Ultrasound-assisted extraction, optimization, isolation, and antioxidant activity analysis of flavonoids from *Astragalus membranaceus* stems and leaves

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**A R T I C L E   I N F O**

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- *Astragalus membranaceus* stems and leaves
- Flavonoids
- Anti-fungal and antioxidant activity
- Ultrasound-assisted extraction and optimization
- Activity-guided isolation
- Molecular docking

**A B S T R A C T**

*Astragalus membranaceus* is a medicinal and edible species in China, with a variety of biological activities. This study evaluated the reuse potential of *A. membranaceus* waste as a source of food antioxidants. Antioxidant and anti-fungal activities of flavonoids, polysaccharides, and saponins from *A. membranaceus* stems and leaves were evaluated. Results showed that inhibition rate of flavonoids on six tested fungi reaches 100% at a concentration of 5 mg/mL, and the antioxidant test demonstrated satisfactory antioxidant activity. On this basis, an extremely economical ultrasonic-assisted extraction of flavonoids from *A. membranaceus* stems and leaves was developed and optimized via response surface methodology (RSM). Optimized conditions included an extraction time of 35 min, ethanol concentration of 75%, liquid–solid ratio of 40 mL/g, and extraction temperature of 58 °C, in which the extraction yield of flavonoids was 22.0270 ± 2.5739 mg/g. The total flavonoids were separated and purified using activity-guided isolation technology, and frac. ccd with strong antioxidant activity were analyzed via HPLC-MS/MS. Results showed that main components are isoquercitrin and astragalin. This study can provide a potential innovative application for the development of natural food antioxidants from *A. membranaceus* waste.

**1. Introduction**

Mycoxin contamination and oxidative rancidity are important causes of food deterioration. According to the United Nations Food and Agriculture Organization (FAO), about 25% of the world’s grains are contaminated with mycotoxins to varying degrees every year [1,2], more than 50 million tons of grains are inedible every year, and direct economic losses reach $140 billion [3]. A variety of poisonous symptoms can be caused by human or animal ingestion of agricultural and livestock products contaminated with mycotoxins as well as inhalation of and skin contact with mycotoxins [4–7]. At the same time, harmful peroxides and free radicals produced in the process of rancidity can cause serious harm to human health, such as increased incidence of inflammation, aging, allergic reactions, and cancer. Antioxidants are often added during food processing, storage, and preservation to achieve long shelf life [8]. At present, synthetic antioxidants, such as butylated hydroxy anisole (BHA) and butylated hydroxy toluene (BHT), are widely used in food production and processing. However, studies have shown that the widespread use of synthetic antioxidants may cause liver damage and cancer [9]. Therefore, natural food antioxidants with high safety, strong antibacterial ability, and absence of side effects have attracted considerable research attention. Many plant extracts have been extensively explored due to their natural antibacterial and antioxidant activities [10–12].

Flavonoids are a type of secondary metabolites created by plants, vegetables, and fruits that consist of a series of compounds formed by two benzene rings with phenolic hydroxyl groups connected by a central three-carbon chains. More than 8,000 flavonoids have been identified [13]. Flavonoids can be classified into flavonoids, flavonols, chalcones, isoflavones, dihydroflavonoids, dihydroflavonols, and others according to characteristics of the linkage location of the B ring, oxidation extent of the central three-carbon chain, and formation of the ring [14]. Flavonoids play an important role in the anti-inflammatory [15], -cancer [16], -viral [17,18], -diabetic [19], and -oxidant properties of many plants.

*Astragalus membranaceus* is a perennial leguminous herb and its root is rich in active substances, such as flavonoids,...
saponins, and polysaccharides, and presents immunomodulatory
[20,21], hypoglycemic [22], anti-inflammatory [23], antioxidant
[24,25], and antiviral activities. On this basis, the above-ground part of
the plant is discarded. However, the treatment of A. membranaceus waste
has become a serious environmental problem due to the large amount
used every year. A. membranaceus stems and leaves are rich in active
substances [26,27]. Hence, recycling strategies may be adopted to
promote the potential innovative application of A. membranaceus hy-
products and develop and utilize of A. membranaceus resources.

Ultrasonic-assisted extraction (UAE) can improve extraction effi-
ciency by combining ultrasonication with traditional solvent extraction.
Compared with other extraction methods, UAE presents the advantages
of high yield, low cost, simple operation, and high efficiency [28] pri-
marily because ultrasonic treatment can produce cavitation effects and
destroy cell walls to promote solute diffusion and increase mass transfer
rate of target compounds from the solid phase to the liquid phase
[29,30]. The ultrasonic method can remarkably reduce the extraction
time and accelerate the extraction rate of active ingredients and the
utilization rate of raw materials [31]. Thus, UAE has been widely used to
extract chemical components from Chinese herbal medicines [32].

