Phytoecdysteroid profiling of *Silene vulgaris* by UPLC-ESI-MS

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***Silene vulgaris* is a wild edible plant consumed in both raw as well as cooked forms in several parts of Europe. The phytoconstituents of *Silene* species include phytoecdysteroids, triterpenoidal saponins, terpenoids, flavonoids and phenolics. *Silene vulgaris* is a relatively unexplored species and the chemical profiling of this plant has not been attempted so far. Hence the UPLC-ESI-MS approach was applied to the extracts of flowers, leaves and roots of *S. vulgaris* for the profiling of phytoecdysteroids. The relative distribution of these compounds varied between flowers and leaves; however, the qualitative composition was similar. Only traces of phytoecdysteroids were present in the roots. The aglycones, sugars and other moieties were determined on the basis of ESI-MS. A total of eight previously known phytoecdysteroids were identified. Partial characterization of eight other phytoecdysteroids was also attempted.**

Keywords: Chemical profiling, chromatographic analysis, phytoecdysteroids, *Silene vulgaris*.

CHEMICAL profiling of unexplored or under-explored plants gives an overview of the phytoconstituents, their relative distribution and chemotaxonomical correlations, if present. It also aids in partial or complete identification of compounds and therefore, gives direction to phytochemical investigations[1]. *Silene vulgaris* (Moench) Garcke (syn. *S. inflata* Sm., bladder campian) is a relatively under-explored species with a few triterpenoid saponins and flavonoids reported from the roots and leaves[2,3]. It is a perennial herb of the family Caryophyllaceae and is used in traditional gastronomy in Turkey, Italy, Austria and Spain[4]. Leaves from the wild *S. vulgaris* plants are used both in raw as well as cooked forms[5,6]. Pharmacologically useful activities of this species include antioxidant, immunomodulatory and anti-arrhythmic[4].

The spectrum of the pharmacological activities of phytoecdysteroids is wide and includes antimicrobial, adaptogenic, antidiabetic, anti-inflammatory, wound healing and hepatoprotective activities[7]. Several species of the genus *Silene* have been screened for the presence of phytoecdysteroids[8]. These compounds were detected in the reproductive organs, leaves and roots of *Silene* plants. Owing to the high levels of phytoecdysteroids, some of the *Silene* species are used at an industrial scale for the production of these compounds[8]. However, *S. vulgaris* has been identified as 'phytoecdysteroid negative' by some researchers[9,10]. During the chemical profiling of *S. vulgaris*, we could detect phytoecdysteroids in the flowers and leaves of this species. The levels of plant secondary metabolites may exhibit inter- or intra-specific variation and several factors, including physiological variations, geographical location, environmental conditions and genetic factors can contribute to the variation in the production as well as composition of secondary metabolites[11]. Therefore, it was germane to conduct a phytochemical analysis of *S. vulgaris* in order to understand the chemistry for the potential health benefits of this plant. The objectives of this study were: (i) to generate and compare the phytoecdysteroid profiles of extracts prepared from the flowers, leaves and roots of *S. vulgaris* using ultra performance liquid chromatography-mass spectrometry (UPLC-MS) and (ii) to identify the major components present in these extracts by their mass fragmentation pattern. As a result, eight previously reported phytoecdysteroids were identified in the extracts of flowers of *S. vulgaris*. Also, eight other phytoecdysteroids have been partially identified on the basis of their molecular weight and fragmentation pattern.

Materials and methods

Reagents and materials

Liquid chromatography mass spectroscopy (LC-MS) grade acetonitrile and water (JT Baker) were used in UPLC and mass analyses. Polytetrafluoroethylene (PTFE) membrane syringe filters (0.22 µm, Whatman) and disc filters (47 mm, Whatman) were procured from GE Healthcare, India. All the solvents used for extraction and fractionation were obtained from CDH, India Pvt Ltd, New Delhi.

