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Preparation, characterization and evaluation of kaempferol phospholipid complex: Improvement of its solubility and biological effect

Dezhi Gao [1†], Honghui Zhao [1†], Fengmao Zou [2], Ming Yang [1], Jing Wang [1], Lina Fang [3], Xu Zhao [1*]

1. Faculty of Functional Food and Wine, Shenyang Pharmaceutical University, Shenyang 110016, China;
2. School of Traditional Chinese Material Medica, Shenyang Pharmaceutical University, Shenyang 110016, China;
3. College of Pharmacy, Shenyang Medical College, Shenyang 110034, China

Abstract Kaempferol (KA), as one of the flavonoids, has extensive pharmacological properties. However, the poor solubility of KA severely limits its clinical application. In our study, the kaempferol phospholipid complex (KA-PC) has been prepared by solvent evaporation for the enhancement of the bioavailability of KA. KA-PC was verified by scanning electron microscope characterization methods. Drug loading, solubility and long-term stability were measured. The characterization results showed that KA-PC was formed through the intermolecular interaction between KA and phospholipids. The solubility of KA-PC in water was 189 times higher than that of KA, and the solubility in *n*-octanol was also significantly improved. Besides, pharmacodynamic studies showed that KA-PC can significantly reduce the level of serum uric acid in mice without causing renal injury. This study expanded the clinical application of KA by preparing KA-PC.

Keywords: kaempferol; phospholipid complex; solubility; uric acid-lowering effect

1 Introduction

Flavonoids are a class of polyphenols widely found in the plant kingdom and are also included in the daily diet as a dietary supplement [1] In recent years, flavonoids have attracted much attention due to their various pharmacological effects. Among them, kaempferol (KA, Fig. 1) is a natural compound widely found in *Kaempferia galanga* L., which is abundant in grapes, onions, tea, and citrus fruits. KA has extensive pharmacological properties, including anti-cancer [2], anti-oxidant [3], anti-bacterial [4], anti-inflammatory [5] and hypoglycemic effects [6]. However, KA falls under the BCS II category of medicines that has low water solubility and high permeability. This leads to low bioavailability, which significantly constrains its future clinical application [7, 8]. Studies in recent years have been conducted to improve the physicochemical properties of KA by nanoparticles [9], liposomes [10], nanosuspension [11] and solid dispersion [12]. However, these dosage forms have certain drawbacks, such as low encapsulation rate, poor stability, and high cost [13]. Therefore, it is necessary to devise a novel dosage form to enhance the water solubility and bioavailability of KA and expand its potential applications.

Phospholipids (Fig. 1) are a crucial component

* Corresponding author: Xu Zhao (zhaoxu_1010@163.com).
† These authors contributed equally to this work;
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of biological membranes and can enhance the transmembrane ability of drugs due to their biocompatibility [14]. Phospholipids are molecules with a hydrophobic alkyl chain at one end and a hydrophilic nitrogen or phosphorus head at the other, making them amphiphilic [15]. In the phospholipid structure, hydroxy oxygen atoms linked by phosphorus atoms tend to gain electrons, while nitrogen atoms tend to lose electrons. Phospholipids can form complexes with drugs of specific structures under certain conditions through hydrogen bonding or van der Waals forces.

