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Isolation of natural compounds from *Phlomis stewartii* showing α-glucosidase inhibitory activity

Bushra Jabeen [a], Naheed Riaz [a,*], Muhammad Saleem [a], Muhammad Akram Naveed [a], Muhammad Ashraf [b], Umber Alam [b], Hafiza Mehwish Rafiq [a], Rasool Bakhsh Tareen [c], Abdul Jabbar [a]

[a] Department of Chemistry, Baghdad-ul-Jadeed Campus, The Islamia University of Bahawalpur, Bahawalpur 63100, Pakistan
[b] Department of Biochemistry and Biotechnology, Baghdad-ul-Jadeed Campus, The Islamia University of Bahawalpur, Bahawalpur 63100, Pakistan
[c] Department of Botany, Baluchistan University Quetta, Quetta, Pakistan

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ABSTRACT

Stewartiiside (**1**), a phenylethanoid glycoside and three 28-nortriterpenoids: stewertiisins A–C [(17*R*)-19(18 → 17)-abeo-3α,18β,23,24-tetrahydroxy-28-norolean-12-ene, **2**; (17*R*)-19(18 → 17)-abeo-2α,16β,18β,23,24-pentahydroxy-28-norolean-12-en-3-one, **3**; (17*R*)-19(18 → 17)-abeo-2α,3α,23,24-tetrahydroxy-28-noroleane-11,13-diene, **4**] together with eight known compounds: lunariifolioside (**5**), notohamosin A (**6**), phlomispentanol (**7**), isorhamnetin 3-(6-*p*-coumaroyl)-β-D-glucopyranoside (**8**), tiliroside (**9**), caffeic acid (**10**), *p*-hydrxybenzoic acid (**11**) and oleanolic acid (**12**) were isolated from the ethyl acetate soluble fraction of the methanolic extract of whole plant of *Phlomis stewartii*. The structures of these isolates (**1**–**12**) were elucidated by the combination of 1D (^{1}H and ^{13}C NMR), 2D (HMQC, HMBC COSY, NOESY) NMR spectroscopy and mass spectrometry (EIMS, HREIMS, FABMS, HRFABMS) and in comparison with literature data of related compounds. All the isolates (**1**–**12**) showed α-glucosidase inhibitory activity with IC_{50} values ranging between 14.5 and 355.4 μM, whereas, compounds **1**, **5**, **9** and **10** showed promising α-glucosidase inhibitory activity with IC_{50} values below 30 μM.

1. Introduction

Phlomis is a large genus of the plant family Lamiaceae, having more than 100 species distributed throughout Euro-Asia and North Africa. The various species of this genus generally used as herbal tea against gastrointestinal troubles and to promote health by protecting liver, kidneys, bones and cardiovascular system (Carmona et al., 2005). Pharmacologically, some species are described to possess antidiabetic (Sarkhail et al., 2007), antinociceptive, antiulcerogenic, anti-inflammatory, antiallergic (Sarkhail et al., 2003), anticancer (Kirmizibekmez et al., 2004), antioxidant and antimicrobial properties (Morteza-Semnani et al., 2006). Literature survey revealed that monoterpenes, sesquiterpenes, aliphatic compounds, fatty acids, flavonoids, iridoids and phenylethyl alcohol have been isolated from the relatives of this genus (Amora et al., 2009), however, *Phlomis stewartii* has never been investigated before for its phytochemicals.

In the present study, the methanolic extract of the whole plant of *P. stewartii* showed inhibitory activity against enzyme α-glucosidase which was further present in ethyl acetate soluble fraction prompted us to carry out bioassay-guided studies on the said fraction. As a result of chromatographic purification of ethyl acetate part led to the isolation of a new phenylethanoid glycoside (**1**), three new 28-nortriterpenoids (**2**–**4**) along with eight known compounds (**5**–**12**) (Fig. 1).

2. Results and discussion

Compound **1** was obtained as a colorless amorphous powder. Its IR spectrum showed the presence of O–H (3438 cm^{-1}), conjugated C=O (1701 cm^{-1}) and aromatic system (1605, 1520, 1450 cm^{-1}). The molecular formula $C_{40}H_{55}O_{23}$ was determined by HRFABMS which showed molecular ion peak $[M+H]^+$ at *m/z* 903.3140 (calcd. for $C_{40}H_{55}O_{23}$, 903.3134). The ^{1}H NMR spectrum of **1** displayed well-separated eight signals in the aromatic region. Due to COSY spectrum and calculation of coupling constants, the three signals [δ 7.06 (1H, d, *J* = 2.0), 6.96 (1H, dd, *J* = 9.2, 2.0), 6.80 (1H, d, *J* = 9.2)] splitted at ABX pattern were attributed to a tri-substituted benzene ring, whereas, another set of three signals [δ 6.68 (1H, d, *J* = 8.0 Hz), 6.66 (1H, d, *J* = 2.0 Hz), 6.55 (1H, dd, *J* = 8.0, 2.0 Hz)] were identified for another aromatic moiety. Due to their coupling constants and chemicals shifts, the remaining two signals resonating at δ 7.60 (1H, d, *J* = 16.0), 6.28 (1H, d, *J* = 16.0) were attested for a conjugated *trans*-olefinic system. This data revealed that at least one caffeoyl moiety is present in **1**, which was further

* Corresponding author. Tel.: +92 3007815194.
E-mail addresses: nrch322@yahoo.com, nrch322@hotmail.com (N. Riaz).

