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Chemosystematically valuable triterpenoid saponins from *Glandularia x hybrida*

Nesma M. Mohamed[a], Makboul A. Makboul[a], Salwa F. Farag[a], Yan-Hong Wang[b], Shaymaa M. Mohamed[a], Samir A. Ross[b,c,*]

[a] Pharmacognosy Department, Faculty of Pharmacy, Assiut University, Assiut, 71526, Egypt
[b] National Center for Natural Products Research, Research Institute of Pharmaceutical Sciences, School of Pharmacy, The University of Mississippi, Mississippi, 38677, USA
[c] Department of BioMolecular Sciences, Division of Pharmacognosy, School of Pharmacy, University of Mississippi, Mississippi, 38677, USA

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ABSTRACT

Phytochemical investigation of the ethanolic extract of *Glandularia x hybrida* roots resulted in the isolation and identification of five previously undescribed saponins, 3-*O*-*β*-D-xylopyranosyl-hederagenin-28-*O*-*β*-D-glucopyranosyl (1→2)-*O*-*β*-D-glucopyranosyl ester, 3-*O*-*β*-D-xylopyranosyl-hederagenin-28-*O*-*β*-D-glucopyranosyl (1→2)-[*β*-D-glucopyranosyl (1→6)]-*β*-D-glucopyranosyl ester, hederagenin-28-*O*-*β*-D-glucopyranosyl (1→2)-[*β*-D-glucopyranosyl (1→6)]-*β*-D-glucopyranosyl ester, 23-*O*-acetyl-3-*O*-*β*-D-xylopyranosyl-pomolic acid-28-*O*-*β*-D-glucopyranosyl ester, and 23-*O*-acetyl-pomolic acid-3-*O*-*β*-D-xylopyranoside, along with eleven structurally diverse compounds. The structural characterizations of the isolated compounds were determined using physical data, comprehensive 1D and 2D NMR spectral analysis, and HRESIMS. All isolated saponins are hederagenin or pomolic acid glycosides conjugated with differentiable sugar units bound to C-3 and/or C-28 of the aglycone through ether and/or ester glycosidic linkages, respectively. Structural diversity of these isolated secondary metabolites would have a great impact on the future chemosystematic studies of this plant. Four saponins, obtained in good yield were evaluated for their anti-inflammatory activities in a rat model using the carrageenan-induced paw edema protocol. Two of these exhibited significant anti-inflammatory activities demonstrated through inhibition of the paw edema by 64 and 60%.

1. Introduction

Several studies have reported various pharmacologically active metabolites with key therapeutic values from different species belong to Verbenaceae (Ingole, 2011; Sousa et al., 2011). *Glandularia* (Verbenaceae) is an important genus comprises approximately 100 species of annual and perennial herbs. *Glandularia* has been considered primarily as a subgenus of *Verbena*, while recent studies have recognized it as a separate genus (O'Leary et al., 2007; Small, 1933; Turner, 1999, 1998; Umber, 1979). *Glandularia* has an ornamental potential owing to its attractive flowers (Lucía et al., 2015). *Glandularia x hybrida* (Groenl. & Rümpler) G. L. Nesom & Pruski (unresolved name: see http://www.theplantlist.org) is an annual ornamental plant known as common garden Verbena (Baile, 1951; Mabberley, 2017; Pruski and Nesom, 1992). Its leaves have been used as diuretic and as a poultice for skin diseases. Additionally, the herb has been used traditionally as anthelminthic, antispasmodic, aphrodisiac astringent, purgative, and tonic (Duke, 1985).

In contrast to genus *Verbena*, genus *Glandularia* has been the subject of very few phytochemical studies (Vestena et al., 2019). Few reports have investigated the phytochemical constituents of *G. x hybrida*; including the isolation of some flavonoids from its flowers, such as flavones, flavonols, and anthocyanins (Togami et al., 2006; Kawashty and El-Garf, 2000; Toki et al., 1995; Stotz et al., 1984). The present study describes the isolation and structural elucidation of the roots' specialized metabolites, together with the evaluation of their anti-inflammatory activities on paw edema induced by carrageenan in a rat model. The *in vivo* anti-inflammatory study has been selected on the basis of the reported pharmacological activities of structurally related saponins. The current study has met its goals through reporting five previously undescribed triterpenoid saponins including a hederagenin monodesmoside **3**, two hederagenin bisdesmosides **1** and **2**, a pomolic acid monodesmoside **6,** and a pomolic acid bisdesmoside **5**, along with other known compounds including triterpenoid sapogenins and saponins, phenylpropanoids, and iridoids. Moreover, compounds **2** and **5**

* Corresponding author. National Center for Natural Products Research, Research Institute of Pharmaceutical Sciences, Department of BioMolecular Sciences, School of Pharmacy, University of Mississippi, Mississippi, 38677, USA.
E-mail address: sross@olemiss.edu (S.A. Ross).

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reduced the paw edema by 64 and 60%, respectively, after 3 h from carrageenan injection reflecting their significant anti-inflammatory properties.

2. Results and discussion

Compound **1** was isolated as a white amorphous powder. It was assigned the molecular formula $C_{47}H_{76}O_{18}$ as determined by positive mode HRESIMS (*m/z* 951.4914 $[M+Na]^+$, calcd for $C_{47}H_{76}O_{18}Na$, 951.4929). The IR spectrum showed absorption bands attributed to a hydroxy group (3400 cm^{-1}), an olefinic group (1612 cm^{-1}), an ester carbonyl group (1736 cm^{-1}), and an ether linkage (1072 cm^{-1}). The 1D NMR spectroscopic data (Tables 1 and 2) demonstrated the characteristic typical pattern of hederagenin (Gao et al., 2015), including six tertiary methyl groups, an olefinic methine, characteristic downfield shifted methine, an oxygenated methine, and a hydroxymethyl group. The downfield shifting of C-3 to δ_C 83.0 and the upfield shifting of C-28 to δ_C 177.8 indicated the bisdesmosidic nature of **1**. Three anomeric protons were observed at δ_H 4.34 (*d*, *J* = 7.2 Hz), 5.46 (*d*, *J* = 7.6 Hz), and 4.81 (*d*, *J* = 8.0 Hz) which were correlated to δ_C 106.1, 93.5, and 103.3, respectively, through the HMQC investigation. The *β*-anomeric configuration of all sugar units was determined based on their large $J_{1\text{-}2}$. The locations of the sugar moieties were deduced from the HMBC correlations (Fig. 2) from H-1′ (δ_H 4.34) of xylose to C-3 (δ_C 83.0) of the aglycone and from H-1″ (δ_H 5.46) of Glc I to C-28 carbonyl group (δ_C 177.8). The carbon atom C-2″ of the Glc I appeared to be down field shifted (δ_C 77.6) compared with that of hederagenin-28-*O*-*β*-D-glucopyranoside derivatives (Kirmizigül and Anil, 2002). Additionally, the proton H-2″ of Glc I (δ_H 3.85) displayed correlations with C-1‴ (δ_C 103.3) of Glc II in the HMBC spectrum (Fig. 2), indicating that Glc II was linked to the C-2″ of the Glc I. Hence, compound **1** was defined as 3-*O*-*β*-D-xylopyranosyl-hederagenin-28-*O*-*β*-D-glucopyranosyl (1→2)-*O*-*β*-D-glucopyranoside, based on the aforementioned thorough discussion and previously published data (Azhar et al., 1993; Breitmaier et al., 1987; Schöpke et al., 1997).

