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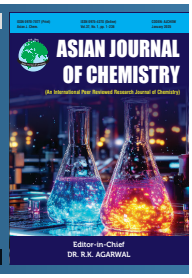

Isolation of Chemical Compounds of *Astragalus galactites* from Mongolia

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We present and discuss our recent findings on the chemical analysis of *Astragalus galactites* (Pall.), a celebrated plant in traditional Mongolian medicine. We aimed to advance the analytical approach to yield precise identification needed for developing new drugs and standardization of active principles and this is the first effort at untargeted metabolite profiling of *A. galactites* in various plant organs (aerial parts, leaves, roots and flowers) as well as previously isolated fractions of chloroform extract using GC-MS/MS and UPC2-QTOF-MS instruments. Twenty-one compounds, including eight phenolic acid derivatives, seven flavonoids, two diterpenoids derivatives, two monoterpenoids, one sesquiterpenoid, two fatty acids and a lignan were identified by UPC2-QTOF-MS in the chloroform fraction of *A. galactites*. Further 16 compounds, which are monosaccharide derivatives and alkaloids were successfully identified by GC-MS/MS analysis in the extract of leaves, flowers, roots and aerial parts of *A. galactites*. Quantification of caffeic acid (7.17 ± 0.003 mg/mL), chlorogenic acid (0.69 ± 0.01 mg/mL) and gallic acid (8.06 ± 0.004 mg/mL) in *A. galactites* was achieved using a conventional HPLC instrument and a corresponding method validated by acceptable precision (RSD < 2%) and good accuracy (98.7-102.7%, RSD 1.15-1.98%). The presence of the above-mentioned compounds supports the pharmacological effects and radical scavenging activities as reported previously.

Keywords: *Astragalus galactites* (Pall.), GC-MS, HPLC, UPC2-QTOF-MS.

INTRODUCTION

The *Astragalus* genus is a large group of family *Fabaceae* [1], predominantly distributed in the temperate regions of the northern hemisphere. Of them, *Astragalus galactites* (Pall.) is a native species to the parts of Mongolia, northern China and other geographical regions such as Siberia [2]. To date, more than 100 *Astragalus* species have been investigated have been intensively analyzed, mainly for three main groups of biologically active metabolites like polysaccharides, flavonoids and saponins [3,4]. The presence of biological active substances such as sesquiterpene-flavonolic complexes [5] sterols, lignans, coumarins and phenolic acids [3,4] have been widely reported. A plethora of bioactive compounds such as triterpenoid saponins, phenolic acids and volatile compounds [6-8] flavonoids, sugars, amino acids and triterpenoid saponins in the flowers of different *A. membranaceus* and *A. mongholicus* [9-11] have been reported to possess the biological activities of interest.

Owing to the activities of the above compounds previously reported, various *Astragalus* species from the Mongolian Flora could be a promising resource for the development of health products to meet the increasing demand of the Traditional Mongolian medicine (TMM) market, by following the recent trend of revival and development of traditional medicines observed throughout many Asian countries. However, the available phytochemical study on some species in *Astragalus* genus is rare and of them is *A. galactites*. This considered to be one of the prominent *Materia medica* in the TMM and reported to contain flavonoids, alkaloids, polysaccharides and saponins [1]. It is use in the traditional Mongolian medicines can be traced back centuries, especially for the reduction of fever, improving cardiac blood flow and the treatment of heart muscle obstruction, myocardial ischemia, *etc*.

In recent years, patients have been consuming herbal medicines extensively for various therapeutic and prophylactic purposes due to their implied safety, efficacy, cultural acceptability

and lesser side effects. Therefore, the chemical profile of bioactive compounds contained in individual floral, faunal and inorganic ingredients and/or the complex traditional mixtures, their substitutions (including counterfeit products penetrating the TMM market) is of great importance in traditional medicine safety. Therefore, a detailed pre-clinical study for assessing the pharmacology, phytochemical, toxic and further biological properties is most important.

