



Full Length Article

Identification and Quantification of Chemical Constituents in *Daphne altaica* and their Antioxidant and Cytotoxic Activities

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Abstract

Daphne altaica is an important Kazakh plant medicine used to cure esophageal cancer, gastric cancer, tracheitis, sore throat, and rheumatism. However, information on the chemical composition of *D. altaica* is limited. The chemical markers are unclear and no quality control method has been established. In this study, we developed a new HPLC-MS/MS method to find out the chemical markers in *D. altaica*. Nine compounds were first clarified based on comparison with their external standards and data recorded in literature. Then, an easy, effective, and feasible HPLC-PAD method was established to concurrently quantify seven chemical markers: daphnin, syringin, daphnetin, daphnodorin B, edgeworthin, daphnoretin, and 7-*O*-[β -D-glucopyranosyl]-6-hydroxy-3-[(2-oxo-2*H*-1-benzopyran-7'-yl)-2*H*-1-benzopyran-2-one. Linearity ($R^2 > 0.9995$), repeatability, recovery (96.30–103.67%) and stability analysis found this method to be reliable. The established method can effectively distinguish *D. altaica* from other *Daphne* species and quantify the chemical constitutions in *D. altaica*. The antioxidant activity and cytotoxic activity of the chemical markers were evaluated. This is the first report on quality assessment of *D. altaica*. © 2019 Friends Science Publishers

Keywords: *Daphne altaica*; HPLC-MS/MS; Chemical profile; Quality control; Antioxidant activity; Cytotoxic activity

Introduction

Daphne altaica Pall., locally known as uwsoyqi in Ili Kazakh Autonomous Prefecture of China, is a upright shrub mainly grows in the north of Jungar Basin of Xinjiang, Altai, Manrak and Tarbagatai Mountains of Kazakhstan, and the Altai region of Russia (Eastwood *et al.*, 2009). Its stem bark is a kind of traditional ethno medicine longtime used to treat esophageal carcinoma, gastric carcinoma, tracheitis, sore throat, the common cold, rheumatism and snakebite (Xu and Kongirkhan, 2009). Recent studies have shown that the ethanol extract of *D. altaica* exhibited growth inhibitory effect on human hepatoma (SMMC-7721), esophageal squamous cancer (Eca-109), cervical cancer (Hela) and gastric carcinoma (AGS) cells (Kizaipek *et al.*, 2011). In addition, *D. altaica* has been found to exert a significant therapeutic effect on bronchitis in clinical use (Yang and Yu, 2015).

Only a few daphnane diterpenoids from *D. altaica* have been isolated and reported thus far (Nugroho *et al.*, 2016); while the main chemical components and active components remain unclear. Moreover, *D. altaica* is a toxic medicinal plant (Xu and Kongirkhan, 2009) and can easily

lead to poisoning in clinical use; however, the quality control of *D. altaica* has not yet been established. Authentication of *D. altaica* is conducted via thin-layer chromatography with daphnetin as the chemical marker (Kongirkhan and Zheng, 2010) and no studies on quantitative analysis have been reported. Thus, characterization of chemical components in the ethanol extract of *D. altaica* would be beneficial in determining the main chemical constituents and identifying the potential bioactive chemical compounds. Furthermore, development of a quantification method for chemical markers of *D. altaica* is urgently needed in clinical applications. The traditional Chinese medicine (TCM) is made up of many components and the therapeutic effects are largely due to the synergic effects of multi-components (Liu *et al.*, 2016). Hence, multi-components analysis of chemical markers has traditionally been used to ensure the TCM quality (Chen *et al.*, 2017). Recently, HPLC-PAD method has been diffusely applied to evaluate TCM due to the higher resolving power and reproducibility (Wang *et al.*, 2017).

Our group has been conducting systematic phytochemical investigation on *D. altaica* and isolated more than 40 compounds recently. In this study, we establish a

HPLC-MS/MS method to identify chemical components in the ethanol extract of *D. altaica*. Furthermore a validated HPLC-PAD method for simultaneous quantitative analysis of the seven chemical markers is also developed. Finally, the newly developed method was used to distinguish *D. altaica* from other *Daphne* genus herbs and quantify the contents of seven compounds in three batch samples of *D. altaica*. At the same time, the antioxidant activity and cytotoxic effect of the chemical markers were evaluated. To our knowledge, this is the first study of certification and quantification of main chemical constituents in *D. altaica*.

Materials and Methods

Plant Material

The stem bark samples of *D. altaica* were harvested from Altai region of Xinjiang China and authenticated by the Food and Drug Administration of Xinjiang Province. Voucher specimens (No. DA201101, DA201301, and DA201501) were preserved in our laboratory.

