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Preparation, physicochemical properties and antioxidant activity of genistein phospholipid complexes

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Abstract Genistein phospholipid complex (GS-PC) was produced in order to increase the solubility and antioxidant activity of genistein (GS), an insoluble natural polyphenol compound. By using the solvent evaporation process, GS-PC was produced. Several characteristics techniques were used to confirm the production of GS-PC, and its physicochemical characteristics and antioxidant activity were investigated. The outcome showed that GS-PC had a recombination rate of 96.84% ± 0.51%. The characterization results confirmed that GS-PC was formed by the intermolecular interaction between GS and phospholipids. *In vitro* antioxidant studies showed that GS-PC had a certain scavenging ability on DPPH free radicals, ABTS free radicals and hydroxyl free radicals. In summary, the results of this study indicated that GS-PC could be used as a formula to improve its solubility and antioxidant activity.

Keywords: genistein; phospholipid complex; physical and chemical properties; antioxidant

1 Introduction

Hyperlipidemia Genistein (GS), also known as genistein flavonoids (Fig. 1), mainly exists in legumes and is a soybean isoflavone component with high active function [1]. As a natural source of flavonoids, GS has many pharmacological activities such as preventing and treating osteoporosis [2], anti-oxidation [3], anti-cancer [4], improving cardiovascular and cerebrovascular diseases [5], and neuroprotection [6-9]. Studies have shown that the aging process is accompanied by an increase in free radicals, leading to oxidative damage to cells, which is considered to be one of the factors in the pathogenesis of many diseases, such as atherosclerosis and diabetes mellitus [10]. GS has multiple phenolic hydroxyl groups and can interact well with free radicals [11]. In rat liver mitochondria, GS was discovered to reduce microsomal lipid peroxidation caused by Fe^{2+}, ADP complex, and NADPH [12]. Some researchers have demonstrated that GS strengthens the cellular antioxidant defense system and prevents apoptosis and necrosis by regulating gene and protein expression [13]. However, due to the poor solubility of GS in water and extremely low oral bioavailability, its application in biomedical and food industry is limited.

Solid dispersions of GS were prepared using solvent rotary evaporation, and the results of *in vitro* release studies showed significantly higher drug release, as well as improved oral bioavailability and reduced effects of lipid accumulation [14]. Some researchers will prepare GS acrylic resin E100

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nanoparticles formulations using nanoprecipitation technique with nanoparticle encapsulation of 50.61% and drug loading of 5.02%, which were found to significantly improve the *in vitro* dissolution and bioavailability of GS [15]. The effect of GS against UV radiation-induced skin damage was investigated by microemulsion technology, which showed a significant increase in the solubility of GS, a significant increase in the skin absorption properties, and the ability of the GS water-in-oil microemulsion carrier to prevent UV-induced oxidative damage [16]. Limitations such as low bioavailability and low water solubility of GS were circumvented by the nanoemulsion technology, which opens up new avenues for alternative epigenetic therapies for osteoporosis [17].

In order to solve these problem, the preparation of GS into different dosage forms to improve its solubility has become a focus of current research [18]. Phospholipid complex is a compound formed by a drug itself through non-covalent interaction with phospholipids, and its physicochemical stability depends on the strength of the interaction between the drug and phospholipid [19, 20]. Therefore, phospholipid complexes usually have a high drug carrying capacity [21]. During the *in vivo* absorption process, the phospholipid complex is similar to the endogenous absorption mechanism of phospholipids through intestinal cells and can pass through the phospholipid bilayer of the cell membrane in an ATP-independent manner, significantly improving the membrane permeability of drugs without damaging the cell membrane, showing good biocompatibility and low toxicity.

In this study, we produced genistein phospholipid complex (GS-PC) by solvent evaporation method to enhance its bioavailability and antioxidant activity in order to address the issue of poor solubility of GS. The physicochemical properties of GS-PC were investigated and a series of characterizations were carried out. Finally, the *in vitro* antioxidant activity of GS-PC was investigated.