Non-targeted mass spectrometry (MS) has become a key method in the fields of metabolomics and environmental research in recent years [12]. Untargeted MS experiments produce complex, multidimensional data that is almost impossible to study manually. For this reason, the computational softwares are needed to extract relevant information from raw spectral data and convert it into a more understandable format. MZmine is an open-source software designed to process raw spectral data generated by different MS platforms, such as liquid chromatography-MS (LC-MS), gas chromatography-MS (GC-MS) and MS-imaging [13].

The chemical composition of *A. galactites* remains largely unexplored, particularly in the context of its traditional medicinal use in Mongolia. Therefore, there is a need to determine the chemical composition of *A. galactites* in traditional medicine. The GC-MS, UPC2-QTOF-MS and HPLC analysis were conducted on endemic *A. galactites* growing in it is natural habitat of Mongolia's region for the first time. These methods provide delicate and efficient analytical performance for the chemical quality evaluation of medicinal materials, which would be suitable for the current quality evaluation.

EXPERIMENTAL

In this work, caffeic acid, ≥ 98.0% (NIFDC, China), chlorogenic acid, ≥ 98.0% (Sigma-Aldrich, Germany), gallic acid, ≥ 99.0% (NIFDC, China), hexane, ≥ 99% (GC) (Sigma-Aldrich, Germany), N,N-*bis*(trimethylsilyl)trifluoroacetamide, BSTFA, ≥ 99% (Sigma-Aldrich, Germany), pyridine, ≥ 99% (Acros Organics), were procured. All other reagents were of analytical grade.

Collection of raw material: The plant *Astragalus galactites* was collected in the Tahiltayn Mountain area (47º91′44″ N, 106º 70′63″ E), Songino Khairkhan District, Mongolia, (May, 2021). The specie was identified by Prof. E. Ganbold (National University of Ulaanbaatar, Ulaanbaatar, Mongolia). The sample was air dried in cool and dry conditions until further analysis.

GC-MS analysis: The chemical profiles of the chloroform extracts of flowers, aerial plants, leaves and roots) of *Astragalus galactites* were investigated by GC-MS technique**.** Each of the accurately weighed 5 mg samples was added into a 1.5 mL short treat vial with a wide opening (Isera GmbH, Germany) and 0.5 mL hexane was added for the ultrasonic (35 kHz,160/640 W) extraction (15 min). Prior to that analysis, hydroxyl containing compounds were derivatized to the corresponding trimethylsilyl forms by reacting with 200 µL of BSTFA in pyridine (9+1, v/v) at 70 ºC for 1 h. After the post-derivatization, the samples were stored at low temperatures (-20 ºC) to maintain the stability. Then, the samples were transferred to 0.1 mL GC vial (Wagner & Munz GmbH, Germany) inlets using a Pasteur pipette. Samples (each 2.0 µL) were injected into the GC-MS/FID (Agilent 5975C apparatus with MSD inert XL TAD and FID) system. Synchronized FID and MS spectra were recorded by a splitter mounted after the column, whereas 1/3 of the flow was sent to the MS and the remaining 2/3 to the FID. The MS trace was used for identification and the FID trace for quantification. The MS detector was operated in the EI mode at 70 eV using a temperature of 280 ºC. The mass scanning range was set to 29–1050 atomic mass units, with solvent cutting after 4 min. The FID was operated at 400 ºC, with an H_2 flow of 30 mL/min, air flow of 400 mL/min and makeup flow (combined) of 25 mL/min. The device was fitted with a DB-5HT capillary column (30 m × 250 µm × 0.1 µm, Agilent J&W). The column temperature program was set as follows: initial T = 60 ºC isothermal for 5 min, ramp to 380 ºC (rate, 10 ºC min^{-1}) and maintain at 380 ºC for 8 min. Helium was used as a carrier gas, with a gas flow of 2.5 mL/min. Injection (1.0 µL) was performed by an autosampler in a cold multimode inlet (MMI), which was kept at 65 ºC for 6 s, increased to 380 ºC at 500 ºC min^{-1} and then held for 5 min (cold split injection). The split ratio was set to 15:1 (split flow, 37.5 mL/min). Enhanced ChemStation (MSD Chemstation F.01.01.2117) was used to record the chromatogram and the MS data. After deconvolution, these were evaluated using the MassHunter Workstation software (Unknowns Analysis). Compounds were putatively identified by comparison with the NIST11 libraries.