Compound **2**, a white amorphous powder, was assigned the molecular formula $C_{53}H_{86}O_{23}$ based upon negative mode HRESIMS (*m/z* 1125.5314 $[M+Cl]^-$, calcd for $C_{53}H_{86}O_{23}Cl$ 1125.5248). The investigation of 1D NMR spectroscopic data (Tables 1 and 2) showed similarity to **1** suggesting the same basic carbon structure. However, compound **2** has an additional hexose unit as deduced from its spectral and HRESIMS profiles. Four anomeric protons resonating at δ_H 5.08 (*d*, *J* = 7.6 Hz), 5.20 (*d*, *J* = 7.6 Hz), 5.69 (*d*, *J* = 7.2 Hz), and 6.20 (*d*, *J* = 8.0 Hz) were observed and correlated via the HMQC experiment to δ_C 107.3, 106.1, 104.9, and 94.1, respectively. Their anomeric *β* configurations were established based on their large vicinal $J_{1\text{-}2}$ values. The downfield chemical shift of C-6″ to δ_C 71.7 indicated that the additional Glc III was linked to C-6″ of Glc I. This was further supported by the HMBC correlations (Fig. 2) from H-6″ (δ_H 4.97) of Glc I to C-1‴ of Glc III (δ_C 106.1) and from H-1‴ of Glc III (δ_H 5.20) to C-6″ (δ_C 71.7) of Glc I. According to the consistent collated data of related glycosides (Azhar et al., 1993; Breitmaier et al., 1987; Kirmizigül and Anil, 2002; Schöpke et al., 1997; Tan et al., 2005), compound **2** was assigned as 3-*O*-*β*-D-xylopyranosyl-hederagenin-28-*O*-*β*-D-glucopyranosyl (1 → 2)-[*β*-D-glucopyranosyl (1 → 6)]-*β*-D-glucopyranosyl ester.

Compound **3** was obtained as a white amorphous powder. Its molecular formula was established to be $C_{48}H_{78}O_{19}$ based on positive HRESIMS (*m/z* 981.5032 $[M+Na]^+$, calcd for 981.5035 $C_{48}H_{78}O_{19}Na$). The ^1H and ^{13}C NMR data summarized in (Tables 1 and 2) resembled that of **2**, except for the absence of the *β*-D-xylopyranoside spectral data. This assumption was confirmed by 1D and 2D NMR analysis including, the upfield shift of C-3 from δ_C 82.3 to 73.5 indicating a free hydroxyl group at C-3 of the aglycone, the HMBC correlations from H-3 (δ_H 4.21) to C-23 and C-24 at δ_C 67.7 and 13.7, respectively, and the ^1H-^1H COSY cross peaks between H-3 (δ_H 4.21) and its neighbor H-2 (δ_H 1.88) (Fig. 2). Comparing the ^1H and ^{13}C NMR values belonging to the sugar part (Tables 1 and 2) with those of **2** indicated the same ester trisaccharide residue. On the basis of complete spectroscopic analysis along with the previously published interrelated data, compound **3** was described as hederagenin-28-*O*-*β*-D-glucopyranosyl (1 → 2)-[*β*-D-glucopyranosyl (1 → 6)]-*β*-D-glucopyranosyl ester (Breitmaier et al., 1987; Kirmizigül and Anil, 2002; Tan et al., 2005).

Compound **5**, a white amorphous powder, showed a characteristic molecular ion peak in negative mode HRESIMS (*m/z* 859.4303 $[M+Cl]^-$, calcd for $C_{43}H_{68}O_{15}Cl$ 859.4246) which is consistent with the molecular formula of $C_{43}H_{68}O_{15}$. Its IR spectrum revealed the presence of hydroxy, acetyl, ester, olefinic, and ether groups owing to their characteristic absorptions at 3403, 1715, 1727, 1618, and 1052 cm^{-1}, respectively. The ^{13}C NMR data (Table 2) showed 43 carbon signals, which were assigned to a triterpenoid aglycone in addition to hexose and pentose moieties along with an acetyl group. The aglycone was characterized in ^1H and ^{13}C NMR spectral data (Tables 1 and 2) by five tertiary methyl signals, a secondary methyl, an oxygenated methine, an olefinic group and the characteristic methine group of Δ^{12} ursane pentacyclic triterpene. Additionally, the ^{13}C NMR spectral data (Table 2) revealed the presence of two carbonyls at δ_C 177.7 (C-28) and 171.3 (Ac. C═O) and an oxygenated quaternary carbon at δ_C 73.3 (C-19). These data are in accordance with the reported ones of pomolic acid (19*α*-ursolic acid) (Sidjui et al., 2015) in which one of the methyl groups oxidized to an oxygenated methylene group which was esterified by an acetyl moiety (Kanchanapoom et al., 2001a). This hypothesis was greatly supported via the ^1H and ^{13}C-NMR signals at δ_H (4.47, *d*, *J* = 11.2 Hz) and (4.64, *d*, *J* = 11.2 Hz) / δ_C 66.4 (CH_2-23), δ_H 2.00, *s* / δ_C 21.3 (Ac. -CH_3), and δ_C 171.3 (Ac. C═O). The acetyl moiety was established to be linked to C-23 based on the HMBC spectrum (Fig. 2) which showed cross peaks from the methylenic protons CH_2-23 (δ_H 4.47 and 4.64) to C-3 (δ_C 82.7), C-4 (δ_C 43.0), C-5 (δ_C 48.8), C-24 (δ_C 13.7) and (Ac. C═O) (δ_C 171.3) and also from methyl protons of the acetyl group (Ac.-CH_3) at δ_H 2.00 to its carbonyl carbon (Ac. C═O) at δ_C 171.3. The NOESY spectrum (Fig. 2) further confirmed the presence of the acetyl group at C-23, through the correlations between H-23 (δ_H 4.47 and 4.64), H-3 (δ_H 3.99) and the anomeric proton H-1′ (δ_H 4.88) which are known to be in the *α* orientation. Also, the absence of correlations between the C-4 methyl protons at δ_H 0.93 and H-3 (δ_H 3.99) and H-1′ (δ_H 4.88) deduces the *α* orientation of the acetyl group (C-23) and the *β* orientation of the methyl group (C-24). The ^1H NMR spectral data (Table 1) showed two anomeric protons at δ_H 4.88 (*d*, *J* = 7.2 Hz) and 6.32 (*d*, *J* = 8.0 Hz) corresponded to δ_C 107.8 and δ_C 96.4 according to the HMQC spectrum. Consequently, compound **5** was deduced to be a pomolic acid bisdesmoside. The downfield chemical shift of C-3 to δ_C 82.7 and the upfield shifting of C-28 to δ_C 177.7 indicated that C-3 and C-28 are the points of glycosylation. The *β*-D-xylose was linked to C-3 of the aglycone based on the HMBC correlations (Fig. 2) from H-1′ (δ_H 4.88) to C-3 (δ_C 82.7). Additionally, the attachment of *β*-D-glucose to the aglycone at C-28 confirmed by the HMBC correlations (Fig. 2) from H-1″ (δ_H 6.32) to the ester carbonyl C-28 (δ_C 177.7) and C-2″ (δ_C 74.6). Thus, compound **5** was deduced as 23-*O*-acetyl-3-*O*-*β*-D-xylopyranosyl-pomolicacid-28-*O*-*β*-D-glucopyranosyl ester, based on the analysis of spectroscopic data and published literature of structurally related saponins (Kanchanapoom et al., 2001a; Wu et al., 2007).

