

Verbascum nigrum: Cytotoxicity Evaluation in A431 Epidermoid Carcinoma Cells and Untargeted LC-HR-MS/MS Metabolite Profiling

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The crude methanolic extract obtained from *Verbascum nigrum* aerial parts (VNE) and its six fractions (VNF1–VNF6) were initially screened regarding their effects on the viability of immortalized HaCaT keratinocytes and A431 epidermoid carcinoma cells (MTT assay, 24 h). None of the tested samples affected the viability of HaCaT cells in a concentration range of 25–150 µg/mL. VNE and VNF4 exhibited significant cytotoxic effects in A431 cells, with IC₅₀ values of 81.92 and 12.27 µg/mL, respectively; the selectivity index was higher than 10 for VNF4. The untargeted LC/HR-MS/MS metabolite profiling led to the tentative annotation of a total number of 23 compounds. Of these, VNE comprised mainly iridoid glycosides (harpagoside, laterioside, acylated aucubin derivatives), whereas VNF4 showed a high abundance of triterpene saponin glycosides (ilwensisaponins A and C, songarosaponins A and B), constituents known for their selective cytotoxic potential.

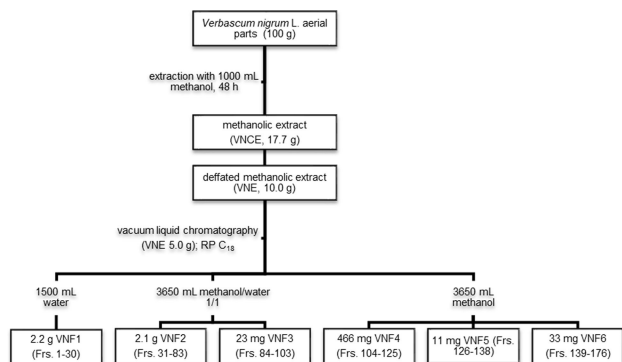
Keywords: *Verbascum nigrum*, cytotoxic activity, saponin glycosides, verbascoside, LC/HR-MS/MS.

Introduction

Native to Europe and Asia, *Verbascum* genus (Scrophulariaceae) comprises more than 360 species distributed not only in the two continents of origin, but also in North America and Northeast Africa.^[1] The roots, leaves and flowers collected from different species have been empirically used since Ancient times in the treatment of a wide range of human ailments.^[2,3] For instance, the aerial parts of *Verbascum nigrum* L. (dark mullein) are traditionally or homeopathically recommended in epilepsy, asthma, acute respiratory infections, gastralgia, diarrhea, dysentery or renal dysfunctions.^[4,5] Regarding previous chemico-biological investigations on this species, the literature is considerably scarce. Several extracts of black mullein were documented to possess anti-inflammatory,^[6]

anticoagulant, antihypotensive and diuretic effects.^[4] These bioactivities might be correlated with the presence of iridoid glycosides (harpagoside, laterioside, aucubin, sinuatol, nigrosides I–V), phenylethanoid glycosides (verbascoside), flavonoids and saponin glycosides (ilwensisaponins A and C) as the main phytochemicals reported in the flowers, leaves or roots of *V. nigrum*.^[5,7,8]

Squamous cell carcinoma, the second most common skin cancer (20% of keratinocyte carcinomas), is associated with a poor prognosis as it easily metastasizes. Both systemic and topical chemopreventive drugs (retinoids, nicotinamide, non-steroidal anti-inflammatory drugs and 5-fluorouracil alone or in combination with calcipotriol, respectively) have limitations with respect to tolerability.^[9,10] Therefore, identification of novel prophylactic and therapeutic



Scheme 1. *Verbascum nigrum* aerial parts: extraction and fractionation.

strategies is of great significance. As part of our continuous research interest in finding new phytochemicals that could be used as potential leads for cancer chemoprevention and therapy,^[2,11,12] some preliminary investigations on *V. nigrum*, a less explored species of mullein, were performed.^[13] Consequently, the current work investigated the *in vitro* cytotoxic activity of a crude methanolic extract obtained from *V. nigrum* and its six fractions in human skin squamous cell carcinoma A431 cells. Furthermore, to potentially correlate the observed biological outcomes with the phytochemical composition, the systematic metabolite profiling of the active samples by liquid chromatography hyphenated with tandem high-resolution mass spectrometry (LC/HR-MS/MS) was performed. The in-depth LC/HR-MS/MS dereplication strategies for the fast metabolite annotation was already established by our group for other *Verbascum* species, such as *V. blattaria* L. and *V. ovalifolium* Donn ex Sims,^[1,2] but not for *V. nigrum*.

Results and Discussion

Cytotoxic Activity

The crude extract of *V. nigrum* aerial parts (VNE) and its six fractions (VNF1–VNF6) obtained by vacuum liquid chromatography (Scheme 1) were initially investigated for their cytotoxic effects by MTT assay (24 h incubation) in spontaneously transformed immortalized HaCaT keratinocytes and A431 epidermoid carcinoma cells. As depicted in Figure 1a, none of the tested samples induced a significant reduction of HaCaT cell viability as compared to vehicle control. A similar trend was observed in A431 tumor cells for VNF1–3 and VNF5–6. However, VNE exhibited impor-

