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Two new bis-iridoids isolated from *Scabiosa stellata* and their antibacterial, antioxidant, anti-tyrosinase and cytotoxic activities

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Abstract

This study presents the chemical profile investigation of a 70% ethanol extract obtained from *Scabiosa stellata*, a medicinal herbaceous traditionally used to treat heel cracks. A ^{13}C NMR-based dereplication methodology was firstly applied on centrifugal partition chromatography-generated fractions in order to quickly identify the major compounds of the extract. The dereplication process was then completed by semi-preparative high-performance liquid chromatography in order to identify unknown or minor compounds. Two new bis-iridoids, namely 7-*O*-caffeoyl-sylvestroside I (**1**) and 7-*O*-(*p*-coumaroyl)-sylvestroside I (**2**), together with ten known compounds (**3**-**12**) were isolated. Their structures were elucidated by spectroscopic methods including NMR and HR-ESI-MS. The antibacterial, anti-tyrosinase and DPPH radical scavenging activities of the crude extract, fractions, and isolated compounds were evaluated. A significant antibacterial activity was observed for nine isolated compounds, particularly **1** and **2** which yielded MIC values of 31.2 µg/mL against *Enterococcus faecalis* and 62.5 µg/mL against *Staphylococcus epidermidis*. The cytotoxic activity of these new bis-iridoids was evaluated on a fibrosarcoma cell line (HT1080) and only compound **1** exhibited a moderate cytotoxic activity (IC_{50} 35.9 µg/mL).

Keywords: *Scabiosa stellata*; Caprifoliaceae; Bis-iridoid glucoside; Antibacterial activity; Cytotoxic activity; dereplication.

1. Introduction

The genus *Scabiosa* belongs to the Caprifoliaceae family and comprises about 100 species. The majority of *Scabiosa* species occurs in the Mediterranean region [1] and among them 12 species grows in Algeria [2]. In Catalonia (Spain), the decoction of the aerial part of *S. columbaria* is traditionally used against diphtheria [3]. In Algeria, *S. arvensis* is used in folk medicine against diarrhea, inflammation, microbial infections and skin disorders [4]. In moroccan folk medicine, the leaves and flowers of *S. stellata* are used against heel cracks [5]. Many extracts obtained from *Scabiosa* species have already demonstrated antibacterial activities, such as *S. atropurpurea* [6], *S. hymettia* [7], *S. columbaria* [8], or *S. arenaria* [9]. The extracts of *S. arenaria* and *S. tschiliensis* have shown antioxidant properties [10,11].

Up to date, chemical investigations of *Scabiosa* species have mainly revealed the presence of saponins [12,13], flavonoids, and coumarins [14]. Previous studies have also reported that the genus *Scabiosa* characteristically contains bis- and mono-iridoid glucosides [15,16].

Scabiosa stellata Cav., known with the common name starflower pincushions, is an herbaceous, bristly-hairy annual plant (20-60 cm). The lower leaves are 7-12 cm long, spoon-shaped in outline, tapered at the base, the margins with blunt teeth irregular in size and placement. The outer florets are light gray-blue, irregularly shaped. The central florets are smaller and subtended each by a rounded translucent bract with a green midrib [4]. In this work, we have investigated the chemical profile of *S. stellata* and the antibacterial, tyrosinase inhibitory, DPPH radical scavenging and cytotoxic activities of the crude extract, fractions and isolated compounds.

2. Results and discussion

The antibacterial activity of the 70% EtOH extract obtained from the whole plant *S. stellata* was evaluated against 22 micro-organisms including 17 Gram-positive and Gram-negative bacteria and 5 yeasts. The MIC determination method in solid media was used [17]. The results are presented in Table 1. The highest antimicrobial activity was observed against *Streptococcus pyogenes* (MIC 1.2 mg/mL), whereas a low to moderate antimicrobial activity was observed against *Bacillus subtilis* (MIC 10 mg/mL), *Staphylococcus epidermidis,* and *Micrococcus luteus* (MIC 2.5 mg/mL). Regarding antifungal activity, the crude extract showed a moderate activity (MIC 2.5 to 5 mg/mL). A moderate DPPH radical scavenging activity was also observed for this extract (IC_{50} 86.0 µg/mL), as well as a moderate tyrosinase inhibitory activity when tested on an *in vitro* mushroom tyrosinase assay (40% inhibition at 1.33 mg/mL) (Table 2).

In order to isolate potentially active compounds, a bioassay-guided fractionation strategy was applied throughout the separation procedure. The 70% ethanol extract of the whole plant *S. stellata* was subjected to a Diaion HP-20 column, eluting with 0%, 25%, 50%, 75% and 100% MeOH, yielding five fractions (A-E, respectively). These fractions were evaluated for their antimicrobial, antioxidant and anti-tyrosinase activities (Tables 1 and 2). Fraction C exhibited the best antimicrobial activity against the 22 microorganisms (MIC 1.2 to 10 mg/mL). Whereas fraction B was active only against the gram-positive bacteria (except *Listeria innocua*) and yeasts (MIC 0.6 to 2.5 mg/mL) (Table 1). Fractions B and C showed also the best DPPH radical scavenging activity (IC_{50} 48.7 and 25 µg/mL, respectively) (Table 2). Fraction C showed also a significant anti-tyrosinase activity (IC_{50} 1.33 mg/mL) and was slightly less active than fraction D (IC_{50} 1 mg/mL) (Table 2). Therefore, the chemical profiles of fractions B and C were investigated in order to determine which compounds were

