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New olean-15-ene type gymnemic acids from *Gymnema sylvestre* (Retz.) R.Br. and their antihyperglycemic activity through α-glucosidase inhibition

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ABSTRACT

A mixture of gymnemic acids was precipitated from the water extract of leaves of *Gymnema sylvestre* (Retz.) R.Br. ex Sm. (Asclepiadaceae) by acidification with 2 N H_2SO_4. The chromatographic separation of the mixture afforded five new gymnemic acids (**1**-**5**). The compounds were characterized as Δ^{15} oleanane glycosides on the basis of extensive spectral data analysis. The compounds (**1**-**5**) showed dose dependent inhibition of α-glucosidase, which was found to be comparable to acarbose (IC_{50} 95 μg/ml). Maximum inhibition was achieved with compound **4** (IC_{50} 57 μg/ml) followed by **3** (IC_{50} 62 μg/ml), **1** (IC_{50} 80 μg/ml), **2** (IC_{50} 120 μg/ml) and **5** (IC_{50} 128 μg/ml). The results revealed that the overall pattern of hydroxyl and acyl substitutions of compounds affected their inhibitory activity. In oral sucrose tolerance test, pre-treatment with crude gymnemic acid mixture and isolated compounds **1** and **4** at a dose of 10 mg/Kg b.w. significantly blunted the effect of sucrose challenge in mice. Based on these results, the antihyperglycemic effect of *G. sylvestre* can be, at least partly, attributed to the inhibition of α-glucosidase by its gymnemic acids. The current study provides relatively more direct evidence explaining the effectiveness of *G. sylvestre* against hyperglycemia.

1. Introduction

Diabetes mellitus is a chronic metabolic disorder characterized by the relative or absolute absence of insulin or its insensitivity resulting in disturbed homeostasis (Alberti and Zimmet, 1998). Post-prandial hyperglycemia (PPHG) is the most important risk factor in the onset of diabetes and development of its comorbidities (Dong et al., 2012). The elevated blood glucose levels can be the cause of several complications associated with diabetes, such as retinopathy, nephropathy, neuropathy and impaired wound healing (Ban and Twigg, 2008). α-Glucosidase is primarily a hydrolysing enzyme located primarily on the epithelial wall of the small intestine. Its inhibition delays the metabolism of carbohydrates as well as their absorption, to consequently suppress PPHG (Krentz and Bailey, 2005). Acarbose, miglitol and voglibose are some of the clinically-used carbohydrate metabolism inhibitors. These agents are associated with common side effects such as abdominal distension, flatulence, meteorism and diarrhoea that can be partly ascribed to the non-specific inhibition of both α-glucosidase and α-amylase enzymes by these agents. Selective α-glucosidase inhibitors are expected to bring about stricter glycemic control while keeping the pancreatic (insulin) pathway undisturbed (Sheliya et al., 2015). Recently several triterpenes have aroused greater interest in tackling disturbances in carbohydrate and lipid metabolism. They are also reported to inhibit the formation of advanced glycation end products that are implicated in the pathogenesis of diabetic nephropathy, neuropathy or atherosclerosis (Nazaruk and Borzym-Kluczyk, 2015). The curative potential of triterpenoids is very high yet still poorly recognised.

Gymnema sylvestre (Retz.) R.Br. ex Sm. (Asclepiadaceae) is a widely distributed medicinal herb in India. It is locally known as *Gur-mar* as it suppresses the ability to taste sweetness. *Sushruta* - an ancient compilation of Indian medicinal plants - refers to *G. sylvestre* as a destroyer of *Madhumeha* (glycosuria). It is thus believed that it neutralizes the excess

Abbreviations: COSY, correlation spectroscopy; DEPT, distortion-less enhancement by polarization transfer; DMSO, dimethyl sulfoxide; FTIR, fourier transform infra red; HR-ES-MS, high resolution electrospray mass spectrometry; MPLC, medium pressure liquid chromatography; MW, molecular weight; NMR, nuclear magnetic resonance; HMBC, heteronuclear multiple bond correlation; HMQC, heteronuclear multi-quantum coherence; PNPG, *p*-nitrophenyl-α-D-glucopyranoside; PPHG, postprandial hyperglycaemia; TLC, thin layer chromatography; TMS, tetramethyl silane

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sugar present in diabetics (Nadkarni, 1986). The plant is popular in Indian systems of traditional medicine and is listed in the Indian Pharmaceutical Codex as a remedy for diabetes (Singh et al., 2008). The leaf extract is reported to reduce hyperglycaemia in humans (Khare et al., 1983) and in experimental animals (Srivastava et al., 1985; Shanmugasundaram et al., 1983, 1990). The blood glucose-lowering effect is postulated to be mediated through the stimulation of insulin release (Persaud et al., 1999), regeneration of β-cells (Ahmed et al., 2010) or inhibition of glucose uptake (Shimizu et al., 1997). The anti-sweet constituents of the plant are loosely referred to as gymnemic acids. A number of triterpenoidal derivatives have been isolated from the plant. The anti-sweet activity of the extracts and some of the saponins from *G. sylvestre* have also been reported (Liu et al., 1992; Sahu et al., 1996; Yoshikawa et al., 1989a, 1989b, 1991; Ye et al., 2000, 2001). The literature reports concerning the anti-diabetic or antisweet effects of triterpenoids from *G. sylvestre* have been compiled in an extensive review (Fabio et al., 2014). It is interesting that there are only a few reports on the gut glucosidase inhibition by individual gymnemic acids (Daisy et al., 2009; Kimura, 2006).