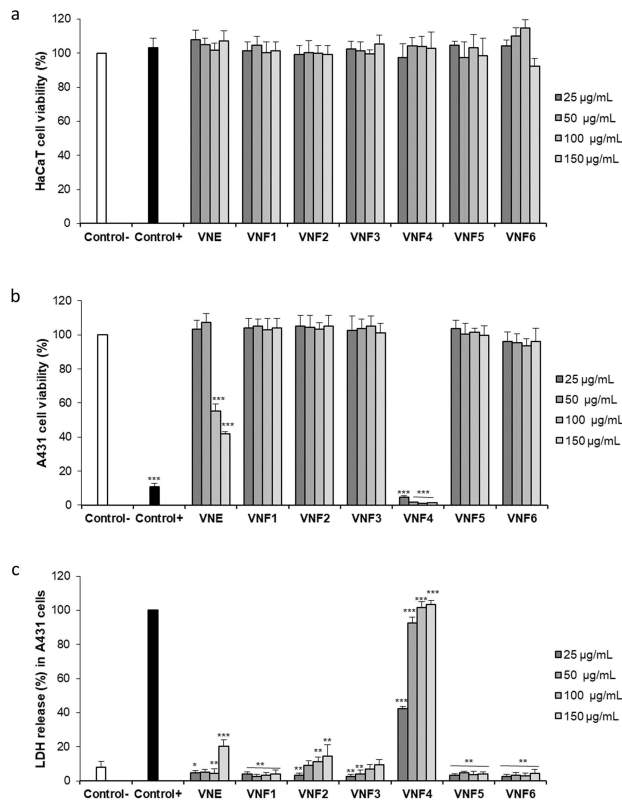


Figure 1. The influence on HaCaT cell viability (a), A431 cell viability (b) and LDH release in A431 cells (c) exhibited by the crude methanolic extract of *V. nigrum* aerial parts (VNE) and its fractions (VNF1–VNF6), after 24 h incubation. The data are expressed as percentage change in viability in comparison to the vehicle treated control group (control–); the positive control (control+) in MTT assays was represented by resveratrol (65 μ M); the positive control (control+) in LDH assay was represented by a cell lysis solution from the CytoTox96[®] assay kit; bars correspond to the standard error of the mean of three individual experiments, each with eight replicates; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. vehicle treated cells (control–).

tant cytotoxic effects of 47% and 58%, especially at higher concentrations (Figure 1b), with $IC_{50} = 81.92 \mu$ g/mL. In contrast to this, VNF4 reduced the viability of A431 cells by more than 95% over the concentration range of 25–150 μ g/mL. As compared to this, the positive control (resveratrol 65 μ M) produced cytotoxic effects of only 90%. Successive dilutions were made in order to obtain the IC_{50} value of 12.27 μ g/mL for VNF4. Taking into consideration that the IC_{50} value for VNF4 in the non-tumor HaCaT cells would be $> 150 \mu$ g/mL, it can be assumed that this fraction exhibited highly selective cytotoxic effects [the selectivity index (SI), calculated by dividing the IC_{50} value in the non-tumor cells to that in the tumor cells, would be > 10 ; samples with $SI > 3$ are considered highly selective].^[10] Previ-

ously we have shown that a crude methanolic extract obtained from the aerial parts of *V. ovalifolium* and its liquid-liquid partitioning fractions exhibited a concentration-dependent reduction of malignant melanoma SK-MEL-2 cell viability over the concentration range of 25–200 $\mu\text{g}/\text{mL}$, with butanol ($\text{IC}_{50} = 77.98 \mu\text{g}/\text{mL}$), hexane ($\text{IC}_{50} = 144.02 \mu\text{g}/\text{mL}$) and ethyl acetate ($\text{IC}_{50} = 189.31 \mu\text{g}/\text{mL}$) fractions as the most active samples.^[2] In another study,^[14] the ethanol, chloroform, hexane and methanolic flower extracts of *V. sinaiticum* Benth. showed IC_{50} values in breast adenocarcinoma MCF-7 cells of 87.30, 95.43 and 119.04 $\mu\text{g}/\text{mL}$, respectively. Moreover, the hydroethanolic leaf extract of the same species exerted a selective cytotoxicity in hepatocellular carcinoma HepG2 cells ($\text{IC}_{50} = 80.6 \mu\text{g}/\text{mL}$) vs. pulmonary MRC-5 fibroblast cells ($\text{IC}_{50} > 500 \mu\text{g}/\text{mL}$).^[15]

Various plant extracts have been previously investigated for their cytotoxic potential against A431 human squamous carcinoma cells. *Vitis vinifera* seed and peel aqueous extracts inhibited the growth of A431 cells with IC_{50} values of 111.11 and 319.14 $\mu\text{g}/\text{mL}$, respectively, showing no cytotoxicity in HaCaT cells.^[16] Another study reported a lower cytotoxicity of grape seed extract against A431 cells ($\text{IC}_{50} = 480 \mu\text{g}/\text{mL}$).^[17] Methanolic extracts of *Catharanthus roseus* and *Calystegia sepium* leaves, methanolic and ethanolic extracts of *Narcissus tazetta* bulbs reduced the viability of A431 cells with IC_{50} values higher than 50 but not exceeding 150 $\mu\text{g}/\text{mL}$. Only the methanolic extract of *Calystegia sepium* leaves showed less cytotoxicity in normal cells, namely human gingival fibroblast (HGF-1) cells ($\text{IC}_{50} > 450 \mu\text{g}/\text{mL}$).^[18] Ethanolic extracts of *Helichrysum odoratissimum* (mixture of leaves and stems) and *Vanilla planifolia* (leaves) were found to be cytotoxic against A431 cells showing IC_{50} values of 15.5 and 31.2 $\mu\text{g}/\text{mL}$, respectively.^[19,20] Moreover, *H. odoratissimum* extract showed a SI of 2.39, expressing its selectivity against A431 cells vs. the non-tumor human embryonic kidney (HEK-293) cells.^[19] Extracts of different polarities obtained from *Cissus quadrangularis* stems were also cytotoxic with the acetone extract and its purified fraction being the most active ($\text{GI}_{50} = 8$ and 4.8 $\mu\text{g}/\text{mL}$, respectively, with GI_{50} standing for growth inhibition 50% value).^[21] As compared to these plant extracts, VNF4, showing an IC_{50} value of 12.27 $\mu\text{g}/\text{mL}$ in A431 cells and higher than 150 $\mu\text{g}/\text{mL}$ in non-tumor HaCaT cells with $\text{SI} > 10$, is undoubtedly a promising candidate for the development of novel therapies against cutaneous squamous cell carcinoma.

