

Preliminary Separation, Purification and Determination of Flavonoids in Aurea Helianthus

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Abstract : The extraction, separation and purification of flavonoids from Aurea Helianthus were performed using various organic solvents and gradient elution methods. Ultraviolet spectrophotometry and high performance liquid chromatography-mass spectrometry were used to analyze flavonoids. Results: UV-Vis spectrophotometer UV 2450 (SHIMADZU) was used to preliminarily identify the content of flavonoids in the extracts of Aurea Helianthus, flavonoid content in the ethyl acetate extract was the highest, $48.3 \pm 0.3\%$. There was a good linear relationship between 0.004 and 0.024 mg/ml ($R^2 = 0.998$). Ethyl acetate extract was used as the test object for high performance liquid chromatography-mass spectrometry analysis. In the linear range 10.0-240 μ g, good precision and reproducibility, stable within 6 hours. The average recoveries of quercetin, Hyperoside, and rutin were 105.32%, 102.25%, and 103.14%. The content of rutin in the gradient elution (EtOAc:MeOH=4:1) was $42.8 \pm 0.3\%$, and the content of hyperin and quercetin in the gradient elution (EtOAc) were $51.2 \pm 0.4\%$ and $12.5 \pm 0.3\%$. The gradient effluent (EtOAc:MeOH=4:1, EtOAc) had a total flavonoid content of $84.2 \pm 0.8\%$ and $75.3 \pm 0.9\%$. Conclusion: This method is simple, rapid, and highly sensitive. It can be used to extract, isolate, purify and determine the content of flavonoids in Aurea Helianthus.

Key words: ultraviolet spectrophotometry; high performance liquid chromatography-mass spectrometry; Aurea Helianthus; flavonoids.

Aurea Helianthus, also known as Golden Hibiscus, Hibiscus syriacus, and Hibiscus, is a family of Malvaceae, Okra, and annual herb. It has functions such as food, medicinal and health care, and has high commercial value. The research found that the flowers, stems, leaves, and seeds of S. lappa are rich in biologically active

substances and have good anti-oxidation, hypolipidemic, hypoglycemic, anti-tumor, analgesic, antipyretic, anti-inflammatory, immune regulation, and anti-resistance. Aging, liver protection and other functions^[1-4]. In recent years, the research on the Aurea Helianthus was mainly focused on the extraction, separation, purification of active ingredients flavonoids, polysaccharides, polyphenols and other aspects^[5-8]. The efficient extraction, purification and purification of flavonoids from Aurea Helianthus are rarely reported. Therefore, in this experiment, the organic solvent extract of Aurea Helianthus was used as the research object to carry out gradient elution to establish a simple, economical and accurate determination method for flavonoid content of Aurea Helianthus: UV spectrophotometry, high performance liquid chromatography-mass spectrometry. The HPLC/MS combination technology is a new technology matured in the 1990s. It has the characteristics of high sensitivity, strong selectivity, separation and structural identification at one time. It has been widely used in the separation analysis of natural products^[9-13]. The authors studied the gradient elution method for the extraction, separation and purification of Aurea Helianthus flavonoids, and established conditions for the separation and analysis of flavonoids from Aurea Helianthus by ultraviolet spectrophotometry and high performance liquid chromatography-mass spectrometry. The establishment of the method has certain guiding significance for the separation and identification of the flavonoids in Aurea Helianthus and the quality control of the traditional medicine.

1. Materials and methods

1.1 Instruments; UV spectrophotometer UV 2450 (Shimadzu SHIMADZU), electronic analytical balance BT 255 (Sartorius), Agilent Technologies 6540 mass spectrometer, DAD detector.

1.2 Reagents; quercetin, hyperoside, rutin standard (Chengdu Pfizer Biotechnology Co., Ltd.); methanol (chromatographically pure) Merck; water is ultrapure water; other reagents are of analytical grade.

1.3 Test materials; Dried flowers for the test flower sunflower were purchased in Beijing-Shunyi.

1.4 Chromatography and Mass Spectrometry Conditions

1.4.1 chromatographic conditions; column: Diamonsil C₁₈(2) column (4.6×250mm, 5μm), mobile phase A is methanol B 0.1% phosphoric acid solution (50:50, V/V), flow rate 1ml/min, detection The wavelength is 360 nm, the column temperature is 25° C., and the injection volume is 10 μL.