Whole plants of *S. vulgaris* were collected from Palampur (lat. 32°11′, long. 76°54′), Himachal Pradesh, India in April 2015. The voucher specimen (PLP17725) is deposited in the Biodiversity Unit of CSIR-Institute of

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Extraction and preparation of extracts

Dried and powdered flowers of *S. vulgaris* (10 g) were extracted with methanol (100 ml) in an ultrasonic bath (60°C) for 90 min (3 × 30 min, adding fresh solvent each time). The combined extracts were filtered and the solvent was removed under reduced pressure. The leaves and roots of *S. vulgaris* were processed in the same way to yield crude extracts. All the procedures were done in triplicate. The crude extracts were partitioned with ethyl acetate followed by *n*-butanol. The *n*-butanol fractions (10 mg) were dissolved in UPLC-grade methanol (1 ml) and analysed by UPLC-MS for the presence of phytoecdysteroids.

LC-MS analysis

Samples were analysed using a UPLC (Acquity™, Waters®) equipped with a sample manager, binary solvent manager and photodiode array and mass spectrometer (Micromass Q-ToF micro™, Waters®) detectors located in the Natural Product Chemistry and Process Development (NPCPD) Division of CSIR-IHBT, Palampur. BEH-C_{18} (2.1 × 100 mm, 1.7 µm) column was used for the separation of compounds. Mass lynx™ or empower™ software were used for data analysis.

The mobile phase consisting of 0.1% formic acid in water (solvent A) and acetonitrile (solvent B) was used in the analysis. The gradient employed for separation of compounds was as follows: 10% B from 0 to 0.5 min, 20% B at 6 min, 80% B at 9 min and 90% B at 10 min. The column was equilibrated to initial conditions for 5 min. Flow rate was set at 0.3 ml/min. The injection volume was 1 µl. Spectral data were collected from 250 to 700 nm.

MS analysis was performed under EI + mode. The electrospray ionization-mass spectrometry (ESI) MS conditions were as follows: nitrogen was used as desolvation gas (at a flow rate of 400 l/h), capillary voltage 3200 V, sample cone voltage 25 V, extraction cone voltage 2.5 V, desolvation temperature 220°C and source temperature 80°C. Full scan for total ion chromatogram (TIC) was performed with a mass range from *m/z* 100 to 2000.

Results and discussion

S. vulgaris is traditionally used in curries and other food preparations. Its nutritional composition and medicinal value have been studied[5,6]. However, no comprehensive data are available regarding the phytoconstituents, especially ecdysteroids of this plant. The objective of this study was to establish a chemical fingerprint of *S. vulgaris* with special focus on phytoecdysteroids.

The *n*-butanol fractions of the methanolic extracts of the flowers, leaves and roots of *S. vulgaris* were prepared in two steps. Extraction by ultrasonication with methanol yielded crude extracts which were further partitioned with ethyl acetate followed by *n*-butanol (Table 1).

The compounds in the *n*-butanol fractions of *S. vulgaris* were resolved by reverse-phase UPLC. Figure 1 *a–c* presents the chromatograms of *n*-butanol fractions of flowers, leaves and roots respectively, of *S. vulgaris*. The mass spectrometric analysis of the resolved peaks allowed the division of the chromatograms into three parts (Figure 1). In the first part, retention time (t_R) 0–3 min, phenolic acids could be detected. Some of the compounds identified on the basis of their molecular weight and fragmentation pattern include (3-, 4- or 5-) caffeoyl quinic acid (t_R 1.3 min, *m/z* 354 $[M + H]^+$, 309, 291, 181)[12], (*o*-, *m*- or *p*-) coumaric acid (t_R 1.4 min., *m/z* 165 $[M + H]^+$, 148, 120)[13] and feruloylquinic acid (t_R 2.2 min, *m/z* 367 $[M + H]^+$, 348, 331)[14]. Due to the possibility of different isomeric structures, these compounds could not be identified unambiguously. However, it is noteworthy that they have not been reported earlier from any *Silene* species. As can be concluded from the area under the peaks in the chromatograms (Figure 1), the proportion of phenolic acids was much less in the extract compared to phytoecdysteroids.