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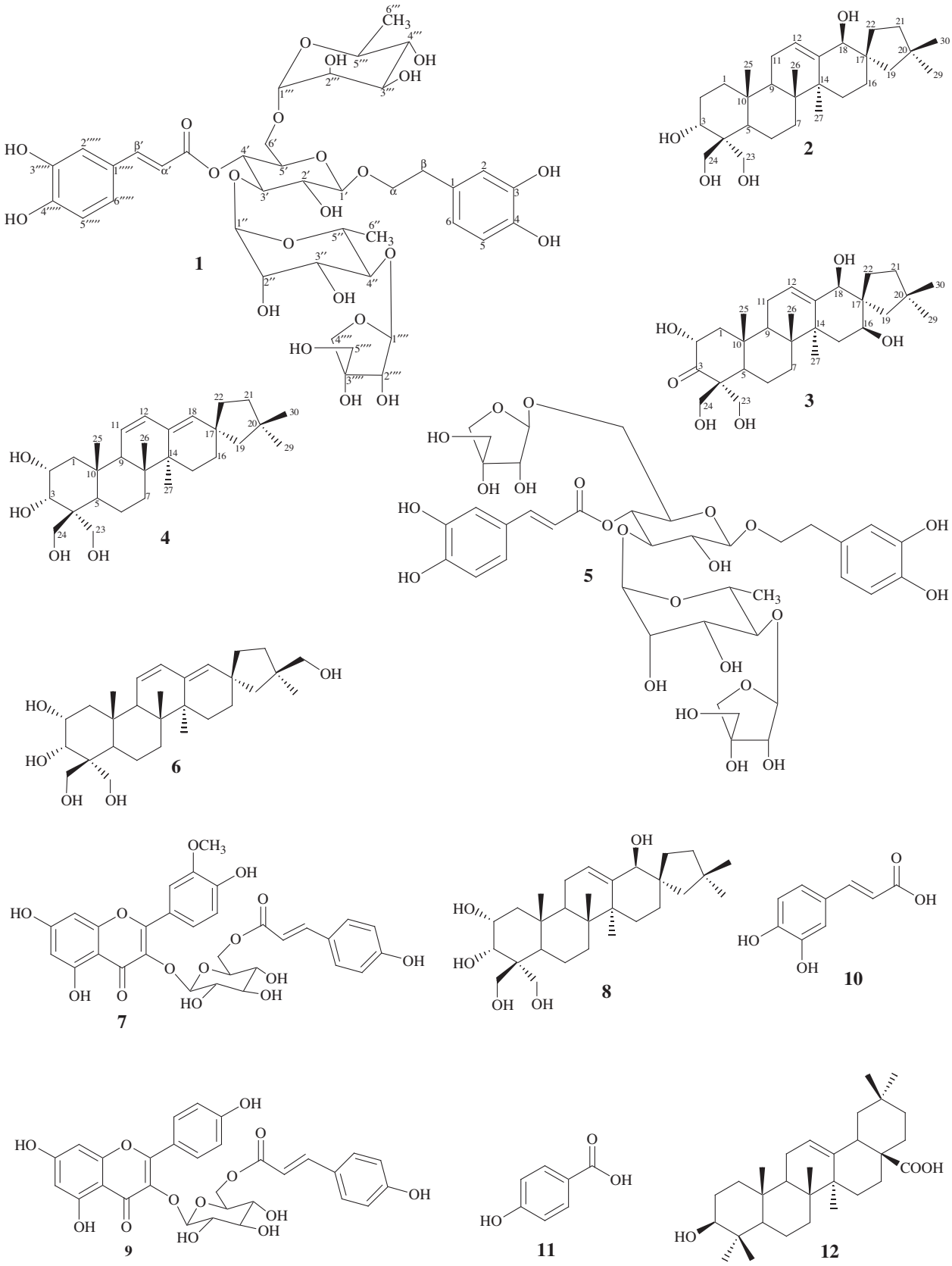

Fig. 1. Structures of compounds (**1**–**12**) isolated from *Phlomis stewartii*.

supported due to the carbon signals at δ 168.1, 149.8, 148.0, 146.0, 127.5, 123.4, 117.1, 116.3 and 116.2 in ^{13}C NMR spectrum.

Another set of COSY relatives resonated in ^{1}H NMR spectrum at δ 4.01 (2H, t, J = 7.0 Hz) and 2.80 (2H, t, J = 7.0 Hz) showed its

attachment with the aromatic system in HMBC spectrum of **1**. This information helped to identify a tyrosol moiety in **1**. Besides the above data, the ^{1}H NMR spectrum showed the presence of four sugar moieties as it afforded signals for four anomeric protons at δ 5.26 (1H, br s), 5.20 (1H, d, J = 6.8 Hz), 5.17 (1H, br s) and 4.36 (1H, d, J = 8.2 Hz) together with overlapped signals for oxymethylene and oxymethine at δ 3.30–4.10. The ^{13}C NMR spectra (BB and DEPT) of **1** was fully supportive of the mass and ^{1}H NMR information as it showed 40 carbon signals for two methyl, five methylene, 25 methine and eight quaternary carbon atoms. The four anomeric carbon signals were observed at δ 111.4, 104.1, 103.0 and 102.1. Careful analysis of both ^{1}H and ^{13}C NMR data for sugar moieties indicated the presence of a glucose unit, two rhamnose moieties and an apiose sugar in **1**.

