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Metabolic differentiations and classification of *Verbascum* species by NMR-based metabolomics

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ABSTRACT

The genus *Verbascum* L. (mulleins) comprises of about 360 species of flowering plants in the Scrophulariaceae family. Mulleins have been used in the traditional folk medicine for centuries, for treatment of a wide range of human ailments, *inter alia* bronchitis, tuberculosis, asthma, and different inflammations. Despite all applications the knowledge of the metabolites, accumulated in different mullein species, is still limited and based mainly on determination of the major compounds. Here we report the application of ^{1}H NMR metabolic fingerprinting in combination with principal component analyses (PCA) in five different *Verbascum* species. Based on the obtained results mulleins were divided in two groups: group A (*Verbascum phlomoides* and *Verbascum densiflorum*) and group B (*Verbascum xanthophoeniceum*, *Verbascum nigrum* and *Verbascum phoeniceum*). Further it was found that the plants in group B accumulate higher amounts of bioactive iridoid and phenylethanoid glycosides. *V. xanthophoeniceum* and *V. nigrum* accumulate higher amounts of the pharmaceutically-important harpagoside (~0.5% on dry weight basis) and verbascoside, forsythoside B and leucosceptoside B (in total 5.6–5.8% on dry weight basis), which underlines the possibility for their application in pharmaceutical industry. To the best of our knowledge this is the first report on the analyses of *Verbascum* sp. leaf metabolome.

1. Introduction

The genus *Verbascum* L., common name mulleins, comprises about 360 species of flowering plants in the Scrophulariaceae family, predominantly distributed in Asia, Europe, and North America (Heywood, 1993; Klimek et al., 2010). Mulleins are biennial or perennial, rarely annual plants, with a deep tap root. At the first year plants form a dense rosette of leaves, which is followed by growth of a stout flowering stem 0.3–2 m tall (Turker and Gurel, 2005). Several records exist for the medical use of mulleins in folk medicine as a remedy for respiratory problems such as bronchitis, dry coughs, whooping cough, tuberculosis, and asthma. In Bulgarian folk's medicine *Verbascum* infusions were used for treating mouth, gullet, stomach and intestine inflammations. The leaves, roots and the flowers possess also anodyne, antimicrobial, anti-inflammatory, and sedative properties (Turker and Gurel, 2005). Although *Verbascum* plants have been used medicinally since ancient times, their popularity increased commercially in the past few years. Today, the dried leaves and flowers, swallow capsules, alcohol extracts and the flower oil of common mullein can be found in the USA in health stores (Turker and Gurel, 2005).

Verbascum plants accumulate several bioactive iridoid and phenylethanoid glycosides (Turker and Gurel, 2005; Klimek et al., 2010; Georgiev et al., 2011), therefore they might serve as an attractive source of potent anti-inflammatory and antioxidant compounds. Except of iridoids and phenylethanoids *Verbascum* plants are reported to produce flavonoid glycosides (7-glycosides of luteolin, quercetin and apigenin) along with other glycosides as diosmin, tamatixetin 7-rutinoside and tamatixetin 7-glucoside (Klimek et al., 2010). The European Pharmacopoeia states that the plant material Verbasci flos may be derived from *V. densiflorum*, *V. phlomoides* or *Verbascum thapsus* (common mullein), however due to some shortcomings of the latter species, common mullein is not frequently used (Klimek et al., 2010).

Metabolomics is defined as both qualitative and quantitative analysis of all metabolites in an organism (Verpoorte et al., 2007). Since the term 'metabolome' has been defined a decade ago several platforms and techniques for high throughput analyses of targeted metabolites have been developed (Villas-Boas et al., 2005). Nuclear magnetic resonance (NMR) has been already proven as a suitable and adequate method to carry out such analyses, because it allows simultaneous detection of diverse groups of secondary metabolites besides abundant primary metabolites

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(Verpoorte et al., 2007; Kim et al., 2010a). Moreover ^{1}H NMR-spectrometry possesses a great advantage over the other metabolomics techniques, as the signal intensity is only dependent on the molar concentration in the solution, which enables the direct comparison of concentrations of all compounds present in the sample (Verpoorte et al., 2008; Kim et al., 2010a,b). Therefore several reports on the evaluation of metabolic differences in *Cannabis sativa*, *Vanilla planifolia*, *Vitis* spp. and *Catharanthus roseus* (among others) and on the classification of *Ilex* species based on their metabolome were published in the recent years (Choi et al., 2004a,b; Ali et al., 2010; Kim et al., 2010b; Palama et al., 2010). A major drawback for the utilization of NMR spectroscopy in metabolomic analyses is signal overlapping, which however can be solved by the use of different 2D techniques (Ali et al., 2010; Kim et al., 2010a,b).

