RSC Advances

PAPER

View Article Online
View Journal | View Issue

Cite this: *RSC Adv.*, 2023, **13**, 17406

Enrichment of the flavonoid fraction from *Eucommia ulmoides* leaves by a liquid antisolvent precipitation method and evaluation of antioxidant activities *in vitro* and *in vivo*

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Eucommia ulmoides leaves originate from the dry leaves of the *Eucommia ulmoides* plant. Flavonoids are the main functional components of *Eucommia ulmoides* leaves. Some flavonoids such as rutin, kaempferol and quercetin are rich in *Eucommia ulmoides*, and they have outstanding antioxidant efficacy. However, the poor water solubility significantly affects the bioavailability of flavonoids. In this study, we used a liquid antisolvent precipitation (LAP) method to enrich the main flavonoid fractions in *Eucommia ulmoides* leaves, and prepared nanoparticles by the LAP method to increase flavonoids' solubility and antioxidant properties. The technological parameters were optimized by Box–Behnken Design (BBD) software and were displayed as follows: (1) total flavonoids (TFs) concentration: 83 mg mL^{-1}; (2) antisolvent–solvent ratio: 11; (3) deposition temperature: 27 °C. Under optimal processing conditions, the purity and recovery rate of TFs were 88.32% ± 2.54% and 88.08% ± 2.13%, respectively. *In vitro* experiments showed that the radical scavenging IC_{50} values for DPPH, ABTS, hydroxyl radicals and superoxide anions were 16.72 ± 1.07, 10.76 ± 0.13, 227.68 ± 18.23 and 335.86 ± 15.98 μg mL^{-1}, respectively. *In vivo* studies showed that the obtained purified flavonoid (PF) (100, 200, 400 mg kg^{-1}) treatment is able to improve CCl_4-induced liver and kidney damage through adjusting, superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), glutathione peroxidase (GSH-Px) and malondialdehyde (MDA) levels. These results demonstrated that the LAP method is capable of extracting TFs from *Eucommia ulmoides* leaves with high bioaccessibility.

Received 6th February 2023
Accepted 27th May 2023

DOI: 10.1039/d3ra00800b

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1. Introduction

Eucommia ulmoides Oliv., a member of the Eucommia ulmoides family, is a large woody plant mainly growing in China.[1] *Eucommia ulmoides* has multiple pharmacological effects on hypertension, diabetes, vertigo, chronic kidney diseases and other diseases.[2] According to the Chinese Pharmacopoeia (National Commission of Chinese Pharmacopoeia, 2020), the medicinal parts of *Eucommia ulmoides* are *Eucommia ulmoides*'s bark and leaves. It is worth noting that *Eucommia ulmoides* is a deciduous tree which can produce large amounts of deciduous leaves as the biological resources.[3] The active ingredients in *Eucommia ulmoides* leaves are polyphenols, sterols, terpenes, phenylpropanoids, polysaccharides and flavonoids.[4,5] Currently, multiple flavonoid components such as rutin, kaempferol and quercetin have been detected in *Eucommia ulmoides* leaves. Their chemical structures were shown in Fig. 1A. Flavonoids have a variety of biological functions, such as anti-cancer, anti-aging, and preventing neurodegenerative diseases.[6–8] Importantly, the free radical removal ability of flavonoids make them the ideal resource for natural antioxidants. Mechanisms have been found to be responsible for antioxidant activities of rutin,[9] kaempferol[10] and quercetin[11] by directly scavenging ROS and increasing the production of glutathione (GSH). In particularly, recent studies demonstrated that kaempferol is capable of maintaining oxidant–antioxidant status through inhibiting the MAPK/AGE-RAGE pathways,[12] and upregulating the levels of NRF-2 and HO-1.[10] Xu *et al.*, reported that quercetin can alleviate oxidative stress by improving Nrf2-ARE pathway or activating PI3K/AKT pathway.[11] In addition, the potentially toxicological properties including acute toxicity, carcinogenicity and mutagenicity of rutin, kaempferol and quercetin were also presented in Fig. 1B by referring to the PubChem database, International Agency for Research on Cancer (IARC) classification, and existing study (**https://ncats.nih.gov/expertise/preclinical/pubchem**).[13]

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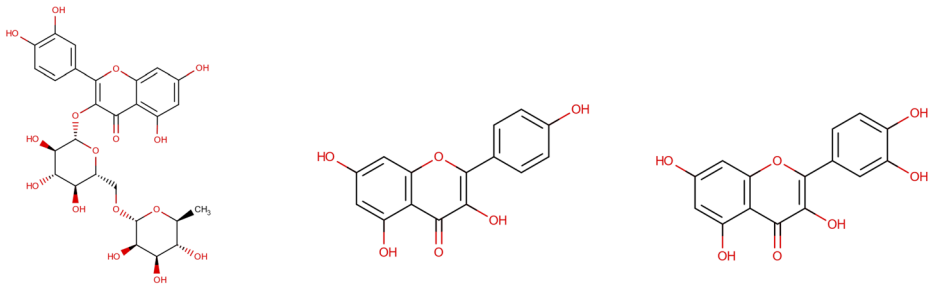
A



B

Compound Name	Rutin	Kaempferol	Quercetin
Antioxidative property	Accelerating ROS removal Increasing GSH production	Accelerating ROS removal Increasing GSH production Inhibiting MAPK/AGE-RAGE pathways Upregulating NRF-2/HO-1 levels	Accelerating ROS removal Increasing GSH production Improving Nrf2-ARE pathway Activating PI3K/AKT pathway
Acute Toxicity	Rat, i.p, LD_{50}: 2 g/kg	Rat, p.o, LD_{50}: 980 mg/kg	Rat, p.o, LD_{50}: 161 mg/kg
Carcinogenicity	N/A	Group 3: Not classifiable as to its carcinogenicity to humans	Group 3: Not classifiable as to its carcinogenicity to humans
Mutagenicity	N/A	Kaempferol is mutagenic in bacteria, insects and mammalian cells in vitro	Quercetin is mutagenic to Drosophila
Reference	Chen, *et al*, 2021; Pubchem	Kluska, *et al*, 2022; Suchal *et al*, 2017; Numair, *et al*, 2015; Pubchem; IARC	Xu, *et al*, 2019; Pubchem; IARC

Fig. 1 (A) Chemical structures of rutin, kaempferol and quercetin; (B) antioxidant properties and toxicological properties (acute toxicity, carcinogenicity and mutagenicity) of rutin, kaempferol and quercetin. Abbreviation: i.p.: intraperitoneal injection; p.o.: *peros*; LD_{50}: half lethal dose; N/A: not available; IARC: International Agency for Research on Cancer.

At present, flavonoids extraction is widely used in food, medicine, cosmetics and other industries. However, the chemical components in *Eucommia ulmoides* leaves are very complex, making the purification process very difficult. The traditional method of flavonoids purification is silica gel column chromatography.[14,15] This method is widely used, but with cumbersome operation, long process cycle, and residual toxic reagents.[16,17] High-performance countercurrent chromatography (HPCCC) and high-speed countercurrent chromatography (HSCCC) have been reported to be used to purify flavonoids, but the amount of phytochemicals obtained is not enough to meet the rapid development of pharmaceutical and health products.[18–21] Macroporous adsorption resin has good adsorption properties and analytical properties and is widely used, but its high organic residues and unsatisfactory purification effect are still need to be solved in the food and pharmaceutical industry.[22–24]

In order to improve the content of medicinal components in the extract, attention focused on the purification methods of *Eucommia ulmoides* leaves.

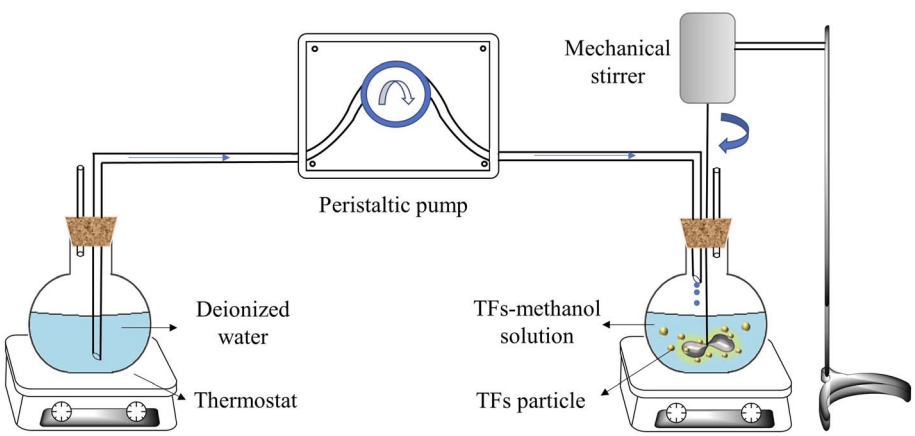


Fig. 2 Schematic description of liquid antisolvent precipitation procedure.

