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Neolignan and Phenylethanoid Glycosides from *Verbascum salviifolium* Boiss

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From the aerial parts of *Verbascum salviifolium* Boiss., 2 neolignan glucosides, dehydrodiconiferyl alcohol-9′-*O*-β-D-glucopyranoside (**1**) and dehydrodiconiferyl alcohol-9-*O*-β-D-glucopyranoside (**2**), along with 5 phenylethanoid glycosides, acteoside (= verbascoside) (**3**), β-hydroxyacteoside (**4**), forsythoside B (**5**), angoroside A (**6**) and martynoside (**7**), were isolated. The structure elucidation of the isolated compounds was established on the basis of spectroscopic evidence. Compounds **1-7** demonstrated scavenging properties toward the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical in TLC autographic and spectroscopic assays.

Key Words: *Verbascum,* Scrophulariaceae, neolignan glucosides, phenylethanoid glycosides, radical scavenging activity.

Introduction

The genus *Verbascum* (Scrophulariaceae) is represented by 228 species in the flora of Turkey; 185 species are recorded as endemic[1]. *Verbascum salviifolium* is a perennial and eglandular herb up to 30-60 cm tall growing on steppes and dry slopes at elevations of 300-1400 m in central Anatolia[1]. It was found that the methanolic extract of the aerial parts of the title plant exhibits antioxidant effects, based on the scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical in a TLC autographic assay. In a previous paper[2], we described the isolation and structure elucidation of 2 new and 5 known iridoid glucosides from the aerial parts of *V. salviifolium*. As a part of our ongoing research on the same plant, we now report

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2 neolignan glucosides, dehydrodiconiferyl alcohol-9′-*O*-β-D-glucopyranoside (**1**) and dehydrodiconiferyl alcohol-9-*O*-β-D-glucopyranoside (**2**), as well as 5 phenylethanoid glycosides, acteoside (= verbascoside) (**3**), β-hydroxyacteoside (**4**), forsythoside B (**5**), angoroside A (**6**) and martynoside (**7**). In addition, the free radical scavenging properties of fractions rich in phenolic compounds as well as the isolates' compounds (**1**-**7**) were evaluated.

Experimental

General experimental procedures: The UV spectra (λ_{max}) were recorded on a Hitachi HP 8452 A spectrophotometer. The IR spectra (υ_{max}) were determined on an ATI Mattson Genesis Series FT-IR spectrophotometer. The ^{1}H and ^{13}C NMR spectra were obtained on a Bruker Avance DRX 500 FT spectrometer operating at 500 MHz for ^{1}H NMR, and 125 MHz for ^{13}C NMR spectra. The chemical shift values are reported as parts per million (ppm) relative to tetramethylsilane (TMS), and the coupling constants are in hertz. For the ^{13}C NMR spectra, multiplicities were determined by DEPT experiment. LC-ESIMS FT data were obtained using a Bruker BioApex FT-MS instrument in the ESI mode. Polyamide (ICN) and reverse-phase material (C-18, sepralyte 40 μm) were used for vacuum liquid chromatography (VLC). Medium pressure liquid chromatography (MPLC) separations were performed on a Labomatic glass column packed with LiChroprep RP-18 (Merck), using a Lewa M5 peristaltic pump. Pre-coated silica gel 60 F_{254} aluminum sheets (Merck) were used for TLC with a developing solvent-system, $CHCl_3$-MeOH-H_2O (61:32:7). Plates were examined by UV fluorescence and sprayed with 1% vanillin in conc. H_2SO_4, followed by heating at 105 ^{o}C for 1-2 min. For the radical scavenging assay, DPPH (= 1,1-diphenyl-2-picrylhydrazyl, Sigma) was used and absorbance at 520 nm was measured using a Hewlet-Packard Hp 5500 /hp compaq spectrometer.

Plant Material: *Verbascum salviifolium* Boiss. was collected from Burdur, Yeşilova, southwest of Burdur Lake, 880 m, in June 2002. A voucher specimen has been deposited in the Herbarium of the Pharmacognosy Department, Faculty of Pharmacy, Hacettepe University, Ankara, Turkey (HUEF 02003).