1.4.2 Mass Spectrometry Conditions. Column: ACQUITY UPLC BEH C18 column (2.1 x 50mm, 1.7um), mobile phase A is acetonitrile B water (containing 0.1% v/v formic acid), flow rate 0.3 ml/min, detection wavelength 360 nm, column temperature 40 °C, injection: 10μL. The ion source is an electrospray ESI ion source with a dryer

flow rate of 8L/min, an ion source temperature of 110°C, a capillary voltage of 3500V, a SKIMMER voltage of 30V, a Fragmentor voltage of 135V, and data acquisition in negative ion mode. Data acquisition range m/z 200- 1000.

1.5 Methods

1.5.1 Preparation of the test solution The sunflower spent dry material is extracted 3 times with a concentration of 75% ethanol at a solid-liquid ratio of 1:10, filtered, and the filtrates are combined. The extract is concentrated under reduced pressure to dryness, and the resulting extract is vacuumed. Dry standby. Weigh the appropriate amount of vacuum-dried extract extract above, and use ultrapure water to make it.

1.5.2 Disperse with ultrapure water 1 L water. The extracted extracts were successively extracted with petroleum ether, ethyl acetate, and n-butanol. Each solvent was extracted 10 times, and the respective extracts were combined and concentrated to obtain a petroleum ether layer extract, an ethyl acetate layer extract, and n-butane. Alcohol layer extract, water layer extract.

1.5.3 Determination of total flavonoids

The aluminum trichloride method is the most commonly used method for the detection of total flavonoids.

Under alkaline conditions, AlCl_3 can form red chelates with flavonoids and have a maximum absorption peak at 510 nm. Accurately take 0.1mg/mL rutin standards 0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 mL in a 25mL glass test tube, add 8mL 1.5% AlCl_3 and 4mL Acetic acid-sodium acetate buffer (pH 5.5), And with 50% aqueous ethanol solution to volume, shaken, and allowed to stand for 30 minutes, and the absorbance was measured at 415 nm. Absorbance was taken as the ordinate, the corresponding final rutin concentration was abscissa, and a standard curve was drawn to obtain a regression curve. Measure a certain amount of each concentration of the sunflower extract solution, according to the standard curve operation method, determine the corresponding absorbance value, calculate the total flavonoid content of Aurea Helianthus.

1.5.4 Select the extract with the highest content of total flavonoids. The extract (1 g) and 1 g (200-300 mesh) of silica gel are mixed well and then subjected to silica gel column chromatography (200-300 mesh silica gel 200 g, 30 (×300 mm) separation, PE:Et OAc (4:1, 1:4), Et OAc, Et OAc:MeO H (4:1, 1:4), MeOH gradient elution gave 6 samples.

1.5.5 Microporous filter (0.22 μm) Each sample was filtered to obtain the test sample. Preparation of quercetin, hyperoside, and rutin standard solutions. Accurate amounts of quercetin, hyperoside, and rutin were prepared and used as a reference solution with a concentration of 1 mg/mL in anhydrous methanol. The injection volume was 10 μL .

2 Results and Analysis

2.1 Trichloro aluminum method to obtain the regression curve equation $Y = 1.5424X + 0.0293$ ($R^2 = 0.998$), the results are shown in Table 1.

□ **Table 1 The contents of total flavonoids in the extracts of Aurea Helianthus (%)**

	Ethanol extract	Petroleum ether extract	Ethylacetate extract	Butanol extract	Extraction water extract
Total flavonoids	30.4±0.3	11.9±0.2	48.3±0.3	28.6±0.2	23.8±0.3

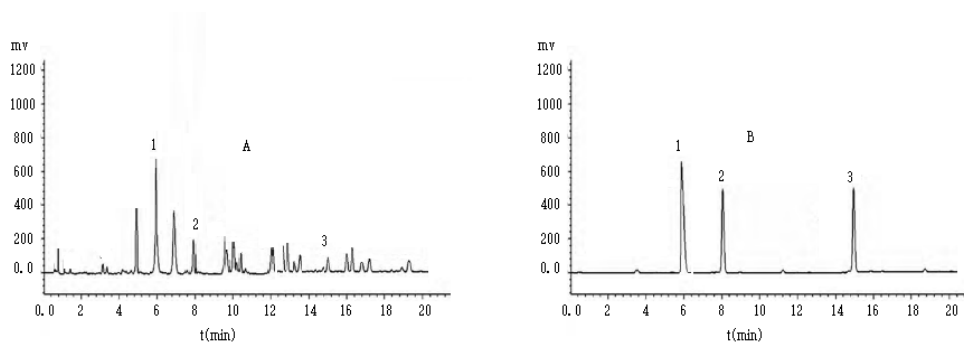
(Note: The values in the table are expressed as mean ± standard deviation, n=5.)