This study evaluates the biological activity of A. membranaceus stems
and leaves for application as a natural source of food antioxidant to
courage recycling and reduce the impact of waste treatment on the
environment.

2. Materials and methods

2.1. Materials and reagents

A. membranaceus stems and leaves were collected from the planting
base of A. membranaceus in Fangshan County, Shanxi Province, China.
Samples were dried and ground into pieces with a diameter of 0.2–0.4
mm. Potato dextrose (PDA) and malt extract (MEA) agars were pur-
chased from Solarbio (Beijing, China). All other chemicals and solvents
used every year. A. membranaceus stems and leaves were extracted under the conditions of 75 % ethanol, liquid
–solid ratio of 20 mL/g, and ultrasonic power of 100 W for 1 h to determine the total
flavonoids and saponins. This process was repeated twice. The crude
extract was dissolved in ddH₂O after rotary evaporation and extracted
with a system solvent to obtain petroleum ether (PEF), ethyl acetate
(EAF), n-butanol (BF), and aqueous (AF) fraction. EAF was isolated and
purified using AB-8 macroporous resin and different ethanol concen-
trations (0 %, 30 %, 50 %, 70 %, and 95 %). The 50 % ethanol elution
material was total flavonoids. BF was isolated and purified using D101
macroporous resin and different ethanol concentrations (0 %, 30 %, 50
%, 70 %, and 95 %). The 50 % ethanol elution material presents total
flavonoids (0.122 g).

Extraction of polysaccharides from A. membranaceus stems and
leaves was carried out using water-extraction and alcohol-precipitation
methods. Dried powder (5 g) of A. membranaceus stems and leaves was
extracted with distilled water for 2 h. The extract was filtered and
concentrated to 20 mL, added with four times the volume of absolute
ethanol, and placed in a refrigerator for overnight precipitation to obtain
the crude polysaccharide (0.657 g).

Flavonoids, saponins, and polysaccharides were dissolved into 100.0
mg/mL of stock solution with absolute ethanol and sterile water and
then diluted to 50×, 25×, 12.5×, and 6.25× via double dilution method
[34].

2.3. Tested fungi

The test strains Aspergillus flavus, Penicillium citrinum, Aspergillus
niger, Fusarium moniliforme, Rhizopus oryzae, and Trichoderma harzianum
used in this study were food-borne pathogens. All fungi were cultured on the
corresponding medium at 28 °C for 48 h and sub-cultured three
times. PDA was the suitable medium for P. citrinum, A. niger, T. harzia-
um, and F. moniliforme, and MEA was the suitable medium for A. flavus,
and R. oryzae.

Anti-fungal activities of extracts were examined using mycelial
growth rate method [35,36]. Different extracts were mixed with a PDA/
MEA medium to obtain solid plates at concentrations of 10, 5, 2.5, 1.25,
0.625, and 0.3125 mg/mL. The PDA/MEA medium was the positive
control, and ketoconazole was the negative control. Colony diameter
(OD) was calculated after the fungus cake was incubated at 28 °C for 48
h. The inhibition rate was calculated as the follows formula:

\[
\text{Inhibition rate (\%) = \left(1 - \frac{\text{OD}_{\text{control}} - \text{OD}_{\text{sample}}}{\text{OD}_{\text{control}}} \right) \times 100%
\]

Half-maximal effective concentration (EC₅₀) of extracts and toxicity
regression equation were calculated according to the biometric-value
conversion chance table [36,37].

2.4. Antioxidant activity

Free radical scavenging activity of A. membranaceus stems and leaves
extracts (polysaccharide, flavonoids, and saponins) was expressed as the
inhibition rate of DPPH and ABTS⁺ free radical formation [38]. Reducing power was carried out according to the method of Fan et al.
[39]. Regression equations were determined using the concentration,
and inhibition rate and IC₅₀ values were calculated. VC was used as the
positive control.

2.5. Optimization of extraction process of total flavonoids from stems and
leaves of A. Membranaceus

2.5.1. Ethanol extraction and ultrasonic-assisted ethanol extraction

Pre-treated powder of A. membranaceus stems and leaves was used to
extract flavonoids using the ethanol extraction method. Extraction
conditions were 50 min of extraction time, extraction temperature of
60 °C, liquid-solid ratio of 20 mL/g, and 70 % ethanol concentration.
The ultrasonic-assisted condition of adding 100 W of ultrasonic power
was based on ethanol extraction, and yield of extracted flavonoids (Y)
was determined as the response. The extraction yield (%) was calculated
according to the mass of the extract (m, g) and the powder used (M, g) as
follows:

\[
\text{Extraction yield (\%) = \frac{m}{M} \times 100\%
\]

2.5.2. Ultrasound-assisted extraction single-factor experiments

Effects of four factors on ultrasonic-assisted extraction were evalu-
at to improve the extraction yield of A. membranaceus flavonoids. Four
independent variables were extraction time (30, 50, 70, and 90 min),
extraction temperature (40 °C, 50 °C, 60 °C, 70 °C, and 80 °C), liquid
–solid ratio (10, 20, 30, and 40 mL/g), and ethanol concentrations (50
%, 60 %, 70 %, 80 %, and 90 %).