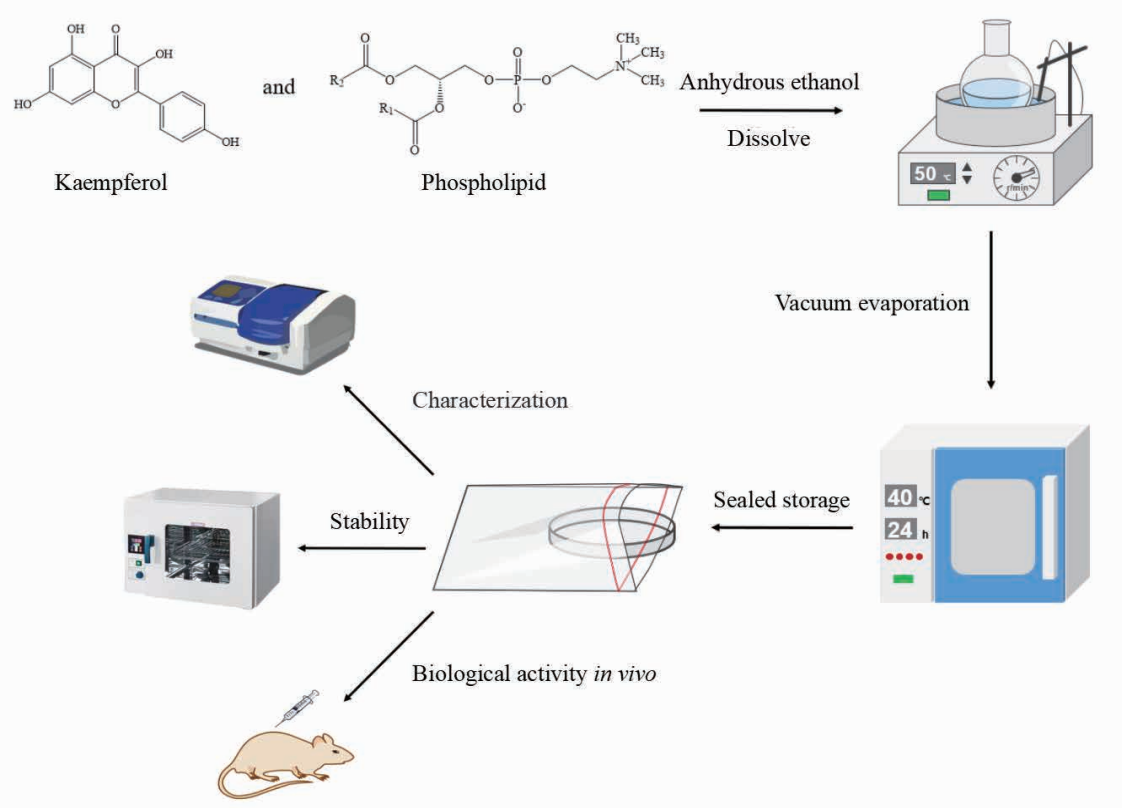

Fig. 1 Diagram of kaempferol phospholipid complex （KA-PC) preparation

Phospholipid complex (PC) is a formulation technique that has undergone extensive research in recent years. Its ability to enhance drug bioavailability by increasing solubility and facilitating absorption is well-documented [16]. PC is a complex of pharmaceuticals and phospholipids formed through intermolecular forces in specific conditions [17]. Phospholipids are utilized as amphiphilic molecules to increase the solubility of drugs, in addition to their ability to fuse with skin lipids [18]. The production process of PC is uncomplicated, making it well-suited for industrial manufacturing and meeting modern fast-formulation demands. Additionally, PC can be utilized as an intermediary component for producing wholesome foodstuffs or pharmaceuticals, or as a finished product for direct use, providing a variety of applicational opportunities [19].

KA-PC was synthesized to enhance the water solubility and fat solubility of KA. The dissolution, stability and anti-hyperuricemia activity were evaluated. The results of this study are of reference importance for the application of other poorly soluble flavonoid compounds.

2 Materials and methods

2.1 Materials and reagents

Lecithin (purity > 98%) and kaempferol (KA, purity > 97%) were obtained from Aladdin Reagent (Shanghai, China). Methylene chloride and ethanol were purchased from Li'an Longbohua Pharmaceutical

Chemistry Co., Ltd (Tianjin, China). The analytical grade of methanol was purchased from Fuyu Fine Chemical Co., LTD (Tianjin, China). *N*-octanol was bought from Yupu Industrial Community (Tianjin, China). Allopurinol, hypoxanthine, potassium oxonate, and paraformaldehyde (4%) were purchased from Meilunbio Co., LTD. Kits for the determination of uric acid (UA), creatinine (Cr), and blood urea nitrogen (BUN) and for Hematoxylin-Eosin staining were bought from Nanjing Jiancheng Bioengineering Research Institute (Nanjing, China).