Compound **6**, a white amorphous powder, was assigned the molecular formula $C_{37}H_{58}O_{10}$ based upon negative HRESIMS (*m/z* 661.3943 $[M-H]^-$, calcd for $C_{37}H_{57}O_{10}$ 661.3953 and 1323.7983 $[2M-H]^-$, calcd for $C_{74}H_{115}O_{20}$1323.7922). The ^{13}C NMR and DEPTQ-135 spectroscopic data (Table 2) displayed 37 carbon atoms, which were attributed to seven methyl groups, eleven methylenes, ten methines and nine quaternary carbons. The analysis of the ^1H NMR spectral data (Table 1), which showed only one anomeric proton at δ_H 4.88 (*d*, *J* = 7.6 Hz) correlated to the anomeric C-1′ at δ_C 107.8 in the HMQC spectrum, in addition to the value of C-28, which was found to be δ_C 181.1 indicating free carboxylic group, revealed the close similarity to **5** except for the absence of the glucose moiety which was linked to the aglycone at C-28.

Table 1
^{1}H-NMR chemical shifts (δ in ppm, J values in Hz) for compounds **1–3**, **5**, & **6**.

Position	Compounds				
	1	**2**	**3**	**5**	**6**
1	1.64 (1H, m)	1.58 (1H, m)	0.99 (1H, m)	2.06 (1H, m)	2.09 (1H, m)
	0.97 (1H, m)	1.08 (1H, m)	1.53 (1H, m)	1.82 (1H, m)	2.18 (1H, m)
2	1.95 (2H, m)	2.24 (2H, m)	1.88 (2H, m)	2.06 (1H, m)	2.07 (1H, m)
				1.99 (1H, m)	1.97 (1H, m)
3	3.62 (1H, br d, 11.4)	4.27, overlap	4.21 (1H, m)	3.98 (1H, dd, 11.6, 4.4)	3.99 (1H, br d, 12.8)
4	–	–	–	–	–
5	1.24 (1H, m)	1.66 (1H, br d, 12.8)	1.49 (1H, m)	1.35 (2H, overlapped)	1.36 (2H, m)
6	1.54 (2H, m)	1.69 (2H, m)	1.65 (2H, m)	1.53, (2H, m)	1.51 (1H, m)
7	1.72 (2H, m)	1.58 (2H, m)	1.60 (2H, m)	1.48 (1H,d, 7.6)	1.42 (1H, m)
				1.68 (1H, m)	1.68 (1H, m)
8	–	–	–	–	–
9	1.64 (1H, m)	1.78 (1H, m)	1.77 (1H, m)	1.88 (1H, m)	1.92 (1H, m)
10	–	–	–	–	–
11	1.92 (2H, m)	1.94 (2H, m)	1.93 (2H, m)	2.05 (2H, m)	2.06 (2H, m)
12	5.25 (1H, br s)	5.45 (1H, br s)	5.47 (1H, t, 3.6)	5.58, (1H, t, 2.4)	5.62 (1H, br s)
13	–	–	–	–	–
14	–	–	–	–	–
15	1.76 (1H, m)	2.21 (1H, m)	1.30 (2H, m)	1.25 (1H, d, 11.6)	1.32 (2H, m)
	1.02 (1H, m)	1.33 (1H, m)		2.49 (1H, t, 14.4)	
16	1.98 (1H, m)	2.45 (1H, m)	2.48 (2H, d, 11.2)	2.03 (2H, m)	2.24 (2H, m)
	1.30 (1H, m)	2.22 (1H, m)			
17	–	–	–	–	–
18	2.82 (1H, dd, 13.2, 3.2)	3.15 (1H, dd, 14.4 & 3.2)	3.16 (1H, dd, 13.2, 4.0)	2.96 (1H, br s)	3.08 (1H, br s)
19	1.76 (1H, m)	1.80 (1H, m)	1.24 (1H, m)	–	–
	1.18 (1H, m)	1.27 (1H, m)	1.80 (1H, m)		
20	–	–	–	1.35 (1H, overlapped)	1.52 (1H, m)
21	1.46 (1H, m)	1.12 (2H, m)	1.10 (2H, m)	1.49 (2H, m)	2.10 (2H, m)
	1.21 (1H, m)				
22	1.52 (1H, m)	2.01 (1H, m)	2.02 (2H, m)	0.99 (1H, dd, 12.8, 3.2)	1.57 (2H, overlapped)
	1.25 (1H, m)			1.60 (1H, d, 12.8)	
23	3.31 (1H, d, 11.6)	3.65 (1H, d, 12.6)	3.65 (1H, d, 10.4)	4.47 (1H, d, 11.2)	4.64 (1H, d, 11.2)
	3.91 (1H, d, 11.6)	4.30 (1H, overlapped)	4.13 (1H, m)	4.64 (1H, d, 11.2)	4.46 (1H, d, 11.2)
24	0.72 (3H, s)	0.89 (3H, s)	0.98 (3H, s)	0.94 (3H, s)	0.91 (3H, s)
25	0.99 (3H, s)	0.89 (3H, s)	0.92 (3H, s)	0.96 (3H, s)	0.92 (3H, s)
26	0.81 (3H, s)	1.04 (3H, s)	1.07 (3H, s)	1.21 (3H, s)	1.11 (3H, s)
27	1.17 (3H, s)	1.22 (3H, s)	1.23 (3H, s)	1.75 (3H, s)	1.78 (3H, s)
28	–	–	–	–	–
29	0.92 (3H, s)	0.87 (3H, s)	0.86 (3H, s)	1.41(3H, s)	1.46 (3H, s)
30	0.94 (3H, s)	0.85 (3H, s)	0.85 (3H, s)	1.08 (3H, d, 6.4)	1.13 (3H, d, 6.4)
β-D-Xyl					
1′	4.34 (1H, d, 7.2)	5.08 (1H, d, 7.6)		4.88 (1H, d, 7.2)	4.88 (1H, d, 7.6)
2′	3.18 (1H, m)	4.05 (1H, m)		4.03 (1H, m)	4.03 (1H, d, 7.2)
3′	3.22 (1H, m)	4.29 (1H, m)		4.13 (1H, t, 9.2)	4.13 (1H, t, 8.0)
4′	3.46 (1H, m)	4.37 (1H, m)		4.22 (1H, m)	4.24 (1H, m)
5′	3.82 (1H, m)	3.72 (1H, d, 11.2)		3.78 (1H, t, 10.4)	4.40 (1H, dd, 11.2, 5.6)
	3.19 (1H, m)	4.38 (1H, m)		4.42 (1H, overlapped)	3.78 (1H, t, 10.0)
β-D-Glc I					
1″	5.46 (1H, d, 7.6)	6.20 (1H, d, 8.0)	6.22 (1H, d, 8.0)	6.32 (1H, d, 8.0)	
2″	3.85 (1H, m)	4.52 (1H, t, 8.0)	4.54 (1H, t, 8.4)	4.23 (1H, m)	
3″	3.33 (1H, m)	4.06 (1H, m)	4.30 (1H, m)	4.33 (1H, m)	
4″	3.37 (1H, m)	4.28, (1H, overlapped)	4.29 (1H, m)	4.23 (1H, m)	
5″	3.23 (1H, m)	4.07 (1H, m)	4.07 (1H, m)	4.33 (1H, m)	
6″	3.81 (1H, m)	4.27 (1H, overlapped)	4.35 (1H, m)	4.39 (2H, m)	
	3.65 (1H, m)	4.97 (1H, d, 11.2)	4.98 (1H, d, 10.8)		
β-D-Glc II					
1‴	4.81 (1H, d, 8.0)	5.69 (1H, d, 7.2)	5.72 (1H, d, 7.6)		
2‴	3.25 (1H, m)	4.05 (1H, m)	4.21 (1H, m)		
3‴	3.37 (1H, m)	3.92 (1H, br s)	3.95 (1H, br s)		
4‴	3.15 (1H, m)	4.23 (1H, m)	4.25 (1H, m)		
5‴	3.31 (1H, m)	4.05 (1H, m)	4.26 (1H, m)		
6‴	3.63 (1H, m)	4.33 (1H, m)	4.34 (1H, m)		
	3.30 (1H, m)	4.41 (1H, dd, 10.8, 3.2)	4.42 (1H, m)		
β-D-Glc III					
1⁗		5.20 (1H, d, 7.6)	5.21 (1H, d, 7.6)		
2⁗		4.11 (1H, m)	4.10 (1H, m)		
3⁗		4.06 (1H, m)	4.30 (1H, m)		
4⁗		4.09 (1H, m)	4.09 (1H, m)		
5⁗		4.16(1H, d, 6.4)	4.15 (1H, m)		
6⁗		4.36 (1H, d, 4.8)	4.37 (1H, m)		
		4.59 (1H, d, 11.6)	4.60 (1H, d, 11.6)		
Ac.-Me				2.00 (3H, s)	2.05 (3H, s)

