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Rapid Profiling and Identification of Triterpene Saponins in Three Different *Cephalaria* Species by UPLC-ESI-MS/MS

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Abstract: Triterpene saponins are bioactive glycosides which are responsible for the defense mechanism of the plants and have extensive structural diversity in many plant species. In this study, profile of triterpene saponins was determined in the crude *n*-BuOH extracts of *Cephalaria hirsuta*, *Cephalaria elazigensis* var. *elazigensis*, and *Cephalaria procera* taxa (Caprifoliaceae) by ultra pressure liquid chromatography coupled with electrospray ionization tandem mass spectrometry. The UPLC-ESI-MS/MS method has been validated to provide linearity, recovery, precision (repeatability), limit of detection and limit of quantification parameters for quantitative analysis. Identification and quantification of the compounds were based on a group of fragment ions and ESI-MS spectra; precursor ions were observed as $[M+Na]^+$ and $[M+H]^+$ in positive mode for molecular mass information. Thirty nine saponins were identified and quantified by reversed-phase liquid chromatography (RP-LC) and they were separated on C18 reverse phase column (50 mm x 2.1 mm, 1.7 μm) by methanol-water gradient containing 0.1 mM ammonium formate solution as a mobile phase. Among the detected compounds, 28 compounds in *C. hirsuta*, 4 compounds in *C. elazigensis* var. *elazigensis* and 22 compounds in *C. procera* were identified and quantified based on their retention times and mass spectra in comparison with the data of references which were isolated in our previous studies. In this study, rapid application of an UPLC-ESI-MS/MS approach for resolving the chemical content of complex crude *n*-BuOH extracts of three *Cephalaria* species were presented, for the first time.

Keywords: *Cephalaria*; saponin; UPLC-ESI-MS/MS.

1. Introduction

Cephalaria Schrad. ex Roem. & Schult. (Caprifoliaceae) spreads out worldwide, mainly in two different regions of South Africa and Holarctic Kingdom [1]. *Cephalaria* species has totally 41 taxa in Turkey and some of them are widely used as traditional medicine and nutrition purposes. For example, yellow flowers of *C. gigantea*, the seeds of *C. syriaca* Schrad. and the fruiting capitula of *C. balansae* Raus. are used for medicinal drug and food conservation in Turkey [2,3].

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According to literatures, *Cephalaria* species have various components, including triterpenes, iridoids, flavonoids, alkaloids, lignans, and their glycosidic compounds [4-8]. Furthermore, essential oil profile, essential fatty acid components [9-11] and biological data have been also reported for various *Cephalaria* species [6-8].

Previous reports indicated that numerous plant species in conventional medicine include saponins, which are biochemically and pharmaceutically appealing compounds [12]. These compounds and plants are regarded as interesting for their pharmacological importance due to their varied biological activities, such as anti-inflammatory, antiviral, antidiabetic, cardiovascular, cytotoxic, antifungal and antitumor activities, [4-7, 12]. For this reason, more rapidly analytical methods are needed to identify and characterize triterpene saponins in this field. Most chromatographic methods, such as thin layer chromatography (TLC), high performance liquid chromatography-evaporative light scattering detection (HPLC-ELSD), liquid chromatography-electrospray ionization mass spectrometry (ESI-LC-MS), liquid chromatography-ultraviolet nuclear magnetic resonance (LC-UV-NMR) and liquid chromatography-photodiode array electrospray ionization tandem mass spectrometry (LC-PDA-ESI-MS/MS) have been reported for quantitative determination of triterpene glycosides [13-15]. Especially, electrospray ionization multi-stage tandem mass spectrometry (ESI-MS^n) and nano-HPLC (nLC) presented a new way for natural product chemistry [16].