2.5.3. Response surface experimental design and statistical analysis

RSM was used to estimate the influence of independent variables,
including liquid–solid ratio (X₁), extraction temperature (X₂), extraction
time (X₃), and ethanol concentration (X₄), on the extraction rate
of flavonoids (mg/g). Box-Behnken design (BBD) with the software pack-
age Design-Expert 8.0.6.1 (Minneapolis, USA) was employed in
designing experimental data. Experiments were randomized to mini-
mize the effects of unexplained variability in the observed responses due
to extraneous factors. The repeatability of the experimental method is
evaluated by designing star and six center points [40]. BBD of three

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levels and four independent variables was carried out after determining the preliminary scope of extracted using the single-factor test. The range and level of independent variables are listed in Table 1. Independent variables and their ranges were chosen according to the preliminary experimental results. The extraction rate (%) of the total flavonoids was the function response value (Y), which is related to variables encoded by the following second-order polynomial equation:

\[
Y(\%) = A_0 + A_1X_1 + A_2X_2 + A_3X_3 + A_4X_4 + A_5X_1^2 + A_6X_2^2 + A_7X_3^2 + A_8X_4^2 + A_9X_1X_2 + A_{10}X_1X_3 + A_{11}X_1X_4 + A_{12}X_2X_3 + A_{13}X_2X_4 + A_{14}X_3X_4
\]

(3)

Coefficients of the polynomial model included the A0 represent the constant term; A1, A2, A3, and A4 represent the linear effects; A11, A22, A33, and A44 represent the quadratic effects; and A12, A13, A14, A23, A24, and A34 represent the interaction effects. The statistical significance of terms in regression equations was examined. Analysis of variance (ANOVA) was used to determine the significance of each response in the model.

2.5.4. Analysis of flavonoid content

Flavonoid contents of the extracted solution were determined through the method of a previous study [41]. Hence, the test solution was mixed with different volumes of 75% ethanol, 5% sodium nitrite, 10% aluminum nitrate, and 4% sodium hydroxide solutions and incubated for 15 min. Absorbance was then measured at 510 nm. The flavonoid content was calculated through the calibration curve of rutin (rutin g/g sample).

2.6. Separation and purification of flavonoids from A. membranaceus stems and leaves

The total flavonoids from A. membranaceus stems and leaves were isolated and analyzed using Sephadex LH-20 column chromatography with guided by antioxidant activity. Ultrasound-assisted extraction of A. membranaceus stem and leaf powder was carried out using the optimized method, and the crude extract was evaporated to dryness under vacuum condition. First, the extract was suspended in water, followed by petroleum ether, ethyl acetate, and n-butanol extraction. The ethyl acetate fraction was separated using Sephadex LH-20 column chromatography and eluted with 50% ethanol to obtain four fractions (Frac. a, b, c, and d). Frac. c was loaded on a Sephadex LH-20 column for chromatography to obtain three fractions (Frac. ca, cb, cc, cd, and cce). Frac. ca was further subjected to Sephadex LH-20 column chromatography to obtain five fractions (Frac. cca, ccb, ccc, ccd, and cee). The fractions (about 15 mL each) were determined via thin layer chromatography (TLC) for merging. The antioxidant activity of each fraction was evaluated with the DPPH method.

2.7. High-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) analysis

According to the procedure described by Wang et al. [36], the flavonoid composition of frac. ccd was analyzed using HPLC-MS/MS. The HPLC separation was carried out using an EXIONLC System (Sciex). Mobile phase A was 0.1% formic acid in water, and mobile phase B was acetonitrile. Column temperature was set to 40°C. Autosampler temperature was set to 4°C, and injection volume was 2 μL. Sciex QTrap 6500+ (Sciex Technologies), was utilized for assay development. Typical ion source parameters were: ion spray voltage: +5,500/-4,500 V, curtain gas: 35 psi, temperature: 400°C, ion source gas 1:60 psi, ion source gas 2: 60 psi, and DP: ± 100 V. Qualitative identification components were based on matching mass spectra with standard compounds available in the Thermo mzCloud online library and Thermo mzVault local library.