Table 2
^{13}C-NMR chemical shifts for compounds **1**, **2**, **3**, **5**, & **6**.

Position	Compounds				
	1	**2**	**3**	**5**	**6**
1	39.1	39.3	39.3	38.3	38.9
2	26.0	26.7	28.2	26.7	27.4
3	83.0	82.3	73.5	82.7	82.6
4	43.5	43.9	43.4	43.0	42.9
5	47.8	48.1	48.9	48.8	48.8
6	18.4	18.6	19.0	19.1	18.9
7	32.6	33.5	33.5	33.8	33.8
8	40.3	40.5	40.5	41.0	40.8
9	48.8	48.7	48.7	48.5	48.5
10	37.3	37.4	37.7	37.4	37.3
11	24.1	24.2	24.4	24.6	24.5
12	123.2	122.9	123.0	128.9	128.4
13	144.7	145.2	145.2	139.8	140.4
14	42.5	42.7	42.7	42.6	42.3
15	29.4	29.7	29.7	29.7	29.7
16	23.1	23.5	23.6	26.7	26.6
17	47.6	47.6	47.6	49.2	48.8
18	42.2	42.3	42.4	55.0	55.1
19	46.8	46.8	46.8	73.3	73.2
20	31.1	31.2	31.3	42.7	42.5
21	34.5	34.6	34.5	27.3	26.8
22	32.6	32.7	32.7	39.2	38.9
23	64.4	64.6	67.7	66.4	66.2
24	13.0	14.1	13.7	13.7	13.6
25	16.1	16.6	16.5	16.6	16.4
26	17.4	18.0	18.0	18.0	17.7
27	26.1	26.7	26.7	25.0	25.0
28	177.8	177.2	177.2	177.7	181.1
29	33.1	33.7	33.7	27.6	27.6
30	23.7	24.3	24.2	17.3	17.2
β-D-Xyl					
1′	106.1	107.3		107.8	107.8
2′	75.4	75.7		75.9	75.9
3′	78.2	78.9		79.1	79.1
4′	70.8	71.7		71.7	71.7
5′	66.4	67.7		67.7	67.6
β-D-Glc I					
1″	93.5	94.1	94.1	96.4	
2″	77.6	79.0	79.0	74.6	
3″	78.4	79.6	79.6	79.4	
4″	70.4	71.2	71.2	71.8	
5″	77.9	79.7	79.0	79.8	
6″	61.9	71.7	71.7	62.9	
β-D-Glc II					
1‴	103.3	104.9	104.9		
2‴	75.1	76.2	75.7		
3‴	78.4	79.8	79.6		
4‴	72.1	72.2	72.2		
5‴	77.8	79.6	78.9		
6‴	63.2	62.5	62.5		
β-D-Glc III					
1⁗		106.1	106.1		
2⁗		76.2	76.2		
3⁗		79.6	79.6		
4⁗		72.8	72.9		
5⁗		77.3	77.4		
6⁗		63.4	63.4		
Ac.-CH_3				21.3	21.2
Ac. C=O				171.3	171.1

The attachment of the *β*-D-xylose at C-3 was confirmed by the downfield chemical shift of C-3 (δ_C 82.6) (Table 2) and the HMBC correlations from the H-3 (δ_H 3.99) to C-1′ (δ_C 107.8) and from H-1′ (δ_H 4.88) to C-3 (δ_C 82.6) (Fig. 2). Therefore, compound **6** was defined as 23-*O*-acetyl-pomolic acid-3-*O*-*β*-D-xylopyranoside (Kanchanapoom et al., 2001a; Wu et al., 2007).

