Supporting Information (SI)

Simple but novel icariin-loaded pro-glycymicelles as functional food: physicochemical characteristics, in vitro biological activities, and in vivo experimental hyperlipidemic prevention evaluations

Qingchen Cui[1], Cuicui Wang[2], Liping Zhou[2], Yanjun Wei[1,3], Zongtao Liu[1,4*], Xianggen Wu[1,3*]

1 College of Chemical Engineering, Qingdao University of Science and Technology, Qingdao, China;

2 Department of clinical laboratory, Qingdao Hospital, University of Health and Rehabilitation Sciences（Qingdao Municipal Hospital）, Qingdao, China;

3 Viwit Pharmaceutical Co., Ltd. Zaozhuang, Shandong, China;

4 Affiliated Qingdao Third People's Hospital, Qingdao University, Qingdao, 266021, China.

***Correspondence:**

*Email: liutao009@163.com (Z.L.)

*Email: wuxianggen@126.com (X.W.)

SI Method

Materials

ICA was purchased from Aladdin Co. LTD (Shanghai, China). Dipotassium glycyrrhizinate (DG; two potassium salt derivatives of glycyrrhizin) was obtained from Shanxi Fujie Pharmaceutical Co., Ltd. (Sanyuan, China). Trypan blue (TB) was obtained from Solarbio, Co. LTD (Beijing, China). Cholesterol esterase, α-glucosidase, pancreatic lipase, 4-nitrophenyl-α-Dglucopyranoside and p-nitrophenyl butyrate were purchased from Shanghai yuanye Bio-Technology Co., LTD (Shanghai, China).

Characterizations of ICA-PG in solid state

In vitro release assay

The in vitro release profiles of ICA from pro-glycymicelles were explored with a previous dialysis bag method[1]. Briefly, 1 mg/ml ICA-PG solution (1 ml) or 1 mg/ml ICA solution (1 ml) was closed in dialysis bags (Mw cut-off 3.5 kDa) and incubated in 100 ml pH 6.8 PBS at 37 °C. At each time point (0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 7, 8, 12, 24 h), 1 ml release medium was extracted, and 1 ml blank buffer solution was used to replenish the release medium. The cumulative release contents of ICA from bare ICA samples and from ICA-PG were measured by the HPLC method[2]. Briefly, an Agilent Technologies 1100 chromatography system had a quaternary pump, a G1314A UV detector (detection at a maximum of 270 nm), and a G1367A Injector. The assay was conducted at 26°C using a 250 × 4.6 mm column filled with 5 μm-reversed phase C18 (Agilent ZORBAX SB-C18, Agilent, US). The flow rate was fixed at 1.0 ml/min of a 70:30 v/v mixture of acetonitrile-water. The injection volume was 20 μl. ICA had a retention time 4.3 ~ 4.6 min. The regression equation for ICA was C = 0.0225A + 0.0035 (R^2 = 0.999) for ICA concentration range 0.0488-0.3906 μg/ml, and C = 0.029A - 0.1693 (R^2 = 1) for ICA concentration range 0.3906-25.00 μg/ml, which was a good linear relationship between ICA concentration and peak area.

Hemolysis evaluation

Hemolysis evaluation was performed with free ICA, DG, DG&ICA, and ICA-PG as reported method[3]. TritonX-100 was set as positive control, and normal saline was set as negative control.

Hen's egg test on chorioallantoic membrane (HET-CAM)

HET-CAM evaluation was performed as reported protocol[4], and 1 mg/ml and 5 mg/ml ICA-PG, DG&ICA (1 mg/ml ICA and 15 mg/ml DG), 15 mg/ml DG solution, 1 mg/ml ICA suspension were tested with 0.9% NaCl solution as negative control and 0.1 M NaOH solution as positive control. After 5-minute exposures, trypan blue (TB) solution (1 mg/ml) was used to quantitatively determine the damages of tested solution to the CAM as reported method[4].