2.8. Molecular docking

UCSF Chimera software was used for molecular docking of superoxide dismutase (SOD: 2COV) with isoquercitrin and astragalin to predict binding affinity. First, the structure of SOD protein and ligand small molecules was prepared by removing water molecules, adding hydrogen atoms and electrons, and minimizing their energies using the Dock Prep program. Second, ligands were docked to the active site of the enzyme using the AutoDock Vina program to construct possible conformations. The grid box with dimensions of 50 points × 70 points × 50 points is centered on the active site of the enzyme. Finally, the stability of the receptor-ligand binding was evaluated with reference to the binding energy. A low score, corresponds to high stability.

2.9. Statistical analysis

All measurements were performed in triplicate. The results were expressed as mean values and standard deviation. Experimental data were processed using GraphPad 8.0. Significant differences between the mean values were determined via Duncan’s multiple range test. P < 0.05 was considered statistically significant.

3. Results

3.1. Anti-fungal activity assay

Inhibitory effects of flavonoids, saponins, and polysaccharide crude extracts were tested in five concentrations (10, 5, 2.5, 1.25, and 0.625 mg/mL) on A. flavus, P. citrinum, A. niger, F. moniliforme, R. oryzae and T. harzianum (Figs. S2 and 1). Fig. 1 shows that flavonoids exhibit significant antifungal activity compared with both saponins and polysaccharides in the tested concentration range. The diameter of fungal colonies treated with flavonoids was significantly smaller than that of the negative control, thereby indicating that the flavonoid extract presents a potential inhibitory effect (Fig. S2). The ANOVA results (Table 2) showed that a significant difference exists at the 0.005 level among the five concentrations of different extracts.

Flavonoids, especially A. flavus with an inhibition rate of 56.73% at 0.625 mg/mL, demonstrate different degrees of inhibition in the tested concentrations (Fig. 2). Moreover, inhibition rates against the six tested fungi at a concentration of 5 mg/mL all reach 100%. These results indicated that A. membranaceus stem and leaf flavonoids present significant inhibitory activity against the tested fungi.

Concentrations of A. membranaceus stem and leaf flavonoids and corresponding inhibition rates were converted into logarithmic and probability values, respectively, to obtain the toxicity regression equation, and calculate the EC50 (Table 3). EC50 refers to the concentration that causes 50% of the maximal effect. A low concentration corresponds to a strong inhibitory effect. The EC50 of flavonoids for A. flavus, P. citrinum, A. niger, F. moniliforme, R. oryzae, and T. harzianum were 0.9027, 1.1107, 1.1013, 1.1779, 1.2616, and 0.9714 mg/mL, respectively. These results indicated that fungi with the strongest and weakest inhibitory effects of A. membranaceus stem and leaf flavonoids were A. flavus and R. oryzae, respectively.

| Table 1 | Independent variables and their levels used in the response surface design. |
|---------|-------------------------------------------------|
| Factor level | Independent variables |
| Liquid-solid ratio (X1/ mL·g⁻¹) | Extraction temperature (X2/ °C) | Extraction time (X3/min) | Ethanol concentration (X4/%) |
| −1 | 20 | 40 | 30 | 70 |
| 0 | 30 | 50 | 30 | 80 |
| 1 | 40 | 60 | 70 | 90 |
3.2. Anti-oxidation activity assay

Anti-oxidation activities of different extracts were tested in five concentrations (5, 2.5, 1.25, 0.625, and 0.3125 mg/mL) on DPPH, ABTS$^\cdot+$, and FRAP (Fig. 3). Fig. 3 presents that flavonoids and saponins exhibit significant antioxidant activity in the tested concentration range. Notably, DPPH, ABTS$^\cdot+$, and FRAP activities of flavonoids were equivalent to those of vitamin C at 1.25, 5, and 1.25 mg/mL, respectively. The ANOVA results (Table 4) showed that a significant difference exists at the 0.005 level among the five concentrations of the different extracts. The regression equation and EC$_{50}$ values are listed in Table 5. EC$_{50}$ of DPPH, ABTS$^\cdot+$ and FRAP flavonoids was 0.1490, 0.0320, and 3.1939 mg/mL, respectively. These results indicated that flavonoids from A. membranaceus stems and leaves show significant anti-oxidant activity.

3.3. Optimization of extraction process of total flavonoids from stems and leaves of A. membranaceus

3.3.1. Comparison of ethanol and ultrasonic-assisted ethanol extraction methods

The results of ethanol and ultrasonic-assisted ethanol extraction methods are illustrated in Fig. 4. Ultrasound-assisted ethanol extraction can significantly improve the yield of flavonoids from A. membranaceus stems and leaves by 10.83 % compared with common ethanol extraction (Fig. 4a). In addition, the anti-oxidant activity of ultrasonic-assisted ethanol extract was stronger than that of common ethanol extract, with a significant difference at the 0.05 level (Fig. 4b, 4c, and 4d).