As part of our ongoing research on the preparative isolation of bioactive natural compounds from *G. sylvestre* (Alkefai et al., 2018), this article describes the isolation and characterization of five new gymnemic acids and delineation of their antihyperglycemic effect. A mixture of gymnemic acids was precipitated from the water extract of leaves of *G. sylvestre* by acidification with 2 N H_2SO_4 The residue was dissolved in minimal amount of methanol and fractionated over silica gel (50–60 µm) column using MPLC. Prep-TLC was used for further purification of eluents from MPLC. This resulted in the isolation of five new gymnemic acids (**1-5**). Their structures were elucidated as Δ^{15} oleanane-type triterpene glycosides by the interpretation of spectral data. The chemical structures of compounds **1-5** are presented in Fig. 1. The orientation of glucopyranosyl and glucuronopyranosyl residues was determined on the basis of ^{1}H and ^{13}C NMR spectral data (Tables 1 and 2, see Supplemental material Fig. S1–S25). Furthermore, D-configuration of the sugar residues was established by TLC comparison of the acid hydrolysate with the reference standards.

2. Results and discussion

Compound **1** was obtained as brown sticky mass from ethyl acetate eluents. Its FTIR spectrum exhibited absorption bands for hydroxyl group (3392 cm^{-1}), carbonyl group (1722 cm^{-1}) and unsaturation (1448 cm^{-1}). Its molecular formula was derived to be $C_{53}H_{84}O_{18}$ (MW 1008) on the basis of ^{13}C/DEPT NMR and mass spectra. Its HR-ES-MS (Fig. S6, see Supplemental material) displayed a fragment ion peak at *m/z* 844.6627 for $[C_{47}H_{72}O_{13}]^{+}$ (calc. 844.4973)arising due to the loss of a glucose residue. A detailed study of 1D and 2D NMR spectra of **1** (Fig. S1-S5, see Supplemental material) led to the assignment of all proton and carbon resonances (Tables 1 and 2). A downfield doublet at δ 5.61 (2H, J = 1.2 Hz, H-15/16) in ^{1}H NMR and a signal at δ 129.2 (C-15/16) in ^{13}C NMR spectra indicated a vinylic linkage between two equivalent carbons that was placed at C-15. This allocation was corroborated by long range correlation observed between C-15/16 and H-21/22 in HMBC spectrum. It also revealed the presence of two vicinal carbinol carbons (C-21 and C-22). A double-doublet at δ 3.21 (1H, J = 1.6, 4.8 Hz) was assigned to H-3 carbinol proton placed in β-orientation due to its lower coupling constant values.

The ^{1}H NMR spectrum of **1** exhibited signals for two methyl butyroyl moieties [δ 2.12 (m, H-2a), 2.17 (m, H-2b), 1.68 (m, H_2-3a), 1.46 (m, H_2-3b), 0.61 (br s, H_3-4a, H_3-4b), 1.29 (d, J = 7.2 Hz, H_3-5a) and 1.27 (d, J = 6.8 Hz, H_3-5b)]. The signals for two anomeric carbons at δ 105.4 and 104.3; and two anomeric protons at δ 4.38 (d, J = 7.6 Hz, H-1′) and 4.30 (d, J = 7.6 Hz, H-1″) suggested the presence of two sugar units. A detailed examination of the saccharide regions of the NMR spectra revealed a significant downfield shift experienced by the C-3′ (δ 89.0 from 76.2) that indicated a 3′→1″ linkage between the sugar units.

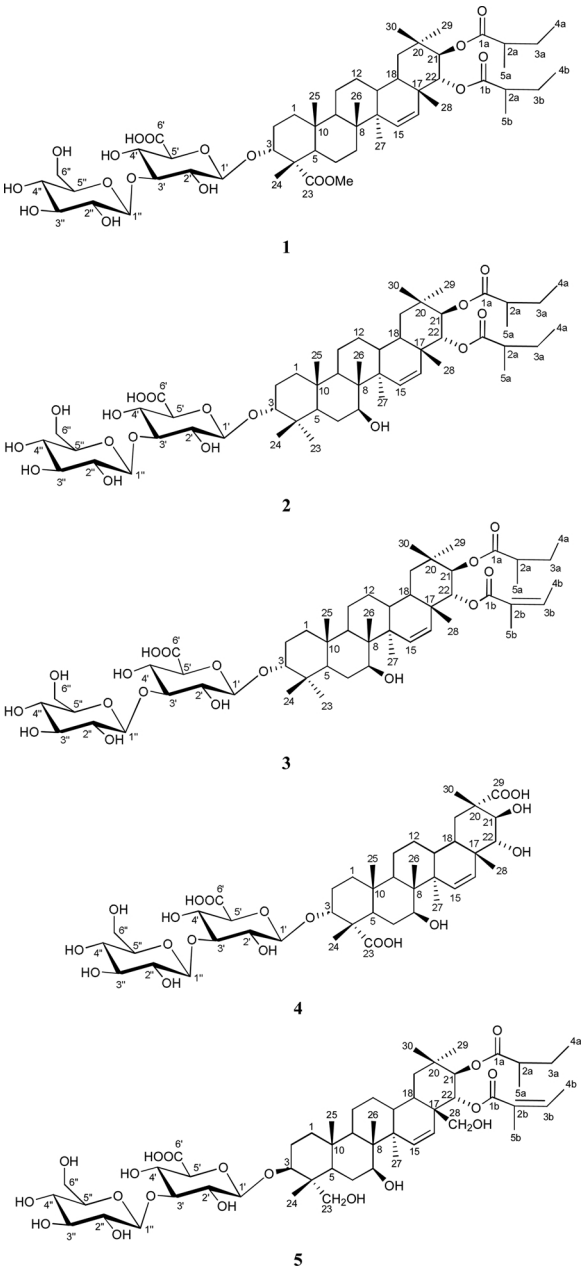

Fig. 1. Structures of compounds **1-5** isolated from *Gymnema sylvestre*.

