Hydrogen bonding between OH- group of Quercetin and polar part of phosphatidylcholine. This interaction makes the long hydrocarbon tail of phosphatidylcholine which turn freely and envelop the polar head of phosphatidylcholine.

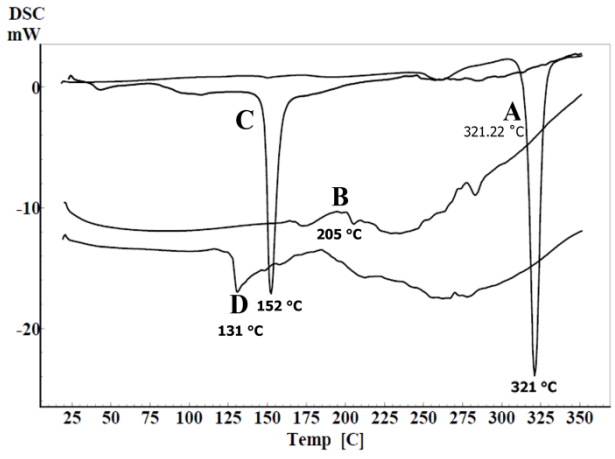

Figure 2. DSC thermogram of pure quercetin (A), phosphatidylcholine (B) cholesterol (C) and Quercetin-loaded nano phytosome (formulation F3) (D).

Quercetin exhibited a high affinity for the phytosomes that resulted from its planar configuration, which can easily introduce into the structured phosphatidylcholine within the phytosome membranes. [41] The decrease in the melting point of cholesterol in nano phytosome compared to the pure cholesterol can be attributed to its incorporation into the bilayer of the phytosome leading to the formation of less ordered structure.

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Conflict of interests

The authors declared that they had no conflict of interests.

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