2.2 Preparation of KA-PC

The preparation of KA-PC was carried out by solvent evaporation and the process of preparation is shown in Fig. 1. KA and phospholipids in a mass ratio of 1:3 were dissolved in a small amount of anhydrous ethanol. The mixture was stirred at 45 °C for several hours [20]. Anhydrous ethanol was then evaporated under vacuum at 40 °C and the residue was dried and sealed and stored.

2.3 Complexation efficiency of KA-PC

The free KA was separated from KA-PC using the difference in dichloromethane solubility of complexed KA and free KA (free KA was insoluble). KA-PC was dissolved in dichloromethane and filtered through a 0.45 μm filter. Then the dichloromethane was evaporated at 40 °C. The residue was dissolved and diluted with anhydrous ethanol [21]. The absorbance of KA was measured at 365 nm using a UV spectrophotometer (UV-5100, Metash, China). The complexation efficiency (F%) was calculated as follows:

$$F\% = \frac{m}{M} \times 100\%$$

Where M is the total mass of KA; m is the mass of complexed KA.

2.4 Scanning electron microscope (SEM)

KA, phospholipids, physical mixture (PM), and KA-PC were fixed on the conductive adhesive with gold sprayed on the surface [22]. Then the morphologies were evaluated by SU8020 SEM (Hitachi, Japan).

2.5 Drug loading

Proper amount of KA-PC was accurately weighed and dissolved with proper amount of methanol in a 10 mL volumetric bottle. Ultrasonic treatment was used to ensure complete dissolution. After quantitative dilution, the content of KA in KA-PC was determined, and the experiment was repeated three times [23]. The drug loading was calculated as follows.

$$\text{Price} = \frac{W_{KA}}{W_{KA\text{-}PC}} \times 100\%$$

W_{KA} is the weight of KA, $W_{KA\text{-}PC}$ is the total weight of KA-PC.

2.6 Solubility

Excess KA and KA-PC were dissolved in 3 mL distilled water and *n*-octanol, respectively, and vibrated at 37 °C for 24 h in an air bath thermostatic oscillator (THZ-82A, Xinrui, China). The rotational speed and time were set at 12 000 rpm for 15 min, the supernatant was taken and the solubility was determined [24].

2.7 Long-term stability

Appropriate amount of KA and KA-PC were placed in a lofting bottle and sealed at 4 ℃ away from light. Samples were taken at the 0, 1, 2, 3, 4 and 6 months, changes in appearance color and physical properties were recorded, and changes in KA content were measured (based on 100% KA content at the 0 month), and the experiment was measured three times in parallel [25].

2.8 Evaluation of biological activity in vivo

2.8.1 Design of experiments and animals

Male KM mice (18 to 22 g) were obtained by Liaoning Changsheng Technology Co. Ltd. All

the mice had free access to water and food. After acclimating for 3 days, the mice were randomly divided into 5 groups according to their weight (n = 10 for each group) as follows: Control, Hyperuricemic model (HU), Allopurinol (ALL, 5 mg/kg), KA (100 mg/kg) and KA-PC (equivalent to 100mg/kg KA) groups. All mice, except the control group, were dosed with hypoxanthine (400 mg/kg) and potassium oxonate (400 mg/kg) orally to induce hyperuricemia. Control group mice were orally administered an equal amount of 0.5% CMC-Na. One hour after hypoxanthine and potassium oxonate administration, mice in the ALL, KA, and KA-PC groups were given allopurinol, KA, and KA-PC by oral gavage, respectively, while mice in the control and HU group were administered 0.5% CMC-Na. The treatment lasted for 7 days.