The above spectral data showed close resemblance to the reported data for lunariifolioside (Calis and Kirmizibekmez, 2004) except the presence of a rhamnose instead of an apiose. All structural assignments were accomplished through interpretation of 2D NMR (COSY, HSQC, HMBC) spectroscopic data.

The acid hydrolysis of **1** provided a binary mixture of aglycones which could be separated and identified as caffeic acid and 3,4-dihydroxyphenylethanol, respectively from organic layer and glycones could be separated through preparative thin layer chromatography (PTLC) using EtOAc–MeOH–H_2O–HOAc; 4:2:2:2 as developing solvent and subsequently identified as D-glucose, L-rhamnose and D-apiose from aqueous layer through sign of their optical rotations and comparison of retention time of their trimethylsilyl (TMS) ethers with those of standards in gas chromatography (GC).

The substitutions and the linkages at various positions in **1** were finally confirmed by HMBC correlations in which the oxymethylene protons at δ 4.01 correlated with the carbon at δ 104.1 (C-1′) of the glucose moiety. The H-4′ (δ 4.90) of glucose moiety showed correlation with ester carbonyl (δ 168.1) confirmed the attachment of caffeolyl group at C-4′. The downfield shift of C-3′ (δ 81.6) of glucose and its correlation with anomeric proton of H-1″ (δ 5.17) of a rhamnose unit established their ether linkage. Further the downfield shift of C-6′ (δ 65.6) of glucose and the HMBC correlation its protons (δ 3.62, 3.28) with C-1‴ (δ 102.1) of other rhamnose moiety confirmed another ether linkage between glucose and the second rhamnose unit. The apiose sugar was fixed at C-4″ (δ 80.1) of the first rhamnose due to the HMBC correlation of H-4″ (δ 3.55) with C-1⁗ (δ 111.4) of apiose. Based on these evidences, the structure of **1** was established as 2-(3,4-dihydroxyphenyl)ethyl *O*-α-rhamnopyranosyl-(1 → 6)-*O*-[*O*-β-apiofuranosyl-(1 → 4)-α-rhamnopyranosyl-(1 → 3)]-4-*O*-(*E*)-caffeoyl-β-glucopyranoside and named as stewartiiside.

Compound **2** was isolated as white amorphous powder, whose IR spectrum exhibited absorption bands for hydroxyl (3410 cm^{-1}) and olefinic (1635 cm^{-1}) functions. The EIMS showed molecular ion at m/z 460, whereas, high resolution (HREIMS) analysis of the same ion (m/z 460.3550) depicted the molecular formula as $C_{29}H_{48}O_4$ with six double bond equivalent (DBE). The ^{1}H NMR spectrum of **2** displayed signals for five tertiary methyl (δ 1.13, 1.06, 1.00, 0.99 and 0.94), two oxygenated methylene [δ 3.83 (2H, br s), 3.85 and 3.75 (1H each, d, J = 10.0 Hz)], two oxygenated methine [δ 4.05 (1H, d, J = 2.0 Hz), 3.86 (1H, s)], a broad singlet olefinic methine (δ 5.72) along with several aliphatic methylenes and methines between δ 0.75 and 2.19. The resonance of two oxymethylenes clearly indicated that two tertiary methyl must have been oxidized to alcohols. The ^{13}C NMR spectra (BB and DEPT) of **2** supported the above data as it displayed 29 carbon resonances for five methyl (δ 30.4, 30.3, 23.4, 18.03, 18.02), 12 methylene (δ 67.3, 63.5, 52.8, 42.9, 42.2, 36.7, 35.1, 30.8, 29.5, 27.8, 24.5, 21.5), five methine (δ 119.8, 76.4, 72.5, 48.8, 45.6) and seven quaternary carbons (δ 142.5, 48.5, 45.3, 43.6, 41.3, 39.8, 38.5). The above spectral data closely related with the data of 28-norterpenoids reported from *Phlomis umbrosa* (Liu et al., 2008, 2007), especially the spiro quaternary carbon displayed its position at δ 43.6 and a methylene of cyclopentano-system appeared at δ 52.8 as a characteristic feature of such a system (Liu et al., 2007). The position of double bond at C-12 was confirmed due to RDA fragments at m/z 240.1730 ($C_{14}H_{24}O_3$) and 220.1830 ($C_{15}H_{24}O$) in HREIMS spectrum of **2**.