In the light of these literatures, chemical composition and biological activity results of *Cephalaria* species prompted us to optimize a new, rapid and easy chromatographic method for saponin determination. Quantitative determination of triterpene saponins by UPLC-ESI-MS/MS makes the prediction of known and possibly new molecules in crude extracts easier. In this study, we mostly motivated on the analysis of known triterpene glycosides in *Cephalaria hirsuta* [1], *Cephalaria elazigensis* var. *elazigensis* [17] and *Cephalaria procera* [1], rather than identification and complete profiling of new compounds. This application on MeOH extracts of three *Cephalaria* species was investigated, for the first time. As a result, totally 40 natural products, 39 saponins and 1 hederagenin aglycone, were determined simultaneously by an easy, sensitive and timesaving chromatographic method. In other words, 28 compounds in *C. hirsuta*, 4 compounds in *C. elazigensis* var. *elazigensis* and 22 compounds in *C. procera* were identified and their contents in *n*-BuOH fractions were determined. With this proposed method, the isolation or purification procedures used to determine these triterpenoid saponins in crude extracts are no more time consuming and expensive. Besides that, we also developed a combined chromatographic technique (UPLC-ESI-MS/MS) which can help the detection process of triterpenoid saponins at minimum valid concentrations.

2. Materials and Methods

2.1. Plant Materials and Chemicals

All studied *Cephalaria* species, which were harvested in vegetative season of 2013 by R.S. Gokturk, were kept in Herbarium Research and Application Centre of Akdeniz University. *Cephalaria hirsuta* was collected from Erzurum, Erzurum-Cat road 5 km (AKDU 4142), *Cephalaria elazigensis* var. *elazigensis* was collected from Elazig-Maden, Maden-Ergani road 8 km (AKDU 1962) and *Cephalaria procera* was collected from Sivas-Zara, Zara-Imranli road (AKDU 3535).

Methanol (95% purity), *n*-butanol (99% purity) and *n*-hexane (99% purity) were purchased from VWR Chemicals Corporation (Radnor, USA). Merck Millipore system (Billerica, MA) was used for purification of tap water to ultra-pure degree. Ammonium formate, LC-MS grade methanol and LC-MS grade water purchased from Sigma–Aldrich (St Louis, MO).

In our previous investigations, 39 saponins and 1 hederagenin aglycone, which are used as reference standards, were isolated from different *Cephalaria* species and identified by our research group in our research laboratory [4-5, 18-24]. Purity of all compounds was calculated from ^{1}H-NMR spectra and results are given in S1 (in Supporting Information).

2.2 Preparation of Standard and Sample Solutions

After harvesting, whole plants were dried and milled. Grinded samples of *C. hirsuta* (2.0 g), *C. elazigensis* var. *elazigensis* (2.0 g) and *C. procera* (2.0 g) were extracted with methanol (3 x 10 mL) by a laboratory mixer (Silverson, L5M-A, USA) for 3 h at room temperature in a polypropylene tube. The extracts were concentrated under reduced pressure till dryness at 40 ^{o}C. MeOH residues were extracted with *n*-BuOH : H_2O (1 : 1, 3×5 mL) solvent system. After the separation and evaporation of *n*-BuOH and H_2O portions, the *n*-BuOH fractions were defatted with *n*-hexane to remove chlorophylls and oily partitions. After that, the saponin-riched *n*-BuOH fractions were concentrated under reduced pressure (Extract mass: for *C. hirsuta* 71.0 mg, for *C. elazigensis* var. *elazigensis* 90.8 mg and for *C. procera* 48.0 mg). 1.0 mg of each residue was dissolved in 10.0 mL MS grade methanol and filtered through a 0.22 μm PTFE syringe filter for the UPLC-ESI-MS/MS analysis. Dilution factor (D.F.) is $1x10^4$ in this operation.

Before validation studies, a trial analyse was performed to estimate the concentrations of saponins in the *n*-BuOH fractions. The highest concentration was approximately 10000 mg/kg for Scoposide G in the *n*-BuOH fraction of *C. hirsuta*. Due to high concentration of Scoposide G, highest level of calibration range was selected as 1.0 mg/L. So, stock solution of forty triterpenoid compounds were prepared by dissolving 1.0 mg of each triterpenoid compounds in the same 10 mL volumetric flask. Six levels (0.025, 0.050, 0.100, 0.250, 0.500, and 1.00 mg/L) of calibration standards were prepared in 5 mL volumetric flask with MeOH. The results were calculated considering dilution factor which is $1x10^4$.

