New Steroidal Saponins from Rhizomes of *Trillium govanianum*: Gram Scale Isolation and Acetylcholinesterase Inhibitory Activity Evaluation

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Abstract

Three previously unknown steroidal saponins named as govanoside C-E (**1**-**3**) along with four known compounds, govanoside B (**4**), protodioscin (**5**), 20*β*-hydroxyecdysone (**6**), and polypodine B (**7**) have been isolated from the rhizomes of *Trillium govanianum* Wall. ex D.Don. The structures of isolated compounds were elucidated by detailed analysis of 1D and 2D NMR, mass and IR spectroscopic data. Compounds **1** and **2** contained a rare sugar moiety *i.e.* 6-deoxy allose, while compound **3** has acetylated rhamnose moiety in its glycone part. Acid hydrolysis of new compounds followed by derivatization for GC analysis of glycone moieties was carried out for the confirmation of monomer saccharides present in each molecule. In addition, we have developed a protocol for the isolation of major steroidal saponin present in the rhizomes of *Trillium govanianum i.e.* borassoside E in gram scale. Parent extract, fractions and all pure molecules were screened to evaluate their antagonistic effects on acetylcholinesterase activity. Among extract and fractions, water fraction (IC_{50} value: 90.2 μg/ mL) was found most active whereas among pure molecules govanoside E (**3**) (IC_{50} value: 8.62 μM) was found most active against acetylcholinesterase. The molecular docking analysis was also carried out to further study the molecular interactions and binding free energy of the pure molecules with acetylcholinesterase.

Keywords: *Trillium govanianum*, steroidal saponins, acetylcholinesterase, molecular docking, enrichment protocol.

1. Introduction

Trillium govanianum Wall. ex D.Don commonly known as "Nag Chhatri" (Melanthiaceae) has been traditionally utilized as Ayurvedic medicine for treating inflammation and pain related ailments (Sharma et al., 2014). The scatter distribution of this herb has been observed at 2500-4000 m altitude of the Himalaya ranging from Hindu Kush mountain ranges of Afganisthna to the North-Eastern part of India (Singh et al., 2017). This plant has not been much phytochemically explored for its pure constituents, as till date merely thirteen molecules are reported (Patil et al., 2021a; Bora et al., 2021). Earlier phytochemical studies reveled that steroidal saponins are the principle phytoconstituents of this plant (Rahman et al., 2015; Singh et al., 2020a). Recently, we also reported that borassoside E, protodioscin, and govanoside B are the three major steroidal saponins in the rhizomes of *T. govanianum* (Singh et al., 2020b). Most of the bioactivity studies on this plant have been carried out with the extract and fractions including anti-inflammatory (Rahman et al., 2016), anticancer (Khan et al., 2016), anti-fertility (Sharma et al., 2018), anti-leishmanial (Khan et al., 2018), antioxidant (Kundra et al., 2020), anti-microbial (Verma et al., 2021), antidiarrheal and antispasmodic (Muhammad et al., 2021). Few reports are documented on the bioactivities evaluation of pure compounds, which includes antifungal (Rahman et al., 2015), insecticidal (Dolma et al., 2021), pro-diabetic enzymes inhibitory (Patil et al., 2021b), and anti-inflammatory (Patil et al., 2021c) activities.

In continuation of our study on phytochemical and pharmacological investigation of *T. govanianum* (Singh et al., 2020a and 2020b; Dolma et al., 2021; Patil et al., 2021b and 2021c) we here with report isolation and characterization of three previously unknown steroidal saponins named as govanoside C (**1**), govanoside D (**2**), and govanoside E (**3**) along with four known compounds, govanoside B (**4**), protodioscin (**5**), 20β-hydroxyecdysone (**6**), and polypodine B (**7**) from the rhizomes of *Trillium govanianum*. The parent extract, fractions, and all pure molecules were *in vitro* evaluated for their antagonistic effects on acetylcholinesterase activity. Molecular docking study was also carried out to analyze the molecular interactions and binding free energy of the pure molecules with acetylcholinesterase. In addition, we have also developed a simple protocol for the gram scale isolation of major compound present in this plant *i.e.* borassoside E with >98% purity.

2. Result and Discussion

2.1 Isolation and characterization of phytomolecules

The water and *n*-butanol fraction was subjected to column chromatography to afford govanoside C (**1**), govanoside D (**2**), govanoside B (**4**), and protodioscin (**5**) from the water fraction, whereas govanoside E (**3**), 20*β*-hydroxyecdysone (**6**), and polypodine B (**7**) were isolated from the *n*-butanol fraction (Fig. 1). The mono-saccharides present in the glycone moieties were confirmed by NMR and then acid hydrolysis followed by GC analysis.

Compound **1** was isolated as a white amorphous powder with specific rotation $[\alpha]^{20}_{D}$= -35.6º (c = 0.003, CH_3OH). The molecular formula for the compound **1** was determined as $C_{50}H_{78}O_{24}$ from its observed sodiated molecular ion peak at *m/z* 1085.4768 $[M+Na]^+$ (calculated for $C_{50}H_{78}O_{24}Na^+$ as 1085.4775) in the HR-ESI-MS spectra (Fig. S11). IR spectrum of compound **1** (Fig. S10) exhibited bands at υ_{max} 3358.07 cm^{-1} (hydroxy group), 2899.01 cm^{-1} (C-H group), 1643.35 cm^{-1} (olefinic group), and 1035.77 cm^{-1} (alcoholic C-O group). ^{1}H NMR spectrum of compound **1** (Fig. S1) exhibited characteristic signals for two methyl groups at δ_H 0.93 (3H, s) and 1.09 (3H, s), one endo-cyclic olefinic proton at δ_H 5.56 (1H, d, *J* = 5.8 Hz), two exo-cyclic methylene proton at δ_H 4.99 (1H, br s) and δ_H 5.10 (1H, br s), respectively. Also the ^{13}C NMR spectrum of compound **1** (Fig. S2) exhibited a total of fifty signals: twenty-seven signals correspond to the aglycone portion and remaining twenty-three signals correspond to the glycone part. Comparison of ^{13}C with DEPT-135 spectrum (Fig. S3) revealed the presence of four methyl, eleven methylene, thirty methine, and five quaternary carbon signals.