Chemicals and Reagents

Reference standards of (1) daphnin, (2) syringin, (3) daphnetin, (4) 7-*O*-[β -D-glucopyranosyl]-6-hydroxy-3-[(2-oxo-2*H*-1-benzopyran-7'-yl)-2*H*-1-benzopyran-2-one (GHBB), (6) daphnodorin B, (7) edgeworthin, and (9) daphnoretin were purified from the stem bark of *D. altaica* for the first time via conventional column chromatography in our laboratory. The chemical structure of each compound was confirmed by ^1H NMR and ^{13}C NMR and is illustrated in Fig. 1. The purity of each compound calculated by HPLC-PAD was over 98%.

HPLC-grade methanol and acetonitrile were provided by Concord Technology Corporation (Tianjin, China). Chromatographic grade formic acid was supplied by Aladdin Industrial Corporation. Analytical grade ethanol and methanol were provided by Beijing Chemical Works (Beijing, China). Ultrapure water was from Wahaha Group (Hangzhou, China). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were both from Sigma-Aldrich Corporation (Saint Louis, USA).

Standard and Sample Solutions Preparation

Standard solutions: Reference standards of 1 (5.17 mg), 2 (8.10 mg), 3 (8.00 mg), 4 (3.50 mg), 6 (9.08 mg), 7 (2.80 mg), and 9 (2.18 mg) were weighed accurately and then dissolved with 1 mL of methanol, respectively. Then pipetted known volume of aforementioned solution to prepare a 2 mL standard mixture solution. Standard working solutions were prepared by a serial twofold dilution of the standard mixture solution. All solutions were saved at 4°C and filtrated by a 0.45 μm filters before use.

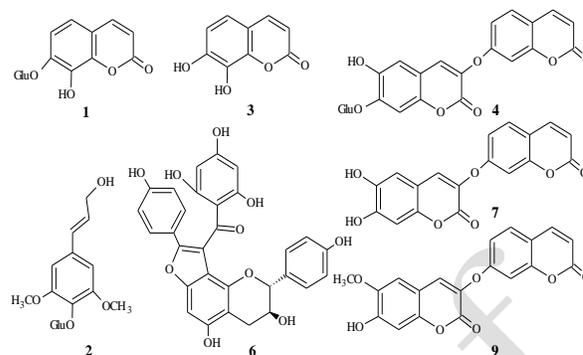


Fig. 1: Structure of analyzed compounds

Sample solutions: A 2.0 g dried stem bark, chopped into small pieces, accurately weighed, was soaked with 100 mL 50% aqueous ethanol for 1 h in a flask and then heated reflux extracted two times (40 min each time). The extracts were collected and evaporated to dryness under reduced pressure after filtration. Remains were then dissolved in 10 mL 80% methanol and centrifuged 10 min at 12,000 rpm. The supernatant was filtered using a 0.45 μm filters for HPLC analysis.

Chromatographic Conditions

The HPLC-PAD method involved a Waters 2695 separation module connected with an auto-sampler, and a 2998 PAD detector set at 280 nm. Separation was conducted using a Zorbax SB-C18 column (Agilent, 4.6 mm \times 150 mm, 5 μm) maintained at 25°C. The mobile phase was made up with methanol (A) and 0.1% formic acid in water (B). The gradient program was listed hereafter: 0–3 min, 5% (A); 3–23 min, 5–30% (A); 23–54 min, 30–42% (A); and 54–68 min, 42–46% (A) with a flow rate of 1.0 mL/min. The injection volume of each analysis was set to 10 μL .

Mass Spectrometry Conditions

HPLC-MS/MS analysis was carried out on a Shimadzu UFLC 20A apparatus (Shimadzu, Japan) interfaced with an API 2000 LC/MS/MS system (AB Sciex, USA) equipped with an APCI and a turbo ion spray (ESI) two ion sources. The mass-spectrometric conditions were as follows: precursor ions scan was executed at an optimized condition: positive and negative ionization mode from m/z 50–1000, ion spray voltage (IS) 4000 V, source temperature (TEM) 320°C, nebulizer gas (GS1) 60 psi, heater gas (GS2) 85 psi and curtain gas 30 psi. The column, flow rate, and time period used were the same as that of HPLC-PAD method throughout the HPLC-MS/MS analyses.

Method Validation

The developed HPLC-PAD method was carefully

validated according to linearity, limit of detection (LOD), limit of quantification (LOQ), precision, stability, repeatability and accuracy.