In vitro digestion assay and bioaccessibility

A protocol for simulating the human digestive system was used to explore the bioaccessibility of ICA-PG[5]. Briefly, ICA-PG and ICA were digested in the in vitro artificial digestive solutions simulating different phases after oral administration at 37 °C with continuous shaking. In the mouth phase, the tested powders 20 mg were dissolved in 10 ml phosphate buffer (pH=6.8) containing 2

mg α-amylase and incubated with 5 min simulating the good mobility solution in the mouth phase. After the mouth phase, the pH value of the tested solution was adjusted to 2, then, 1,330 mg pepsin was added and completely dissolved to simulate stomach phase. And this solution was incubated for 1 h subsequently. After the stomach phase, the pH value was adjusted to 7.0, followed by dissolving 250 mg lipase and 50 mg bile salts to simulate intestinal phase, and then this solution was incubated for 2 h. Finally, all the samples before and after each phase were collected and centrifuged at 10,000 rpm for 30 min. The supernatant was mixed with methanol, and then a second centrifugation was performed. The level of ICA in the supernatant was measured with HPLC.

Bioaccessibility was calculated as the following equation.

Bioaccessibility(%) = $M_{ss}/M_{st} \times 100\%$.

Where M_{ss} was the amount of ICA in supernatant and M_{st} was the amount of ICA in total.

Antioxidant activity evaluations in HepG2 cells

Cell viability after different concentrations of H_2O_2 (0.4 mM, 0.5 mM, 0.6 mM, 0.7 mM, 0.8 mM, 0.9 mM, 1 mM, 2 mM, 4 mM, 8 mM) 24 h incubation was tested with MTT assay[6]. Concentrations of Vc 31.3 μg/ml, DG 468.8 μg/ml, ICA 31.3 μg/ml, ICA 31.3 μg/ml&DG 468.8 μg/ml, and ICA-PG 31.3 μg/ml in MEM were used in this evaluation as no obvious cytotoxicity were observed after 48 h incubation based on the results of cytotoxicity assay. Cells were pre-treated with these solutions for 24 h, then H_2O_2 was added into each well with the final concentration of 1 mM. A further 24 h incubation was performed to evaluate the protection abilities of these tested against H_2O_2-induced oxidative damages. Cells treated with MEM in the whole test were set as negative control, and cells treated with MEM for 24 h and followed by H_2O_2 for the next 24 h were set as positive control. The protection abilities of these tested solutions against H_2O_2-induced oxidative damages were evaluated with cell viability evaluation, and the intracellular ROS level and SOD level were also measured with assay kits.

ICA levels in plasma after ICA-PG oral given in rats

ICA levels in plasma after ICA-PG was orally given were assessed in SD rats. ICA-PG with an ICA:DG mass ratio of 1:15 was tested here. Before the experiment, we fasted the animals for 12

h but allowed them free access to water. Then, we separated the rats randomly and equally into two groups of four animals per group. A concentration of 60 mg/ml of bare ICA and ICA-PG were orally given to the rats with oral doses of 60 mg/kg ICA. After the oral administration, we drew blood samples of 0.3 ml from the tail vein into a heparinized tube at 0.25, 0.75, and 1 h post-administration. We obtained the rats' blood plasma by centrifuging the samples at 8000 rpm for 10 min. We then preserved the plasma at -20 °C until using it for further analysis.

ICA concentration in plasma samples was analyzed by the HPLC method. The instrument information of HPLC was consistent with the above in vitro release determination. Briefly, we used 100 µl of an acetonitrile solution containing 1 mg/ml chlorzoxazone as an internal standard and then added the solution to 100 µl of plasma. Next, using a vortex mixer, we mixed the samples for 2 min. Then, all samples were centrifuged at 8000 rpm for 10 min to obtain the supernatant for the HPLC analysis. The flow rate was fixed at 1.0 ml/min of a 50:50 v/v mixture of acetonitrile-water. The injection volume was 20 µl. The standard curve for ICA was $C = 506.29A - 0.0738$ ($R^2 = 0.9999$) for the ICA concentration range 21.8 – 15600.0 ng/ml. ICA had a retention time of ~ 9.2 min, and chlorzoxazone (internal standard) had a retention time of ~ 6.2 min.