As can be seen from Table 1, the total flavonoid content in the extracts of Aurea Helianthus is different, which are: ethyl acetate extract > ethanol extract > n-butanol extract > raffinate aqueous extract > petroleum ether extract. The above results showed that the total flavonoid content in the ethyl acetate extract was 48.3±0.3% in each of the five species of Aurea Helianthus extract. Therefore, the ethyl acetate extract was a gradient elution subject to gradient elution to obtain 6 samples.

2.2 Qualitative identification of ethyl acetate extracts and mass spectrometry detection mode

The rutin, quercetin and hyperin were identified by HPLC and HPLC/MS. First, the retention time comparison method and the standard addition method were used for preliminary characterization, and then the ultraviolet spectrum of each chromatographic peak was collected using a diode array UV detection technique. The shape, absorption band position, and relative intensity of the obtained spectrum were compared with the standard product spectrum. The mass spectrum obtained by HPLC/MS was compared with the standard mass spectrum. The chromatograms and mass spectra of Aurea Helianthus extract of ethyl acetate were shown in Figures 1 and 2, respectively.

From Figures 1 and 2, it can be seen that the molecular ions $[MH]^-$ of quercetin, hyperoside, and rutin are 301.0352, 463.0963, and 609.1448, and the extracts of rutin, quercetin, and gold are contained in the ethyl acetate extract of Aurea Helianthus. Hormone has a high content.



A Ethyl acetate extract of Aurea Helianthus ; B Mix reference product

1-Rutin (360 nm); Hyperoside (360 nm); 3-Quercetin (360 nm).

Fig 1 HPLC chromatogram for Ethyl acetate extract of Aurea Helianthus and Mix reference product

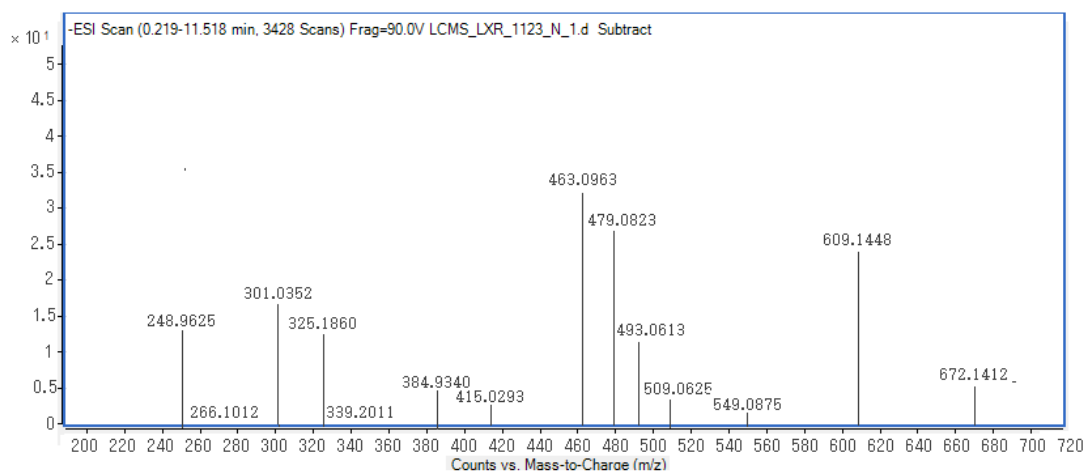


Fig 2 Mass spectrum of Aurea Helianthus.

2.3 The results of qualitative identification of each gradient elution are shown in Figure 3.

From Figures 3-1 and 2, it can be seen that the quercetin, hyperoside, and rutin contents in the gradient elution were very low when gradient elution was performed with PE and EtOAc. From Figures 3-3, 4, 5, and 6, it can be seen that the content of quercetin, hyperoside, and rutin in each gradient elution is different. The greater the polarity of the gradient eluent, the greater the content of rutin. The smaller the content of hyperin and rutin.