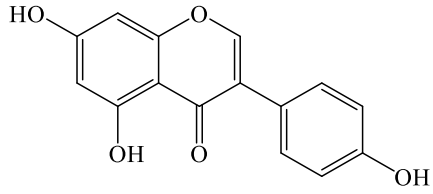

Fig. 1 The chemical structure of genistein

2 Method

2.1 Materials

Genistein and lecithin were purchased from Aladdin Reagents Ltd. Tetrahydrofuran and trichloromethane of analytical grade were purchased from Lianlong Bohua (Tianjin) Pharmaceutical Chemical Co. Octanol was acquired at Tianjin Hengxing Chemical Reagent Co. Ltd.

2.2 Preparation of phospholipid complex

A 100 mL round-bottomed flask containing precisely weighed amounts of 100 mg GS, 200 mg lecithin (PDC), and 50 mL of tetrahydrofuran was placed in a magnetic heating stirrer and heated to 50 °C for 2 h while the reaction was stirred. At the end of the reaction, tetrahydrofuran was removed by rotary evaporation (45 ℃, 150 rpm), 10 mL of trichloromethane was added and redissolved, and the unreacted GS was extracted using 0.45 μm organic microporous filtration membranes. After the filtrate was removed by rotary evaporation of solvent trichloromethane, the obtained product was dried in a vacuum oven at 50 ℃ for 12 h, that is, the required GS-PC sample was obtained.

2.3 Recombination rate

According to the solubility of GS and GS-PC lipid complex in trichloromethane, GS is insoluble and GS-PC is soluble. A previously prepared 10 mg of GS-PC was added to 10 mL of trichloromethane

and dissolved. By separating the unrecombined GS, 0.45 μm microporous membrane filtration was used. Using a rotary evaporator at 40 °C, trichloromethane was eliminated. The residue was dissolved with 50 mL of anhydrous ethanol, and after dilution, the absorbance was detected at 262 nm by ultraviolet spectrophotometry, and the content of GS was calculated to determine the recombination rate, which was calculated according to the following formula:

$$\text{Recombination rate (\%)} = \frac{W_1}{W_0} \times 100\%;$$

In this formula, W_1 is the mass of the combined genistein, and W_0 is the mass of the initial input genistein.

2.4 Fourier infrared spectroscopy (FTIR)

First grounded potassium bromide into fine powder, and then took appropriate amounts of GS, PDC, physical mixture (PM) of GS and PDC, GS-PC, and mixed them with ground fine potassium bromide powder at a ratio of 1:100−1:200. After grinding again, the tablets were pressed, and the samples were scanned by IR spectra in the range of 4 000−400 cm^{-1}.

2.5 Scanning electron microscopy (SEM)

Appropriate amounts of GS, PDC, PM, and GS-PC were respectively coated on the conductive adhesive, and the surface was sprayed with gold coating under vacuum conditions, and then placed in the scanning electron microscope testing chamber to observe the surface morphology of the above samples [22].

2.6 X-ray diffraction (XRD)

Apply appropriate amounts of GS, PDC, PM and GS-PC to the sample disk of the X-ray diffraction analyzer, using the Cu target as the ray emitter. The tube voltage was 40 kV, the tube current was 40 mA, the step size is 0.02°, and the scanning range (2θ) was 5°−60°. X-ray diffraction analysis was performed at a scanning rate of 4°/min.

2.7 Determination of drug load

Six portions of GS-PC were precisely weighed in 10 mL volumetric flasks, 4 mg per portion, diluted with ethanol to the degree of accuracy, then 0.2 mL of each was taken into six 10 mL EP tubes, 7 mL of ethanol was added, mixed, and at the wavelength of 262 nm. The concentration of GS in the complex was estimated after the absorbance value was measured using a UV-visible spectrophotometer, followed by the drug loading calculation.

$$\text{Drug loading (\%)} = \frac{W_{GS}}{W_{GS-PC}} \times 100\%;$$

"W_{GS}" is the weight of GS and "$W_{GS\text{-}PC}$" is the total weight of GS-PC.

