



# Efficient extraction, excellent activity, and microencapsulation of flavonoids from *Moringa oleifera* leaves extracted by deep eutectic solvent

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## Abstract

A deep eutectic solvent (choline chloride (ChCl)-urea) was chosen to extract flavonoids from *Moringa oleifera* leaves (FMOL), the condition of extraction was tailor-made, under the optimal extraction conditions (material-to-liquid ratio of 1:60 g/mL, extraction time of 80 min, extraction temperature of 80 °C), the highest extraction efficiency reached  $63.2 \pm 0.3$  mg R/g DW, and nine flavonoids were identified. Then, the biological activities including antioxidant activities, antibacterial activities, and anti-tumor activities were systematically studied. FMOL was superior to positive drugs in terms of antioxidant activity. As to DPPH investigation, the  $IC_{50}$  of FMOL and Vc were  $64.1 \pm 0.7$  and  $176.1 \pm 2.0$   $\mu$ g/mL; for the ABTS, the  $IC_{50}$  of FMOL and Vc were  $9.5 \pm 0.3$  and  $38.2 \pm 1.2$   $\mu$ g/mL, the FRAP value of FMOL and Vc were  $15.5 \pm 0.6$  and  $10.2 \pm 0.4$  mg TE/g, and ORAC value of FMOL and Vc were  $4687.2 \pm 102.8$  and  $3881.6 \pm 98.6$   $\mu$ mol TE/g. The bacteriostatic (MICs were  $\leq 1.25$  mg/mL) activities of FMOL were much better than propyl p-hydroxybenzoate. Meanwhile, FMOL had comparable inhibitory activity with genistein on tumor cells,  $IC_{50}$  was 307.8  $\mu$ g/mL, and could effectively induce apoptosis in HCT116. Microcapsules were prepared with xylose-modified soybean protein isolate and gelatin as wall materials; after that, the intestinal release of modified FMOL microcapsules was 86 times of free FMOL. Therefore, this study confirmed that FMOL extracted with ChCl/urea has rich bioactive components, and microencapsulated FMOL has potential application in food industry.

**Keywords** Flavonoids · Deep eutectic solvent · *Moringa oleifera* leaves · Identification · Microcapsule

## 1 Introduction

*Moringa oleifera* is a traditional plant in India, and its flowers, fruits, seeds, leaves, and roots are almost all edible and have high nutritional value [1]. Some studies have reported that *Moringa oleifera* exhibits growth inhibitory activity against various pathogens [2]. *Moringa oleifera* leaves

(MOL) also contains polysaccharides, polyphenols, water-soluble proteins, and different kinds of flavonoids. Extracts of MOL have potential benefits in the antioxidant, anticancer, and hypoglycemic activities [1]. Flavonoids are one of the main biologically active components of *Moringa oleifera*. Various solvents such as water [3], methanol/water [4, 5], methanol [6, 7], ethanol [7, 8], ethanol/water [9, 11], n-hexane [12, 13], ethyl acetate [6, 12], and chloroform fraction [13] combined with assisted extraction methods (homogenizer [4], ultrasound [14, 15], microwave [14] and supercritical fluid extraction [16], etc.) have been used for extracting flavonoids from *Moringa oleifera* leaves.

Traditional organic solvents are the most commonly used to extract flavonoid compounds. But they are volatile, toxic, highly consumed, which largely lead to excessive solvent residue in the extraction and unfriendly to environment. Deep eutectic solvents (DESSs) have emerged at the historical moments to address the disadvantage of traditional solvents.

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DESs have been widely used as catalyst, reaction media, and solvent to extract natural products [17]. As DESs with low raw material price, easy to achieve, low volatility, safe nontoxicity, convenient to store, and lack of byproducts, they have been exploited to extract bioactive substances such as flavonoids [18]. Koutsoukos et al. extracted phenolic and carotenoid compounds from propolis, almond, and red shrimp using DES, prepared by choline chloride/tartaric acid [19]. Cui et al. prepared 12 kinds of DESs to extract flavonoids from sea buckthorn leaves [17]. Yu and Bulone prepared five kinds of choline chloride – based DESs to extract quercetin derivatives from apple pomace [20]. Wu et al. attempted to use 13 kinds of DESs, based choline chloride, betaine, L-proline as hydrogen bond acceptors and malic acid, glycerol, oxalic acid, levulinic acid as hydrogen bond donors with different molar ratios, to extract total phenolic and flavonoid of *MOL* [21]. The extraction ability of choline chloride/citric acid as DES also has been demonstrated on dried *MOL*, two of glucosinolates, eight of chlorogenic acids, and twenty of flavonoids have been extracted [22]. Our previous study investigated the extraction rate of *FMOL* by choline chloride/lactic acid, and the *FMOL* can be reached 54.8 mg/g DW, which much higher than that of extracted by water and ethanol [23]. Different DESs have various extraction capacities; therefore, it is valuable to attemptable to extract *FMOL* using deep eutectic solvent and systematic studies on the biological activity of the extracted products.

The flavonoids can be broken down into other compounds with lower bioavailability after gastrointestinal digestion, which may affect by many factors such as pH values, bile salts, and digestive enzymes in the gastrointestinal tract [24]. Therefore, in addition to optimize the efficient extraction of green solvents, it is also necessary to further improve the bioavailability of flavonoids. Microencapsulation as a versatile technique is commonly used for isolation, protection, and controlled delivery of active core materials to enhance the bioavailability and stability of compounds and to improve their controlled release [25, 26]. Soy protein isolate (SPI) and gelatin (GE) are commonly used as wall materials for the preparation of microcapsules. Jia et al. reported that Maillard reaction modification of whey protein and xylooligosaccharide could help to improve the embedding rate and bioavailability [27]. Zhong et al. used a variety of oligosaccharides to modify soybean protein isolate through Maillard reaction as the antioxidant wall material for embedding *Lactobacillus casei* [28]. In order to improve the bioavailability of *FMOL* in intestine, xylose-modified soy protein isolate could be used as wall material for microencapsulation of *FMOL*.

In this study, flavonoids were extracted from the leaves of *Moringa oleifera* using DES (ChCl/urea). The stirring extraction conditions were optimized, and the flavonoids

were isolated from ChCl/urea. The composition of the extracted flavonoids was qualitatively identified. Then, systematically studied the biological activities of the extracted flavonoids, including antioxidant activity, antibacterial activity, and antitumor activity. Moreover, the isolated *Moringa oleifera* flavonoids were microencapsulated in order to improve its bioavailability in small intestine.

## 2 Materials and methods

### 2.1 Reagents and materials

See supplementary materials for materials and reagents.

### 2.2 Preparation of the DES

The DESs list in Table S1 were prepared according to the report [17] with minor modifications. See supplementary materials for specific operation.

### 2.3 The extraction of *FMOL* by DES

The flavonoids from *Moringa oleifera* leaves (*FMOL*) were extracted by a simple stirring method, and the extraction rate of flavonoids was determined by ultraviolet spectrophotometry as Wang's report [29]. The specific method was that 0.25 g dry *MOL* added to 5 mL DES, stirring at 60 °C, 200 rpm for 60 min. As shown in Table S1, the solvent with the highest extraction rate of *FMOL* was selected from the six solvents and compared with ethanol and deionized water. Each group of experiments was carried out three times in parallel.

ChCl/urea (1:2, mol/mol) was selected as extraction solvent; the effects of material-to-liquid ratio (1:10–1:80 g/mL), extraction time (20–120 min), and temperature (40–90 °C) on the extraction rate were further studied at the stirring velocity of 200 rpm.

### 2.4 Experiment of response surface methodology

On the basis of single factor experiment, the extraction process was optimized by Box-Behnken design (BBD) with three factors and three levels by using the Design-Expert 8.0.6 statistical software. The experimental scheme was shown in Table S2, and Table S3 for factor level table. The variables were coded as – 1, 0, and + 1 levels respectively, in which the material-to-liquid ratio (X1) were 1:70, 1:80, and 1:90 g/mL, the extraction duration (X2) were 70, 80, and 90 min, and the extraction temperature (X3) were 70, 80, and 90 °C. Taking the extraction rate of total flavonoids in *FMOL* (Y1) as the response variable, 17 groups of experiments were designed.

## 2.5 Detection and identification of FMOL components

An ultra-performance liquid chromatography orbital ion trap mass spectrometry (UPLC-Q-Orbitrap MS) platform was used for the qualitative study. See supplementary materials for specific operation.