Acid hydrolysis of **1** yielded D-glucose and D-glucuronic acid that were identified by TLC comparison of the hydrolysate with the respective standards. The ^{13}C/DEPT NMR spectra of **1** revealed the presence of three oxygenated methine carbons [δ 82.0 (C-3), 72.7 (C-21) and 69.7 (C-22) associated with its aglycone. Comparison of NMR data of **1** with those of substituted oleananes (Liu et al., 1992; Mahato and Kundu, 1994; Sahu et al., 1996), established its aglycone as 3α, 21β,22α-trihydroxy olean-15-ene. For determining the position of attachment of acyl/sugar groups, ^{1}H-^{13}C chemical shift correlation spectra were examined in detail. Key correlational cross peaks were exhibited between H-1′ and C-3; H-1″ and C-3′; H-21 and C-1a; and H-22 and C-1b in HMBC spectrum. In ^{13}C NMR spectrum, a downfield shift of +3.3 ppm was observed for C-3 (δ 82.0 compared to δ 78.7 for oleanolic acid) due to its glycosylation (Kasai et al., 1979). The signals for C-21/22 carbons also showed a downfield shifts that were attributed to their acylation (Ishii et al., 1978). Based on the foregoing account, the structure of **1** was elucidated as 3α,21β,22α-trihydroxy-21,22-bis(2-methyl-1-oxobutoxy)-olean-15-en-23-methyl carboxylate-3yl-3-*O*-β-D-

Table 1
^{1}H NMR spectroscopic data (δ in ppm, *J* in Hz) for compounds **1-5**.

H	1	2	3	4	5
3	3.21 dd (1.6, 4.8)	3.20 dd (1.6, 4.8)	3.33 dd (1.6, 4.8)	3.33 dd (1.5, 3.0)	3.75 dd (2.0, 8.5)
7	–	4.26 dd (6.8)	4.21dd (7.0)	4.26 dd (7.0)	4.49 d (7.5)
15	5.61 d (1.2)	5.61 br s	5.73 br s	5.74 br s	5.73 br s
16	5.61 d (1.2)	5.61 br s	5.73 br s	5.74 br s	5.73 br s
21	3.70 d (4.8)	3.69 d (4.8)	3.80 d (4.5)	3.81 d (5.0)	3.84 d (4.5)
22	4.05 d (4.8)	4.04 d (4.8)	4.15 d (4.5)	4.17 d (4.5)	4.16 d (4.5)
23	–	0.89 s	0.90 s	–	3.43 d (10.5)
24	0.96 s	0.91 s	0.91 s	0.91 s	0.90 s
25	1.26 s	1.26 s	1.30 s	1.26 s	1.26 s
26	1.27 s	1.27 s	1.27s	1.28 s	1.29 s
27	0.91 s	0.93 s	0.90 s	0.94 s	0.94 s
28	1.29 s	1.29 s	1.30 s	1.28 s	3.29 d (9.5)
29	1.01 s	1.01 s	1.02 s	–	1.01 s
30	1.38 s	1.38 s	1.39 s	1.38 s	1.31 s
Glc 1′	4.38 d (7.6)	4.48 d (7.6)	4.29 d (7.0)	4.31 d (8.5)	4.51 d (6.5)
2′	3.98	3.79	4.15-3.67	4.30-3.53	4.40-3.90
3′	4.42	3.63	4.15-3.67	4.30-3.53	4.40-3.90
4′	3.63	3.72	4.15-3.67	4.30-3.53	4.40-3.90
5′	3.41	3.53	4.15-3.67	4.30-3.53	4.40-3.90
Glu 1″	4.30 d (7.6)	4.38 d (6.8)	4.19 d (6.5)	4.23 d (8.0)	4.41 d (7.0)
2″	3.78	3.78	4.16-3.67	4.30-3.53	4.40-3.90
3″	3.49	3.44	4.16-3.67	4.30-3.53	4.40-3.90
4″	3.32	3.24	4.16-3.67	4.30-3.53	4.40-3.90
5″	3.59	3.56	4.16-3.67	4.30-3.53	4.40-3.90
6″	3.50 dd (4.0, 10.8)	3.44 br d (6.8)	3.63 d (10.0)	3.43 d (9.0)	3.43 d (9.5)
First acyl	**Mba**	**Mba**	**Mba**	–	**Mba**
2a	2.12 m	2.54 m	2.59 m	–	2.48 m
3a	1.68 m	1.54 m	1.54 m	–	1.60 m
4a	0.61 br s	0.60 br s	0.71 br s	–	0.72 s
5a	1.29 d (7.2)	1.25 d (7.2)	1.21 d (6.0)	–	1.19 d (7.0)
Other acyl	**Mba**	**Mba**	**Tig**	–	**Tig**
2b	2.17 m	2.20 m	–	–	–
3b	1.46 m	1.43 m	6.78 m	–	7.01 br m
4b	0.61 br s	0.60 br s	1.85 m	–	1.61 br d
5b	1.27 d (6.8)	1.27 d (6.8)	2.08 s	–	2.03 s
COOMe	3.68 s	–	–	–	–

Glc: glucuronic acid, Glu: glucose, Mba: methyl butyroyl, Tig: tigloyl. Overlapped signals are indicated without designated multiplicity.

Table 2
^{13}C NMR spectroscopic data (δ in ppm) for compounds **1-5**.