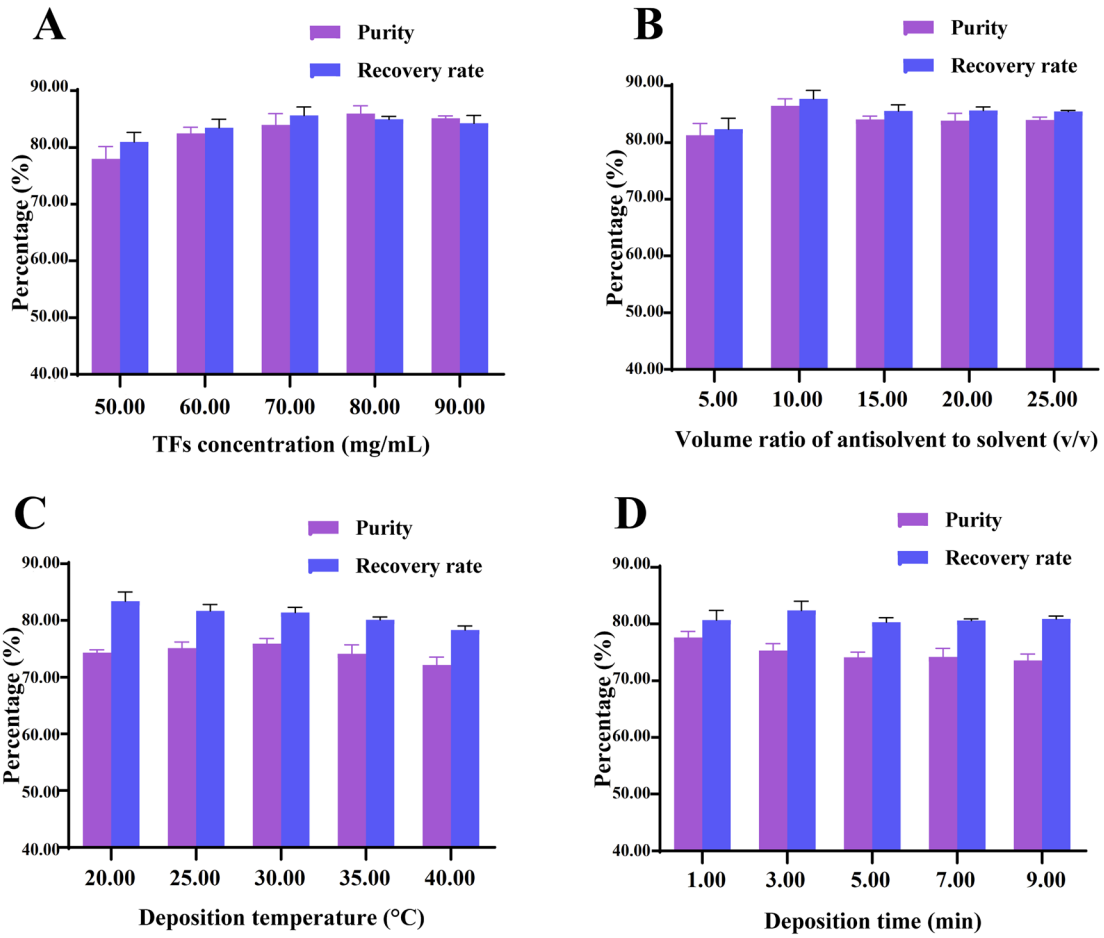


Fig. 3 Optimization of purification TFs method by single factor design. (A) Effect of TFs concentration, (B) antisolvent–solvent ratio, (C) deposition temperature and (D) deposition time.

Recently, new isolation and purification techniques have been developed with significant advantages over conventional methods. These techniques include supercritical CO_2 extraction method, membrane separation technique, metal ion complex purification, and recrystallization technique.[25,26] Among them, recrystallization technique can be a purification technology with potential application value because of its simple and easy operation.

Liquid antisolvent precipitation (LAP) method is to make it saturated with a chemical in a mixed system containing solvent and antisolvent, to precipitate a substance with high solubility.[27] In recent years, LAP technology has been widely used in changing the crystal form such as reducing particle size and improving water solubility. For example, Yu *et al.*[28] produced Daidzein microparticles by LAP method. The minimum average particle size of was 181 ± 2 nm. Wu *et al.*[29] used the LAP method to improve the water solubility of silibinin, with 180.81 ± 5.32 μg mL^{-1} in artificial gastric juice. This method has the advantages of simple preparation process, low cost, easy operation and high yield. However, few reports about LAP application in purification of the active ingredient of *Eucommia ulmoides*, but none considering purification of flavonoids in *Eucommia ulmoides* leaves until now.

Recently, our research team reported on the use of microwave-assisted micelle solution to extract total flavonoids in *Eucommia ulmoides* leaves, with an extraction rate of 1.45%.[30] In this study, we continued the previous work by using the LAP method to purify crude flavonoids (CFs) from *Eucommia ulmoides* leaves, to simultaneously obtain purified flavonoid (PF) particles, and explore the effect of micronized flavonoids on antioxidant activity. We used methanol as the solvent and deionized water as the antisolvent. The influencing factors in the purification process are the concentration of TFs, ratio of antisolvent to solvent, deposition time and temperature. The optimal purification process was obtained by optimizing the factor conditions. In addition, the extracted micronized flavonoids components were studied for antioxidant activity *in vitro* and *in vivo*. The schematic diagram of the purification process is shown in Fig. 2.

2. Materials and methods

2.1 Plant and materials

Fresh *Eucommia ulmoides* leaves were purchased from Bozhou Chinese herb market (Anhui, PR China). The *Eucommia ulmoides* leaves were dried in a cool and ventilated place at room

Table 1 The response surface test design and results for purifying TFs by LAP method[a]

	Box–Behnken design				
No.	X_1	X_2	X_3	Y_1	Y_2
1	70.00	5.00	25.00	82.54	80.11
2	90.00	5.00	25.00	83.78	82.03
3	70.00	15.00	25.00	83.81	84.15
4	90.00	15.00	25.00	86.37	86.14
5	70.00	10.00	20.00	82.76	82.11
6	90.00	10.00	20.00	85.55	84.12
7	70.00	10.00	30.00	84.62	84.35
8	90.00	10.00	30.00	87.46	87.16
9	80.00	5.00	20.00	83.03	79.61
10	80.00	15.00	20.00	86.26	82.07
11	80.00	5.00	30.00	84.74	82.26
12	80.00	15.00	30.00	87.34	86.22
13	80.00	10.00	25.00	89.26	88.11
14	80.00	10.00	25.00	87.12	88.43
15	80.00	10.00	25.00	88.43	87.91
16	80.00	10.00	25.00	88.95	87.15
17	80.00	10.00	25.00	88.42	86.87

[a] Abbreviations: TFs, total flavonoids; LAP, liquid antisolvent precipitation. X_1 is TFs concentration (mg mL^{-1}), X_2 is volume ratio of antisolvent to solvent (v/v), X_3 is deposition temperature (°C), Y_1 is purity (%), Y_2 is recovery rate (%).

temperature for 20 days, and then stored in a dry place until use.

2.2 Chemicals and reagents

Sodium dodecyl benzene sulfonate (SDBS, analytical purity) was acquired from Wuxi Ya Tai United Chemical Co., Ltd. (Jiangsu, PR China). 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) and 2,2′-azinobis-(3-ethyl benzthiazoline-6-sulfonic acid) (ABTS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ascorbic acid (Vc) and butylated hydroxy toluene (BHT) were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.3 Extraction of TFs

TFs in *Eucommia ulmoides* leaves were extracted by microwave ultrasonic combined with alkaline sodium dodecyl benzene sulfonate solution.[30,31] Briefly, 6 g of dried powdered *Eucommia ulmoides* leaves were fully mixed with 1.5% sodium dodecyl benzene sulfonate solution 300 mL in a round bottom flask and soaked for 6 h at room temperature. The mixture system was placed in the microwave ultrasonic extraction device for an extraction time of 30 min at a microwave power of 700 W. The filtrate and the filter residue were separated by centrifugation. The filtrate was then stored until the next purification step.