The second part of the chromatogram (t_R 3–7 min) was rich in phytoecdysteroids. It is worth mentioning here that phytoecdysteroids were the major constituents of flowers and leaves. Also, the profiles of the flowers and leaves were comparable qualitatively. On the other hand, these compounds were present only in traces in the roots of *S. vulgaris*. In the third part of the chromatogram (t_R 7–10 min), triterpene saponins were detected. The compounds (peaks) in the second part of the chromatogram were intensively studied using their ESI-MS data and are the main focus of this study.

Assignment and fragmentation of reported phytoecdysteroids

In general, the mass spectra of phytoecdysteroids presented a molecular ion peak, an intense fragment corresponding to the aglycone (in case of glycosides) and

Table 1. The extractive values and weights of *n*-butanol fractions of flowers, leaves and roots of *Silene vulgaris*

Plant part	Crude extract (g)[a]	*n*-Butanol extract (g)[a]
Roots	3.23 ± 0.08	0.396 ± 0.01
Leaves	1.29 ± 0.02	0.209 ± 0.02
Flowers	1.27 ± 0.04	0.659 ± 0.01

[a]Results are expressed as mean ± SD (n = 3).

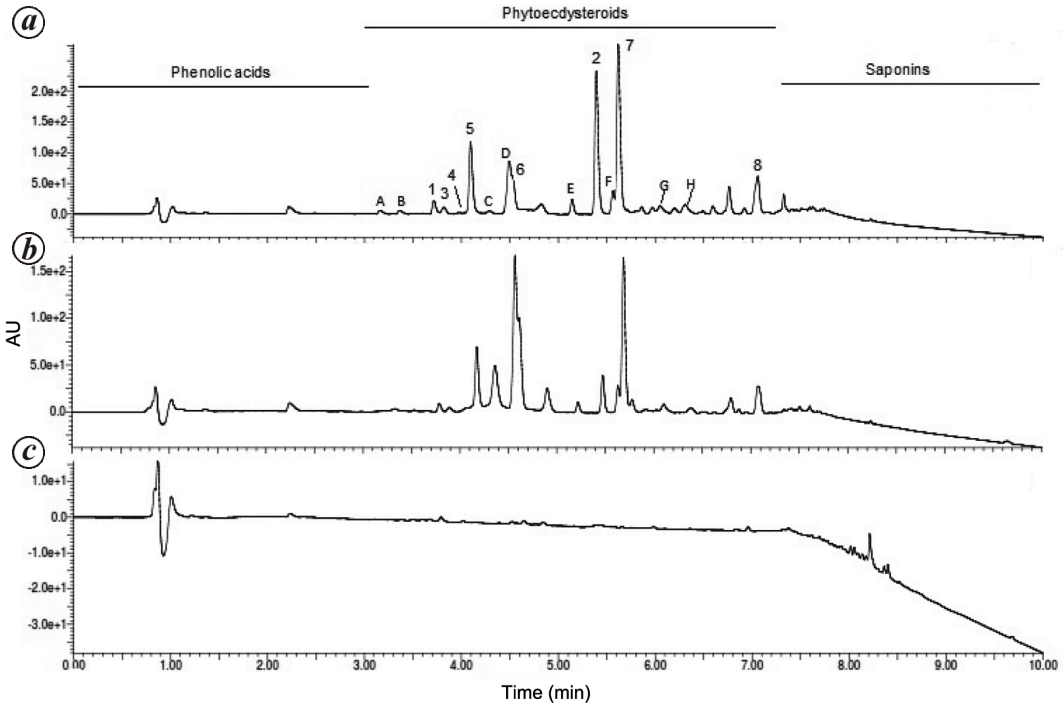

Figure 1. Chromatograms of *n*-butanol fractions of (***a***) flowers, (***b***) leaves and (***c***) roots of *Silene vulgaris*. Various identified compounds are marked: **1** (2,22-dideoxyecdysone 25-*O*-*β*-D-glucopyranosyl-(1 → 2)-*O*-*β*-D-glucopyranoside); **2** (2,22-dideoxyecdysone 25-*O*-*β*-D-glucopyranoside); **3** and **4** (isomeric pair of 20,26-dihydroxyecdysone 2,22-diacetate and 20,26-dihydroxyecdysone 3,22-diacetate); **5** (silenoside E); **6** (2-deoxy-20-hydroxyecdysone 3-[4-(1-*β*-D-glucopyranosyl)]-ferulate); **7** (2-deoxyecdysone 3-[4-(1-*β*-D-glucopyranosyl)]-ferulate); **8** (2-deoxy-20-hydroxyecdysone 3-[4-(1-*β*-D-glucopyranosyl)]-caffeate). Compounds **A**–**H** have been assigned with tentative identities.

prominent fragments after the loss of water molecules from the polyhydroxylated phytoecdysteroids. An additional signal corresponding to $[2M + Na]^+$ was observed in some cases.