Extraction and Isolation: The air-dried and powdered aerial parts of *Verbascum salviifolium* (339.08 g) were extracted twice with MeOH (2 x 2000 mL) at 40^{o}C. After evaporation of the combined extract in vacuo, 40.84 g of MeOH extract was obtained. The crude extract was dissolved in water and partitioned in $CHCl_3$. The lyophilized H_2O phase (29.49 g) was fractionated over a polyamide column (VLC, 250 g), eluting with H_2O (400 mL) and gradient MeOH-H_2O mixtures (25-100%) to afford 8 main fractions (A-H) to be studied and 7 iridoid glycosides isolated from fractions B-D were reported previously[2]. Fractions A-H were tested for their scavenging properties toward the DPPH radical in a TLC autographic assay [3,4] and frs. C-H demonstrated scavenging properties by expressing active yellow spots on a purple background. Therefore, frs. C-H were investigated. Fraction C (855.06 mg) was subjected to LiChroprep C_{18}-VLC. Employment of H_2O-MeOH (0-75 % MeOH) and MeOH afforded 5 fractions (Frs. C_{1-5}). Purification of fr. C_3 (244.8 mg) by C_{18}-MPLC (20-70 % MeOH) furnished 2 fractions (Frs. $C_{3a,b}$). Fraction C_{3b} (106.1 mg) was rechromatographed on a silica gel column ($CHCl_3$-MeOH, 90:10 to 80:20 v/v) to obtain **6** (5.4 mg). Fraction D (1.74603 g) was likewise subjected to C_{18}-MPLC using stepwise gradients of MeOH (0-70 %) in H_2O to yield **5** (53.3 mg) and an additional fraction D_3. Fraction D_3 (271.0 mg) was then purified by silica gel CC using gradient $CHCl_3$-MeOH mixtures (90:10 to 85:15 v/v) to afford a mixture of **1** and **2** (14

mg). Repeated chromatography of fr. E (484.1 mg) using a similar method (C_{18}-MPLC; 20-70 % MeOH) gave **3** (130.8 mg), **4** (12.4 mg) and **7** (15.0 mg). The remaining fractions rich in flavonoids are still being investigated.

Results

Dehydrodiconiferyl alcohol-9′-O-β-D-glucopyranoside (**1**): Positive ion LC-ESIMS m/z 543 $[M+Na]^+$; negative ion LC-ESIMS m/z 519 $[M-H]^-$ (calc. for $C_{26}H_{32}O_{11}$).^{1}H NMR (500 MHz, DMSO-d_6) and ^{13}C NMR (125 MHz, DMSO-d_6): Table 1.

Table 1. ^{13}C NMR (125 MHz, DMSO-d_6) and ^{1}H NMR (500 MHz, DMSO-d_6) data and HMBC of **1**.*

C/H Atom		δ_C	$\delta_H J$ (Hz)	HMBC
1	C	132.5		
2	CH	110.9	7.00 br s	C-3, C-6, C-7
3	C	148.2		
4	C	147.3		
5	CH	115.8	7.13 br s	C-1, C-2, C-3, C-4, C-8
6	CH	119.1	6.80 †	C-2, C-4, C-7
7	CH	88.0	5.54 d (6.0)	C-1, C-2, C-6, C-8, C-5′
8	CH	51.1	3.62 t (7.5)	C-1, C-7, C-9, C-5′
9	CH_2	62.4	3.68 t (7.5)	C-7, C-8
			3.44 †	
3-OMe	CH_3	56.4	3.77 s	C-3
1′	C	129.8		
2′	CH	111.1	6.95 †	C-3′, C-4′, C-6′, C-7′
3′	C	144.0		
4′	C	147.7		
5′	C	131.0		
6′	CH	115.7	6.77 †	C-2′, C-4′, C-5′
7′	CH	129.3	6.50 d (16.0)	C-1′, C-2′, C-6′, C-9′
8′	CH	128.5	6.24 d (15.0)	C-1′, C-9′
9′	CH_2	71.1	4.10 d (5.0)	C-7′, C-8′, C-1″
			3.69 †	
3′-OMe	CH_3	56.7	3.82 s	C-3′
1″	CH	103.3	4.28 d (7.5)	C-9′
2″	CH	74.1	3.01-3.05 †	
3″	CH	77.4	3.08-3.20 †	
4″	CH	70.7	3.07-3.14 †	
5″	CH	77.5	3.08-3.20 †	
6″	CH_2	61.7	3.70 d (11.5)	
			3.46 dd (11.5/5.5)	

* The ^{13}C and ^{1}H assigments were based on HMQC, HMBC and COSY experiments.
†Unclear due to signal overlapping.