2.4 Purification of TFs

2.4.1 Pretreatment of extraction solution. The flavonoid extraction solution was added to the same volume of petroleum ether to extract and defatted, and the water layer was concentrated at 45 °C in a rotary evaporator. The concentrated solution was separated by chromatography on a glass column (80 mm × 700 mm, containing 650 g of D101 macroporous resin). The bed volume (BV) was 1.5 L and the adsorption time was 12 h. Subsequently, distilled water was used to wash the elution solution until almost colourless. The highly polar components were removed by elution with 10% aqueous ethanol of 3BV. Finally, rinse the adsorbent with 12 BV 70% aqueous ethanol and collect 70% aqueous ethanol, concentrate, and then dried. The recovery and purity of the TFs were 89.24% ± 1.26% and

Table 2 The results analysis of variance for regression equation of purity for purifying TFs by LAP method

Source	Sum of squares	Degrees of freedom	Mean square	*F*-value	*P*-value
Model	77.34	9	8.59	16.26	0.00070
X_1	11.12	1	11.12	21.03	0.0025
X_2	11.74	1	11.74	22.21	0.0022
X_3	5.38	1	5.38	10.18	0.015
X_1X_2	8.52	1	0.44	0.82	0.39
X_1X_3	0.00063	1	0.00063	0.0012	0.97
X_2X_3	0.099	1	0.099	0.19	0.68
X_1^2	21.85	1	21.85	41.35	0.00040
X_2^2	17.40	1	17.40	32.93	0.00070
X_3^2	4.74	1	4.47	8.96	0.020
Residual	3.70	7	0.53	—	—
Lack of fit	1.02	3	0.34	0.51	0.70
Pure error	2.68	4	0.67	—	—
Cor total	81.04	16	—	—	—

Credibility analysis of the regression equations

R^2	Adjust R^2	Predicted R^2	Adequacy precision
0.95	0.90	0.75	11.43

Table 3 The results analysis of variance for regression equation of recovery rate for purifying TFs by LAP method

Source	Sum of squares	Degrees of freedom	Mean square	*F*-value	*P*-value
Model	128.86	9	14.32	40.05	<0.0001
X_1	9.53	1	9.53	26.65	0.0013
X_2	26.54	1	26.54	74.22	<0.0001
X_3	18.24	1	18.24	51.02	0.0002
X_1X_2	0.0012	1	0.0012	0.0034	0.96
X_1X_3	0.16	1	0.16	0.45	0.53
X_2X_3	0.56	1	0.56	1.57	0.25
X_1^2	7.63	1	7.63	21.33	0.0024
X_2^2	44.22	1	44.22	123.68	<0.0001
X_3^2	15.41	1	15.41	43.11	0.0003
Residual	2.50	7	0.36	—	—
Lack of fit	0.77	3	0.26	0.59	0.65
Pure error	1.74	4	0.43	—	—
Cor total	131.37	16	—	—	—

Credibility analysis of the regression equations

R^2	Adjust R^2	Predicted R^2	Adequacy precision
0.98	0.96	0.89	17.69

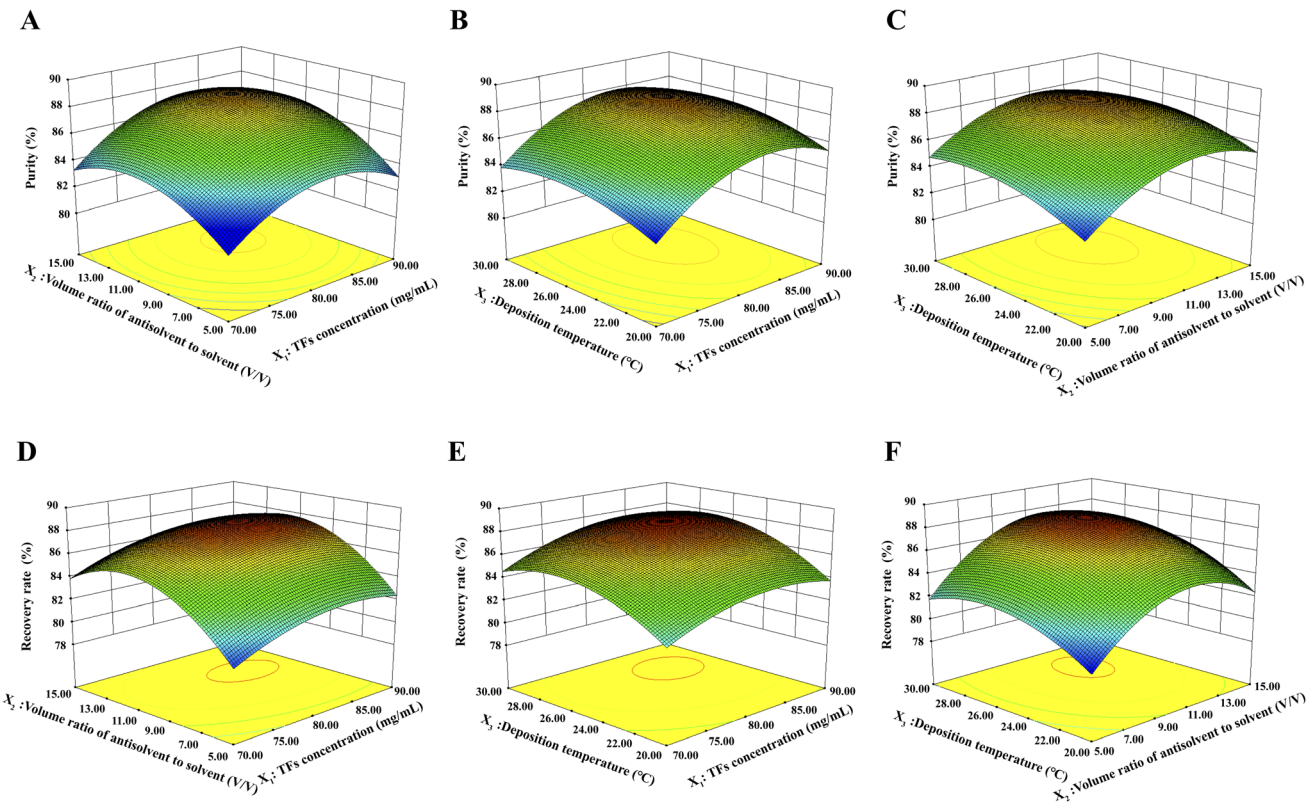


Fig. 4 Response surface for the interactions of independent variables on purity and recovery rate of total flavonoids. (A) The interaction of concentration of TFs and antisolvent–solvent ratio, (B) the interaction of concentration of TFs and deposition temperature and (C) the interaction of antisolvent–solvent ratio and deposition temperature on TFs purity, (D) the interaction of concentration of TFs and antisolvent–solvent ratio, (E) the interaction of concentration of TFs and deposition temperature and (F) the interaction of antisolvent–solvent ratio and deposition temperature on TFs recovery rate.

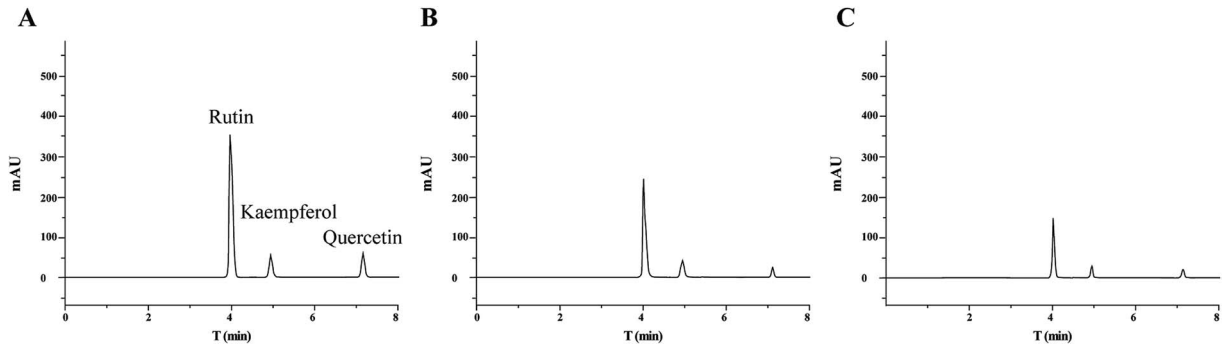


Fig. 5 HPLC chromatogram: (A) reference standards of three flavonoids, (B) the main flavonoids in PFs, and (C) CFs.

69.13% ± 0.96%, respectively. The purified TFs (96.78 mg) was stored at 4 °C and used in the next experiment.