3.3.2. Effects of operation parameters on the extraction yield of flavonoids from stems and leaves of A. membranaceus

Fig. 5 shows the effects of different ethanol concentrations, solid–liquid ratios, extract times and extract temperatures on the yield of flavonoids from A. membranaceus stems and leaves. The maximum yield was 18.7653 mg/g when the ethanol concentration was 80 % (Fig. 5a). Flavonoids began to decline when the ethanol concentration was greater than 80 %. Fig. 5b presents the effect of different liquid–solid ratios on the extraction rate of flavonoids. The maximum yield was 19.6343 mg/g when the liquid-to-solid ratio was 30 mL/g. Note that extraction time is another parameter that affects the extraction rate. Fig. 5c depicts the effect of extraction time on the extraction rate of flavonoids. The maximum yield of flavonoids was achieved at 50 min. Fig. 5d illustrates the effect of temperature on the extraction rate of flavonoids. The maximum extraction rate of the total flavonoids was 19.6154 mg/g at 50 °C.

3.3.3. Statistical analysis and model fitting

Thirty runs were used to optimize the four parameters in the current BBD. The experimental conditions and the extraction yield of flavonoids according to the factorial design are presented in Table 6. The maximum extraction yield of flavonoids was 24.3904 mg/g under the experimental conditions.
conditions of extraction temperature of 58.1 °C, extraction time of 34.5 min, liquid–solid ratio of 40 mL/g, and ethanol concentration of 73.15 %. Response and test variables were related on the basis of multivariate regression analysis of the experimental data using the following second-order polynomial equation:

\[ Y(\%) = 21.49 + 2.51X_1 + 0.30X_2 - 0.20X_3 - 1.32X_4 - 0.31X_1^2 - 0.40X_2^2 - 0.12X_3^2 - 1.10X_4^2 + 0.25X_1X_2 + 0.29X_1X_3 - 0.18X_1X_4 - 0.22X_2X_3 + 0.13X_2X_4 + 0.14X_3X_4 \]  

ANOVA was applied to test the importance and adequacy of the model. The ANOVA results of the fitted models for all investigated responses are listed in Table 7. F-test and p-value are used to evaluate the statistical significance of the regression model. A p-value of < 0.05 generally indicates the significance of model terms. The p-value of the regression model is smaller than 0.05, thereby confirming the significance of model terms. Moreover, linear (X_1 and X_4) and quadratic (X_4^2) coefficients are significant (P < 0.05). The liquid–solid ratio and ethanol concentration are clearly related to the extraction yield. Moreover, failure of the model to represent experimental data, in which points in the regression or differences in random error are excluded from the model, can be reflected by the lack of fit. The lack of fit of the equation in our experiment is insignificant (P greater than 0.05). This finding indicated that the equation matched properly with the test and can be used to analyze and predict the result of ultrasonic-assisted extraction for flavonoids. The order of factors affecting the yield from high to low is: X_1 > X_4 > X_2 > X_3.

Table 3
Toxicity regression equations and EC\(_{50}\) values of the total flavonoids from A. membranaceus stems and leaves against six tested fungi.

| Tested fungi         | Toxicity regression equations | Correlation coefficient | EC\(_{50}\) (mg/mL) |
|----------------------|-------------------------------|-------------------------|----------------------|
| Aspergillus flavus   | \(Y = 2.7239x + 5.1211\)     | 0.7608*                 | 0.9027               |
| Penicillium citrinum | \(Y = 4.0785x + 4.814\)      | 0.8499*                 | 1.107                |
| Aspergillus niger    | \(Y = 4.0159x + 4.8317\)     | 0.8626*                 | 1.0103               |
| Fusarium             | \(Y = 4.2974x + 4.6944\)     | 0.8905*                 | 1.1779               |
| moniliforme          | \(Y = 4.4018x + 4.5558\)     | 0.8727*                 | 1.2616               |
| Rhiopus oryzae       | \(Y = 3.8615x + 5.0487\)     | 0.8641*                 | 0.9714               |

Fig. 2. Anti-fungal activities of total flavonoids from A. membranaceus stems and leaves. Different letters indicate significant differences at the 0.05 level.

Fig. 3. Anti-oxidation activities of A. membranaceus stem and leaf extracts: (a) DPPH, (b) ABTS\(^{-}\), and (c) FRAP. Different letters indicate significant differences at the 0.05 level.
3.3.4. Optimization of extraction conditions of flavonoids and data diagnosis

Response surfaces (three-dimensional) were depicted using Design-Expert software (8.0.6.1) to illustrate the interactions of factors for the optimal response value. Data were generated by maintaining the three variables at their respective zero levels and changing other variables in the experimental range of the response surface. Fig. S3 depicts information about the interaction between two independent variables.