The aim of the present study was to determine the metabolic differences between five *Verbascum* species. Leaves of plants grown in a greenhouse were analyzed by means of 1D ^{1}H NMR based metabolomics and the species were classified by means of principal component analyses (PCA). To the best of our knowledge there are no reports on the analysis of the whole metabolome of *Verbascum* species.

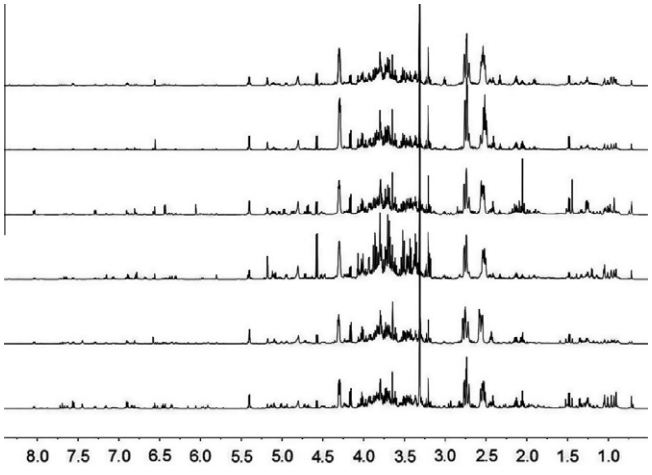

Fig. 1. Six hundred megahertz ^{1}H NMR spectra of extracts of *Verbascum* leaves. From bottom to top: *V. xanthophoeniceum*, *V. nigrum*, *V. phlomoides* 7, *V. phoeniceum*, *V. phlomoides* 33 and *V. densiflorum*.

2. Results and discussion

2.1. Identification of metabolites in Verbascum extracts by 1D and 2D NMR

Verbascum plants are known to possess a wide spectrum of biological activities and therefore have been used for centuries in the folk's medicine. However, the knowledge of the metabolites, accumulated in different mullein species is still limited and based mainly on determination of the major compounds. Considering that different species maybe used as herbal medicine a generally applicable method to distinguish between the species would be very useful.

In the present report we describe the analysis of five different *Verbascum* species: *V. nigrum* (dark mullein), *V. phoeniceum* (purple mullein), *V. phlomoides* (orange mullein; two varieties, designated as 7 and 33), and *V. densiflorum* (dense flowered mullein) and *V. xanthophoeniceum* (an endemic plant species for the Balkan region, as well as for Northwestern and Southern parts of Turkey). All seeds were germinated under the same conditions in the green house of Institute of Biology Leiden (Leiden University). After three months of growth *Verbascum* plants formed ~12–18 cm (in diameter) dense rosettes.

We applied ^{1}H NMR in combination with some 2D NMR techniques, following the protocol developed in our laboratory (Kim et al., 2010a). The obtained results revealed both qualitative and quantitative differences between the *Verbascum* samples (Fig. 1). In total we were able to assign the signals to 22 compounds in different *Verbascum* species, including amino acids, organic acids, carbohydrates, phenolics and iridoid glycosides (Table 1). The amino acids alanine, leucine, valine and glutamine were identified from the aliphatic region (δ 0.5–3.0) in all mullein species by comparison with the reference spectra of these compounds. Also some strong signals were assigned to formic acid, citric acid, malic and ascorbic acid (Table 1). Moreover in this region of the spectrum choline and 2,3-butanediol were identified. Two phenolic acids – gallic acid and chlorogenic acid – were found to be present in all

Table 1
^{1}H NMR chemical shifts (δ) and coupling constant (J, Hz) of *Verbascum* metabolites, identified by references and by 1D and 2D NMR spectra (CD_3OD-KH_2PO_4 in D_2O, pH 6.0).