2.4.2 Purification of TFs. Total *Eucommia ulmoides* flavonoids were purified by liquid antisolvent precipitation (LAP) method with methanol as solvent and deionized water as antisolvent. When the two solvent solution systems were mixed the solvent was dissolved by the antisolvent, and the solute in the solvent was purified by reverse crystallization (Fig. 2). Put the powder obtained in Section 2.4.1 into the beaker and add a certain amount of methanol. The TFs were properly dissolved using ultrasound, and insoluble substances were removed by centrifugation to obtain a clear solution. Deionized water was gradually added into the system of methanol solution containing TFs through the syringe pump during accompanying agitation. After a time period, all the solutions were removed by filtration and the precipitates were obtained. The precipitates were washed three times with deionized water, dried and collected at constant mass in a 50 ± 1 °C oven. The effect of different factors on the purity and recovery of PFs were investigated using univariate experiments: concentration of TFs (50, 60, 70, 80, and 90 mg mL^{-1}), ratio of antisolvent to solvent (5, 10, 15, 20, and 25 v/v), deposition temperature (20, 25, 30, 35, and 40 °C) and the deposition time (1, 3, 5, 7, and 9 min). The content of purified total flavonoids was determined using the method described in Section 3.5.

2.4.3 Process optimization. Interactions between each factor are crucial for obtaining higher purity and recovery of TFs. After selecting the most important factors for TFs purification, the Box–Behnken Design (BBD) was used to determine the optimal level of these variables. Based on BBD, the effects of the concentration of TFs, ratio of antisolvent to solvent and deposition temperature on the purity and recovery were optimized. The scope of the variables studied were listed as followed: 70–90 mg mL^{-1} concentration of TFs, 5–15 v/v ratio of antisolvent to solvent and 20–30 °C deposition temperature. Predicting the response through the full second-order polynomial equation is as shown in equation:

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} {x_i}^2 + \sum_{i<j}^{k} \beta_{ij} x_i x_j$$

In the above formula, y is the predicted response value, β_0 is the coefficient constant, β_i is the linear coefficient, β_{ii} is the quadratic equation coefficient, and β_{ij} is the interaction coefficient. Three different independent variables are defined as X_1, X_2 and X_3.

2.5 Determination of the main flavonoids

2.5.1 Determination of TFs. The TFs were measured by the colorimetric-based method assay with a slight modification.[32] Briefly, The TFs solution (1.0 mL) was added to 70% aqueous ethanol (4.0 mL) and mixed evenly, and then the $NaNO_2$ (0.5 mL, 5%, w/v) added to it was reacted with 6 min. Then, $AlCl_3$ (0.5 mL, 10%, w/v) and NaOH (3.0 mL, 1 M) were added to the above solution, followed by the addition of distilled water to reach 10.0 mL, which was fully mixed to incubate 15 min at room temperature. The 3 mL sample was placed in the quartz cuvettes (1.0 cm) (Shimadzu, Japan) and the absorbance at 510 nm wavelength was detected by UV-1700 spectrophotometer. The control contained all the reaction reagents except for the test sample. Standard curve regression equations were: $Y = 13.75X + 0.0772R^2 = 0.9992$ (where Y is the absorption and X is the rutin concentration in mg mL^{-1}). The flavonoid content was calculated from the calibration curve and expressed as rutin equivalents.

2.5.2 Determination of main flavonoids by HPLC. The contents of the main flavonoids (Rutin, kaempferol and

Table 4 The content of main flavonoids in CFs and PFs by HPLC ($n = 3$)[a]

Samples	Rutin (%)	Kaempferol (%)	Quercetin (%)	Total flavonoids (%)
CFs	20.61 ± 1.21	0.82 ± 0.09	1.22 ± 0.12	55.31 ± 3.12
PFs	27.14 ± 1.14	1.51 ± 0.21	2.32 ± 0.24	88.32 ± 2.54

[a] Data presents as mean ± standard deviation.

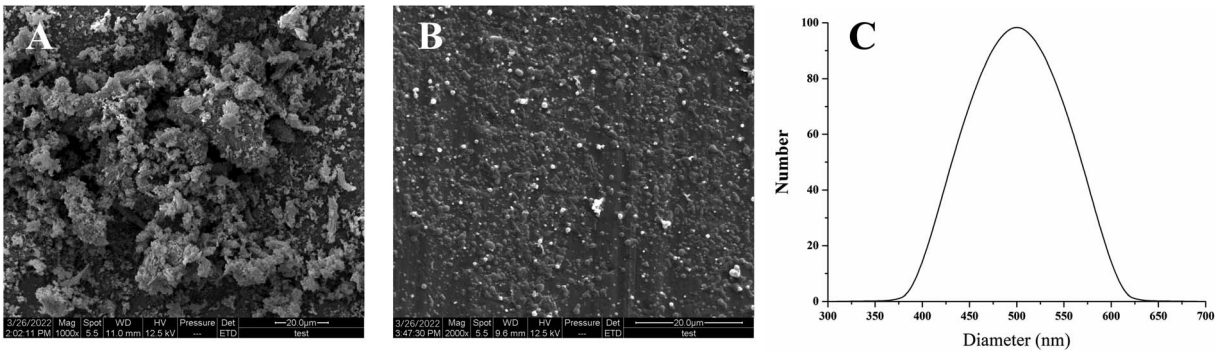


Fig. 6 SEM images and particle size distribution of sample morphology: (A) CFs; and (B) PFs; (C) particle size distribution.

quercetin) in *Eucommia ulmoides* leaves were determined. A modified high-performance liquid chromatography (HPLC) method was performed on a Waters chromatographic instrument (Waters Delta 600 pump and a 2487 UV detector).[33] The chromatographic column uses a C_{18} reverse-phase column (250 mm 4.6 mm, 5 μm, China). The 0.1% aqueous formic acid (A) and 0.1% formic acid in acetonitrile (B) were used as the mobile phase with a flow rate of 0.6 mL min^{-1}. The gradient elution conditions were as follows: 0–2 min, 10–20% (B); 2–5 min, 20–28% (B); 5–8 min, 28–50% (B); 8–10 min, 50–95% (B); and 10–12 min, 95–10% (B). Test conditions were column temperature at 25 °C with an injection volume of 10 μL and detected wavelength at 354 nm.

2.6 Characterization of morphology

The morphology of the extracted and purified total flavonoids samples was examined by SEM (Quanta 200, FEI, Hillsboro, OR, USA). The dry samples were fixed on the silicon wafer and sputtered with gold to a thickness of about 150 nm.

2.7 Antioxidant activity *in vitro*

2.7.1 DPPH and ABTS radical scavenging assay. The scavenging activities of DPPH and ABTS free radicals of PFs and CFs were determined using previously reported method.[34–36] A certain mass of samples were dissolved in 95% ethanol and configured as solutions with different concentration gradients (samples concentrations of 2, 5, 10, 15 and 20 mg mL^{-1}). The sample (2 mL) was fully mixed with 2 mL DPPH solution of 95% ethanol (0.1 mmol L^{-1}). Similarly, the samples (2 mL) were fully mixed with 2 mL ABTS solution of 95% ethanol (7 mmol L^{-1}). The suspension was thoroughly mixed for 30 minutes under normal temperature and dark conditions. The absorbance at 517 (DPPH) and 734 (ABTS) nm wavelengths was detected by spectrophotometer (Hitachi, Ltd., Tokyo, Japan). Ascorbic acid (Vc) and butylated hydroxy toluene (BHT) were used as positive control. The formula for the determination of radical scavenging activity (RSA) (ABTS and DPPH) is as follows:

$$\mathrm{RSA}(\%) = (A_0 - A_1/A_0) \times 100\% \quad (1)$$

where, antioxidant capacity is the AC, absorbance of the blank sample is the A_0, and absorbance of the test compound is A_1

2.7.2 Scavenging activity of hydroxyl radicals (·OH) assay. The experimental method of hydroxyl radical scavenging activity was slightly modified with reference to previous literature report.[37] Different concentrations of samples solution (1.5 mL) were successively added to phosphate-buffer solution (1.5 mL, 0.2 M, pH 7.4), 1,10-phenanthroline (0.75 mL, 0.75×10^{-3} M), $FeSO_4$ (0.75 mL, 0.75×10^{-3} M) and 0.75 mL 0.3% H_2O_2 (v/v) were fully mixed. The suspension was heated at 37 °C for 30 min, and the absorbance (A_1) of the sample was measured at 510 nm. The formula for calculating the ability of the sample to scavenge ·OH is as follows:

$$\cdot\mathrm{OH}\ \text{scavenging activity}\ (\%) = [1 - (A_1 - A_2)/A_0] \times 100\% \quad (2)$$

where, A_0 is absorbance of the blank sample (the same volume of distilled water was used instead of PFs ethanol solution), A_2 is the absorbance of distilled water instead of $FeSO_4$ solution under 510 nm.

2.7.3 Scavenging activity of the superoxide anion ($O_2^{\cdot-}$) assay. Different concentrations of PFs solution of 0.5 mL were mixed with 4.5 mL Tris–HCl buffer (50 mM, pH 8.2). The suspension was placed at 25 °C to incubate 20 min. Then, 10 μL pyrogallol (45 mM) was added to the suspension for 6 min at 25 °C. The absorbance of the sample was measured at 320 nm. The scavenging capacity of superoxide anion is calculated by the following formula.

$$O_2^{\cdot-}\ \text{scavenging activity}\ (\%) = [1 - (A_1 - A_2)/A_0] \times 100\% \quad (3)$$

where, A_1 is the absorbance of the added PFs, A_2 is the absorbance of without pyrogallol, A_0 is absorbance of the blank sample (without PFs).