SI Results and Figures

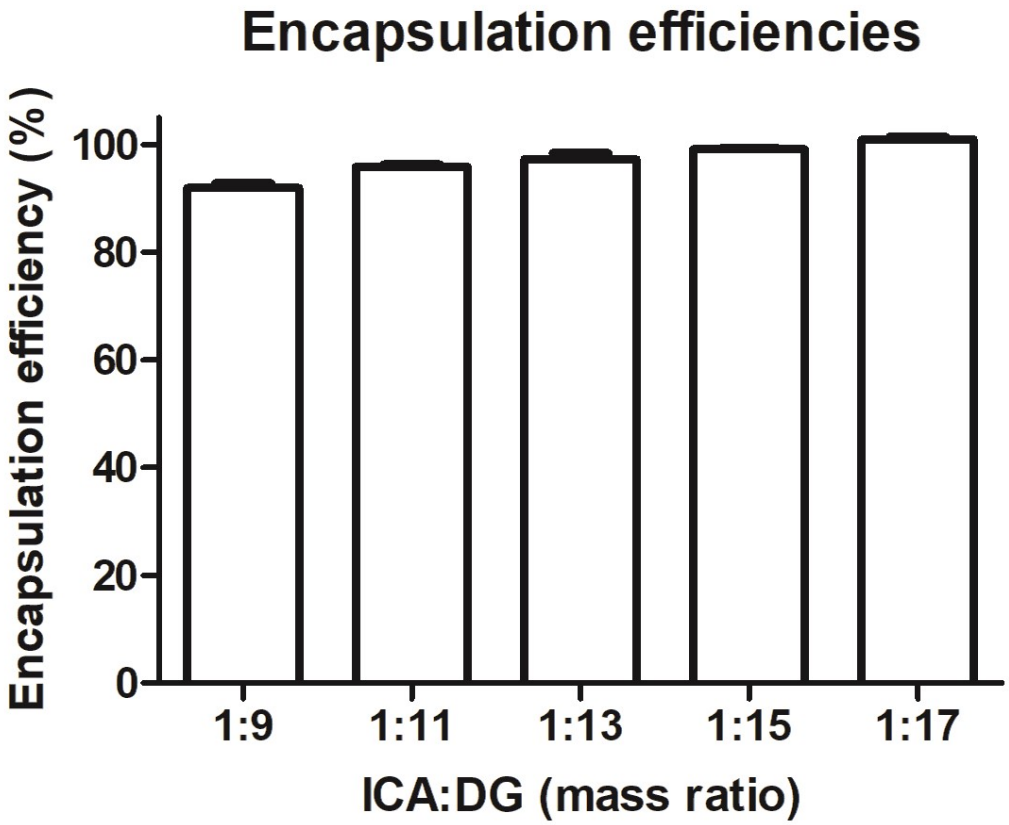

Figure S1 Encapsulation efficiency vs. mass ratio of ICA and DG. DG: dipotassium glycyrrhizinate; ICA: icariin.