Concerning the stereochemistry, the conformational features of the parent sapogenins (hederagenin and pomolic acid) are consistent with those reported in the literature. In addition, the relative configurations of all asymmetric centers and the different substituents (Fig. 1) were determined based on the earlier established data from previous studies (Büchi, 1958; Joshi et al., 1999).

The identities as well as the absolute configurations of the sugar moieties were elucidated using UHPLC-UV/MS. The hydrolysates of the isolated compounds were treated according to the reported procedure (Wang et al., 2012). From the chromatograms and mass spectra of the derivatives of compounds **1–3**, **5**, and **6** in addition to the published NMR data, the sugar moieties were identified as D-glucose and D-xylose.

The structures of compounds (**4, 7–16**) were established through comparing their NMR spectral data with the published ones. These compounds were identified as colchiside A (**4**) (Mshvildadze et al., 2001), 3-*O*-*β*-D-xylopyranosyl-pomolic acid-28-*O*-*β*-D-glucopyranosyl ester (**7**) (Büchi, 1958; Mshvildadze et al., 2001; Wu et al., 2007; Zhao et al., 2013), oleanonic acid (**8**) (Uddin et al., 2011), ursolic acid (**9**) (Lee et al., 2005), 3*α*, 24*β*-dihydroxy-olean-12-ene-28-oic acid (**10**) (Morota et al., 1995), and hederagenin (**11**) (Joshi et al., 1999), three phenylpropanoids: verbascoside (**12**) (Kanchanapoom et al., 2001b), martynoside (**13**) (Yalçin et al., 2003) and leucosceptoside (**14**) (Ono et al., 2006), and two iridoids: lamiide (**15**) and ipolamiide (**16**) (Güvenalp et al., 2006).

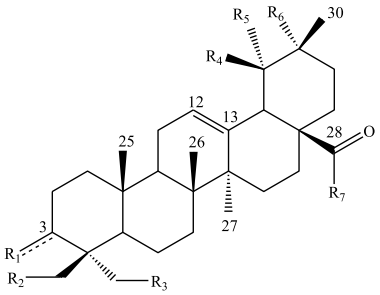
The phytochemical profile of *Glandularia* species has not yet thoroughly investigated. Nevertheless, flavonoids, iridoids, and phenylpropanoids along with their glycosides have been reported from the closely related genus *Verbena* (Vestena et al., 2019). Moreover, triterpenoid saponins have not been encountered in genus *Verbena*. Consequently, the results presented in the current study revealed the commonalities and uniqueness in the distribution of the secondary metabolites among the closely related genera. This would have a remarkable contribution to the chemotaxonomic evaluation of these genera and confirm the identity of the plant under investigation (cf. the previous report that described it as *Verbena canadensis*) (Turner, 1998).

Pronounced anti-inflammatory activity has been attributed to several triterpenoid saponins, especially hederagenin glycosides which are considered to have anti-nociceptive, and anti-rheumatic activity (Kim et al., 1999; Veeranjaneyulu et al., 2005). Therefore, the carrageenan-induced paw edema assay was conducted to establish the anti-inflammatory effect of the major isolated saponins. The injection of carrageenan suspension into the right paws of the rats resulted in the swelling of rat's paws as an inflammatory response. This response was reduced by the prophylactic administration of the tested saponins in a dose-dependent manner comparable to that of the positive control indomethacin as observed from the collected data listed in Table 3 hereinafter. In brief, the pretreatment of Gr.4–11 with compounds **2**, **4**, **5**, and **7** at doses of 25 and 50 mg/kg resulted in inhibition of the carrageenan-induced edema after 3 h of the carrageenan injection. Compounds **2** and **5** (at doses of 50 mg/kg), showed significant anti-inflammatory activities with 64% and 60% reduction of edema after 3 h of the carrageenan injection, respectively.

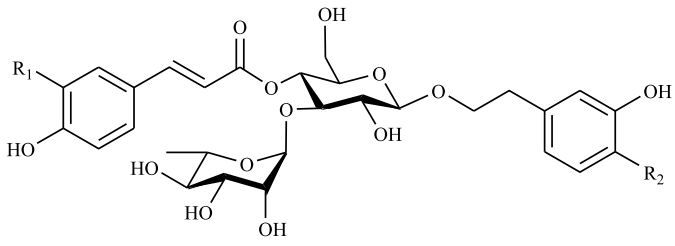
3. Experimental

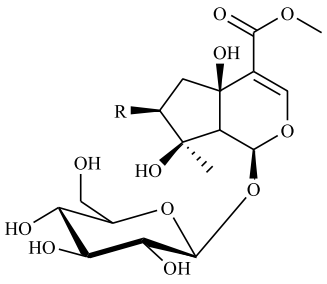
3.1. General procedures

Specific rotations were measured using a Rudolph Research AutoPol IV polarimeter (Rudolph Research Analytical, Hackettstown, NJ, USA) at room temperature; UV spectra were recorded by a Hewlett-Packard 8452A UV–Vis spectrometer (American Laboratory Trading, CT, USA); IR spectra were acquired using Bruker Tensor 27 and MIRacle ATR FT − IR spectrometers (Bruker Scientific Instruments, Bruker Optics, MA, USA). All NMR experiments were carried out on Varian - Mercury AS 400 MHz (^{1}H) and 100 MHz (^{13}C) (Varian Inc., NC, USA) and Bruker Avance DRX spectrometer at 400 MHz (^{1}H) and 100 MHz (^{13}C), 500 MHz (^{1}H) and 125 MHz (^{13}C) and 600 MHz (^{1}H) and 150 MHz (^{13}C) (Bruker Scientific Instruments, MA, USA). High-resolution mass spectra were obtained using an HRESI-TOFMS spectrometer with the Analyst QS software for data acquisition and processing (Agilent Series