The HMBC correlation of two oxymethylens (δ 3.75, 3.85 H-24 and 3.83 H-23) with each other and with the carbons at δ 72.5 (C-3), 48.5 (C-4) and 45.6 (C-5) confirmed that geminal methyl at C-4 are oxidized to alcoholic groups. The HMBC correlation of H-12 with oxymethine resonated at δ 76.4 confirmed the presence of a hydroxyl function at C-18. The smaller coupling constant of H-3 (J = 2.0 Hz) and its NOESY correlation with OCH_2-24 (δ 3.75, 3.85) confirmed hydroxyl group at C-3 as axial and α in orientation. The geometry of OH at C-18 was confirmed as β and equatorial due to NOESY correlation of H-18 with Me-27 (δ 1.13) (Luo et al., 2003) and through molecular model. The above discussion led to the structure of **2** as (17*R*)-19(18 → 17)-abeo-3α,18β,23,24-tetrahydroxy-28-norolean-12-ene and named as stewertiisin A.

Compound **3** was also isolated as white amorphous powder. In addition to alcoholic and olefinic absorption bands, the IR spectrum afforded a strong band for carbonyl function at 1719 cm^{-1}. The molecular formula $C_{29}H_{46}O_6$ of **3** was deduced by HREIMS due to molecular ion peak at m/z 490.3290 with eight DBE. Most of the signals appeared in ^{1}H NMR spectrum of **3** were similar to the signals observed for **2** with few differences. The ^{1}H NMR spectrum of **3** showed three oxymethine protons at δ 3.80 (1H, dd, J = 9.2, 3.8), 3.55 (1H, s) and 3.35 (1H, br s) instead of two. The cleavage of ring C through RDA fragmentation resulted into fragments at m/z 254.1520 ($C_{14}H_{22}O_4$) and 236.1780 ($C_{15}H_{24}O_2$) indicated the presence of four oxygen atoms in ring A or B and two in ring D or E. The ^{13}C NMR spectra of **3** was also supportive of these differences as it displayed three oxymethines at δ 78.1, 76.2 and 67.0, whereas, the signal for a ketonic function appeared at δ 208.4. However, the spectrum afforded two methylene less when compared to that of the data of compound **2**. The HMBC correlation of two oxymethylene (δ 3.82, H-23 and 3.86, H-24) with the carbonyl carbon (δ 208.4) revealed that 3-OH has been oxidized to ketonic function. The HMBC correlations of H-5 (δ 1.58) with carbonyl carbon confirmed the above observation. Further the long range interaction of an oxymethine resonating at δ 3.80 (H-2) with carbonyl carbon and two quaternary carbons at δ 59.8 (C-4) and 38.8 (C-10) fixed one hydroxyl group at C-2, whereas, other two alcoholic functions were fixed at C-16 and C-18 due to the HMBC correlations of H-16 (δ 3.35) and H-18 (δ 3.55) with the spirocarbon (C-17). Further, H-16 exhibited HMBC interaction with C-18 (δ 76.2) and H-18 interacted with C-16 (δ 78.1) and C-12 (δ 119.0).

The relative stereochemistry at various chiral centers was established through NOESY spectrum, in which H-2 (δ 3.80) correlated with Me-25 (δ 1.10), H-16 (δ 3.35) with H-18 (δ 3.55) and Me-27 (δ 1.17) indicated the orientation of OH-2 as α and equatorial, whereas, OH-16 and OH-18 were placed as β and equatorial. These deductions were further substantiated with the help of molecular model. The above discussion and comparative study finally led to the structure of compound **3** as (17*R*)-19(18 → 17)-abeo-2α,16β,18β,23,24-pentahydroxy-28-norolean-12-ene-3-one, which is named as stewertiisin B.

Compound **4** was also found to be a spiro-nor-triterpenoid as most of its spectral data were comparable with that of compounds **2** and **3**. The notable difference was observed through UV spectrum that exhibited an absorption peak at 236 nm for a heterodienic system. The ^{1}H NMR spectrum also attested this information as it displayed three olefinic methines at δ 5.90 (1H, d, J = 8.0 Hz), 5.55 (1H, d, J = 8.0 Hz) and 5.36 (1H, s). Although, the ^{1}H NMR spectrum of **4** afforded signals for two oxymethines as were observed in **2**, but

they were found vicinal to each other at C-2 and C-3 due to their COSY correlations with each other and HMBC interaction with C-4 (δ 48.5). Further, H-3 (δ 3.83) was correlated in HMBC spectrum with C-4 and two oxymethylenes at δ 72.3 (C-23) and 62.3 (C-24).

The NMR data of **4** looked similar to the data reported for notohamosin A (Luo et al., 2003). The conjugated double bonds were fixed at C-11 and C-13 due to various HMBC interactions. The stereochemistry of 2-OH and 3-OH was established due to their coupling constants, molecular model and NOESY spectrum in which H-2 (δ 3.89) and H-3 (δ 3.83) were correlated with each other as well as with Me-24 (δ 3.78) and Me-25 (δ 1.02) confirming OH-2 as α and equatorial and OH-3 as α and axial in orientation. Finally, compound **4** was identified as (17*R*)-19(18 → 17)-abeo-2α,3α,23,24-tetrahydroxy-28-norolean-11,13-diene and named as stewertiisin C.