## 2.6 Antioxidant activities

### 2.6.1 DPPH free radical scavenging activity

The DPPH radical scavenging rate of the sample was determined as previous report [23]. See supplementary material for specific operation.

### 2.6.2 ABTS free radical scavenging activity

The clearance rate of ABTS<sup>+</sup> free radicals was determined according to the previous report [23]. See supplementary material for specific operation.

### 2.6.3 Iron reducing antioxidant capacity (FRAP)

FRAP capability of FMOL was determined by previous report [23]. See supplementary material for specific operation.

### 2.6.4 Oxygen Free Radical Absorption Capacity (ORAC)

The oxygen radical absorbance capacity (ORAC) was evaluated by comparison with Trolox using the method [30]. See supplementary material for specific operation.

## 2.7 Antimicrobial activity

The inhibition zone was determined by the filter paper agar diffusion method. Added 300  $\mu\text{L}$  of test bacteria suspension ( $1.5 \times 10^6$  CFU/mL) to agar medium, took 6 mm diameter sterile filter paper with sterile forceps and stuck it, dropped 10  $\mu\text{L}$  of sample to be tested, cultured upside down in 37 °C incubator for 24 h, and measured and counted the diameter of a bacteriostatic circle.

The minimum inhibitory concentration (MIC) has been determined. 5  $\mu\text{L}$  sample was mixed with 195  $\mu\text{L}$  of test bacterial suspension ( $1.5 \times 10^6$  CFU/mL) and incubated at 37 °C for 24 h. The OD was measured at 610 nm with multifunctional microplate reader. The positive control was propyl hydroxybenzoate, and the negative control was DMSO. All tests were performed in triplicate.

## 2.8 Antiproliferative activity assay

### 2.8.1 Antitumor cell activity

The inhibition rate of tumor cells was determined by the MTT method. Three types of tumor cells with higher inhibition rates were selected from human breast cancer cells (MCF-7), human colon cancer cells (HCT116), human cervical cancer cells (HeLa), human lung cancer cells (A549), human liver cancer cells (Hep G-2), and mouse melanoma cells (B16), and their IC<sub>50</sub> were calculated. See supplementary material for specific operation. Cell inhibition rate (%) =  $(\text{OD}_{\text{control}} - \text{OD}_{\text{sample}}) / \text{OD}_{\text{control}} \times 100\%$ . All tests were performed in sextuplicate.

Acridine orange (AO) staining experiment: 1 mL/well cell suspension ( $5 \times 10^5$  cells/mL) was added into each cell culture plate, and cultured in 5% CO<sub>2</sub> at 37 °C for 24 h. Then each well was added 1 mL FMOL sample (diluted with culture medium) and further incubated for 3 h. After the incubation, each sample was stained with 20  $\mu\text{L}$  AO fluorescent dye solution (5  $\mu\text{g}/\text{mL}$ ) for 0.5 h, and then washed with PBS for 3 times, after that 1 mL of culture medium was added to each well. Then, the samples were observed under a fluorescence inverted microscope or laser confocal microscope.

### 2.8.2 The effect of FMOL on apoptosis

The apoptosis experiment was determined according to the reported method with slightly modified [31]. See supplementary material for specific operation.

## 2.9 Preparation of microcapsules

Xylose (xyl) was selected to modify soybean protein isolate (SPI) by Maillard reaction [27], and FMOL microcapsules were prepared by combining gelatin (GE) as crosslinking agent with modified SPI by composite coagulation method [28]. Microcapsule embedding rate (EE) =  $(A - A_0) / A \times 100$ , where A is the content of FMOL in microcapsules, A<sub>0</sub> is the surface flavonoids attachment of FMOL microcapsules.

The factors of microcapsule preparation included exploring the effects of different wall material concentration, core-wall ratio, emulsifying power, and emulsifying time on microcapsule embedding rate and morphology. The specific was shown in supplementary material.

## 2.10 Characterization of FMOL microcapsules

The characterization methods of microcapsules were shown in supplementary material, include Fourier transform infrared spectroscopy (FT-IR), X-ray diffraction (XRD) [32], and scanning electron microscope (SEM) [33].

## 2.11 In vitro digestion of FMOL microcapsules

The simulated in vitro digestion of FMOL microcapsules was investigated and the general procedure was followed Brodkorb's study [34]. The simulated oral digestive juice, gastric digestive juice, and small intestinal digestive juice were shown in Table S4 and preheated at 37 °C before use. The digestive stabilities of FMOL or FMOL microcapsules in different digestive stages, that is, bioavailability, were studied by simulating the digestion of oral, gastric, and intestinal fluids. The release rate of flavonoids (%) =  $C/C_0 \times 100$ , where  $C$  was the content of flavonoids in the solution after in vitro digestion, and  $C_0$  is the content of flavonoids in the coating extract before in vitro digestion.

## 2.12 Statistical analysis

The data were processed with Origin 2019, and the final results were expressed as the mean  $\pm$  standard deviation of three values ( $n = 3$ ). The Design-Expert 8.0.6 (STAT ease Inc., Minneapolis, Minnesota, USA) was used for response surface methodology (RSM) as an optimization tool. The IC50 results were statistically analyzed using GraphPad prism version 8.0 (GraphPad Software Inc., La Jolla, CA), and the significance level was 5%.

## 3 Results and discussions

### 3.1 The screening of DES

Six deep eutectic solvents were selected as extraction solvents. Figure 1(a) compares the extraction rate of flavonoids between six DESs (composed of choline chloride and various hydrogen bond donors) and traditional solvents. The extraction rates of FMOL conducted with DESs were all higher than those obtained with ethanol and DI water. DESs composed of diverse hydrogen bond donors and choline chloride had different extraction effects on FMOL. This could be attributed to the different physical and chemical properties of various DESs, including solubility, viscosity, and polarity, which could affect the extraction efficiency of FMOL [35]. The DES prepared by ChCl/urea had the highest rate for FMOL extraction in the investigated solvents.

### 3.2 The effects of different conditions on the FMOL extraction rate

The control variable method was used to study three single factors. As Fig. 1(b) shows, with the material-to-liquid ratio of 1:10 to 1:80 g/mL, the extraction rate of FMOL increased from  $34.0 \pm 0.5$  to  $51.7 \pm 0.2$  mg R/g DW, while the extraction rates of the material-to-liquid ratio were

1:60 and 1:80 g/mL; the rates have little difference, namely  $50.7 \pm 0.9$  and  $51.7 \pm 0.2$  mg R/g DW, respectively, showing that the extraction approached to platform stage, so the best material-to-liquid ratio was regarded as 1:60 g/mL. Such a result could contribute to that when the material-to-liquid ratio was too low, the MOL powder was not fully dispersed and mixed insufficiently with the DES, leading to an insufficient dissolution of flavonoids. However, an excessively high material-to-liquid ratio would cause the solvent to absorb a certain amount of heat and increase the viscosity, which is averse to the breaking of MOL cell's wall, decreasing mass transfer, and results in low extraction rate of flavonoids. In the meantime, it could also cause solvent waste and problems of subsequent test treatment [36]. Therefore, MOL powder's optimal material-to-liquid ratio to ChCl/urea solvent was considered to be 1:60 g/mL.

Under the material-to-liquid ratio of 1:60 g/mL, the effect of extraction time on the extraction rate of flavonoids was studied. As Fig. 1(c) depicts, with extraction time increasing from 20 to 120 min, the extraction rate of flavonoids increased from  $29.4 \pm 0.04$  to  $56.1 \pm 1.3$  mg R/g DW. When the extraction time reached 80 min, the highest extraction rate ( $60.6 \pm 0.9$  mg R/g DW) was obtained, but when the extraction time was increased continuously to 120 min, the extraction rate decreased to  $56.1 \pm 1.3$  mg R/g DW. This could be allied to the extraction time being too long and thus the flavonoids being destroyed.