In order to identify the known phytoecdysteroids, the detected molecular ions were matched with known compounds of *Silene* as well as other plants. Of the total of 16 assigned compounds having retention times between 3 and 7 min, the molecular weight and fragmentation characteristics of eight could be correlated with reported phytoecdysteroids. Detailed study of the fragmentation pattern of compounds **1–8** aided to distinguish compounds of same molecular weight and led to unambiguous identification. Compound **1** was identified as 2,22-dideoxyecdysone 25-*O*-*β*-D-glucopyranosyl-(1 → 2)-*O*-*β*-D-glucopyranoside on the basis of its molecular ion peak at *m/z* 756 and two major fragment ions at *m/z* 594 and 432, resulting from the successive neutral loss of the two glucopyranosyl moieties. The fragment at *m/z* 432 corresponded with a trihydroxylated phytoecdysteroid core that was further emphasized by the fragment ions at *m/z* 355 and 303. Compound **2** had a molecular weight 162 units less than compound **1**, indicating the presence of only one hexose unit in the structure. The fragmentation pattern of compound **2** was very similar to compound **1** and depicted a 2,22-dideoxyecdysone core. It was identified as 2,22-dideoxyecdysone 25-*O*-*β*-D-glucopyranoside. Both compounds **1** and **2** have earlier been reported from *Froelichia floridana*[15]. Figure 2 presents the fragmentation of compounds **1** and **2**.

Compounds **3** and **4** were characterized as an isomeric pair of 20,26-dihydroxyecdysone 2,22-diacetate and 20,26-dihydroxyecdysone 3,22-diacetate (identities interchangeable). The molecular ion of these compounds was present at *m/z* 580, and the loss of H_2O and CO units gave fragments at *m/z* 563 and 553 respectively. The loss of 59 units from the molecular ion along with a water molecule demonstrated the presence of an acetate moiety. Further, the C-23, C-24 cleavage accompanied with loss of acetate (59 units) gave a fragment at *m/z* 432. Figure 2 presents detailed fragmentation of compounds **3** and **4**. Both compounds have earlier been isolated from *Silene virdiflora*[16].