Next, the cytotoxicity of VNE and its fractions was evaluated by quantifying intracellular LDH released to the cell culture supernatant (marker of cellular dam-

age) in A431 tumor cells. As compared to the negative control (untreated cells), only VNF4 produced significant cytotoxic effects ($\text{IC}_{50} = 28.83 \mu\text{g}/\text{mL}$), whereas VNE and all the other fractions were considered inactive ($\text{IC}_{50} > 150 \mu\text{g}/\text{mL}$). At concentrations $> 50 \mu\text{g}/\text{mL}$, the LDH release induced by VNF4 was comparable to the positive control, in which the cell lysis solution was added (Figure 1c).

LC/HR-MS/MS Metabolite Profiling

To find the possible correlations between the observed cytotoxic effects exhibited by VNE and VNF4 in A431 cells and their phytochemical composition, untargeted LC/HR-MS/MS metabolite profiling was next performed. It was noticed that VNE comprised mainly iridoid glycosides, whereas VNF4 showed a high abundance of saponin glycosides (Table 1). Figure 2 presents the base peak chromatograms of the analyzed samples, whereas Figure 3 depicts the possi-

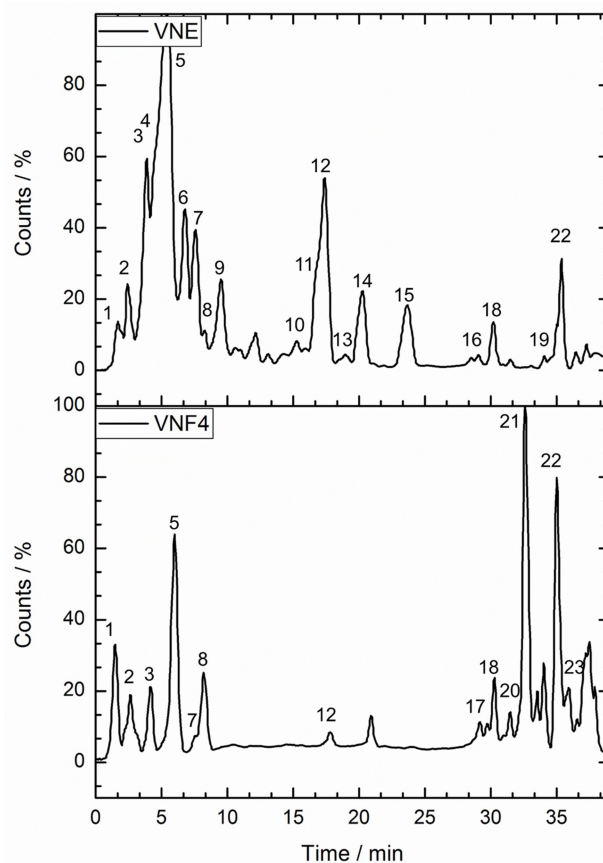
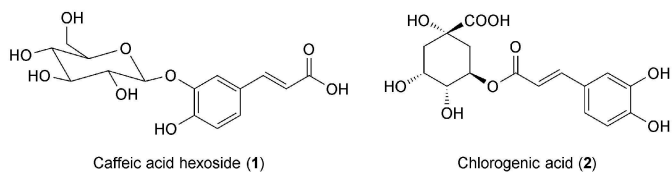
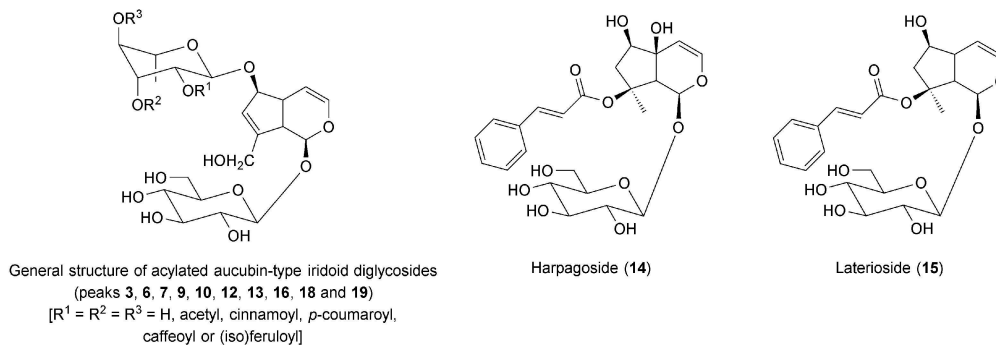
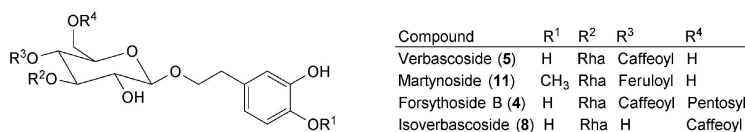
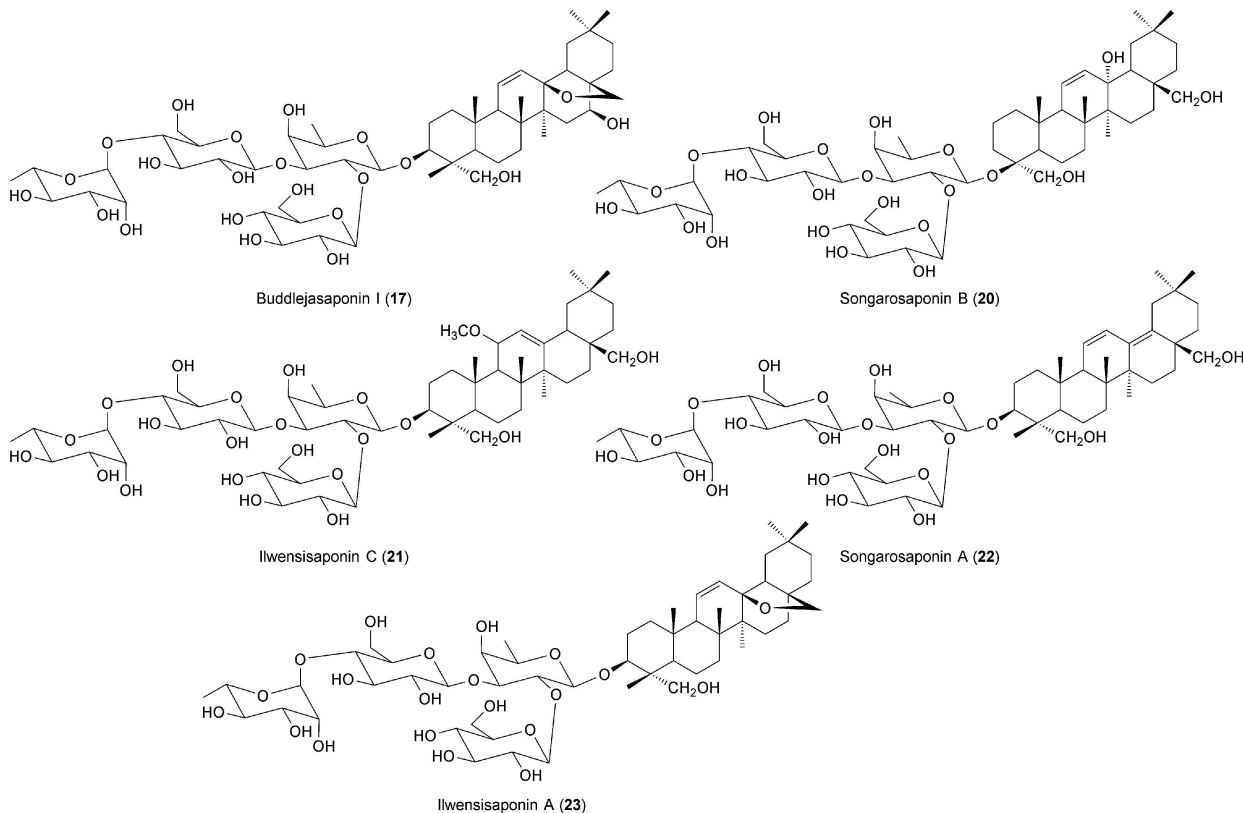