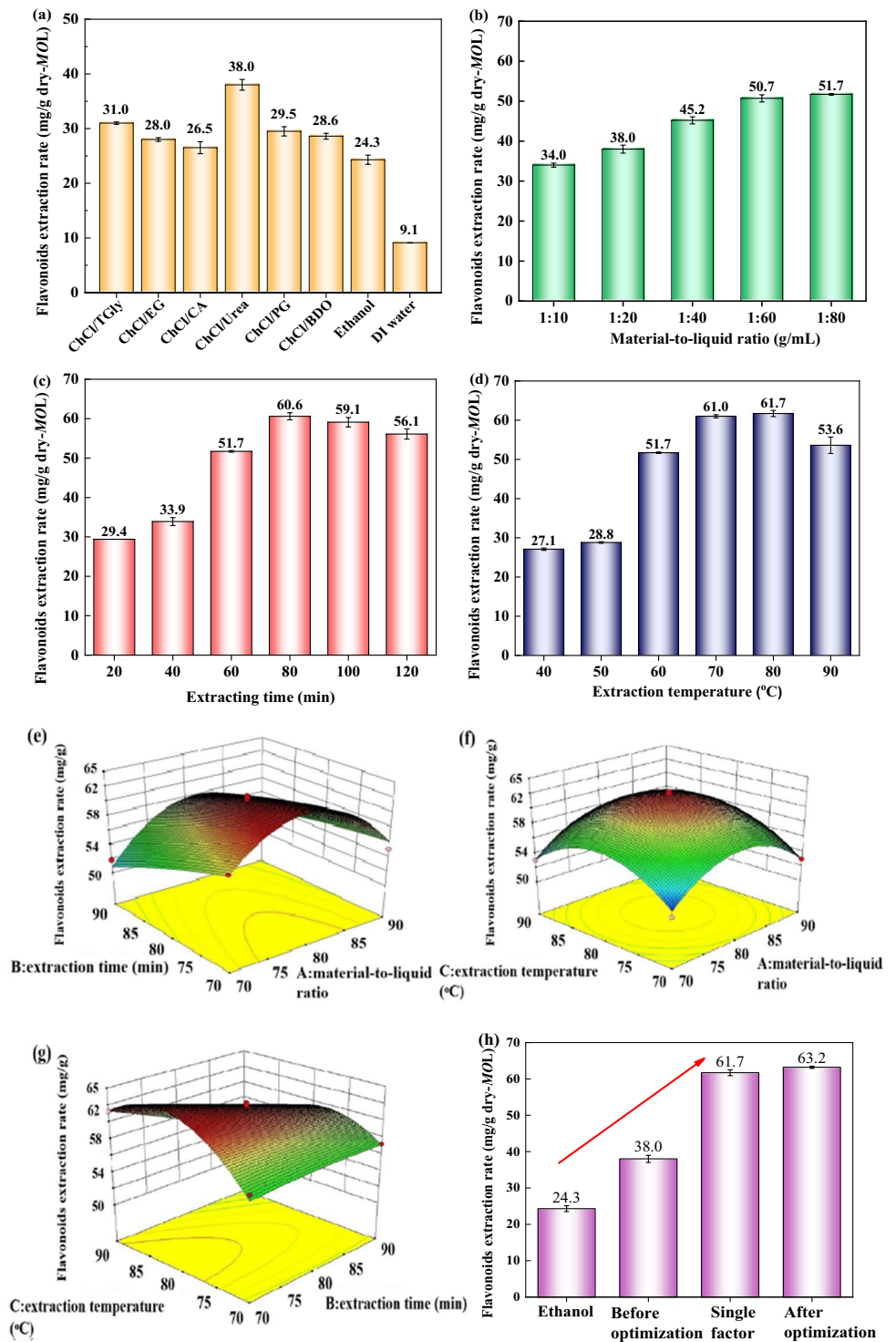
Then, the optimal extraction temperature of MOL powder extracted with ChCl/urea was further investigated under the as-obtained optimum material-to-liquid ratio and extraction time. As shown in Fig. 1(d), as the temperature improved from 20 to 80 °C, the extraction rate increased from  $27.1 \pm 0.3$  to  $61.7 \pm 0.8$  mg R/g DW. In comparison, when the temperature was increased continuously to 90 °C, the extraction rate descended to  $53.6 \pm 2.1$  mg R/g DW. This may be owing to when the extraction temperature was too low, the molecular movement was slow and led to a low dissolution, diffusion, and penetration rate, which goes against to the dissolution of flavonoids. High temperature could reduce the viscosity of DES, increase the diffusion coefficient and improve the extraction efficiency. However, high temperature could lead to the structure damage of flavonoids, resulting in a reduction in the extraction rate.

The variance analysis was conducted on the test data, and the bias regression coefficients of each factor were tested. The analysis of variance results is summarized in Table S5. The larger the  $F$  value, the greater the influence. It could be seen that the order of factors affecting the content of flavonoids was B (extraction time) > C (extraction temperature) > A (material-to-liquid ratio).

The  $P$  value of this model was  $< 0.01$ , extremely significant, and the  $P'$  value of lack of fit was  $0.0967 > 0.01$ , not significant, indicating that the regression equation fit well and could



**Fig. 1** **a** Screening and comparison of extraction solvents. Extraction rate of flavonoids using ChCl/urea under different **b** material-to-liquid ratios, **c** extraction times, and **d** extraction temperatures. Response surface diagram of interaction, **e** A: material-to-liquid ratio and B: extraction time, **f** A: material-to-liquid ratio and C: extraction temperature, **g** B: extraction time and C: extraction temperature, **h** comparison of extraction rate before and after optimization



predict the content of flavonoids under different extraction conditions. The correlation coefficient  $R^2$  of the correction model was 0.9804, and the correction determination coefficient  $R_{adj}^2$  was 0.9551, indicating that the fitting of the equation was good. The model could explain the change of 98.04% response value. Therefore, this model could analyze the real changes in response values and determine the optimal extraction process conditions.

To further investigate the interaction of relevant variables and determine the best advantage, a response surface graph was drawn using the Design-Expert 8.0.6 software for visual analysis. The results were consistent with the results of mathematical model analysis in Fig. 1(e)–(g), which could better understand the interaction between the two variables and its optimal range [35].

**Model verification:** The optimal extraction conditions, including material-to-liquid ratio, extraction time, and temperature, were 1:79 g/mL, 70 min, and 83 °C. Under these conditions, the predicted flavonoid content in *MOL* was  $63.3 \pm 0.1$  mg R/g DW. The experimental results showed that the measure value of total flavonoids in *MOL* was  $63.2 \pm 0.3$  mg R/g DW, and the error was only 0.1 mg R/g DW. Furthermore, it can be seen from Fig. 1(h) that the extraction rate of flavonoids after optimization was  $63.2 \pm 0.3$  mg mg R/g DW, which was 1.7 and 2.6 times to that of before optimization ( $38.0 \pm 0.9$  mg R/g DW) and ethanol extraction ( $24.3 \pm 0.8$  mg R/g DW). The optimized value was significantly higher than the screening value ( $38.0 \pm 0.9$  mg R/g DW), indicating that the extraction conditions of *FMOL* were successfully optimized via this model. Additionally, the optimal extraction rate of *FMOL* ( $63.2 \pm 0.3$  mg R/g DW) extracted by ChCl/urea from *Moringa oleifera* leaves was much higher than that of those extracted with other solvents, such as the extract yields extracted with 50% aqueous ethanol solution ( $29.5 \pm 0.3$  mg/g DW) [11], methanol (35.19 mg/g DW) [37], L-proline/ethylene glycol (30.93 mg GAE/g DW) [38], and L-proline/glycerol (29.8 mg GAE/g DW) [21].

### 3.3 Identification of *FMOL*

The composition of flavonoids extracted with ChCl/urea was identified using LC–MS, the results were presented in Fig. 2 and Table S6. Nine of flavonoids were identified, including hyperoside, vitexin, quercetin, cynaroside, quercetin 3- $\beta$ -D glucoside, kaempferol, taxifolin, luteolin, and fisetin; the MS diagram of the nine flavonoids could be seen from Fig. S5–S13, in which hyperoside, vitexin, quercetin,

cynaroside, quercetin 3- $\beta$ -D glucoside, kaempferol, luteolin, and taxifolin have been reported in *Moringa oleifera* leaves extracted by hot water [22], ethanol [9], methanol or methanol/water [30, 39], choline chloride/lactic acid [23], 20% ammonium sulfate and ethanol [22], choline chloride/citric acid [22], L-proline/levulinic [21], and L-proline/glycerol [21], and fisetin has been reported in *Moringa oleifera* leaves for the first time.