responsible for these activities (Figure 1). Fractions B (2 g) and C (3 g) were subjected separately to CPC fractionation. The biphasic solvent system M*t*BE/CH_3CN/water (3/3/4, v/v/v) was selected to recover moderately polar compounds. After pooling the collected fractions on the basis of TLC profile similarities, adjacent sub-fractions B_1-B_{23} and C_1-C_{21} containing simplified mixtures or even pure compounds were obtained. Fractions B_1-B_{23} and C_1-C_{21} were all analyzed by ^{13}C-NMR for dereplication [18]. Automatic peak picking and binning of ^{13}C signals across spectra resulted in two tables (one table for each CPC separation of B and C) which were independently submitted to Hierarchical Clustering Analysis (HCA) for pattern recognition. In this way, statistical correlations between ^{13}C NMR signals belonging to individual structures within the fraction series were visualized as "chemical shift clusters" on the resulting two-dimensional HCA correlation heat maps in front of the corresponding dendrograms. As illustrated in Figure 2, several well-defined clusters were intensely colored in yellow. After entering the chemical shift values of cluster 1 located in sub-fractions B_{4-8} and C_{7-14} into the database, the molecular structure of isoorientin (**8**) [19] was proposed. By means of the same database search strategy, cluster 2 present in sub-fractions B_{2-4} and C_{3-6} was identified as hyperin (**10**) [20], cluster 3 present in sub-fractions B_{20-23} and C_{11-14} was identified as eustomoruside (**6**) [21], cluster 4 present in sub-fractions C_{8-9} corresponded to a bis-iridoid structure containing loganic acid and caffeic acid moieties, cluster 5 present in sub-fractions B_{19-22} and C_{15-21} was identified as sweroside (**5**) [22], cluster 6 present in sub-fractions C_{6-8} was identified as swertiajaponin (**9**) [19]. Cluster 7 present in sub-fractions C_{2-3} corresponded to a mixture of 3,5-dicaffeoylquinic acid (**11**) and 4,5-dicaffeoylquinic acid (**12**) [11]. Cluster 8 present in sub-fractions B_{13-15} corresponded to eustomoside (**7**) [23]. Cluster 9 present in sub-fractions B_9-B_{12} was identified as caffeic acid. For the sub-fractions B_{4-9}, B_{20-23} and C_{8-9}, the database proposed a complex mixture of bis-iridoid derivatives, partially composed of loganin, demethylsecologanol and sweroside units,

which could not be identified unambiguously (cluster 10). Further purifications of the fractions containing the products corresponding to the remaining unidentified clusters were performed using silica gel flash chromatography, RP-18 flash chromatography and semi-preparative RP-18 HPLC, leading to the identification of two previously undescribed bis-iridoid glucosides (**1**-**2**) and two known bis-iridoid glucosides; sylvestroside I (**3**) [24] and septemfidoside (**4**) [25] (Figure 1).

Compound **1** was obtained as white amorphous powder with a molecular formula of $C_{42}H_{54}O_{22}$, deduced from the positive HR-ESI-MS analysis (*m/z* 933.2997, $[M+Na]^+$). The IR spectrum indicated the presence of hydroxyl groups (3420 cm^{-1}) and *α*, *β*-unsaturated ester carbonyl groups (1697 cm^{-1}). The UV spectrum showed the characteristic absorption of *β*-alkoxyacrylic acid or its ester at 228 nm and absorption at 326 nm characteristic of a cinnamic acid derivatives. The ^1H and ^{13}C NMR spectroscopic data of compound **1** showed two distinct parts, units A and B (Fig. 1). The ^1H NMR spectrum showed signals for unit A that indicated the presence of an olefinic proton *β*-alkoxyacrylic acid at δ_H 7.42 (s, H-3a), a set of three protons on vinyl group at δ_H 5.84 (ddd, J = 17.3, 10.3, 8.7 Hz, H-8a), 5.36 (d, J = 17.3 Hz, H-10a_1) and 5.30 (d, J = 10.3 Hz, H-10a_2), and an acetal proton at δ_H 5.60 (d, J = 6.5 Hz, H-1a) which were characteristic of a secoiridoid moiety. The ^{13}C NMR, spectrum showed signals for carboxyl carbon (δ_C 167.8, C-11a), two alkenyl carbons [δ_C 151.3 (C-3a) and 110.4 (C-4a) due to the *β*-alkoxyacrylic ester group, and two others δ_C 134.3 (C-8a) and 118.2 (C-10a) due to the vinyl group], one acetal carbon (δ_C 96.3, C-1a) and one hydroxyl-methyl carbon (δ_C 62.7, C-7a). 2D-NMR analysis indicated that unit A was an open form of the lactone moiety of sweroside. Furthermore, two anomeric protons resonances corresponding to *O*-linked sugars were observed in the ^1H NMR spectrum of **1** as two doublets at δ_H 4.74 (J = 7.9 Hz) and 4.68 (J = 7.9 Hz). The sugar units were elucidated as two *β*-D-glucopyranoses [glc-A ($\delta_{H\text{-}1'}$ 4.74 and $\delta_{C\text{-}1'}$ 96.8) and glc-B ($\delta_{H\text{-}1'''}$ 4.68 and $\delta_{C\text{-}1'''}$ 96.8) (Table 3) based on the results of

the acid hydrolysis of fraction C from which compound **1** was obtained, by the magnitudes of their $J_{1,2}$ coupling constants, interpretation of COSY, HSQC and HMBC spectra, and by comparing the ^{13}C NMR chemical shifts with those of related systems reported in the literature [26]. The glc-A unit was linked to C-1a as deduced from the long-range correlation observed between H-1′ and C-1a in the HMBC spectrum. The spectroscopic data of unit A were almost identical to demethylsecologanol [22,27,28] except for the low-frequency shift of H-7a (δ_H 4.21 and 4.26) and C-7a (δ_C 62.7) and the high-frequency shift of C-11a (δ_C 167.8). In the ^{1}H NMR spectrum of **1**, a set of signals belonging to a *E*-caffeoyl moiety was detected with two coupled *trans* double-bond protons [δ_H 6.24, H-8′′ and 7.55, H-7′′, each d, J = 16.0 Hz] and three coupled aromatic protons [δ_H 6.80 (d, J = 8.1 Hz, H-5′′), 6.96 (dd, J = 8.1, 2.0 Hz, H-6′′) and 7.05 (d, J = 2.0 Hz, H-2′′)]. The presence of an *E*-caffeoyl moiety was evident by the connectivities observed in the HMBC spectrum between the *trans* double-bond and the 1,3,4-substituted aromatic ring as well as to an ester carbonyl (δ_C 167.9, C-9′′) and its ^{1}H and ^{13}C resonances were assigned using 2D-NMR experiments (COSY, HSQC, and HMBC) (Table 3). An HMBC correlation was observed between H-7a and the carbonyl carbon C-9′′, indicating that demethylsecologanol was esterified by the *E*-caffeoyl moiety. Thus, unit A was identified as grandifloroside [29]. The second half, unit B, was easily assigned to a loganic acid-type iridoid [22,27,28] due to a methyl group at δ_H 1.10 (d, J = 6.9 Hz, H-10b), olefinic signal at δ_H 7.53 (s, H-3b) together with δ_C 152.3, 111.6 and 166.9 which were ascribed to C-3b, C-4b and C-11b, respectively (Table 3). This was verified by the correlations in ^{1}H-^{1}H COSY spectrum observed between the protons signals at δ_H 3.14 (H-5b), 2.08 (H-9b), 5.28 (H-1b) and 2.19 (H-8b), between the methyl group at δ_H 1.10 (H-10b) and H-8b and between signal at δ_H 5.24 (H-7b), and H-8b. The structure of loganic acid moitiey was confirmed by the HMBC correlations observed between H-3b/C-1b, C-4b, C-5b and C-11b, H-7b/C-5b and H-1b/C-10b and the anomeric carbon C-1′′′ of Glc B.