2.7.4 Ferric-reducing antioxidant power (FRAP) assay. The sample was prepared in the same manner as described in the "DPPH and ABTS radical scavenging assay" section. Different concentrations of samples solution 2 mL were added to 2.5 mL phosphate buffer (0.2 M, pH 6.6), and 2.5 mL 1% potassium ferricyanide solution was added. The suspension was incubated at 50 °C for 30 min. Subsequently, 10% trichloroacetic acid of 2.5 mL was added, and the solution mixture was further

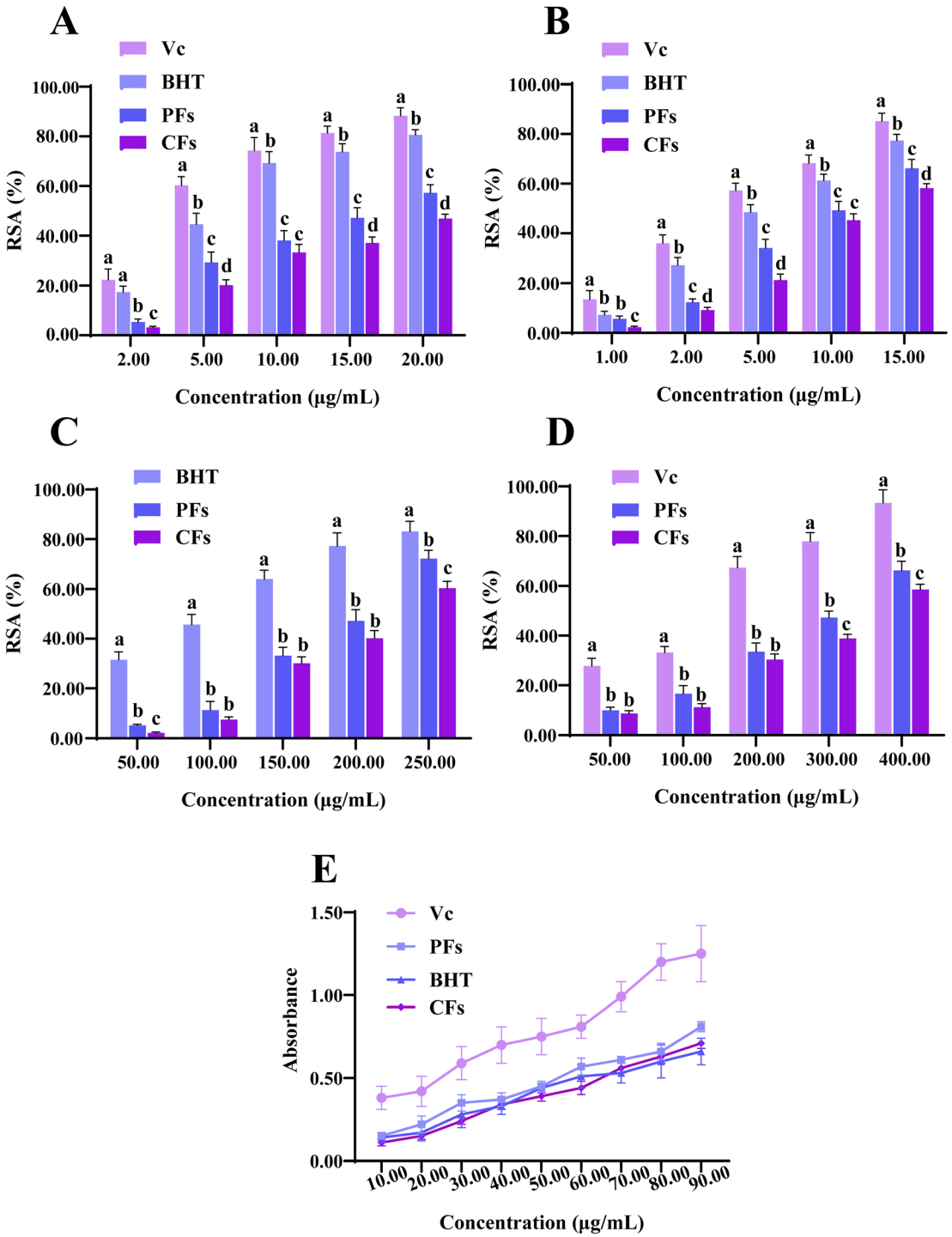


Fig. 7 *In vitro* radical scavenging activity RSA (%) assay. Comparison of different concentrations of Vc BHT and PFs. (A) Free radical scavenging activity of DPPH; (B) scavenging activity of ABTS; (C) $^{\bullet}OH$ radical scavenging activity; (D) $O_2^{\bullet-}$ radical scavenging activity; (E) Ferric Reducing Power (FRAP) assay. Data are presented as mean ± SEM ($n = 3$). Means with different letters (a–d) are significantly different from one another ($p < 0.05$). Abbreviation: RSA, radical scavenging activity; BHT, butylated hydroxy toluene; PFs, purified flavonoids; CFs, crude flavonoids.

incubated at room temperature for 10 min. Take the above mixed solution 2.5 mL and mix it with 0.1% ferric chloride and 2.5 mL distilled water of 0.5 mL and incubate for 10 min. The absorbance A_1 of PFs mixed solution and the absorbance of blank sample A_0 were determined at 700 nm.

2.8 *In vivo* testing antioxidant activity of PFs

2.8.1 Experimental animals. Sprague–Dawley (SD) rats (160 g ± 20 g) were obtained from Beijing HFK Bioscience Co., Ltd. (Beijing, China). All rats were fed with standard pellet feed and water and fed indoors at 25 °C ± 2 °C, 60–70% relative

Table 5 The *in vitro* antioxidant activities of PFs ($n = 3$)[a]

Sample	DPPH (IC_{50}) μg mL^{-1}	ABTS (IC_{50}) μg mL^{-1}	$^{\cdot}OH$ (IC_{50}) μg mL^{-1}	$O_2^{\cdot -}$ (IC_{50}) μg mL^{-1}	FRAP (SRP) μg^{-1}
V_c[b]	4.15 ± 0.06	3.76 ± 0.09	—	164.27 ± 9.72	0.0113 ± 0.0034
BHT[b]	6.58 ± 0.11	5.13 ± 0.10	114.25 ± 12.63	—	0.0080 ± 0.0015
PFs	16.72 ± 1.07[c]	10.76 ± 0.13[c]	227.68 ± 18.23	335.86 ± 15.98[c]	0.0091 ± 0.0018
CFs	19.13 ± 0.23	13.12 ± 0.11	243.22 ± 8.625	377.52 ± 10.23	0.0083 ± 0.0012

[a] Data presents as mean ± standard deviation. Abbreviations: V_c, ascorbic acid; BHT, butylated hydroxy toluene; PFs, purified flavonoids. SRP: slope of trend line in reducing power assay (μg^{-1}). IC_{50}: the IC_{50} value represents the concentration when free radical scavenging inhibits 50% (μg mL^{-1}). [b] Positive control. [c] $p < 0.05$ compared to CFs.

humidity and a 1a 12/12 h light/dark cycle. All animal operations were implemented in accordance with the Guidelines for the Care and Use of Experimental Animals of Northeast Forestry University. Animal experiments were approved by the Ethics Committee of the Northeast Forestry University.

2.8.2 Carbon tetrachloride-induced oxidative stress model. The SD rats were divided into 6 groups (6 rats in each group, half male and female). Group I was treated with 0.3% sodium carboxymethyl cellulose (CMC-Na) (1 mL kg^{-1} in sterile distilled water, p.o. https://www.bio-rad.com/en-us/product/sterile-distilled-water?ID=LS5B7ODFX) for 5 days and olive oil (2 mL kg^{-1}, i.p.) on day 2 and day 3. Group II was treated with 0.3% CMC-Na (2 mL kg^{-1}, p.o.) for 5 days and carbon tetrachloride (CCl_4)–olive oil (1 : 1, 2 mL kg^{-1} in olive oil, i.p.) on day 2 and day 3. Group III was administrated with vitamin E (100 mg kg^{-1} in 0.3% CMC-Na, p.o.) daily for 5 days and CCl_4 (1 mL kg^{-1} in olive oil, i.p.) on day 2 and day 3. Group IV, Group V and Group VI were low dose (100 mg kg^{-1}), middle dose (200 mg kg^{-1}) and high dose (400 mg kg^{-1}) of PFs, respectively.[38] Group IV–VI (test groups) were treated with different concentrations of PFs in 0.3% CMC-Na by oral gavage for 5 days and CCl_4–olive oil (1 : 1, 2 mL kg^{-1}) on day 2 and 3. On the 6th day, all the rats were sacrificed under anesthesia, and kidneys and livers were obtained by dissection. The liver and kidney homogenates (10.0%, w/v) were prepared with phosphate buffer solution (50 mM, pH 7.4). The supernatants were obtained for biochemical detection by centrifugation at 5000 rpm and 4 °C for 15 min.