Dehydrodiconiferyl alcohol-9-O-β-D-glucopyranoside (**2**): Positive ion LC-ESIMS m/z 543 $[M+Na]^+$; negative ion LC-ESIMS m/z 519 $[M-H]^-$ (calc. for $C_{26}H_{32}O_{11}$). ^{1}H NMR (500 MHz, DMSO-d_6): aglycon: δ 7.00 (1H, *br s*, H-2), 7.03 (1H, *br s*, H-5), 6.79 (1H, *overlapped*, H-6), 5.49 (1H, *d*, J= 7.5 Hz, H-7), 3.62 (1H, *t*, J = 7.5 Hz, H-8), 4.00 (1H, *t*, J = 7.5 Hz, H-9a), 3.75 (1H, *overlapped*, H-9b), 6.95 (1H, *overlapped*,

H-2′), 6.77 (1H, *overlapped*, H-6′), 6.50 (1H, *d*, J = 16.0 Hz, H-7′), 6.24 (1H, *d*, J = 15.0 Hz, H-8′), 3.68 (1H, *overlapped*, H-9′a), 3.44 (1H, *t*, J = 5.5 Hz, H-9′b), 3.77 (3H, *s*, 3′-OMe), 3.81 (3H, *s*, 3-OMe), glucose moiety: 4.26 (1H, *d*, J = 7.5 Hz, H-1″), 3.01-3.20 (each 1H, *overlapped*, H-2″/5″), 3.70 (1H, *d*,J = 11.5 Hz, H-6″a), 3.46 (1H, *dd*,J = 11.5/5.5 Hz, H-6″b); ^{13}C NMR (125 MHz, DMSO-d_6): aglycon: δ 132.0 (*s*, C-1), 110.9 (*d*, C-2), 148.2 (*s*, C-3), 147.2 (*s*, C-4), 115.8 (*d*, C-5), 118.8 (*d*, C-6), 87.6 (*d*, C-7), 51.4 (*d*, C-8), 71.1 (*t*, C-9), 56.4 (*q*, OMe), 129.7 (*s*, C-1′), 111.1 (*d*, C-2′), 143.9 (*s*, C-3′), 147.8 (*s*, C-4′), 131.1 (*s*, C-5′), 115.7 (*d*, C-6′), 129.3 (*d*, C-7′), 128.5 (*d*, C-8′), 62.3 (*t*, C-9′), 56.7 (*q*, OMe); glucose moiety: 103.4 (*d*, C-1″), 74.2 (*d*, C-2″), 77.5 (*d*, C-3″), 70.7 (*d*, C-4″), 77.6 (*d*, C-5″), 61.7 (*t*, C-6″).

Reduction of DPPH radical: Methanolic solutions (0.1%) of compounds **1-7** were chromatographed on a Si gel TLC plate using $CHCl_3$-MeOH-H_2O (61:32:7). After drying, TLC plates were sprayed with a 0.2% DPPH (Sigma) solution in MeOH. Compounds showing a yellow-on-purple spot were regarded as antioxidants [3,4].

DPPH assay in vitro: The radical scavenging activity of the neolignan glucosides (**1-2**) and the phenylethanoid glycosides (**3-7**) was examined with the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical as described previously [5,6]. (±)-α-tocopherol (= vitamin E) (Sigma) and 3-*t*-buthyl-4-hydroxy-anisole (= 3-BHA) (Sigma) were used as controls. MeOH solutions of compounds **1-7** at various concentrations (10, 25, 50, 100, 200 μM) were added to 1.5 x 10^{-5}M DPPH in MeOH. The reaction mixture was shaken vigorously and the absorbance at 520 nm was determined after 30 min incubation at room temperature. Decreasing DPPH solution absorbance indicates an increase in DPPH radical-scavenging activity. This activity is given as % DPPH radical-scavenging and is calculated in the equation

$$\%\ \text{DPPH radical-scavenging} = \frac{(\text{Control absorbance} - \text{sample absorbance})}{\text{Control absorbance}} \times 100$$

The DPPH solution without sample solution was used as a control.

Discussion

The water soluble extract obtained from the methanolic extract of the aerial parts of *V. salviifolium* was fractionated over polyamide-VLC followed by reverse-phase vacuum liquid chromatography (RP-VLC, LiChroprep C_{18}), Si gel CC and C_{18}-MPLC to yield compounds **1-7**.