2.4 The standard curve is drawn

Accurately prepared mass concentrations were 10.0, 20.0, 40.0, 80.0, 160.0, 240.0, $\mu\text{g/mL}$, rutin, quercetin, hyperoside reference substance solution, and the peak area of each component was measured under the above chromatographic conditions. The equation is: $Y= 11203X-2039$ ($R^2= 0.9975$) (quercetin), $Y= 13613X- 1308$ ($R^2= 0.9987$) (hyperoside), $Y= 10286X-1431$ ($R^2= 0.9983$) (rutin) . The linear range is 10.0-240 μg

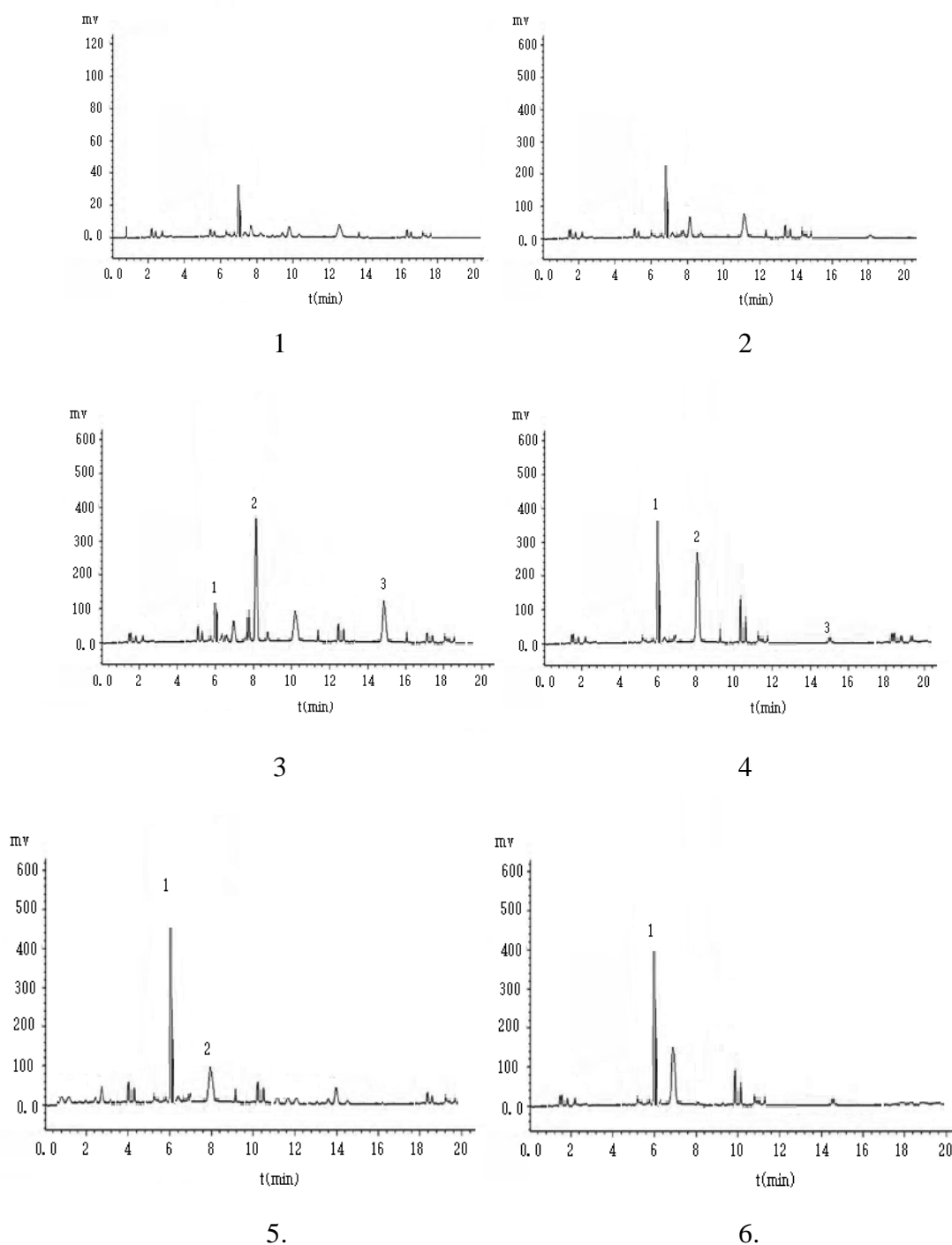


Fig 3 HPLC chromatogram of each gradient elution.

1-(PE:EtOAc=4:1) , 2- (PE:EtOAc =1:4) , 3-EtOAc , 4-(EtOAc:MeOH =4:1) ,

5-(EtOAc:MeOH =1:4) , 6-MeOH

2.5 Precision test

The 40.0, 160.0, and 240.0 $\mu\text{g/mL}$ quercetin, hyperoside, and rutin control were repeatedly injected 5 times according to the above chromatographic conditions, and

the peak area was measured. The results showed that the precision was good, RSD were quercetin (0.95%, 0.88%, 1.05%), hyperoside (1.15%, 0.85%, 0.91%), rutin (1.21%, 0.96%, 1.13%, respectively). It shows that the precision is good.