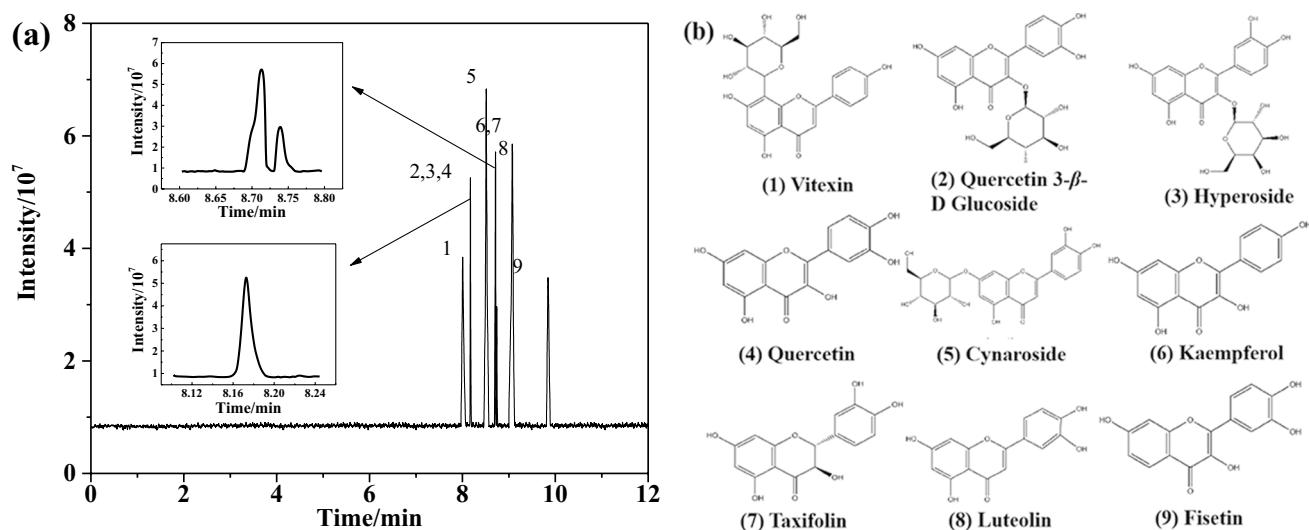
Further analysis of the extracted products obtained with different solvents revealed various flavonoids. Table S7 shows part of the reported results of extracted flavonoids, indicated that the DES is a good solvent for the extraction of natural products. Different DESs could sometimes obtain various extracted products, and new compounds from *Moringa oleifera* leaves.

### 3.4 Bioactivity of *FMOL*

#### 3.4.1 Antioxidant activities

**DPPH free radical scavenging activity of *FMOL*** Flavonoids from *Moringa oleifera* have a significant antioxidant capacity related to its immune enhancement activity [40], anti-inflammatory activities [41], antioxidant activities [23], and anticancer activities [1]. Previous studies showed that the determination of antioxidant activity depended on the reaction mechanism, and a variety of tests must be combined to accurately assess the antioxidant capacity of samples [42]. In this study, DPPH, ABTS, FRAP, and ORAC methods were used to assess the antioxidant activity of *FMOL* samples reliably.

The DPPH radical scavenging ability of the samples was determined. As Fig. 3(a) depicts, it was gratifying that the



**Fig. 2** a LC–MS chromatogram of *FMOL* extract, b molecular structure formula of flavonoids

*FMOL* extracted with ChCl/urea showed excellent DPPH radical scavenging activity, superior to Vc in the range of 12.5–800  $\mu\text{g/mL}$ . The scavenging rate was  $81.4\% \pm 1.1$  at 800  $\mu\text{g/mL}$ . The IC<sub>50</sub> of Vc and *FMOL* values were  $170.6 \pm 2.0$  and  $64.1 \pm 0.7$   $\mu\text{g/mL}$ , respectively. *FMOL* samples' DPPH free radical scavenging activity was more than twice as high as Vc. The satisfactory DPPH scavenging activity of *FMOL* was found to be far better than those of many flavonoid extracts from plants, such as *Moringa oleifera* leaf extract (149.8  $\mu\text{g/mL}$ ) [43], and hawthorn samples (80  $\mu\text{g/mL}$  and 120  $\mu\text{g/mL}$ ) [30].

**ABTS free radical scavenging activity of *FMOL*** ABTS free radical solution had high absorption at 734 nm when *FMOL* was added to ABTS free radical solution, the absorbance at 734 nm decreased, that is, ABTS free radicals were removed by *FMOL*, indicating that *FMOL* had the activity of scavenging free radicals [44]. As Fig. 3(b) shows, the scavenging activity of *FMOL* extracted with ChCl/urea was much better than that of Vc at low concentrations (12.5 ~ 50  $\mu\text{g/mL}$ ). In contrast, the scavenging activity of *FMOL* was equal to that of Vc at high concentrations (100 ~ 400  $\mu\text{g/mL}$ ). *FMOL* exhibits higher activity in scavenging free radicals even at low concentrations, further demonstrating the excellent antioxidant capacity of the as-obtained *FMOL*. In the meantime, the IC<sub>50</sub> of *FMOL* was  $9.5 \pm 0.3$   $\mu\text{g/mL}$ , which was much better than Vc ( $38.2 \pm 1.2$   $\mu\text{g/mL}$ ).

**Ferric reducing antioxidant power (FRAP) of *FMOL*** It is important to estimate the total antioxidant activity by diverse methods for the total antioxidant potential of any food matrix, so the FRAP of *FMOL* was also studied.

The FRAP of *FMOL* extracted with ChCl/urea was measured, the value obtained was  $15.5 \pm 0.6$  mg TE/g (Table 1), and the corresponding value of the positive control Vc was  $10.2 \pm 0.4$  mg TE/g. Rocchetti et al. investigated the FRAP of flavonoid samples from *Moringa oleifera* leaves extracted by ethyl acetate, and the corresponding value was 13.77 mg TE/g [4]. The FRAP of *FMOL* extracted with ChCl/urea ( $15.5 \pm 0.6$  mg TE/g) was not only higher than that of Vc ( $10.2 \pm 0.4$  mg TE/g) but also superior to that of flavonoids extracted via ethyl acetate (13.77 mg TE/g), indicating that the DES-extracted *FMOL* has outstanding ferric reducing antioxidant capacity.

**Oxygen Free Radical Absorption Capacity (ORAC) of *FMOL*** The ORAC was measured and the results was shown in Table 1; the value was  $4687.2 \pm 102.8$   $\mu\text{mol TE/g}$ , superior to that of the positive control Vc ( $3881.6 \pm 98.6$   $\mu\text{mol TE/g}$ ). Recently, the ORAC values of flavonoids extracted from *Moringa oleifera* leaves using water /methanol (20:80, v/v) and water/ ethanol (20:80, v/v) were 108.1  $\mu\text{mol TE/g}$  and 3560.5  $\mu\text{mol TE/g}$  [45], respectively, far below the

ORAC value of *FMOL* ( $4687.2 \pm 102.8$   $\mu\text{mol TE/g}$ ), which undoubtedly demonstrated that the flavonoids extracted by DES have superior antioxidant activity than those extracted by the traditional solvent.

Through the above comprehensive evaluation of the antioxidant capacity of *FMOL* by the DPPH, ABTS, FRAP, and ORAC, the excellent antioxidant capacity of *FMOL* extracted via ChCl/urea was demonstrated better than Vc.

### 3.4.2 Antimicrobial activity

With the abuse of antibiotics, plants have attracted considerable attention as a potential source of antibacterial drugs [40]. Bacterial growth inhibition is one of methods to study antimicrobial activity assessment, which is low cost and can provide meaningful effect within a short period of time. Therefore, it was of great significance to explore the inhibitory effect of *FMOL* on different bacteria.