C	1	2	3	4	5
1	38.1	38.2	38.0	38.8	38.4
2	26.3	26.0	27.5	27.1	26.6
3	82.0	82.3	79.4	82.2	81.9
4	39.7	39.6	39.4	39.7	41.5
5	55.4	55.3	54.9	54.9	55.4
6	17.3	18.3	20.2	18.4	16.7
7	33.8	66.5	66.3	66.3	66.0
8	41.5	46.0	46.4	41.5	39.7
9	46.5	51.8	52.1	51.8	51.8
10	39.7	38.2	41.0	36.0	38.8
11	23.3	23.4	23.5	29.2	29.1
12	31.7	31.7	33.1	31.6	31.5
13	46.6	46.6	46.7	41.2	46.6
14	38.8	36.9	36.5	36.2	36.4
15	129.2	129.2	129.4	129.2	129.2
16	129.2	129.2	129.4	129.2	129.2
17	42.0	42.5	41.3	40.2	42.1
18	41.2	41.1	40.3	46.6	31.7
19	45.4	46.5	47.3	35.6	38.8
20	37.4	36.8	36.7	36.6	29.4
21	72.7	72.7	73.3	72.3	73.9
22	69.7	69.1	69.0	67.8	68.4
23	170.2	26.5	26.0	174.2	61.3
24	13.2	13.2	13.5	13.2	13.3
25	16.1	16.5	15.4	15.7	15.9
26	18.4	18.3	18.1	18.2	18.5
27	27.1	27.5	28.4	26.6	27.2
28	19.4	19.3	19.2	15.4	63.2
29	28.5	28.6	28.3	177.1	29.1
30	19.6	19.4	19.3	19.4	19.7
Glc 1′	105.4	104.3	110.0	105.4	105.4
2′	76.1	75.1	75.7	75.8	76.1
3′	89.0	89.1	89.7	89.7	89.7
4′	71.8	72.9	73.3	73.8	73.6
5′	78.8	76.9	76.4	76.5	77.7
6′	172.4	171.7	174.9	172.9	171.9
Glu 1″	104.3	114.3	115.0	104.3	104.3
2″	73.7	73.7	73.4	76.0	76.0
3″	76.5	76.5	75.4	77.7	77.7
4″	71.2	71.8	70.3	72.3	72.0
5″	78.2	78.2	77.2	75.0	75.0
6″	62.9	62.4	63.2	63.2	63.4
First acyl	**Mba**	**Mba**	**Mba**	–	**Mba**
1a	177.1	177.2	175.1	–	176.1
2a	41.5	41.5	41.5	–	39.7
3a	24.9	24.7	24.5	–	26.6
4a	10.8	10.9	11.2	–	10.9
5a	15.7	15.4	15.1	–	15.9
Other acyl	**Mba**	**Mba**	**Tig**	–	**Tig**
1b	174.2	175.7	168.5	–	167.4
2b	41.9	41.9	127.9	–	129.2
3b	23.3	23.4	131.1	–	138.8
4b	12.1	12.2	13.2	–	12.1
5b	14.8	14.4	14.4	–	16.2
COOMe	51.6	–	–	–	–

Glc: glucuronic acid, Glu: glucose, Mba: methyl butyroyl, Tig: tigloyl.

glucopyranosyl(1→3) *O*-β-D-glucuronopyranoside.

Compound **2** was obtained as yellowish brown amorphous powder from ethyl acetate-methanol (98:2 v/v) eluents. Its FTIR spectrum displayed absorption bands for hydroxyl group (3391 cm^{-1}), carbonyl group (1726 cm^{-1}) and unsaturation (1441, 1267 cm^{-1}). Its molecular formula was derived to be $C_{52}H_{84}O_{17}$ (MW 980) on the basis of ^{13}C/DEPT and mass spectra. Its HR-ES-MS (Fig. S13, see Supplemental material) displayed a diagnostic fragment ion peak at *m/z* 878.3750 for $[C_{47}H_{74}O_{15}]^{+}$ (calc. 878.4028)arising due to the loss of a methyl butyroyl residue. Intensive study of 1D and 2D NMR spectra of **2** (Fig. S7-S12, see Supplemental material) led to assignment of all the protons and carbon resonances unambiguously (Tables 1 and 2). The presence of downfield broad singlet at δ 5.61 (2H, H-15/16) in ^{1}H NMR, and a signal at δ 129.2 (C-15/16) in ^{13}C NMR spectra supported a vinylic linkage between two equivalent carbons that was placed at C-15. This allocation was supported by long range correlations observed between C-15/16 and H-21/22 in HMBC spectrum. It also indicated the presence of two vicinal carbinol carbons (C-21 and C-22). Two double doublets at δ 3.20 (*J* = 1.6, 4.8 Hz) and 4.16 (*J* = 6.8, 6.8 Hz), each integrating for one proton, were assigned correspondingly to H-3 and H-7 carbinol protons placed in α- and β-orientation based on their coupling constant values. The ^{13}C/DEPT NMR spectra of **2** also supported the presence of four oxygenated methine carbons [δ 82.3 (C-3), 66.5 (C-7), 72.7 (C-21) and 69.7 (C-22)] in its aglycone. Comparison of NMR data of **2** with those of substituted oleananes (Mahato and Kundu, 1994; Ye et al., 2001) established 3α, 7β, 21β, 22α-tetrahydroxy olean-15-en as its aglycone. A close examination of ^{1}H and ^{13}C NMR chemical shift of the saccharide portion indicated the presence of the same sugar chain at C-3 position of the aglycone as in **1**. Acid hydrolysis of **2** afforded D-

glucuronic acid and D-glucose that were identified by comparison of TLC of the hydrolysate with the respective standards. For determination of the linkage of acyl/sugar chain, ^{1}H-^{1}H and ^{1}H-^{13}C chemical shift correlation were examined in detail. Key cross peaks between H-3 and H-1′; H-16 and H-22; and H-21 and H-22 in COSY; along with correlations between H-1′ and C-3; H-1″ and C-3′; H-21 and C-1a; H-22 and C-1b in HMBC spectra were observed. In ^{13}C NMR spectrum, a glycosylation shift of +3.6 ppm was observed for C-3 (δ 82.3 from δ 78.7 for oleanolic acid). The signals for C-21/22 also exhibited downfield shifts due to acylation of these carbons (Ishii et al., 1978). These finding led to determination of **2** as 3α, 7β, 21β, 22α-tetrahydroxy-21,22-bis(2-methyl-1-oxobutoxy)-olean-15-en-3yl-3-*O*-β-D-glucopyranosyl (1→3)-*O*-β-D-glucuronopyranoside.