UPC2-QTOF-MS analysis: The aerial parts of *Astragalus galactites* plant (8.6 kg) were extracted with 95% and 50% ethanol for 2 h, respectively. A total of 1.6 kg of ethanol extract was obtained from the extraction. An initial column chromatography separation of the extracts was performed using Sephadex LH20 and solvents of various polarities such as ethyl acetate, petroleum ether, chloroform and *n*-butanol. After screening with the TLC method, the chloroform fraction that contained the most biologically active substances was selected for further instrumental analysis.

Characterization of individual compounds in chloroform fractions by UPC2-QTOF-MS was carried out on the Acquity UPC2 system coupled with Acquity UPC2 XEVO G2-XS Mass Spectrometer (Acquity Technologies, Inc., USA). The analyses were made in the negative mode and MS (mass spectrometry) system was utilized in the electrospray ionization (ESI) technique. The MS operating mode is the 2.0 GHz extended dynamic range. Agilent Mass Hunter Software B06.00 and Metlin Metabolite database were used for data evaluation and analysis. For the separation, Torus™ 2-PIC column (130 Å, 1.7 µm, 2.1 mm × 100 mm) (Waters, USA) was used. The column temperature was 45 ºC; the injection volume was 1.00 µL; the flow rate was 1.2 mL/min; the analysis time was 15 min. The mobile phase was set using solvent A (CO_2) and solvent B (MeOH/ACN/H_2O/formic acid 70/28/2/0.1). The flow program was as follows: 1% to 30% B in 10 min hold at 40% for 2 min and re-equilibrate for 3 min.

HPLC analysis: Plant material (1.0 g) was homogenized with 80% methanol at 25-30 ºC, ultrasonicated for 30 min and centrifuged at 3500 rpm followed by filtration using 0.45 µm filter membrane. The reversed-phase HPLC was performed

by using the Welchrom C18 column (250 mm × 4.6 mm, 5 µm). Gradient elution was carried out with acetonitrile (B) and 0.1% phosphoric acid in distilled water (A) (75:25 %v/v, 85:15%, v/v). The flow rate was 0.5 mL/min and the detection wavelength was either 272 nm and 320 nm.

Statistical analysis: In this study, the data were expressed using basic biostatistical methods (arithmetic mean, standard deviation and relative standard deviation) with GraphPad Prism software and the Origin program. A *p* value of 0.05 was regarded as statistically significant.

RESULTS AND DISCUSSION

In the field of phytochemistry and analysis, the chemical constituents from more than 200 kinds of *A. galactites* have been studied. Although 21 bioactive compounds, including phenolic acids, terpenoids, polysaccharides and flavonoids, were determined by using the MZ mine 3.28 software program from them, the systematic phytochemistry research needs to be improved. The root and flowers could be used as the raw materials for the polysaccharides, while the aerial part and leaves may be better materials for polyphenolic acids. With this research on *A. galactites*, a key medicinal plant used in Mongolia, we aimed to identify the prevailing phenolics contain in the selected fractions, using HPLC, GC-MS/MS and UPC2-QTOF-MS techniques. Shotgun metabolite profiling showed the presence of the numerous compounds, providing the first such data for this species, representing an increase by an order of magnitude over previous studies. The number and content of specialized metabolites found in the chloroform fraction of the aerial part were significantly higher compared with other samples.

UPC2-QTOF-MS analysis: A simple, non-assisted extraction of flavonoids, phenolic acids and saponins from various plants with solvents such as methanol, is a well-established technique [12-14]. The column chromatography separation yielded three fractions with chloroform and the contents of 21 compounds were used as indicators for evaluating the extraction efficiency (Table-1). The MS/MS data of the study were analyzed using the MZmine program. The major compounds in chloroform fractions were identified as dihydrosinapic acid, sinapic acid, methyl pinolenate, (+)-gallocatechin, catechin, hippuric acid, 3′-O-methyl-catechin, α-bisabolol, naringin, apigenin, hydroxycaffeic acid, 7-O-glucoside, *cis*-verbenol, irisolidone, manool, γ-terpinene, dihydroferulic acid, irisolidone, quercetin 3-O-arabinoside, (+)-lariciresinol, 13-epi-manoyl oxide and 7,11,14-eicosatrienoic acid. According to the present results, it was determined more polyphenolic compounds in the chloroform fractions of *A. galactites.*