Fig. 6 shows the data diagnosis of optimized parameters to evaluate the feasibility of the method. Fig. 6a presents the perturbation plot of the yield of extracting flavonoids from stems and leaves of A. membranaceus. The extraction yield response was plotted by changing only one factor over its range while other factors were command constant. Fig. 6a depicts the influence of all factors at a central point in the design space, including (A) liquid–solid ratio, (B) extraction temperature, (C) extraction time, and (D) ethanol concentration. Relative flatline of the extraction time indicated its minimal effect on the extraction rate of flavonoids. The steep curvature within the extraction time and ethanol concentration demonstrated that the extraction rate of flavonoids is rapidly responsive to these factors. Therefore, the order of the positive effect of single terms on the extraction rate response was liquid-to-solid ratio, ethanol concentration, extraction temperature, and extraction time. Fig. 6b illustrates the normal plot of residuals. Data close to a straight line indicate an enhanced effect. The satisfactory linear relationship shown in this experiment indicated the acceptable

Table 4
Results of ANOVA for the anti-oxidant activity of A. membranaceus stem and leaf extracts.

| Source of variance | Square sum | df | Mean square sum | F value | Sig. |
|--------------------|------------|----|----------------|---------|------|
| DPPH               | 12,205     | 3  | 4068           | 16.19   | <0.005 |
| Treatment          |            |    |                |         |      |
| Total variance     | 16,226     | 19 |                |         |      |
| ABTS               | 15,128     | 3  | 5043           | 8.213   | <0.005 |
| Treatment          |            |    |                |         |      |
| Total variance     | 24,953     | 19 |                |         |      |
| FRAP               | 7905       | 3  | 2635           | 14.88   | <0.005 |
| Treatment          |            |    |                |         |      |
| Total variance     | 10,738     | 19 |                |         |      |

Table 5
Regression equations and EC_{50} values of anti-oxidant activity of flavonoids from A. membranaceus stems and leaves.

| Methods     | Regression equations | Correlation coefficient | EC_{50} (mg/mL) |
|-------------|----------------------|-------------------------|-----------------|
| DPPH        | Y = 0.8586x + 5.7099 | 0.9779*                 | 0.1490          |
| ABTS        | Y = 0.6953x + 6.039  | 0.9491*                 | 0.0320          |
| FRAP        | Y = 1.389x + 4.2995  | 0.9993*                 | 3.1939          |

Fig. 4. Comparison of ethanol extraction and ultrasonic-assisted ethanol extraction: (a): Flavonoid yield, (b): DPPH, (c): ABTS·, and (d): FRAP. Different letters indicate significant differences at the 0.05 level.

Fig. 5. Effects of (a) ethanol concentration, (b) liquid–solid ratio, (c) extraction time, and (d) extraction temperature on the extraction yield of total flavonoids from stems and leaves of A. membranaceus (%).
actual yields. Experimental data scattered around the theoretical line.

flavonoids. L. Cui et al. and leaves included the following: ethanol concentration of 73.15 %, optimal extraction process of flavonoids from

| Source | Degrees of freedom | Coefficient | Sum of squares | Mean sum of squares | F-value | p-value(N) |
|--------|--------------------|-------------|----------------|-------------------|---------|------------|
| Model  | 14                 | 281.91      | 201.14         | 11.11             | <0.0001 |            |
| X1     | 1                  | 140.68      | 140.68         | 77.61             | <0.0001 |            |
| X2     | 1                  | 1.90        | 1.90           | 1.05              | 0.3216  |            |
| X3     | 1                  | 0.91        | 0.91           | 0.50              | 0.4988  |            |
| X4     | 1                  | 32.83       | 32.83          | 18.11             | 0.0007  |            |
| X1X2   | 1                  | 0.86        | 0.86           | 0.48              | 0.5005  |            |
| X1X3   | 1                  | 1.27        | 1.27           | 0.70              | 0.4150  |            |
| X1X4   | 1                  | 0.41        | 0.41           | 0.23              | 0.6402  |            |
| X2X3   | 1                  | 0.69        | 0.69           | 0.38              | 0.5473  |            |
| X2X4   | 1                  | 0.20        | 0.20           | 0.11              | 0.7463  |            |
| X3X4   | 1                  | 0.24        | 0.24           | 0.13              | 0.7219  |            |
| X1X2X3 | 1                  | 2.95        | 2.95           | 1.63              | 0.2217  |            |
| X1X2X4 | 1                  | 4.37        | 4.37           | 2.41              | 0.1413  |            |
| X1X3X4 | 1                  | 0.40        | 0.40           | 0.22              | 0.6459  |            |
| X2X3X4 | 1                  | 33.42       | 33.42          | 18.44             | 0.0006  |            |
| Residual| 15                | 27.19       | 1.81           |                   |         |            |
| Lack of Fit | 10       | 23.34       | 2.33           | 3.03              | 0.1165  |            |
| Pure Error | 5        | 3.85        | 0.77           |                   |         |            |
| Cor Total | 29      | 309.11      |                |                   |         |            |

*p-values < 0.05 means significant difference; p-values < 0.001 was extremely significant.

reproducibility of the method. Fig. 6c shows the plot of predicted and actual yields. Experimental data scattered around the theoretical line indicated that the results are consistent with those of the model.