Metabolite	Selected characteristic signals in NMR
Alanine	δ 1.48 (d, J = 7.4)
L-Leucine	δ 0.96 (d, J = 7.5), δ 0.98 (d, J = 7.5)
DL-Valine	δ 1.00 (d, J = 7.0), δ 1.05 (d, J = 7.0)
Glutamine	δ 2.15 (m), δ 2.47 (m)
α-Glucose	δ 5.18 (d, J = 3.73)
β-Glucose	δ 4.57 (d, J = 7.9)
Fructose	δ 4.16 (d, J = 8.6)
Sucrose	δ 5.40 (d, J = 3.82)
2,3-Butanediol	δ 1.14 (d, J = 6.8)
γ-Amino-butyrate (GABA)	δ 1.9 (m), δ 2.31 (t, J = 7.5), δ 3.01 (t, J = 7.5)
Formic acid	δ 8.45 (s)
Choline	δ 3.21 (s)
Citric acid	δ 2.56 (d, J = 11.96), δ 2.74 (d, J = 4.3)
Malic acid	δ 2.65 (dd, J = 17.3, 9.2), δ 2.75 (dd, J = 16.0, 4.2), δ 4.30 (dd, J = 7.7, 4.2)
Ascorbic acid	δ 4.52 (d, J = 2.0)
Chlorogenic acid	δ 6.88 (d, J = 6.6), δ 7.15 (d, J = 2.8), δ 7.55 (d, J = 9.5)
Gallic acid	δ 7.04 (s)
Fumaric acid	δ 6.53 (s)
Harpagide	δ 1.1 (s), δ 1.71 (d, J = 14.1), δ 2.71 (s), δ 6.06 (d, J = 0.98), δ 6.28 (d, J = 6.4)
Ajugol	δ 5.29 (dd, J = 3.3, 1.75), δ 5.72 (d, J = 1.3)
Aucubin	δ 4.62 (d, J = 5.8), δ 5.13 (dd, J = 6.14), δ 5.89 (br d), δ 5.23 (d, J = 5.5), δ 6.35 (d, J = 6.3)
Harpagoside	δ 1.52 (s), δ 2.26 (d, J = 15.5), δ 5.01 (dd, J = 6.5, 1.5), δ 6.15 (d, J = 0.97), δ 6.46 (d, J = 6.4), δ 6.53 (d, J = 16.0), δ 7.45 (m), δ 7.63 (m), δ 7.67 (d, J = 16.0), δ 4.72 (d, J = 8.2)

Verbascum species. The signals in the carbohydrate region (δ 3.0–5.5) are in general highly clustered and frequently overlapping (Ali et al., 2010; Kim et al., 2010a). Despite of that we were able to identify the anomeric protons of α-glucose at δ 5.18 (d, J = 3.73), β-glucose at δ 4.57 (d, J = 7.9), fructose at δ 4.16 (d, J = 8.6) and sucrose at δ 5.40 (d, J = 3.82), which were in fact the major compounds in *Verbascum* extracts (Fig. 1).

Among other metabolites we were also able to identify in the aromatic region (δ 5.5–9.0) four iridoid glycosides (Table 1). By comparison with authentic standards, aucubin (**1**), ajugol (**2**) and harpagide (**4**) were found in all *Verbascum* species, while harpagoside (**3**; Fig. 2) was detected only in *V. xanthophoeniceum* and *V. nigrum* plants. 2D-^{1}H–^{1}H *J*-resolved spectra of *V. xanthophoeniceum* and *V. phoeniceum* showed characteristic signals of common iridoids – ajugol, harpagide and aucubin, along with the presence (in *V. xanthophoeniceum*) and absence (in *V. phoeniceum*) of harpagoside signals (Fig. 3). The harpagoside H-1 signal was reported as a singlet in some old literature, probably due to the lower resolution of NMR apparatus used (Zhang et al., 1994; Li et al., 1999). However, our results (ob-

Fig. 2. Chemical structures of major iridoid and phenyletanoid glycosides in *Verbascum* leaves.