Additionally, in the HMBC spectrum, the proton signal at δ_H 7.53 (H-3b), 3.68 (-OCH_3, H-12b) and 3.14 (H-5b) showed long-range correlations with the carbon resonance at δ_C 166.9 (C-11b) indicating the location of a methoxy group at C-11b. The spectroscopic data of unit B were almost identical to loganin [28,29] except for the downfieled shifts of H-7b (δ_H 5.24) and C-7b (δ_C 77.0). The connectivity between A and B units was found to be an ester linkage between the C-7b hydroxyl group of unit B and the carboxyl group (C-11a) as deduced by the HMBC correlation observed between H-7b and C-11a. The relative configuration of **1** was further determined on the basis of coupling constants and by the ROESY experiment. The ROESY correlations of δ_H 2.95 (H-5a) with 2.72 (H-9a), 2.08 (H-9b) with 1.10 (H-10b), 3.14 (H-5b) with 2.08 (H-9b) and 5.24 (H-7b) with 2.19 (H-8b) revealed the relative configuration of **1**. Therefore, the structure of **1** was elucidated as 7-*O*-(*E*-caffeoyl)-sylvestroside I shown in Fig. 1 and differs from sylvestroside I, reported in *Dipsacus laciniatus* [24], with an additional *E*-caffeoyl group at C-7a.

Compound **2** displayed an $[M+Na]^+$ ion peak at *m/z* 917.3063, corresponding to the molecular formula $C_{42}H_{54}O_{21}$ (calcd for $C_{42}H_{54}O_{21}Na$, 917.3055), suggesting the lack of one hydroxyl group compared to **1**. Comparison of ^1H and ^{13}C NMR values and the analysis of the ^1H-^1H-COSY, ROESY, HSQC and HMBC showed that **1** and **2** contained the same–skeleton (sylvestroside I) (Table 3). Thus, the difference between **1** and **2** should be located at the cinnamoyl derivative substituant. This latter was represented on the ^1H NMR spectrum by two *trans*-coupled double-bond protons at δ_H 7.61 and 6.30 (each 1H, d, J = 16.0 Hz) assignable to H-7″ and H-8″, respectively and four aromatic protons at δ_H 7.47 and 6.83 (each 2H, d, J = 8.6 Hz) assignable to H-2″/6″ and H-3″/5″ respectively, as well as to an ester carbonyl (δ_C 167.9) in the ^{13}C NMR spectrum. These data were consistent with the presence of an *E*-coumaroyl moiety (Table 1) [30] and its ^1H and ^{13}C resonances were assigned using 2D-NMR experiments (COSY, HSQC, and HMBC). The coumaroyl moiety was linked to the C-7a of

the seco-loganin moiety as deduced from the HMBC correlation between the H-7a (δ_H 4.21 and 4.26) and the carbonyl carbon of coumaroyl group (δ_H 167.9, C-9′′). Thus, the structure of compound **2** was concluded to be 7-*O*-*(E*-*p*-coumaroyl)-sylvestroside I as shown in Fig.1.

In order to screen the antibacterial potential of the compounds **1**-**12** isolated from the active fractions B and C, a bioautography assay was applied on a sensitive strain of *Staphylococcus aureus* (*S. aureus* CIP 53.154). *S. aureus* is a Gram-positive cocci bacterium frequently found on the skin and in the respiratory tract, and can be responsible for nosocomial infections. Compounds **1**-**4**, **6**, **8**, **9**, **11** and **12** were the only active compounds as revealed by the white inhibition zones observed on the TLC plate around the compound spots. A serial liquid dilution technique in 96-well microtiter plates was used to determine the minimum inhibitory concentration (MIC) of these eight active compounds against *Enterococcus faecalis* ATCC 1034, *Staphylococcus aureus* CIP 53.154, *Escherichia coli* CIP 54.127, *Staphylococcus epidermidis*, and *Pseudomonas aeruginosa* ATCC 9027 (Table 4). The results showed a good inhibitory effect of compounds **1** and **2** against *E. faecalis* (MIC 31.2 µg/mL), *S. epidermidis* (MIC 31.2 µg/mL), and *S. aureus* (MIC 62.5 µg/mL). Compound **6** exhibited a good inhibitory effect against *E. faecalis* and *S. aureus* (MIC 62.5 µg/mL). Only compound **3** showed an antibacterial activity against *E. coli* in addition to *S. aureus* (MIC 62.5 µg/mL). Compounds **4**, **8**, **9** and **11** showed a low antibacterial activity against the five tested bacteria with MIC values ranging from 125 to 500 µg/mL (Table 4).

All isolated compounds (**1**-**12**) were then evaluated for their DPPH radical scavenging effect. As summarized in table 2, only compounds **6**, **7**, **8** and **10** exhibited IC_{50} values ranging from 7.1 ± 0.2 to 16.0 ± 0.4 µg/mL whereas for the other compounds the 50 % DPPH inhibition could not been reached even at 200 µg/mL. These values were very close to that obtained with ascorbic acid used as positive control (IC_{50} 6.3 ± 0.1 µg/mL). Compounds **1**-**12** were also evaluated for their mushroom tyrosinase inhibitory activity. None of the isolated compound **1**-

12 was active at the concentration of 665 µg/mL (Table 2). Finally, the cytotoxic activity of previously undescribed compounds **1** and **2** was evaluated *in vitro* using a fibrosarcoma cell line (HT1080). Only compound **1** exhibited a moderate cytotoxic activity with an IC_{50} value of 35.9 ± 0.06 µg/mL, while compound **2** was inactive (IC_{50} > 100 µg/mL).

3. Experimental

3.1. General experimental procedures

Optical rotations of pure compounds were measured in CH_3OH using a Perkin-Elmer 341 Polarimeter. HR-ESI-MS experiments were performed using a Micromass Q-TOF micro instrument (Manchester, UK). Thin-layer chromatography (TLC) was carried out on silica gel 60 F_{254} pre-coated aluminum plates (0.2 mm, Merck), using the system $CHCl_3$/MeOH/H_2O (14/6/1, v/v) as the mobile phase. The spots were visualized under UV light (254 and 366 nm) and sprayed with 50% H_2SO_4 followed by heating. Flash chromatography was carried out on a Grace Reveleris system equipped with dual UV and ELSD detection and using Grace® cartridges (Silica gel or RP-C_{18}).

3.2. Plant material

The plant *Scabiosa stellata* Cav. (Caprifoliaceae) was collected from Djebel El-Ouahch at Constantine (North eastern of Algeria) in June 2015 and authenticated by Mr. Kamel Kabouche. A Voucher specimen (LOST.Cs.06.15) was deposited at the Constantine University Herbarium (Algeria).