3.3.5. Validation of the model

According to the results of the response surface optimization test, the optimal extraction process of flavonoids from A. membranaceus stems and leaves included the following: ethanol concentration of 73.15 %, liquid–solid ratio of 40 mL/g, ultrasonication time of 34.5 min, and extraction temperature of 58.1°C. The yield of flavonoids was 24.3904 mg/g under optimal conditions. Optimum extraction conditions in the actual operation were as follows: liquid–solid ratio of 40 mL/g, ethanol concentration of 75 %, extraction time of 35 min, and extraction temperature of 58 °C. The applicability of optimal extraction parameters in this case was verified with three validation experiments. The average value of the actual experiment of 22.0270 ± 2.5739 mg/g (n = 3) verified the effectiveness of the RSM model and the accuracy of the regression model. Hence, the model is suitable for extracting the total flavonoids from the leaves and stems of A. membranaceus.

3.4. Sephadex LH-20 column chromatography and HPLC-MS/MS analysis

The total flavonoids from A. membranaceus stems and leaves were isolated and purified via atmospheric-pressure Sephadex LH-20 column chromatography and TLC. First, four fractions (frac. a, b, c, and d) were obtained from the ethyl acetate phase, and frac. c showed the strongest antioxidant activity, with a DPPH scavenging rate of 45.60 % at 1 mg/mL (Fig. 7a). Second, frac. c was eluted to obtain three fractions (frac. ca, cb, and cc), of which frac. cc presented the strongest antioxidant activity, with a DPPH scavenging rate of 89.25 % at 1 mg/mL (Fig. 7b). Finally, five fractions (frac. cca, ccb, ccc, ccd, and cee) were obtained from frac. cc, and the antioxidant activity of frac. ccd was the strongest, with a DPPH scavenging rate of 92.27 % at 1 mg/mL (Fig. 7c). The analysis of frac. ccd using HPLC-MS/MS identified 54 flavonoids, which accounted for 68.64 % of the total detected components. Among them, isoorientin and astragalin were the most abundant at 42.33 % and 19.02 %, respectively. The chromatogram is shown in Fig. S4.

3.5. Molecular docking studies

The binding mode of SOD with isoorientin and astragalin is presented in Fig. 8. The binding energy calculation revealed that binding energies of isoorientin and astragalin to SOD are −9.6 and −9.5 kcal/mol, respectively. Binding modes of astragalin and isoorientin with SOD are illustrated in Fig. 8a and 8b, respectively. The binding site of isoorientin with SOD forms a suitable spatial complementarity that creates five hydrogen bonds. The fifth oxygen atom forms one hydrogen bond with the H atom of ASN53 (B chain) as the hydrogen bond
acceptor, the seventh oxygen atom forms one hydrogen bond with the H atom of VAL148, the eighth oxygen atom forms one hydrogen bond with the H atom of ASN53 (A chain), and the ninth oxygen atom forms two hydrogen bonds with the H atom of ASN53 (A chain) and LYS9. Similarly, the interaction of astragalin with SOD results in the formation of three hydrogen bonds.

4. Discussion

The safety of chemically synthesized food additives has become a primary concern with the improvement of people’s standard of living and the increasing awareness of food safety. Safe, non-toxic, natural, and efficient food antioxidants have become an important research direction. We explore the likelihood of recycling *A. membranaceus* waste as a major source of food antioxidants in this study.