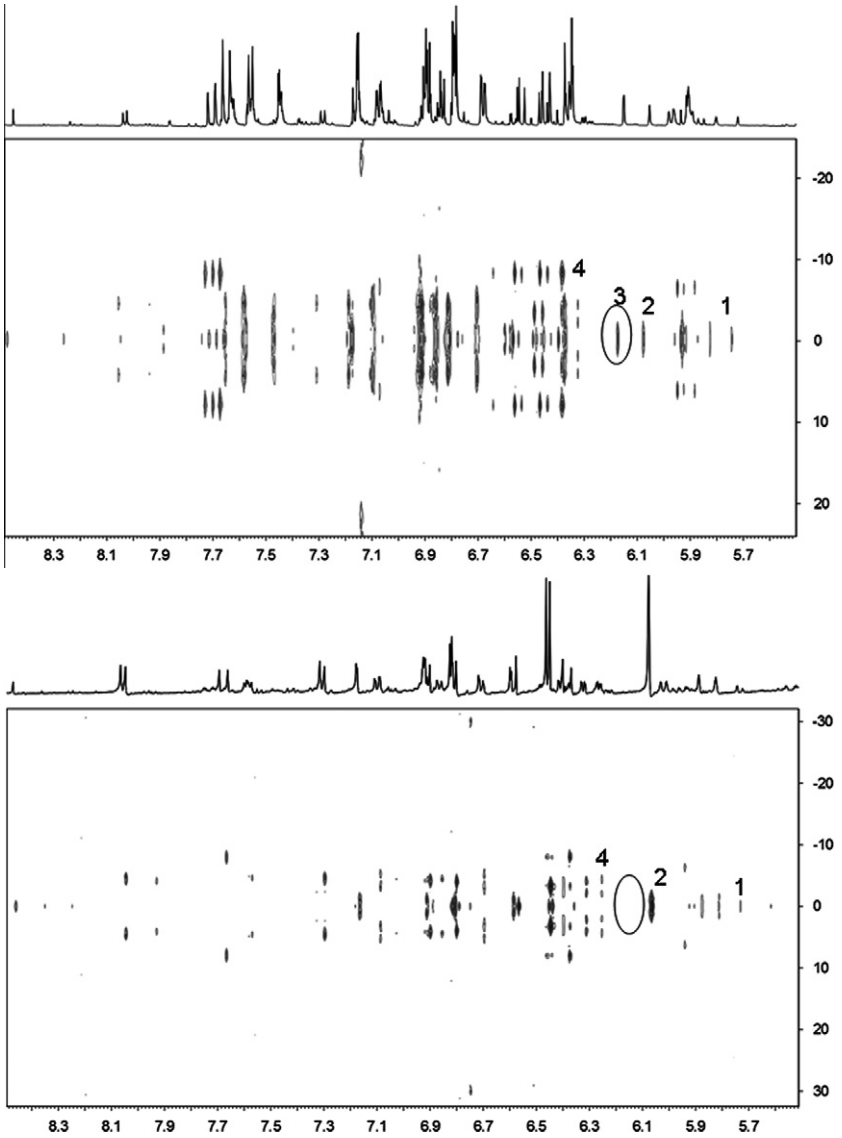

Fig. 3. *J*-resolved spectra of *V. xanthophoeniceum* (top) and *V. phoeniceum* (bottom) leaves extracts. Signals labelled as 1, 2, 3 and 4 correspond to H-1 of ajugol, H-1 of harpagide, H-1 of harpagoside and H-3 of aucubin, respectively. Ellipses are drawn to show the presence and absence of harpagoside signals.