Figure 2. Base peak chromatograms of the crude methanolic extract of *Verbascum nigrum* aerial parts (VNE) and its VNF4 fraction (identity of peaks as in Table 1).

Table 1. Chromatographic and spectral data of compounds identified in the crude extract of *Verbascum nigrum* aerial parts (VNE) and its VNF4 fraction.

No.	t_R (min)	$[M-H]^-_{exp.}$ (m/z)	$[M-H]^-_{calc.}$ (m/z)	δ (ppm)	MF	MS/MS fragment ions (exp. m/z)	Proposed identity	Sample
1	1.7	341.0885	341.0878	2.05	$C_{15}H_{18}O_9$	179.0579, 161.0474	Caffeic acid hexoside	VNE, VNF4
2	2.5	353.0887	353.0878	2.53	$C_{16}H_{18}O_9$	191.0484, 161.0140, 135.0374	Chlorogenic acid*	VNE, VNF4
3	3.8	653.2066	653.2087	3.22	$C_{30}H_{38}O_{16}$	473.1602, 325.0894, 203.0241, 165.0485, 161.0243, 135.0403	Caffeoyl rhamnosylaucubin	VNE, VNF4
4	3.9	755.2281	755.2251	3.91	$C_{34}H_{44}O_{19}$	593.1977, 461.1512, 315.1023, 161.0190	Forsythoside B	VNE
5	5.8	623.2004	623.1981	3.61	$C_{29}H_{36}O_{15}$	461.1678, 315.1041, 179.0314, 161.0259, 135.0451	Verbascoside*	VNE, VNF4
6	6.8	667.2271	667.224	4.10	$C_{31}H_{40}O_{16}$	487.1658, 339.0900, 235.0561, 193.0499, 165.0570	(Iso)feruloyl rhamnosylaucubin	VNE
7	7.6	637.2151	637.2138	2.05	$C_{30}H_{38}O_{15}$	457.1544, 309.0927, 205.0507, 187.0357, 165.0563, 163.0392, 145.0310, 119.0497	<i>p</i> -Coumaroyl rhamnosylaucubin	VNE, VNF4
8	8.3	623.2007	623.1981	4.09	$C_{29}H_{36}O_{15}$	461.1694, 315.1069, 179.0341, 161.0235, 135.0447	Isoverbascoside	VNE, VNF4
9	9.5	695.2173	695.2193	2.83	$C_{32}H_{40}O_{17}$	653.2120, 515.1621, 473.1681, 367.0982, 307.0754, 203.0269, 165.0558, 161.0207, 135.0408	Caffeoyl acetyl rhamnosylaucubin	VNE
10	15.3	679.2259	679.2244	2.27	$C_{32}H_{40}O_{16}$	499.1530, 457.1468, 351.1136, 309.1010, 205.0501, 165.0510, 163.0360, 145.0259, 119.0259	<i>p</i> -Coumaroyl acetyl rhamnosylaucubin	VNE
11	16.1	651.2286	651.2294	1.29	$C_{31}H_{40}O_{15}$	475.1896, 329.1375, 193.0518, 175.0393	Martynoside	VNE
12	17.4	621.2212	621.2189	3.73	$C_{30}H_{38}O_{14}$	459.4366, 293.1264, 179.0548, 165.0579, 147.0430	Cinnamoyl rhamnosylaucubin	VNE, VNF4
13	18.9	799.2466	799.2455	1.39	$C_{39}H_{44}O_{18}$	653.2192, 637.2227, 473.1393, 443.1601, 351.0771, 205.0636, 165.0526, 161.0246, 145.0278	Caffeoyl <i>p</i> -coumaroyl rhamnosylaucubin	VNE
14	20.3	493.1729	493.1715	2.76	$C_{24}H_{30}O_{11}$	345.1312, 183.0874, 165.0708, 147.0456, 103.0556	Harpagoside*	VNE
15	23.7	477.1760	477.1766	1.30	$C_{24}H_{30}O_{10}$	329.2338, 185.1390, 147.0493	Laterioside	VNE
16	29.1	783.2517	783.2506	1.44	$C_{39}H_{44}O_{17}$	653.1986, 635.1982, 621.2256, 603.1880, 473.1501, 455.1473, 439.1523, 341.2520, 325.0920, 165.0552, 147.0279, 145.0279, 135.0452, 103.0505	Caffeoyl cinnamoyl rhamnosylaucubin	VNE
17	29.2	1087.5724	1087.5694	2.71	$C_{54}H_{88}O_{22}$	941.5382, 925.5241, 779.4741, 617.4171, 471.2766	Buddlejasaponin I	VNF4
18	30.2	663.2269	663.2294	3.83	$C_{32}H_{40}O_{15}$	603.1944, 515.1820, 473.1758, 353.1405, 179.0507, 165.0533, 147.0424	Cinnamoyl acetyl rhamnosylaucubin	VNE, VNF4
19	31.5	767.2564	767.2557	0.96	$C_{39}H_{44}O_{16}$	619.2084, 587.2047, 473.1264, 307.0677, 265.0632, 205.0436, 177.0490, 165.0529, 163.0341, 147.0395, 145.0247	Cinnamoyl <i>p</i> -coumaroyl rhamnosylaucubin	VNE
20	31.6	1089.5803	1089.5851	4.40	$C_{54}H_{90}O_{22}$	927.5213, 781.5595, 619.4619, 473.2166	Songarosaponin B	VNF4
21	32.7	1103.5974	1103.6007	2.99	$C_{55}H_{92}O_{22}$	957.5255, 941.5368, 795.4787, 633.4248, 487.2539	Ilwensisaponin C	VNF4
22	34.0	1071.5714	1071.5745	2.92	$C_{54}H_{88}O_{21}$	909.5233, 763.4699, 601.4123, 455.2497	Songarosaponin A	VNE, VNF4
23	35.0	1071.5733	1071.5745	1.12	$C_{54}H_{88}O_{21}$	909.5149, 763.4780, 601.4071, 455.2410	Ilwensisaponin A	VNE, VNF4

 t_R retention time, δ mass error; *identified based on standard.

Potential structures of phenolic acids tentatively identified in *V. nigrum*

Potential structures of iridoid glycosides tentatively identified in *V. nigrum*

Potential structures of phenylethanoid glycosides tentatively identified in *V. nigrum*

Potential structures of saponin glycosides tentatively identified in *V. nigrum*

Figure 3. Potential chemical structures of compounds tentatively identified in *Verbascum nigrum* aerial parts.

ble structures of the compounds annotated in *V. nigrum* aerial parts.

Two phenolic acid derivatives, namely caffeic acid hexoside (**1**) and chlorogenic acid (**2**) were tentatively annotated.^[1,2] Next, a total number of 12 iridoid glycosides were identified in the analyzed samples, with ten acylated aucubin-type iridoid diglycosides. The molecular formulas of these distinguishable derivatives were suggested from the HRMS data (with mass errors < 5.0 ppm), whereas several diagnostic ion fragments were observed in their MS/MS spectra. For instance, the sequential neutral elimination of glucosyl/glucose (162/180 Da) and aucubigenyl/dehydroaucubigenyl (166/148 Da) afforded ion fragments composed of the acyl unit(s) attached to the rhamnose group. Further intramolecular cleavage of the latter group would eventually give rise to the deprotonated cinnamic acid-derived ions [caffeate, *p*-coumarate, (iso) ferulate, cinnamate]. These latter ions can suffer dehydration or decarboxylation, generating specific smaller fragment ions. Furthermore, the ion fragment with m/z 165.0485 was essential for the diagnostic of the aucubigenin nature of the aglycon part of these derivatives. Consequently, four monoacyl aucubin-type iridoid diglycosides (**3**, **6**, **7** and **12**) and six diacyl aucubin-type iridoid diglycosides (**9**, **10**, **13**, **16**, **18**