2.8.2 Biochemical analysis

Mice were fasted overnight before blood and tissue sample were collected. Blood samples were taken from the orbital venous plexus and centrifuged at 4 000 rpm for 10 min at 4 °C to separate the serum on day 7, 0.5 h after drug administration. The serum samples were stored under 2−8 °C until analysis. Then the mice were sacrificed, and the left kidney was taken and transected for a pathological section. Serum UA, Cr, and BUN levels were determined with biochemical kits within 36 h after the serum was obtained [26-28].

2.8.3 Histopathological examinations

The kidney tissue was fixed with 4% paraformaldehyde and stored in 75% ethanol. Then the biopsies were dehydrated with ethanol solutions with series-graded concentrations and embedded in paraffin. The sections of 3 μm were stained with hemoglobin and eosin, and the morphological evaluation were carried out under a light microscope at 400 × magnification [29].

2.9 Statistical analysis

Unpaired Student's t-test was used to express data as mean ± SD. $P < 0.05$ values were considered significant using SPSS 20.0.

3 Results and Discussion

3.1 Optimization of KA-PC preparation

When the mass ratio of KA to phospholipid was 1:3, the reaction time was 3 h, and the reaction temperature was 50 ℃, the maximum complexation efficiency was 96.67%.

3.2 SEM

SEM has the characteristics of large depth of field, high resolution, visual imaging and wide magnification range. KA (Fig. 2A) was a rectangular crystal. Phospholipid (Fig. 2B) was an amorphous structure with a smooth surface. Clear KA crystals can be seen in PM (Fig. 2C), indicating that PM was a simple mixture of KA and phospholipid. The morphology of KA-PC (Fig. 2D) had changed significantly, with a smooth surface and disappearance of KA crystals, indicating that KA was dispersed in phospholipid to form an amorphous structure [30].

3.3 Drug loading

The drug loading results of KA-PC prepared under the optimal conditions were 21.43%, 20.96%, 21.26%, 21.53%, and 21.22%, respectively. The average drug loading of KA-PC was (21.28 ± 0.22)% (n = 5).

3.4 Solubility

Equilibrium solubility refers to the solubility of the solute after the system reaches equilibrium, and the solubility of one substance in another substance can be examined by equilibrium solubility. The water solubility of KA-PC increased from (1.59 ± 0.34) μg/mL to (326.69 ± 6.57) μg/mL, which increased by 205 times. The solubility in n-octanol increased from