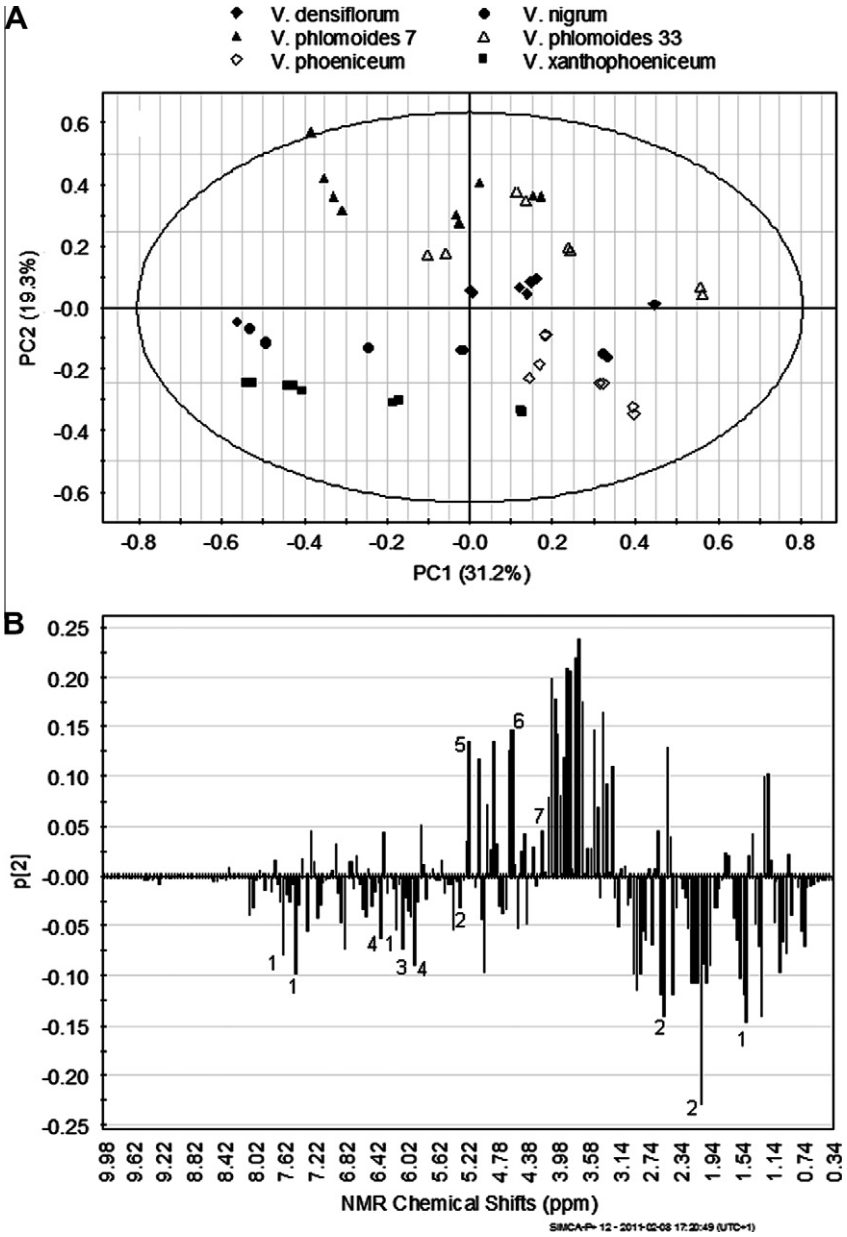

Fig. 4. Score plot of principal component analysis (PCA) results obtained from all ^{1}H NMR data showing PC1 and PC2 (A) and its corresponding loading column plot (B). The latter shows signals of compounds found higher in the extracts f leaves of *V. phlomoides* 7 and 33, and *V. densiflorum* (upper) and *V. xanthophoeniceum*, *V. nigrum* and *V. phoeniceum* (down). 1, harpagoside; 2, ajugol; 3, harpagide; 4, aucubin; 5, α-glucose; 6, β-glucose; 7, fructose.

tained in 600 MHz NMR) indicate that the H-1 signal of harpagoside is a small doublet (J = 0.97), which is also in agreement with the obtained spectral data of pure harpagoside (purchased from Extrasynthese, Genay, France). Iridoids are a large group of natural compounds, characterized by skeletons in which a six-membered ring containing an oxygen atom is fused to a cyclopentane ring (Fig. 2). A wide spectrum of biological activities of iridoid glycosides has been reported, however, they are most frequently applied as anti-inflammatory agents (Villasenor, 2007). Harpagoside (**3**), a very active member of the iridoid family, is the major iridoid glycoside of *Harpagophytum procumbens* (commonly known as devil's claw, Pedaliaceae family), an important South African medicinal plant. Extracts of the devil's claw tubers (containing 0.5–1.6% harpagoside) have been found to be effective in the treatment of degenerative rheumatoid arthritis, osteoarthritis, tendonitis, kidney inflammation and heart disease (Stewart and Cole, 2005; Grant et al., 2007). Currently, in the USA *Harpagophytum* extracts are undergoing phase II clinical trials for treating hip and knee osteoarthritis (www.clinicaltrials.gov/ct2/archive/NCT00295490).

2.2. Multivariate data analyses and metabolic classification of *Verbascum* plants

The ^{1}H NMR data set was subjected to PCA and HCA aiming to highlight the similarities or differences among *Verbascum* species studied (Figs. 4 and 5). A 13-components model explained 98.1% of the variance, with the first three components covering 64.2%. By using component 2 all *Verbascum* plants were clustered in two groups (Fig. 4A). Group A, having positive component 2, was formed by *V. densiflorum* and the two *V. phlomoides* varieties 7 and 33, while in the Group B with negative component 2,

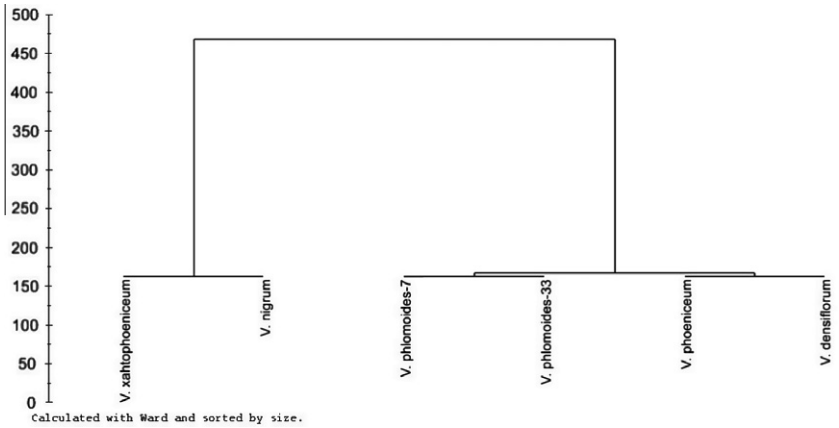

Fig. 5. Dendrogram of hierarchical cluster analysis of *Verbascum* species.

V. xanthophoeniceum, *V. nigrum* and *V. phoeniceum* were placed. Fig. 4B shows the loading column plot for the component 2. In general plants in the Group A contain more sugars (α-glucose, β-glucose and fructose), while the *Verbascum* plants in the Group B accumulate higher amounts of iridoid glycosides (harpagoside, ajugol, harpagide and aucubin).

Although the PCA score plot provides some ideas for grouping, the available principal components are limited because only three of them can be graphically presented. Also, the score plots do not provide any information on the closeness between groups (Kim et al., 2010b). Therefore we applied HCA to reveal the similarities between *Verbascum* species. As shown in Fig. 5 two groups were obtained from HCA; *V. xanthophoeniceum* and *V. nigrum* were clearly separated from the other mullein species. As expected the metabolome of two varieties of *V. phoeniceum* (7 and 33) were found to be most similar as wells as *V. densiflorum* and *V. phoeniceum* were closed.

^{1}H NMR metabolomics data and multivariate data analyses revealed that *V. xanthophoeniceum* and *V. nigrum* species are quite different from the other mullein species, recognized by the European Pharmacopoeia.