2.8.3 Determination of antioxidant enzyme activity and malondialdehyde content *in vivo*. The contents of antioxidant enzyme catalase (CAT) (SH007-2), malondialdehyde (MDA) (SH003-1), superoxide dismutase (SOD) (SH001-2), glutathione (GSH) (SH006-1) and glutathione peroxidase (GSH-Px, NADPH nethod) (SH005) in liver and kidney were detected by the kits provided by Nanjing Njbiogene Biotechnology Ltd. (Nanjing, China).

2.8.4 Determination of adverse effects of PFs on liver and kidney. The SD rats were divided into 3 groups (6 rats in each group, half male and female). A control group treated with sterile distilled water; A solvent group treated with 0.3% (w/v, in sterile distilled water) CMC-Na https://macro.lsu.edu/howto/solvents/Solvent%20Group.htm; A test group treated with 400 mg kg^{-1} PFs in 0.3% CMC-Na. All the rats were administrated daily by oral gavage for 2 weeks. At the end of the 2nd week, the blood samples were collected and left to stand for 1 h

Table 6 Antioxidant capacity of PFs in liver and kidney ($n = 6$)[a]

	Liver					Kidney				
No.	SOD	CAT	GSH	GSH-Px	MDA	SOD	CAT	GSH	GSH-Px	MDA
Group I	207.41 ± 8.73[c]	42.52 ± 3.52[c,d]	1.31 ± 0.35	1187.64 ± 106.32[c,d]	6.92 ± 0.53[c,d]	201.62 ± 4.31[c]	38.44 ± 4.12[c]	2.21 ± 0.13[c]	323.24 ± 32.52[c]	4.35 ± 0.42[c,d]
Group II	157.32 ± 9.34[b,d]	11.32 ± 2.73[b,d]	0.87 ± 0.12	452.32 ± 58.74[b,d]	10.45 ± 1.12[b,d]	146.35 ± 5.32[b,d]	10.45 ± 1.52[b,d]	1.04 ± 0.21[b,d]	235.21 ± 24.62[b,d]	7.98 ± 0.86[b,d]
Group III	184.82 ± 7.24[c]	20.14 ± 4.32[b,c]	1.24 ± 0.31	745.35 ± 66.24[b,c]	1.45 ± 0.42[b,c]	197.61 ± 7.23[c]	30.24 ± 4.62[c]	2.04 ± 0.16[c]	346.71 ± 47.63[c]	2.45 ± 0.32[b,c]
Group IV	188.72 ± 11.45[c,e]	27.64 ± 4.63[b,c,e]	1.37 ± 0.25[c,e]	703.25 ± 73.51[b,c,e]	7.12 ± 0.84[c,d,e]	195.14 ± 10.42[c,e]	22.41 ± 2.85[b,c,e]	2.56 ± 0.32[c,e]	346.26 ± 32.51[c,e]	3.03 ± 0.21[b,c,e]
Group V	193.22 ± 7.23[c,e]	28.72 ± 3.85[b,c,e]	1.43 ± 0.31[c,e]	668.47 ± 76.33[b,c,e]	7.25 ± 0.48[c,d,e]	194.21 ± 7.54[c,e]	21.48 ± 3.27[b,c,e]	2.77 ± 0.42[c,e]	312.50 ± 25.62[c,e]	2.56 ± 0.52[b,c,e]
Group VI	196.85 ± 10.22[c,e]	26.17 ± 2.52[b,c,e]	1.48 ± 0.24[c,e]	721.51 ± 83.52[b,c,e]	7.72 ± 1.04[c,d,e]	194.51 ± 6.26[c,e]	25.24 ± 1.85[b,c,e]	2.64 ± 0.35[c,e]	334.52 ± 31.42[c,e]	2.22 ± 0.32[b,c,e]

[a] Data presents as mean ± standard deviation. Group I (control group) was treated with CMC-Na and olive oil. Group II (CCl_4-induced damage group) was treated with CMC-Na and CCl_4. Group III (positive control group) was treated with vitamin E and CCl_4. Group IV (test group I) was treated low dose of PFs and CCl_4. Group V (test group II) was middle dose of PFs and CCl_4. Group VI (test group III) was High dose of PFs and CCl_4. Vitamin E and PFs suspended in 0.3% (w/v) CMC-Na was administrated by oral gavage; CCl_4 dissolved in olive oil was intraperitoneally injected. Abbreviations: SOD: Ssuperoxide dismutase, U mg^{-1} protein; CAT: catalase, U mg^{-1} protein; GSH: glutathione, mg GSH per g protein; GSH-Px: glutathione peroxidase, U mg^{-1} protein; MDA: malondialdehyde, nmol MDA per mg protein. [b] $p < 0.05$ compared to Group I. [c] $p < 0.05$ compared to Group II. [d] $p < 0.05$ compared to Group III. [e] $p > 0.05$ comparison among Group IV, Group V and Group VI.

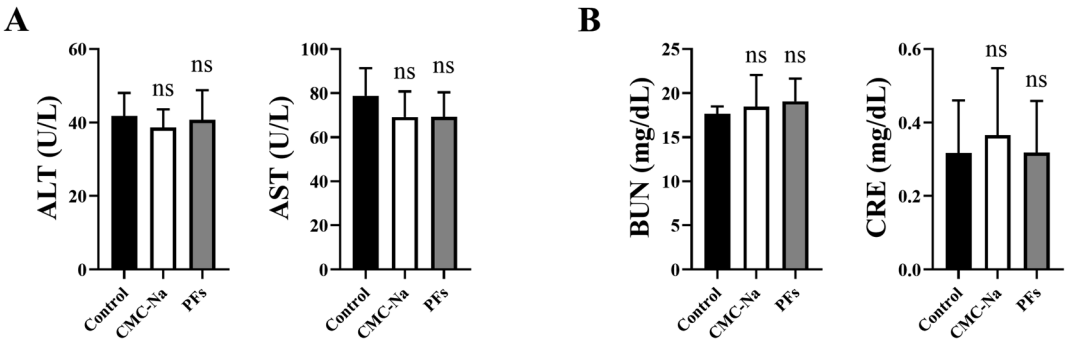


Fig. 8 The potential adverse effects of PFs on liver and kidney. Serum levels of (A) liver damage indicators (ALT and AST), and (B) kidney damage indicators (BUN and CRE). Control: sterile distilled water; CMC-Na: 0.3% (w/v, in sterile distilled water) CMC-Na; PFs: 400 mg kg^{-1} PFs in 0.3% CMC-Na. Data represent the mean ± S.D. ns $p > 0.05$.

at room temperature. Subsequently, serum was separated by centrifuging at 3000 rpm for 15 min at 4 °C. Then, the serum levels of alanine aminotransferase (ALT) (BC1555, Beijing Solarbio Science & Technology Co., Ltd., Beijing, China), aspartate aminotransferase (AST) (BC1565, Solarbio), blood urea nitrogen (BUN) (BC1530, Solarbio) and Creatinine (CRE) (BC4910, Solarbio) were detected following the manufacturer's protocols.

2.9 Statistical analysis

Statistical analysis of data using GraphPad Prism 8.0 software. The data are shown as mean ± SD. Total variation of data was estimated by *t*-test and one-way ANOVA, $p < 0.05$ can be considered statistically significant ($*p < 0.05$, $**p < 0.01$, and $***p < 0.001$).

3. Results and discussion

3.1 Optimization of purification process for PFs

3.1.1 Effects of the TFs concentration. Different concentrations of TFs (50.00, 60.00, 70.00, 80.00, and 90.00 mg mL^{-1}) combined with fixed conditions (10 : 1, v/v antisolvent to solvent ratio; 25 °C deposition temperature; 3 min deposition time) was used to purify the sample. As shown in Fig. 3A, the purity and recovery rate of TFs gradually increased (78.11 ± 2.22, 82.51 ± 1.13 and 84.13 ± 2.02%; 81.22 ± 1.73, 83.53 ± 1.47 and 85.73 ± 1.54%) as the content of TFs increased from 50.00 mg mL^{-1} to 70.00 mg mL^{-1}. However, when the concentration of TFs increased to 90 mg mL^{-1}, there was a decrease in the purity and recovery rate of TFs (85.23 ± 0.44%; 84.33 ± 1.42%). The phenomenon may be due to more TFs reached supersaturation as the increase of TFs concentration. The purity and recovery rate of TFs decreased after reaching the maximum value, which may be related to the precipitation of more impurity component content.