The aglycon moiety was confirmed by the hetero-nuclear correlations (HSQC, HMBC) and homo-nuclear correlations (COSY). The olefinic proton at 5.56 (1H, d, *J*= 5.8Hz) showed the HSQC correlation with δ_C 126.0 (C-6) (Fig. S6), and HMBC correlations (Fig. S5) with δ_C 32.6 (C-7), δ_C 34.1 (C-8), and δ_C 43.3 (C-10). The proton signal at δ_H 3.50 (H-1) showed the HMBC correlation with δ_C 43.3 (C-10), δ_C 15.3 (C-19) δ_C 69.1 (C-3), and δ_H 2.08 (H-2) with δ_C 84.7 (C-1), δ_C 69.1 (C-3), δ_C 43.4 (C-4) (Fig. 2). Also the positions of protons were confirmed by COSY ^{1}H-^{1}H correlations for δ_H 3.50 (H-1) with 1.73 (H-2a)/δ_H 2.08 (H-2b), and δ_H 3.37 (H-3) with δ_H 2.21 (H-4a)/δ_H 2.23 (H-4b), respectively (Fig. 2). The downfield shifts in the values at δ_C 84.7 (C-1); δ_H3.50 (H-1) and δ_C 69.1 (C-3); δ_H 3.37 (H-3) indicated the presence of hydroxyl or glycosidic substitutions at these positions. The quaternary carbon at δ_C 111.9 (C-22) showed the HMBC correlation with H-21 (δ_H 3.52), H-23 (δ_H 3.74), and H-24 (δ_H 4.29). The deshielded value at these positions indicated the presence of hydroxyl groups at C-21 (δ_C 62.8), C-23 (δ_C 72.1), and C-24 (δ_C 83.3). The two singlet protons at δ_H 4.99 (H-27a) and δ_H 5.10 (H-27b, CH_2) correlating with 114.0 in HSQC and with δ_C 83.3 (C-24), δ_C144.4 (C-25), δ_C 62.1 (C-26) in

HMBC indicated the presence of exo-olefin bond at C-25 position (Fig. 2). Similarly, the relative configuration and orientation of the aglycone moiety was determined by homo-nuclear NOE correlations. The relative configuration of aglycone was assigned with respect to H-3, whose *β* -orientation was confirmed after literature analysis (Mimaki et al., 2008). The NOE correlations for H-1/H-3 and H-1/H-9 indicated that both the hydroxyl groups at C-1 and C-3 positions are *β*-orientated. Similarly, the orientation of methyl groups was determined by their NOE correlations with H-8 proton, which confirmed that both methyls are *β*-orientated as no NOE correlation was observed between H-1, H-9 with H-19. Similarly, the NOE correlations between H-9/H-14, H-14/H-17, and H-16/H-17 (Fig. 2) confirmed that ring fusion for B/C and C/D rings is *trans* and for D/E is *cis*, respectively (Mimaki et al., 2008, Rahman et al., 2015). The *S*-configuration of hydroxyl groups at C-23 and C-24 was determined by NOE correlations between H-20/H-23 and H-23/H-24 (Mimaki et al., 2008, Rahman et al., 2015, Singh et al., 2020a).

After analysis of ^{1}H and ^{13}C NMR spectrum of compound **1** and on comparison with literature, the aglycone moiety structure was assigned as 1*β*,3*β*-(23S,24S)-spirosta-5,25-(27)-diene-1,3,21,23,24- pentol (Mimaki et al., 2008, Rahman et al., 2015, Singh et al., 2020a).

Moreover, the presence of four anomeric protons at δ_H 5.41(1H, br s), δ_H 4.74 (d, J = 8.2 Hz, 1H), δ_H 4.38 (t, J = 7.8 Hz, 2H) along with two methyl groups at δ_H 1.25 (3H, overlapped), and δ_H 1.12 (3H, d, J = 6.6 Hz) in the ^{1}H spectrum of compound **1** indicated the presence of four monosaccharide units including two deoxy-hexose sugars. In the ^{13}C NMR resonances, four anomeric carbon signals were observed at δ_c 105.3, δ_c 103.3, δ_c 101.6, and δ_c 100.3.

The attachment of different sugar moieties and their respective anomeric proton-carbon correlations were determined by hetero-nuclear single quantum correlation (HSQC) and hetero-nuclear multiple bond correlations (HMBC). The overlapped anomeric protons at δ_H 4.38 (t, J = 7.8 Hz, 2H) showed two HSQC correlation with δ_c 100.3 (C-1') and δ_c 105.3(C-1'''). Similarly, their HMBC correlation with δ_C 84.7 (C-1) and δ_C 76.9 (C-2'), indicated the attachment of these sugars at C-1 and C-2' positions, respectively**.** The HMBC correlation of anomeric proton at δ_H 4.74 (H-1'''') with the δ_C 83.3 (C-24) indicating the linkage of the sugar at the C-24 position of the aglycone moiety. Furthermore, the remaining anomeric proton at δ_H 5.41 (1H, br s) was correlated with δ_C 88.9 (C-3') in HMBC, confirming the linkage with C-3'. The spectral data analysis of compound **1** also revealed that it contains 6-deoxy-*β*-D-allose which is a C-3 epimer of 6-deoxy-*β*-D-glucose, linked at C-24 position of aglycone moiety. The presence of 6-deoxy-*β*-

D-allose was confirmed by 1D-TOCSY (Fig. S8), 1D-NOE (Fig. S9) spectrum analysis and by determining their coupling constant values. The characteristic triplet at H-3'''' (δ_H 3.99) and its low coupling value (J = 3.4 Hz) with respect to H-2'''' (δ_H 3.67, dd, J = 8.3, 3.3 Hz) and H-4'''' (δ_H 3.42 (overlapped)) indicated its *cis*-conformation (Lenherr et al., 1987). However, in case of glucose, H-3 conformation observed to be *trans* having coupling constant value >7 Hz (Duus et al, 2000). The *cis* conformation was further supported by 1D NOE correlations of H-3'''' proton at δ_H 3.99 with δ_H 3.67 (H-2''''), δ_H 3.42 (H-4'''') and δ_H 3.90 (H-5''''), respectively. Further the presence of tertiary methyl at H-6'''' (δ_H 1.12, d, J = 6.6 Hz) confirmed it as a deoxy-hexose sugar (Kobayashi et al., 1992).