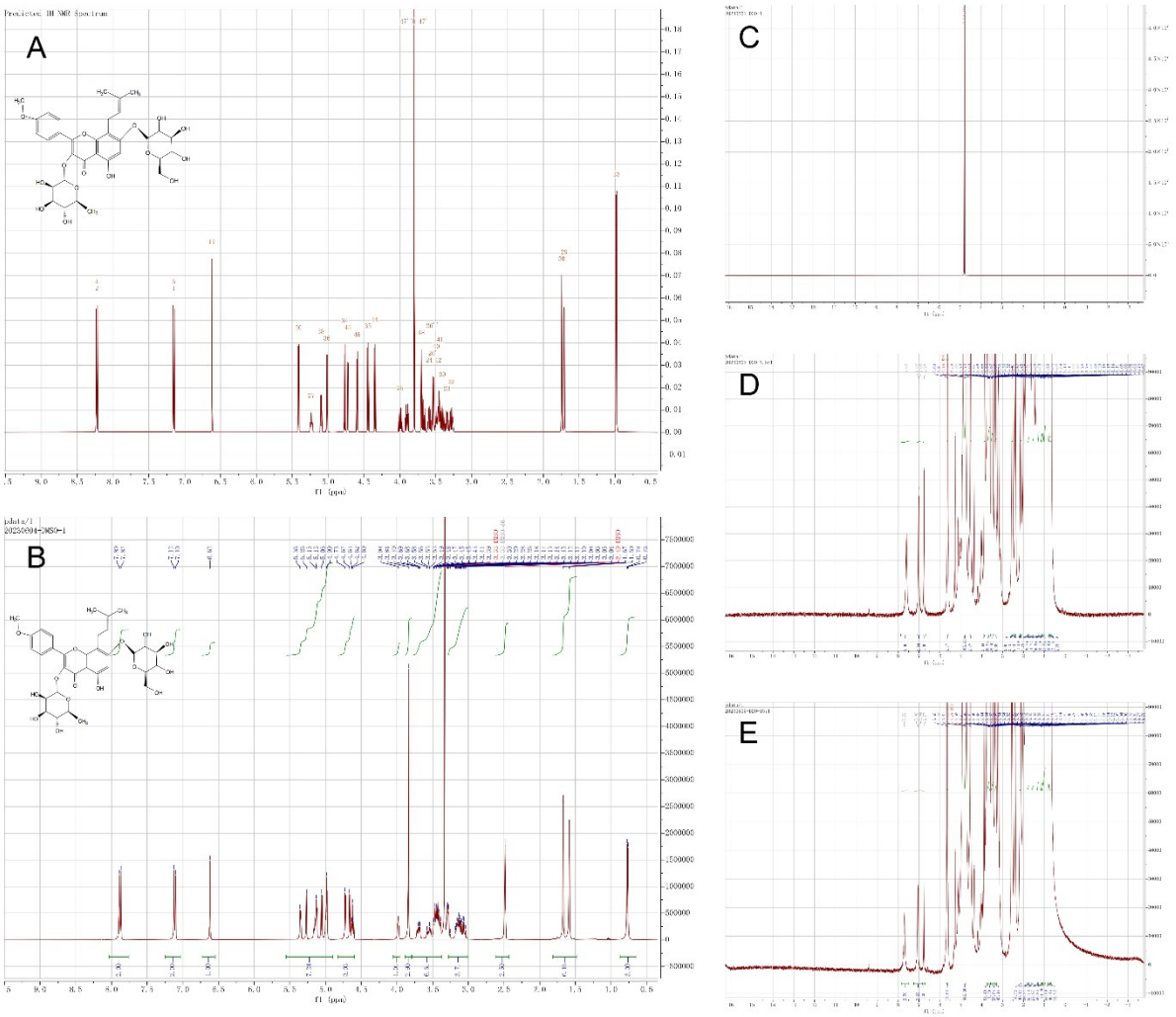

Figure S2 ^{1}H NMR spectra of (A) predicated ICA (B) ICA in DMSO-d_6, (C) in D_2O; (D) 1:7.5 ratio of ICA-PG in D_2O, and (E) 1:15 ratio of ICA-PG in D_2O. All NMR spectra were recorded at 27 °C.