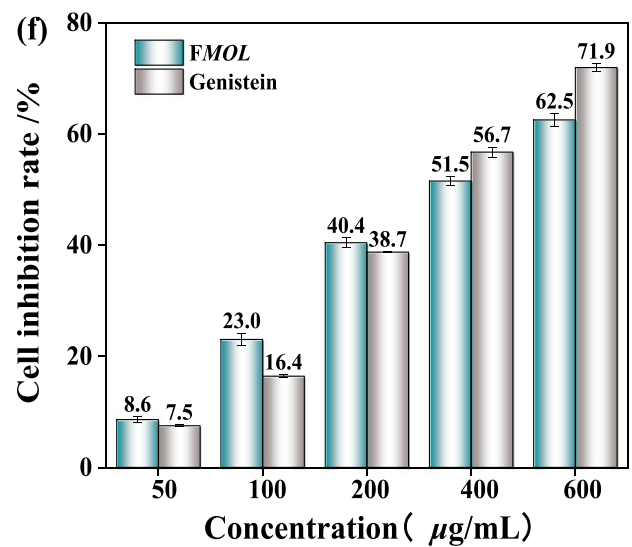
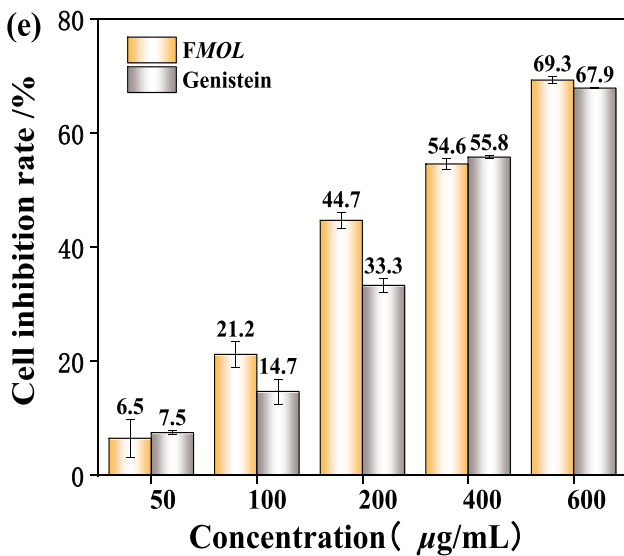
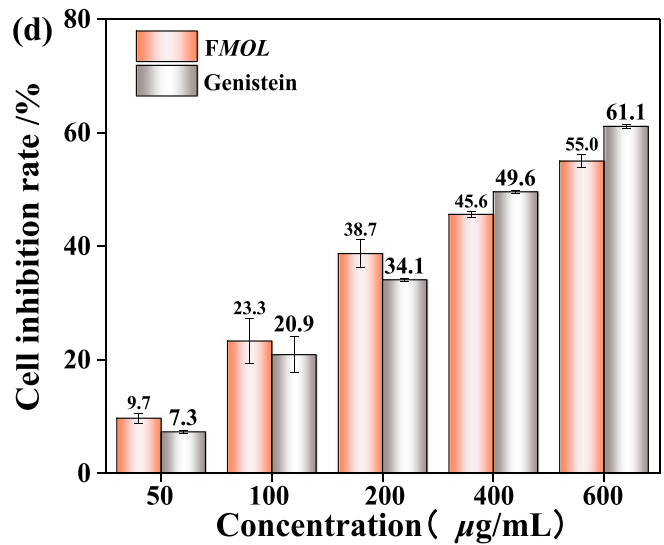
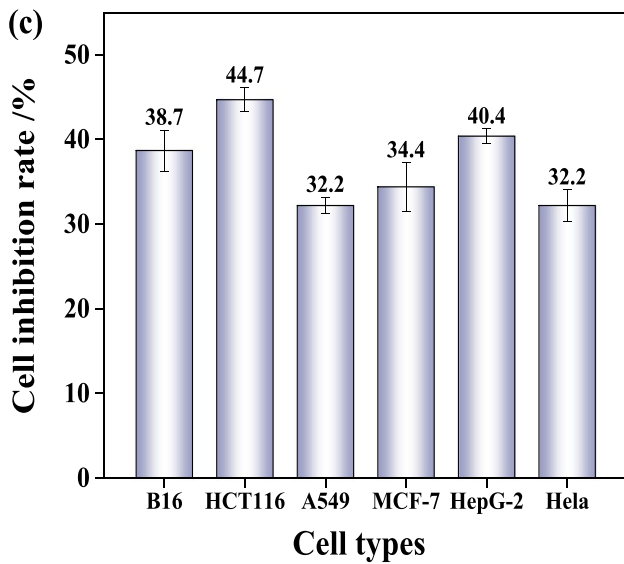
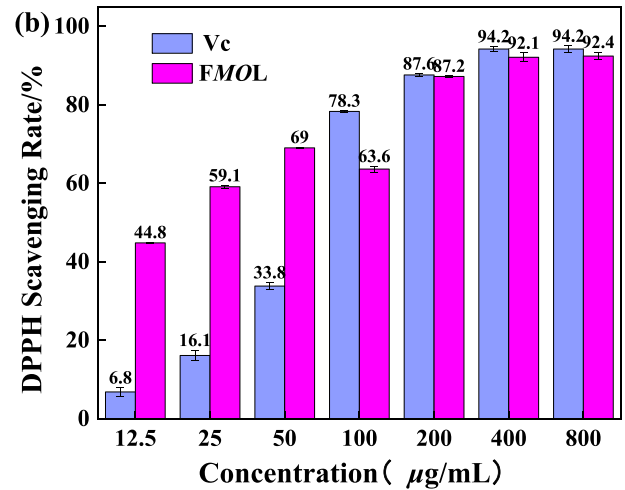
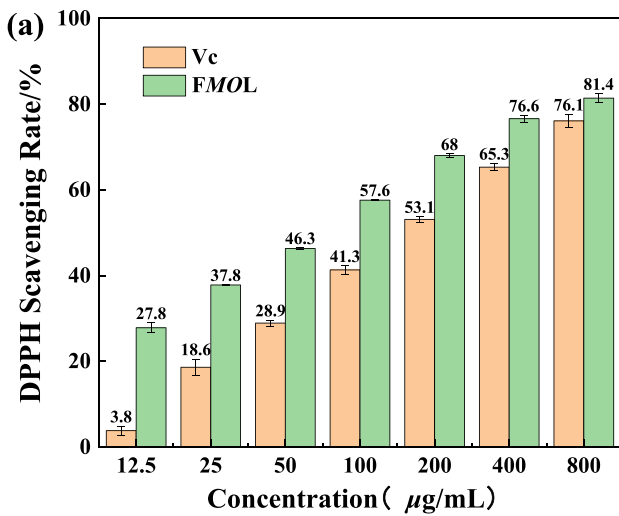
The experimental results in Table 2 and Fig. S1 show that the diameter of the inhibition zone produced by *FMOL* at a concentration of 2.5 mg/mL ( $15.6 \pm 0.4$ – $18.7 \pm 0.6$  mm) was even more significant than that of the positive drug at 10 mg/mL ( $13.6 \pm 0.4$ – $15.2 \pm 1.3$  mm), which indicates that the inhibitory effects of *FMOL* on five bacteria were better than that of propyl p-hydroxybenzoate (the positive control). The inhibitory effect on *Proteus common* was showing best among the investigated bacteria.

To further explore the antimicrobial capacity of *FMOL* extracted with ChCl/urea, the minimum inhibitory concentration (MIC) was investigated by the above five bacteria. As shown in Table 2, the MICs of *FMOL* to *E. coli*, *Staphylococcus aureus*, *Bacillus subtilis*, and *Pseudomonas aeruginosa* were 1.25 mg/mL and to *Proteus common* was 0.625 mg/mL. The MIC values of the positive drug (propyl p-hydroxybenzoate) were twice to that of *FMOL*, further revealing the strong bacteriostatic ability of the as-prepared *FMOL*. In addition, the MIC results were consistent with the inhibitory zone experiment results.

### 3.4.3 Antiproliferative activity

Epidemiological studies have shown that various parts of *Moringa oleifera* have beneficial effects on health, and the leaves are potential source of antitumor activity [39]. Biological evaluation of *FMOL* was performed with an in vitro antitumor MTT assay via six types of tumor cells (MCF-7, HCT116, HeLa, A549, B16, Hep G-2).

According to the MTT experimental results in Fig. 3(c), *FMOL* showed relatively high inhibitory activity against B16, HCT116, and Hep G-2 cells. Different concentrations of *FMOL* were used to the three kinds of cells to obtain different cell inhibitory activities, as shown in Fig. 3(d)–(f).





**Fig. 3** **a** DPPH radical scavenging activities of Vc and FMOL; **b** ABTS radical scavenging activities of Vc and FMOL. **c** Inhibition of FMOL on various tumor cells, **d** inhibitory activity of FMOL/genistein at different concentrations on B16 cells, **e** inhibitory activity of FMOL/genistein at different concentrations on HCT116 cells, **f** inhibitory activity of FMOL/genistein at different concentrations on Hep G-2 cells

The IC<sub>50</sub> was for the inhibition rates of the three kinds of cells. The results displayed that the IC<sub>50</sub> of FMOL on B16, HCT116, and Hep G-2 cells were  $415.0 \pm 3.3$ ,  $307.8 \pm 5.8$ , and  $349.6 \pm 8.1$   $\mu\text{g/mL}$ , respectively. The IC<sub>50</sub> of genistein (the positive control) on B16, HCT116, and Hep G-2 cells were  $398.2 \pm 10.1$ ,  $281.7 \pm 4.2$ , and  $335.8 \pm 6.7$   $\mu\text{g/mL}$ , respectively. Based on the results of the IC<sub>50</sub> of antitumor proliferation in the three kinds of cells, FMOL had the best inhibitory effect on HCT116 cells, and it was slightly lower than that of genistein. Recently, Yang et al. revealed that peanut extract has a strong antiproliferative effect on HCT116 cells, with IC<sub>50</sub> of 1.39~9.33 mg dry extract/mL [46], far higher than that of as-extracted FMOL, which indicated that FMOL has a considerable inhibitory effect on tumor cells.