Structures of all the known compounds were established due to spectroscopic analysis and comparison with the reported values as lunariifolioside (**5**) (Calis and Kirmizibekmez, 2004), notohamosin A (**6**) (Luo et al., 2003), phlomispentanol (**7**) (Liu et al., 2008), isorhamnetin 3-(6-*p*-coumaroyl)-β-D-glucopyranoside (**8**) (Joua et al., 2004), tiliroside (**9**) (Kaouadji, 1990), caffeic acid (**10**) (Olennikov et al., 2012), *p*-hydrxybenzoic acid (**11**) (Aldrich Library, 1992a,b) and oleanolic acid (**12**) (Mousa et al., 1994) (Fig. 1).

2.1. α-Glucosidase inhibition of compounds 1–12

All the isolates **1**–**12** were evaluated for their enzyme inhibition activity against the yeast α-glucosidase. All the tested compounds exhibited some α-glucosidase inhibitory activity with IC_{50} values ranging between 14.5 ± 0.1 to 355.4 ± 0.9 μM (Table 1). Compounds **1**, **5**, **9** and **10** showed significantly better inhibitory potential (IC_{50} = 26.1 ± 0.3, 26.6 ± 0.2, 14.5 ± 0.1, 27.4 ± 0.2 μM, respectively) than the standard drug acarbose IC_{50} = 38.3 ± 0.1 μM. Tiliroside (**9**) was the most active (IC_{50} = 14.5 ± 0.1 μM) where as its methoxy derivative phlomispentanol (**7**) showed the least activity (IC_{50} > 500 μM), which indicats that the presence of hydroxyl group in ring C of **9** has an important role in enzyme inhibition. The activity of stewartiiside (**1**, IC_{50} = 26.1 ± 0.3 μM) is comparable with that of lunariifolioside (**5**, IC_{50} = 26.6 ± 0.2 μM), which revealed that the glycone part is not playing important role in enzyme inhibition. Stewertiisin A (**2**, IC_{50} = 38.0 ± 0.2 μM) was found to be a good inhibitor among other analogues (**3**, **4**, **6**, **8**). This indicates that 3-OH and 18-OH played important role in enzyme inhibition.

Table 1
α-Glucosidase inhibition of compounds **1**–**12**.

Compound	Inhibition (%)	Conc (mM)	IC_{50} (μM)
1	95.8 ± 0.3	0.5	26.1 ± 0.3
2	97.1 ± 0.8	0.5	38.0 ± 0.2
3	79.6 ± 0.4	0.5	315.8 ± 0.2
4	95.2 ± 1.6	0.5	355.4 ± 0.9
5	95.4 ± 0.3	0.5	26.6 ± 0.2
6	42.1 ± 1.0	0.5	318.5 ± 0.3
7	20.2 ± 0.75	0.5	>500
8	90.8 ± 1.3	0.5	305.2 ± 1.0
9	99.0 ± 0.7	0.5	14.5 ± 0.1
10	98.1 ± 0.2	0.5	27.4 ± 0.2
11	41.1 ± 0.1	0.5	297.7 ± 0.0
12	88.4 ± 0.1	0.5	231.3 ± 0.1
Methanolic extract**	80.2 ± 1.2	–	–
Acarbose	92.2 ± 0.1	0.5	38.3 ± 0.1

*All compounds were prepared in methanol with a concentration of 0.5 mM. All the measurements were done in triplicate and statistical analysis was performed by Microsoft Excel 2003. Results are presented as mean ± SEM.
**1.0 mg/ml methanolic extract/assay volume.

α-Glucosidase inhibitors (e.g., acarbose, miglitol, voglibose, 1-deoxynojirimycin) are widely used in the treatment of type-2 diabetes. These inhibitors delay the absorption of carbohydrates from the small intestine and thus have a lowering effect on postprandial blood glucose. These undigested oligosaccharides pass into the large intestine and cause the gastrointestinal disorders that are the most frequent adverse effect of α-glucosidase inhibitors (Alain, 1998; Floris et al., 2005). The inhibitors of α-glucosidase are also of interest due to their promising therapeutic potential against the diseases such as HIV infection, metastatic cancer, and lysosomal storage diseases. Amongst the natural α-glucosidase inhibitors, the metabolites like polyhydroxyacetylenes (Chan et al., 2010), biflavonoids (Zhou et al., 2010), stilbenes (Lam et al., 2008), triterpenoids (Wang et al., 2013) are comparably better α-glucosidase inhibitors than the standard acarbose. In the present studies, compounds **1**, **5**, **9** and **10** are also phenolics and showed promising α-glucosidase inhibitory activity with IC_{50} values lower than the standard acarbose. It has already been reported that anti-α-glucosidase activity is increased due to presence of phenolic chromophore (Lam et al., 2008). Therefore, the said compounds may be considered as potential candidates for the development of new anti-diabetic drugs in future.

3. Conclusion

One new phenylethanoid glycoside (**1**) and three new 28-nortriterpenoids: stewertiisins A–C (**2**–**4**), together with eight known alkaloids (**5**–**12**) were isolated from the ethyl acetate soluble fraction of the methanolic extract of *P. sterwartii*. Their structures were elucidated by intensive spectroscopic and spectrometric analyses. Previously, phenylethanoid glycoside (Calis and Kirmizibekmez, 2004) and 28-nortriterpenoids (Liu et al., 2008, 2007) were isolated from the genus *Philomis*. Nearly all the isolates exhibited good to moderate activity with IC_{50} values ranging between 14.5 and 355.4 μM. Compounds **1**, **5**, **9** and **10** showed better inhibitory potential (IC_{50} = 26.1, 26.6, 14.5, 27.4 μM, respectively) than the standard drug acarbose. Therefore, we can conclude that some of these α-glucosidase inhibitors obtained from *P. stewartii* may act as leads for the future development of anti-diabetic drugs.