	R_1	R_2	R_3	R_4	R_5	R_6	R_7
1	*O*-*β*-D-Xyl	H	OH	H	H	CH_3	*O*-*β*-D-gluc.-(1→2)-*O*-*β*-D-gluc.
2	*O*-*β*- D-Xyl	H	OH	H	H	CH_3	*O*-*β*-D-gluc.(1→2)-[*β*-D-gluc.-(1→6)]-*β*-D-gluc. ester
3	OH	H	OH	H	H	CH_3	*O*-*β*-D-gluc.(1→2)-[*β*-D-gluc.-(1→6)]-*β*-D-gluc. ester
4	*O*-*β*-D-Xyl	H	OH	H	H	CH_3	OH
5	*O*-*β*-D-Xyl	H	*O*-Acetyl	CH_3	OH	H	*O*-*β*-D-glucopyranosyl
6	*O*-*β*-D-Xyl	H	*O*-Acetyl	CH_3	OH	H	OH
7	*O*-*β*-D-Xyl	H	H	CH_3	OH	H	*O*-*β*-D-glucopyranosyl
8	O	H	H	H	H	CH_3	OH
9	*O*-*β*-OH	H	H	CH_3	H	H	OH
10	*O*-*α*-OH	OH	H	H	H	CH_3	OH
11	*O*-*β*-OH	H	OH	H	H	CH_3	OH





	R_1	R_2
12	H	H
13	CH_3	H
14	CH_3	CH_3

	R
15	OH
16	H

Fig. 1. Compounds 1–16, isolated from *Glandularia x hybrida*.

1100 SL, ESI source model #G1969A, Agilent Technologies, Palo Alto, CA, USA). The UPLC used for UPLC-UV/MS method was an Acquity UPLCTM BEH C18 column (100 mm × 2.1 mm I.D., 1.7 μM) and was used on Waters Acquity UPLCTM system (Waters Corp., Milford, MA, USA) that includes a binary solvent manager, sample manager, heated column compartment, photodiode array (PDA) detector, and single quadrupole detectors (SQD). Column chromatography was carried on Silica gel G60 (60–120 mesh, Merck, Darmstadt, Germany), Sephadex LH-20 (Mitsubishi Kagaku, Tokyo, Japan), and polyamide (50-160 μm, Sorbent Technologies, Norcross, GA, USA). Solid phase extraction (SPE) cartridges (supelco, reversed phase C-18, 10 g, St. Louis, MO, USA). TLC was conducted on pre-coated aluminum sheets silica 60 F254, 0.25 mm (E-Merck, Darmstadt, Germany) and detected by spraying with 1% w/v vanillin sulfuric followed by heating. Solvents used for extraction and isolation were analytical grade, purchased from Fisher Scientific (Fair Lawn, NJ, USA).

3.2. Plant material

The air-dried roots of *G. x hybrida* (Groenl. & Rümpler) G.L.Nesom & Pruski (unresolved name: see The Plant List http://www.theplantlist.org) (Verbenaceae) were collected during its flowering stage in April 2013 from El Nile nursery, El-Manial, Cairo, Egypt. The plant was kindly identified by the late Prof. Naeem E. Keltawy, Prof. of Ornamental Horticulture and Floriculture, Faculty of Agriculture, Assiut University, Assiut, Egypt. A voucher specimen number GHV-2013 has been deposited at the Herbarium of Pharmacognosy Department, Faculty of Pharmacy, Assiut University, Assiut, Egypt.

3.3. Extraction and isolation

The air-dried powdered roots (900 g) were grounded and extracted with ethanol (3 L× 3 days × 4 times) at room temperature, followed

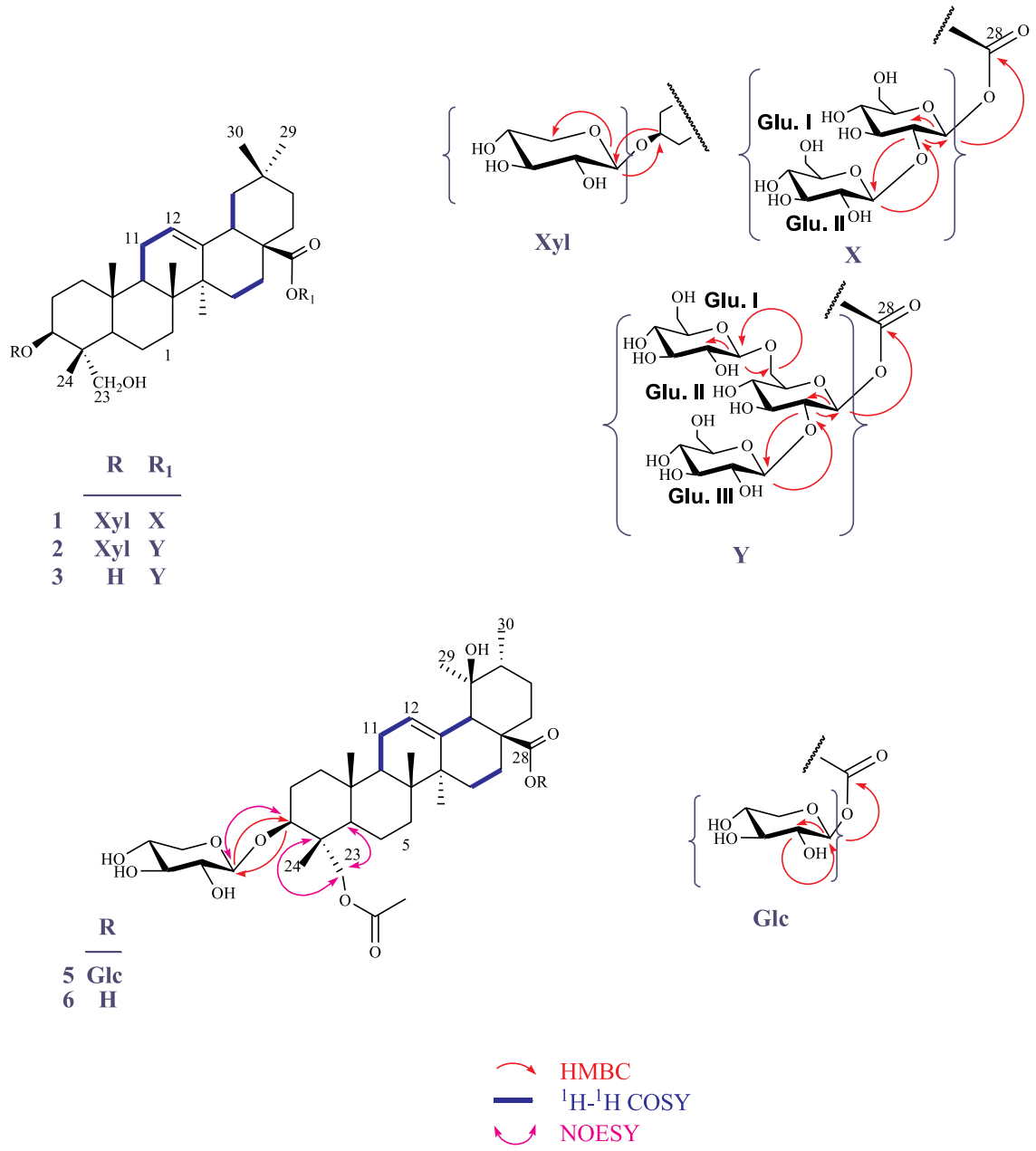


Fig. 2. Important HMBC, ^{1}H-^{1}H COSY and NOESY correlations of compounds 1–3, 5, 6.