and **19**) were tentatively labeled in the two extracts (Table 1). Furthermore, the MS/MS fragmentation pathways for compound **3** were proposed in Figure 4, together with general structure of the ten aucubin derivatives. Aucubin-type iridoid derivatives have been previously reported in *V. nigrum*, with nigrosides I [6-*O*-(3-*O*-cinnamoyl- α -L-rhamnopyranosyl)aucubin], II [6-*O*-(2-*O*-cinnamoyl- α -L-rhamnopyranosyl)aucubin], III [6-*O*-(2-*O*-*p*-cumaroyl- α -L-rhamnopyranosyl)aucubin], IV [6-*O*-(3-*O*-isoferuloyl- α -L-rhamnopyranosyl)aucubin] and V [6-*O*-(2-*O*-isoferuloyl- α -L-rhamnopyranosyl)aucubin] as some suggestive congeners.^[7,8] Also, harpagoside (**14**) and laterioside (**15**) were observed in VNE. Harpagoside was identified by comparing its chromatographic and spectral data with those of a reference compound, whereas laterioside was tentatively labeled based on a previous report.^[2] Both harpagoside and laterioside are acylated iridoid monoglycosides, with cinnamoyl as their acyl group, being already reported in *V. nigrum*.^[7,22] It is worth mentioning that, from the 12 iridoid glycosides, only a limited number (**3**, **7**, **12** and **18**) was spotted in VNF4; this could be correlated with the relatively polar nature of these derivatives which gives them a lower affinity for high concentrations of organic solvents (fraction VNF4 was eluted with 100% methanol, Scheme 1).

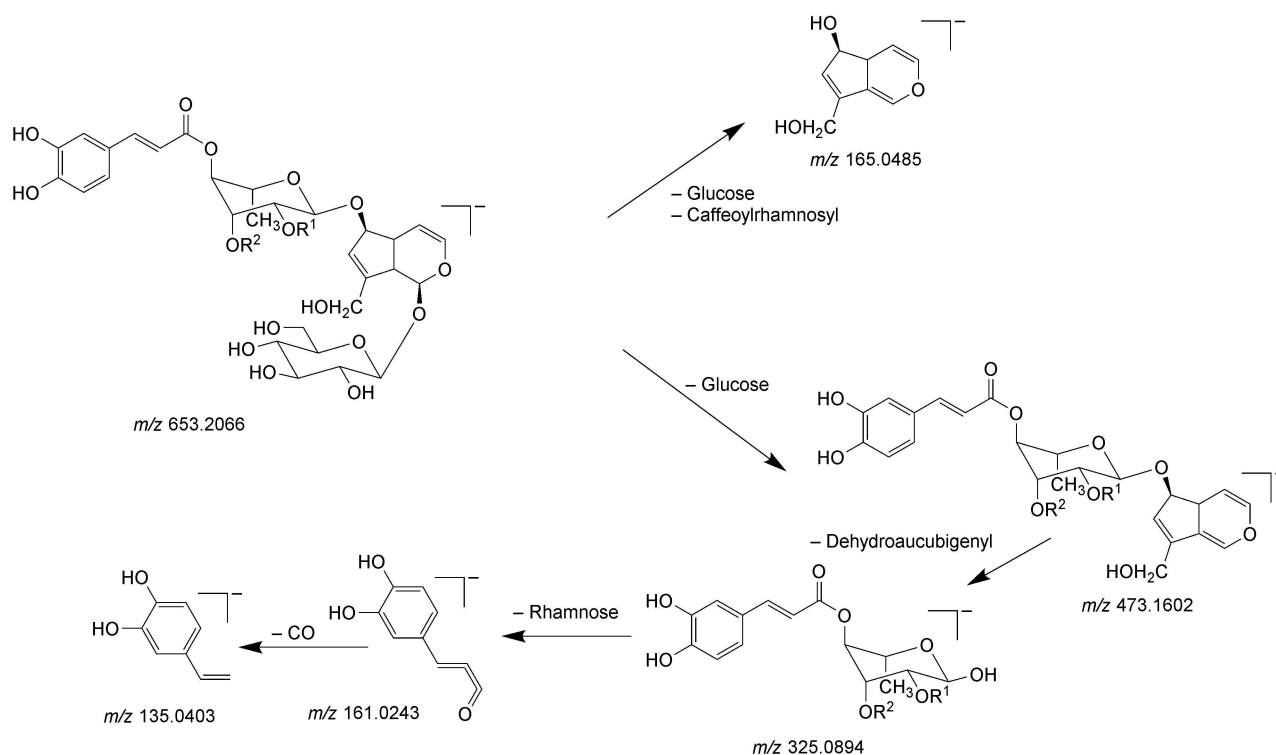


Figure 4. Proposed MS/MS fragmentation pathways for acylated aucubin-type iridoid diglycosides (e.g., compound **3**).