2.6 Stability Test

According to the method for the determination of the test solution, 10 μ L of the same test solution was injected at 0, 2, 4, 6, 8 and 6 h respectively. The RSD of quercetin, hyperoside and rutin in the sample was 1.15 each. %, 0.98%, 1.03%. It shows that the sample solution is stable within 6h.

2.7 Reproducibility test

Five test samples were accurately weighed and determined according to the above chromatographic conditions. The RSDs of quercetin, hyperin, and rutin in the samples were 1.23%, 1.12%, and 1.38%, respectively. This shows that the method is reproducible.

2.8 Recovery Test

Precisely take 5 mL of the test solution containing the assayed solution and set it in a 10 mL volumetric flask. Add the appropriate amount of quercetin, hyperoside, and rutin reference solution (0.6 mL, 3 parts each). Dilute with methanol to the mark. , Shake well, and determine the content according to the above method; The average recoveries of quercetin, hyperin, and rutin were 105.32%, 102.25%, and 103.14%, respectively.

2.9 Determination of Content

2.9.1 Use the regression equation to calculate the content of flavonoids in the ethyl acetate extract of *Aurea Helianthus*.

The results of the measurements are shown in Table 2.

Table 2 The contents of flavonoids in Ethyl acetate extract of *Aurea Helianthus* (%)

	Quercetin	Hyperin	Rutin
content	5.6 \pm 0.2	12.7 \pm 0.3	21.5 \pm 0.3

(Note: The values in the table are expressed as mean \pm standard deviation, n=3.)

From Table 2, the total flavonoid content in the ethyl acetate extract of *Aurea Helianthus* was 39.8 \pm 0.8%, and the value determined by spectrophotometry (48.3 \pm 0.3%) was small. The reason may be that there are other flavonoids in the ethyl acetate extract of *Aurea Helianthus*.

2.9.2 Use the regression equation to calculate the content of flavonoids in each gradient of sunflower.

The results of the measurements are shown in Table 3

Table 3 The content of flavonoids in each gradient of Aurea Helianthus(%)

	PE:EtOAc= 4:1	PE:EtOAc =1:4	EtOAc	EtOAc:MeOH =4:1	EtOAc:MeOH =1:4	MeOH
Quercetin	-	2.6±0.3	12.5±0.3	5.3±0.2	1.5±0.1	-
Hyperin	2.4±0.3	16.4±0.2	51.2±0.4	36.1±0.3	5.8±0.3	3.5±0.3
Rutin	2.1±0.2	5.2±0.2	11.6±0.2	42.8±0.3	38.6±0.3	23.2±0.5
Total content	4.5±0.5	24.2±0.7	75.3±0.9	84.2±0.8	45.9±0.7	26.7±0.8

(Note: The values in the table are expressed as mean ± standard deviation, n=3.)

As can be seen from Table 3, the content of flavonoids in each gradient elution of Aurea Helianthus is different, which are as follows: gradient elution EtOAc: MeOH=4:1> EtOAc> EtOAc: MeOH=1:4> MeOH> PE: EtOAc =1:4>PE:EtOAc=4:1. With a gradient of EtOAc:MeOH=4:1, the rutin content was 42.8±0.3%, the highest. With a gradient elution of EtOAc, the hyperoside and quercetin contents were 51.2±0.4% and 12.5±0.3%, respectively. In comparison with the acetic acid ethanol extract flavonoid content (39.8±0.8%), the flavonoid content of the gradient elution (EtOAc:MeOH=4:1, EtOAc) was 84.2±0.8% and 75.3±0.9%, respectively.

3 Conclusion

This paper establishes a new method for the extraction, separation and purification of flavonoids from Aurea Helianthus. UV spectrophotometry and high performance liquid chromatography-mass spectrometry were used to separate and determine the flavonoids in Aurea Helianthus. The results showed that the use of gradient elution method can increase the content of flavonoids in Aurea Helianthus and achieve the purpose of separation and purification of flavonoids in Aurea Helianthus. The use of UV spectrophotometry and HPLC/MS method to separate and determine the flavonoids in Aurea Helianthus has the characteristics of short analysis time, less samples and high sensitivity, and reduces the cumbersome process of sample separation, purification and preparation. Separation, identification and quantitative analysis of flavonoids in Aurea Helianthus and other edible and medicinal plant resources provide a simple and rapid method.

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