3.1.2 Effect of the antisolvent–solvent ratio. The effect of the volume ratio of antisolvent to solvent on the purity and recovery rate of TFs was verified. Under the conditions of different volume ratio of antisolvent to solvent (5 : 1, 10 : 1, 15 : 1, 20 : 1 and 25 : 1), the sample was purified at 80.00 mg mL^{-1} TFs concentration, 25 °C deposition temperature and 3 min deposition time. The results (Fig. 3B) displayed that the purity and recovery rate of TFs increased with the increase of volume ratio of antisolvent to solvent from 5 : 1 to 10 : 1. It could be attributed to supersaturation of LFs in the mixed system with the addition of antisolvent. However, the purity and recovery rate of the TFs did not change significantly when the antisolvent–solvent volume ratio increased from 10 : 1 to 25 : 1. It may be due to the substantial nucleation rate of TFs and the increased other components with the increasing antisolvent–solvent volume ratio. Nevertheless, constant increase of the antisolvent–solvent volume ratio can lead to the similar nucleation rate of the TFs and the other components.

3.1.3 Effect of the deposition temperature and deposition time. Under the conditions of different deposition temperature (20.00, 25.00, 30.00, 35.00 and 40.00 °C), the sample was purified with 80.00 mg mL^{-1} TFs concentration, 10 : 1 volume ratio of antisolvent to solvent and 3 min deposition time. The purity of TFs rose with the increase of deposition temperature, but the purity of TFs slightly decreased when the temperature exceed 25 °C (Fig. 3C). The reason is that the solubility of TFs decreases when the deposition temperature is low, making the saturated solubility of TFs in the solvent system larger and therefore easier to precipitate. Meanwhile, the precipitation of the impurity component causes a decrease in the purity of the TFs. With the gradual increase of the deposition temperature, the solubility of TFs increases, so the TFs are not easy to precipitate and thereby leading to a decreased recovery.

Under the conditions of different deposition time (1.00, 3.00, 5.00, 7.00 and 9.00 min), the sample was purified at 80.00 mg mL^{-1} TFs concentration, 10 : 1 volume ratio of antisolvent to solvent and 25 °C deposition temperature. The purity of TFs tended to decrease as the deposition time increased from 1 min to 5 min (Fig. 3D). The reason is that at the deposition time of 1 min, the TFs are more supersaturated than other impurity components, and the nucleation rate is higher than the other components. Over time, other impurities continuously precipitate to reduce the purity of TFs. There is no significant difference in the recovery of TFs with time changes, so time has little effect on the recovery of TFs.

3.2 Optimization of purification technology of TFs by BBD software

According to the univariate results, the deposition time has little effect on the purity and recovery rate of TFs. Therefore, TFs concentration, antisolvent to solvent volume ratio and deposition temperature were selected as independent variables for the BBD design. The interactions of various factors (the concentration of TFs: X_1, antisolvent–solvent ratio: X_2 and deposition temperature: X_3) in the quadratic polynomial model for the purification of TFs was further explored. The experimental design and experimental results are shown in Table 1. Experimental data were fitted to secondary multiple regression by Design-Expert 8.0.6 software. The results of analysis of variance for regression equation purity and recovery rate are given in Table 2 and Table 3. The "Lack of fit P value" for purity and recovery was 0.70 and 0.65 were both >0.05, respectively, revealed that the quadratic regression model fitted with the actual situation. The R^2 values for the TFs purity and recovery equations were 0.95 and 0.98, respectively, with the correlation coefficients both close to 1. The correction determination coefficient ${R_{adj}}^2$ was 0.90 and 0.96, respectively, suggesting that a good linear correlation between the independent variables. The quadratic polynomial equation of the correlation between the response variables and the test variables is as follows:

$$Y_{(\text{purity})} = 88.44 + 1.18X_1 + 1.21X_2 + 0.82X_3 + 0.33X_1X_2 + 0.012X_1X_3 - 0.16X_2X_3 - 2.28{X_1}^2 - 3.24{X_2}^2 - 1.91{X_3}^2$$

$$Y_{(\text{recovery rate})} = 87.69 + 1.09X_1 + 1.82X_2 + 1.51X_3 + 0.017X_1X_2 + 0.2X_1X_3 + 0.38X_2X_3 - 1.35{X_1}^2 - 2.03{X_2}^2 - 1.06{X_3}^2$$

The 3D surface maps obtained according to the above formula are shown in Fig. 4. With the TFs purified by LAP, the significant factors of the purity analysis were ranked as: antisolvent–solvent ratio > TFs concentration > deposition temperature, and that of the significant factors of the recovery rate analysis were ranked as: antisolvent–solvent ratio > deposition temperature > TFs concentration. The best conditions for TFs purification by LAP were predicted by BBD software: the TFs concentration of 83.00 mg mL^{-1}, antisolvent–solvent ratio of 11, and deposition temperature of 27 °C. Under the conditions of the point prediction, the purity and recovery rate of the final PFs were 88.93% and 88.53%, respectively. Three replicate experiments were performed according to the optimal purification conditions. The average purity and recovery rates of the PFs were 88.32% ± 2.54% and 88.08% ± 2.13%, respectively, indicating that the test results match the model well. The parameters of LAP purified TFs optimized by response surface analysis were more reliable and feasible.

3.3 The content of TF and main flavonoid compounds

The peak-out position of the main flavonoids was determined by comparing the HPLC chromatogram with the standard. Fig. 5 shows the HPLC profiles of reference standards, PF and CF. The peaks in Fig. 5A are the standard rutin, kaempferol and quercetin. The suitability tests of the HPLC method including linearity, reproducibility, precision, detection limit, recovery, and stability have been carried out before the extracts were determined. The limits of detection of rutin, kaempferol and quercetin were 0.95, 1.60, and 2.80 μg mL^{-1}, respectively. The linear regression equations of rutin, kaempferol and quercetin were $y = 18\,008.49x - 115.99$ ($R^2 = 0.999$), $y = 14\,244.22x - 5117.00$ ($R^2 = 0.999$), and $y = 12\,740.97x - 2888.90$ ($R^2 = 0.999$), respectively.

In previous studies, the actual yield and purity of total flavonoids obtained by microwave extraction were 1.45% and 55.31%, respectively.[30] The purification method of LAP method increased the purity of the total flavonoid up to 88.32% under the optimal process conditions (Table 4). In addition, rutin, kaempferol and quercetin increased at approximately 1.31 times, 1.87 times, and 1.91 times in PF, respectively.

The improved purity of the main flavonoids is because the flavonoids were firstly dissolved in methanol during the purification process. In addition, other impurities in methanol solution cannot be recrystallized when the solvent was added into deionized water.

3.4 Morphology analysis

As shown in Fig. 6, the morphological characteristics of CFs and PFs were examined by SEM. Fig. 6A showed the morphology of lyophilized CFs powder obtained from *Eucommia ulmoides* leaves treated by microwave extraction. The lyophilized CFs powder has a large particle size (approximately 0.9–21.0 μm) and presents irregular block particles. The morphology of the lyophilized PFs powder purified by the LAP method was shown in Fig. 6B. The particles are uniformly distributed spherical, with a particle size of about 500 nm. This result indicates that the PF particle size produced by the LAP method is smaller than the CF particle size, which is due to the slow recrystallization of total flavonoids after nucleation in the water–methanol system. The particle size distribution of the PF was shown in Fig. 6C, with an average particle size of 505.3 nm, and it is consistent with the SEM. TFs was purified by LAP method to obtain PFs powder with smaller particle size. Due to the TFs particle size change, smaller PFs particle sizes were expected to increase the degree of dispersion in the aqueous phase, thus contributing to its bioavailability.[39,40]

3.5 Effects of PFs on *in vitro* antioxidant activities

Antioxidants from natural sources have been shown to have protective functions of oxidative damage and are associated with decrease the risk of chronic diseases.[41] The conventional method for evaluating the free radical scavenging ability of antioxidants is UV/visible absorption spectrophotometer. The DPPH and ABTS can be purchased from merchants, easy to operate results credible. Therefore, DPPH and ABTS radical scavenging methods have been widely used to evaluate the radical scavenging activity of plant-derived antioxidant material samples.

