doi.org/10.36721/PJPS.2024.37.4.REG.881-890.1

Enrichment and purification of flavonoids from *Euphorbia hirta* L. and antioxidant activity evaluation

Xuexue Xue[1,2], Liyang Guo[1,2], Liwen Ai[1,2], Lulu Li[1,2], Weixue Liu[1,2], Peng Li[1]* and Zunlai Sheng[1,2]*

[1]College of Biological and Pharmaceutical Engineering, Jilin Agricultural Science and Technology University, Jilin, PR China
[2]College of Veterinary Medicine, Northeast Agricultural University, Harbin, PR China

Abstract: This study aimed to enrich flavonoids from *Euphorbia hirta* L. (*E. hirta*) extracts, and the enrichment parameters were optimized by adsorption and desorption tests. The HPD-300 resin was chosen after a comparison of the flavonoids from *E. hir15ta*'s adsorption and desorption capabilities on nine different types of macro porous resin. The optimal enrichment for purification of *E. hirta* extracts were determined as sample concentration of 3.0mg/mL, pH of 2.0 and a desorption solvent of 50% ethanol. The optimal dynamic parameters were loading 2.5 BV of sample at a feeding flow rate of 2 BV/h, cleaning the column with 5 BV of water and then eluting 50.0% ethanol at a 2 BV/h elution flow rate using 5 BV of eluent. Following a single treatment cycle with HPD-300 resin, the product's total flavonoid content rose from 6.32% to 28.8%, with an 80.01% recovery yield. Then, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-Azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) and hydroxyl radical scavenging ability were used to evaluate the antioxidant properties of the purified flavonoids. The main chemical components of purified flavonoids from *E. hirta* are astragalin, quercetin-3β-D-glucoside, 9,16-dioxo-10,12,14-octadeca-trienoic acid and gallic acid. The results showed that purified flavonoids from *E. hirta* had a strong antioxidant effect, which indicated that it represented a valuable natural antioxidant source.

Keywords: *Euphorbia Hirta* L., flavonoids, enrichment, macroporous resins, antioxidant capacity.

INTRODUCTION

The Euphorbiaceae family includes the small annual herb *Euphorbia Hirta L.* (*E. hirta*). It normally has an erect, creeping stem that is 80 cm tall (Basma *et al*., 2011). The plant has a rich, traditional crimson or purple color. The seeds are oblong, quadrangular, prismatic, somewhat folded and transversely grooved. It usually grows in lowland areas with disturbed farming and sandy soil, as well as in gardens, paddy fields and along roadsides. It prefers to grow in arid, sunny conditions. Typically, each plant only blooms once a year. Its distribution is primarily found in tropical and subtropical areas. It is commonly used as an old-fashioned herbal remedy to treat digestive, cutaneous and skin problems (Chandel *et al*., 2023). It can be available in a variety of ways depending on the setting. It is used as a soup and granule in China to cure a wide range of illnesses, such as diarrhea, asthma and stomach ulcers. It was previously used to treat hypertension in Australia. It can be consumed as a snack in Java and India. People in the Philippines generally use the full *E. hirta* to make tea.

E. hirta has numerous pharmacological effects, such as sedation (Lanhers *et al*., 1990), anti-inflammatory (dev Sharma *et al*., 2023), antibacterial (Iskandar *et al*., 2022), and anti-asthma (Parmar *et al*., 2018). Rich active ingredients include flavonoids, triterpenoids (Ragasa & Cornelio, 2013), inositol, and gallic acid (L. Zhang *et al*., 2021). In *E. hirta,* flavonoids are the main active ingredients. As of right now, while several research have been carried out to assess the biological activities of *E. hirta*, none of them have involved with the efficient purification of flavonoids from the plant.

Enriching active ingredients from plant extracts in advance is a crucial step in the manufacture of phytochemical-rich products. The purity of flavonoids has a positive correlation with their activity (Jiang *et al*., 2019). *E. hirta* must be purified using the proper technique following extraction. Research describes a number of techniques for enriching and separating active components, including liquid-liquid extraction (Jiang *et al*., 2023), ion exchange (Ngere *et al*., 2023) and expanded bed adsorption (Q. Li *et al*., 2023). These well-established techniques have a number of drawbacks, including limited recovery, low capacity, high cost, solvent waste, costly procedures, the need for specialized equipment, and environmental contamination. Among the existing methods for separating flavonoids, macro porous resin adsorption is a suitable choice due to its low cost, great adsorption performance, high reuse rate, and safety (Liu *et al*., 2020). The aim of this investigation was to determine the viability of purifying *E. hirta* using macro porous resin and for determining the necessary purification parameters (sample volume, pH, eluent

**Corresponding authors:* e-mails: lp@jlnku.edu.cn; shengzunlai@neau.edu.cn

concentration, washing volume, sample loading flow rate, elution flow rate).

It has been established that human illness and physiology are intimately linked to free radicals. A tiny quantity of free radicals has the potential to seriously harm people's health. According to the free radical theory, the majority of flavonoids have a multi-hydroxyl structure that allows them to respond to the many types of free radicals generated by the body's metabolism and limit the damage that free radicals can do to healthy human cells (Mustafa, 2023). Three techniques served to contrast the overall flavonoid content antioxidant properties from *E. hirta* beforehand and following purification in order to achieve this goal: 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-Azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS), and hydrogen radicals. This study is assumed to be the first to report the enrichment technology and production of total flavonoids from *E. Hirta*.

MATERIALS AND METHODS

Apparatus

The concentration of the samples was collected using a RE-52AA rotary evaporator (Shanghai Yarong Biochemistry Instrument Factory, China), whereas the analysis of total flavonoids was done using a UV-1700 spectrophotometer (Shimadzu Corporation, Japan).

Plant materials

E. hirta was collected in Sichuan province of China in September 2021. Heilongjiang University of Chinese Medicine Professor Junkai Wu identified and verified the plants. The sample was completely cleaned with deionized water, allowed to air dry until the humidity reached equilibrium, ground, and then put through a 40-mesh filter in preparation for additional testing. For reference, a specimen (voucher number. 20210901) was added to the herbarium of Northeast Agricultural University (Heilongjiang, China), the Department of Veterinary Medicine.