3.3. Extraction and isolation

The dried and powdered *S. stellata* whole plant (653 g) was macerated in 70% EtOH (3×3 L, 24h) at room temperature. The combined 70% EtOH solution was concentrated under vacuum to provide the 70% EtOH extract (118 g). This extract was dissolved in water (1 L) and then subjected to a Diaion HP-20 macroporous resin column (4.3 × 40 cm, internal diameter),

eluting successively with a mixture of MeOH-H_2O (2 L each for 0%, 25%, 50%, 75% and 100% MeOH solution,) to afford five fractions A-E, respectively.

3.3.1. Centrifugal partition chromatography

Centrifugal partition chromatography (CPC) experiments were carried out using a lab-scale FCPE300® column of 303 mL capacity (Rousselet Robatel Kromaton, Annonay, France) containing 7 circular partition disks and engraved with a total of 231 partition twin-cells (≈1 mL per twin cell). The liquid phases were pumped by a KNAUER Preparative 1800 V7115 pump (Berlin, Germany). The column was coupled on-line with a UVD 170 S detector set at 210, 254, 280 and 366 nm (Dionex, Sunnivale, CA, USA). Fractions of 15 mL were collected by a Pharmacia Superfrac collector (Uppsala, Sweden). The solvent system was M*t*BE / CH_3CN / water in the ratio 3/3/4 (v/v/v). The column was filled with the lower phase of the biphasic solvent system at 500 rpm. The rotation speed was then increased up to 1200 rpm. Fractions B and C were subjected separately to CPC; each one was dissolved in 30 mL of a mixture of both lower phase (20 mL) and upper phase (10 mL). For the separation of fraction B (1.8 g injected), the upper phase of the biphasic solvent system was pumped for 100 min in the ascending mode at 20 mL/min. Then the column was extruded by pumping the organic phase in the descending mode at 20 mL/min. Fractions of 20 mL were collected over the whole experiment. For the separation of fraction C (3 g), the CPC method was identical, except for the extrusion step which was performed between 80 and 100 min. All fractions were analyzed by TLC and HPLC and then pooled, giving sub-fractions B_1-B_{23} and C_1-C_{21}.

3.3.2. NMR analyses and dereplication of the major metabolites

As a first step in this developed ^{13}C NMR-based dereplication method [18], structures and names of metabolites already described in the genus *Scabiosa* were collected from the reports available in the literature. In total, 12 metabolites were found. The predicted ^{13}C NMR chemical shifts of each one was then stored into a local database already comprising 2700

structures of natural compounds (NMR Workbook Suite 2012, ACD/Labs, Ontario, Canada). In the second step, all the sub-fractions of the both CPC experiments were dried under vacuum and each aliquot (≈20 mg) was dissolved in 600 µL of methanol-d_4 and analyzed by ^{13}C NMR. NMR spectra were recorded at 298 K on a Bruker Avance AVIII-600 spectrometer (Karlsruhe, Germany) equipped with a TXI cryoprobe. ^{13}C NMR spectra were acquired at 150.91 MHz. A standard zgpg pulse sequence was used with an acquisition time of 0.9 s and a relaxation delay of 3 s. For each sample, a total of 1024 scans were added to obtain a satisfactory signal-to-noise ratio. The spectral width was 240 ppm and the receiver gain was set to the highest possible value. Spectra were then manually phased, baseline corrected using the TOPSPIN3.2 software (Bruker), and calibrated on the central resonance of methanol-d_4 (δ 49.10 ppm).

The last step consisted in the binning of all ^{13}C NMR signals followed by the visualization of the whole dataset as a heat map. For this purpose, the absolute intensities of all ^{13}C NMR signals detected in the spectra of the fraction series were automatically collected and each resulting peak list was stored as a text file. The binning step was performed by using a locally developed computer script written in Python language. Its principle was to divide the ^{13}C spectral width (from 0 to 240 ppm) into regular chemical shift windows ($\Delta\delta$ = 0.2 ppm), and to associate the absolute intensity of each peak to the corresponding bin. The resulting table was imported into the PermutMatrix version 1.9.3 software (LIRMM, Montpellier, France) and submitted to Hierarchical Clustering Analysis (HCA) for data visualization. Then the chemical shifts clusters regrouped with the HCA were compared to a database to identify the compounds. In order to confirm the structures of the identified compounds, additional 1D and 2D NMR experiments (^{1}H NMR, HSQC, HMBC, and ^{1}H-^{1}H-COSY).

3.3.3. HPLC, Flash chromatography and column chromatography analyses

Analytical HPLC experiments were performed using a Thermofisher Ultimate 3000 (Thermo Fischer Scientific, Villebon sur Yvette, France), equipped with a 4 ways pump LPG 3400 SD, an automatic injector WPS 3000 SL and a UV/Visible diode array detector 3000. The mobile phase was composed of H_2O with TFA (0.0025% v/v) and CH_3CN. A gradient elution method was applied from 10% to 50% of CH_3CN in 30 min with a flow rate of 1 mL/min. The chromatographic column used was a Kinetex C_{18} (4.6×100 mm 2.6 µ, Phenomenex, France). A prepacked RP-C_{18} column (Phenomenex Luna 250×15 mm, 5 µ) was used for semi-preparative HPLC. The mobile phase consisted of H_2O with TFA (0.0025%) and CH_3CN with a flow rate of 5 mL/min and the chromatogram was monitored at 210, 254, 280 and 366 nm. Sub-fractions $C_{2\text{-}4}$ were purified through RP-C_{18} semi-prep HPLC with gradient system (18-45% CH_3CN, 25 min) to yield compounds **8** (t_R 7.9, 6 mg), **11** (t_R 13.4, 6 mg) and **12** (t_R 14.2, 16 mg). Compounds **1** (7 mg) and **2** (23 mg) were purified by a flash chromatography from the sub-fraction $C_{20\text{-}21}$ (293 mg) on reversed phase using a gradient 10-50 % CH_3CN in water as a mobile phase. Compounds **10** (36 mg) and **9** (7 mg) were obtained as a yellow precipitate from the subfractions C_{10} and C_{13} respectively. Sub-fractions C_{18} (398 mg) was subjected to flash chromatography over silica gel, eluted by gradient system of $CHCl_3$-MeOH (10:0-7:3) to give compounds **3** (15 mg) and **4** (3 mg). Compounds **5** (4 mg), **6** (6 mg) and **7** (5 mg) were obtained from sub-fractions C_{20} using both the same method and eluent as for the sub fraction $B_{21\text{-}22}$.