The free radical scavenging activity of PFs and CFs *in vitro* were investigated by DPPH, ABTS, $^{\cdot}OH$, $O_2^{\cdot -}$ and FRAP methods

in this study. The positive control resistance as a reference showed as ascorbic acid (Vc) consistent with concentrations of BHT. Fig. 7A showed the scavenging activity of different samples to DPPH radicals at different concentrations. The ability of PFs to scavenge DPPH free radicals is closely related to its concentration. The concentrations of Vc, BHT, PFs and CFs scavenging inhibition DPPH 50% free radical activity (IC_{50}), as shown in Table 5, were 4.15 ± 0.06, 6.58 ± 0.11, 16.72 ± 1.07 and 19.13 ± 0.23, respectively. Among them, the IC_{50} value of PFs was lower than that of CFs and showed significant variability ($p < 0.05$). The reason is that the PFs obtained after purification by LAP method are more pure than the CFs, thus bringing with the enhanced free radical scavenging ability.

The ABTS analysis result is shown in Fig. 7B, and free RSA gradually decreased in the following order: ascorbic acid > BHT > PFs > CFs. The IC_{50} value of ABTS free radical scavenging ability of PFs and CFs were 10.76 ± 0.13 μg mL^{-1} and 13.12 ± 0.11 μg mL^{-1}, respectively. The IC_{50} values significantly change between PFs and CFs ($p < 0.05$).

$^{\bullet}OH$ is one of the strongest oxidants that can react unselectively with surrounding chemicals, including a variety of organic compounds and redox sensitive elements.[42] The scavenging activity of different concentrations of samples to $^{\bullet}OH$ were shown in Fig. 7C, where BHT is a positive control. The free radical scavenging activity of PFs and CFs on $^{\bullet}OH$ increased dose-dependently in the measured range from 50 μg mL^{-1} to 250 μg mL^{-1}. The IC_{50} value of $^{\bullet}OH$ free radical scavenging ability of PFs and CFs were 227.68 ± 18.23 μg mL^{-1} and 243.22 ± 8.625 μg mL^{-1}, respectively. In conclusion, the PFs showed better $^{\bullet}OH$ free radical scavenging activity compared with CFs.

Under physiological conditions, $O_2^{\bullet -}$ is the product of constant production during normal cell metabolism, but excessively accumulated concentrations will damage biofilm, tissue and organic systems.[43] Fig. 7D showed the radical scavenging activity of $O_2^{\bullet -}$ at different concentrations of PFs and CFs, with Vc as a positive control group. When the sample concentration was 300 μg mL^{-1}, the RSA of PFs, CFs and Vc were 47.32 ± 2.61%, 42.41 ± 2.33% and 77.93 ± 3.54%, respectively. Although the radical scavenging activity of PFs (IC_{50} value of 335.86 mg mL^{-1}) was lower than the control Vc (IC_{50} value of 164.27 mg mL^{-1}), the radical scavenging activity of PFs increased with the PFs concentration throughout the experimental concentration range. The $O_2^{\bullet -}$-radical scavenging activity of PFs was always higher than that of CFs, and the IC_{50} of PFs was significantly different from the IC_{50} of CFs ($p < 0.05$).

The FRAP has been used as an important assessment of the antioxidant capacity of natural antioxidants. In the FRAP assay, the absorbance of Vc, PFs, BHT and CFs increased with the sample concentration. As shown in Fig. 7E, ascorbic acid showed a significantly stronger antioxidant capacity than PFs, BHT, and CFs ($p < 0.05$), whereas there is no significant difference in antioxidant capacity among the PF, CFs and BHT sample groups ($p > 0.05$). The PFs, CFs and BHT had equal antioxidant capabilities in the FRAP assay. Meanwhile, the SRP value showed an increased concentration dependence along with the sample concentration.

3.6 Antioxidant enzymes activities and MDA levels in the liver and kidney *in vivo*

Reactive oxygen species (ROS) are produced by oxidative stress reactions in organisms at the core of aging-related diseases. The living organisms already has an antioxidant defense system that includes the presence of non-enzymatic antioxidants, such as GSH, uric acid, vitamin C, and enzymes such as SOD, CAT and GSH-Px. Free radicals are known to cause damage to the liver, and many antioxidants have proven to protect the liver against hepatotoxicants.[44] *In vivo* experiments with PFs containing 88.32% ± 2.54% flavonoids, including rutin, kaempferol and quercetin were 27.14% ± 1.14%, 1.51% ± 0.21% and 2.32% ± 0.24, respectively.

CCl_4 is a classical toxicant that causes oxidative stress mediated toxicity.[45] Therefore, CCl_4 was used to establish the oxidative stress model in the study. The antioxidant activity of PFs was investigated in rats. The *in vivo* antioxidant activity test results were shown in Table 6. Experimental results showed that SOD, CAT, and GSH-Px activities in the CCl_4-intoxicated group (Group II) was significantly reduced when compared to the blank control group (Group I) ($p < 0.05$) in the liver. Furthermore, the SOD, CAT, GSH and GSH-Px activities in the CCl_4-intoxicated group (Group II) was significantly decreased compared to the control group ($p < 0.05$) in the kidney. The MDA levels in the liver and kidney have also increased significantly. When the PFs (100, 200, and 400 mg kg^{-1} body weight) were given along with CCl_4, the PFs showed the ability to significantly increase the levels of SOD, CAT, GSH and GSH-Px ($p < 0.05$). Furthermore, the liver and kidney MDA levels decreased significantly as compared to the CCl_4-intoxicated group ($p < 0.05$). The reason is that PFs can restore the activity of these antioxidant enzymes/or activate the enzyme activity in the damage caused by CCl_4. There was no significant difference in enzyme activity in the three doses of PFs (100, 200 and 400 mg kg^{-1} body weight) and the vitamin E group (100 mg kg^{-1} body weight) at SOD, CAT, GSH and GSH-Px ($p > 0.05$). Moreover, the three dose groups of PFs (100, 200, and 400 mg kg^{-1} body weight) differ significantly from the blank control group at MDA levels in the liver ($p < 0.05$). Vitamin E (Group III) in the positive control group showed elevated SOD, CAT, and GSH-Px levels and a significant decrease in liver and kidney MDA levels ($p < 0.05$). In addition, we evaluated the potential adverse effects of PFs on rat liver and kidney by blood biochemical indicators. As shown in Fig. 8, compared to other two groups, we observed that 400 mg kg^{-1} PFs did not significantly alter serum levels of ALT, AST, BUN and CRE under a two-week administration, indicating that the current dose of PFs is safe for rat liver and kidney.

ROS is a metabolite of various cells and plays an important role in the pathogenesis of various serious diseases.[46] Recently natural antioxidants have been shown to have antioxidant or free radical removal activity.[47] Briefly, antioxidants provide protective effects by removing numerous ROS (including peroxyl radicals, hydroxyl radicals, superoxide anions and hypochlorite acids). In particular, the multiple antioxidant-related activities of flavonoids in cardiovascular diseases, anti-cancer, liver preservation, anti-bacterial, anti-viral and anti-

inflammatory have been widely described.[48] CCl_4 as a potent liver toxin as an important drug research substance is widely used in the study of liver disease caused by liver toxins. CCl_4 is metabolized *in vivo* as a $\cdot CCl_3$ free radical by cytochrome P450, further involved in oxidation to form trichloromethyl peroxide free radicals ($CCl_3O_2^{\cdot}$). $CCl_3O_2^{\cdot}$ radicals covalently bind to cellular macromolecules and biofilms, triggering lipid peroxidation, resulting in increased MDA levels, and reductions in SOD, CAT, GSH and GSH-Px.

In vivo studies in rats induced by carbon tetrachloride (CCl_4) showed that PFs can effectively improve antioxidant enzymes (SOD, CAT and GSH-Px) activities, reducing the substance of lipid peroxide reaction product (MDA) in the liver and kidney.

4. Conclusions

In conclusion, a new technology for purifying flavonoids from *Eucommia ulmoides* leaves by LAP was proposed in this study. The results based on the scavenging activity of DPPH, ABTS, and $O_2^{\cdot -}$ radicals showed that PFs have better scavenging ability than CFs. *In vivo* antioxidant studies showed that PFs purified by LAP method exhibited outstanding antioxidant activity in SD rats, which protected the liver and kidney from oxidative stress. Therefore, the LAP method may have great potential value and is expected to be a new technique for the purification *Eucommia ulmoides* leaves of total flavonoids.

Author contributions

Data curation, Yaya Peng; Formal analysis, Yaya Peng and Fei Luo; Investigation, Fei Luo; Methodology, Mingfang Wu; Project administration, Qian Zhang; Software, Qianli Zhuang; Supervision, Mingfang Wu; Writing–original draft, Qianli Zhuang and Umar Farooq; Writing–review & editing, Mingfang Wu.

Conflicts of interest

No potential conflict of interest was reported by the authors.

Acknowledgements

This work was supported by the University-Industry Collaborative Education Program (grant number: 202102242031) and Fundamental Research Funds for the Zhejiang University of Science and Technology (grant number: 2023QN030).

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