Reagents

We acquired nine macro porous resins from Nankai Hecheng S&T Co., Ltd. located in Tianjin, China. The physical and chemical characteristics of these macro porous resins were displayed in table 1. Prior to usage, each resin was immersed in 95% ethanol for a full day and then thoroughly cleaned to remove any remaining material. In Beijing, China, the Chinese Institute for the Control of Pharmaceutical and Biological Products is the source of rutin. Sigma (St. Louis, MO, USA) provided the ascorbic acid (VC), DPPH and ABTS. Hangzhou Reagent Company (Hangzhou, PR China) provided salicylic acid, hydrogen peroxide, potassium persulfate, ferrous sulfate, and ethanol. Additional analytical-grade chemicals were acquired from Heilongjiang Reagent Company (Heilongjiang, PR China) and utilized in this investigation.

Preparation of crude extraction

The *E. hirta* (2.0kg) was powdered (400-500μm) and refluxed with 70% ethanol at 80°C in a material-liquid ratio of 1:30 under reflux twice for 180min. The extraction was performed twice. 371.2g of crude extract were obtained by combining and condensing all filtrates using rotary evaporation at 60°C and lower pressure. To be employed in the ensuing macro porous resin separation, the crude extract was kept refrigerated. There were 6.32% flavonoids in the extracts. To obtain sample solutions, deionized water was added to crude extracts. The starting concentrations of the sample solutions were 3.0mg/mL.

Determination of total flavonoids content

With slight adjustments, an approach described by Kim *et al*. was used to estimate the total flavonoids concentration (Kim *et al*., 2003). In short, 1.0mL of the diluted sample solution was combined with 0.3mL of 5% $NaNO_2$ solution for six minutes. Following this, 0.3mL of 10% $AlCl_3$ solution was added and thoroughly mixed. 4.0mL of 4.0mol/L NaOH was added six minutes later. After the mixture was let to stand for 10 minutes without the sample serving as a control, a spectrophotometer was used to measure the absorbance at 511 nm in about the same mixture. The overall content of flavonoids was represented by a standard calibration curve composed of various rutin concentrations. The calibration curve's regression equation was as follows: y=5.7359x+0.0828 (R^2=0.9911), y represented the absorbance value and x represented the flavonoid concentration.

Selection of suitable resin for cleaning-up

The adsorption tests were determined by the modified methods of Toor *et al* (Toor & Jin, 2012). Nine different types of resins in table 1 were investigated to find the best suitable macroporous resin for enriching flavonoids from *E. hirta*. 30mL of crude *E. hirta* extracts (3.00 mg/mL) were added to each resin, weighing 1.0g (dry weight), and the flask was covered. The resins were then allowed to adsorb for 12h at 25°C while being shaken at 120 rpm. All resins were washed out with 5BV of distilled water after reaching adsorption equilibrium and then transferred to a fresh flask with 30mL of a 70% ethanol solution. The flask was then shaken (120 rpm) at 25°C for a duration of 12h to facilitate desorption. Using the following formulas, the adsorption capacities (Q_e), desorption capacities (Q_d), and desorption rate (D) of resins were calculated in order to assess their selectivity:

$$Q_e = (C_o - C_e) \times \frac{V_i}{W} \quad (1)$$

$$Q_d = \frac{C_d \times V_d}{W} \quad (2)$$

$$D = \frac{C_d \times V_d}{(C_o - C_e) \times V_i} \times 100\% \quad (3)$$

where C_o, C_e and V_i are the original, adsorption equilibrium amounts of total flavonoids in the sample solutions (mg/mL) and the volume of the original solution (mL), respectively; Q_e is the adsorption capacity (mg/g), and W is the resin's weight (g). The volume of the desorption solution (mL), the desorption capacity (mg/g), and the desorption concentration (mg/mL) are represented by the values V_d, Q_d, and C_d, Desorption ratio (%) is represented by *D*.

Effects of adsorption time and pH on the adsorption capacities

By mixing 50mL sample solutions (3.0 mg/mL) with pre-weighed volumes of hydrated resin (equivalent to 1 g dry HPD-300 resin) in 100mL flasks, the impact of adsorption time on the adsorption capacities was evaluated. The static adsorption curve was then obtained (Jiang *et al*., 2020). Subsequently, the flasks were subjected to a 12h shake at 25°C (120 rpm). Up to equilibrium, the concentrations of each flavonoid in the adsorption buffer were continuously observed. 1g (dry weight) of HPD-300 resin was mixed with 50mL of each sample solution in the pH range of 1.0 to 7.0 in order to investigate the impact of pH on the adsorption capabilities of total flavonoids.

Effects of ethanol concentration on the adsorption capacities

The resins were taken out of the solution and allowed to dry on filter paper following the establishment of the adsorption equilibrium. And then 30.0mL of various ethanol concentrations were employed for adsorption at 25°C.

Tests of dynamic adsorption and desorption

A glass column of 300 mm x 20 mm in diameter was used for the testing, and it was filled with 10.0g of dry weight HPD-300 resin. The loading amount was first determined using the dynamic breakthrough experiment. Following that, the impacts of the loading flow rate (1-5 BV/h), eluent volume and elution flow rate (1-5 BV/h) were examined. And the adsorption ratio was calculated as follows:

$$E = \frac{C_o - C_e}{C_o} \times 100\% \quad (4)$$

Where C_o and C_e stand for the total flavonoid concentrations (mg/mL) at the beginning and adsorption equilibrium in the sample solutions (mg/mL). *E* is the adsorption ratio (%).

Laboratory preparative-scale separation

Separation on a laboratory preparative scale can be carried out under the previously indicated ideal circumstances (Du *et al*., 2012). The 2.5 BV sample solution (3.0mg/mL) was added to a glass column (100.0 cm x 7.5cm i.d.) with 1.0kg of dry HPD-300 macro porous resin. The entire flavonoid-rich fraction was obtained by eluting the column with 5 BV of 50% ethanol after it had been cleaned with 5 BV of deionized water. About 2 BV/h was the elution flow rate, and 50% of the ethanol eluate was obtained and freeze-dried.

$$\text{Flavonoid recovery }\% = \frac{X_1 V_1}{X_2 V_2} \times 100\% \quad (5)$$

Where X_1 and X_2 are the concentration of flavonoids in the recovered solution and the sample solution (mg/mL). V_1 and V_2 are the volumes of the recovered liquid and the sample liquid (mL).

Antioxidant capacity in vitro

The sample's antioxidant capability was assessed using DPPH, ABTS and hydroxyl radicals both before and after purification. Because of its potent antioxidant properties, L-ascorbic acid was employed as a positive control. All sample solutions were diluted with 75% ethanol. Every experiment was operated in triplicate.