The orientation of the sugars moieties was determined by their characteristic anomeric proton values and their respective coupling constants. The high coupling constant values (J > 7 Hz) for three anomeric protons confirmed β-orientated sugars. Similarly, the broad singlet peak at δ_H 5.41 (1H, br s) confirmed the presence of one α-oriented sugar (Duus et al., 2000). The configurations of the sugars were identified as β-D-6-deoxy-allopyranoside, α-L-rhamnopyranosyl, β-D-xylopyranosyl, and β-D-glucopyranosyl. The presence of these moieties were further supported by ESI-MS/MS spectrum of compound **1** which showed the fragment ion peaks at *m/z* 1085 $(M+Na)^+$, 953 $(M+Na\text{-}xyl)^+$, 807 $(M+Na\text{-}xyl\text{-}rha)^+$ and 663 $(M+Na\text{-}xyl\text{-}rha\text{-}glu+H_2O)^+$ due to the continuous breakdown of sugar units, respectively.

Moreover, the identity of α-L-rhamnopyranosyl, β-D-xylopyranosyl, and β-D-glucopyranosyl were also confirmed by acid hydrolysis followed by derivatization, GC analysis and comparison of their retention time with derivatized standards. While β-D-6-deoxy-allopyranoside was confirmed based on 1D-TOCSY (Fig. S8) and 1D-NOE (Fig. S9) spectral analysis and coupling constant values. Based on detailed analysis of observed data from NMR (1D & 2D), HR-ESI-MS, FT- IR and GC analysis of compound **1,** its structure was assigned as 24-*O*-β-D-6-deoxy-allopyranoside-1β,3β-(23S,24S)-3,21,23-trihydroxyspirosta-5,25-dienyl-*O*-α-L-rhamnopyranosyl -(1→2)-[β-D-xylopyranosyl-(1→3)]-β-D-glucopyranosyl and named as govanoside C (Fig. 1).

Compound **2** was also obtained as white amorphous powder with specific rotation $[\alpha]^{20}_D$= -26.0 (c = 0.003, CH_3OH). Its molecular formula was established as $C_{61}H_{96}O_{33}$ based on its observed ion peak at *m/z* 1379.5735 $[M+Na]^+$ (calculated for $C_{61}H_{96}O_{33}Na^+$ as 1379.5726) in the HR-ESI-MS (Fig. S23). IR spectrum analysis exhibited the presence of hydroxyl group at υ_{max} 3350.35 cm^{-1}, C-H stretching at 2889.37 cm^{-1}, olefinic group at υ_{max}1637.56 cm^{-1}, and alcoholic C-O group at υ_{max} 1035.77 cm^{-1} (Fig. S22). The analysis and comparison of the ^1H (Fig. S14),

^{13}C (Fig. S15), and DEPT (Fig. S16) spectrum of compound **2** with compound **1** indicated that compound **2** is almost super imposable to compound **1** (Mimaki et al., 2008, Rahman et al., 2015, Singh et al., 2020a), but the major differences were observed in the E ring of the aglycone and the number of sugar units in its glycon portion. The downfield shift observed for C-21 (from 62.8 ppm in compound **1** to 70.8 ppm in compound **2**) and upfield shift for C-20 (from 46.5 ppm to 44.2 ppm) suggested an extra glycosylation at C-21 of compound **2**.

Compound **2** exhibited six anomeric protons at δ_H 5.38 (1H, br s), δ_H 5.19 (d, J = 2.9 Hz, 1H) δ_H 4.74 (1H, d, J = 8.3 Hz), δ_H 4.40 (2H, d, J = 7.6 Hz), δ_H 4.20 (d, J = 7.3 Hz, 1H) and two tertiary methyl groups at δ_H 1.12 (3H, d, J = 6.5 Hz) and 1.25 (3H, d, J = 6.1 Hz), which indicated the presence of six monosaccharide units including two deoxy-hexose sugars. Similarly, the ^{13}C spectrum exhibited six anomeric carbons at δ_c 111.9, 105.4, 105.2, 103.2, 101.5, and 100.3. The linkages of sugars were determined after the complete analysis of HMBC correlations. The HMBC correlation of anomeric protons at δ_H 4.40, 2H (H-1', H-1'''') with 84.8 (C-1) and 88.6 (C-3') confirmed the attachment of two sugars at C-1 and C-3' positions in the structure. Similarly, the major HMBC correlations of anomeric protons at δ_H 5.38 and δ_H 4.74 with 77.1 (C-2') and 83.4 (C-24) established their attachments at these positions. The attachments of these four sugars were similar as in compound **1**. Two extra anomeric protons at δ_H 4.20 and δ_H 5.19 in the spectrum of compound **2** suggested the presence of a total six sugars as compared to compound **1**.

The HMBC correlation of anomeric proton at δ_H 4.20 (H-1'''') with the hydroxy-methylene carbon at δ_C 70.8 (C-21) suggested the glycosylation at C-21 position, which was further supported by the deshielded value at C-21 position. One major difference in the structure of compound **2** is the presence of β-D-apiofuranosyl linked at C-3'' position, which was confirmed by the HMBC correlation of anomeric proton at δ_H 5.19 with δ_C 80.32 (C-3'') and vice-versa (Rahman et al., 2015). The presence of β-D-apiofuranosyl was confirmed by its characteristic signals at δ_C 111.9 (C-1''') correlating with δ_H 5.19 (d, J = 2.9 Hz, 1H), one methylene signal at δ_C 65.4 (C-3''') correlating with δ_H 3.61 s (2H) in HSQC and one quaternary carbon at δ_C 80.30 (C-4''') (Rahman et al., 2015).

The coupling constant values for anomeric protons at δ_H 4.74 (1H, d, J = 8.3 Hz), δ_H 4.40 (2H, d, J = 7.6 Hz), δ_H 4.20 (d, J = 7.3 Hz, 1H) and δ_H 5.19 (d, J = 2.9 Hz, 1H) suggested that five sugar units are β-oriented, while anomeric proton at δ_H 5.38 (1H, br s) is α-oriented (Duus et al., 2000, Rahman et al., 2015).

The configurations of sugars were identified as *β*-D-6-deoxy-allopyranoside, *β*-D-apiofuranosyl, *α*-L-rhamnopyranosyl, *β*-D-xylopyranosyl, and two *β*-D-glucopyranosyl, which were further supported by ESI-MS/MS and GC analysis of derivatized product. The ESI-MS/MS spectrum of compound **2** exhibited fragment ion peaks at *m/z* 1379 $(M+Na)^+$, 1217 $(M+Na\text{-}glu)^+$, 1085 $(M+Na\text{-}glu\text{-}apiose)^+$, 953 $(M+Na\text{-}glu\text{-}apiose\text{-}xyl)^+$, 807 $(M+Na\text{-}glu\text{-}apiose\text{-}xyl\text{-}rha)^+$ and 663 $(M+Na\text{-}glu\text{-}apiose\text{-}xyl\text{-}rha+H_2O)^+$ due to simultaneous loss of sugar units, respectively. Similarly acid hydrolysis was done to get the glycone moiety. The comparison of derivatized hydrolyzed glycone product and available sugar standards also confirmed the presence of *α*-L-rhamnopyranosyl, *β*-D-xylopyranosyl, and *β*-D-glucopyranosyl in the structure, while the other two sugars were determined by their literature based NMR data values as discussed above.