2.8 Equilibrium solubility measurement

The equilibrium solubility was determined using a previously reported method. Extra GS and GS-PC were measured out and added to 4 mL of distilled water or n-octanol, respectively. The samples were then filtered after being shaken at 37 °C on a constant temperature shaker (100 rpm/min) for 24 h. A calculation of solubility was made.

2.9 Determination of oil-water distribution coefficient

The method of determining the oil-water distribution coefficient has been slightly modified from the previous report [23]. The n-octanol and distilled water were taken separately in an air-constant temperature shaker, shaken, and centrifuged, and the upper oil phase and the lower aqueous phase were separated. An appropriate amount of sample was weighed and added to the oil phase, after filtration, the filtrate was taken and diluted with ethanol, and the concentration of GS was calculated and noted as C_0. The other filtrate was taken and added to the aqueous phase, shaken centrifuged, and filtered, and the concentration of GS was calculated and noted as C_1. The following equation was used to compute the oil-water partition coefficient (P).

$$P = C_1 / (C_0 - C_1).$$

2.10 Dissolution measurement

The blade method, as described in the People's Republic of China Pharmacopoeia 2020 edition, was used to determine the *in vitro* dissolution of GS and GS-PC. As the dissolving media, two phosphate buffer solutions with pH values of 6.8 and 7.4 were used. Appropriate amounts of GS and GS-PC were weighed in the dissolution apparatus, samples were taken after solution treatment and filtered. The cumulative *in vitro* dissolution was calculated after the GS concentration was established.

2.11 DPPH, ABTS, and hydroxyl radical scavenging capacity

The treated DPPH, ABTS, and hydroxyl radical chromogenic solutions faded in color, and absorbance values decreased when antioxidants were added to them. Depending on the degree to which the samples absorbance had decreased, the antioxidant activity of the samples was calculated.

In this experiment, 80% methanol was used as the solvent, and the solutions of GS and GS-PC were configured separately. The concentration gradients of the solutions required for DPPH, and ABTS radical assay were 0.5, 1.0, 1.5, 2.0, and 2.5 mg/mL, and the concentration gradients of the solutions required for hydroxyl radical assay were 0.3, 0.6, 0.9, 1.2, 1.5 mg/mL. Then the levels of antioxidant indexes were detected according to the instructions of detection kit, respectively.

3 Results

3.1 Fourier infrared spectroscopy (FTIR)

The infrared spectral scanning results of GS, PDC, physical mixture (PM), and GS-PC were shown in Fig. 2. It could be seen from the figure that the infrared spectrum of PM was the superposition of the main peaks of GS and PDC, while the infrared spectrum of GS-PC was similar to that of PM but has some differences, indicating that the formation of phospholipid complex did not produce new chemical bonds between molecules. Compared with the PM spectrum, in the GS-PC spectrum, the stretching vibration absorption peak of -OH at 3 413 cm^{-1} of GS tended to disappear, indicating that -OH changed in the complex. The C=C vibration absorption peak of GS at 1 618 cm^{-1} and 1 583 cm^{-1} also changed, moving to the low wave level of 1 613 cm^{-1} and 1 577 cm^{-1}, respectively. The C-H stretching vibration absorption peaks of PDC at 2 925 cm^{-1} and 2 854 cm^{-1} did not change. The P=O stretching vibration at 1 246 cm^{-1} and the P-O-C stretching vibration absorption peak at 1 093 cm^{-1} shift to the low wave digit of 1 240 cm^{-1} and 1 083 cm^{-1}, respectively, indicating that the P=O bond in the complex also changed. These results demonstrated that the hydroxyl and benzene rings of GS and the polar phosphate groups of phospholipids interacted via hydrogen bonds, ionic bonds, chemical bonds, and van der Waals forces [24].