Compound **3** was obtained as brownish amorphous powder from ethyl acetate-methanol (98:2 v/v) eluents. Its FTIR spectrum exhibited absorption bands for hydroxyl (3450 cm^{-1}), carbonyl (1723 cm^{-1}) and olefinic (1650, 1441 cm^{-1}) functionalities. Based on ^{13}C/DEPT and mass spectra (Fig. S15-S17, see Supplemental material), the molecular weight of **3** was determined to be 978, consistent with molecular formula $C_{52}H_{82}O_{17}$ (calc. 978.5052, obsd. 978.4949). The ^{1}H NMR spectrum of **3** (Fig. S14, see Supplemental material) exhibited signals for a tigloyl moiety [δ 6.78 (m, H-3b), 1.85 (m, H_3-4b) and 2.08 (s, H_3-5b)] and a methyl butyroyl moiety [δ 2.59 (m, H-2a), 1.54 (m, H_2-3a), 0.71(brs, H_3-4a) and 1.21 (d, J = 6.0 Hz, H_3-5a)]. The signals for two anomeric carbons at δ 110.0 (C-1′) and 115.0 (C-1″), and two anomeric protons at δ 4.29 (d, J = 7.0 Hz, H-1′) and 4.19 (d, J = 6.5 Hz, H-1″) revealed the presence of two sugar units. A detailed examination of NMR spectra particularly that of the saccharide region supported the presence of the same sugar chain in **3** as in **2**. Acid hydrolysis of **3** afforded D-glucuronic acid and D-glucose that were identified by comparison of TLC of the hydrolysate with the respective standards. Significant downfield shift were observed for C-3 of glycone to δ 79.4 and for C-3′ of glucuronic acid to δ 89.7 due to glycosylation. The signals for C-21 and C-22 also exhibited downfield shifts to δ 73.3 and δ 69.0, respectively that can be attributed to acylation f these carbons (Ishii et al., 1978). ^{13}C NMR spectrum of **3** also supported the presence of four oxygenated carbons [δ 79.4 (C-3), 66.3 (C-7), 73.3 (C-21) and 69.0 (C-22)] in its aglycone. Comparison of NMR data of compound **3** with that of compound **2** revealed that **3** has one tigloyl and one methyl butyroyl unit compared to two methyl butyroyl units in **2**. Thus compound **3** was identified as 3α, 7β, 21β, 22α-tetrahydroxy-21-(2-methyl-1-oxobutoxy)-22-[(2-methyl-1-oxobutenyl)oxy] olean-15-en-3yl-3-*O*-β-D-glucopyranosyl(1→3) *O*-β-D-glucuronopyranoside.

Compound **4** was obtained as white amorphous powder from ethyl acetate-methanol (90:10 v/v) eluents. Its FTIR spectrum showed absorption bands for hydroxyl (3399 cm^{-1}), carbonyl (1724 cm^{-1}), olefinic (1643, 1455 cm^{-1}) functionalities. Its HR-ES-MS (Fig. S21, see Supplemental material) displayed a molecular ion peak at *m/z* 872.5179 that accounted to the molecular formula $C_{42}H_{64}O_{19}$ (calc. 872.4997). The formula indicated the presence of eleven double bond equivalents, five of which were attributed to pentacyclic framework, three to carboxylic groups, two to sugar units and the remaining one to a vinylic linkage. A two-proton broad signal δ 5.74 in ^{1}H NMR spectrum and a single signal in the olefinic region of the ^{13}C NMR spectrum at δ 129.2 indicated the presence of the only vinylic linkage between two equivalent carbons that was consequently placed at C-15. The presence of two anomeric proton signals at δ 4.31 (d, J = 8.5 Hz, H-1′), and 4.23 (d, J = 8.0 Hz (H-1″), and two anomeric carbon signals at δ 105.4 (C-1′) and 104.3 (C-1″) further supported presence of two sugar units (Fig. S18 and S19, see Supplemental material). TLC comparison of the hydrolysate of **4** revealed the sugars to be D-glucuronic acid and D-glucose. Further, ^{13}C NMR spectrum showed significant downfield shift experienced by C-3 to δ 82.7 and by C-3′ to δ 89.7, indicating the glycosylation at δ C-3 of aglycone and C-3′ of glucuronic acid. ^{13}C/DEPT NMR spectra (Fig. S19, S20, see Supplemental material) of **4** indicated the presence of four oxygenated carbons [δ 82.2 (C-3), 66.3(C-7), 72.3 (C-21) and 67.8 (C-22)] in its aglycone. The spectra also revealed the presence of three carboxylic carbons [δ 174.2 (C-23), 177.1 (C-29) and 172.9 (C-6′)]. Comparison of NMR data of **4** with oleanane derivatives (Mahato and Kundu, 1994; Ye et al., 2001) and the other compounds reported here clearly indicated the absence of any acyl substitution. Thus the structure of **4** was deduced to be 3α, 7β, 21β, 22α-tetrahydroxy olean-15-en-23, 29-dioic acid-3-yl-3-*O*-β-D-glucopyranosyl (1→3) *O*-β-D-glucuronopyranoside.

Compound **5** was obtained as light green amorphous powder from ethyl acetate-methanol (90:10 v/v) eluents. Its FTIR spectrum showed absorption bands for hydroxyl (3418 cm^{-1}), carbonyl (1723 cm^{-1}) and olefinic (1645, 1448 cm^{-1}) functionalities. Based on ^{13}C/DEPT and mass spectra (Fig. S23-S25, see Supplemental material), the molecular weight of **5** was established to be 1010 corresponding to the molecular formula $C_{52}H_{82}O_{19}$. Its HR-ES-MS showed a diagnostic fragment ion peak at *m/z* 846.5386 for $[C_{46}H_{70}O_{14}]^{+}$ (calc. 846.4766) arising due to the loss of a glucose residue from the molecular ion.

The ^{1}H NMR spectrum of **5** (Fig. S22, see Supplemental material) exhibited signals for one tigloyl moiety [δ 7.01 (brm, H-3b), 1.61 (br d, H_3-4b) and 2.03 (s, H_3-5b)] and one methyl butyroyl moiety [δ 2.48 (m, H-2a), 1.60 (m, H_2-3a), 0.72 (s, H_3-4a) and 1.19 (d, J = 7.0 Hz, H_3-5a)]. The presence of two anomeric proton signals at δ 4.51 (d, J = 6.5 Hz, H-1′) and 4.40 (d, J = 7.0 Hz, H-1″), and two anomeric carbon signals at δ 105.4 (C-1′) and 104.3 (C-1″) supported the presence of two sugar units. Comparison of TLC of the hydrolysate of **5** with reference standards confirmed the presence of D-glucuronic acid and D-glucose. Further, ^{13}C NMR spectrum showed significant downfield shift experienced by C-3 to δ 81.9 and by C-3′ to δ 89.7 supporting the glycosylation at δ C-3 of aglycone and C-3′ of glucuronic acid. The signals for C-21 and C-22 also displayed downfield shift to δ 73.9 and 68.4, respectively due to acylation at these positions (Ishii et al., 1978).