The pseudomolecular ion peak of compound **5** appeared at *m/z* 1244 $[2M + Na]^+$. The sodiated ion at *m/z* 633 corresponded to $[M + Na]^+$ and the base peak at *m/z* 610 gave the molecular weight of the compound. The product ion at *m/z* 448 depicted phytoecdysteroid core and the loss of 162 units (hexose unit) from the molecular ion. This pattern pointed towards the presence of a phytoecdysteroid monosaccharide. The molecular weight could be matched with silenoside E (syn. Blechnoside A) as well as blechnoside B (Figure 2). In the case of silenoside E, the *β*-D-glucopyranosyl moiety was present at C-3 while in blechnoside B, C-25 was glycosylated. The mass

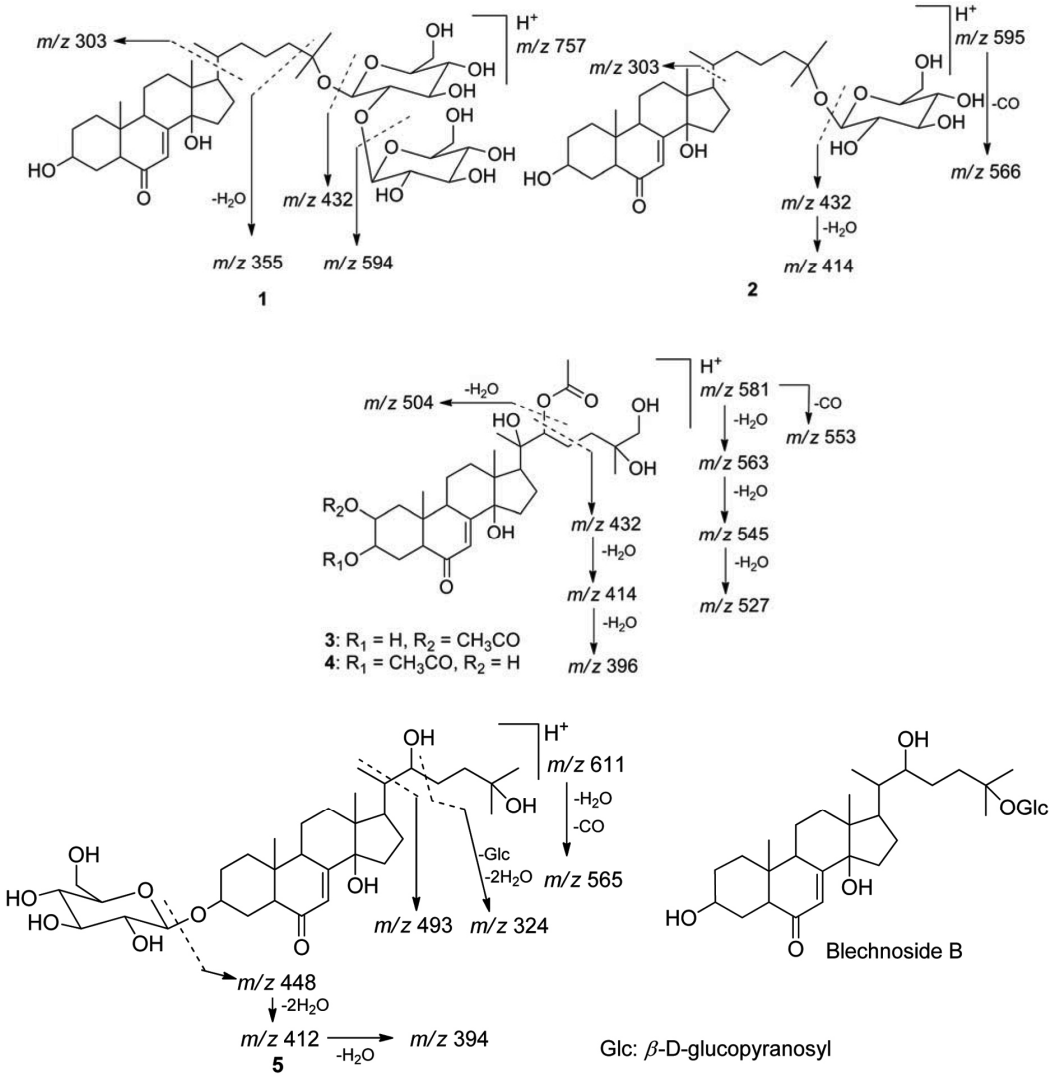

Figure 2. Proposed mass fragmentation of compounds **1**–**5** and the structure of blechnoside B.