Four phenylethanoid glycosides were identified in the analyzed samples. Verbascoside was unequivocally assigned as peak **5** by comparing its retention time, HR-MS and MS/MS spectra with those of a commercially available standard. Having the same suggested molecular formula and fragment ions, peak **8** was ascribed as isoverbascoside. Lastly, compounds **4** ($[M-H]^-$ at m/z 755.2281) and **11** ($[M-H]^-$ at m/z 651.2294) were putatively considered as forsythoside B and martynoside, respectively.^[1,2] Verbascoside and forsythoside B were previously described in *V. nigrum*,^[22] whereas isoverbascoside and martynoside are very well-known constituents of *Verbascum* genus.^[23]

A number of five oleanane-type triterpene tetradecanoid saponin glycosides, namely buddlejasaponin I, songarosaponins A and B, ilwensisaponins A and C were tentatively identified in VNF4, with only one of these present in VNE (Table 1). Their MS dereplication was performed as previously described by our group.^[1] For instance, peak **17** with the deprotonated ion $[M-H]^-$ at m/z 1087.5724 ($C_{54}H_{87}O_{22}^-$) dissociated into the fragment ions at m/z 941.5382 $[M-H\text{-rhamnosyl}]^-$, 925.5241 $[M-H\text{-glucosyl}]^-$, 779.4741 $[M-H\text{-rhamnosyl-glucosyl}]^-$, 617.4171 $[M-H\text{-rhamnosyl-2}\times\text{glucosyl}]^-$ and 471.2766 $[M-H\text{-rhamnosyl-2}\times\text{glucosyl-fucosyl}]^-$. These spectral data were in agreement with those reported by Hartleb and Seifert^[24] for the structure of buddlejasaponin I. The same sequential neutral losses of rhamnosyl, two glucosyl and fucosyl groups were observed in the case of peaks **20** (m/z 1089.5803), **21** (m/z 1103.5944), **22** (m/z 1071.5714) and **23** (m/z 1071.5733) leading to proposing their tentative structures as songarosaponin B, ilwensisaponin C, songarosaponin A and ilwensisaponin A, respectively.^[25] Ilwensisaponins A and C were previously reported in *V. nigrum*,^[5] whereas the other saponins might be new for *V. nigrum*, being formerly shown in *V. songaricum* Schrenk.^[24,26]

The fact that VNF4 showed higher cytotoxic effects in A431 tumor cells could be attributed to the predominance of saponins, as compared to VNE in which iridoids and phenylethanoids were found its main constituents. For instance, ilwensisaponins A and C were shown to reduce the viability of human myelomonocytic HL-60 cells, with IC_{50} values of 4 and 33 $\mu\text{g/mL}$, respectively, after an incubation time of 18 h. On the other hand, verbascoside was also tested under the same conditions, but its IC_{50} value was $> 62.5 \mu\text{g/mL}$.^[27] Furthermore, in another study, buddlejasaponin IV and songarosaponin D decreased the viability of human renal cell adenocarcinoma ACHN

($IC_{50} = 7.9$ and $32.7 \mu\text{M}$, respectively), malignant melanoma A375 ($IC_{50} = 14.6$ and $12.4 \mu\text{M}$, respectively), amelanotic melanoma C32 ($IC_{50} = 26.9$ and $14.0 \mu\text{M}$, respectively), lung carcinoma A549 ($IC_{50} = 38.3$ and $41.1 \mu\text{M}$, respectively), lung large cell carcinoma COR-L23 ($IC_{50} = 0.4 \mu\text{M}$ for both saponins) and colon adenocarcinoma Caco-2 ($IC_{50} = 40.7$ and $16.7 \mu\text{M}$, respectively) cells, after 48 h exposure. Nevertheless, the viability of human skin fibroblast 142BR cells was not affected (IC_{50} values $> 50 \mu\text{M}$); this indicates the selective cytotoxic profile of *Verbascum* saponins,^[28] which is in agreement with the highly selective cytotoxicity exhibited by VNF4, a saponin-enriched fraction of *V. nigrum*.

Conclusions

In this study, a crude methanolic extract obtained from the aerial parts of *V. nigrum* and its six fractions were evaluated for their effects in immortalized HaCaT cells and tumor A431 cells by MTT and LDH assay. Since the crude extract (VNE) and VNF4 showed the highest cytotoxic effects, their metabolite profiling by LC/HR-MS/MS was subsequently performed. The aim was to putatively identify the constituents that could be responsible for the observed bioactivities. The analysis revealed that VNE comprised mainly iridoid glycosides, whereas VNF4 showed a high abundance of saponin glycosides, suggesting that the latter category of specialized metabolites might be endowed with an important selective cytotoxicity as compared to other types of constituents present in *V. nigrum*. To conclude, fraction VNF4 is a good candidate for isolation of phytochemicals with selective cytotoxicity against human skin squamous cell carcinoma A431 cells.

Experimental Section

Chemicals

Methanol, acetonitrile, diethyl ether, formic acid, verbascoside, harpagoside, chlorogenic acid, dimethyl sulfoxide (DMSO), staurosporine from *Streptomyces* sp., Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Gam (DMEM/F12-Ham), Dulbecco's Phosphate Buffer Saline (DPBS), trypan blue, trypsin-EDTA, glycine, sodium chloride and heat inactivated fetal calf serum (FCS) were purchased from Sigma-Aldrich (Steinheim, Germany). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was provided by

AppliChem GmbH (Darmstadt, Germany). L-Glutamine and Hoechst 33342 trihydrochloride trihydrate were purchased from Lubioscience (Lucerne, Switzerland). CytoTox 96® non-radioactive cytotoxicity kit was acquired from Promega AG (Dübendorf, Switzerland).

Plant Material

The aerial parts of *Verbascum nigrum* L. (stems, leaves and inflorescences) were collected in June 2014 from Hlinaia village (Edinet District, Republic of Moldova) and identified by Nina Ciocarlan and Veaceslav Ghendov (The Botanical Garden of Chisinau, Academy of Sciences of Moldova, Chisinau, Republic of Moldova). A voucher specimen (VN3006/2014) can be found in the Department of Pharmacognosy, Faculty of Pharmacy, Grigore T. Popa University of Medicine and Pharmacy Iasi, Romania.