After the complete analysis including 1D, 2D-NMR (HSQC, HMBC, COSY, NOE), HR-ESI-MS, FT- IR and GC analysis of compound **2,** the structure was established as 24-*O*-*β*-D-6-deoxy-allopyranoside-21-*O*-*β*-D-glucopyranosyl-1*β*,3*β*-(23*S*,24*S*)-3,23-dihydroxyspirosta-5,25-dienyl-*O*-*β*-D-apiofuranosyl-(1→3)-*α*-L-rhamnopyranosyl-(1→2)-[*β*-D-xylopyranosyl-(1→3)]-*β*-D-glucopyranosyl and named as govanoside D (Fig 1).

Compound **3** was obtained as white amorphous powder with an optical rotation $[\alpha]^{20}_D$= - 44.0 (c = 0.003, CH_3OH). Its molecular formula was calculated as $C_{47}H_{74}O_{19}$ from its observed sodiated ionic peak at *m/z* 965.4712 $[M+Na]^+$ (calculated for $C_{47}H_{74}O_{19}Na^+$ as 965.4717) from HR-ESI-MS (Fig. S34). IR spectrum showed bands at υ_{max} 3365.78 cm-1, 2931.80 cm-1, 1720.50 cm-1, 1641.42 cm-1 and 1037.70 cm-1,which attributed for O-H stretching, C-H stretching, C=O stretching, olefinic stretching and C-O stretching, respectively (Fig. S33).

^1H (Fig. S26), and ^{13}C (Fig. S27) spectrums revealed that the basic skeleton of compound **3** was quite different from the compound **1** and compound **2.** The ^1H spectrum of compound **3** showed the presence of one olefinic proton at δ_H 5.36 (d, *J* = 4.6 Hz) and three methyl signals at δ_H 0.95 s, δ_H 1.08 s, and δ_H 1.26 (d, *J* = 7.1 Hz) for aglycone portion. The presence of one endo-cyclic olefinic bond, two quaternary and one tertiary methyl signals in the aglycone portion indicated it as 5,6-spirostanol skeleton. Similarly, the ^{13}C spectrum exhibited a total of forty seven carbons signals. The analysis of these signals and comparison of ^{13}C with DEPT-135 concluded that among these signals, six signals were attributed to methyl groups, twelve for methylene, and twenty three for methine carbons, while remaining six were quaternary carbon signals.

The presence of methine carbon values at δ_C 90.5 (C-16), along with a quaternary carbon at δ_C 90.6 (C-17) showing HMBC correlation with the neighboring protons at δ_H 1.54 (m) (H-15a) and 2.22 (m) (H-15b) indicated it as pennogenin type moiety (Ju et al., 1992) (Fig. 4). But an unusual behavior in the NMR values at δ_C 64.4 (C-26) and δ_C 64.8 (C-27) indicated slight change in the structure from basic Pennogenin skeleton as the DEPT-135 spectrum confirmed two methylene carbons at C-26 and C-27 positions. Also the deshielded value at δ_C 64.8 (C-27), indicated the hydroxyl substitution at this position (Ju et al., 1992). The NOE correlations with respect to H-3 were analyzed and used to determine the relative configuration of aglycone skeleton. The methyl groups were determined to be *β*-oriented after analyzing the NOE correlation of H-8 with H-18 and H-19. Similarly the ring fusion orientation were determined to be similar as previously reported skeleton (Ju et al., 1992). After the NMR analysis (1D, 2D including HSQC, HMBC, COSY, (Fig. 4) and NOE (Fig. 5) and comparison with literature reports aglycone moiety of compound **3** was identified as (25*S*)-spirost-5-en-3*β*,17*α*,27-triol (Ju et al., 1992).

The ^{1}H spectrum of compound **3** exhibited three anomeric protons at δ_H 6.38 s, δ_H 5.89 s, and δ_H 5.01 (d, J = 7.6 Hz), and two methyl signals at δ_H 1.80 (d, J = 6.0 Hz) δ_H 1.66 (d, J = 6.0 Hz), attached to tertiary carbons indicated the presence of three sugars including two deoxy-hexose sugars in the structure. The anomeric proton at δ_H 5.01 (d, J = 7.6 Hz) correlated with δ_C100.4 in HSQC and having HMBC correlation with δ_C 78.5 (C-3) indicated the glycosidic substitution at C-3 position of aglycone moiety. The presence of two singlets at δ_H 6.38 s, and δ_H 5.89 s and two methyl groups confirms the presence of two deoxy sugars in the structure. Anomeric protons at δ_H 6.38 s (H-1''), 5.89 s (H-1'''') correlated with δ_C102.5, δ_C 103.3 in HSQC and in HMBC the anomeric proton at δ_H 6.38 s (H-1'') showed correlation with the δ_C 78.7 (C-2') and 5.89 s (H-1'''') with δ_C 78.9 (C-4'), respectively, which confirmed the linkage of the deoxy sugars at C-2' and C-4' positions (Y. Ju. *et. al.,* 1992). Furthermore, one highly deshielded carbon at δ_C 171.5 (C-1'''), identified as quaternary carbon after DEPT-135 analysis, showed HMBC correlation with the singlet methyl signal at δ_H 1.91s (H-2''') indicated the presence of an acetyl group. The HMBC correlation of acetyl carbon at δ_C 171.5 with δ_H 5.93 (dd, J = 9.7, 2.7 Hz) (H-3'') and the deshielded value at δ_C 77.2 (C-3'') confirmed the acetylation at C-3'' position of first deoxy sugar. Anomeric proton at δ_H 5.01 (d, J = 7.6 Hz) with a coupling constant value >7.0 Hz indicated the presence *β*-oriented sugar, while anomeric protons at δ_H 6.38 s, and 5.89 s indicated two *α*-oriented sugars (Duus et al., 2000). The ESI-MS/MS spectrum of compound **3** exhibited fragment ion peaks at *m/z* 965 $(M+Na)^+$, 758 $(M+Na\text{-acetyl rha-}H_2O)^+$, and 612 $(758\text{-rha})^+$, due

to simultaneous loss of acetylated rhamnose and rhamnose sugar moieties, respectively. The sugars were identified as one *β*-D-glucopyranoside, and two *α*-L-rhamnopyranosyl monosaccharides which were further confirmed by comparing their retention time in GC analysis after acid hydrolysis with standards.