DPPH free radical assay

The scavenging capacity of the sample solution for DPPH radical refers to some methods reported (Lu *et al*., 2022; Zhang *et al*., 2019; M. Zhang *et al*., 2021) and modified. Completely blend 2.0mL of DPPH (0.2 mmol/L) solution with 2.0 mL of sample solutions at varying concentrations. The mixture's absorbance at 517nm was measured next 30 min reaction in darkness at the temperature of the room.

$$\text{Clearance rate}\% = [1 - \left(\frac{A_1 - A_2}{A_o}\right) \times 100]\ \% \quad (6)$$

Where A_1 was the absorbance of 2.0mL sample and 2.0mL DPPH mixture; A_2 was the absorbance of 2.0mL sample and 2.0mL 75% ethanol mixture; A_0 was the absorbance of 2.0mL DPPH and 2.0mL 75% ethanol mixture.

ABTS free radical assay

For the determination of the $ABTS^+$ clearance rate, the method of Re *et al*. (Re *et al*., 1999) was used and modified. Combine 2.45 mmol/l potassium persulfate and 7.00 mmol/L ABTS in equal amounts and let stand at room temperature for 16 hours without exposure to light. When in use, dilute ABTS reserve solution with 75% ethanol into the working solution so that its absorbance at 734nm is 0.800 ± 0.005. After 30 min of mixing 2.0 mL of the ABTS working solution with 2.0 mL of samples at various concentrations, the absorbance at 734 nm was measured, and the clearance rate was calculated.

$$\text{The clearance of ABTS }\% = [1 - \left(\frac{A_1 - A_2}{A_o}\right) \times 100]\% \quad (7)$$

Where A_1 and A_2 respectively represent the absorbance of 2.0mL of working solution, 2.0mL of 75% ethanol, and 2.0mL of the sample mixture. A_0 is the absorbance of 2.0 mL of ethanol and 2.0mL of the working solution mixture.

Hydroxyl radical assay

With minor adjustments, antioxidant capacity of total flavonoids to hydroxyl radicals is based on the

methodology of Zhou *et al*. (Zhou *et al*., 2018). In short, 1.0mL sample solution of different concentrations, 3.0 mL of 1.6 mmol/L salicylic acid-ethanol solution, 2.0mL of 1.6mmol/L ferrous sulfate solution and 2.0mL of distilled water were added in a test tube. Following that, added 2.0mL of 1.5mmol/L hydrogen peroxide (H_2O_2) to the above mixture and measured the absorbance at 510 nm.

The clearance of hydroxyl radical% $=[1-\left(\frac{A_1 - A_2}{A_o}\right)\times 100]\%$

Where A_0 is the absorbance of 75% ethanol instead of the sample, A_2 is the absorbance of 75% ethanol instead of H_2O_2 and A_1 is the absorbance of the sample combined with the reaction solution.

Liquid Chromatography-mass spectroscopy (LC-MS)

The 20μL sample was delivered into a C18 column (150mm×2.1mm, 1.7um). Water was used as the A phase, acetonitrile was used as the B phase, and both phases received additions of 0.2mmol/L ammonium acetate and 0.1% formic acid. The following is the gradient elution procedure: 10% B for 0-1.0min; 10% B for 1.0-9.0 min; 90% -100% B for 9.0-11.0 min; 100% B - 10% B for 11.0 -11.1 min and 10% B for 11.1-13.0 min. The column temperature was 30°C and the flow rate was 0.25 mL/min for 13 min.

The following were the source conditions for the mass analysis. During the MS/MS acquisitions, both positive and negative electrospray ionization types were employed for the mass range of 150 to 1500m/z. The nebulizer pressure was 40 psi, the drying gas flow was 12 L/min, and the drying gas temperature was 350°C. Agilent Mass Hunter workstations were used for data capture, and Mass Hunter Qualitative Analysis software was used for data processing. Additionally, automatic MS/MS low-energy collision dissociation (CID) at 5-8 eV collision energy was carried out when doing MS/MS investigations in the multiple reaction monitoring (MRM) mode. The process of identifying peaks involved analyzing data from literary and internet sources.

Ethics approval

The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

STATISTICAL ANALYSIS

All experiments were conducted three times, and the data are presented as mean values ± standard deviation (n=3). Mean values were considered statistically significant if $p < 0.05$. The statistical analysis for the antioxidant assay data was performed using one-way ANOVA (analysis of variance), followed by Tukey's post-hoc test. Data analysis was conducted using SPSS software, version 18.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Macro porous resins selection

Nine different types of macroporous resins with a range of physical characteristics were assessed in order to find the resin with optimal adsorption and desorption capabilities. Fig. 1a showed that the non-polar resins (HPD-300) demonstrated superior adsorption and desorption capabilities compared to other resins, which indicated that non-polar and substantial surface area resin was favored for the enrichment of *E. hirta* flavonoids (Ren *et al*., 2019). Thus, the HPD-300 was chosen as the model with the optimum adsorption and desorption capacity.

Effects of adsorption time and pH on the adsorption capacities

Fig. 1b illustrates that the adsorption capacity of the flavonoids in *E. hirta* increased as adsorption time increased, reaching equilibrium for HPD-300 at around 6 h. The adsorption capacity grew quickly in the first 4 hours, and after 4 hours, the slopes approached equilibrium at 6 hours, showing that the adsorption capacity fluctuated little over time.

The effect of the sample solution's initial pH on the adsorption capacity is shown in fig. 1c. It demonstrates that the adsorption capacity decreases as the pH of the original sample solution reduced. Particularly, the adsorption capacity drastically reduced when the pH was greater than 3. Therefore, a pH value of 2 was selected for the next step of testing.

Effects of ethanol concentration on the adsorption capacities

Fig. 1d displays the result of ethanol concentration on resolution. At ethanol concentrations of 50%, the desorption capacity was higher than in other concentrations.

Dynamic adsorption and desorption analysis

In this case, the solute concentration and effluent liquid volume were used for building the dynamic breakthrough curve for HPD-300 resin. Before 2.5 BV, the whole flavonoids in the solution were almost completely absorbed by the HPD-300 resin. To find a breakthrough point, the 10% proportion of the exit to the input solute concentration was commonly employed (Liu *et al*., 2023). The total breakthrough volume of flavonoids on HPD-300 resin, as per this standard, was 25 mL (2.5 BV). Fig. 2a showed that the number of total flavonoids in the leak solution increased rapidly until it plateaued at 30mL. Therefore, the loading amount for the subsequent experiments was 2.5-bed volumes (2.5 BV).