Extraction and Fractionation

For extraction and fractionation, we used a procedure described in a previous work^[13] with some modifications. The air-dried aerial parts of *Verbascum nigrum* were powdered using a mill and then, 100 g were extracted with methanol at room temperature (1000 mL, 48 h). The liquid extract was then filtered and evaporated to dryness in a rotary vacuum evaporator at temperatures below 40 °C, affording the crude methanolic extract (VNCE, 17.7 g). Next, 15.0 g of VNCE were suspended in water (300 mL) and partitioned between water and diethyl ether (5 × 300 mL). After concentration and lyophilization, the aqueous phase yielded a defatted extract (VNE, 10.0 g). 5 g of this extract were subjected to vacuum liquid chromatography on C₁₈ Polyogrep column (50–60 µm, 2 × 40 cm, Macherey-Nagel) and eluted at a flow-rate of 10 mL/min, with 1500 mL water, 3650 mL water/methanol 1/1 (v/v) and 3650 mL methanol. Fractions of 50 mL were collected and they were pooled together based on their TLC profile: Silica gel 60 F₂₅₄ (Merck) as stationary phase; ethyl acetate/methyl ethyl ketone/water/formic acid 50:30:10:10 as mobile phase; derivatization with anisaldehyde-sulfuric acid reagent (105 °C, 5 min) and visualization under normal light. Six fractions (Scheme 1) were eventually pooled, as follows: VNF1 (Frs. 1–30, 2.2 g), VNF2 (Frs. 31–83, 2.1 g), VNF3 (Frs. 84–103, 22.7 mg), VNF4 (Frs. 104–125, 466 mg), VNF5 (Frs. 126–138, 11 mg) and VNF6 (Frs. 139–176, 33 mg).

Cell Lines

Human immortalized keratinocyte HaCaT cells (obtained from Cell Line Service, CLS, Heidelberg, Germany) and human epidermoid carcinoma A431 cells (ATCC CRL-1555) were grown in DMEM/F12 medium, supplemented with 1% GlutaMAX™ and 10% FBS. Cells were cultured at a density of 10⁵ cells/mL, 37 °C, in a 5% CO₂ atmosphere, with 95% humidity. When cells reached 70–80% confluence, they were harvested using trypsin-EDTA and DPBS. Cell counting was performed by staining with trypan blue solution and visualization on a hemocytometer under a microscope.

MTT Assay

Cell viability was measured using a tetrazolium-based colorimetric assay (MTT assay).^[13,29] Briefly, cells were seeded in 96-well plates (10⁴ cells/well), allowed to grow for 24 h and then treated with different concentrations of VNE and its fractions (VNF1–VNF6) for 24 h. At the end of the incubation time, MTT (10 µL/100 µL) was added to each well and cells were further incubated for 4 h; the medium was then removed and DMSO (150 µL/well) was added for 10 min to solubilize the formazan crystals. The absorbance was recorded at 570 nm, using with a BioTek™ microplate reader. Results were expressed as:

$$\text{cell viability (\%)} = \frac{\text{Absorbance of cells treated with extracts}}{\text{Absorbance of untreated cells}} \times 100$$

and as half maximal inhibitory concentration (IC₅₀) determined by a sigmoidal fit with Boltzmann function in OriginPro2020 software.

LDH Assay

The levels of lactate dehydrogenase (LDH) were evaluated in the culture medium, using CytoTox 96® assay kit (Promega AG, Dübendorf, Switzerland). The assay was performed according to the manufacturer's instructions, with cells being incubated with VNE and its VNF4 fraction for 24 h. Results were expressed as:

$$\text{LDH release (\%)} = \frac{\text{Absorbance of cells treated with extracts (experimental LDH release)}}{\text{Absorbance of control cells treated with a cell lysis solution (maximum LDH release)}} \times 100$$

LC/HR-MS/MS Metabolite Profiling

The untargeted LC/HR-MS/MS metabolite profiling was performed on an Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a binary pump (G1312C), column oven (G1316A), auto-sampler (G1329B), diode array detector (DAD, G1315D) and quadrupole-time-of-flight (Q-TOF) spectrometer (G6530B) with a dual jet stream electrospray ionization source. The elution was carried out on a Phenomenex Gemini C₁₈ column (100 mm × 2 mm, 3 μm i.d.) with a mobile phase composed of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) and the following gradient: 15% B (0 min), 25% B (13–20 min), 40% (25 min), 60% B (30 min) and 100% B (35–40 min). The flow-rate was 0.2 mL/min and the injection volume was 10 μL. The operating ESI-Q-TOF parameters in negative ionization mode were: mass range *m/z* 100–1700, gas temperature 350 °C, gas flow 12 L/min, nebulizer 40 psi, capillary voltage –4000 V, fragmentor 120 V and fixed collision energy 40 V. Data were analyzed with a Mass Hunter Qualitative Navigator B.08.00 software.

Statistical Analysis

Values are presented as means ± standard error of mean (SEM) of three individual experiments, each with eight replicates. Statistical analysis was performed by comparing treated samples with untreated controls. The statistical significance was evaluated using Student's *t*-test for unpaired samples, with *p* < 0.05 considered statistically significant. All statistical tests were conducted using OriginPro2020.

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Author Contribution Statement

I.A.I. carried out the extraction and fractionation of the extracts and the bioassays. I.A. contributed to the bioassays. S.V.L. performed the phytochemical experiments. S.P. contributed to the phytochemical analysis. A.M. and E.W. were responsible for the funding acquisition, study design and project coordination. K.S.W. was involved in study design and project coordination. I.A.I. and S.V.L. prepared the draft, while critical revision of the manuscript was performed by all authors.

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