Antioxidant Activity Test

The antioxidant activities of the compounds were determined by the DPPH radical scavenging method with a slight modification (Zhang *et al.*, 2017a). Briefly, 50 μ L sample solution with different concentrations prepared in methanol was added to 1950 μ L DPPH methanol solution (100 μ M). The final concentrations of the compounds in the mixture were 25, 12.5, 6.25, 3.12 and 1.56 μ g/mL. The absorbance of the mixture at 517 nm was determined after a 30 min chemical reaction in darkness at room temperature. Ascorbic acid was processed like the sample same and used as a positive control. The negative group was 50 μ L methanol instead of the sample solution. The scavenging effect (%) was calculated as: $(1 - \text{experiment group OD}_{517} / \text{negative group OD}_{517}) \times 100\%$. The concentrations of samples scavenged 50% of the DPPH radical was IC_{50} values. All the procedures were carried out at least three times.

Cell Culture

The human cervical cancer (Hela), lung cancer (A549), gastric carcinoma (SGC-7901), hepatocellular carcinoma (HepG2), immortalized cervical epithelial (H8), embryonic lung (MRC-5), gastric epithelial (GES-1) and normal liver (L02) cells were provided by Shanghai Institutes for Biological Sciences of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 medium supplemented 10% fetal bovine serum (FBS, Ausbian), 100 units penicillin and 100 μ g/mL streptomycin (Hyclone, USA) at 37°C in 5% CO₂.

MTT Assay

The cytotoxic effect of compounds was measured by MTT assay. A total of 8000 Cells were placed in a 96-well plate and cultured for 24 h. The tested compounds at concentrations of 0, 0.1, 1, 10, 100 μ g/mL were added and then cultured for 48 h in the 3% serum medium. Subsequently, 20 μ L of 5 mg/mL MTT dissolved in PBS solution was added, the cells were kept at 37°C for another 4 h. The supernatant was carefully discarded and 100 μ L DMSO was put in to make the formazan crystals dissolved by shaking the plates for at least 10 min. Finally, the absorbance of each well was tested at 570 nm using a microscope reader (Thermo election, USA). The inhibition ratio on cell viability (%) was determined as: $(1 - \text{experiment group OD}_{570} / \text{negative control group OD}_{570}) \times 100$. The inhibitory concentration values of compounds (IC_{50}) were calculated through SPSS software (IBM statistics version 21.0).

Results

Identification of Constituents in *D. altaica*

Nine compounds, including three coumarins (daphnetin, edgeworthin and daphnoretin); three coumarin glycosides (daphnin, GHBB and edgeworoside E); two biflavones (daphnodorin I and daphnodorin B) and one syringin (syringin) were clarified by the comparison of mass data, UV spectra and retention time with that of reference standards and data reported in papers. The total ion chromatograms of reference standards and *D. altaica* sample are displayed in Fig. 2 and the obtained mass spectrum data are summarized in Table 1. Our systematic phytochemical isolation and HPLC-MS/MS analysis of *D. altaica* suggested that daphnin, syringin, daphnetin, daphnodorin B, edgeworthin, daphnoretin and GHBB were high content chemical constituents in *D. altaica*. These compounds were analyzed as detail below.

Coumarins and their glycosides: As shown in Table 1, coumarins and their glycosides manifested primarily as precursor ions ($[M + H]^+$ and $[M - H]^-$) in positive and negative ion modes. In addition, coumarin glycosides usually lose their glucose residue (162 Da) to generate other fragments. The mass spectrum of daphnin is shown in Fig. 3A. Daphnoretin showed ion at m/z 335.8 besides the deprotonated molecular ion ($[M - H]^-$) at m/z 350.9 in negative ion mode due to neutral loss of CH₃ (Fig. 3B). Similarly, peak 8 displayed the precursor $[M + H]^+$ at m/z 659.3 and yielded the fragment $[M + H - C_{12}H_{18}O_9]^+$ at m/z 353.1 by losing the substituent. Data showed that peak 8 was assigned as edgeworoside E.

Bioflavonoids: Table 1 indicates that bioflavonoids displayed $[M + H]^+$ at m/z 542.9 in positive ion mode and $[M - H]^-$ at m/z 540.9 in negative ion mode. This cracking way was similar with that of flavan compounds, which generated a characteristic fragment m/z 406.9 in positive ion mode (Fig. 3C). Peak 6 was definitively identified as daphnodorin B by comparison with the reference compound. Peak 5 (UV λ_{max} [MeOH] nm: 287.5, 218.9) was temporarily identified as daphnodorin I.

Syringin: As listed in Table 1, parent ions ($[M + H]^+$ or $[M - H]^-$) of syringin were not detected both in negative and positive ion mode. In negative ion mode, these ions merely appeared as adduct molecular ions, $[M + COOH]^-$ (m/z 417.0) and $[M - H - \text{glc}]^-$ (m/z 208.9); they appeared as $[M + Na]^+$ (m/z 395.1) and $[2M + Na]^+$ (m/z 767.0) in positive ion mode (Fig. 3D–E).