GC-MS analysis: The bioactive compounds in *A. galactites* were separated and identified by gas chromatography-mass spectrometry (GC-MS) in the samples of the whole above-ground parts, flower, leaves, roots, and flower after extracting with chloroform (Fig. 1). Enhanced *Chem*Station software, was used for recording the chromatograms. Resulted data files were exported in AIA (*.cdf) file format, using the MSD *ChemStation* Enhanced Data Analysis software (Agilent Technologies, Santa Clara, CA, USA) as a pipeline. Resulted chromatograms were processed using MZmine-based MZmine dashboard 3.28 [13] for simultaneous deconvolution and integration. The tentative identity of individual unknowns was accepted by comparison of their mass-spectral data with those of the NIST17 mass spectral library (NIST) data after comprehensive peak alignment, manual interval selections and feature detections (Table-2). According to the results, it was determined more monosaccharides compounds in the root of *A. galactites* plant.

HPLC analysis: Tentative identification of gallic acid, chlorogenic acid and caffeic acid contained in *A. galactites*, was achieved using a conventional HPLC technique as desc-

TABLE-1
RESULTS OF UPC2-QTOF-MS ANALYSIS IN THE CHLOROFORM FRACTION OF *A. galactites*

No.	RT (min)	*m/z*	Ion mode	Compounds name	m.f.	RSD (%)
1	4.36	225	ESI-	Dihydrosinapic acid	$C_{11}H_{14}O_5$	8.12
2	4.47	221	ESI-	Sinapic acid	$C_{11}H_{12}O_5$	
3	6.04	291	ESI-	Methyl pinolenate	$C_{19}H_{32}O_2$	
4	6.43	305	ESI-	(+)-Gallocatechin	$C_{15}H_{14}O_7$	
5	9.89	178	ESI-	Hippuric acid	$C_9H_9NO_3$	
6	10.95	289	ESI-	Catechin	$C_{15}H_{14}O_6$	
7	12.02	303	ESI-	3'-O-Methylcatechin	$C_{16}H_{16}O_6$	
8	3.33	221	ESI-	α-Bisabolol	$C_{15}H_{26}O$	
9	3.95	195	ESI-	Hydroxycaffeic acid	$C_9H_8O_5$	
10	5.68	419	ESI-	Apigenin 7-O-glucoside	$C_{21}H_{24}O_9$	
11	5.03	579	ESI-	Naringin	$C_{27}H_{32}O_{14}$	
12	10.96	289	ESI-	Manool	$C_{20}H_{34}O$	
13	4.29	151	ESI-	*cis*-Verbenol	$C_{10}H_{16}O$	
14	4.57	135	ESI-	γ-Terpinene	$C_{10}H_{16}$	
15	6.40	195	ESI-	Dihydroferulic acid	$C_{10}H_{12}O_4$	
16	6.96	313	ESI-	Irisolidone	$C_{17}H_{14}O_6$	
17	8.68	433	ESI-	Quercetin 3-O-arabinoside	$C_{20}H_{18}O_{11}$	
18	9.26	359	ESI-	(+)-lariciresinol	$C_{20}H_{24}O_6$	
19	10.30	519	ESI-	5,3',4'-Trihydroxy-3-methoxy-6:7-methylenedioxy flavone 4'-O-glucuronide	$C_{21}H_{20}O_{14}$	
20	11.12	289	ESI-	13-epi-Manoyl oxide	$C_{20}H_{34}O$	
21	11.62	305	ESI-	7,11,14-Eicosatrienoic acid	$C_{20}H_{34}O_2$	