An acridine orange (AO) staining experiment has been performed for FMOL to verify the results from the MTT assay. The overlap effect of AO staining results for B16 (Fig. S2), HCT116 (Fig. S3), and Hep G-2 cells (Fig. S4) became more evident with increasing FMOL concentration, and its variation effect was consistent with the IC<sub>50</sub> results. This result indicated that the pH value in cells increased under the action of FMOL, and it was speculated that FMOL had a similar effect on alkaline lysosomes [47]. Figure 4 (a)–(d) shows confocal laser imaging of HCT116 cells stained with AO after 3 h of treatment with FMOL. The overlapping effect of AO staining revealed dose-dependent effect on cell viability for

the as-extracted FMOL; furthermore, HCT116 cells exhibited increasing detached and dead cells with the increasing dosage of FMOL, indicating the excellent antiproliferative activity of FMOL extracted with ChCl/urea.

In order to further demonstrate the bioactivity of FMOL on the tumor cells, the effect of FMOL on cellular apoptosis was investigated. HCT116 cells were treated with FMOL at varying concentrations for 24 h by employing an Annexin V-FITC/PI assay. As shown in Fig. 4(e)–(j), incubation with FMOL and genistein drastically increased the proportion of apoptotic cells compared with the control group. After treatment with FMOL, the proportions of early apoptotic cells were 1.8% at 100  $\mu\text{g/mL}$ , 5.1% at 500  $\mu\text{g/mL}$ , and 6.0% at 1000  $\mu\text{g/mL}$ , respectively, whereas the corresponding proportions of late apoptotic cells reached 4.6%, 9.4%, and 11.2%, respectively. These results illustrated that FMOL significantly induced cellular apoptosis in a dose-dependent manner and thus could effectively induce apoptosis in HCT116 cells.

### 3.5 Characterization of FMOL microcapsules

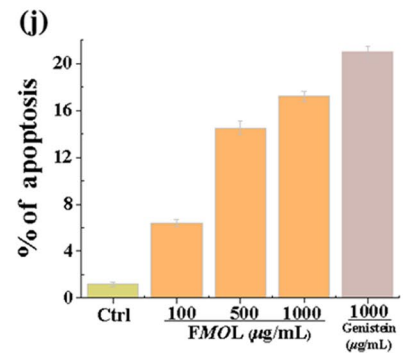
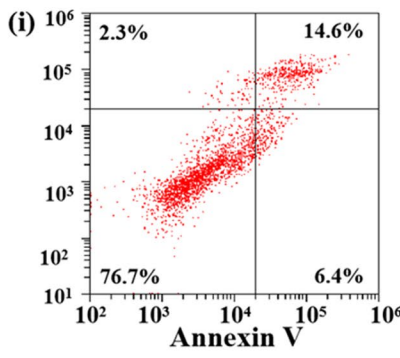
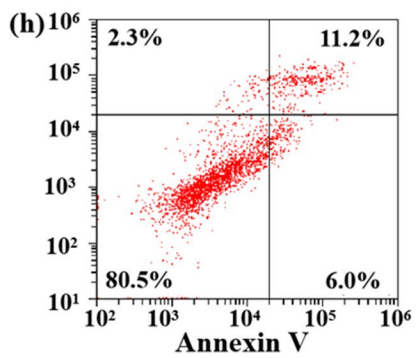
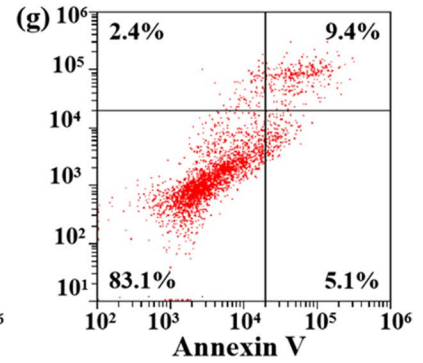
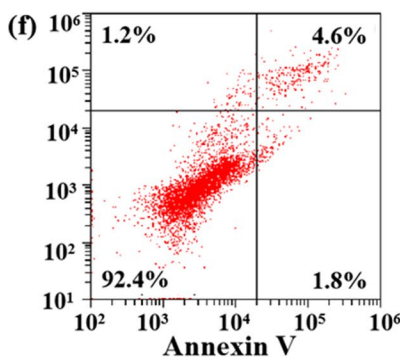
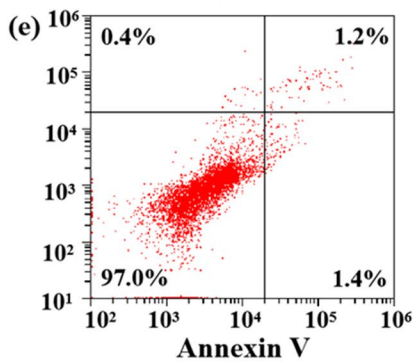
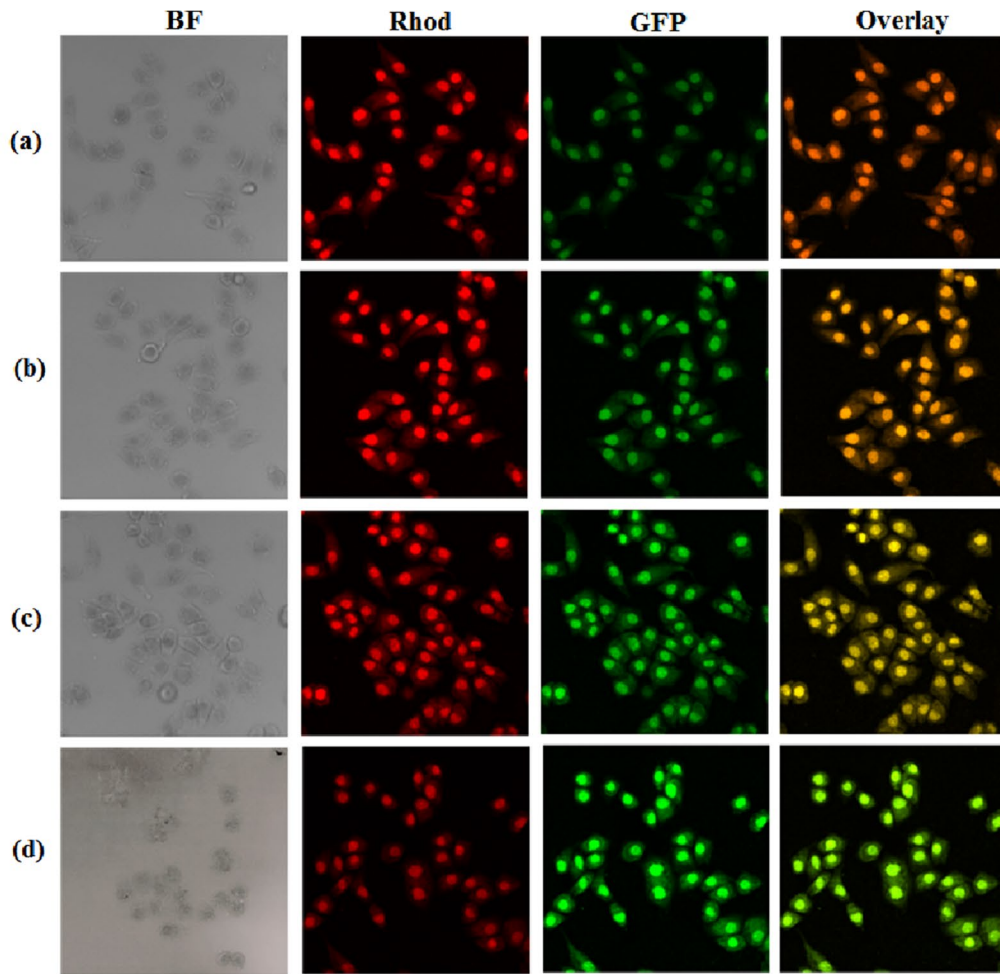
The preparation process is shown in Fig. 5(a). As depicts in Fig. S14, the optimal wall material concentration, core-wall ratio, and stirring rate and time were of 1.8% SPI-3.6% xyl and 1.0% GE, 1:6, 500 rpm, and 15 min, under which conditions, the embedding rate of FMOL could reach 66.5%, and the FMOL microcapsule particle size distribution was shown in Fig. S15, 94% of the FMOL microcapsule diameter were < 100  $\mu\text{m}$ , observing the morphology of FMOL microcapsule through microscope and SEM. The microscope diagram of wet microcapsule is shown in Fig. 5(b), and the SEM results are shown in Fig. 5(c)–(d). The morphology of microcapsules showed a certain bonding block, there was no obvious particle dispersion state, and only a few cystic protrusions could be seen in the sample. The reason for this phenomenon may be that some residual wall material polymers did not form microcapsules during the preparation of microcapsules, or the water loss during the freeze drying process after the preparation of microcapsules may cause the adhesion on the surface