The ^{13}C/DEPT NMR spectra of **5** also displayed signals for three hydroxy methyl carbons at δ 61.3 (C-23), 63.2 (C-28) and 63.4 (C-6″); and four carbinol carbons at δ 81.9 (C-3), 66.4 (C-7), 73.9 (C-21) and 68.4 (C-22). Comparison of spectra data of **5** with that of **1** and other hydroxylated gymnemic acids (Yoshikawa et al., 1989a, 1989b), supported **5** to be a hexahydroxy oleanane derivative. A doublet of doublets at δ 3.75 (J = 2.0, 8.5 Hz) was assigned to H-3 carbinol proton that was placed in α-orientation based on high coupling constant value. On the basis of the foregoing account, the structure of **5** was elucidated as 3β, 7β, 21β, 22α, 23,28-hexahydroxy-21-(2-methyl-1-oxobutoxy)-22-[(2-methyl-1-oxobutenyl)oxy]-olean-15-en-3-yl-3-*O*-β-D-glucopyranosyl(1→3) *O*-β-D-glucuronopyranoside. It is noteworthy that the compounds **1**-**5** represent Δ^{15} oleanane-type gymnemic acids (all acylated except **4**) compared to the arylated Δ^{12} oleanane series of gymnemic acids reported earlier by our group (Alkefai et al., 2018).

Compounds **1**-**5** were screened for α-glucosidase inhibitory activity at concentrations ranging from 31.25–500 μg/ml. All the compounds showed dose-dependent inhibition of α-glucosidase as shown in Fig. 2. The maximum inhibition was achieved with compound **4** (IC_{50} 57 μg/ml) followed by **3** (IC_{50} 62 μg/ml), **1** (IC_{50} 80 μg/ml), **2** (IC_{50} 120 μg/ml) and **5** (IC_{50} 128 μg/ml). IC_{50} values for acarbose and crude gymnemic acid (CGA) were found to be 95 μg/ml and 170 μg/ml, respectively. It is noteworthy that the compounds **3** and **4** were about three fold more potent than the CGA (Table S1, see Supplemental material). Compounds **3** could not be screened further for the lack of sufficient quantity. Compounds **1** and **4** were screened for their effect on blood glucose in oral sucrose tolerance test in mice at a dose of 10 mg/kg b.w. after oral administration.

Oral administration of sucrose (4 g/kg, b.w.) produced a significant increase ($p < 0.01$) in blood glucose level (BGL) of sucrose-challenged control animals, resulting in carbohydrate induced hyperglycemia. It is evident from Fig. 3 that a pre-treatment compounds **1** and **4** (each10 mg/kg, b.w., p.o.) blunted the effect of sucrose challenge as signified by less than 65 mg/dL increase in peak blood glucose level after 30 min of sucrose overload compared to about 95 mg/dL increment in sucrose

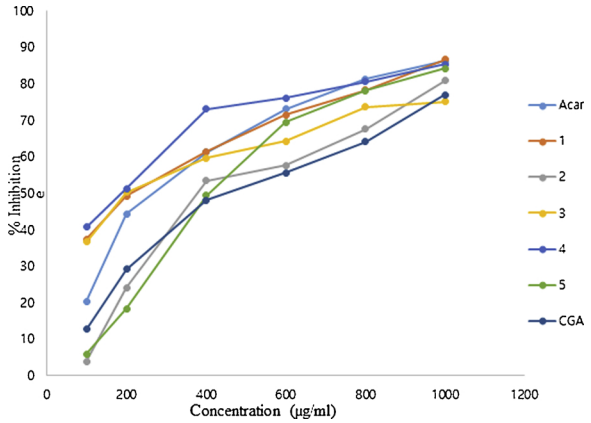

Fig. 2. α-Glucosidase inhibitory activity of compounds **1**-**5** and crude gymnemic acid mixture from *Gymnema sylvestre vs* acarbose.

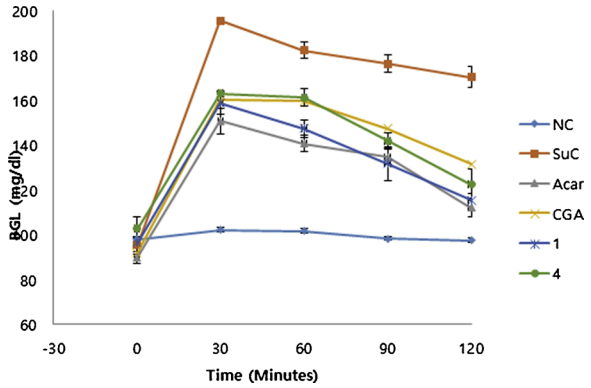

Fig. 3. Antihyperglycemic effect of crude gymnemic acid mixture and compounds **1** and **4** from *Gymnema sylvestre vs* acarbose in sucrose challenged mice.

challenged animals at the same time level. Acarbose restricted the increment in peak BGL to less than 50 mg/dL. Pre-treatment with both the isolated compounds exhibited significant reduction in peak BGL compared to the sucrose-challenged group ($p < 0.01$). Pre-treatment with CGA and acarbose (10 mg/kg, b.w.) significantly ($p < 0.01$) lowered peak BGL compared to sucrose-challenged animals, respectively (Table S2, see Supplemental material). From the above discussion it is clear that the antihyperglycemic effect of **1** and **4** was comparable to that of acarbose.