4. Experimental

4.1. General experimental procedures

Optical rotations were measured on a JASCO DIP-360 polarimeter. UV spectra were obtained in methanol on U-3200 Schimadzu UV-240 spectrophotometer. Infrared (IR) spectra were recorded on Shimadzu 460 spectrometer. ^{1}H (400, 500 MHz), ^{13}C NMR (100, 125 MHz) and 2D NMR (HMQC, HMBC and COSY; 400, 500 MHz) spectra were recorded on Bruker spectrometer. The chemical shift values (δ) are reported in ppm and the coupling constant (*J*) are in Hz. EIMS and HREIMS were recorded on Finnigan (Varian MAT) JMS H × 110 with a data system and JMSA 500 mass spectrometers, respectively. Chromatographic separations were carried out using aluminium sheets pre-coated with silica gel 60 F_{254} (20 × 20 cm, 0.2 mm thick; E. Merck) for thin layer chromatography (TLC) and silica gel (230–400 mesh) for column chromatography. TLC (Thin Layer Chromatography) plates were visualized under UV at 254 and 366 nm and by spraying with ceric sulphate reagent solution (by heating).

4.2. Plant material

The whole plant material *P. stewartii* Hk. was collected from District Ziarat (Baluchistan) in September 2011 and was identified

by Prof. Dr. Rasool Bakhsh Tareen, Plant Taxonomist, Department of Botany, Baluchistan University Quetta, Quetta, Pakistan where a voucher specimen is deposited (PS/RBT-91/11).

4.3. Extraction and isolation

The shade-dried whole plant material of *P. stewartii* (10 kg) was extracted thrice with methanol (3 × 30 L) at room temperature. The crude methanolic extract (670 gm) was suspended in water and extracted with *n*-hexane and ethyl acetate. The ethyl acetate soluble fraction (70 gm) was subjected to column chromatography over silica gel using *n*-hexane/EtOAc, EtOAc, EtOAc/MeOH and MeOH as eluent resulted into six fractions E_1–E_6. The fractions E_1 (1.5 gm) on gradient elusion using 40% EtOAc in *n*-hexane to get oleanolic acid (**12**) and *p*-hydroxybenzoic acid (**11**) from the head and tail fractions, respectively. The fraction E_2 (1.7 gm) on gradient elusion using 60% EtOAc in *n*-hexane to get caffeic acid (**10**). The fraction E_3 (3.5 gm) on gradient elusion using 5% MeOH in EtOA to get tiliroside (**9**) and isorhamnetin-3-glycoside (**8**), respectively. The fraction E_4 (2.9 gm) on gradient elusion using 10% MeOH in EtOA to get notohamosin A (**6**) and stewertiisin B (**3**), respectively. The fraction E_5 (1.7 gm) on gradient elusion using 15% MeOH in EtOA to get stewertiisin A (**2**), stewertiisin B (**3**) and phlomispentanol (**7**) from the head, middle and tail fractions, respectively. The fraction E_6 (3.9 gm) on gradient elusion using 25% MeOH in EtOA to get stewartiiside (**1**) and lunariifolioside (**5**), respectively.