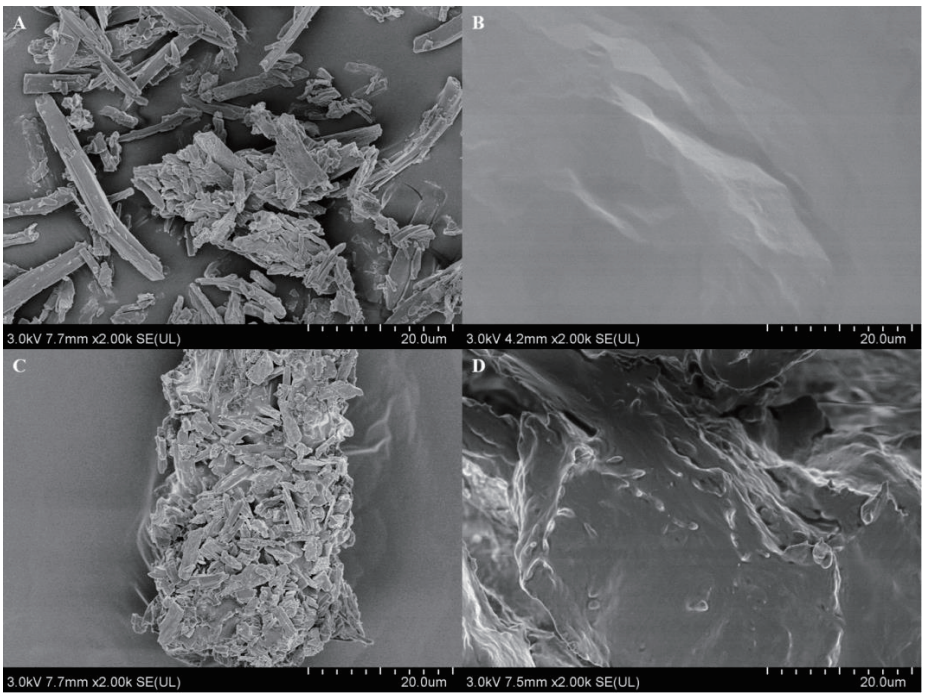

Fig. 2 SEM images of (A) KA, (B) phospholipids, (C) PM, and (D) KA-PC

(942.85 ± 12.58) μg/mL to (2 874.41 ± 24.93) μg/mL, which increased by 3 times. These results indicated that KA-PC increased the solubility of KA significantly.

3.5 Long-term stability

Stability is a crucial evaluation criterion for pharmaceutical preparations. It provides the basis for determining subsequent storage and transportation conditions. According to the guiding principles for the stability test of Raw Material Drugs and Preparations in the fourth part of Chinese Pharmacopoeia (2020 Edition), the long-term stability was investigated by using appearance traits and KA content in KA-PC as indicators. The KA-PC was sealed and stored at 4 ℃ away from light to investigate its long-term stability. The results were shown in Table 1 and Fig. 3. The results showed that the color of KA-PC changed from light yellow to yellow in the third month, and the appearance character did not change significantly during the storage time of 6 months, and its relative content decreased to 96.82%. The results indicated that the KA-PC could maintain basic stability when stored at 4 ℃, away from light and sealed for 6 months. The long-term stability results of KA were consistent with those of KA-PC, with no significant changes in appearance during the 6-month storage period and the relative content decreased to 95.67%, as shown in Table 2 and Fig. 4.

Table 1 Long-term stability results of KA-PC

Time (month)	Appearance	Content (%)
0	Light yellow flake crystals	100.00
1	Light yellow flake crystals	98.96
2	Yellow flake crystal	98.41
3	Yellow flake crystal	98.04
4	Yellow flake crystal	97.94
6	Yellow flake crystal	96.82

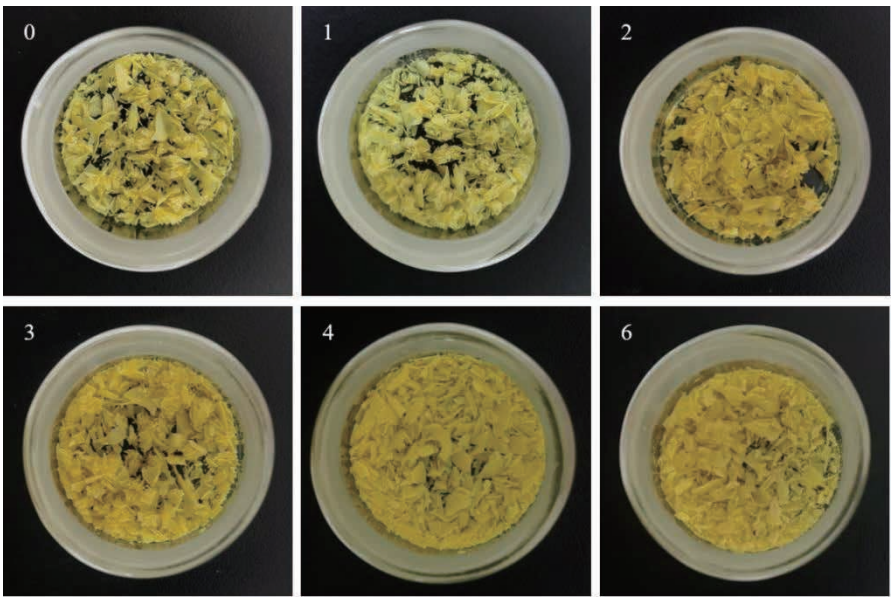

Fig. 3 KA-PC long-term stability sample appearance

Table 2 Long-term stability results of KA

Time (month)	Appearance	Content (%)
0	Yellow powder	100
1	Yellow powder	99.14
2	Yellow powder, caked	98.84
3	Yellow powder, caked	98.12
4	Yellow powder, caked	97.24
6	Ginger powder, caked	95.38