Extraction Method Development

In this work, we assessed the effects of extraction methods and extraction solvents on the chemical composition to determine an efficient extraction program capable of extracting more compounds. Six extraction solvents

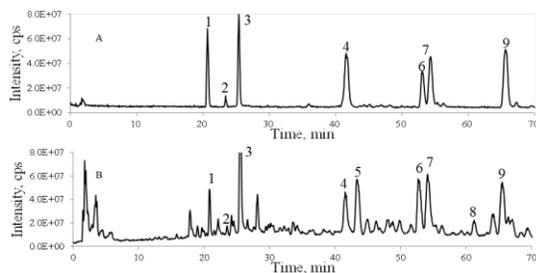


Fig. 2: Total ion chromatograms of (A) standard solution and (B) extract of *D. altaica*

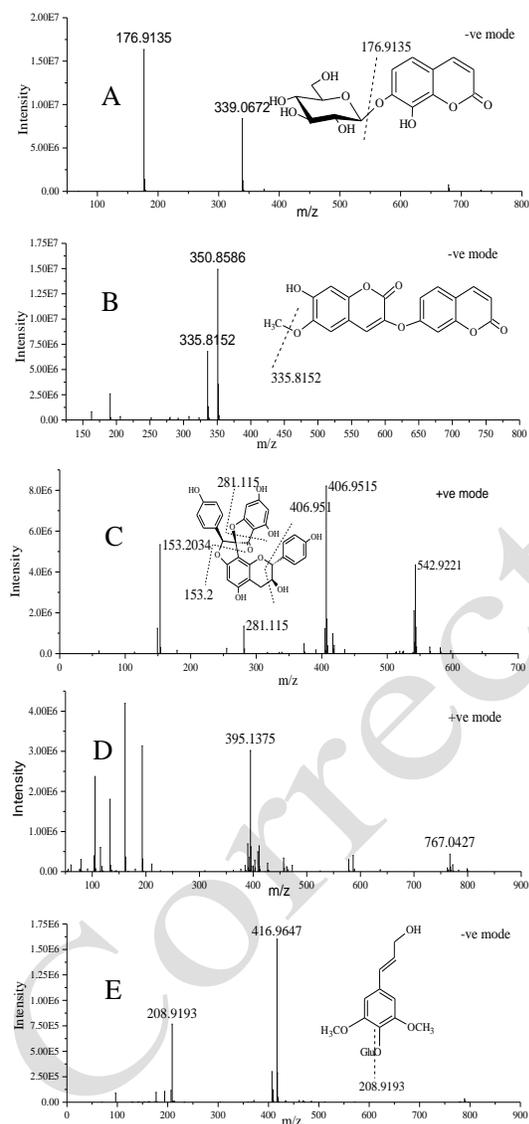


Fig. 3: Mass spectrum and proposed fragment pathways of (A) daphnin, (B) daphnoretin, (C) daphnodorin I and (D-E) syringin

(water, methanol, 95, 70, 50 and 30% ethanol) were applied in reflux extraction, which was performed using

the procedure described in section “Sample solutions”. Peak area analysis of the obtained chromatograms revealed that 50% ethanol elicited more peaks and demonstrated optimal extraction efficiency.

Conventional solvent reflux extraction is laborious, requiring a relatively high temperature, long time window and large amount of solvent. Ultrasound extraction has been identified as a simple and convenient extraction method. Thus, the ultrasound extraction and reflux extraction with 50% ethanol for 40 min were compared and results are displayed in Fig. 4. Ultrasonication showed lower the extraction efficiency for most compounds, particularly compound 6; which may be related to properties of the compound. Therefore, two cycles of 40 min heat-reflux extraction with 50% methanol (2 g samples/100 mL) were used to characterize the chemical profile of *D. altaica* comprehensively.

Optimization of HPLC-PAD Condition

In the HPLC-PAD analysis, system conditions including chromatographic columns, mobile phase compositions, detection wavelength, and gradient program were optimized respectively for better separation. Coumarins have similar structures and a broad range of polarity. Four different types of chromatographic columns (Zorbax SB-C18, Eclipse XDB-C8, Eclipse XDB-C18, 80A Extend-C18 column) with the same dimension (4.6 mm × 150 mm, 5 μm) were tested for acceptable separation. Findings revealed that Zorbax SB-C18 demonstrated the least tailing effect and best baseline separation. Methanol-water and acetonitrile-water are common solvent systems in HPLC separation. Besides, acid can alter the pH values of the mobile phase and reduce tailing. Thus, methanol/water and acetonitrile/water mobile phase systems with different concentrations (0.5, 0.1 and 0.05%) of formic acid were evaluated. Ultimately, methanol and 0.1% formic acid in ultrapure water was chosen for better shape of peaks and more stable baseline. The concentration of formic acid exerted no significant effect on separation. To achieve better detection sensitivity for more classes of compounds, chromatograms at 280, 238, 254, 265, 299, 310, 330 and 365 nm were compared. Finally, the chromatogram at 280 nm displayed more peaks and information. The final HPLC condition was described in “Chromatographic Conditions” section. Representative chromatograms of standard compounds and extract of *D. altaica* are displayed in Fig. 5.