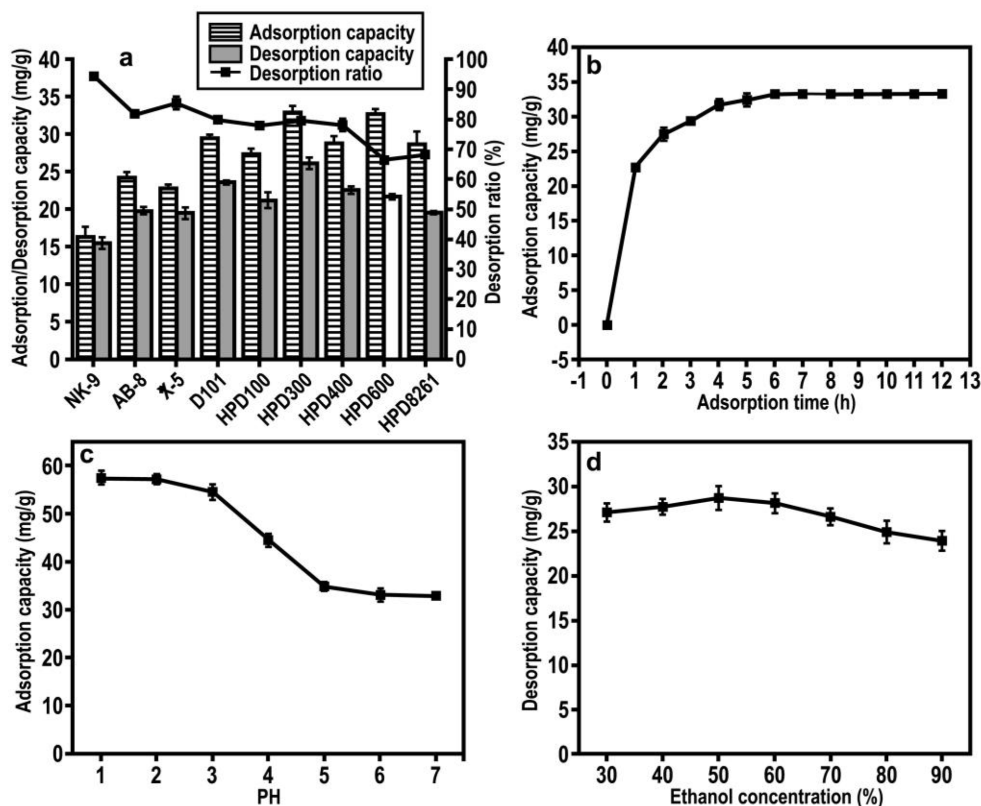

Fig. 1: Adsorption/desorption capacity and desorption ratio of total flavonoids on the nine different resins (a). Static adsorption kinetic curves (b) of HPD300. The effect of pH value on adsorption capacity (c) and ethanol concentration on analytical capacity (d).

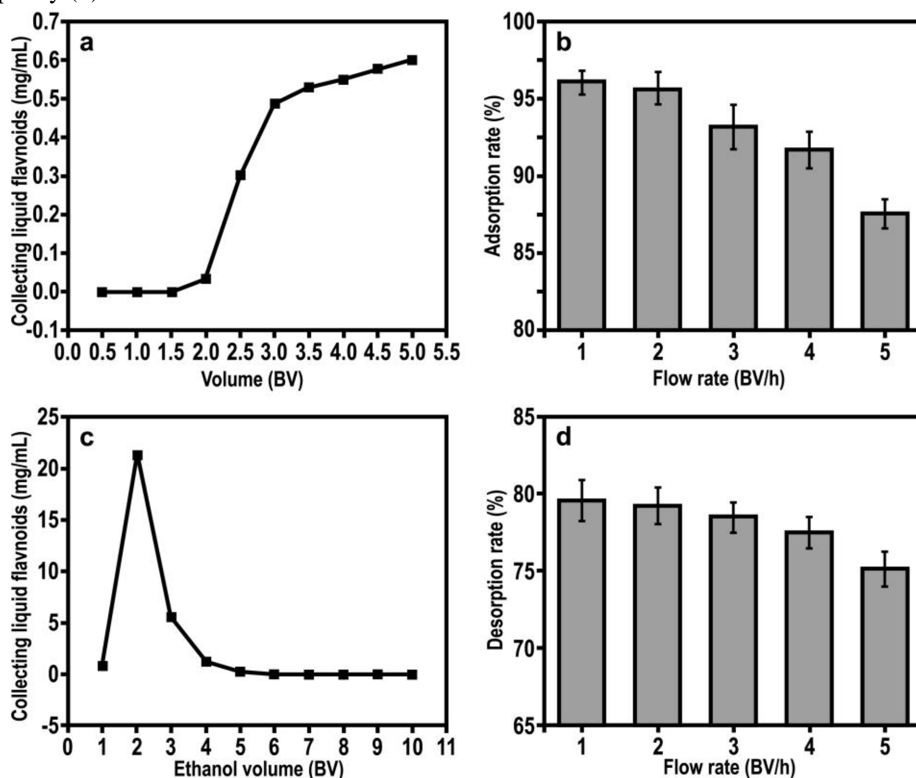

Fig. 2: Dynamic breakthrough curve (a), sample loading flow rate (b), desorption curve (c), and elution flow rate (d) of the total flavonoids on a column packed with HPD300 resin. Data represented mean ± SD (n=3).