2.3 Instrumentation

Determination of compounds was performed on an ultra-high-performance liquid chromatography (UPLC) combined with electrospray ionization (ESI) tandem mass spectrometer (MS/MS) (Waters, Milford, MA). The mass spectrometer was controlled by MassLynx 4.1 software. The samples were separated on a BEH C18 column (50 mm x 2.1 mm, 1.7 μm particle size; Waters).

2.3.1 Chromatographic Conditions

The mobile phases were 0.1 mM ammonium formate solution in H_2O (A) and 0.1 mM ammonium formate solution in methanol (B). Gradient programme was adjusted to 0.4 mL/min flow rate at 40 ^{o}C. After 5 μL injection, (B) linearly increased from 5% to 50% in 2 min, linearly increased to 75% in 1 min, linearly increased to 95% in 1.5 min and stayed at this concentration for 0.5 min. Then, the system was returned to the initial conditions in 1 min.

2.3.2 Mass Spectrometry Conditions

Tandem mass spectrometer with an ESI system operated in positive-ion mode. The desolvation gas (N_2) flow and cone gas (Ar) flow were set at 500 L/hr and 100 L/hr, respectively. Capillary energy was set at 3.0 kV. Cone energies, collision energies, precursor and transition ions were set up individually considering their response and peak shapes. Ion source and desolvation gas (N_2) were heated at 120 ^{o}C and 350 ^{o}C, respectively. The scan range was *m/z* 50–2000, and the dwell time was 0.100 second.

2.4 Method Validation

Linearity parameter for each compound was determined by analyzing standard solutions. The linearity range was determined as 0.025 to 1.0 mg/L for all compounds. External calibrations were applied to calculate the concentration of triterpenoid compounds in the *n*-BuOH fractions of three plant extracts. The recovery experiments were evaluated at two fortification levels (500.0 mg/kg and

1000.0 mg/kg) and three times injection. Dilution factor (D.F.= $1x10^4$) was used to calculate the results. The precision (repeatability) of the reported method was evaluated by repeating the measurements 6 times at 500.0 mg/kg and 1000.0 mg/kg spiked the concentrations in the same day. The LOD and LOQ values of triterpenoid compounds were calculated *via* signal to noise (S/N) ratio.

3. Results and Discussion

3.1 Optimization of UPLC Conditions

UPLC conditions were optimized considering peak shapes, resolutions and responses of reference substances. Varied UPLC parameters were examined and compared. Different brand chromatographic columns (same adsorbent, particle size, length and ID) didn't provide an advantage to the BEH C18 column. Methanol-water gradient was used as mobile phase which is more effective than acetonitrile-water gradient in resolving peaks. Under the optimized UPLC-MS/MS conditions, all compounds could be identified in the crude extract solutions by comparing their retention times and transition ions with reference solutions.

3.2 Application for ESI-MS/MS

To identify *m/z* ratio of precursor and transition ions for quantitative analysis, forty individual reference substances were infused directly to the mass spectrometer in ESI positive mode. The cone energies were optimized individually according to peak heights and shapes for the precursor ions. The collision energies were adjusted to achieve adequate sensitivity for the transition ions and the list is given in S2 (Supporting Information).