Validation of Analytical Method

The calibration curves were established according to the ratio of peak areas against concentrations of the standard analytes (Table 2). Regression equations of the seven standards possessed favorable linearity ($R^2 > 0.9995$) within the analytical range. The LOD and LOQ were set at a signal-to-noise (S/N) ratio of 10 and 3, respectively. The

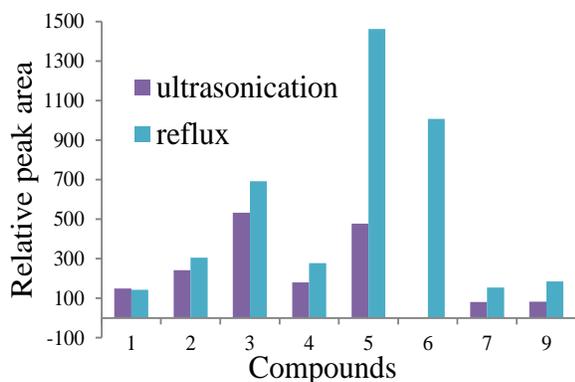
Table 1: Characterization of compounds from extract of *D. altaica*

Comp. No.	RT (min)	Compound	Formula	Precursor (m/z)	MS/MS (m/z)	Pathways
1	20.79	Daphnin	C ₁₅ H ₁₆ O ₉	341.1 [M + H] ⁺ 339.1 [M - H] ⁻	179.1 (100) 176.9 (100)	[M + H - glc] ⁺ [M - H - glc] ⁻
2	23.49	Syringin	C ₁₇ H ₂₄ O ₉	395.1 [M + Na] ⁺ 417.0 [M + COOH] ⁻	767.0 (14) 208.9 (48)	[2M + Na] ⁺ [M - H - glc] ⁻
3	25.48	Daphnetin	C ₉ H ₆ O ₄	179.1 [M + H] ⁺		
4	41.51	GHBB	C ₂₄ H ₂₀ O ₁₂	501.1 [M + H] ⁺ 499.1 [M - H] ⁻	339.1 (100) 336.9 (48)	[M + H - glc] ⁺ [M - H - glc] ⁻
5	43.34	Daphnodorin I	C ₃₀ H ₂₂ O ₁₀	542.9 [M + H] ⁺	407.0 (100) 281.1 (16)	[M + H - C ₈ H ₈ O ₂] ⁺ [M + H - C ₁₄ H ₁₄ O ₅] ⁺
6	52.75	Daphnodorin B	C ₃₀ H ₂₂ O ₁₀	542.9 [M + H] ⁺	406.9 (64) 281.1 (100)	[M + H - C ₂₂ H ₁₄ O ₇] ⁺ [M + H - C ₈ H ₈ O ₂] ⁺ [M + H - C ₁₄ H ₁₄ O ₅] ⁺
7	54.13	Edgeworthin	C ₁₈ H ₁₀ O ₇	339.1 [M + H] ⁺		
8	64.07	Edgeworoside E	C ₃₁ H ₃₀ O ₁₆	659.3 [M + H] ⁺ 657.1 [M - H] ⁻	353.1 (100) 350.9 (88)	[M + H - C ₁₂ H ₁₈ O ₉] ⁺ [M - H - C ₁₂ H ₁₈ O ₉] ⁻
9	65.49	Daphnoretin	C ₁₉ H ₁₂ O ₇	350.9 [M - H] ⁻	335.8 (45)	[M - H - CH ₃] ⁻

Table 2: Linear regression data, LOD, LOQ and precision of seven quantified compounds