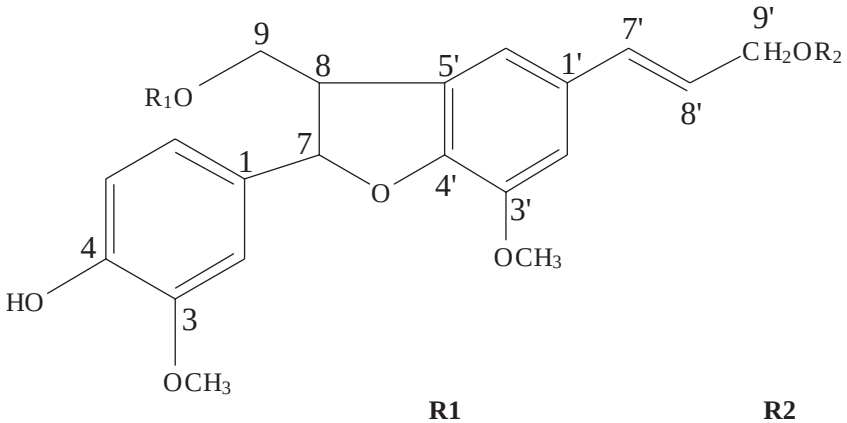
Compounds **3-7** were obtained as amorphous powders. The structures were identified as acteoside (**3**)[7], β-hydroxyacteoside (**4**)[8], forsythoside B (**5**)[7], angoroside A (**6**)[9]and martynoside (**7**)[10]by comparing their ^{1}H and ^{13}C NMR and DEPT-135 data with previously published data.

Compounds **1** and **2** were obtained as an inseparable mixture in a ratio of 2:3, $[\alpha]_{20}^{D}$ +17.1 (c 7.0, $CHCl_3$). The positive LC-EISMS of this mixture exhibited the molecular ions $[M+Na]^+$ at m/z 543, while the negative LC-EISMS showed the ions $[M-H]^-$ at m/z 519 for **1** and **2**. These data were compatible with the molecular formula $C_{26}H_{32}O_{11}$for **1** and **2,** which was supported by ^{1}H NMR, ^{13}C NMR and DEPT-135 data (Table 1). However, most NMR signals appeared double, indicating a close structural similarity between **1** and **2**. ^{1}H-^{1}H COSY, ^{1}H-^{13}C HMQC and ^{1}H-^{13}C HMBC experiments allowed one to pick the signals for **1** and **2** individually.

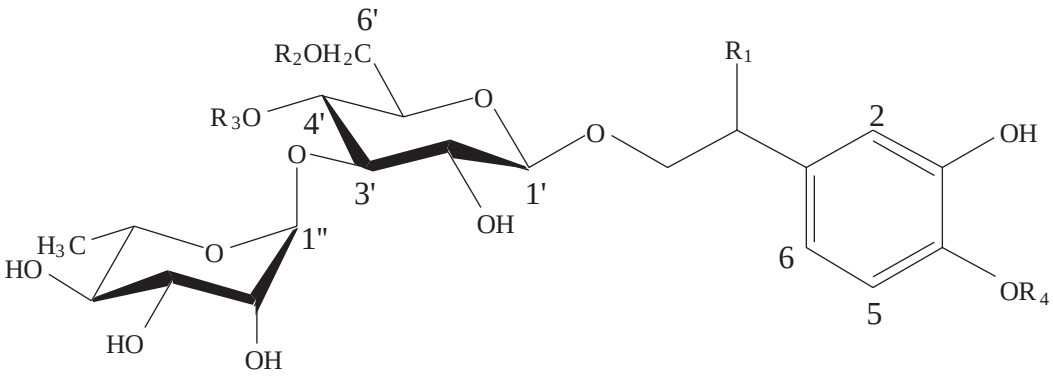
In the ^{1}H-NMR spectrum, 5 aromatic proton signals were recognized for **1** (Table 1). Of these, the proton resonances at δ_H 7.00 (1H, *br s*, H-2), 7.13 (1H, *brs*, H-5) and 6.80 (1H, *overlapped*, H-6) were