Calculated ions of compounds have taken from literature data of references [4-5, 18-24]. The corresponding $[M+Na]^+$ and $[M+H]^+$ ions at *m/z* 1406.00 for davisianoside A [19], *m/z* 1243.61 for aytachoside A [20], *m/z* 627.16 for anemoclemoside A [25], *m/z* 951.25 for akebia saponin D [26], *m/z* 1391.96 for gazipashoside B [23], *m/z* 1537.70 for aristatoside B [22], *m/z* 1568.00 for cilicicoside I [18], *m/z* 1421.14 for macranthoidin B [27], *m/z* 1567.70 for aristatoside A [22], *m/z* 1229.87 for decaisoside E [28], *m/z* 1259.60 for macranthoidin A [29], *m/z* 1230.50 for elmalienoside C [4], *m/z* 1259.60 for elmalienoside A [4], *m/z* 1375.60 for balansoid D [30], *m/z* 1097.55 for dipsacoside B [31], *m/z* 1098.56 for elmalienoside B [4], *m/z* 1375.60 for balansoid B [30], *m/z* 1375.46 for gazipashoside A [23], *m/z* 1507.8 for scoposide C [5], *m/z* 1213.60 for balansoid C [30], *m/z* 1097.55 for 3-O-*β*-D-glucopyranosyl-(1→3)-*α*-L-rhamnopyranosyl-(1→2)-*α*-L-arabinopyranosyl hederagenin 28-O-*β*-D- glucopyranosyl ester [32], *m/z* 1375.66 for scoposide B [5], *m/z* 1067.14 for decaisoside D [28], *m/z* 935.49 for 3-O-*α*-L-rhamnopyranosyl-(1→2)-*α*-L-arabinopyranosyl hederagenin 28-O-*β*-D-glucopyranosyl ester [33], *m/z* 1200.09 for aristatoside C [22], *m/z* 1213.50 for scoposide F [24], *m/z* 1068.40 for sapindoside C [34], *m/z* 1213.59 for scoposide G [24], *m/z* 1082.50 for scoposide A [5], *m/z* 773.44 for *α*-hederin [35], *m/z* 1051.54 for scoposide D [5], *m/z* 1375.60 for isacoside [21], *m/z* 905.48 for sapindoside B [36], *m/z* 1051.70 for lycicoside II [18], *m/z* 935.50 for macranthoside A [37], *m/z* 1082.61 for davisianoside B [19], *m/z* 1051.8 for balansoid A [30], *m/z* 495.30 for hederagenin [32], *m/z* 890.47 for scoposide E [5] and *m/z* 1175.78 for *β*-sitosterol glucoside [38] were selected as precursor (parent) ions. Manual integration of peaks at infusion stage created differences between *m/z* of calculated ions and *m/z* of observed precursor ions. Cone energies were between 60 V and 160 V for all observed precursor ions. Collision energies were between 40 V and 90 V for all observed transition ions.

3.3 Method Validation

Method validation was applied with linearity, precision (repeatability), recovery, limit of detection (LOD) and quantification (LOQ) parameters. Due to the rich content of *n*-BuOH fraction,

Cephalaria hirsuta plant was chosen to assess recovery, precision (repeatability), limit of detection (LOD) and limit of quantification (LOQ) parameters. These parameters are not needed for other fractions because of the same chemical properties.

3.3.1 Linearity

The linearity parameter for triterpene compounds were examined by analyzing standard solutions of six-point linear plot in the range of 0.025–1.0 mg/L. Correlation coefficients for all analytes in the working range were calculated as ≥ 0.9974. The linear regression equations and correlation coefficients are given in S1 (in Supporting Information).

3.3.2 Recovery and Precision (Repeatability)

The recovery parameter was accomplished with *n*-BuOH fraction of *Cephalaria hirsuta* at 500.0 mg/kg and 1000.0 mg/kg concentration levels for each analytes. Due to content similarities between three species just *n*-BuOH fraction of *Cephalaria hirsuta* was performed as representative matrix. For the preparation of 500.0 mg/kg concentration level, 5 µL of the standard stock solution (100 mg/L) was added on 1.0 mg *n*-BuOH fraction. Then, 10 µL of the standard stock solution was added on 1.0 mg *n*-BuOH fraction to prepare 1000.0 mg/kg concentration level. Then, this spiked fractions was diluated to 10 mL with methanol in a volumetric flask (Dilution Factor : $1x10^4$). The unspiked *n*-BuOH fraction was also analyzed to determine the triterpene saponin concentrations in blank samples. The recoveries of each saponin at *n*-BuOH extract were evaluated. Recoveries were calculated according to the following formula **(**Eq. 1), which changed in between 90.0% and 105.9%.