4.3.1. Stewartiiside (*1*)

Pale yellow amorphous powder (48 mg); $[\alpha]_D^{25}$ − 80.5° (*c* = 0.0012, MeOH); UV (CH_3OH) λ_{max} nm (log ε): 219 (3.01), 328 (3.09); IR (KBr) ν_{max} cm^{-1}: 3438, 1701, 1605,1520, 1450; ^1H (500 MHz, CD_3OD): 7.60 (1H, d, *J* = 16.0 Hz, H-β′), 7.06 (1H, d, *J* = 2.0 Hz, H-2′′′′′), 6.96 (1H, dd, *J* = 9.2, 2.0 Hz, H-6′′′′′), 6.80 (1H, d, *J* = 9.2 Hz, H-5′′′′′), 6.68 (1H, d, *J* = 8.0 Hz, H-5), 6.66 (1H, d, *J* = 2.0 Hz, H-2), 6.55 (1H, dd, *J* = 8.0, 2.0 Hz, H-6), 6.28 (1H, d, *J* = 16.0 Hz, H-α′), 5.26 (1H, br s, H-1′′′), 5.20 (1H, d, *J* = 6.8 Hz, H-1′′′′), 5.17 (1H, br s, H-1′′), 4.90 (1H, t, *J* = 8.2 Hz, H-4′), 4.36 (1H, d, *J* = 8.2 Hz, H-1′), 4.01 (2H, t, *J* = 7.0 Hz, H-α), 3.90 (1H, m, H-2′′), 3.89 (1H, m, H-3′′′), 3.82 (1H, m, H-2′′′), 3.79 (1H, t, *J* = 8.2 Hz, H-3′), 3.71 (1H, m, H-5′,3′′), 3.70 (1H, s, H-4′′′′), 3.65 (1H, m, H-6′), 3.62 (1H, d, *J* = 6.8 Hz, H-2′′′′), 3.61 (1H, m, H-4′′′), 3.55 (1H, m, H-4′′), 3.54 (1H, m, H-5′′′), 3.48 (1H, m, H-5′′), 3.37 (1H, t, *J* = 8.2 Hz, H-2′), 3.28 (2H, s, H-5′′′′), 3.26 (1H, m, H-6′), 2.80 (2H, t, *J* = 7.0 Hz, H-β), 1.11 (3H, d, *J* = 6.4 Hz, H-6′′), 1.07 (3H, d, *J* = 6.2 Hz, H-6′′′); ^{13}C NMR (125 MHz, CD_3OD): 168.1 (C═O), 149.8 (C-4′′′′), 148.0 (C-β′), 146.8 (C-3), 146.0 (C-3′′′′), 144.6 (C-4), 131.5 (C-1), 127.5 (C-1′′′′), 123.4 (C-6′′′′), 121.3 (C-6), 117.1 (C-5′′′′), 116.5 (C-2), 116.3 (C-α′), 116.2 (C-2′′′′), 114.6 (C-5), 111.4 (C-1′′′), 104.1 (C-1′), 103.0 (C-1′′), 102.1 (C-1′′′), 81.6 (C-3′), 80.6 (C-3′′′), 80.1 (C-4′′), 78.6 (C-2′′′), 76.1 (C-2′), 75.1 (C-4′′′), 75.0 (C-5′), 74.6 (C-4′′′), 72.5 (C-α), 72.3 (C-2′′,2′′′), 72.1 (C-3′′′), 72.0 (C-3′′), 70.4 (C-5′′′), 70.1 (C-4′), 68.7 (C-5′′), 65.6 (C-6′,5′′′), 36.5 (C-β), 18.7 (C-6′′′), 18.4 (C-6′′); HRFABMS *m*/*z*: 903.3140 $[M+H]^+$ (calcd. for $C_{40}H_{55}O_{23}$, 903.3134).

4.3.2. Stewertiisin A [(17R)-19(18 → 17)-abeo-3α,18β,23,24-tetrahydroxy-28-norolean-12-ene; *2*]

Amorphous white powder (40 mg); $[\alpha]_D^{25}$ + 42.6 (*c* = 0.0011, MeOH); IR (KBr) ν_{max} cm^{-1}: 3410, 1635, 1378, 1034; ^1H (500 MHz, CD_3OD): 5.72 (1H, br s, H-12), 4.05 (1H, d, *J* = 2.0 Hz, H-3), 3.86 (1H, s, H-18), 3.85 (1H, d, *J* = 10.0 Hz, H-24), 3.83 (2H, br s, H-23), 3.75 (1H, d, *J* = 10.0 Hz, H-24), 1.99 (2H, m, H-11), 1.96 (1H, d, *J* = 12.2 Hz, H-19), 1.15 (1H, d, *J* = 12.2 Hz, H-19), 1.13 (3H, s, H-27), 1.06 (3H, s, H-25), 1.00 (3H, s, H-29), 0.99 (3H, s, H-30), 0.94 (3H, s, H-26); ^{13}C NMR (125 MHz, CD_3OD): 142.5 (C-13), 119.8 (C-12), 76.4 (C-18), 72.5 (C-3), 67.3 (C-23), 63.5 (C-24), 52.8 (C-19), 48.8 (C-9), 48.5 (C-4), 45.6 (C-5), 45.3 (C-14), 43.6 (C-17), 42.9 (C-21), 42.2 (C-1), 41.3 (C-8), 39.8 (C-20), 38.5 (C-10), 36.7 (C-16), 35.1 (C-7), 30.8 (C-22), 30.4 (C-29), 30.3 (C-30), 29.5 (C-2), 27.8 (C-15), 24.5 (C-11), 23.4 (C-27), 21.5 (C-6), 18.03 (C-26), 18.02 (C-25); HREIMS *m*/*z*: 460.3550 $[M]^+$ (calcd. for $C_{29}H_{48}O_4$, 460.3545).