observed as an ABX system, suggesting that **1** contains a trisubstituted aromatic moiety. The proton signals at δ_H 6.95 (1H, *overlapped*, H-2′) and δ_H 6.77 (1H, *br s*, H-6′) were indicative of the presence of an additional tetrasubstituted aromatic moiety in the structure of **1** . Moreover, the ^{1}H NMR spectrum of **1** displayed 2 methoxy singlets at δ_H 3.82 and 3.77, 2 *trans* double bond protons at δ_H 6.50 (1H, *d*, J = 16.0 Hz, H-7′) and 6.24 (1H, *d*,J = 15.0 Hz, H-8′), an oxymethine proton at δ_H 5.54 (1H, *d*,J= 6.0 Hz, H-7) and an anomeric proton signal at δ_H 4.28 (1H, *d*, J = 7.5 Hz, H-1″), suggesting the presence of a β-glucopyranose unit within **1**[11]. Apart from 6 signals due to a β-glucopyranoside unit and 2 OMe signals, the ^{13}C NMR spectrum of **1** (Table 1) contained 18 skeletal carbon resonances, which were classified as 2 methylenes, nine methines and 7 quaternary carbon atoms by DEPT-135 experiment. From a detailed inspection of these data, associated with the interpretation of 2D NMR data, compound **1** was predicted to be dehydrodiconiferyl alcohol-9′-*O*-β-D-glucopyranoside. Accordingly, an oxymethine proton at δ_H 5.54 (1H, *d*, J = 6.0 Hz), which coupled to the methine proton δ_H 3.62 (1H, *t*, J = 7.5 Hz) in the COSY spectrum, and these were ascribed to H-7 and H-8 of the benzofuran ring, respectively. H-8 showed additional coupling with the oxymethylene protons (δ_H 3.44, *overlapped*, 3.68, *t*, J = 7.5 Hz, H_2-9). The methoxy function was placed at C-3 of the trisubstitued aromatic ring on the basis of prominent HMBC correlations (Figure 2, Table 1). Additional long-range couplings between H-7/C-1, H-7/C-2, H-7/C-6, H-7/C-8, H-8/C-7, H-8/C-9, H_2-9/C-7 and H_2-9/C-8 showed the aromatic substitution to take place at C-7. The assignments of the second methoxy group (C-3′) as well as H-2′ and H-6′ of the benzofuran ring were also possible using the cross-peak observed in the HMBC spectrum (Figure 2). Thus the unsaturated side chain and the β-glucopyranoside unit remained to be positioned. In the COSY spectrum, the proton signal of H-7′ (δ_H 6.50, 1H, *d*, J= 16.0 Hz) coupled with the H-8′ proton (δ_H 6.24, 1H, *d*, J =15.0 Hz). The latter showed further couplings with the hydroxymethyl protons (δ_H 4.10, 1H,*d*, J =5.0 Hz, H-9′a and 3.69, 1H, *overlapped*, H-9′b). Heteronuclear HMBC cross coupling between H-7′/C-1′, H-7′/C-2′, H-7′/C-6′, H-7′/C-9′, H-8′/C-1′, H-8′/C-9′, H_2-9′/C-7′ and H_2-9′/C-8′ indicated the attachment of a side chain at C-1′, as expected. The glycosidic linkage was determined to be at C-9′ due to the downfield shift of C-9′ (δ_C 71.1, $\Delta\delta$ + 9.6 ppm)[12], in addition to the HMBC correlation observed from H-1″ (δ_H 4.28) of the glucose moiety to C-9′ atom. The stereochemistry at C-7 and C-8 could not be established from the available data, but the positive optical rotation value of the mixture ($[\alpha]_{20}^{D}$ + 17.1) suggested that **1** may have a (+) stereoisomeric structure [12]. Consequently, compound **1** was almost identical to (+)-dehydrodiconiferyl alcohol-9′-*O*-β-D-glucopyranoside, previously isolated from *Cynomorium songaricum* (Cynomoriaceae)[13].

The complete analysis of the remaining ^{1}H and ^{13}C NMR signals, assigned by 2D NMR experiments (COSY, HMQC and HMBC) again, revealed that **2** was almost identical to **1**. However, the major difference between **1** and **2** was concluded to be the attachment site of the glucosyl unit on the aglycone moiety. When the NMR data of compound **2** were compared with those of **1**, the effects of glucosylation of C-9 in **2**,i.e. the upfield shifts of C-9′ (δ_C 62.3) and the downfield shifts of C-9 (δ_C71.1), were observed. This was confirmed by the HMBC experiment. The stereochemistry of **2** was also assumed to be (+) as in the case of **1**. Therefore, the structure of **2** was established as (+)-dehydrodiconiferyl alcohol-9-*O*-β-D-glucopyranoside, previously isolated from *Verbascum thapsus* (Scrophulariaceae)[14].