After the complete analysis of NMR (1D & 2D), HR-ESI-MS, FT-IR and GC analysis, the structure of compound **3** was assigned as (25*S*)-spirost-5-en-3*β*,17*α*,27-triol-3-O-(3-O-acetyl-*α*-L-rhamnopyranosyl-(1-2)]-[*α*-L-rhamnopyranosyl(l-4)]-*β*-D-glucopyranoside and named as govanoside E (Fig. 1).

In addition to these three previously unknown molecules, four known molecules govanoside B (**4**) [Singh et al., 2020a] (Fig. S37-S41), protodioscin (**5**) [Abdel-Sattar et al., 2008] (Fig. S42-S46), 20*β*-hydroxyecdysone (**6**) [Maliński et al., 2021] (Fig. S47-S51), and polypodine B (**7**) [Malińskiet al., 2021] (Fig. S52-S56), were also isolated from the water and *n*-butanol fractions and identified by the NMR analysis and comparison of their observed spectral data with those reported in the literature. The purity of all the isolated molecules were found >95% based on UPLC analysis.

2.3 Protocol for extraction of total steroidal saponins and gram scale isolation of borassoside E

The ultrasonic-assisted technique was used for the extraction and enrichment of total steroidal saponins (TSS) from the rhizomes of *T. govanianum* (Fig. 6). Extraction was performed using green solvents such as water and ethanol. Three final sub-fractions were collected *i.e.*, pellet-1, pellet-2, and supernatant-2 (Fig. S63). The TSS content in the collected sub-fractions was estimated using the anisaldehyde-sulphuric acid-based spectroscopic method. This analysis revealed that pellet-1 has approximately 5.1 ± 1.23% of TSS with 0.062% recovery, supernatant-2 has 10.6 ± 1.17% of TSS with 0.012% recovery, and pellet-2 has 88.3 ± 2.59% of TSS with 91.7% recovery (Table 3).

The result of the TSS estimation has indicated that pellet-2 is enriched with steroidal saponins so this steroidal saponins enriched fraction (pellet-2) was further analyzed by using MALDI-TOF mass spectrometry. The MALDI-TOF spectra showed that almost all the molecules were found to be coupled with sodium ion (Na, 22.989 Dal), and the major ion peaks were observed at 745.378 (borassoside D); 891.433 (borassoside E); 1053.484 (pennogenin tetraglycoside); 1071.501 (protodioscin); 1217.474 (govanoside B), along with several other minor peaks (Fig. S64 and Table S1).

The steroidal saponins enriched fraction (pellet-2) was further processed using column chromatography to yield 2.46 g of borassoside E with 98.38% of purity (UPLC-ELSD) (Fig. 7). The gram scale isolated borassoside E contain minor impurity of protodioscin (0.69%) and pennogenin tetraglycoside (0.91%).

2.4 Acetylcholinesterase activity and Molecular docking

2.4.1 Effects on acetylcholinesterase activity

The inhibitory effect of the extract, fractions, and isolated molecules on acetylcholinesterase (AChE) activity is expressed as percentages of inhibition and IC_{50} values (Table S2), calculated based on the concentration-dependent inhibitory activity. The results of AChE inhibition by the extract, fractions, and isolated molecules revealed that among the extract and fractions, WF (IC_{50} value: 90.2 μg/mL) exhibited highest inhibitory effect (Fig. S65). However, among the pure molecules govanoside E (IC_{50} value: 8.62 μM) and protodioscin (IC_{50} value: 8.98 μM) showed highest inhibitory effect on AChE activity in a concentration-dependent manner (Fig. 8). The calculated IC_{50} value of Donepezil is approximately 5.40 μM. The PE (percentage of inhibition: 19.35 ± 1.62 at 100 μg/ mL) was found least active while WF (percentage of inhibition: 55.65 ± 7.39 at 100 μg/ mL) was found most active against AChE. Moreover, govanoside E (percentage of inhibition: 57.97 ± 1.61 at 10 μM) and protodioscin (percentage of inhibition: 55.65 ± 1.40 at 10 μM) were 30.47 % and 32.79 % less active than Donepezil (percentage of inhibition: 88.44 ± 0.41 at 10 μM), respectively. The activity of WF may be attributed to the presence of protodioscin (77.6 ± 0.8 mg/g).

2.4.2. Molecular docking

From molecular docking of steroidal saponins isolated from *T. govanianum* against acetylcholinesterase, govanoside E (-10.849 kcal/mol), pregna-chacotrioside (-10.471 kcal/mol), protodioscin (-10.425 kcal/mol) were found better as compared to donepezil (-8.083 kcal/mol) (Table 4 and Fig. 8). Steroidal saponins bound with acetylcholinesterase as govanoside E *via* Val73, Thr75, Asp283, and Arg296; pregna-chacotrioside *via* Thr75, Asp283, His287, and Phe295; and protodioscin *via* Ser293 residues.

3. Conclusion

In summary, total seven steroidal saponins including three undescribed steroidal saponins named govanoside C (**1**), govanoside D (**2**), and govanoside E (**3**) along with four known

compounds (**4-7**), were isolated from the rhizomes of *T. govanianum*. The structures of isolated compounds were elucidated using the analysis of 1D and 2D NMR spectroscopic data, and the relative stereochemistry of compounds was characterized using 1D TOCSY and NOE NMR experiment. The antagonistic effects of the extract, fractions, and isolated molecules on acetylcholinesterase activity were evaluated and further validated by molecular docking study to predict binding free energy and molecular interactions. Govanoside E and protodioscin have exhibited promising antagonistic effects on AChE activity. In addition, the easy and reliable method was developed for the direct extraction of the total steroidal saponins and gram scale isolation of bioactive borassoside E. This finding suggests, further phytochemical exploration of *T. govanianum* has necessary to investigate bioactive and unique steroidal saponins. It was also speculated that govanoside E and protodioscin might be the potential anti-acetylcholinesterase agents and further *in vitro* and *in vivo* studies are essential to validate their inhibitory potential. The gram scale isolated borassoside E, might be a potential source for the development of bio-non-ionic detergent like *digitonin*.