2.3. Relative quantification of metabolites in Verbascum extracts and HPLC-DAD analyses

The ^{1}H NMR data set (bucket table) was subjected to ANOVA in order to verify the results from multivariate data analyses. The one-way ANOVA analyses confirmed the participation of different metabolites in the discrimination between *Verbascum* plants ($p < 0.005$). To determine the (relative) quantity of ajugol, harpagide and aucubin in *Verbascum* plant leaves we used the mean peak area of their characteristic signals (shown in Fig. 4B). The harpagide relative quantity significantly varies between mullein plants showing highest amounts in *V. phoeniceum*, followed by *V. nigrum* and *V. xanthophoeniceum*, while its content in both *V. phoeniceum* species (7 and 33) and *V. densiflorum* was significantly lower (Fig. 6). Ajugol levels were also higher in *V. xanthophoeniceum*, *V. nigrum* and *V. phoeniceum*. Aucubin was present in comparable amounts in *V. xanthophoeniceum* and *V. nigrum*, being several times higher compared to the other *Verbascum* species (Fig. 6). Aucubin, isolated from *Verbascum lasianthum*, was reported to possess significant antinociceptive and anti-inflammatory activity (Kupeli et al., 2007). Therefore, its high content in *V. xanthophoeniceum* and *V. nigrum* might offer another attractive possibility for utilization of these plants.

Harpagoside content in both *V. xanthophoeniceum* and *V. nigrum* plants was quantified through an HPLC-DAD assay (Fig. 7). Both *Verbascum* species showed similar contents of harpagoside (5.8–6.4 mg/g dry weight). These harpagoside amounts are slightly higher, compared to the content in *H. procumbens* leaves (data not shown), which indicates that *V. xanthophoeniceum* and *V. nigrum* could serve as an alternative source of this anti-inflammatory molecule.

Another major group of compounds, which was found to be accumulated in *Verbascum* plants (especially in their leaves) are phenylethanoid glycosides (Klimek et al., 2010; Georgiev et al., 2011). Phenylethanoid glycosides are natural, water-soluble secondary metabolites that characteristically have a hydroxyphenylethyl moiety linked to a β-pyranose (apiose, galactose, rhamnose or xylose) via a glycosidic bond (Fig. 2). These substances are widely distributed throughout the plant kingdom and have a wide spectrum of biological activities (Dembitsky, 2005). For instance, verbascoside (also known as acteoside) is a phenylethanoid glycoside that has been shown to have strong anti-leukemic and cytotoxic activity against a murine cell line, as well as anti-inflammatory activity and antioxidant properties (Pettit et al., 1990; Diaz et al., 2004; Georgiev et al., 2010; Gyurkovska et al., 2011).

All *Verbascum* species were found to accumulate verbascoside (**5**), forsythoside B (**6**) and leucosceptoside B (**7**). Because of their closely related structures (Fig. 2) it was not possible to distinguish clearly between their relative content by ^{1}H NMR. Therefore, their presence was confirmed by LC-APCI-MS (forsythoside B: *m/z* 755.2 $[M-H]^-$; verbascoside: at *m/z* 623.2 $[M-H]^-$; and leucosceptoside

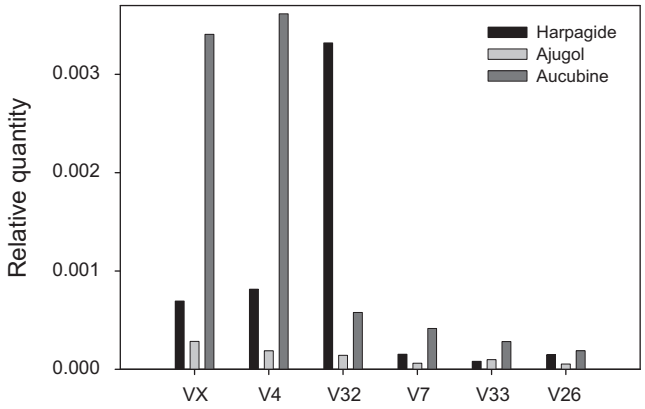

Fig. 6. Relative quantification of iridoid glycosides based on the mean peak area of the associated signals from 600 MHz ^{1}H NMR spectra of extracts of *Verbascum* leaves. VX, *V. xanthophoeniceum*; V4, *V. nigrum*; V32, *V. phoeniceum*; V7, *V. phlomoides* 7; V33, *V. phlomoides* 33; V 26, *V. densiflorum*.