Natural products are forms of secondary metabolites created by organisms within the evolution process to adapt to the environment that have established numerous chemical structures and distinctive biological functions. The extraction and separation technology of natural products is a crucial research direction within the field of natural product chemistry. Compared with traditional solvent extraction, ultrasonic-assisted extraction (UAE) has been widely used in extracting active ingredients from Chinese officinal herbs because of its advantages of high production, cost effectiveness, simple manipulation, and high efficiency [28,42,43,44]. The yield of flavonoids from *A. membranaceus* stems and leaves extracted via UAE in this work was 10.83% higher than that of traditional extraction. In addition, the extraction solvent, solid-liquid ratio, extraction time, and extraction temperature are the main factors affecting extraction efficiency. Flavonoids are insoluble in water due to their unique molecular structure. Ethanol is a green organic solvent for extracting target flavonoids, and its concentration affects the solubility and extraction yield of components by affecting the polarity of...
the solvent and the hydrophobicity and hydrogen bond strength of target components [45]. According to the inference of similarity and mutual solubility, high similarity in the polarity indicates fast dissolvability of the solute from the cell [46]. The underlying mechanism of this phenomenon may be the effect of the ethanol concentration on the hydrogen bond or van der Waals force between the target component and ethanol. Different liquid-to-solid ratios are an important factor that affects the extraction rate; an insufficient liquid-to-solid ratio will lead to incomplete extraction, whereas an excessive liquid-to-solid ratio will result in low extraction rate and solvent waste [47]. Extraction time can affect the interaction between the extractant and the powder, and sufficient extraction time can accelerate the dissolution of the target compound [48]. Excessive extraction time will oxidize flavonoids and reduce the extraction efficiency [48]. An appropriate heat treatment increases the solubility of flavonoids in the solution by reducing the viscosity of the solvent and increasing the speed of molecules in the substance [47].

Response surface methodology (RSM) is a set of mathematical and empirical techniques that can build models and optimize processes in the presence of complex interactions [49]. This program presents the advantages of determining the interaction between parameters and reducing the number of experiments, development time, and total cost [50]. The response surface optimization of flavonoids extracted from A. membranaceus stems and leaves was carried out in this study using UAE. Optimal extraction conditions were as follows: extraction time of 35 min, ethanol concentration of 75%, liquid–solid ratio of 40 mL/g, and extraction temperature of 58 °C. Moreover, signal-to-noise ratio (SNR) of 11.352 and coefficient of variation (CV) of 6.73% (Table 7) indicated the reproducibility of the model. Hence, this quadratic model is suitable for this experimental setup.

Activity-guided isolation is an economical technique that has been successfully in the identification, isolation, and purification of plant metabolites, which demonstrate antibacterial, antioxidant, and antitumor properties. Compared with the crude extract, the fraction with activity-guided separation showed higher biological activity [51–53]. The total flavonoids from A. membranaceus stems and leaves in this work were isolated and purified on the basis of antioxidant activity, and two flavonoids with strong antioxidant activity, namely, isoquercitrin and astragalin, were screened. Moreover, plant-derived flavonoids are a sort of natural product that has attracted widespread attention for their antibacterial effects. Previous studies showed that plant-derived flavonoids, such as Calendula officinalis L. [54] and saffron [36] extracts, present satisfactory antifungal effects. This study investigated the inhibitory activity of A. membranaceus stem and leaf extracts against six food-borne fungi, and clarified its potential application in the development of natural food preservatives.

The metabolism of the body produces many reactive oxygen (ROS) and reactive nitrogen (RNS) species, which exert adverse effects on enzymes, proteins, and DNA, thereby resulting in physiological imbalances that eventually lead to numerous diseases. Antioxidants, such as superoxide dismutase (SOD) and catalase (CAT), can scavenge free radicals within the body. However, antioxidants in the body fail to remove free radicals completely once the body’s immunity is weakened or invaded by toxic substances, such as Cd and Hg, and the intake of foods rich in antioxidants can make up for this deficiency. Notably, the extensive application of artificial antioxidants in the food industry may cause liver damage and cancer [9]. This study assessed the antioxidant and antifungal activities of A. membranaceus stem and leaf extracts to provide a possible innovative application for the development of natural antioxidants from A. membranaceus by-products, encourage recycling, and reduce the environmental impact of waste disposal.

Fig. 8. Three-dimensional docking models of (a) astragalin and (b) isoquercitrin with SOD.
5. Conclusions

*U. membranaceus* is a species of medicinal and edible homology in China that presents a variety of biological activities. The reuse potential of *A. membranaceus* waste as a source of food antioxidants was evaluated in this work. The response surface methodology was used to determine optimum processing conditions and obtain the maximum extraction yield of flavonoids from *A. membranaceus* stems and leaves. Optimal extraction conditions for flavonoids were an ethanol concentration of 75 %, liquid–solid ratio of 40 mL/g, extraction time of 35 min, and extraction temperature of 58°C. The total flavonoids were separated and purified using activity-guided separation technology. Frac. ccd composition with strong anti-oxidant activity were obtained, with isoquercitrin and astragalin as the main components. The result of this study can provide a potential innovative application for the development of natural food antioxidants from *A. membranaceus* waste.

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### CRediT authorship contribution statement

Liyan Cui: Data curation, Visualization, Writing – original draft. Zhenhan Ma: Software, Data curation, Visualization. Defu Wang: Resources, Writing – review & editing. Yanbing Niu: Conceptualization, Supervision, Funding acquisition.

### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

### Data availability

Data will be made available on request.

### Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ulsoch.2022.106190.

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