*3.4. 7-O-(E-caffeoyl)-sylvestroside I (**1**)*

Yellowish solid; $[\alpha]_D^{25}$ -36.5 (*c* 0.23, MeOH); UV_{max} (MeOH): 228, 326; ^{1}H- and ^{13}C-NMR data, see Table 3; HR-ESI-MS *m/z*: 933.2997 $[M+Na]^+$ (calcd for $C_{42}H_{54}O_{22}Na$, 933.3004).

*3.5. 7-O-(E-p-coumaroyl)-sylvestroside I (**2**)*

Yellowish solid; $[\alpha]_D^{25}$ -77.2 (*c* 0.25, MeOH); IR (KBr) ν_{max} (cm^{-1}): 3420, 1697; UV_{max} (MeOH): 228, 312; ^1H- and ^{13}C-NMR data, see Table 3; HR-ESI-MS *m/z*: 917.3063 $[M+Na]^+$ (calcd for $C_{42}H_{54}O_{21}Na$, 917.3055).

3.6. Bioassay procedure

3.6.1. Antimicrobial Activity

3.6.1.1. Antimicrobial screening of the 70% EtOH extract and fractions A-E of S. stellata against a panel of 22 microbial strains.

The antimicrobial screening was performed on a total of 17 bacteria obtained from the Laboratory of Microbiology, faculty of pharmacy from the University of Reims Champagne-Ardenne including the following Gram-positive bacteria: *Bacillus subtilis*, *Enterococcus faecalis* ATCC 1034, *Staphylococcus aureus* 8325-4, *Staphylococcus aureus* CIP 53.154, *Micrococcus luteus*, *Listeria innocua*, *Streptococcus pyogenes* and *Staphylococcus epidermidis*, and the following Gram-negative bacteria: *Escherichia coli* CIP 54.127, *Enterobacter cloacae*, *Salmonella enterica*, *Serratia marcescens*, *Proteus vulgaris*, *Klebsiella pneumoniae*, *Providencia stuartii*, *Pseudomonas aeruginosa* ATCC 9027, *Shigella soneii*, as well as five yeasts obtained from the Laboratory of Parasitology-Mycology (Transmission Vectorielle et Epidémio-surveillance des Maladies Parasitaires, EA 4688) of the University of Reims Champagne-Ardenne: *Candida glabrata*, *C. tropicalis*, *C. kefyr*, *C. albicans* and *Cryptococcus neoformans*. The 17 bacteria and 5 yeasts were incubated overnight at 37 °C in tubes containing Mueller-Hinton (MH) broth medium. The bacteria were then diluted with MH-broth by means of serial dilution to finally reach a concentration of 10^5 bacteria/mL. The same process was performed for bacteria and yeasts. The minimum inhibitory concentration (MIC) of the crude ethanol extract of *S. stellata* was studied using MH agar in square Petri dishes seeded by multiple inoculators as described in a previous work [17,31]. The crude

extract was tested at six final concentrations (10, 5, 2.5, 1.2, 0.6, and 0.3 mg/mL) against the 22 microorganisms. The agar plates were incubated for 24 h at 37 °C. The activity was then visually estimated by the presence or absence of colonies. MIC values were recorded as the lowest concentrations of compounds enabling growth inhibition. Solvents were checked for absence of antimicrobial activity. Positive antimicrobial controls were used for bacteria (gentamicin and vancomycin) and yeasts (amphotericin B).

3.6.1.2. Evaluation of the antibacterial activity of the isolated compounds against Staphylococcus aureus by bioautography

As described in a previous work [31], an aliquot of each compound (2 mg) was solubilized in 1 mL methanol. A part of the resulting solutions (25 µL) were spotted onto Merck 60 F_{254} pre-coated silica gel plates (10×10 cm). Methanol and Gentamicin (50 µg) were also spotted on the plates as negative and positive control, respectively. The TLC plates were directly dried without migration and sterilized. The plates, placed in square Petri dishes were then covered by Mueller-Hinton (MH) agar medium containing a *Staphylococcus aureus* 53.154 suspension of 10^5 bacteria/mL. After incubation for 24 h at 37 °C, bacterial growth was revealed by a 2 mg/mL solution of thiazolyl blue tetrazolium bromide (MTT) and growth inhibition zones were measured. White stains indicated where reduction of MTT to the colored formazan did not take place due to the absence of bacterial growth.

3.6.1.3. MIC determination of the most active compounds against E. faecalis, S. aureus, E. coli, S. epidermis and P. aeruginosa by broth microdilution

A serial liquid dilution technique in 96-well microliter plates was used to determine the MIC values of the most promising compounds as revealed by bioautography [32]. For this purpose, nine concentrations of the most active fractions, from 500 µg/mL to 2 µg/mL were tested in

presence of bacterial suspensions (10^5 bacteria/mL) giving a final volume of 200 µL in MH media. Two wells were represented as bacteria culture control (positive control) and medium sterility control (negative control). The plates were incubated overnight at 37 °C, sprayed with a 0.2 mg/mL MTT solution and incubated again at 37 °C for 30 min. Bacterial growth was indicated by a violet color whatever the color intensity, while bacterial growth inhibition was admitted only for wells which remained clear. MIC values were determined as the lowest concentrations of samples having an inhibitory effect on bacteria growth (clear wells).

3.6.2. Free radical scavenging activity

The antioxidant activity of crude extracts, fractions and purified compounds was measured in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH method [33]. 5 µL of different concentrations of the samples were added to 95 µL of a DPPH solution (158 µM, dissolved in EtOH 50%). The reaction proceeded for 30 min at 37 °C on a 96-well microplate and the absorbance was then read at 515 nm. The DPPH inhibition percentage was calculated as followed: % inhibition $[(Ab_{control}-Ab_{sample})/Ab_{control}]\times100$. A DPPH solution in EtOH 50% was used as a control. The curve of the % scavenging activity against the concentration of sample was prepared by MSExcel based program to obtain the IC_{50}. Samples were prepared at concentrations of 100, 50, 25, 6.2 and 3.1 µg/mL. Ascorbic acid was used as a positive control. All the tests were conducted in triplicate. Ascorbic acid was used as a positive control agent.