4. Experimental

4.1 General experimental procedures

^{1}H (600 MHz), ^{13}C (150 MHz) and 2D NMR experiments were performed on a Bruker Avance-600 spectrometer using CD_3OD and pydidine-d_5 deutrated solvents, which were purchased from Sigma-Aldrich™ (St. Louis, MO, USA). HR-ESI-MS spectra were taken on high-resolution 6560 Ion Mobility Q-TOF LC/MS (Agilent, Santa Clara, USA) mass spectrometer equipped with an ESI source). IR data were recorded on Shimadzu IR Prestige-21with ZnSe Single reflection ATR accessory. Silica gel of 60-120 mesh size and reverse-phase fully end capped C-18 were used for column chromatography. Precoated TLC sheets of silica gel 60 F_{254} were used for thin-layer chromatography. Spot and separated compounds visualization on TLC were performed by firstly under UV light and then after spraying visualization agent para-anisaldehyde-H_2SO_4 followed by the heating of TLC plate at 100˚C. HPLC solvents used were purchased from Sigma-Aldrich™ (St. Louis, MO, USA). Specific rotation and melting point were determined on MCP 100 Modular circular Polarimeter and Visual Melting Range Apparatus (MR-VIS), respectively.

4.2 Plant material

The rhizomes of *T. govanianum* were collected from Bharmour, Chamba district, Himachal Pradesh, India (2580 m) in 2018. The plant material was identified by Dr. Amit Chawla, High Altitude Biology, Department, CSIR-Institute of Himalayan Bioresource Technology, Palampur, India. The voucher specimen (voucher no. PLP 13037) was deposited in the herbarium of CSIR-Institute of Himalayan Bioresource Technology, Palampur, India.

4.3 Extraction and isolation of metabolites

The procedure for extraction and fractionation from rhizomes of *T. govanianum* was reported in our previous research paper (Singh et al., 2020a). Water fraction (221.0 g) was subjected to column chromatography over Diaion® HP-20 resin eluted with H_2O: MeOH (100:00-00:100), yielding 10 sub-fractions (WFA-WFJ; each of 500 mL). Fraction WF-D (12.220 g), obtained from 50% methanol, was subjected to column chromatography over silica gel (230-400 mesh) eluted with chloroform: methanol (100:00 to 00:100), resulting 10 different sub-fractions (WF-D-1 to WF-D-10). WF-D-6 (4.9 g), obtained at 50% methanol, was applied to column chromatography over RP C-18. Compound **1** (540 mg) was obtained at polarity 25% MeOH: H_2O**.** WF-A to WF-C (10.024 g) was mixed by comparing TLC analysis and then subjected to RP C-18 column chromatography eluted with solvent system H_2O: MeOH (100:00-00:100), to give five sub-fractions. WF-AC-1 to WF-AC-5. Further WF-AC-4 (2.0 g), obtained from 30 and 40% methanol, was subjected to RP C-18 column chromatography eluted with H_2O:MeOH (80: 20), result in the isolation of compound **2** (997 mg) and compound **4** (498 mg). Similarly the other sub-fraction WF-AC-2 subjected over RP C-18 yielded compound **5** (943 mg).

Other three compounds were isolated from *n*-butanol fraction of *T. govanianum.* The *n*-butanol fraction (150.0g) yielded 10 sub-fractions after column chromatography (silica-gel 60-120 mesh) as described in our previous research paper (Singh et al., 2020a). Sub-fraction BF-E (906 mg) subjected over reverse phase column chromatography yield compound **3** (75 mg). Similarly BF-C and BF-D collectively (2.60 g) subjected for CC on C-8 silica resulting in the isolation of compound **6** (503 mg) and compound **7** (14 mg), respectively.

4.3.1 Govanoside C (1)

White amorphous solid; (540 mg); mp 240-242 ˚C; $[\alpha]^{20}_D$= -35.6º (c = 0.003, CH_3OH); HR-ESI-MS (Positive) *m/z* 1085.4768 $[M+Na]^+$ (cal for $C_{50}H_{78}O_{24}Na^+$,1085.4775); IR (ZnSe) ν_{max}: 3358.07 cm^{-1} (O-H stretching), 2899.01 cm^{-1} (C-H stretching), 1643.35 cm^{-1} (Olefinic

stretching), 1035.77 cm^{-1} (C-O stretching); ^{1}H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) data in Table 1.

4.3.2 Govanoside D (2)

Amorphous solid; (997 mg); mp 252-255 ˚C; $[\alpha]^{20}_{D}$= -26.0º (c = 0.003, CH_3OH); HR-ESI-MS (Positive) *m/z* 1379.5735 $[M+Na]^+$ (cal for $C_{61}H_{96}O_{33}Na^+$, 1379.5726); IR (ZnSe) ν_{max}: 3350.35 cm^{-1} (O-H stretching), 2889.37 cm^{-1} (C-H stretching), 1637.56 cm^{-1} (C=C stretching), 1035.77 cm^{-1} (C-O stretching); ^{1}H-NMR (CD_3OD, 600 MHz)and ^{13}C-NMR (CD_3OD, 150 MHz) data in Table 1.

4.3.3 Govanoside E (3)

Amorphous solid; (75 mg); mp 195-198 ˚C; $[\alpha]^{20}_{D}$= -44.0º (c = 0.003, CH_3OH);HR-ESI-MS (Positive) *m/* 965.4712 $[M+Na]^+$ (cal for $C_{47}H_{74}O_{19}Na^+$, 965.4717); IR (ZnSe)ν_{max}: 3365.78 cm^{-1} (O-H stretching), 2931.80 cm^{-1} (C-H stretching), 1720.50 cm^{-1} (C=O stretching), 1641.42 cm^{-1} (C=C stretching, 1037.70 cm^{-1} (C-O stretching); ^{1}H-NMR (pyridine-d5, 600 MHz) and ^{13}C-NMR (pyridine-d5, 150 MHz) data in Table 2.

4.3.4 Govanoside B (4)

White amorphous solid; (498 mg); mp 268–270 ˚C; HR-ESI-MS (Positive) *m/z* 1217.5197 $[M+Na]^+$ (cal. for $C_{55}H_{86}O_{28}Na^+$, 1217.5198); ^{1}H-NMR (CD_3OD, 600 MHz)and ^{13}C-NMR (CD_3OD, 150 MHz). Observed data was compared with literature and compound was identified as govanoside B [Singh et al., 2020a].