$$\text{Recovery (\%)} = \frac{\text{Calculated Conc. - Blank Sample Conc.}}{\text{Spiked Conc.}} \times 100 \qquad \text{(Equation 1)}$$

For the precision (repeatability) parameter, *n*-BuOH extract spiked at two concentration levels (500.0 mg/kg and 1000.0 mg/kg) and analysed in six replicate during one day. The sample preparation was same as recovery procedure. Relative standard deviations (RSD) of precision were as determined as between 1.2% and 8.9%. The representative data for recovery and RSD (%) are given in S1 (in Supporting Information).

3.3.3 Limit of Detection (LOD) and Limit of Quantification (LOQ)

The limit of detection (LOD) of triterpene saponins were obtained by multiplying the S/N ratio by 3. Then, LOQ values were calculated by multiplying the S/N ratio by 10. The calculated values for LOD and LOQ are given in S1 (in Supporting Information).

3.3.4 Measurement of Uncertainty

Linearity, repeatability (precision) and recovery parameters were evaluated to calculate uncertainty [39]. All results which have confidence level % 95 were given with measurement of uncertainty.

3.4 Determination and Quantification of Saponins in n-BuOH Fractions

Quantitative determination of the compounds was applied by comparing reference and *n*-BuOH extracts solutions based on retention times and transition ions.

Table 1. Contents of saponins as mg/kg in *n*-BuOH fractions of *C. hirsuta*, *C. elazigensis* var. *elazigensis* and *C. procera.*

Peak No	Compounds	*C. hirsuta*	*C. elazigensis* var. *elazigensis*	*C. procera*
1	Davisianoside A	<LOQ	<LOQ	<LOQ
2	Aytachoside A	<LOQ	<LOQ	<LOQ
3	Anemoclemoside A	<LOQ	<LOQ	<LOQ
4	Akebia Saponin D	<LOQ	<LOQ	<LOQ
5	Gazipashoside B	545.35±49.6	<LOQ	36.90±3.4
6	Aristatoside B	88.05±8.5	<LOQ	<LOQ
7	Cilicicoside I	<LOQ	<LOQ	<LOQ
8	Macranthoidin B	<LOQ	<LOQ	46.89±3.0
9	Aristatoside A	37.80±1.8	<LOQ	<LOQ
10	Decaisoside E	336.33±18.8	<LOQ	146.23±8.2
11	Macranthoidin A	229.83±15.6	<LOQ	151.91±10.3
12	Elmalienoside C	439.69±42.2	<LOQ	131.33±12.6
13	Elmalienoside A	108.54±4.9	<LOQ	22.33±1.0
14	Balansoid D	<LOQ	<LOQ	<LOQ
15	Dipsacoside B	1063.22±62.7	<LOQ	994.00±58.6
16	Elmalienoside B	308.35±12.0	<LOQ	290.93±11.3
17	Balansoid B	<LOQ	<LOQ	<LOQ
18	Gazipashoside A	111.68±6.6	<LOQ	<LOQ
19	Scoposide C	43.43±3.7	<LOQ	<LOQ
20	Balansoid C	<LOQ	<LOQ	<LOQ
21	3-*O*-*β*-D-glucopyranosyl-(1→3)-*α*-L-rhamnopyranosyl-(1→2)-*α*-L-arabinopyranosyl hederagenin 28-*O*-*β*-D- glucopyranosyl ester	148.40±13.9	<LOQ	92.71±8.7
22	Scoposide B	81.25±7.2	<LOQ	62.20±5.5
23	Decaisoside D	733.27±64.5	<LOQ	242.28±21.3
24	3-*O*-*α*-L-rhamnopyranosyl-(1→2)-*α*-L-arabinopyranosyl hederagenin 28-*O*-*β*-D-glucopyranosyl ester	227.33±19.8	<LOQ	120.57±10.5
25	Aristatoside C	200.30±16.2	<LOQ	<LOQ
26	Scoposide F	972.94±47.7	8.84±0.4	6.49±0.3
27	Sapindoside C	95.95±5.5	<LOQ	13.02±0.7
28	Scoposide G	10060.69±814.9	<LOQ	651.58±52.8
29	Scoposide A	3062.46±303.2	41.23±4.1	214.00±21.2
30	α-Hederin	243.35±21.7	<LOQ	152.69±13.6
31	Scoposide D	152.96±119	<LOQ	8.57±0.7
32	Isacoside	<LOQ	<LOQ	<LOQ
33	Sapindoside B	386.30±22.8	<LOQ	170.89±10.1
34	Lycicoside II	208.42±11.0	<LOQ	15.73±0.8
35	Macranthoside A	629.21±42.2	8.54±0.6	214.70±14.4
36	Davisianoside B	66.71±3.3	<LOQ	<LOQ
37	Balansoid A	<LOQ	<LOQ	<LOQ
38	Hederagenin	<LOQ	<LOQ	<LOQ
39	Scoposide E	156.44±9.9	<LOQ	<LOQ
40	*β*-Sitosterol glucoside	1024.20±88.1	254.26±21.9	166.97±14.4