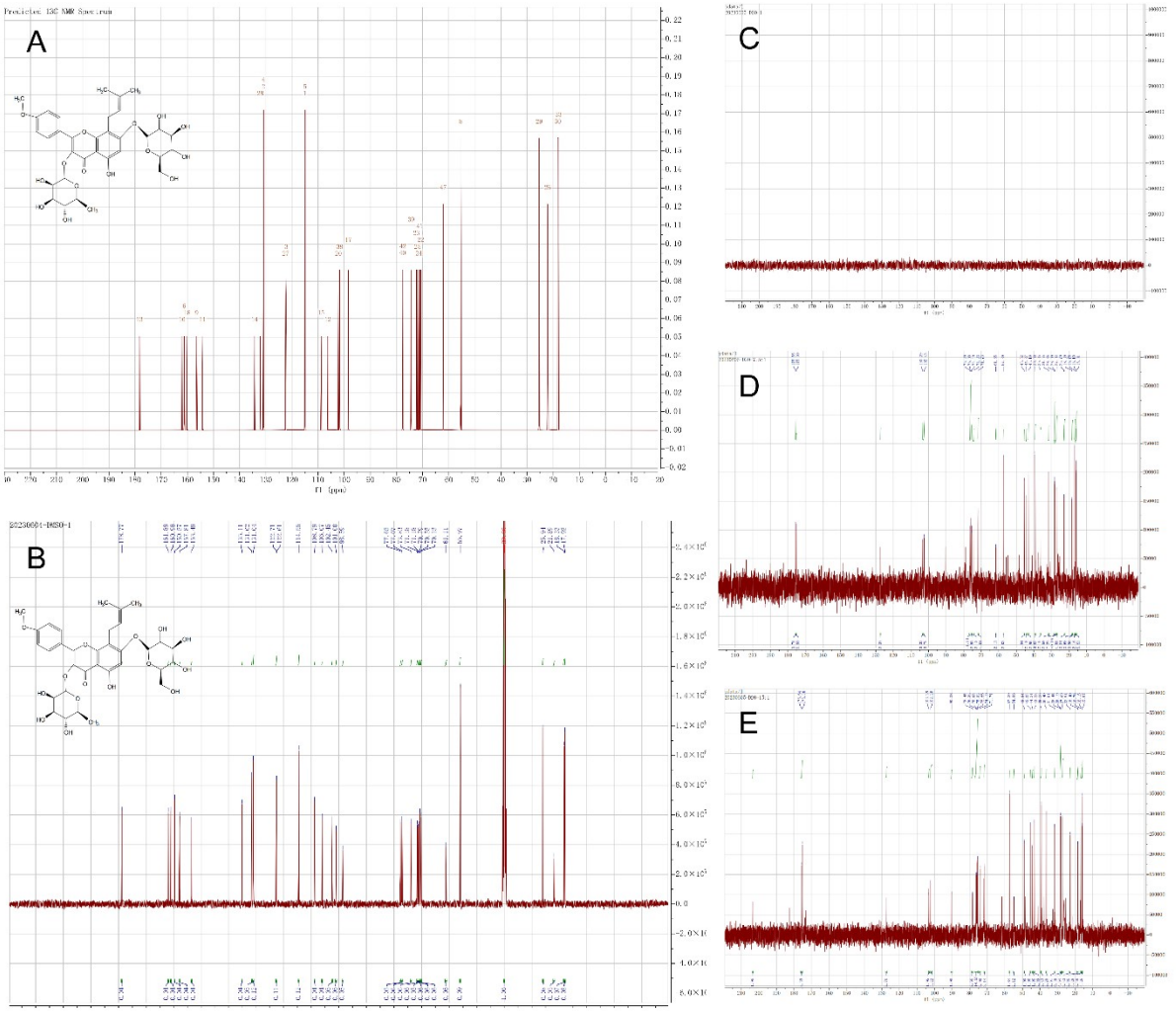

Figure S3 ^{13}C NMR spectra of (A) predicated ICA (B) ICA in DMSO-d_6, (C) in D_2O; (D) 1:7.5 ratio of ICA-PG in D_2O, and (E) 1:15 ratio of ICA-PG in D_2O. All NMR spectra were recorded at 27 °C.

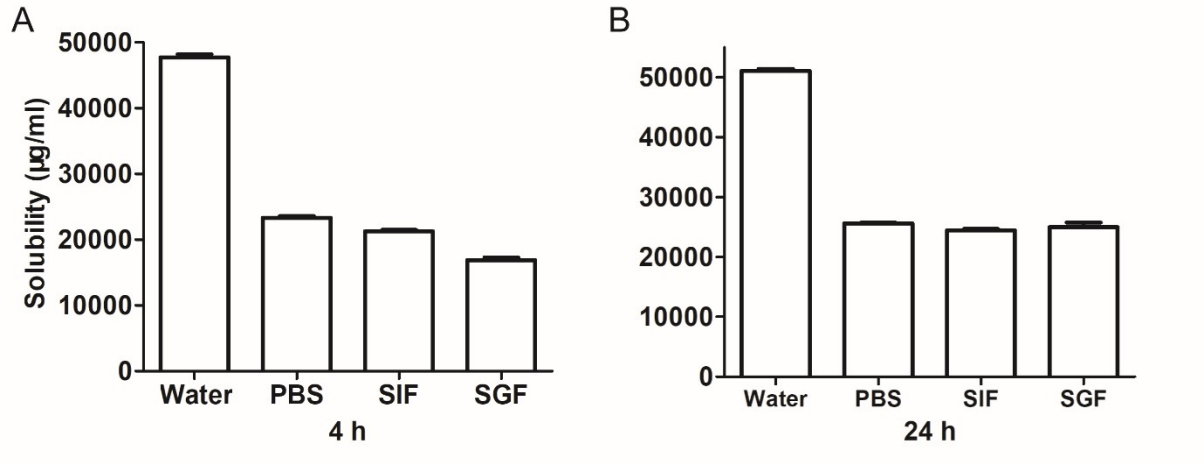

Figure S4 Solubility profiles of ICA&PG. Apparent solubility of ICA in ICA&PG in different solutions after (A) 4 h and (B) 24 h of incubation (n = 3).