4.3.5 Protodioscin (5)

Amorphous solid; (943 mg); mp 220-222 ˚C HR-ESI-MS (Positive) *m/z* 1071.5360 $[M+Na]^+$ (cal for $C_{51}H_{84}O_{22}Na^+$, 1071.5346); ^{1}H-NMR (CD_3OD, 600 MHz) and ^{13}C-NMR (CD_3OD, 150 MHz). Observed data was compared with literature and compound was identified as protodioscin [Abdel-Sattar et al., 2008].

4.3.6 20β-hydroxy ecdysone (6)

White powder; (503 mg); mp 243–244 °C; HR-ESI-MS (Positive) *m/z* 481.3164 $[M+H]^+$ (cal for $C_{27}H_{45}O_7^+$, 481.3160) ;^{1}H-NMR (CD_3OD, 600 MHz) and ^{13}C-NMR (CD_3OD, 150

MHz). Observed data was compared with literature and compound was identified as 20*β*-hydroxy ecdysone [Maliński et al., 2021].

4.3.7 Polypodine B (7)

Amorphous white solid; (14 mg); mp 225–227 °C; HR-ESI-MS (Positive) *m/z* 519.2895 $[M+Na]^+$ (cal for $C_{27}H_{44}O_8Na^+$, 519.2928); ^{1}H-NMR (CD_3OD, 600 MHz) and ^{13}C-NMR (CD_3OD, 150 MHz). Observed data was compared with literature and compound was identified as polypodine B [Maliński et al., 2021].

4.4 Purity analysis of isolated molecules by UPLC-ELSD

The purity of all the isolated molecules was analyzed by using Ultra high performance liquid chromatography (UPLC) with evaporative light scattering detector (ELSD). Sample concentration (1mg/ mL) was prepared by dissolving each compound in HPLC grade methanol. Our previously reported quantification method was applied for the purity determination (Singh et al., 2020b).

4.5 Acid hydrolysis and GC analysis for sugar confirmation

4.5.1 Acid hydrolysis

Compound **1** (15 mg) was dissolved in 1 mL MeOH and then 10 mL 2N HCl (diluted in 1,4 dioxane:H_2O (1:1) was added in the solution. The solution was heated on an oil bath at 95 ^oC for 3 hours. After the completion of the reaction, the reaction mixture was cooled down at room temperature. After cooling, the reaction mixture was neutralized by passing through Amberlyst®A21 free base (Sigma-Aldrich). The neutralized solution was then fractionated with water and methylene chloride to separate the glycone and aglycone moieties. The aqueous layer containing sugar units was dried on a rotary evaporator under reduced pressure at temperature 50 ^oC.

Compound **2** (15 mg) and compound **3** (15 mg) were subjected for acid hydrolysis followed by the same procedure as done for the compound **1.**

4.5.2 Derivatization and GC analysis

The fully dried aqueous layer was dissolved in 100 µL pyridine and then .1 mL N, O-bis(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane was added. The reaction mixture was incubated on magnetic stirrer at 40 ^oC for 12 hours. After the completion of reaction, 50 µL

from each sample was taken and diluted with GC grade DCM for GC analysis. The sugar units were confirmed from GC analysis by comparing their retention time with available sugar standards after derivatization. Peaks were identified for compound **1, 2** and **3** at 18.721 (*β*-D-glucose), 16.992 (*β*-D-xylose), and 16.048 (*α*-L-rhamnose). Other sugars were determined based on their characteristic NMR signals values reported in literature.

4.6 Extraction of total steroidal saponins and isolation of borassoside E

The ultrasound-assisted extraction was employed for the extraction of steroidal saponins. The 100 g of rhizome powder was suspended into the ethanol: water (80:20) and ultrasonicated with 250 watt (3 sec on/ 1sec off) at 50 ˚C for 1 hour with constant stirring. Followed by filtered with marceline cloth and the filtrate was subsequently dried using a rotary evaporator. The dried filtrate was suspended in 100 mL of ethanol and centrifugation at 5000 rpm for 10 min and which separate out as supernatant-1 and pellet-1. The supernatant-1 was dried using rotary evaporator followed by suspending into the 100 mL of distilled water and centrifugation at 5000 rpm for 10 min to yielded supernatant-2 and pellet-2. The pellet-1 and pellet-2 were passed through the Diaion® HP-20 resin and eluted with water and ethanol respectively and dried using lyophilizer. The extraction was performed thrice. The total steroidal saponin content in the pellet-1, supernatant-2, and pellet-2 was estimated using previously developed anisaldehyde-sulphuric-acid-ethyl acetate method (Patil et al., 2021c). The further chemical constituents of pellet-2 were analyzed using MALDI-TOF.

Pellet-2 (5 g) was subjected to column chromatography over silica gel 100-200 mesh size and eluted with gradient of methanol: chloroform (00:10 up to 05:05). At the gradient of 03:07 yielded borassoside E which was further qualitatively and quantitatively analyzed by ELSD-UPLC (Singh et al., 2020b).

4.7 Acetylcholinesterase activity inhibition

The inhibitory effects of the extract, fraction, and isolated steroidal saponins (Singh et al., 2020a), from *T. govanianum* on AChE activity was evaluated using Acetylcholinesterase inhibitor screening kit (Sigma-Aldrich) based on an improved Ellman method (Ka et al., 2020). The three concentrations were taken for the extract and fractions *i.e.*, 100-300 µg/mL and three for pure molecules 10-30 µM. Donepezil has taken as standard inhibitor. The experiment was performed according to the standard protocol. AChE hydrolyzes acetylthiocholine (substrate) into thiocholine which react with 5,5`-dithiobis(2-nitrobenzoic acid) (DTNB) to form a yellow

colour complex. The intensity of colour product was recorded at 412 nm by using Synergy H1 BioTek microplate reader. The experiment was performed in triplicate.

4.7.1 Molecular docking Study

The chemical structure of donepezil and isolated steroidal saponins (Singh et al., 2020a), from *T. govanianum* were drawn using ChemDraw. All the ligands are optimized and minimized by OPLS3e (Optimized Potentials for Liquid Simulations) force field in Ligprep, Schrodinger, 2020.3. The template of *Electrophorus electricus* acetylcholinesterase (PDB ID: 1C2O) with resolution 4.20 Å was taken from the RCSB-Protein Data Bank. The template of acetylcholinesterase was pre-processed, optimized, and minimized by protein preparation wizard and receptor grid was generated at top site after sitemap. The docking study was performed using extra-precision (XP) with flexible mode to predict the binding efficiency of steroidal saponins with acetylcholinesterase. Donepezil was used as a standard inhibitor.