The appropriate two transition ions were selected for determination and the transition ions have more response were integrated to calculate concentrations of triterpene saponins in *n*-BuOH fractions. The concentrations of *n*-BuOH fractions were presented in Table 1. Major components of *n*-BuOH fractions of three species were shown as Figures 1-3.

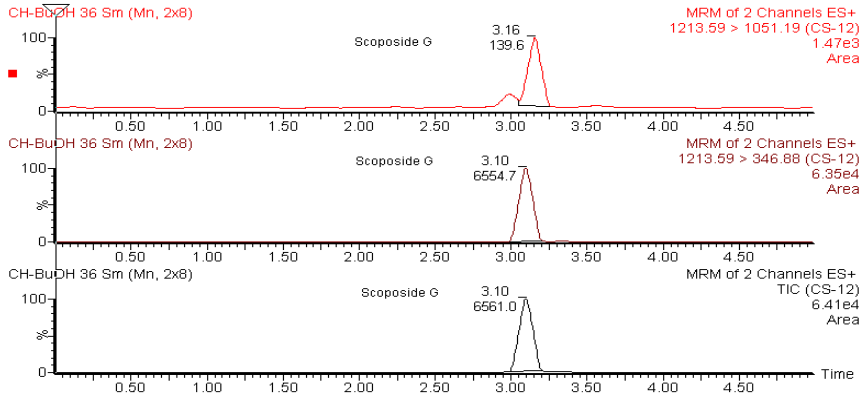

Figure 1. Chromatogram of scoposide G (10060.69 mg/kg) as major component in *C. hirsuta.*

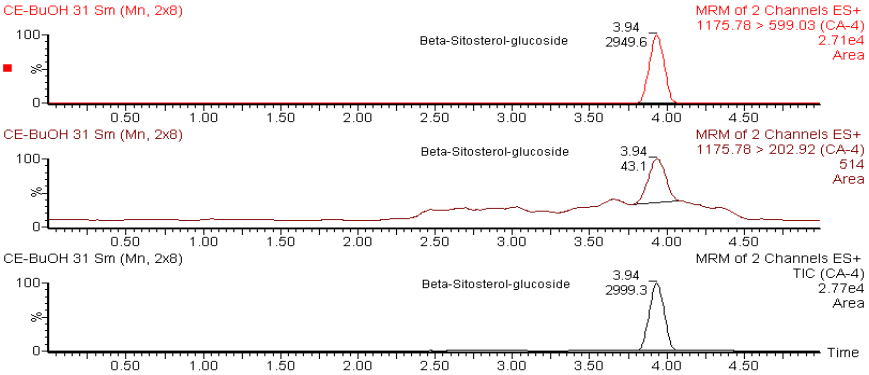

Figure 2. Chromatogram of *β*-sitosterol-glucoside (254.26 mg/kg) as major component in *C. elazigensis* var. *elazigensis.*