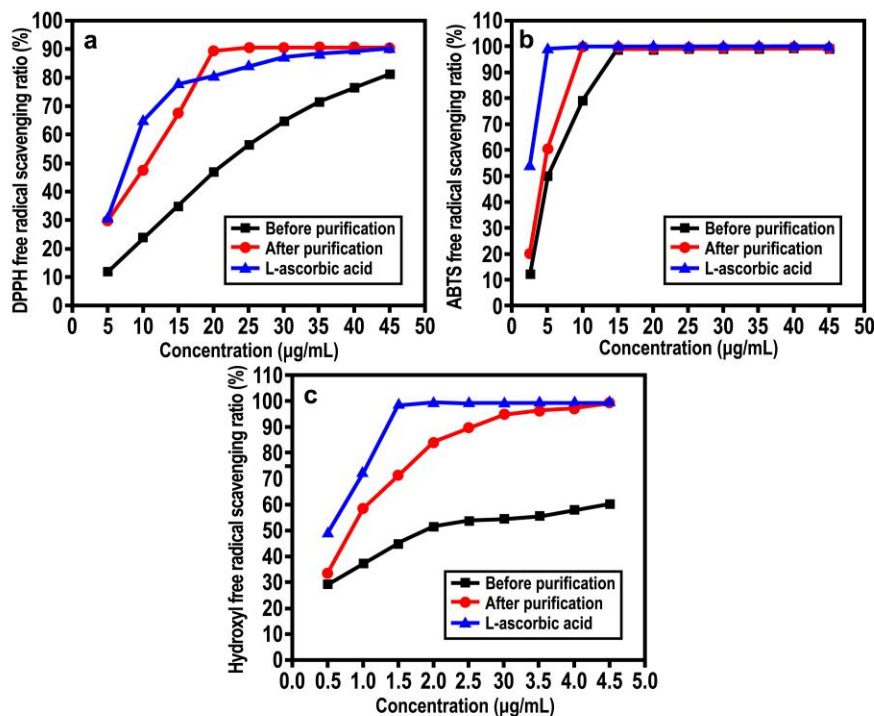

Fig. 3: Scavenging effects on DPPH radical (a), ABTS (b) and hydroxyl radical (c) of pre- and post-enrichment total flavonoids and ascorbic acid. Data represented mean ± SD (n=3).

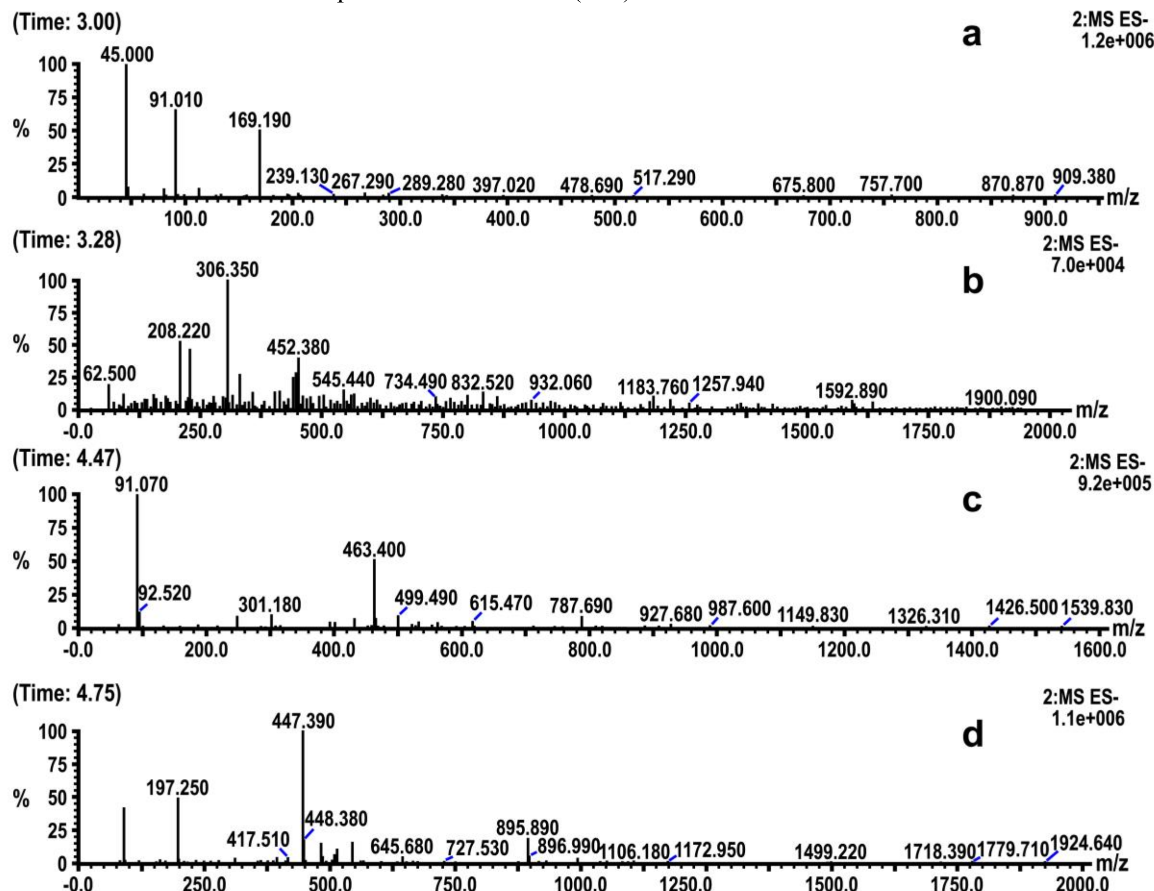

Fig. 4: Mass spectrogram of purified flavonoids from *E. hirta*: Astragalin (a), quercetin-3β-D-glucoside (b), 9,16-dioxo-10,12,14-octadeca-trienoic acid (c) and gallic acid (d).

Table 1: Specifications of the tested macroporous adsorption resins.

Resin	Particle size (mm)	Surface area (m2\g)	Average pore diameter (nm)	Polarity
NKA-9	0.3-1.25	170-250	15.5-16.5	Polar
HPD826	0.3-1.25	500-600	9.0-10.0	Polar
HPD-600	0.3-1.25	550-600	8.0-9.0	Polar
HPD-400	0.3-1.25	500-550	7.5-8.0	Middle polar
AB-8	0.3-1.25	450-530	13.0-14.0	Weak-polar
D101	0.3-1.25	600-700	10.0-12.0	Non-polar
HPD-300	0.3-1.25	800-870	5.0-5.5	Non-polar
X-5	0.3-1.25	500-600	21.0-23.0	Non-polar
HPD-100	0.3-1.25	650-700	8.5-9.0	Non-polar

Table 2: Comparison before and after enrichment

Process	Concentration of total flavonoids (%)	Recovery of the total flavonoids (%)	Purification fold
Crude extract	6.32±0.47	80.01±1.59	4.80
After purification	28.80±0.63		

Table 3: Identification of purified flavonoids from *E. hirta*

No	Compound name	Formula	R.T. (min)	Expected Mass(m/z)	Precursor type	Observed Mass(m/z)
1	Astragalin	$C_{21}H_{20}O_{11}$	4.75	448.37	$[M-H]^-$	447.39
2	Quercetin-3β-D-glucoside	$C_{21}H_{20}O_{12}$	4.47	464.38	$[M-H]^-$	463.4
3	9,16-dioxo-10,12,14-octadeca-trienoic acid	$C_{18}H_{26}O_{3}$	3.28	306.62	$[M-H]^-$	306.35
4	Gallic acid	$C_{7}H_{6}O_{5}$	3.00	170.12	$[M-H]^-$	169.19

Fig. 2b illustrated how the loading flow rate affected the adsorption ratio. The adsorption ratio decreased with the increase in flow rate, which was due to the shortening of the interaction time between flavonoids and resin surface active parts. Thus, the flow rate of loaded samples was chosen at 2 BV/h, taking efficiency into account.