**Table 1** FRAP and ORAC values of FMOL and Vc

Sample	FRAP (mg TE/g)	ORAC ( $\mu\text{mol TE/g}$ )
FMOL	$15.5 \pm 0.6$	$4687.2 \pm 102.8$
Vc	$10.2 \pm 0.4$	$3881.6 \pm 98.6$

**Table 2** Inhibitory results of FMOL against five strains

Strains	DMSO		Propyl p-hydroxybenzoate		FMOL	
	Diameter of inhibition zone (D/mm)	MIC (mg/mL)	Diameter of inhibition zone (D/mm)	MIC (mg/mL)	Diameter of inhibition zone (D/mm)	MIC (mg/mL)
<i>E. coli</i>	—	—	$13.6 \pm 0.4$	2.5	$16.6 \pm 0.2$	1.25
<i>Staphylococcus aureus</i>	—	—	$15.2 \pm 1.3$	2.5	$16.3 \pm 0.7$	1.25
<i>Bacillus subtilis</i>	—	—	$14.4 \pm 1.4$	2.5	$17.3 \pm 0.5$	1.25
<i>Proteus vulgaris</i>	—	—	$14.8 \pm 1.1$	1.25	$18.7 \pm 0.6$	0.625
<i>Pseudomonas aeruginosa</i>	—	—	$14.2 \pm 0.8$	2.5	$15.6 \pm 0.4$	1.25



**Fig. 4** Confocal laser imaging of HCT116 cells stained with acridine orange after 3 h of **a** cells without sample treatment, **b–d** the cells treated with different concentrations of FMOL were extracted by ChCl/urea: **b** 50  $\mu\text{g}/\text{mL}$ , **c** 100  $\mu\text{g}/\text{mL}$ , **d** 200  $\mu\text{g}/\text{mL}$ . Under different treatments: **e** cells without sample treatment, **f–h** the cells treated with different concentrations of FMOL: **f** 100  $\mu\text{g}/\text{mL}$ , **g** 500  $\mu\text{g}/\text{mL}$ , **h** 1000  $\mu\text{g}/\text{mL}$ , **i** Genistein (1000  $\mu\text{g}/\text{mL}$ ), induced HCT116 cell apoptosis for 24 h, and **j** the proportions of apoptosis cells

of film-forming wall materials, resulting in agglomeration and shrinkage of microcapsules [28].

Then, the FMOL microcapsules were characterized. It can be seen from Fig. 5(e) that the characteristic absorption of amide C=O stretching and amide II bands (N–H and C–N) in protein was around 1650 and 1540  $\text{cm}^{-1}$ , and the characteristic absorption of carbohydrates (including C–C and C–O stretching vibration and C–H bending) was mainly concentrated at 1180–953  $\text{cm}^{-1}$  [28]. Herein, the peak enhancement of wave number 1650  $\text{cm}^{-1}$  and the peak attenuation of 1540  $\text{cm}^{-1}$  probably be the result of glycosylation between SPI and xylose. FMOL microcapsules contained the characteristic peaks of SPI, xyl, GE, and FMOL in the range of 500–4000  $\text{cm}^{-1}$ , and two characteristic peaks of FMOL (about 3440  $\text{cm}^{-1}$  and 2950  $\text{cm}^{-1}$ ) could be found in the infrared spectrum of FMOL microcapsules. Therefore, it could be seen that the wall material had been wrapped into FMOL to form FMOL microcapsules.

In general, crystalline materials exhibit spikes, while amorphous products exhibit wider peaks. As shown in Fig. 5 (f), in the XRD patterns of all materials, a characteristic diffraction peak appears at about 20°. However, xylose, as a crystalline substance, showed many peaks between 10 and 80°, but the characteristic peaks of xylose after maillard reaction did not appear in the characteristic peaks of FMOL microcapsules. The reason may be that xylose could carry out maillard reaction efficiently at 80 °C and 30 min, which completely changed its structure [32].

### 3.6 In vitro digestion of FMOL microcapsules

Microcapsules have been widely used in the field of food, and many of microencapsulation of food bioactive substances are to release active ingredients in the digestive system in a way of effective digestion and absorption, and give play to their physiological and health effects.

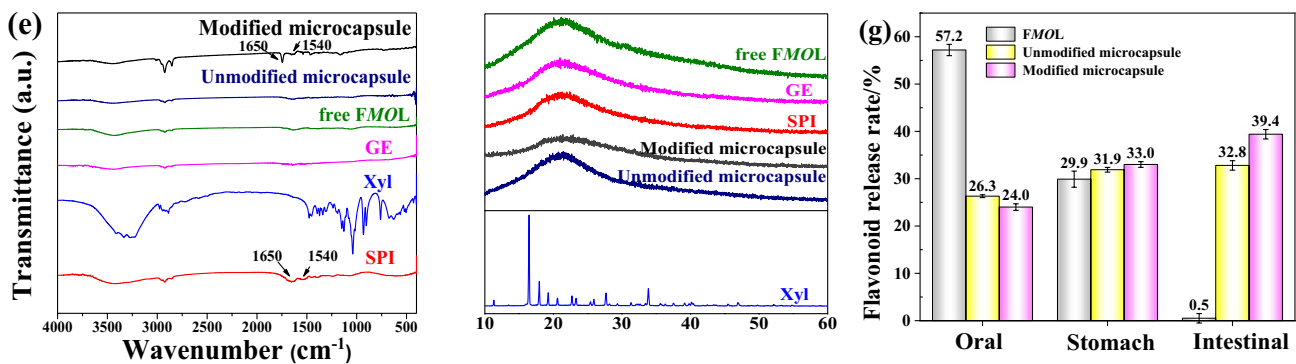
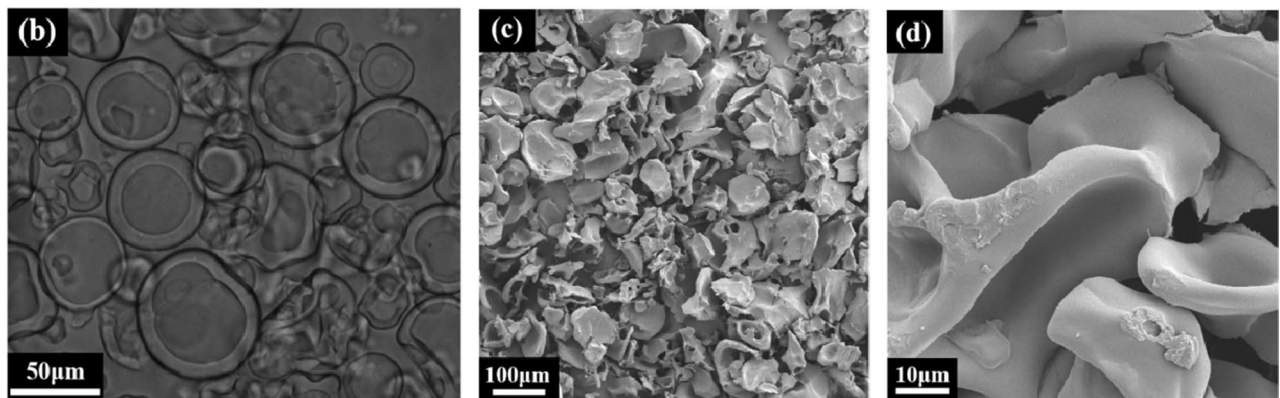
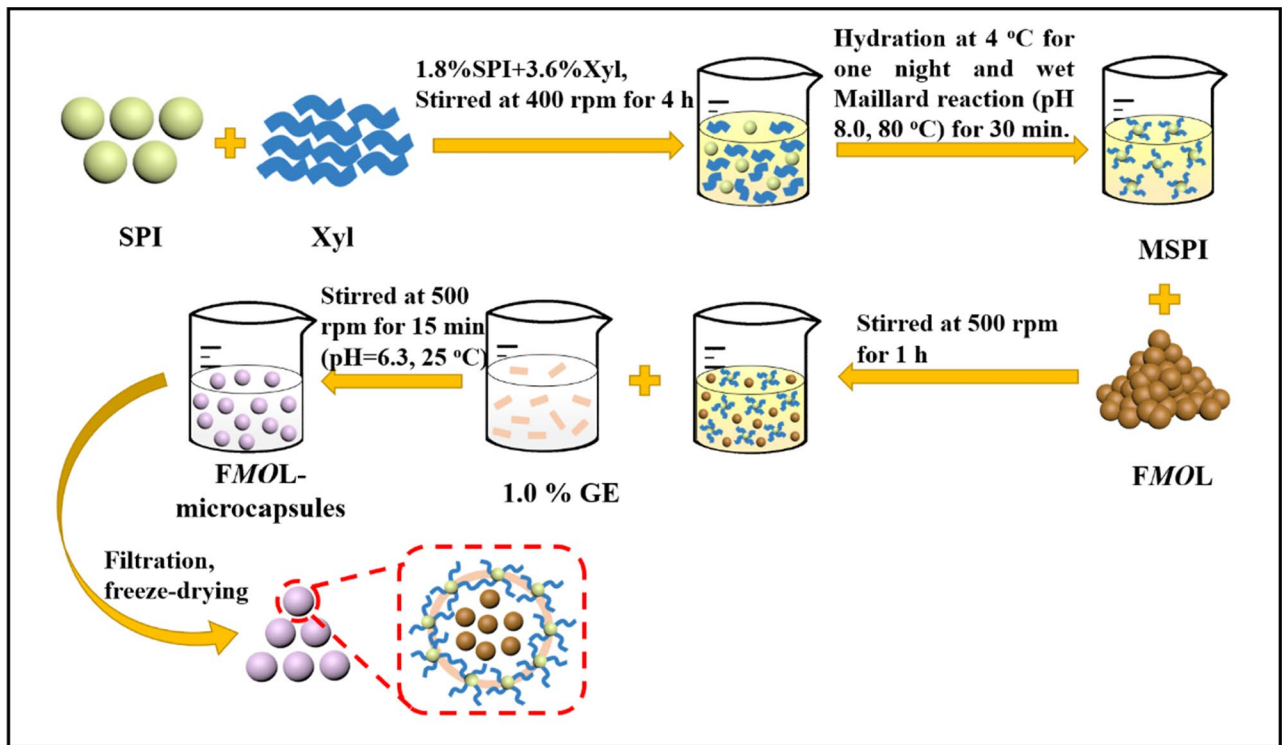
Table 3 shows that the flavonoid release and bioavailability of free FMOL, modified FMOL microcapsules, and unmodified FMOL microcapsules in the simulated oral, gastric, and intestinal stages. The results showed that the bioavailability of modified FMOL microcapsules increased 9.1% and unmodified FMOL microcapsules increased 2.4% compared with the free FMOL. Therefore, the application of modified microcapsules of FMOL could improve the bioavailability of *Moringa* flavonoids.