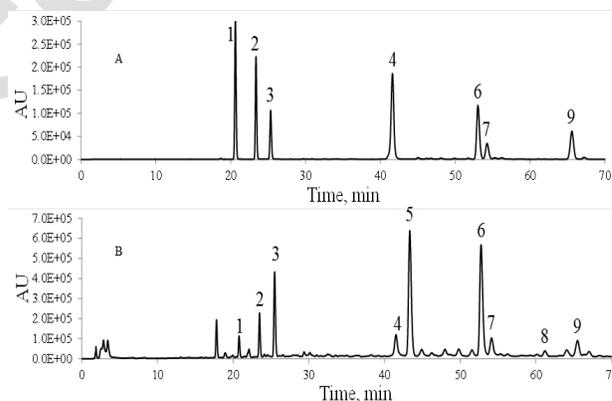
Comp. No	Calibration curve ^a	R ^{2b}	Linear range ($\mu\text{g/mL}$)	LOD ($\mu\text{g/mL}$)	LOQ ($\mu\text{g/mL}$)	Precision ($n=6$, RSD%)
1	Y=10.731x+0.624	0.9995	6.5-207.0	0.205	0.682	2.98%
2	Y=12.461x-48.364	0.9998	20.3-648.0	0.225	0.752	2.93%
3	Y=11.289x-23.576	0.9995	20.0-640.0	0.228	0.769	1.99%
4	Y=14.147x-54.812	0.9997	21.9-700.0	0.304	1.011	2.18%
6	Y=22.933x-337.76	0.9997	49.7-1590.0	0.204	0.681	1.34%
7	Y=8.880x+69.447	0.9995	17.5-560.0	0.437	1.458	2.47%
9	Y=15.838x-76.968	0.9996	15.3-490.5	0.306	1.013	2.84%

^a y, peak area/1000; x, the concentration of reference compound ($\mu\text{g/mL}$)

^b R², correlation coefficient of regression equations

Fig. 4: Effects of extraction techniques on extraction efficiency of target compounds

ranges of LOD and LOQ values were 0.204–0.437 $\mu\text{g/mL}$ and 0.681–1.458 $\mu\text{g/mL}$, respectively (Table 2). Precision was analyzed at one concentration with six replicates of the extracted sample in 1 day. Table 2 shows the relative standard deviation (RSD) values of the seven tested compounds for precision. The RSD values were from 1.34 to 2.98%.

Six replicates from one batch sample were performed and tested to appraise repeatability. The results of repeatability are given in Table 3. The RSDs were less than 3%, suggesting good repeatability of this method. During


Fig. 5: HPLC chromatograms of (A) standard solution and (B) extract of *D. altaica*

stability test, the prepared sample was kept at room temperature and respectively measured at 0, 2, 4, 8, 12 and 24 h. All RSDs were less than 3% and all compounds in the solution were stable within 24 h at room temperature (Table 3). The data revealed good accuracy of this method with average recoveries ranging from 96.30 to 103.67% and RSD values less than 3.0% ($n = 6$) for all seven chemicals. These validation results implied that the established HPLC-PAD method was trustworthy and accurate.

Table 3: Repeatability, stability and recovery of seven quantified compounds

Comp. No.	Repeatability		Stability		Recovery ^b	
	Mean±SD (μg/mL)	RSD ^a (%) (n=6)	Mean±SD (μg/mL)	RSD (%) (n=6)	Mean±SD (%)	RSD (%) (n=6)
1	123.46±3.30	2.67	104.65±2.96	2.83	102.31±2.46	2.41
2	207.76±3.66	1.76	182.48±5.16	2.83	98.63±3.94	2.87
3	527.94±10.28	1.95	471.92±10.07	2.13	96.74±2.31	2.39
4	233.82±6.10	2.61	214.79±5.28	2.46	100.21±2.92	2.92
6	702.98±13.4	1.91	624.86±11.80	1.89	103.67±1.50	1.44
7	327.53±9.88	3.00	280.25±6.17	2.20	96.30±0.68	0.71
9	161.35±4.61	2.86	142.43±2.55	1.79	100.42±1.04	1.03

^aRSD (%) = (SD/mean) × 100%, ^bRecovery (%) = [(detected-original)/spiked] × 100

Table 4: Contents of seven compounds in different samples of *D. altaica*

Sample No.	Contents (mg/g)							Similarity value ^a
	1	2	3	4	6	7	9	
1	0.61±0.01	1.03±0.02	2.63±0.08	1.19±0.03	3.54±0.03	1.66±0.05	0.82±0.02	0.815
2	11.61±0.07	5.53±0.05	3.90±0.04	3.70±0.02	4.49±0.08	1.80±0.02	1.93±0.03	0.991
3	10.12±0.11	5.07±0.04	3.58±0.02	4.54±0.03	2.98±0.03	1.90±0.03	1.86±0.01	0.996
A	-	-	-	-	-	-	-	0.035
B	-	-	-	-	-	-	-	0.511

A: *Daphne giraldii*, B: *Daphne genkwa*, - No relevant data

a: The similarity of each chromatogram to *D. altaica* simulative mean chromatogram