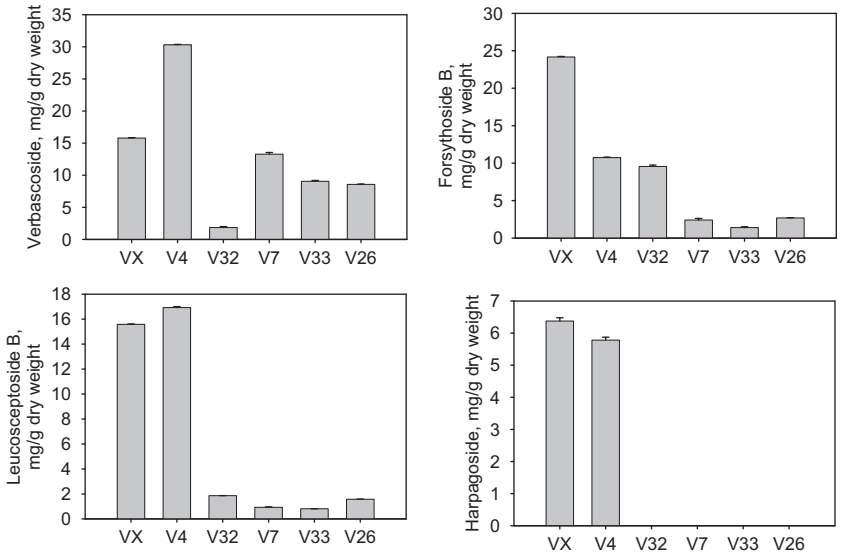

Fig. 7. HPLC-DAD quantification of phenylethanoid (verbascoside, forsythoside B and leucosceptoside B) and iridoid (harpagoside) glycosides content in *Verbascum* leaves. VX, *V. xanthophoeniceum*; V4, *V. nigrum*; V32, *V. phoeniceum*; V7, *V. phlomoides* 7; V33, *V. phlomoides* 33; V 26, *V. densiflorum*.

B at *m/z* 783.2 $[M-H]^-$) and their content was determined by HPLC-DAD assay (Fig. 7). Verbascoside content varies significantly in *Verbascum* leaves (between 0.18% and 3.03% of the dry weight), showing highest amounts in *Verbascum nigrum* (30.31 mg/g dry weight), followed by *V. xanthophoeniceum* and *V. phoeniceum* 7 (15.79 and 13.28 mg/g dry weight, respectively). Forsythoside B was highest in *V. xanthophoeniceum* (24.18 mg/g dry weight), followed by *V. nigrum* and *V. phoeniceum* (10.75 and 9.56 mg/g dry weight, respectively), whilst its content in *V. phoeniceum* 7 and 33, and *V. densiflorum* was bellow 0.3% of the dry weight. Similarly, leucosceptoside B was accumulated in higher amounts in *V. xanthophoeniceum* and *V. nigrum* (15.58–16.93 mg/g dry weight).

3. Concluding remarks

NMR-based metabolomics approach has been successfully applied to study metabolic differentiations of 5 mullein species. ^{1}H NMR fingerprinting in combination with PCA and HCA allows classification of *Verbascum* species in two groups. Two mullein species – *V. xanthophoeniceum* and *V. nigrum* – were found to accumulate high amounts of phenylethanoid glycosides (5.6–5.8% of the leaves dry weight). This fact along with the relative high harpagoside amounts (~0.5% of the leaves dry weight) revealed that these plant species could serve as attractive source for the pharmaceutical industry, although it has yet to be shown that leaves extracts have pharmacological potential (experiments are currently undergoing).

4. Experimental

4.1. Plant material

The *V. nigrum* L., *V. densiflorum* Bertol., *V. phoeniceum* L., *V. phlomoides* L. (varieties 7 and 33) seeds were provided with courtesy of IPK, Gatersleben (Gatersleben, Germany). The *V. xanthophoeniceum* Griseb seeds were collected from different habitats (Lesovo village, Yambol district) by Dr. S. Bancheva (Institute of Botany, Bulgarian Academy of Sciences). Voucher specimens were deposited in the Herbarium of the Institute of Botany, Sofia, Bulgaria (SOM). All *Verbascum* seeds were germinated in decontaminated soil and grown in the green house, at 25 °C, with an illumination period of 16 h light: 8 h dark. After three months of growth *Verbascum* plants were used for analyses. The leaves were always harvested at the same day time point, freeze-dried and stored at −80 °C prior to use.

4.2. Standards and chemicals

CH_3OH-d_4 (99.9%) and D_2O (99.9%) were purchased from Cambridge Isotope Laboratories Inc. (Miami, FL, USA), while trimethyl silylpropionic acid sodium salt-d_4 (TMSP-d_4) was from Sigma–Aldrich. Harpagoside was obtained from Extrasynthese (Genay, France), verbascoside was a generously provided from Dr. I. Koleva (University of Food Technology, Plovdiv, Bulgaria), while forsythoside B and leucosceptoside B were isolated before by Dr. K. Alipieva. All chemicals used apart from those mentioned above were of HPLC grade.