The bioavailability of free FMOL and microencapsulated FMOL have been investigated; the release amounts of FMOL, unmodified FMOL microcapsules, and modified FMOL microcapsules in different simulated digestion stages were further analyzed. Figure 5(g) depicts that the release amounts of free FMOL, unmodified FMOL microcapsules, and modified FMOL microcapsules in simulated oral digestion stages were 57.2%, 26.3%, and 24.0%, respectively. In the simulated gastric digestion stage, the release amounts were 29.9%, 31.9%, and 33.0%, respectively. In the simulated intestinal digestion stage, the release rates were 0.5%, 32.8%, and 39.4%, respectively. The experimental results showed that the oral release of FMOL was twice of microcapsules, which was not conducive to the embedding protection of flavonoids. There was little difference in the release of the three samples in the simulated gastric digestion stage. The release of unmodified FMOL microcapsules was  $49.0 \pm 0.5 \mu\text{g}/\text{mL}$ ; the release rate was 32.8%, which was 70 times to that of free FMOL. In the simulated intestinal digestion stage, the release of free FMOL was only  $0.7 \pm 0.3 \mu\text{g}/\text{mL}$ ; the release rate was only 0.5%. The release amount of modified FMOL microcapsule was  $60.2 \pm 0.6 \mu\text{g}/\text{mL}$ ; the release rate was 39.4%, which greatly increased the intestinal release of free FMOL (about 86 times). The intestinal release rate of modified FMOL microcapsules was about 6.6% higher than that of unmodified FMOL microcapsules. Therefore, the microencapsulation of FMOL was conducive to protect the slow release of FMOL in the process of digestion, and the microcapsule prepared by soybean protein isolate modified by xylose could further retard the release of FMOL in the intestine.

## 4 Conclusion

Different DESs were investigated to extract FMOL, and ChCl/urea was found to be the best DES for obtaining FMOL, with an optimized extractive effect ( $63.2 \pm 0.3 \text{ mg R/g DW}$ ). Nine flavonoids were identified by LC–MS and luteolin was previously unreported from *Moringa oleifera* leaves. Then, its antioxidant, antibacterial, and antitumor activities were systematically studied. FMOL was superior to positive drugs in antioxidant activity (DPPH free radical scavenging, ABTS free radical scavenging, FRAP, ORAC) and antibacterial activity in vitro. The FMOL extracted with ChCl/urea had exciting antineoplastic activity and was comparable with that of the positive control. The FMOL has certain antitumor activity, and the overlap effect of AO staining and cell apoptosis results revealed a dose-dependent effect on cell viability for the as-extracted FMOL. The microencapsulation has enhanced the slow release rate of FMOL, the release amounts of FMOL, unmodified FMOL microcapsules, and modified FMOL microcapsules were  $0.7 \pm 0.3$ ,  $60.2 \pm 0.6$ , and  $49.0 \pm 0.5 \mu\text{g}/\text{mL}$  respectively.





**Fig. 5** a Microcapsule preparation flow chart. b microcapsule micrograph. SEM images of microcapsules: c 100 μm, d 10 μm. e Infrared characterization of microcapsules. f XRD characterization of microcapsules. g Simulating the release rate of flavonoids during digestion

**Table 3** Content and bioavailability of total flavonoids in different digestion stages

Samples Digestive stage	Free FMOL ( $\mu\text{g/mL}$ )	Unmodified microcapsule ( $\mu\text{g/mL}$ )	Modified microcapsule ( $\mu\text{g/mL}$ )
Initial concentration	133.9 $\pm$ 1.8	149.5 $\pm$ 1.3	152.7 $\pm$ 1.3
Oral	76.6 $\pm$ 1.6	39.4 $\pm$ 0.5	36.7 $\pm$ 1.0
Stomach	116.6 $\pm$ 2.2	87.1 $\pm$ 0.8	87.1 $\pm$ 0.9
Intestine	117.3 $\pm$ 1.3	136.1 $\pm$ 0.3	147.3 $\pm$ 1.5
Intestinal release ( $\mu\text{g/mL}$ )	0.7 $\pm$ 0.3	49.0 $\pm$ 0.5	60.2 $\pm$ 0.6
Biological accessibility/%	87.6	91.0	96.5

The intestinal release of unmodified FMOL microcapsules and modified FMOL microcapsules were 70 and 86 times of that of free FMOL. Therefore, the microencapsulation can improve the bioavailability of FMOL. In conclusion, FMOL extracted by ChCl/urea has excellent biological activity, and its microencapsulation is helpful to FMOL slower release, which could broaden the application prospects of FMOL in food or pharmaceutical industry.

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**Author contribution** Ping Wei: conceptualization, investigation, formal analysis, writing—original draft, writing—editing. Yue Zhang: methodology, investigation, data treatment, formal analysis. Yao-Ying Wang: methodology, software, investigation, writing—original draft. Jin-Feng Dong: formal analysis, validation. Bi-Ni Liao: writing—editing. Zhi-Cheng Su: formal analysis. Wu Li: writing—editing. Ju-Cai Xu: writing—editing. Wen-Yong Lou: writing—editing. Hui-Hui Su: writing—editing. Chao Peng: project administration, funding acquisition, supervision, writing—review.

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## Declarations

**Ethical approval** With no need for ethical approval.

**Competing interests** The authors declare no competing interests.

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