	R1	R2
1	H	β-D-glucopyranosyl
2	β-D-glucopyranosyl	H



3	R1=H,	R2=H,	R3=caffeoyl,	R4=H
4	R1=OH,	R2=H,	R3=caffeoyl,	R4=H
5	R1=H,	R2=apiose,	R3=caffeoyl,	R4=H
6	R1=H,	R2=arabinose,	R3=caffeoyl,	R4=H
7	R1=H,	R2=H,	R3=feruloyl,	R4=CH_3

Figure 1. Neolignan glucosides and phenylethanoid glycosides.

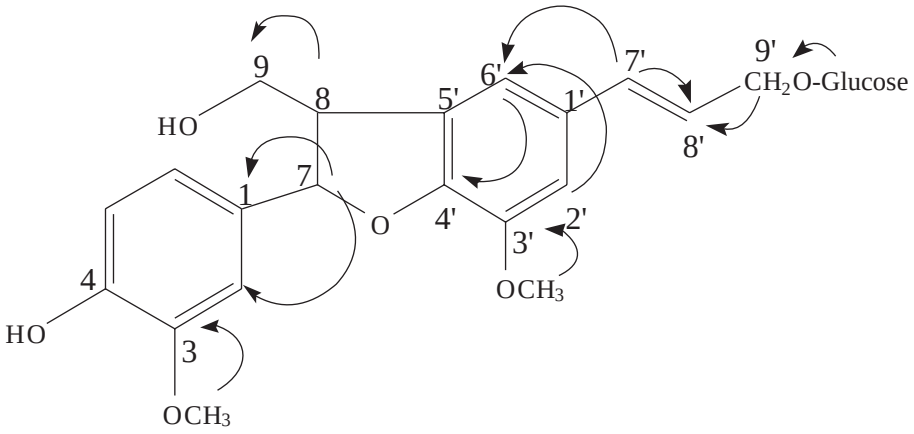


Figure 2. Selected HMBC correlations of **1.**

Table 2. Free radical scavenging effects of compounds **1-7**, 3-BHA and (±)-α- tocopherol on the DPPH radical (1.5×10^{-4} M).

Compounds	1×10^{-5} M	2.5×10^{-5} M	5×10^{-5} M	1×10^{-4} M	2×10^{-4} M
1-2	3.85[a]	7.69	18.15	19.92	62.89
3	-	23.08	40.95	89.86	73.89
4	-	11.83	51.70	46.82	64.98
5	-	13.15	-	52.11	81.44
6	-	27.71	28.74	46.20	38.78
7	10.77	9.23	45.80	71.12	76.22
3-BHA[b]	-	-	18.91	30.19	39.39
(±)-α-Tocopherol[b]	2.31	13.08	25.44	32.59	91.88

- : Not recorded.
[a] % DPPH radical-scavenging
[b] Reference compounds

Isolated compounds **1-7** were also screened for their antioxidant activities by a TLC autographic assay with the DPPH radical and showed antioxidant properties based on their ability to scavenge free radicals [3,4]. Then the free radical scavenging effects of the isolates (**1-7**), corresponding to the intensity of quenching of the DPPH radical, were evaluated by a spectroscopic assay[5,6]. Compounds **1-7** exhibited a dose-dependent reduction on DPPH. The results are given in Table 2.

Conclusion

To date, there has only been one report on the isolation of neolignan glucosides from the genus *Verbascum*[14]. Therefore, this is the second paper on this type of glycosides in this genus. This is the first demonstration of the occurrence of dehydrodiconiferyl alcohol-9′-*O*-β-D-glucopyranoside (**1**) as well as β-hydroxyacteoside (**4**) and the second report on the isolation of dehydrodiconiferyl alcohol-9-*O*-β-D-glucopyranoside (**2**)[14], angoroside A (**6**)[9]and martynoside (**7**)[10] in the genus *Verbascum.*

The neolignan glucosides (**1-2**) and the phenylethanoid glycosides (**3-7**) isolated from *V. salviifolium* are potential natural free radical scavengers based on the experiments, and their activity against the DPPH radical is closely associated with their chemical structure.

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