Then, the resins were cleaned with 5 BV distilled water followed by a 50% ethanol solution. The eluent was collected in portions of 10mL for a total of 10 portions (fig. 2c). As a result, 50.0mL of eluent was needed to completely elute the flavonoid on the HPD-300 resin. The influence of elution flow rate on the desorption ratio was shown in fig. 2d. As the elution rate increased, the analytical amount also decreased. The final flow rate was calculated to be 2BV/h for efficiency. Thus, the following requirements for complete flavonoid separation and purification were established:

Adsorption conditions included 3.0 mg/mL of total flavonoids in the sample solution; pH 2.0; sample loading amount of 2.5 BV; flow rate of 2 BV/h. Desorption included deionized water 5 BV, then 50% ethanol 5 BV, flow rate 2 BV/h.

Laboratory preparative-scale separation

Perform laboratory preparation and separation based on the above experimental parameters to obtain a flavonoid recovery rate of 80.01±1.59%. Following enrichment, the product's total flavonoid content increased by 4.80 times to 28.80% ±0.63. This indicates that HPD-300 macro porous resin has an excellent purification effect on the total flavonoids of *E. hirta* and is suitable for separating flavonoids.

Antioxidant capacity

Half inhibitory concentration (IC50) is the amount of a sample required to scavenge 50% of DPPH, ABTS, and hydrophobicity free radicals. It is used as a measure to compare antioxidant activity. Fig. 3 showed that the sample content and capacity to scavenge free radical were related in a dose-dependent manner. In an experiment comparing the DPPH radical scavenging capacity (fig. 3a). At 45.00μg·mL^{-1} crude extract and purified, the scavenging ratios were 81.40% and 90.70%, respectively. L-ascorbic acid, crude extract and purified extract had IC_{50} values of 22.15, 10.30 and 7.80μg·mL^{-1}, respectively. The IC_{50} values for L-ascorbic acid, crude extract, and purified extract were 2.26μg·mL^{-1}, 5.03 and 4.34 μg·mL^{-1} in fig. 3b. Every test reagent had an ABTS scavenging ratio above 99.0%, ranging from 15.00 to 45.00μg·mL^{-1}. With an IC^{50} value of 0.831mg·mL^{-1}, the antioxidant capacity of pure flavonoids increased from 33.5% to 99.0% when the quantity of flavonoids raised from 0.5 to 4.50 mg·mL^{-1}. At 4.5mg·mL^{-1}, the crude

extract's hydroxyl radical scavenging ratio was 60.4%, and its IC_{50} value was 1.86 mg·mL^{-1} (fig. 3c).

Identification of purified flavonoids from E. hirta

The chemical components of *E. hirta* purified solution were qualitatively identified through LC-MS/MS analysis performed in negative ionization modalities. fig. 4 shows the base peak chromatogram (negative ionization mode). The compounds underwent analysis to determine their retention times, UV-vis absorption spectrum, mass spectrum acquired using MS-ESI, fragmentation profile, and comparison with prior literature reports (Abu Bakar *et al*., 2020; Zhao *et al*., 2023). Based on the LC-MS/MS analysis results, four chemical constituents were tentatively assigned using the negative ionization mode: astragalin ($[M-H]^-$ ion at m/z 447.39), quercetin-3β-D-glucoside ($[M-H]^-$ ion at m/z 463.4), 9,16-dioxo-10,12,14-octadeca-trienoic acid ($[M-H]^-$ ion at m/z 306.35), and gallic acid ($[M-H]^-$ ion at m/z 169.19) as shown in table 3.

DISCUSSION

The *E. hirta* extract's total flavonoids were enhanced using macro porous resin chromatography. Both non-polar and polar resins are appropriate for the adsorption of flavonoids because flavonoids include both polar polyhydroxy groups and non-polar phenyl groups (Wan *et al*., 2014). Therefore, nine resins with varying polarities were selected for screening. PH has an impact on solute ionization, which can alter the solute and solution adsorption affinity (R. Li *et al*., 2023). As a result, the processes of adsorption and desorption are greatly influenced by pH. Similar findings were made by Fu *et al* (Fu *et al*., 2007), who discovered that the pH's effect on vitexin and isovitexin adsorption was reduced. These sudden decreases in adsorption capacity are the result of the dissociation of the phenolic hydroxyl groups in flavonoids, which produced H^+ and their related anions with an increasing pH. Based on these results, the sample pH of 2 was selected for both adsorption and desorption. The molecular force that holds the resin and all the flavonoids together can be weakened by ethanol. A decrease in the desorption capability could occur from some alcohol-soluble contaminants dissolving in a high concentration of ethanol when the concentration of ethanol above 50% (Hou *et al*., 2020; Xi *et al*., 2015).

Tsaia *et al*.(Tsai & Lin, 2019) found that the antioxidant properties of flavonoids from *Glycyrrhiza glabra L.* leaf increased by two to three times after purification by macro porous resin. According to Wan *et al*. (Wan *et al*., 2014), the purity of flavonoids derived from *Flos Populi* plants was enhanced by 3.17 times and ultimately reached 81.62% following purification using macroporous resin. HPD300 macro porous resin was employed for enrichment in this investigation. With a purity of 80.01%, the content of flavonoids in *E. hirta* was enhanced by 4.80 times. The outcomes demonstrated that enriching and purifying flavonoids in raw materials may be accomplished using macro porous resin.