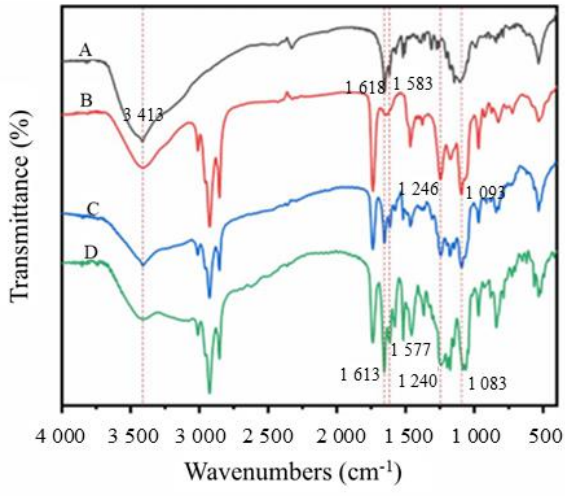

Fig. 2 FTIR spectra of (A) GS, (B) PDC, (C) PM and (D) GS-PC

3.2 Scanning electron microscopy (SEM)

The SEM results of GS, PDC, PM, and GS-PC were shown in Fig. 3. GS was a crystalline substance with uneven size, and PDC was an amorphous amorphous substance with an irregular shape. The PM was small granular with a certain crystalline morphology, while GS-PC, in contrast to PM, showed the same amorphous characteristics as phospholipids, indicating that the complex formation was not a simple physical mixing of the two raw materials.

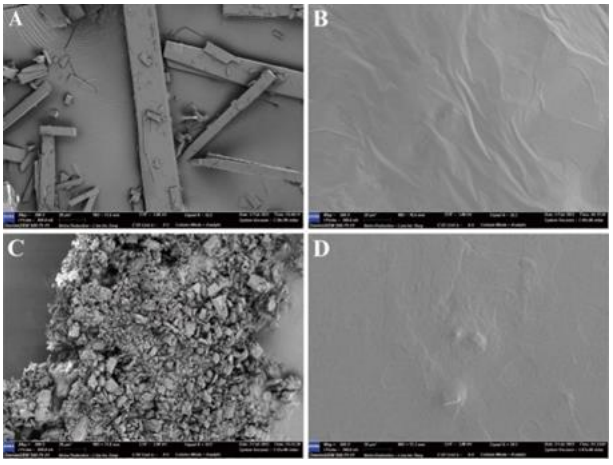

Fig. 3 SEM images of (A) GS, (B) PDC, (C) PM and (D) GS-PC

3.3 X-ray diffraction (XRD)

The X-ray powder diffraction results of GS, PDC, PM, and GS-PC were shown in Fig. 4. GS had sharp crystal diffraction peaks in many places, which indicated that GS existed in the form of crystal structure. There was no crystal diffraction peak in PDC, which indicated that PDC existed in an indeterminate state. PM still had a low intensity crystal diffraction peak, which indicated that it had a certain crystal structure. In GS-PC, the diffraction peak of the crystal belonging to GS completely disappeared, indicating that it existed in an indeterminate state.

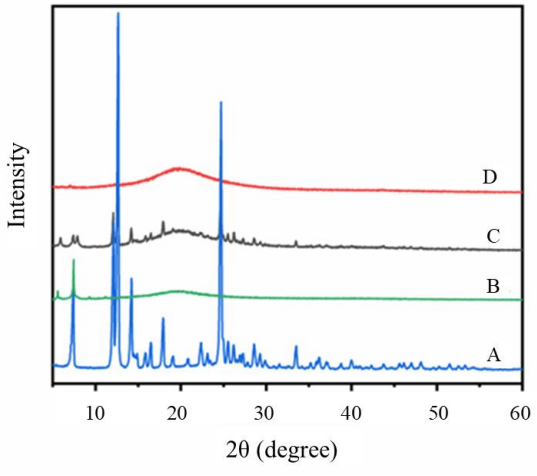

Fig. 4 XRD spectra of (A) GS, (B) PDC, (C) PM and (D) GS-PC

3.4 Drug load

The results of the drug loading assay of GS-PC showed that the drug loading of the prepared GS-PC was 32.48% ± 0.40%.