3.6.3. Tyrosinase enzyme assay

The tyrosinase inhibitory activity was determined according to the method described previously [34]. L-DOPA was used as the substrate in this experiment. Samples were prepared at concentrations of 400, 100, 50, 25 and 12.5 µg/mL in 10% DMSO in aqueous solution and

100 µL of each concentration were added to 96-well plate and then 100 µL of 135 U/mL fungal tyrosinase in phosphate buffer solution (PBS, pH 6.8) were added. After pre-incubation at 25 °C for 10 min, 100 µL of L-DOPA (0.5 mM, PBS pH 6.8) were added into 96-well plate. The reaction mixture was incubated for another 5 min at 25 °C. The amount of dopachrome in the mixture was determined by the measurement of the absorbance of each well at 475 nm. Kojic acid was used as positive control agent. The inhibitory percentage of tyrosinase was calculated according to the following equation: % inhibition = {[(*A*-*B*)-(*C*-*D*)]/(*A*-*B*)}×100 (*A*: Ab without test substance; *B*: Ab without test substance and tyrosinase; *C*: Ab with test substance; *D*: Ab with test substance, but without tyrosinase). All the tests were conducted in triplicate and IC_{50} was determined by interpolation of concentration % inhibition curve obtained by MSExcel based program.

3.6.4. Cell proliferation assay

The fibrosarcoma cells (HT1080) were cultured in Minimum Essential Media (MEM) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin Streptomycin (PS) at 37 °C with 5% CO_2 and harvested every three days for maintenance. Compounds **1** and **2** were dissolved in DMSO. For treatment, cells were plated at a density of 10^4 cells/mL in 24-well plates at 37 °C. After 24h, the culture medium was discarded and cells were treated with the compounds in a fresh culture medium at various concentrations for 72h, while the same dilution volume of DMSO was added in negative control wells. The concentration of DMSO did not exceed 0.1% to avoid significant toxicity on the tested cells. Therefore, the cells were washed once with 1 mL of D-PBS and then detached with 0.2% Trypsin/EDTA. Cell counting was carried out on a KOVA® slide and with a phase contrast microscope as indicated by the manufacturer. Cell growth was calculated in percentage as the fraction of cell number in

treated and control cells. IC_{50} was determined as the concentration of each compound which induced 50% inhibition of cell growth.

4. Conclusions

Twelve compounds were identified and isolated from crude 70% EtOH crude extract of *S. stellata* among them seven showed good to moderate inhibitory activity against *S. aureus*, *E. coli, P. aeruginosa, E. faecalis* and *S. epidermidis*. And only four compounds showed DPPH radical scavenging activity. A new bis-iridoid showed a cytotoxic activity against fibrosarcoma cell line (HT1080). All structures were unambiguously assigned based on NMR, HRESIMS data which afforded the new bis-iridoids (**1**-**2**) and known compounds **3**-**12** structures. Iridoids and bis-iridoids, found in a large number of plants of the Caprifoliaceae family and the major components were derived from loganin or secologanin with a sugar unit at C-1 and bis-iridoids heterodimers [11,35]. Interestingly; in the present work, all bis-iridoids (**1**-**4**) isolated from *S. stellata* were found to possess secoiridoid/iridoid-subtype skeletons, which are closely related to the bis-iridoids catleyoside isolated from *Scabiosa variifolia* [15] and dipsanoside **C**-G isolated from *Dipsacus asper* [36]. Also, sylvestroside I (**3**) was previously isolated from the genus *Dipsacus* [24]. This indicates a close relationship between the two genera *Scabiosa* and *Dipsacus*. Septemfidoside (**4**), eustomorusside (**6**) and eustomoside (**7**) are reported for the first time in the Caprifoliaceae family.

Conflict of interest.

The authors declare no conflict of interest

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Appendix A. Supplementary data.

Supplementary data to this article can be found online at

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Table 1.
Antimicrobial activity of 70% ethanol extract of *S. stellata* and fractions A-E (solid medium).

Micro-organisms	**70% EtOH extract** MIC (mg/mL)	Fractions MIC (mg/mL)					Positive controls MIC (µg/mL)		
		A	**B**	**C**	**D**	**E**	**G**	**V**	**Am**
Gram positive bacteria									
Bacillus subtilis	5	> 10	2.5	2.5	10	> 10	0.12	2	NT
Enterococcus faecalis ATCC 1034	> 10	> 10	1.2	2.5	10	> 10	16	> 64	NT
Staphylococcus aureus 8325-4	> 10	> 10	0.6	1.2	10	> 10	0.5	4	NT
Staphylococcus aureus CIP 53.154	> 10	> 10	0.6	1.2	10	> 10	4	> 64	NT
Staphylococcus epidermidis	2.5	10	1.2	1.2	5	5	0.25	4	NT
Micrococcus luteus	2.5	10	1.2	2.5	5	5	0.5	4	NT
Listeria innocua	> 10	> 10	> 10	10	> 10	> 10	0.5	≤ 4	NT
Streptococcus pyogenes	1.2	10	1.2	2.5	5	5	2	1	NT
Gram negative bacteria									
Escherichia coli CIP 54.127	> 10	> 10	> 10	10	> 10	> 10	≤4	>16	NT
Enterobacter cloacae	> 10	> 10	> 10	10	> 10	> 10	≤4	>16	NT
Salmonella enterica	> 10	> 10	> 10	10	> 10	> 10	≤4	>16	NT
Serratia marcescens	> 10	> 10	> 10	10	> 10	> 10	0.5	> 64	NT
Proteus vulgaris	> 10	> 10	> 10	10	> 10	> 10	≤4	>16	NT
Klebsiella pneumoniae	> 10	> 10	> 10	10	> 10	> 10	> 64	> 64	NT
Providencia stuartii	> 10	> 10	> 10	10	> 10	> 10	2	> 64	NT
Pseudomonas aeruginosa ATCC 9027	> 10	> 10	> 10	10	> 10	> 10	8	> 64	NT
Shigella sonnei	> 10	> 10	> 10	10	> 10	> 10	0.5	8	NT
Yeast									
Candida albicans	5	10	0.6	1.5	5	10	> 64	> 64	0.5
Candida glabrata	2.5	10	2.5	2.5	10	10	> 64	> 64	0.25
Candida tropicalis	5	> 10	1.5	1.5	5	10	> 64	> 64	0.25
Candida kefyr	2.5	5	1.2	1.2	5	5	> 64	> 64	0.25
Cryptococcus neoformans	2.5	5	1.2	1.2	5	5	> 64	> 64	0.5

MIC: minimum inhibitory concentration, NT: not tested. Positive controls: G: **G**entamicin, V: **V**ancomycin and Am: **Am**photericin B.