The results revealed that the overall pattern of hydroxyl and acyl substitution in compounds affected their α-glucosidase inhibitory activity. As is clear from Fig. 1, the structure of compound **4** has three OH groups that can form hydrogen bonds at the catalytic site of α-glucosidase enzyme. Even the two COOH groups in **4** are amenable to hydrogen bonding. These groups in **4** provide points of stronger interaction with α-glucosidase compared to other compounds that either have one or no OH group. On the other hand, a comparable inhibitionof α-glucosidase by acylated ymnemic acids **1** and **3** point towards the role of hydrophobic interaction as well. Thus, it would be safe to infer that inhibition involved both the hydrophobic and hydrophilic interactions of the compounds at the catalytic site. The results of the inhibitory studies corroborated the earlier studies involving triterpenoids and their synthetic analogues (Ali et al., 2002; Gowri et al., 2007; Hou et al., 2009; Kuang et al., 2011; Uddin et al., 2012). A comparison of the findings here and that of our earlier report highlights the fact that the non-acylated gymnemic acid (**4**) is a more effective α-glucosidase inhibitor than the acylated (**1**-**3** and **5**) and the arylated gymnemic acids (Alkefai et al., 2018). *G. sylvestre* has been used as a traditional remedy for diabetes in many countries. The current study lends credence to this ethno-medical practice. Moreover, our study highlights the effectiveness of gymnemic acids against hyperglycemia.

3. Materials and methods

3.1. General experimental procedures

Melting points were determined using one end open capillary tubes on Melting Point M-560 apparatus (Perfit, India) and are uncorrected. UV spectra were measured on UV-160 spectrometer (Shimadzu, Japan). Infra-red spectra were recorded on IR Affinity1 Spectrophotometer (Shimadzu, Japan) using KBr pellets. ^{1}H NMR (400 or 500 MHz), ^{13}C NMR (100 or 125 MHz) and 2D NMR spectra were recorded on DRX NMR instruments (Bruker, Switzerland). Deuterated methanol was used as solvent and TMS an internal standard. ES-Mass spectral data were recorded on Synapt mass spectrometer (Waters, UK) equipped with direct inlet probe system.

3.2. Chemicals

Acarbose was obtained as gift sample from Medley Pharmaceuticals Ltd, India. α-Glucosidase (EC 3.2.1.20), *p*-nitrophenyl-α-D-glucopyranoside (PNPG) and methanol-d_4 (NMR grade) were purchased from Merck (Mumbai, India). Hexane, ethyl acetate and methanol of analytical grade were procured from S.D. fine chemicals (Mumbai, India). Silica gel Si60 (50–60 µm) for column chromatography, prep-TLC silica gel Si60 plates and pre-coated HPTLC silica gel Si60 F_{254} plates from Merck (Germany) were used for chromatographic separations.

3.3. Plant material

The leaves of *G. sylvestre* were procured from the local crude drug market, Delhi (M/s Universal Biotic, Delhi, India) and identified by Dr. H. B. Singh, Chief Scientist (RHMD), NISCAIR, New Delhi, India. A voucher specimen (NISCAIR/RHMAD/Consult/-2011-12/1856/02) was deposited in RHMD, NISCAIR, New Delhi, India.

3.4. Preparation of crude gymnemic acids mixture

Leaves of *G. sylvestre* were cleaned, washed under water and dried in an oven at 45 °C. Dried leaves were pulverized to a coarse powder using a grinder. About 5 kg of powder was defatted with hexane and then the defatted leaves were air dried and extracted with water (10 l) under air-reflux for 6 h with occasional stirring. This was followed by filtration. The filtrate was adjusted to pH 3.0 by adding 2 N H_2SO_4 and centrifuged at 5000 rpm for 10 min to yield a crude gymnemic acids mixture (60 *g*, 1.2% *w/w*) (Kurihara, 1969).

3.5. Isolation of compounds

The mixture of gymnemic acids was subjected to normal-phase MPLC. The isolation was carried out on a Sepacore Easy Purification System (Buchi Labortechnik, Flawil, Switzerland) consisting of two C-605 pump modules, a C-615 control unit and a C-640 UV detector. Fractionation was carried out with a 70 × 460 mm plastic-glass column (Buchi, Switzerland) packed with silica gel Si60 (50–60 µm). Sample solution was prepared in methanol. The solution was homogenized, filtered and loaded on to the column through the injector loop.

The eluents from the column were detected by an on-line UV detector set at 225, 245, 290 and 325 nm for simultaneous detection. Eluents were manually collected based on the changes in absorbance and aliquots were analysed by TLC on silica gel 60 F_{254} pre-coated plates. Chromatographically identical eluents were combined and concentrated to yield five compounds. Compound 1 was obtained as a brownish sticky mass from ethyl acetate fractions. Compounds 2 and 3 were obtained as amorphous powders from 2% methanol in ethyl acetate fractions. Elution with 10% methanol in ethyl acetate afforded a

mixture of compounds 4 and 5 that were purified using prep-TLC (BuOH-EtOAc-H_2O, 20:0.5:0.5) as white and light green powders, respectively. The purity of compounds was ascertained by HPTLC performed on pre-coated silica gel Si60 F_{254} plates.