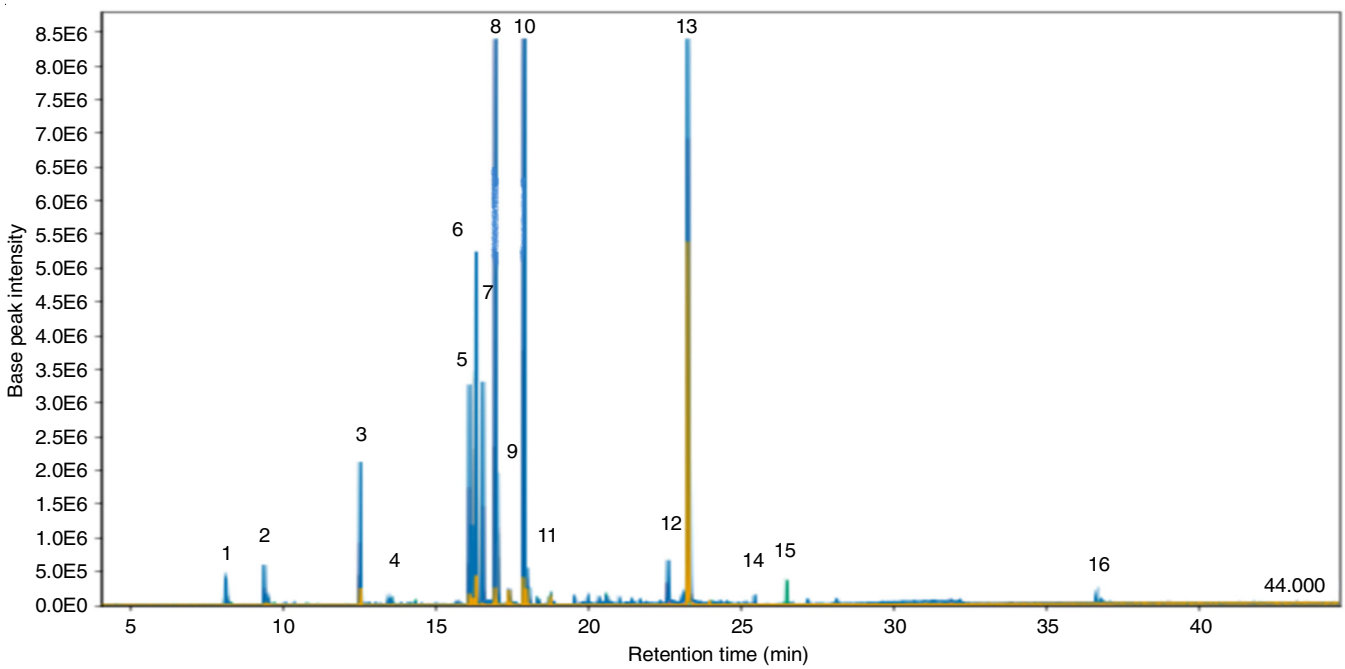

Fig. 1. GC-MS total ion chromatogram of *Astragalus galactites* (5 groups)

TABLE-2
RESULTS OF THE GC-MS ANALYSIS OF *A. galactites*

No.	Peak RT[a] (min)	Compound detected*	m.f.	Part of the plant	RSD (%)
1	8.3	N-Benzyl-1*H*-benzimidazole	$C_{14}H_{12}N_2$	Aerial, leaves, flower, flower[b]	
2	9.4	Silanol, trimethyl-, phosphate	$C_9H_{27}O_4PSi_3$	Root, flower[b], leaves, aerial, flower	
3	12.5	Malic acid, 3TMS derivative	$C_{13}H_{30}O_5Si_3$	Root, flower[b], aerial, flower	
4	13.4	2,3,4-Trihydroxybutyric acid *tetrakis*(trimethylsilyl) threonic acid	$C_{16}H_{40}O_5Si_4$	Leaves, flower[b]	
5	16.1	D-(-)-Fructofuranose, *pentakis*(trimethylsilyl) ether (isomer 2)	$C_{21}H_{52}O_6Si_5$	Root, flower[b], aerial, flower	
6	16.3	D-Pinitol, pentakis(trimethylsilyl)ether	$C_{22}H_{54}O_6Si_5$	Root, aerial, leaves, flower, flower[b]	
7	16.5	β-D-Galactofuranose	$C_{21}H_{52}O_6Si_5$	Root, aerial, flower, flower[b]	
8	16.9	D-(+)-Galactopyranose, 5TMS derivative	$C_{21}H_{52}O_6Si_5$	Root, aerial, leaves, flower, flower[b]	5.69
9	17.4	D-Sorbitol, 6TMS derivative	$C_{24}H_{62}O_6Si_6$	Root, aerial, leaves, flower	
10	17.8	β-D-Glucopyranose, 5TMS derivative	$C_{21}H_{52}O_6Si_5$	Root, aerial, leaves, flower, flower[b]	
11	18.7	Myo-Inositol, 6TMS derivative	$C_{24}H_{60}O_6Si_6$	Root, aerial, leaves, flower, flower[b]	
12	22.5	Docosanol, TMS derivative	$C_{25}H_{54}Osi$	Aerial, flower, flower[b]	
13	21.2	Sucrose, 8TMS derivative	$C_{36}H_{86}O_{11}Si_8$	Root, aerial, flower, flower[b]	
14	25.4	Silane, dimethyl(4-(2-phenylprop-2-yl)phenoxy) decyloxy-	$C_{27}H_{42}O_2Si$	Leaves, flower[b]	
15	26.4	1-[(4-Methylbenzene) sulfonyl]pyrrole	$C_{11}H_{11}NO_2S$	Leaves	
16	36.5	D-(+)-Cellobiose, *octakis*(trimethylsilyl) ether, methyloxime	$C_{37}H_{89}NO_{11}Si_8$	Leaves, flower[b]	