4.3. Extraction and NMR analyses

Extraction of *Verbascum* plant leaves was performed, following the protocol described by Kim et al. (2010a). In brief, 50 mg freeze-dried sample was transferred to a 2 mL Eppendorf tube, to which 0.75 mL CH_3OH-d_4 and 0.75 mL D_2O (KH_2PO_4 buffer, pH 6.0), containing 0.005% (w/v) TMSP-d_4 were added. The mixture was vortexed at room temperature for 1 min, ultrasonicated for 20 min and then centrifuged at 13,000 rpm at room temperature for 20 min. The supernatant (~0.75 mL) was transferred to a 5 mm NMR tube and used for the NMR analyses.

^{1}H NMR and 2D *J*-resolved spectra were recorded at 25 °C on a 600 MHz Bruker DMX-600 spectrometer (Bruker, Karlsruhe, Germany), operating at a proton NMR frequency of 600.13 MHz, as described before (Ali et al., 2010). CH_3OH-d_4 was used as the internal lock. The resulting spectra were manually phased and baseline corrected, and referenced to internal standard TMSP at 0.0 ppm, using XWIN NMR (version 3.5, Bruker). 2D *J*-resolved NMR spectra were acquired using 8 scans per 128 increments of F_1 and 8 k for F_2 using spectral widths of 500 Hz in F_2 (chemical shift axis) and 66 Hz in F_1 (spin–spin coupling constant axis). The *J*-resolved spectra were tilted by 45°, symmetrized about F_1, and then calibrated, using XWIN NMR software.

4.4. High performance liquid chromatography with diode array detection (HPLC-DAD) and liquid chromatography-atmospheric pressure chemical ionization mass spectrometry (LC-APCI-MS) analyses

Freeze-dried *Verbascum* leaves (~100 mg) were extracted with methanol (solid:liquid ratio 1:50) in an ultrasonic bath (3 × 20 min), pooled, filtered through 0.2-μm filters and directly injected into an HPLC-DAD system, to quantify harpagoside and phenylethanoid glycosides content. The HPLC-DAD system consisted of an 1200 Series instrument equipped with a G1310A pump, a G1329A autosampler, a G1322A degasser, a G1316A column oven, a G1315D diode array detector controlled by ChemStation (all from Agilent Technologies, Inc., Santa Clara, CA, USA) and a Luna reverse phase (C_{18}) column (150 mm × 4.6 mm i.d., 5 μm particle size; Phenomenex, Utrecht, The Netherlands). The metabolites were then separated using a mobile phase consisting of methanol/water 10:90 at a flow rate of 1.0 mL/min, followed by a linear gradient to 50:50 (A:B) at a flow rate of 1.1 mL/min for 10 min, then to 100 (B) at a flow rate of 0.8 mL/min for 5 min, followed by decrease of B to 10 for 5 min for determination of harpagoside and methanol/water 5:95 (A:B) for 3 min, followed by linear gradients to 25:75 (A:B) after 7 min then to 60:40 (A:B) after 13 min and the flow rate was 1 mL/min for determination of phenylethanoid glycosides (Gyurkovska et al., 2011). Eluting harpagoside and phenylethanoids (forsythoside B, verbascoside and leucosceptoside B) were detected, and quantified, by monitoring the eluate at a wavelength of 278 and 330 nm, respectively.

The extracts of *Verbascum* leaves (10 μL) were injected into an LC-APCI-MS system consisting of an 1100 Series instrument equipped with a G1312A binary pump, a G1367A autosampler, a G1379A degasser, a G1316A column oven, a G1315B diode array detector, a simple quadrupole SL mass spectrometer driven by ChemStation software, all supplied by Agilent Technologies. The same column and mobile phases as described above were used. The eluent was monitored by the UV detector at 278 nm (for harpagoside) and 330 nm (for phenylethanoid glycosides), and by the MS from mass-to-charge ratio (m/z) 300–850 (negative ionization mode) with drying gas at 10 mL min^{-1}, 350 °C, 50 psig and capillary voltage at 3000 V.

4.5. Data analyses

The ^{1}H NMR and the *J*-resolved data files were processed as described by Kim et al. (2010a). Both projection spectra were automatically reduced to ASCII files with AMIX software (version 3.7, Bruker). Spectral intensities were scaled to TMSP and reduced to integrated regions of equal width of 0.04 ppm, corresponding to the region of δ 0.3–10.0. The regions of δ 3.28–3.34 and δ 4.85–4.95 were excluded from the analyses, because of the residual signal of CH_3OD and D_2O, respectively. Principal component analysis (PCA) and hierarchical cluster analysis (HCA) were performed with SIMCA-P software (version 12.0, Umetrics, Umeå, Sweden). Both Pareto and unit variance (UV) scaling methods were applied to PCA. The ANOVA analysis for the ^{1}H NMR signals was performed by MuliExperiment Viewer (version 4.0; Saeed et al., 2003).

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