spectrum of compound **5** had a fragment ion at *m/z* 493 resulting from the cleavage of C-20, C-22 bond. This fragment was only possible if the sugar unit was attached with a hydroxyl of phytoecdysteroid core and the hydroxyls of C-17 side chain were free. Similarly, the fragment ion at *m/z* 324 supported the structure of compound **5** as silenoside E (Figure 2)[17].

The molecular ion peak of compound **6** appeared at *m/z* 802 $[M]^+$ and its molecular weight corresponded to 2-deoxy-20-hydroxyecdysone 3-[4-(1-*β*-D-glucopyranosyl)]-ferulate. A loss of 162 units depicted the cleavage of glucopyranosyl moiety and gave a fragment at *m/z* 639. Further, the cleavage of feruloyl unit was presented by fragments at *m/z* 410 and 302. The phytoecdysteroid core was observed at *m/z* 488 after the loss of glucopyranosyl and ferulate moieties. The hydroxyl substitution pattern on C-17 side chain was confirmed by the fragment ions at *m/z* 668 and 566 (Figure 3). Compounds **7** and **8** had a molecular weight of 786 (16 units less than compound **6**) and 788 (14 units less than compound **6**) respectively. On the basis of their fragmentation patterns, these were characterized as 2-deoxyecdysone 3-[4-(1-*β*-D-glucopyranosyl)]-ferulate (**7**) and 2-deoxy-20-hydroxyecdysone 3-[4-(1-*β*-D-glucopyranosyl)]-caffeate (**8**; Figure 3). These compounds (**6**–**8**) have earlier been isolated from *Microsorum membranifolium*[18,19].

Structure elucidation of other phytoecdysteroids

The information obtained from the assignment of compounds **1** to **8** and the fragmentation pattern of these compounds were utilized to assign the aglycone, sugar(s) and other structural motifs present in remainder of the compounds. This resulted in seven phytoecdysteroid glycosides and an acetonide. Overall, four different types of aglycones were depicted by *m/z* 464 (ecdysone or phytoecdysteroid core with five hydroxyls), *m/z* 448