by filtration. The ethanolic extract was concentrated under reduced pressure to afford a dark brown viscous residue (32 g) which was suspended in distilled water and defatted using *n*-hexane, to yield *n*-hexane fraction (10 g) and aqueous fraction (21 g). The *n*-hexane fraction was chromatographed on silica gel CC (4 × 120 cm, 250 g) using *n*-hexane-acetone mixtures in increasing order of polarities to get five main fractions (H-I to H-V). Fraction H-II (0.9 g) was chromatographed over silica gel CC (1.5 × 60 cm, 30 g) using *n*-hexane-EtOAc in a gradient elution manner. Fractions eluted with *n*-hexane-EtOAc (90:10) were combined and compound **8** (2 mg) was attained thereof by re-crystallization from MeOH. Fraction H-III (2.2 g) was subjected to silica gel CC (2 × 120 cm, 60 g) using *n*-hexane-EtOAc mixtures in a gradient elution manner. Fractions eluted with *n*-hexane-EtOAc (80:20) were combined to provide compound **9** (5 mg). Fraction H-IV (2.5 g) was subjected to silica gel CC (2 × 120 cm, 60 g) using *n*-hexane-EtOAc mixtures in gradient elution manner. Two subfractions were obtained; H-IV-1 (0.7 g, eluted with *n*-hexane-EtOAc 80:20) and H-IV-2 (0.5 g, eluted with *n*-hexane-EtOAc 70:30). Subfraction H-IV-1 was subjected to sephadex LH-20 CC (2 × 150 cm, 100 g) using CH_2Cl_2-MeOH (60:40) as eluent to yield compound **10** (4 mg). Subfraction H-IV-2 was further purified by re-crystallization to afford compound **11** (7 mg).

The aqueous fraction was fractionated using polyamide CC (4 × 120 cm, 300 g) which was eluted with H_2O and H_2O-MeOH mixtures to give five fractions; A-I to A-V. Fraction A-II (2.0 g) was subjected to sephadex LH-20 CC (4 × 140 cm, 200 g) using H_2O-MeOH (50:50) in an isocratic elution manner, resulting in two main subfractions A-II-1 (0.8 g) and A-II-2 (1.1 g). Subfraction A-II-1 was subjected to silica gel CC (1.5 × 60 cm, 30 g) using a gradient of CH_2Cl_2-MeOH. Fractions eluted with systems CH_2Cl_2-MeOH (80:20) and (70:30) were collected and concentrated to furnish compounds **2** (8 mg) and **3** (3 mg), respectively. Fraction A-III (2.2 g) was subjected to sephadex LH-20 CC (4 × 140 cm, 200 g) using MeOH as an eluent, and resulted

Table 3
The effect of the isolated compounds **2**, **4**, **5**, & **7** on rat paw volume in carrageenan-induced rat paw edema model.

Group	1 h		2 h		3 h	
	Paw Vol. (mL)	% Edema Inhibition	Paw Vol. (mL)	% Edema Inhibition	Paw Vol. (mL)	% Edema Inhibition
Control	0.41 ± 0.05	–	0.40 ± 0.05	–	0.40 ± 0.05	–
Carrageenan	0.62 ± 0.01	–	0.65[a] ± 0.03	–	0.72[a] ± 0.04	–
Indomethacin	0.55 ± 0.02	22.7	0.50 ± 0.01	56.0	0.45[b] ± 0.01	81.2
Compound **2** (25 mg)	0.62 ± 0.01	4.5	0.58[a] ± 0.01	36.6	0.53[a,b] ± 0.01	57.2
Compound **2** (50 mg)	0.60 ± 0.02	9.1	0.55[a] ± 0.04	46.4	0.50[a,b] ± 0.02	63.6
Compound **4** (25 mg)	0.62 ± 0.05	0	0.63[a] ± 0.05	8.3	0.65[a] ± 0.03	8.8
Compound **4** (50 mg)	0.62 ± 0.02	0	0.61[a] ± 0.02	9.2	0.63[a] ± 0.02	10.7
Compound **5** (25 mg)	0.60 ± 0.02	4.5	0.55[a] ± 0.02	36.2	0.55[a,b] ± 0.02	52.8
Compound **5** (50 mg)	0.61 ± 0.03	9.1	0.53[a] ± 0.02	39.8	0.50[a,b] ± 0.02	59.5
Compound **7** (25 mg)	0.62 ± 0.02	2.8	0.64[a] ± 0.02	2.6	0.62[a] ± 0.02	12.3
Compound **7** (50 mg)	0.62 ± 0.05	4.0	0.62[a] ± 0.02	5.4	0.61[a] ± 0.02	13.9

Data are presented as mean ± S.D, $n = 6$.
[a] Significantly different from the corresponding control group at $p < 0.05$.
[b] Significantly different from the corresponding carrageenan-treated group at $p < 0.05$.

in three main subfractions A-III-1 (0.6 g), A-III-2 (0.25 g) and A-III-3 (0.7 g). Subfraction A-III-1 was subjected to SPE-RP-C18 (10 g) using H_2O-MeOH mixtures in a gradient elution manner, fractions eluted with system H_2O-MeOH (90:10) were combined to give compound **1** (2.5 mg), while fractions eluted with system H_2O-MeOH (80:20) afforded compound **5** (14 mg) and fractions eluted with system H_2O-MeOH (75:25) resulted in compound **7** (4.5 mg). Subfraction A-III-2 was subjected to silica gel CC (1 × 30 cm, 15 g) using mixtures of CH_2Cl_2-MeOH as eluent, fractions eluted with system CH_2Cl_2-MeOH (90:10) resulted in compound **13** (4 mg) while fractions eluted with system CH_2Cl_2-MeOH (80:20) afforded compound **12** (25 mg). Subfraction A-III-3 was subjected to silica gel CC (1.5 × 60 cm, 25 g) and mixtures of CH_2Cl_2-MeOH as eluent, fractions eluted with system CH_2Cl_2-MeOH (80:20) were combined to give compound **15** (90 mg), while fractions eluted with system CH_2Cl_2-MeOH (75:25) afforded compound **16** (4.7 mg). Fr. A-IV (3.4 g) was subjected to silica gel CC (4 × 140 cm, 100 g) using EtOAc-CH_2Cl_2-MeOH-H_2O mixture in a gradient elution manner, fractions eluted with system EtOAc-CH_2Cl_2-MeOH-H_2O (80:40:11:2) were collected and concentrated to afford compound **14** (3.8 mg) and those which were eluted with system EtOAc-CH_2Cl_2-MeOH-H_2O (15:8:4:1) were collected and concentrated to yield a residue of 1.2 g. This residue was subjected to silica gel CC (1.5 × 60 cm, 30 g) using CH_2Cl_2-EtOAc mixtures as eluent. The fractions eluted with CH_2Cl_2-EtOAc (80:20) were collected and concentrated to afford compound **4** (17 mg), while those eluted with system CH_2Cl_2-EtOAc (70:30) were combined to afford compound **6** (2.7 mg).