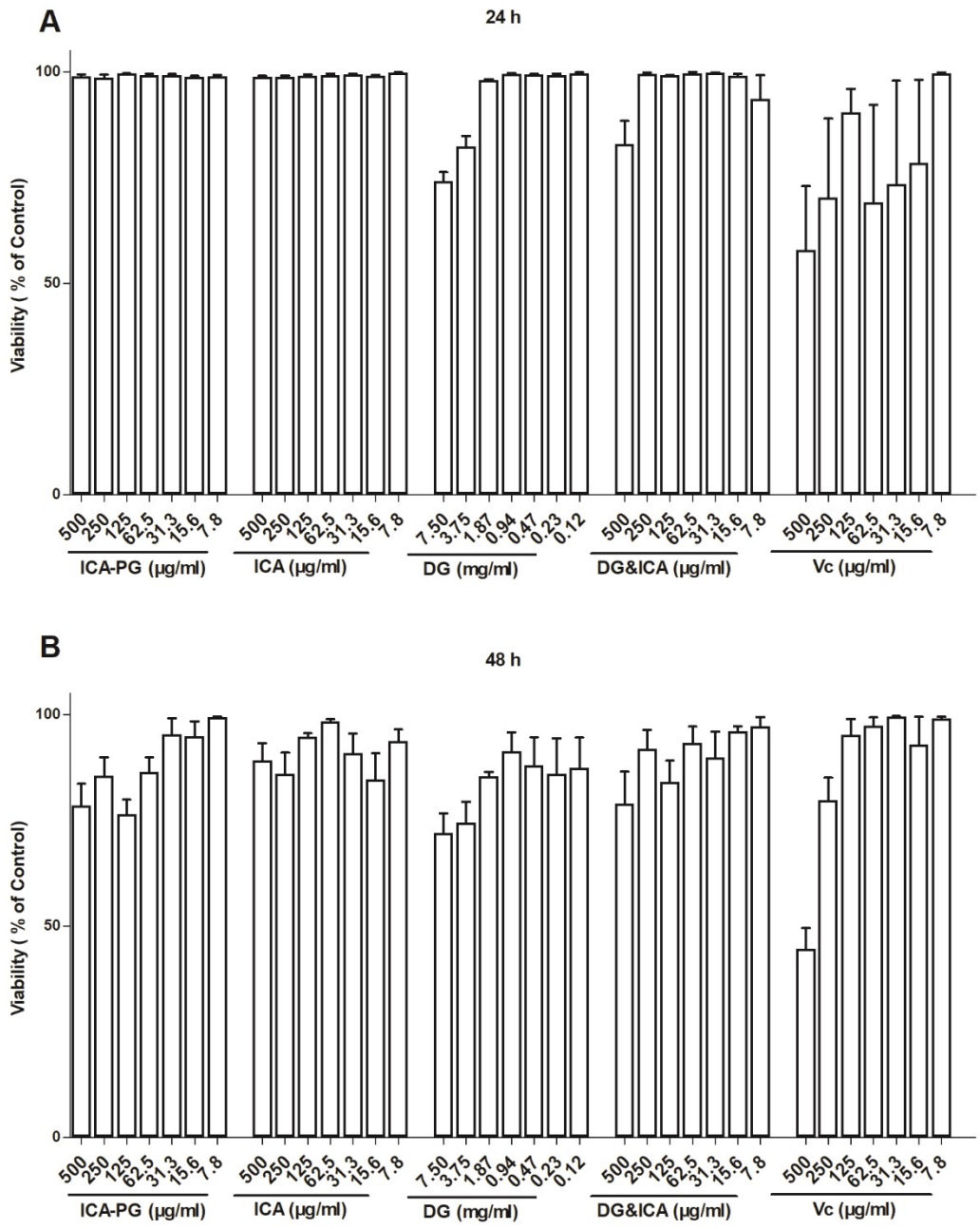

Figure S5 Cytotoxicity evaluation of ICA-PG in HepG2 cells. n = 3. Cytotoxicity evaluation of tested solutions with (A) 24 h and (B) 48 h incubation.