3.5 Equilibrium solubility

The results of equilibrium solubility measurements of GS and GS-PC showed that after the formation of phospholipid complexes with PDC, GS showed a significant increase in its solubility in all solutions, with a 7.48-fold increase in solubility in water compared to GS, a 2.90-fold increase in solubility in n-octanol compared to GS, and a 5.13-fold increase (pH = 1.2) in solubility in solutions of varying pH compared to GS, respectively, 5.00 (pH = 4.5), 7.91 (pH = 6.8), 8.52 (pH = 7.4) times in different pH solutions, respectively.

3.6 Oil-water distribution coefficient

The results of oil-water partition coefficient measurements of GS and GS-PC showed that the Log*P* of GS was 2.144, which was poor in hydrophilicity and unfavorable for oral absorption and utilization (Table 1). After the formation of phospholipid complexes between GS and PDC, the Log*P* decreased to 1.294, indicating a change in its solubility characteristics and an increase in hydrophilicity, which was expected to improve its oral bioavailability *in vivo*.

Table 1 Oil/water partition coefficient of GS and GS-PC

Sample	*P* ± SD	Log*P* ± SD
GS	139.46 ± 2.90	2.144 ± 0.032
GS-PC	19.69 ± 1.57	1.294 ± 0.035

3.7 Dissolution

The dissolution determination results of GS and GS-PC were shown in Fig. 5. In PBS with pH of 6.8 and 7.4, the dissolution behavior of GS-PC was significantly better than that of GS, and a higher cumulative dissolution degree could be achieved in a shorter time. This indicated that GS-PC had better absorption in the gastrointestinal tract, which was conducive to the improvement of GS bioavailability.

3.8 DPPH, ABTS, and hydroxyl radical scavenging capacity

The results of DPPH, ABTS, and hydroxyl radical scavenging ability of GS and GS-PC were shown in Fig. 6. Both GS and GS-PC have a certain degree of antioxidant capacity, which increases correspondingly with the increase of concentration. Compared with GS, GS-PC has a stronger scavenging ability for DPPH radicals, a scavenging ability for ABTS radicals that is basically equivalent to that of GS, and a hydroxyl radical scavenging ability that is slightly lower than that of GS.

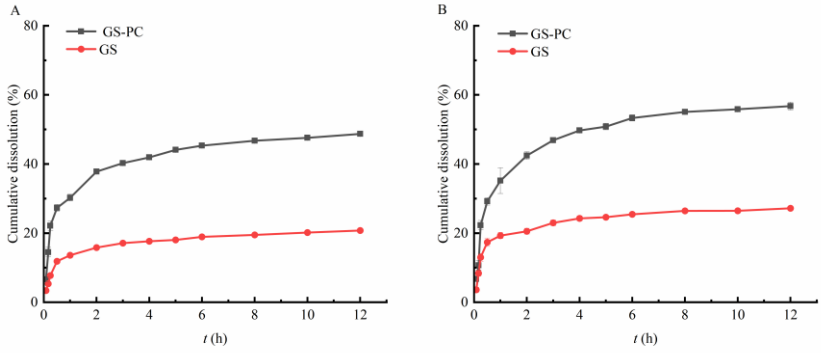

Fig. 5 *In vitro* release curves of the sample in PBS with pH = 6.8 (A) and pH = 7.4 (B) (*n* = 3)