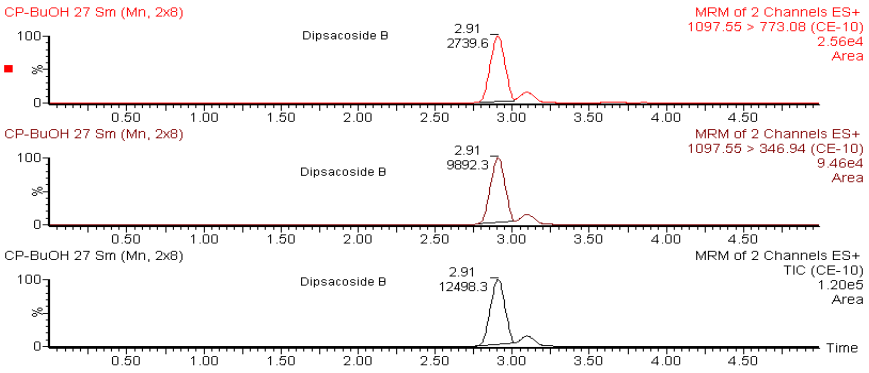

Figure 3. Chromatogram of dipsacoside B (994.00 mg/kg) as major component in *C. procera.*

According to results, *C. hirsuta* was the richest species which has twenty-eight saponins. While scoposide G (10060.69 mg/kg) was the major compound for this species, aristatoside A (37.80 mg/kg) was the minor compound. Twenty-two compounds were detected in *n*-BuOH extract of *C. procera* which is resembled to *C. hirsuta* in terms of triterpene saponins. While dipsacoside B was the major compound (994.00 mg/kg), scoposide F (6.49 mg/kg), was the minor compounds in *n*-BuOH extract of

C. procera. *C. elazigensis* var. *elazigensis* was the poorest species in terms of saponin content that we investigated. Only four saponins were detected in *n*-BuOH extract of this species. The major component was *β*-sitosterol glucoside (254.26 mg/kg) and the minor was macranthoside A (8.54 mg/kg).

In supporting information section [S3-S96], related figures show the retention times and peaks of all triterpene saponins which were detected in *C. hirsuta*, *C. elazigensis* var. *elazigensis* and *C. procera*. In the present study, saponin contents of *C. hirsuta*, *C. elazigensis* var. *elazigensis*, and *C. procera* were determined by an UPLC-ESI-MS/MS method, for the first time. The method was applied in this study to perform a quantitative determination of 40 saponins in crude extracts. We identified and tentatively characterized 28 compounds in *C. hirsuta*, 4 compounds in *C. elazigensis* var. *elazigensis* and 22 compounds in *C. procera* based on their retention times and mass spectra in comparison with the data from reference standards which were isolated in our previous studies. This validated quantitative method presents content of triterpene saponins as mg/kg in *n*-BuOH fractions. These results prompted us to consider on the saponin contents of these *Cephalaria* species which were not studied yet. According to our ongoing biological activity studies, saponins exhibit different activities such as immunomodulator, hemolytic and cytotoxic activities which will have important usage areas in the near future [30]. As a result of this study, we found that among three species *C. elazigensis* var. *elazigensis* has only 4 saponins in its *n*-butanol fraction. Because it is known that isolation procedures are a difficult, long and costly way, the studies on this plant are not preferable. On the contrary, a more detailed investigation on the *C. hirsuta* and *C. procera* species including 28 and 22 saponins, respectively is needed. So, analysis of these compounds in crude extracts provides an idea before isolation and purification studies. This easy, sensitive and timesaving chromatographic method provides rapid identification of saponins in *n*-BuOH extracts of other plants.

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Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

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