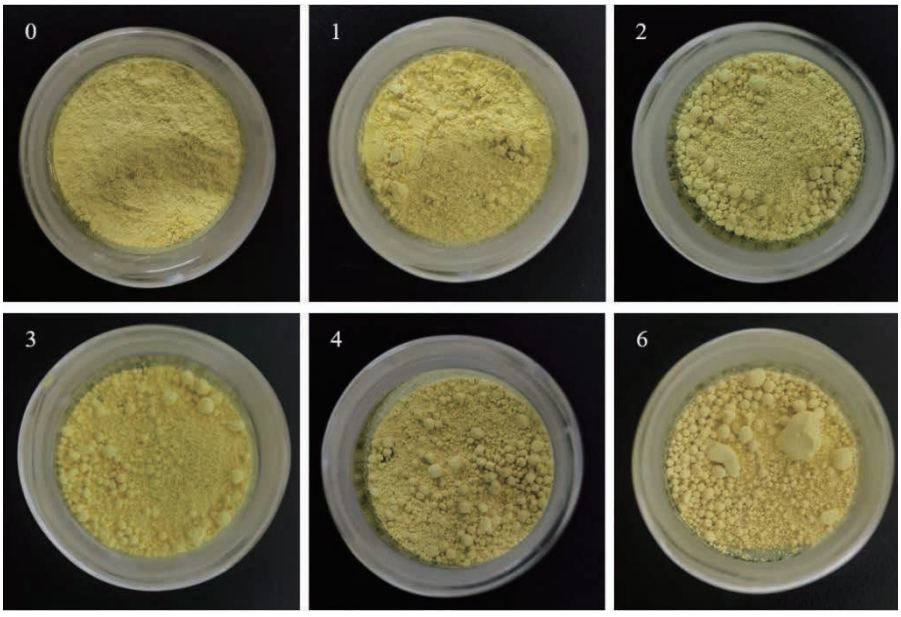

Fig. 4 KA long-term stability sample appearance

3.6 Evaluation of biological activity

3.6.1 Uric acid-lowering effect of KA-PC in a hyperuricemic mouse model

The results of the UA assay performed were shown in Fig. 5A. UA levels in HU group were higher than those in control group ($P < 0.01$) after administering hypoxanthine and potassium oxonate, indicating good establishment of hyperuricemic model [31]. There was no significant difference in UA level between the KA group and model group. The possible reason was that the poor solubility of KA limits its oral bioavailability [32]. Following KA-PC treatment, the level of UA was significantly lower than in the HU group ($P < 0.01$) and lower than in the KA group ($P < 0.01$), suggesting KA-PC was effective in improving the oral bioavailability of KA and demonstrated the effect of UA reduction *in vivo*. The results of previous pretrial had shown that treatment with KA (150 mg/kg, 200 mg/kg) were effective in reducing serum uric acid levels in hyperuricemic mice. In this study, a dose of KA of 100 mg/kg to highlight the advantages of KA-PC in UAreducing effect by oral administration was designed.