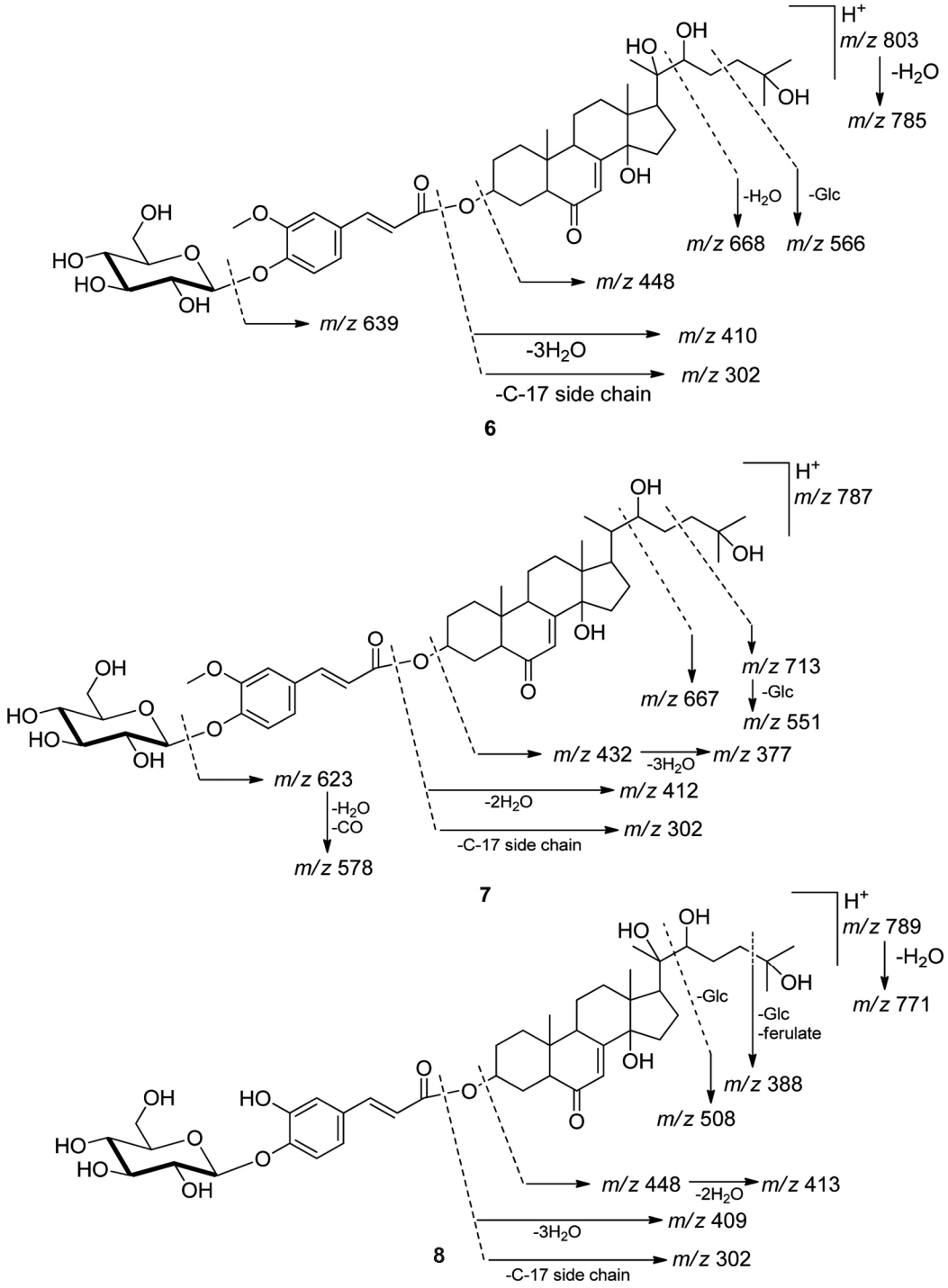

Figure 3. Mass fragmentation pattern of compounds **6**–**8**.

(deoxyecdysone or dideoxy hydroxyecdysone), *m/z* 432 (dideoxyecdysone) and *m/z* 462 (dehydroecdysone). It is interesting to note that **A**, **B** and **D** showed a neutral loss of 146 units, indicating a rhamnose in their structures. Rhamnosides of phytoecdysteroids have been isolated from a few ferns[20]; however, they have not been detected earlier in *Silene*. Table 2 presents details of structure and fragmentation of compounds **A**–**H**. The molecular weight and mass fragmentation pattern of **D**, **E**, **G** and **H** corresponded to 2-deoxyecdysone 25-*O*-α-L-rhamnopyranoside[18], 2,22-dideoxy-5β-hydroxyecdysone 25-*O*-β-D-glucopyranosyl-(1 $\rightarrow$ 2)-β-D-glucopyranoside[15], brainesteroside A (ref. 21) and stachysterone A 20,22-acetonide[22] respectively. However, the position of linkage of sugar with the aglycone, monodesmosidic or bidesmosidic nature of the glycoside and other structural features such as the

Table 2. Assignment of the aglycone, sugar(s) and other structural motifs present in compounds **A–H**