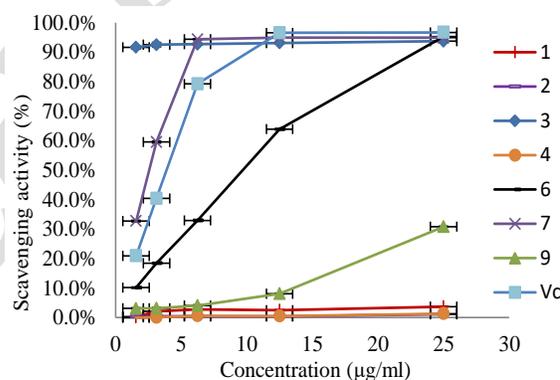
Sample Analysis

Three batches of *D. altaica* samples collected in different years and two other *Daphne* species (*D. giraldii* and *D. genkwa*) were analyzed according to the newly proposed HPLC-PAD method. The HPLC chromatograms were compared by the software of *Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine* (Version 2004A). The similarity values of all the samples were determined by a correlation coefficient method. The closer the value of correlation coefficient is to 1, the more similar the two fingerprints are. As presented in Table 4, the similarity values of three batches of *D. altaica* samples were above 0.8, while *D. giraldii* and *D. genkwa* were 0.035 and 0.511. *D. giraldii* and *D. genkwa* are all common traditional Chinese medicines, the barks of which were very similar to that of *D. altaica*. The proposed HPLC-PAD method was effective to identify and difference of *D. altaica* with other related herbs.

The contents of chemical markers in three batches of *D. altaica* samples were analyzed and are presented in Table 4. The data showed that all *D. altaica* samples contained the seven compounds. These seven compounds were highly abundant and the contents ranged from 0.61 to 11.61 mg/g, all above 1.0 mg/g except compound 1 in sample 1.

Antioxidant Activity

The results of the DPPH free radical scavenging effect are presented in Fig. 6, which showed that compound 3, 6 and 7 exhibited noticeable radical scavenging abilities. Moreover, compound 3 showed the most DPPH scavenging activity (> 90%). The compound 9 exhibited less potency with 30.88% at 25 μg/mL. The antioxidant activities IC₅₀ values of compound 3, 6, 7 and 9 were 0.63 ± 0.01, 7.81 ± 0.07,

**Fig. 6:** The scavenging effect of compounds on DPPH

2.27 ± 0.03 and 58.63 ± 1.30 μg/mL respectively compared with the IC₅₀ value 3.17 ± 0.01 μg/mL of the positive control (ascorbic acid). However, almost no DPPH free radical scavenging effect was observed for compound 1, 2 and 4.

The antioxidant capacity of the coumarins was also found to be associated with hydroxyl groups' number and location. The coumarins with ortho-hydroxyl had better antioxidant potency (compound 3 and 7), the coumarin with one hydroxyl and one methoxy group at the ortho position had poor activity (compound 9) and the coumarin with one hydroxyl and one glycoside group exhibited almost no antioxidant effect (compound 1 and 4).

Cytotoxic Activity

We investigated the cytotoxic activity of the main constituents in *D. altaica*. The IC₅₀ values of these

Table 5: Cytotoxic activity of compounds on human cancer cell and normal cell lines

Cell lines	IC ₅₀ values of compounds (μg/mL)							
	1	2	3	4	6	7	9	
Hela	>200	>200	19.17±2.54	>200	No effect	11.09±1.73	50.79±5.29	
H8	-	-	51.77±5.63	-	-	42.64±3.87	45.16±4.29	
A549	>200	>200	38.77±1.80	>200	No effect	65.26±2.92	54.51±4.53	
MRC-5	-	-	54.52±4.65	-	-	146.66±1.78	44.01±1.69	
SGC-7901	>200	>200	16.68±2.04	>200	No effect	18.61±1.76	28.95±0.73	
GES-1	-	-	43.11±2.03	-	-	146.18±8.15	>200	
HepG2	>200	>200	37.65±3.86	>200	>200	48.23±4.20	58.06±4.97	
L02	-	-	154.16±3.92	-	-	>200	>200	

compounds on cancer cell and normal cell lines are shown in Table 5. Among these main compounds, compounds 3, 7 and 9 were active constituents with relatively less IC₅₀ values on cancer cell lines. These compounds showed different inhibitory effects on four human cancer cells (Hela, A549, SGC-7901, HepG2). The stronger inhibition effects of these compounds on SGC-7901 and Hela indicated a better treatment effects on gastric carcinoma and cervical cancer. On the other hand, the inhibition ratio of compounds 3, 7 and 9 on four normal cell lines was examined to evaluate the cytotoxic to normal human cells. As displayed in Table 5, the obtained IC₅₀ values of these compounds on normal cell lines were much higher than aforementioned cancer cells except compound 9 (H8, 45.16 ± 4.29 μg/mL; MRC-5, 44.01±1.69 μg/mL). It indicated that compounds 3, 7 and 9 were less cytotoxic to normal cell lines.