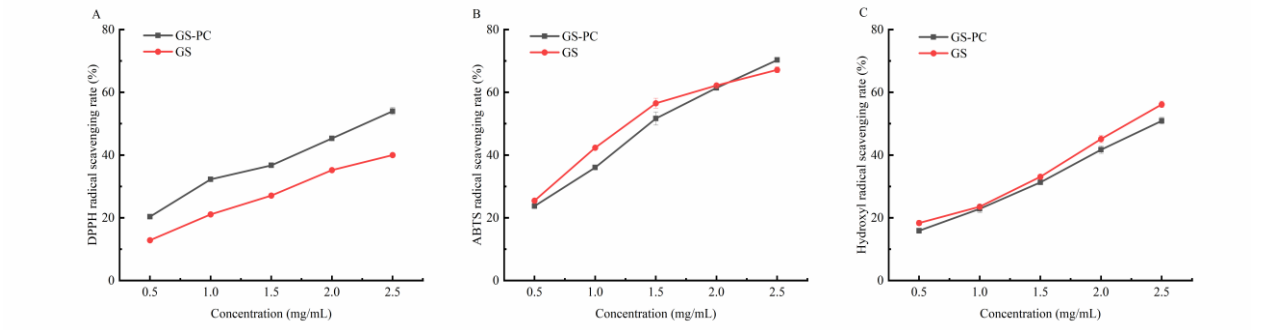

Fig. 6 Scavenging activities of GS and GS-PC on DPPH (A), ABTS (B), and hydroxyl radicals (C)

4 Discussion

Solvent evaporation [25], antisolvent precipitation [26], co-solvent lyophilization [27], supercritical fluid process, and co-grinding [28] are some of the techniques for the manufacture of phospholipid complexes that have been established to date. GS-PC was made by solvent evaporation and then evaluated using several techniques. As shown by FTIR, GS-PC was created as a result of the intermolecular contact between the hydroxyl and conjugated flavonoid skeleton of GS and the phosphate group of PDC by hydrogen bonding. SEM and XRD results showed that GS had a crystalline form, the PM had a certain crystal form, and GS-PC had a great change compared with them, showing an amorphous form. Studies have shown that amorphous drugs have higher solubility [29].

Researchers found that the solubility and cumulative dissolution rate of the phospholipid complexes were greatly improved compared to bulk drugs, greatly increasing their bioavailability [8]. Through drug loading, equilibrium solubility, oil-water partition coefficient, and dissolution studies, the physicochemical properties of GS-PC were evaluated in this work. These results suggested that oral bioavailability of GS is improved by being prepared as a phospholipid complex.

The results of *in vitro* antioxidant activity assays revealed that GS-PC had some scavenging ability on DPPH, ABTS, and hydroxyl free radicals, and the scavenging rate of each free radical rose with the rise in concentration within the experimental range. The overall comparison showed that GS-PC had similar antioxidant activity to GS monomer *in vitro*, indicating that the formation of phospholipid complex did not affect the antioxidant activity of GS *in vitro*. Studies had found that GS could reverse the decrease of SOD and GSH-Px activities of endothelial cells caused by Ox-LDL, improve lipid peroxidation, morphological damage, and apoptosis, and play a protective role in oxidative damage of endothelial cells [30].

GS-PC developed in this study effectively improved the physicochemical properties of GS, but collectively, its solubility and dissolution still had much potential for improvement. Therefore, GS-PC can be used as an intermediate dosage form in the future and its physicochemical properties can be further improved using formulation techniques such as cyclodextrin encapsulation or solid dispersion.

5 Conclusion

Solvent evaporation was successfully used in this study to create GS-PC and boost its bioavailability. The FTIR, SEM, and XRD measurements all verified the synthesis of GS-PC. Through intermolecular interactions, GS binds to phospholipid phosphate groups to form phospholipid complexes. The increase in GS solubility was supported by studies on equilibrium solubility, oil-water partition coefficient, and dissolution. The antioxidant activity of GS-PC was similar to that of GS monomer *in vitro*. This study serves as a foundation for enhancing GS's bioavailability and creating additional formulations based on GS.

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