3.4. Spectroscopic data of compounds (1–3, 5, 6)

3.4.1. Compound 1

Amorphous powder; $[\alpha]_D^{20}$ + 14.9 (*c* 0.05, MeOH); IR (NaCl/film) ν_{max} 3400, 1612, 1736 and 1072 cm^{-1}; UV (MeOH) λ_{max} 212 nm. For ^1H NMR (CD_3OD, 400 MHz) and ^{13}C NMR (CD_3OD, 100 MHz) data, see Tables 1 and 2. HRESIMS *m/z* 951.4914 $[M+Na]^+$ (calcd 951.4929).

3.4.2. Compound 2

Amorphous powder; $[\alpha]_D^{20}$ + 16.2 (*c* 0.05, MeOH); IR (NaCl/film) ν_{max} 3420, 1727, 1626 and 1090 cm^{-1}; UV (MeOH) λ_{max} 209 nm; For ^1H NMR (C_5D_5N, 400 MHz) and ^{13}C NMR (C_5D_5N, 100 MHz) data, see Tables 1 and 2. HRESIMS *m/z*: 1125.5314 $[M+Cl]^-$ (calcd 1125.5248).

3.4.3. Compound 3

Amorphous powder; $[\alpha]_D^{20}$ - 23.4 (*c* 0.05, MeOH); IR (NaCl/film) ν_{max} 3393, 1737 and 1623 cm^{-1}; UV (MeOH) λ_{max} 213 nm. For ^1H NMR (C_5D_5N, 400 MHz) and ^{13}C NMR (C_5D_5N, 100 MHz) data, see Tables 1 and 2. HRESIMS *m/z*: 981.5032 $[M+Na]^+$ (calcd 981.5035).

3.4.4. Compound 5

Amorphous powder; $[\alpha]_D^{20}$ + 34.7 (*c* 0.05, MeOH); IR (NaCl/film) ν_{max} 3403, 1715, 1727, 1618 and 1052 cm^{-1}; UV (MeOH) λ_{max} 218 nm. For ^1H NMR (C_5D_5N, 400 MHz) and ^{13}C NMR (C_5D_5N, 100 MHz) data, see Tables 1 and 2. HRESIMS *m/z*: 859.4303 $[M+Cl]^-$ (calcd 859.4246).

3.4.5. Compound 6

Amorphous powder; $[\alpha]_D^{20}$ + 39.9 (*c* 0.05, MeOH); IR (NaCl/film) ν_{max} 3430, 1715, 1737, 1622 and 1122 cm^{-1}; UV (MeOH) λ_{max} 220 nm. For ^1H NMR (C_5D_5N, 400 MHz) and ^{13}C NMR (C_5D_5N, 100 MHz) data, see Tables 1 and 2. HRESIMS *m/z*: 661.3943 $[M-H]^-$ (calcd 661.3953), 1323.7983 $[2M-H]^-$ (calcd 1323.7922).

3.5. Carrageenan-induced acute inflammatory model

Anti-inflammatory activities were evaluated using the reported procedure for the carrageenan-induced rat paw edema assay (Abdel Motaal et al., 2016; Eldahshan and Azab, 2012). Adult male rats (130–150 g) were used. They were housed at room temperature, alternating 12 h light–dark cycle and with the normal access to water and food. Rats were randomly divided into eleven equal groups (Gr. 1–11), six animals per group. Gr. 1 and 2 were given the vehicle (0.5% carboxymethylcellulose). Animals in group Gr. 3 received indomethacin (10 mg/kg) as a standard anti-inflammatory drug, whereas the other eight groups (Gr. 4 – Gr. 11) were orally treated with the compounds **2**, **4**, **5**, and **7** (each compound was tested at doses of 25 and 50 mg/kg). The dosing volume was kept constant (10 mL/kg) for all the orally treated groups. Thirty minutes after oral treatment, Gr.1 received 0.05 mL saline, whereas Gr. 2–11 received 0.05 mL of carrageenan (1% solution in saline) subcutaneously on the plantar surface of the right hind paw. The right hind paw volume was measured immediately after carrageenan injection by water displacement using a plethysmometer (model 7140, Ugo Basile, Comerio, Italy). The paw volume was re-measured at 1, 2, and 3 h following the carrageenan injection (Matsumoto et al., 2015; Winter et al., 1962). Reduction in the paw volume compared to the vehicle-treated control animals was considered as anti-inflammatory response. Percent inhibition paw volume was calculated using the formula:

$$\% \text{ inhibition} = (1 - V_t/V_c)x\ 100$$

where, V_t represents mean increase in paw volume after treatment with test and standard drug and V_C represents mean increase in paw volume of control group.

3.6. Statistical analyses

All the provided numerical data were expressed as the means ± S.D. One-way analysis of variance (ANOVA) and Dunnett's test were used to compare groups against control. A value of $p < 0.05$ was considered significant.

3.7. Determination of the sugars identities and configurations

The identities and configurations of the conjugated sugars were determined according to the reported method by Wang and co-workers (Wang et al., 2012). In brief, 1 mg of each compound was hydrolyzed using 200 μL of 2 M HCl at 90 °C for 2 h. After hydrolysis, the reaction mixture was neutralized with 200 μL of 9 M NH_4OH and dried with N_2 gas of high purity. Monosaccharide standards, including D-(+)-xylose, L-(−)-xylose, D-(+)-glucose and L-(−)-glucose and the hydrolysis products of the compounds were derivatized. The final products of each sample were analyzed by UHPLC-UV-MS. An Acquiry UPLCTM BEH C-18 column (100 mm × 2.1 mm I.D., 1.7 μM) was used on Waters Acquity UPLCTM system. The mobile phase consisted of water with 0.05% formic acid (A) and acetonitrile/methanol/isopropanol (50:25:25, v/v) with 0.05% formic acid (B). Analysis was performed using gradient elution at a flow rate 0.3 mL/min: 14% B to 16.5% B in 22 min, and increasing B to 100% B in the following 0.5 min. The PDA detection wavelength was 254 nm and ESI source of SQD was used in the positive mode. The configurations of the sugar units were determined through comparing retention times and mass spectra with that of the reference standards.

Declaration of competing interest

The authors declare no conflicts of interest.

Acknowledgments

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