*The compounds that were identified and compared with a similar mass fragmentation in the NIST database library. RT[a]: Retention time; flower[b]: Sample of the flower extracted with chloroform solvent.

ribed previously [15,16] with minor improvements. Each of 20 µL samples were injected into the HPLC system according to the chromatographic conditions. The recorded chromatogram of *A. galactites* indicated the presence of gallic acid, caffeic acid and chlorogenic acid each with a retention time of 5.34, 5.53 min and 4.67 min compared with corresponding reference substances. The optimized chromatograms were obtained for the standards and test samples of gallic acid, caffeic acid and chlorogenic acid. The regression equation obtained from the calibration curves can be used to determine the gallic acid, caffeic acid and chlorogenic acid contents in *A. galactites* (Fig. 2). From these results, it can be observed that gallic acid, caffeic acid and chlorogenic acid content in *A. galactites* is 8.06 mg/mL, 7.17 mg/mL and 0.69 mg/mL, respectively.

Method of validation: The HPLC method for the estimation of gallic acid, caffeic acid and chlorogenic acid was developed and validated according to ICH Q2 (R1) guidelines (Table-3) [17]. The results of the validation parameters described above are satisfactory in HPLC methods. According to the results, the content of gallic acid was relatively higher than

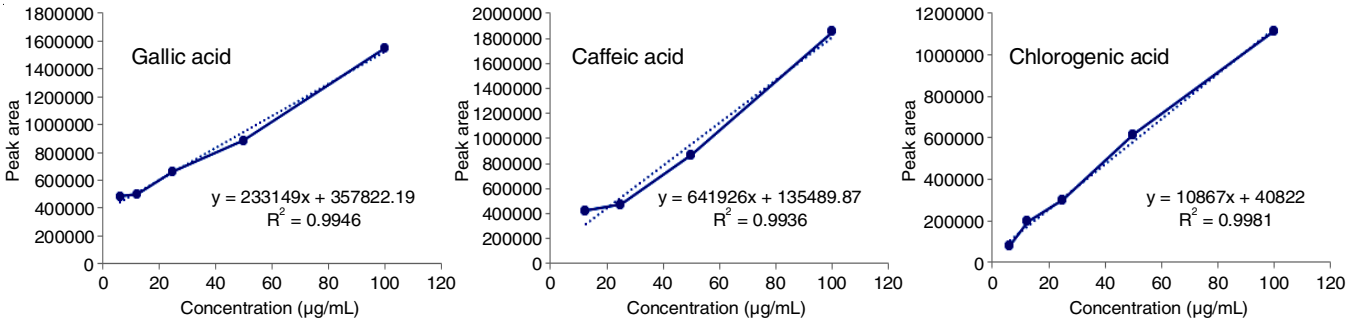

Fig. 2. Calibration curve of gallic acid, caffeic acid and chlorogenic acid

TABLE-3
VALIDATION PARAMETERS OF THE PROPOSED HPLC METHOD

Parameters	Gallic acid	Caffeic acid	Chlorogenic acid
Wavelength of detection	272 nm	272 nm	320 nm
Retention time (min)	5.34 min	5.53 min	4.67 min
Beer's law limit (µg/mL)	6.25-100	6.25-100	6.25-100
Regression equation (Y = ax + b)	Y = 213149x + 357822.19	Y = 641926x + 135489.87	Y = 40821.67 + 10867.20x
Slope	213149	641926	10867.20
Intercept	357822.19	135489.87	40822
Coefficient of correlation	0.9946	0.9936	0.9981
Limit of detection	0.8 µg/mL	0.05741 µg/mL	13.05 µg/mL
Limit of quantification	2.45 µg/mL	0.17397 µg/mL	39.55 µg/mL
Accuracy (% RSD)	99.11-101.34%, RSD 1.15%	98.8-101.7%, RSD 1.44%	98.7-102.7%, RSD 1.98%
Precision (% RSD)	Inter-day = 1.32%	Inter-day = 1.27%	Inter-day = 1.11%
	Intra-day = 1.93%	Intra-day = 0.13%	Intra-day = 1.32%
Mean (mg/mL)	8.06 ± 0.004	7.17 ± 0.003	0.69 ± 0.01

% RSD = Relative standard deviation

other compounds, at 8.06 ± 0.004 mg/mL. However, the content of chlorogenic acid was the lowest at 0.69 ± 0.01 mg/mL.