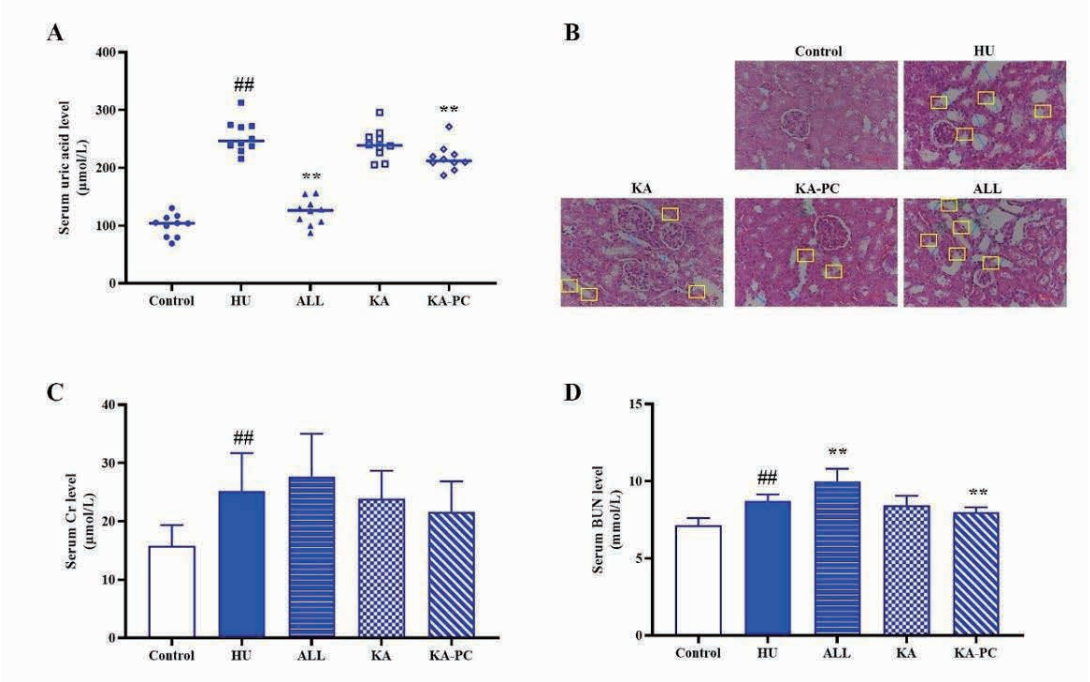

Fig. 5 Pharmacodynamics study

Note: Effect of KA-PC on levels of UA in HU mice (A), Cr (C), and BUN (D). Renal cortex sections for H&E staining (magnification, 400×) (B): Arrow: Dilated renal tubules; Box: Damaged or detached epithelial cell; green circle: Pyknotic or necrotic nuclei. Data were expressed as mean ± SD for 10 mice. ## $P < 0.01$, compared with control group; ** $P < 0.01$, compared with HU group.

3.6.2 KA-PC exhibited nephroprotective effect in vivo

To evaluate the nephroprotective effect of KA-PC, relative renal function indexes such as serum Cr and BUN levels were detected. As shown in Fig. 5C and 5D, the serum level of Cr and BUN of HU mice were remarkably higher than those in the control group. KA showed no statistical differences in reducing Cr and BUN levels in HU mice. But treatment with KA-PC lowered the BUN level after hypoxanthine and potassium oxonate administration ($P < 0.01$).

H&E staining of kidney was performed (Fig. 5B). Compared to the control group, the renal tubules shrank and some epithelial cells remarkably damaged or disappeared in hyperuricemic mice, whereas these

pathologies were improved to some degree after treatment with KA-PC [33]. This was consistent with the results of Wang Wei, et al. 's study on improving the inflammatory response and oxidative stress induced chronic kidney disease after hyperuricemia by preparing PC of gardenoside [34]. These results demonstrated the efficacy of KA-PC to reduce renal damage and provide a glimpse into the possible renoprotective effects of KA-PC in the processes of hyperuricemia and gout.

4 Conclusion

In this study, KA-PC was successfully prepared to improve the solubility of KA. KA-PC was verified by SEM, FTIR, ^{1}H-NMR, DSC, and XRD. The intermolecular interaction caused KA to change from a crystallized state to an amorphous state. KA-PC was more soluble in water and *n*-octanol than KA. The stability results showed that KA-PC should be preserved to avoid high temperature, high humidity, and light. Compared with KA, the anti-hyperuricemia activity of KA-PC was significantly improved. This study provides a basis for improving the bioavailability of KA and the development of other flavonoids.

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