Table 2. DPPH radical scavenging and mushroom tyrosinase inhibition of 70% MeOH extract, fractions A-E and compounds isolated from *S. stellata.*

	DPPH radical scavenging activity IC_{50} (µg/mL)	Mushroom tyrosinase inhibition IC_{50} (µg/mL)
70% MeOH extract	86.0 ± 1.8	(40%)*
Fraction A	133 ± 2.6	(45%)*
Fraction B	48.7 ± 1.1	(15%)*
Fraction C	25.0 ± 0.8	1330 ± 23
Fraction D	64.3 ± 1.5	1000 ± 19
Fraction E	> 200	1330 ± 22
6	7.1 ± 0.4	> 665
7	7.2 ± 0.4	> 665
8	8.5 ± 0.5	> 665
10	16.0 ± 0.6	> 665
Ascorbic acid[a]	6.3 ± 0.1	
Kojic acid[a]		6.8 ± 0.1

*****% Inhibition at 1330 µg/mL.
[a]The positive control.

Table 3
NMR spectroscopic data of the compounds **1-2** in CD_3OD

	1		**2**	
	δ_H m (*J* in Hz)	δ_C	δ_H m (*J* in Hz)	δ_C
Unit A				
1a	5.60 d (6.5)	96.3	5.60 d (6.5)	96.3
3a	7.43 s	151.3	7.42 s	151.2
4a	-	110.4	-	110.3
5a	2.95 q (6.2)	30.1	2.95 q (6.2)	30.3
6a	1.92 ddd (13.8, 7.8, 6.6)	28.9	1.92 ddd (13.8, 7.5, 6.8)	28.9
	2.10 m		2.10 m	
7a	4.21 m	62.7	4.21 m	62.7
	4.26 m		4.26 m	
8a	5.84 ddd (17.3, 10.3, 8.7)	134.3	5.82 ddd (17.3, 10.5, 8.0)	134.3
9a	2.72 m	44.0	2.71 dt (8.0, 6.2)	44.0
10a	5.30 d (10.3)	118.2	5.30 d (10.5)	118.2
	5.36 d (17.3)		5.36 d (17.3)	
11a	-	167.8	-	167.8
1a-*O*-glc				
1′	4.74 d (7.9)	96.8	4.74 d (7.9)	98.8
2′	3.23 t (8.0)	73.3	3.23 t (8.0)	73.3
3′	3.39 t (8.8)	76.6	3.39 t (9.0)	76.6
4′	3.29 t (8.9)	70.2	3.29 t (9.5)	70.2
5′	3.33 m	76.9	3.33 m	76.9
6′	3.68 dd (12.0, 5.6)	61.4	3.68 dd (12.0, 5.6)	61.4
	3.92 dd (12.0, 2.2)		3.92 dd (12.0, 2.2)	
	7a-*O*-caffoyl		**7-*O*-*p*-Coumaryol**	
1″	-	126.3	-	125.7
2″	7.05 d (2.0)	113.7	7.47 d (8.6)	129.8
3″	-	145.4	6.83 d (8.6)	115.4
4″	-	148.2	-	148.2
5″	6.80 d (8.1)	115.1	6.83 d (8.6)	115.4
6″	6.96 dd (8.1, 2.0)	121.6	7.47 d (8.6)	129.8
7″	7.55 d (16.0)	145.5	7.61 d (16.0)	145.1
8″	6.24 d (16.0)	113.7	6.30 d (16.0)	113.8
9″	-	167.9	-	167.9
Unit B				
1b	5.28 d (5.3)	96.3	5.28 d (5.3)	96.3
3b	7.53 s	152.3	7.53 s	152.3
4b	-	111.6	-	111.7
5b	3.14 q (8.2)	31.5	3.14 q (8.2)	31.5
6b	1.76 ddd (13.8, 8.2, 5.1)	38.9	1.76 ddd (13.5, 8.0, 5.2)	38.9
	2.34 dd (13.8, 8.1)		2.34 dt (13.8, 8.0)	
7b	5.24 t (5.1)	77.0	5.24 t (4.5)	77.0
8b	2.19 m	39.7	2.16 m	39.7
9b	2.08 m	45.7	2.08 m	45.7
10b	1.10 d (6.9)	12.6	1.10 d (6.9)	12.6
11b	-	166.9	-	166.9
OCH_3	3.68 s	50.3	3.68 s	50.3
1b-*O*-glc				
1‴	4.68 d (7.9)	96.8	4.68 d (7.9)	98.7
2‴	3.23 t (8.0)	73.2	3.23 t (8.0)	73.2
3‴	3.39 t (9.0)	76.6	3.39 t (9.0)	76.6
4‴	3.31 t (8.8)	70.1	3.31 t (8.8)	70.1
5‴	3.33 m	77.0	3.33 m	77.0
6‴	3.68 dd (12.0, 5.6)	61.4	3.68 3.68 dd (12.1, 5.6)	61.4
	3.92 dd (12.0, 2.2)		3.92 dd (12.1, 2.0)	

Table 4.
Minimum inhibitory concentration (MIC) values of compounds isolated from *S. stellata* in liquid medium.[a]

	MIC (µg/mL)				
Compounds	*E. faecalis* (ATCC 1054)	*S. aureus* (CIP53.154)	*E. coli* (CIP 54.127)	*S. epidermis*	*P. aeruginosa* (ATCC9027)
1	31.2	62.5	250	31.2	125
2	31.2	62.5	125	31.2	125
3	500	62.5	62.5	125	125
4	125	250	500	250	250
6	62.5	62.5	125	250	125
8	125	250	125	125	125
9	250	250	125	125	125
11	250	250	>500	250	125
12	125	125	500	125	62.5

[a] No bacterial growth inhibition observed in bioautography assay for compounds **5**, **7** and **10.**