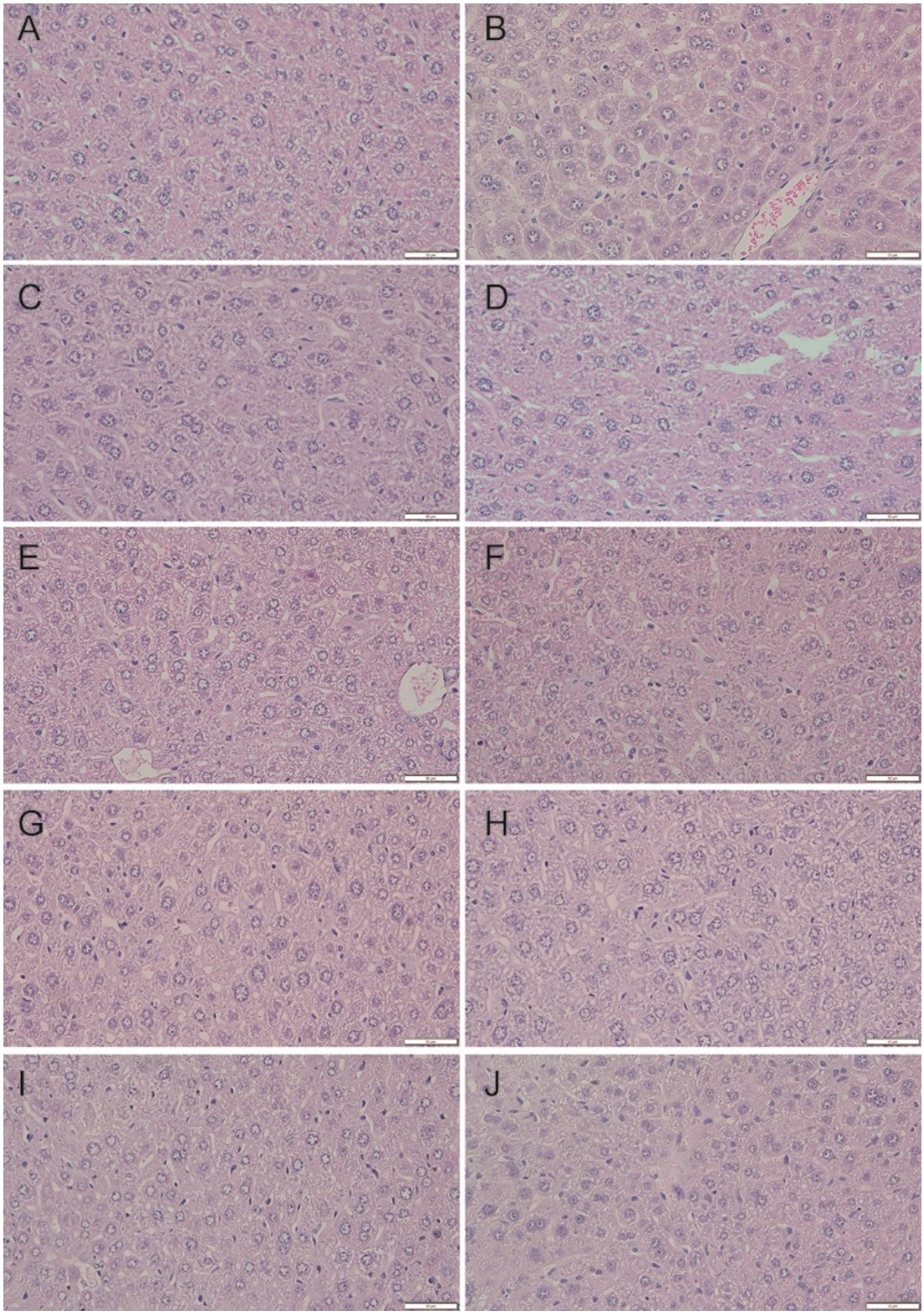

Figure S6 H&E staining of liver tissue. (A) Normal control group; (B) NS group; (C) FEN group; (D) SIM group; (E) ICA group; (F) DG group; (G) DG&ICA; (H) 20 mg/kg ICA-PG; (I) 40 mg/kg ICA-PG; (J) 60 mg/kg ICA-PG.