3.6. Characterization of compounds

3.6.1. Compound 1

Brown sticky mass (0.25 g, 0.05% yield); mp 48-51 °C; UV (MeOH) λ_{max} 213, 282 nm; IR (KBr) ν_{max} 3392, 2941, 1722, 1640, 1512, 1448, 1383, 1031 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; +ve ES-MS *m/z* (*rel. int.*): 1008 $[M]^+$ $C_{53}H_{84}O_{18}$ (N.O.), 844.6627 (calc. for $C_{47}H_{72}O_{13}$, 844.4973) (8), 353 (60), 182 (65)

3.6.2. Compound **2**

Yellowish brown amorphous powder (0.2 g and 0.04% yield); mp 176–178 °C; UV (MeOH) λ_{max} 210, 282 nm; IR (KBr) ν_{max}: 3391, 2929, 1726, 1644, 1514, 1441, 1267, 1031 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; -ve ES-MS *m/z* (*rel. int.*): 980 [M]- $C_{52}H_{84}O_{17}$ (N.O.), 878.3750 (calc. for $C_{47}H_{74}O_{15}$, 878.4028) (20), 399 (100)

3.6.3. Compound **3**

Brownish amorphous powder (0.26 g, 0.05% yield); mp 144–147 °C; UV (MeOH) λ_{max} 209, 278 nm; IR (KBr) ν_{max}: 3450, 2861, 1723, 1549, 1441, 1383, 1267, 1031 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; +ve ES-MS *m/z* (rel. int.): 978.4949 $[M]^+$ (calc. for $C_{52}H_{82}O_{17}$ 978.5052) (3), 845 (16), 831 (25), 685 (45)

3.6.4. Compound **4**

White amorphous powder (0.3 g, 0.06% yield); mp 111–113 °C; UV (MeOH) λ_{max} 212, 283, 318 nm; IR (KBr) ν_{max} 3399, 2946, 2850, 1724, 1643, 1455, 1383, 1269, 1032 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; +ve ES-MS *m/z* (*rel. int.*): 872.5179 $[M]^+$ (calc. for $C_{42}H_{64}O_{19}$ 872.4997) (5), 869 (20), 831 (50), 789 (100)

3.6.5. Compound **5**

Light green amorphous powder (0.5 g, 0.1% yield); mp 131–135 °C; UV (MeOH) λ_{max} 216, 286, 319 nm; IR (KBr) ν_{max} 3418, 2947, 1723, 1645, 1448, 1270, 1043 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; +ve ES-MS *m/z* (*rel. int.*): 1010 $[M]^+$ $C_{52}H_{82}O_{19}$ (N.O.), 846.5386 (calc. for $C_{46}H_{70}O_{14}$, 846.4766) (5), 685 (25), 663 (100)

3.6.6. Acid hydrolysis of compounds

The isolated compounds (each 15–20 mg) were refluxed with 15% HCl in 60% ethanol (5 ml) for 6 h. Each reaction mixture was diluted with water and neutralized with NaOH, and extracted with ethyl acetate. The organic was concentrated under reduced pressure to afford aglycone. The neutral hydrolysate revealed the presence of glucuronic acid and/or glucose by HPTLC ($CHCl_3$-CH_3OH-H_2O, 6.5:3.5:1) when compared with authentic samples [Rf 0.42 (D-glucose) and 0.36 (D-glucuronic acid)].

3.7. In-vitro α-glucosidase inhibition

The enzyme inhibition assays were carried out with compounds **1–5** and crude gymnemic acids mixture and were compared with acarbose at doses ranging from 31.25–500 μg/ml as per the method given by Dong et al. (2012), with slight modifications. Briefly, a volume of 60 μl of sample solutions in DMSO of test samples or acarbose and 50 μl of 0.1 M phosphate buffer (pH 6.8) containing α-glucosidase solution (0.2 U/ml) was incubated in 96 well plate at 37 °C for 20 min. After pre-incubation, 50 μl of 5 mM *p*-nitrophenyl-α-D-glucopyranoside (PNPG) solution in 0.1 M phosphate buffer (pH 6.8) was added to each well and incubated again at 37 °C for another 20 min. Then the reaction was stopped by adding 160 μl of 0.2 M Na_2CO_3 into each well, and the absorbance (A) recorded at 405 nm by a micro-plate reader and compared to a control which had 60 μl of buffer solution in place of the extract. For blank incubation (to allow for absorbance produced by the extracts), the enzyme solution was replaced with a buffer solution and absorbance recorded. The α-glucosidase inhibitory activity was expressed as a percentage inhibition and was calculated as follows:

$$\%\ Inhibition = \frac{\text{A control} - (\text{A } test - A\ background)}{\text{A control}} \times 100$$

where $A_{control}$, A_{test}, $A_{background}$ are defined as the absorbance of 100% enzyme activity, test sample with the enzyme and test sample without the enzyme, respectively.

The concentration of inhibitors required for inhibiting 50% of enzyme activity under assay conditions was presented as IC_{50} value.

3.8. Antihyperglycemic activity

3.8.1. Animals

Wistar albino mice (30–50 g) were obtained from Central Animal Facility, Jamia Hamdard, New Delhi and maintained under controlled condition of illumination (12 h light/12 h darkness) and temperature 20–25 °C. They were housed under ideal laboratory conditions, maintained on standard pellet diet (Lipton rat feed Ltd., Pune, India) and water *ad-libitum* throughout the experimental period. Animals were acclimatized to the conditions before the start of experiments. The experimental study was approved by the Institutional Animal Ethics Committee (IAEC) of Jamia Hamdard, New Delhi, India (JH/CAHF/173/CPCSEA/28th January/2000, approval no. 2017/986). All the test compounds and the standard drugs were administered orally.

3.8.2. Oral sucrose tolerance test

The animals were randomly divided into six groups, each consisting of six mice (n = 6). The animals were fasted overnight before the start of the experiment. Group I served as normal control which received 1 ml/kg b.w. vehicle (0.5% CMC in distilled water). Group II served as sucrose challenged control that received sucrose (4 g/kg b.w.). Group III received acarbose as a standard drug (10 mg/kg b.w.). Groups IV, V and VI were administered crude gymnemic acid mixture, compound **1** and **4** (10 mg/kg, p.o., each), respectively. This was followed by sucrose challenge (4 g/kg, b.w.) 20 min after the treatment in Groups III-VI. Blood was withdrawn from the tail vein at 0, 30, 60, 90 and 120 min after the carbohydrate challenge and blood glucose level determined using a one-touch glucometer (myLifePura, Switzerland).

3.8.3. Statistical analysis

All data were presented as mean ± standard error of mean (SEM). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Dunnett's test (GraphPad® InStats version 3.06, USA). *P*-values less than 0.05 were considered to be significant.

Conflict of interest

The authors report no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.phytol.2019.05.005.

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