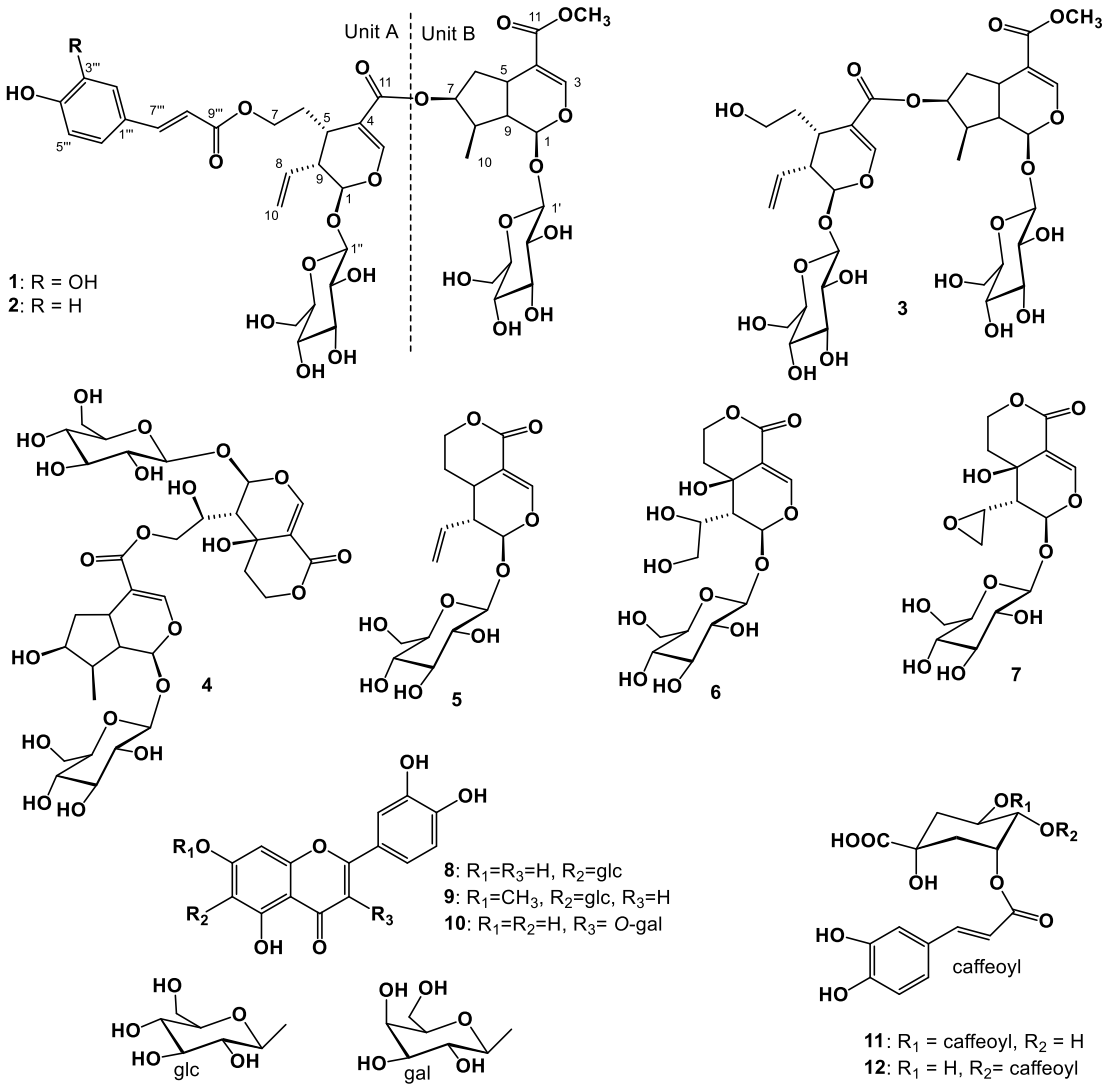

Figure 1. Chemical structures of compounds **1**-**12** isolated from *Scabiosa stellata*.

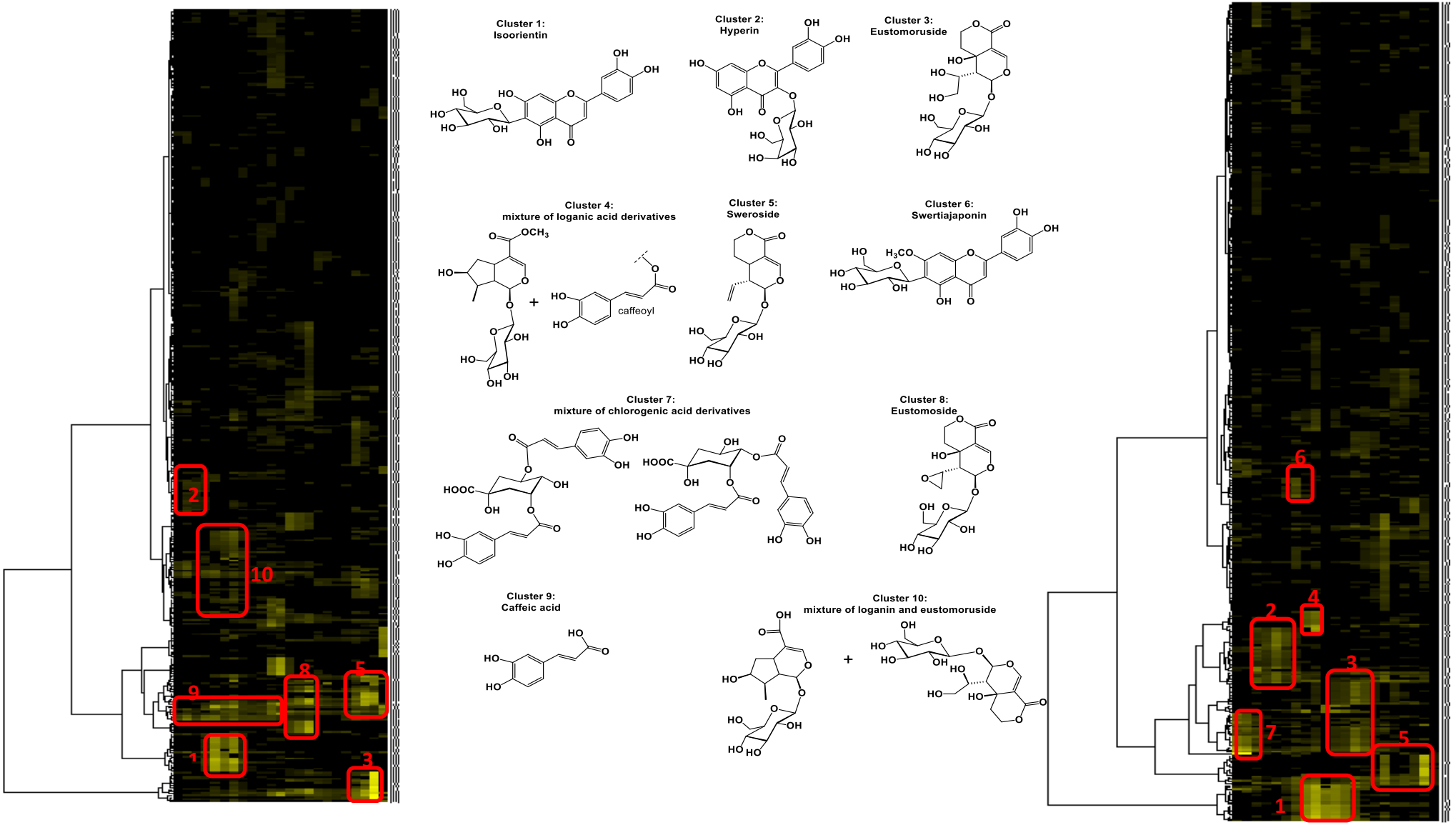

Fig. 2. ^{13}C NMR chemical shift clusters obtained by applying HCA on CPC fractions B (left) and C (right) of *Scabiosa stelatta*.