In this work, we could detect phenolic acids, sesquiterpenoids, monoterpenoids lignans and isoflavonoids in *A. galactites* with the help of automated data processing with MassHunter Workstation software (unknowns analysis). More importantly, by combining some chloroform fraction analysis, some novel flavonoid derivatives, fatty acids and polyphenols were isolated. In addition, the *A. galactites* root and flower (extracted with chloroform solvent) were found to contain more polysaccharides, which is consistent with the source data suggesting that this plant has anti-immune effects.

Based on HPLC methods, the bioactive compounds in *A. galactites* are gallic acid, caffeic acid and chlorogenic acid. The amount of gallic acid, caffeic acid and chlorogenic acid constituents determined by HPLC in *A. galactites* (Pall.) were 8.06 ± 0.004 mg/mL, 7.17 ± 0.003 mg/mL and 0.69 ± 0.01 mg/mL, for methanol, respectively. In all validation experiments, the % RSD values are less than 2% indicating the high reliability and validity of the developed methods. Previous research on the root of *A. membranaceus* has revealed that the main bioactivities depend on the presence of non-volatile components, such as polysaccharides, saponins and phenolics [18,19].

We have previously investigated for *A. galactites* that DPPH scavenging assay, methanol and ethanol extracts established antioxidant properties with an IC_{50} concentration of 91.04 µg/mL and 93.13 µg/mL, in the ABTS scavenging assay, IC_{50} concentrations of 387.2 µg/mL (methanol extract) and 436.2 µg/mL (ethanol extract), respectively [20]. The results of this study showed that the methanol extract showed more antioxidant activity. Therefore, we used the methanol extract in the HPLC method for the determination of gallic acid, chlorogenic acid and caffeic acid compounds.

Conclusion

The non-targeted qualitative analysis of the representative biologically active principles extracted from *A. galactites* was performed using different analytical techniques. Polyphenolic compounds of interest were identified with the help of HPLC method and % RSD values of below 2% indicating the reliability and validity of the method for further rapid testing of similar Traditional Mongolian medicine (TMM) ingredient and product by providing a basis for its quality control, rational development and utilization of resources. Further 21 compounds were identified with the help of UPC2-Q-TOF-MS, which can be used as indicators for evaluation of the extraction efficiency. In addition, GC-MS/MS studies revealed that the roots, leaves, aerial parts, and flowers of *A. galactites* contain monosaccharide compounds and alkaloids. Collectively, this study provides a reference on the polyphenolic compounds and saponins of *A. galactites*, a medicinal plant with anti-inflammatory properties, supporting the pharmacological effects and radical scavenging activities as reported earlier.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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