The reaction between the antioxidant and the DPPH free radical was realized by electron transfer or accepting hydrogen atoms, and the methods of ABTS and hydroxyl radical were based on electron transfer (Ge *et al*., 2021). As organic antioxidants, flavonoids can give free radicals hydrogen atoms and change into more stable phenolic radicals to stop lipids from oxidizing further (Apak, 2019). Oancea *et al*. (Oancea *et al*., 2020) found that 81% of the peony crude extract was cleared of DPPH free radical. The clearance matched the findings of this investigation. After enrichment, the clearance rate is 90.70%, a considerable improvement over the crude extract. As the quantity of pure flavonoids increases, their antioxidant ability surpasses that of ascorbic acid, indicating that purified flavonoids have significant scavenging capacity on DPPH radicals. Due to the hydrophobicity of DPPH, water-soluble ABTS was utilized to assess the antioxidant potential of hydrophilic compounds (Chen *et al*., 2019). Within the experimental concentration range, all groups demonstrated strong ABTS free radical scavenging capabilities. Purified flavonoids have a considerable ability to scavenge ABTS radicals, as seen by their reduced IC50 value when compared to crude extracts. According to Zhao *et al*. (Zhao *et al*., 2013), hydroxyl radicals are extremely harmful to the body and hazardous to cells. Examining the samples' ability to scavenge hydroxyl radicals was crucial. Ascorbic acid, purified flavonoids, and crude extract all have hydroxyl radical-scavenging properties that increase with concentration. Ascorbic acid and pure flavonoids hydroxyl scavenging rates are nearly equal up to a concentration of 4.50mg/mL. Compared to crude extract, purified flavonoids shown a greater ability to scavenge hydroxyl radicals. The findings demonstrate that pure flavonoids had a discernible ability to scavenge hydroxyl radicals.

CONCLUSION

This work provided a gentle technique for the effective purification of *E. hirta*. The suitable macro porous resin in this investigation for enriching total flavonoids from *E. hirta* extracts was the HPD-300 resin. The optimum conditions for HPD-300 resin column enrichment were as follows: For adsorption, the concentration, flow rate, and volume of the sample were 3 mg/mL (PH value of 2), 2 BV/h and 2.5 BV, respectively; for adsorption, 5 BV of 50% ethanol was used to desorb the flavonoids-loaded HPD-300 resin column at a rate of 2 BV/h. After enrichment, the content of flavonoids increased by up to 4.8-fold from 6.32% ± 0.47 to 28.80% ± 0.63 with a recovery yield of 80.01% ± 1.59. The vitro antioxidant properties of the purified flavonoids were assessed, encompassing their capacity to scavenge

hydroxyl, DPPH and ABTS radicals. When compared to the crude extract from *E. hirta*, the purified flavonoids showed higher antioxidant activity. Further investigation in the purified extract of *E. hirta* using LC-MS analysis revealed the presence of astragalin, quercetin-3β-D-glucoside, 9,16-dioxo-10,12,14-octadeca-trienoic acid and gallic acid. The findings can serve as a theoretical foundation for the full use of *E. hirta* and the flavonoid extraction as a possible antioxidant source.

Confidentiality of data

The authors declare that no patient data appears in this article.

ACKNOWLEDGMENTS

This research was supported by Key Subject of Biology operated by Jilin Agricultural Science and Technology University.

REFERENCES

Abu Bakar FI, Abu Bakar MF, Abdullah N, Endrini S and Fatmawati S (2020). Optimization of extraction conditions of phytochemical compounds and anti-gout activity of *Euphorbia hirta* L. (Ara Tanah) using response surface methodology and liquid chromatography-mass spectrometry (LC-MS) analysis. *Evid-Based. Compl. Alt.*, **2020***:* 4501261.

Apak R (2019). Current issues in antioxidant measurement. *J. Agric. Food Chem.*, **67**(33): 9187-9202.

Basma AA, Zakaria Z, Latha LY and Sasidharan S (2011). Antioxidant activity and phytochemical screening of the methanol extracts of *Euphorbia hirta* L. *Asian. Pac. J. Trop. Med.*, **4**(5): 386-390.

Chandel S, Das S, Mazumder A and Chauhan VS (2023). An overview on phytoconstituents and multiple biological activities of *Euphorbia hirta*. *Plant Sci. Today*, **10**(3): 281-299.

Chen CC, Lin C, Chen MH and Chiang PY (2019). Stability and quality of anthocyanin in purple sweet potato extracts. *Foods*, **8**(9): 393.

Dev Sharma A, Kaur I, Chuhan A, Kumar S and Singh N (2023). Phytochemical profile, *in-vitro* antioxidant, anti-diabetic and anti-inflammatory activities of traditionally used *Euphorbia hirta* (L.) growing under wild conditions of Northern Punjab. *Drug Anal. Res*, **7**(1): 27-40.

Du Z, Wang K, Tao Y, Chen L and Qiu F (2012). Purification of baicalin and wogonoside from *Scutellaria baicalensis* extracts by macroporous resin adsorption chromatography. *J. Chromatog. B*, **908**: 143-149.

Fu Y, Zu Y, Liu W, Hou C, Chen L, Li S, Shi X and Tong M (2007). Preparative separation of vitexin and isovitexin from pigeonpea extracts with macroporous resins. *J. Chromatogr. A*, **1139**(2): 206-213.

Ge X, Jing L, Zhao K, Su C, Zhang B, Zhang Q, Han L, Yu X and Li W (2021). The phenolic compounds profile, quantitative analysis and antioxidant activity of four naked barley grains with different color. *Food Chem*, **335**: 127655.

Hou M, Hu W, Xiu Z, Shi Y, Hao K, Cao D, Guan Y and Yin H (2020). Efficient enrichment of total flavonoids from *Pteris ensiformis* Burm. extracts by macroporous adsorption resins and in vitro evaluation of antioxidant and antiproliferative activities. *J. Chromatogr. B*, **1138**: 121960.

Iskandar B, Lukman A, Syaputra S, Al-Abrori UNH, Surboyo MDC and Lee CK (2022). Formulation, characteristics and anti-bacterial effects of *Euphorbia hirta* L. mouthwash. *J Taibah Univ Med Sci*, **17**(2): 271-282.

Jiang H, Li J, Chen L and Wang Z (2020). Adsorption and desorption of chlorogenic acid by macroporous adsorbent resins during extraction of *Eucommia ulmoides* leaves. *Ind. Crop. Prod.*, **149**: 112336.