Compound	t_R (min)	Aglycone	Saccharide*	$m/z^{\#}$ I	II	III	IV	V	VI	VII
A	3.15	Ecdysone or like ($C_{27}H_{44}O_6$)	Hex, Rha	796 $[MH + Na]^+$	772 $[M]^+$	626 $[M\text{-}Rha]^+$	564 $[M\text{-}Rha\text{-}CO\text{-}2H_2O]^+$	**464** $[M\text{-}Rha\text{-}Hex]^+$	412 $[M\text{-}Rha\text{-}Hex\text{-}3H_2O]^+$	
B	3.38	Deoxy-ecdysone ($C_{27}H_{44}O_5$)	Hex, Rha	756 $[M]^+$	610 $[M\text{-}Rha]^+$	593 $[M\text{-}Rha\text{-}H_2O]^+$	**448** $[M\text{-}Rha\text{-}Hex]^+$			
C	4.32	Dideoxy-ecdysone ($C_{27}H_{44}O_4$)	Hex	617 $[M + Na]^+$	594 $[M]^+$	577 $[MH\text{-}H_2O]^+$	**432** $[MH\text{-}Hex]^+$	414 $[MH\text{-}Hex\ H_2O]^+$		
D	4.49	Deoxy-ecdysone ($C_{27}H_{44}O_5$)	Rha	617 $[M + Na]^+$	594 $[M]^+$	577 $[MH\text{-}H_2O]^+$	566 $[M\text{-}CO]^+$	**448** $[MH\text{-}Rha]^+$	430 $[MH\text{-}Rha\text{-}H_2O]^+$	303 $[M\text{-}Rha\text{-}C\text{-}17\ side\ chain]^+$
E	5.16	Deoxy-ecdysone ($C_{27}H_{44}O_5$)	2 Hex	772 $[M]^+$	610 $[MH\text{-}Hex]^+$	**448** $[MH\text{-}2Hex]^+$	371			
F	5.56	Dehydro-ecdysone or the like ($C_{27}H_{42}O_6$)	Hex, DHex	779 $[MH + Na]^+$	756 $[M]^+$	624 $[M\text{-}DHex]^+$	**462** $[M\text{-}DHex\text{-}Hex]^+$			
G	6.05	Dehydro-ecdysone or the like ($C_{27}H_{42}O_6$)	Hex	648 $[MH + Na]^+$	625 $[MH]^+$	624 $[M]^+$	580 $[MH\text{-}H_2O\text{-}CO]^+$	567 $[M\text{-}C_4H_9]^+$	480 $[MH\text{-}C17\ side\ chain]^+$	**462** $[MH\text{-}Hex]^+$
H	6.25	Dehydro-ecdysone or the like ($C_{27}H_{42}O_6$)	–	502 $[M]^+$	485 $[MH\text{-}H_2O]^+$	430 $[MH\text{-}C_4H_9O]^+$	341 $[MH\text{-}C_3H_6O_2\text{-}C_5H_{11}O]^+$	324 $[341\text{-}H_2O]^+$	310 $[MH\text{-}C_3H_6O_2\text{-}C_4H_9O\text{-}CO\text{-}H_2O]^+$	

*Hex, Hexose; Rha, Rhamnose; DHex, Deoxyhexose; #M, Molecular ion; Aglycones are shown in bold.

positioning of double bonds cannot be ascertained by mass spectrometry alone.

Conclusion

The present study was designed to understand the chemistry of *S. vulgaris*, a herb consumed in fresh as well as cooked forms. The phytoecdysteroid fingerprints of the extracts of *S. vulgaris* were generated using UPLC-MS approach. The chromatographic analysis revealed that the aerial parts, viz. leaves and flowers had similar constitution whereas the roots had an entirely different profile. A total of 16 compounds could be assigned to the leaves as well as flowers of *S. vulgaris*. Among these, eight phytoecdysteroids could be identified and the remaining were partially characterized on the basis of their molecular weight and mass fragmentation pattern. This methodology is rapid and can help generate chemical profiles of other *Silene* species.

Conflict of interest: The authors declare no conflict of interest.

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