4.3.3. Stewertiisin B [(17R)-19(18 → 17)-abeo-2α,16β,18β,23,24-pentahydroxy-28-norolean-12-ene-3-one; *3*]

Amorphous white powder (37 mg); $[\alpha]_D^{25}$ − 52.6 (*c* = 0.001, MeOH); IR (KBr) ν_{max} cm^{-1}: 3410, 1719, 1635; ^1H (500 MHz, CD_3OD): 5.78 (1H, s, H-12), 3.86 (2H, s, H-24), 3.82 (2H, br s, H-23), 3.80 (1H, dd, *J* = 9.2, 3.8 Hz, H-2), 3.55 (1H, s, H-18), 3.35 (1H, br s, H-16), 2.0 (2H, d, *J* = 10.0 Hz, H-11), 1.17 (3H, s, H-27), 1.12 (2H, m, H-19), 1.10 (3H, s, H-25), 1.07 (3H, s, H-29), 1.02 (3H, s, H-30) 0.98 (3H, s, H-26); ^{13}C NMR (125 MHz, CD_3OD): 208.4 (C-3), 143.6 (C-13), 119.0 (C-12), 78.1 (C-16), 76.2 (C-18), 67.0 (C-2), 66.2 (C-23), 62.2 (C-24), 59.8 (C-4), 53.0 (C-19), 48.2 (C-5), 45.2 (C-14), 44.1 (C-17), 43.6 (C-9), 43.0 (C-1), 42.2 (C-21), 40.2 (C-8), 38.8 (C-10), 35.1 (C-7), 30.8 (C-22), 30.5 (C-20), 30.3 (C-30), 28.1 (C-15), 26.5 (C-27), 24.9 (C-11), 19.1 (C-6), 18.1 (C-25), 17.6 (C-29), 17.5 (C-26); HREIMS *m*/*z*: 490.3290 $[M]^+$ (calcd. for $C_{29}H_{46}O_6$, 490.3285).

4.3.4. Stewertiisin C [(17R)-19(18 → 17)-abeo-2α,3α,23,24-tetrahydroxy-28-norolean-11,13-diene; *4*]

Amorphous white powder (51 mg); $[\alpha]_D^{25}$ − 50.1 (*c* = 0.0015, MeOH); UV (CH_3OH) λ_{max} nm: 236 (1.6); IR (KBr) ν_{max} cm^{-1}: 3415, 1635, 1035; ^1H (500 MHz, CD_3OD): 5.90 (1H, d, *J* = 8.0 Hz, H-12), 5.55 (1H, d, *J* = 8.0 Hz, H-11), 4.30 (1H, d, *J* = 10.0 Hz, H-23), 4.00 (1H, d, *J* = 10.0 Hz, H-23), 3.89 (1H, m, H-2), 3.83 (1H, br s, H-3), 3.78 (2H, br s, H-24), 2.14 (1H, d, *J* = 9.0 Hz, H-9), 1.51 (1H, m, H-19), 1.36 (3H, s, H-30), 1.35 (3H, s, H-29), 1.33 (1H, m, H-19), 1.02 (3H, s, H-25), 0.94 (3H, s, H-27), 0.75 (3H, s, H-26); ^{13}C NMR (125 MHz, CD_3OD): 139.7 (C-13), 137.5 (C-18), 131.7 (C-12), 126.3 (C-11), 72.3 (C-23), 72.0 (C-3), 66.9 (C-2), 62.3 (C-24), 57.0 (C-19), 55.7 (C-9), 48.5 (C-4), 47.0 (C-5), 45.5 (C-17), 42.4 (C-1), 41.8 (C-8), 41.4 (C-21), 41.1 (C-14), 40.5 (C-22), 40.2 (C-20), 39.0 (C-10), 34.3 (C-16), 33.5 (C-7), 31.4 (C-29), 30.7 (C-30), 27.2 (C-15), 20.4 (C-27), 20.3 (C-6), 19.4 (C-25), 17.0 (C-26); HREIMS *m*/*z*: 458.3027 $[M]^+$ (calcd. for $C_{29}H_{46}O_4$, 458.3081).

4.4. Acid hydrolysis of compound *1*

Compound **1** (8 mg) was dissolved in 2 ml 2 N HCl and heated at 100 °C for 3 h, cooled and extracted with EtOAc two times (each 8 ml). The aqueous phases were concentrated under reduced pressure and subjected to preparative thin layer chromatography using solvent system (EtOAc–MeOH–H_2O–HOAc; 4:2:2:2) and the sugars were identified as glucose, rhamnose and apiose by comparing signs of their optical rotation {$[\alpha]_D^{20}$ + 51.0° (*c* = 0.001), $[\alpha]_D^{20}$ + 8.0° (*c* = 0.0012), $[\alpha]_D^{20}$ + 4.0° (*c* = 0.0011)}, respectively. It was also confirmed by comparison of the retention time of their TMS ethers in GC (D-glucose 7.8 min, L-rhamnose 8.6 min, D-apiose 5.7 min) with the standards.

4.5. α-Glucosidase inhibition assay

The α-glucosidase inhibition assay was performed with slight modifications as done by Pierre et al. (1978). Total volume of 100 μL reaction mixture contained 70 μL 50 mM phosphate buffer, pH 6.8, 10 μL (0.5 mM) test compound, followed by the addition of 10 μL (0.0234 units, Sigma Inc.) enzyme. The contents were mixed, preincubated for 10 min at 37 °C and pre-read at 400 nm. The reaction was initiated by the addition of 10 μL of 0.5 mM substrate (*p*-nitrophenyl glucopyranoside, Sigma Inc.). After 30 min of incubation at 37 °C, absorbance of the yellow color produced due to the formation of *p*-nitrophenol was measured at 400 nm using Synergy HT (BioTek, USA) using 96-well microplate reader.

Acarbose was used as positive control. The percent inhibition was calculated by the following equation

$$\text{Inhibition } (\%) = (\text{abs of control} - \text{abs of test}/\text{abs of control}) \times 100$$

IC_{50} values were calculated using EZ-Fit Enzyme Kinetics Software (Perrella Scientific Inc. Amherst, USA).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2013.09.015.

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