Jiang H, Yang S, Tian H and Sun B (2023). Research progress in the use of liquid-liquid extraction for food flavour analysis. *Trends Food Sci. Technol.*, **132**: 138-149.

Jiang J, Yan L, Shi Z, Wang L, Shan L and Efferth T (2019). Hepatoprotective and anti-inflammatory effects of total flavonoids of Qu Zhi Ke (peel of *Citrus changshan-huyou*) on non-alcoholic fatty liver disease in rats via modulation of NF-κB and MAPKs. *Phytomedicine*, **64**: 153082.

Kim DO, Jeong SW and Lee CY (2003). Antioxidant capacity of phenolic phytochemicals from various cultivars of plums. *Food Chem.*, **81**(3): 321-326.

Lanhers MC, Fleurentin J, Cabalion P, Rolland A, Dorfman P, Misslin R and Pelt JM (1990). Behavioral effects of *Euphorbia hirta* L.: sedative and anxiolytic properties. *J. Ethnopharmacol.*, **29**(2): 189-198.

Li Q, Peng L, Shi J, Chu J, Zhang X and Shi Q (2023). Valorization of lotus leaf by *in situ* expanded- bed adsorption and ultrasonic- assisted desorption. *Chem. Eng. Technol.*, **46**(4): 803-812.

Li R, Xia Z, Tian Y, Guan M, Zheng Y, Bin L, Dong J, Jiang Q, Du L and Li M (2023). Purification of total flavonoids from *Ginkgo biloba* flowers with resin column chromatography and evaluation of antioxidant activities *in vitro*. *Prep. Biochem. Biotech*., **53**(3): 308-316.

Liu B, Liu J, Huang D, Wei J and Di D (2020). Boric acid modified macroporous adsorption resin and its adsorption properties for catechol compounds. *Colloid. Surface A*, **595**: 124674.

Liu X, Yan S, Zhou H, Wu H, Wang S, Yong X and Zhou J (2023). Separation and purification of glabridin from a deep eutectic solvent extract of *Glycyrrhiza glabra*

residue by macroporous resin and its mechanism. *Sep. Purif. Technol.*, **315**: 123731.

Lu J, Huang Z, Liu Y, Wang H, Qiu M, Qu Y and Yuan, W (2022). The optimization of extraction process, antioxidant, whitening and antibacterial effects of fengdan peony flavonoids. *Molecules*, **27**(2): 506.

Mustafa YF (2023). Harmful free radicals in aging: A narrative review of their detrimental effects on health. *Indian J. Clin. Biochem.*, **39**: 154-167.

Ngere JB, Ebrahimi KH, Williams R, Pires E, Walsby-Tickle J and McCullagh JS (2023). Ion-exchange chromatography coupled to mass spectrometry in life science, environmental, and medical research. *Anal. Chem.*, **95**(1): 152-166.

Oancea S, Perju M and Olosutean H (2020). Influence of enzyme-aided extraction and ultrasonication on the phenolics content and antioxidant activity of *Paeonia officinalis* L. petals. *J. Serb. Chem. Soc.*, **85**(7): 845-856.

Parmar G, Pundarikakshudu K, Balaraman R and Sailor G (2018). Amelioration of anaphylaxis, mast cell degranulation and bronchospasm by *Euphorbia hirta* L. extracts in experimental animals. *Beni-Suef. U. J. Basic*, **7**(1): 127-134.

Ragasa CY and Cornelio KB (2013). Triterpenes from *Euphorbia hirta* and their cytotoxicity. *Chin. J. Nat. Medicines*, **11**(5): 528-533.

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M and Rice-Evans C (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic. Biol. Med.*, **26**(9-10): 1231-1237.

Ren J, Liao L, Shang S, Zheng Y, Sha W and Yuan E (2019). Purification, characterization, and bioactivities of polyphenols from *Platycladus orientalis* (L.) Franco. *J. Food Sci.*, **84**(3): 667-677.

Toor M and Jin B (2012). Adsorption characteristics, isotherm, kinetics and diffusion of modified natural bentonite for removing diazo dye. *Chem. Eng. J.*, **187**: 79-88.

Tsai C and Lin L (2019). Camellia DPPH scavenging capacity of extracts from seed dregs using polyol compounds as solvents. *Heliyon*, **5**(8): e02315.

Wan P, Sheng Z, Han Q, Zhao Y, Cheng G and Li Y (2014). Enrichment and purification of total flavonoids from *Flos populi* extracts with macroporous resins and evaluation of antioxidant activities *in vitro*. *J. Chromatogr. B,* pp.945-946: 68-74.

Xi L, Mu T and Sun H (2015). Preparative purification of polyphenols from sweet potato (*Ipomoea batatas* L.) leaves by AB-8 macroporous resins. *Food Chem.*, **172**: 166-174.

Zhang L, Jiang Y, Pang X, Hua P, Gao X, Li Q and Li Z (2019). Simultaneous optimization of ultrasound-assisted extraction for flavonoids and antioxidant activity of *Angelica keiskei* using response surface methodology (RSM). *Molecules*, **24**(19): 3461.

Zhang L, Wang XL, Wang B, Zhang LT, Gao HM, Shen T, Lou HX, Ren DM and Wang XN (2021). Lignans from *Euphorbia hirta* L. *Nat. Prod. Res.*, **36**(1): 26-36.

Zhang M, Ma W, Wang C, Yang X, Lou Y, Xia X and Xu H (2021). Optimization of enzyme-assisted extraction and purification of flavonoids from *Pinus koraiensis* nut-coated film and antioxidant activity evaluation. *Molecules*, **26**(7): 1950.

Zhao X, Li B, Wang J, Zhu L and Zhang G (2023). Advances in chemical components, pharmacological activities and clinical applications of *Euphorbia hirta* L. and *Euphorbia thymifolia* L. *J. Shenyang Pharm. Sci.*, **40**(03): 381-393.

Zhao Z, Xu X, Ye Q and Dong L (2013). Ultrasound extraction optimization of *Acanthopanax senticosus* polysaccharides and its antioxidant activity. *Int. J. Biol. Macromol.*, **59**: 290-294.

Zhou J, Zhang L, Li Q, Jin W, Chen W, Han J and Zhang Y (2018). Simultaneous optimization for ultrasound-assisted extraction and antioxidant activity of flavonoids from sophora flavescens using response surface methodology. *Molecules*, **24**(1): 112.