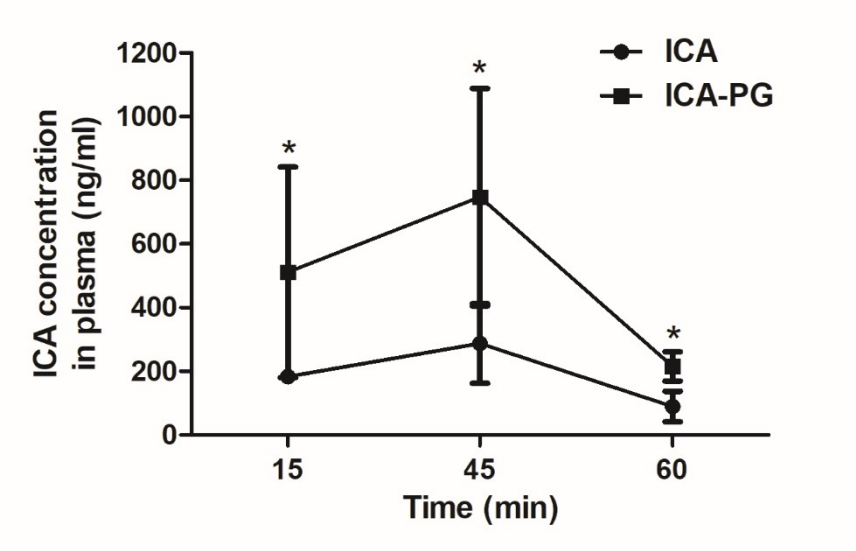

Figure S7 ICA levels in plasma after ICA-PG oral given in rats (n=4).

The levels of ICA in plasma after oral administration of bare ICA and ICA-PG are shown in Figure S7. At 0.25, 0.75, and 1 h after given, the ICA levels were 1.78, 1.59, and 1.41 times higher in the ICA-PG group than in the bare ICA group, respectively (P<0.05). These data indicated that ICA-PG enhanced the oral absorption of ICA in rats.

Reference

1. X. Tao, L. Zhan, Y. Huang, P. Li, B. Liu and P. Chen, Preparation, characterization and evaluation of capsaicin-loaded indica rice starch nanoparticles, *Food Chem*, 2022, **386**, 132692.
2. D. Xu, Y. R. Lu, N. Kou, M. J. Hu, Q. S. Wang and Y. L. Cui, Intranasal delivery of icariin via a nanogel-thermoresponsive hydrogel compound system to improve its antidepressant-like activity, *Int J Pharm*, 2020, **586**, 119550.
3. B. Sun, H. Jing, M. T. Mabrouk, Y. Zhang, H. Jin and J. F. Lovell, A surfactant-stripped cabazitaxel micelle formulation optimized with accelerated storage stability, *Pharm Dev Technol*, 2020, **25**, 1281-1288.
4. A. Lagarto, R. Vega, I. Guerra and R. Gonzalez, In vitro quantitative determination of ophthalmic irritancy by the chorioallantoic membrane test with trypan blue staining as alternative to eye irritation test, *Toxicol In Vitro*, 2006, **20**, 699-702.
5. J. Yang, J. Lin, X. Chen, L. Rong, M. Shen, Y. Wang and J. Xie, Mesona chinensis polysaccharide/zein nanoparticles to improve the bioaccesibility and in vitro bioactivities of curcumin, *Carbohydr Polym*, 2022, **295**, 119875.
6. W. Xiong, Y. Li, Y. Yao, Q. Xu and L. Wang, Antioxidant mechanism of a newly found phenolic

compound from adlay (NDPS) in HepG2 cells via Nrf2 signalling, *Food Chem*, 2022, **378**, 132034.