Discussion

Daphne altaica is a traditional Kazakh herbal medicine with well treatment for gastric carcinoma and tracheitis etc. HPLC-MS/MS analysis above and our previous systematic phytochemical isolation revealed that, *D. altaica* is rich in coumarins with variety of structural variations *i.e.*, monomeric coumarins, bis-coumarins and their glycosides. Coumarin is a class of compound with the oxygen heterocyclic structures and shows the $[M + H]^+$ ion in the MS (Zhong *et al.*, 2015) and most of their substituent will be lost if any. Bioflavonoids were another representative compound with similarity structures in *D. altaica*. Positive ion mode produced more abundant and specific signals than the negative ion mode in MSⁿ experiments.

Daphnetin and its glucosides daphnin are widespread in *Daphne* genus and have chemotaxonomic relevance (Venditti *et al.*, 2017). GHBB was an uncommon class of C₃-C₇-linked bis-coumarins, which was only separated from the roots of *D. oleoides* and could be considered a characteristic compound of *D. altaica*. From a biological activity standpoint, the bioactivity of daphnetin has been extensively investigated. Findings have revealed: anti-inflammatory effects (Li *et al.*, 2017), anti-cancer activity *in vitro* (Kumar *et al.*, 2016) and antioxidant effects against oxidative damage (Lv *et al.*, 2017). Daphnin has also shown wound healing properties by increasing collagen production and expression (Kim *et al.*, 2017). The antidiabetic effect of

edgeworthin demonstrated potent inhibitory activity against α-glucosidase (Zhao *et al.*, 2015). Daphnoretin exhibited predominant antiproliferative activity on HepG2 and Hep3B cancer cell line (Yang *et al.*, 2016). Bioflavonoids were another representative compound in *D. altaica* possessing anti-cancer (Zheng *et al.*, 2007) and anti-viral (Hu *et al.*, 2000) activities. Syringin, a phenolic glycoside, was abundant in *D. altaica* and reported protective effects against acute lung injury induced by lipopolysaccharide (Zhang *et al.*, 2017b). Taken together, daphnin, syringin, daphnetin, daphnodorin B, edgeworthin, daphnoretin, and GHBB were main chemical constituents in *D. altaica* and possessed a wide range of activities. Thus, these seven compounds could be used as chemical markers to conduct the chemical evaluation and quality analysis of *D. altaica*.

Macroscopic and microscopic authentications of herbs are the usually used standard methods, but these methods are limited in scope. Chemical identification and quantification of herbs are essential means for quality control as it is directly related to medicinal functions (Lu *et al.*, 2005). The newly established HPLC-PAD method successfully differentiated the *D. altaica* samples from two other related species. The contents of chemical markers in three batches were all at relatively high levels, which is consistent with the results of HPLC-MS/MS analysis. However, actual amounts of the seven compounds in the three samples showed obvious variation with the largest variation for compound 1. The considerable variability the herb shown may be related to the time of harvest and growth conditions, such as soil and climate etc. (Wu *et al.*, 2018). Standardization of planting is an important aspect for ensuring the quality of herbal medicines.

There are few reports on the biological properties of *D. altaica* (Kizaibek *et al.*, 2011). The antioxidant activity of chemical markers of *D. altaica* was investigated through the DPPH radical scavenging assay. The lower antioxidant activities IC₅₀ values of compounds suggest that *D. altaica* has strong antioxidant bioactivity. Daphnetin, daphnodorin B, edgeworthin, and daphnoretin were the bioactive components. For the structure-activity relationship, the results demonstrated that the ortho-hydroxyl was the main contributor of radical scavenging activity of coumarins (Thuong *et al.*, 2010). The cytotoxic activity results indicated that compounds Daphnetin, edgeworthin, and daphnoretin were the main anti-cancer active ingredients in

D. altaica and have selectivity against cancer cells whilst less toxic to human normal cells.

Conclusion

The phytochemical profile of *D. altaica* was studied by the proposed HPLC–MS/MS method. Nine components, including three coumarins, three coumarin glycosides, two biflavones and one syringin were identified for the first time. Seven chemical markers of *D. altaica* were simultaneously determined using a validated HPLC–PAD method, which was successfully used for identification and quantitative assessment of three batches of *D. altaica*. Daphnetin, daphnodorin B, edgeworthin and daphnoretin were the bioactive components for antioxidant and cytotoxic activity of *D. altaica*. This study thus provided reference for further quality control and pharmacological studies of *D. altaica*.

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