4.8 Data analysis and statistics

The enzyme inhibition results were expressed as mean ± standard deviation (SD). Further, experimental graphs plotting and the IC_{50} values were calculated by unpaired *t*-tests and one-way ANOVA by using Tukey-Kramer post hoc analysis to compare data sets, using GraphPad Prism Software (GraphPad Software, La Jolla, CA, USA). Differences between means were considered to be significant if $p < 0.05$.

Supplementary material

The spectroscopic data relating to this paper

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Disclosure statement

The authors report no conflict of interest.

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Figures

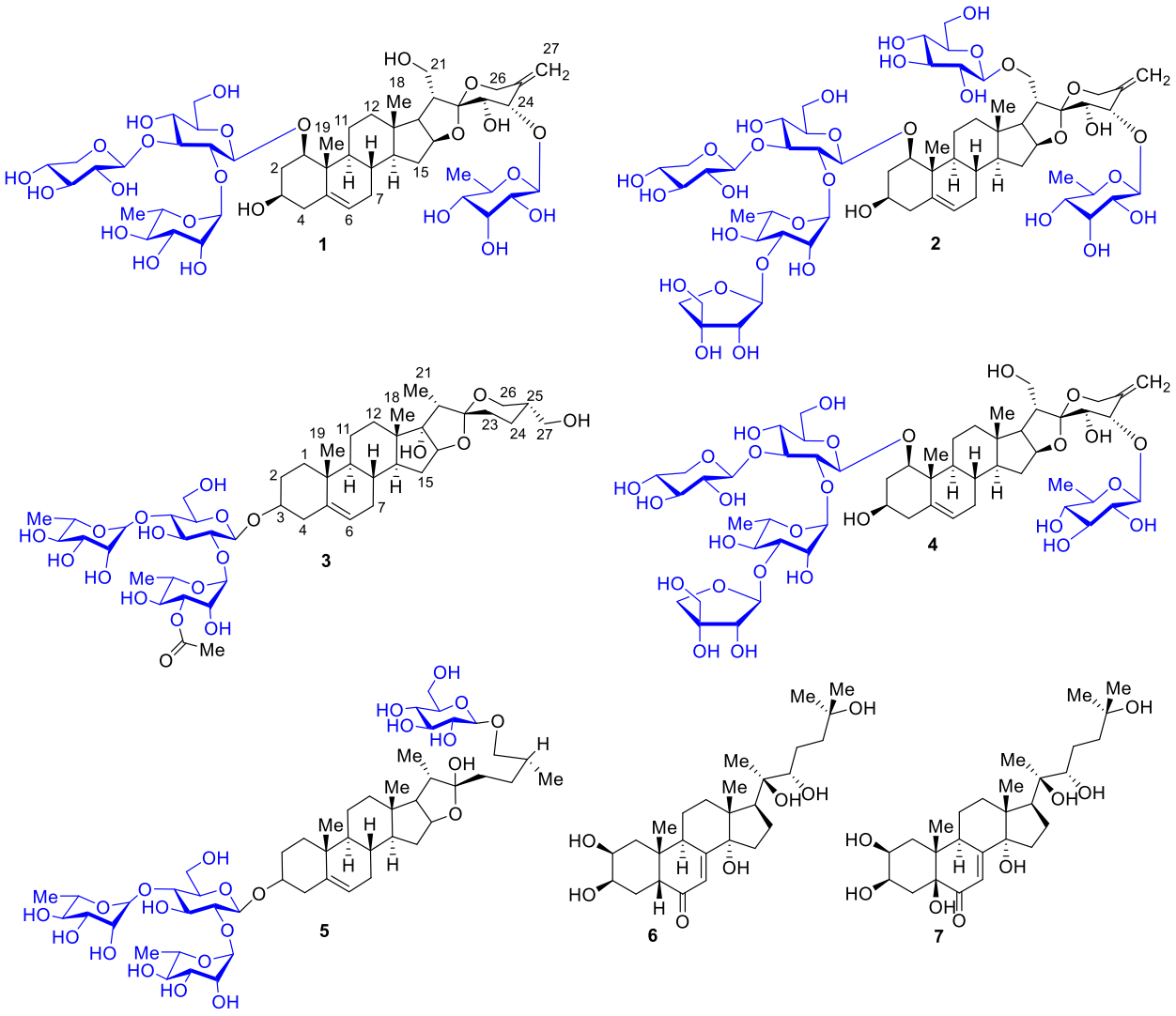

Fig. 1 The structures of isolated compounds from *Trillium govanianum*

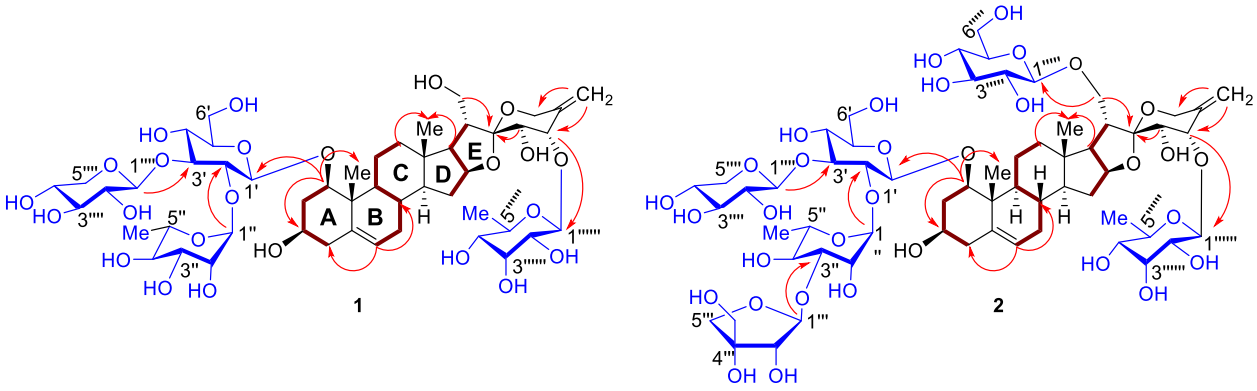

Fig. 2 Key HMBC (→) and COSY (▬) correlations of compounds **1** and **2**.

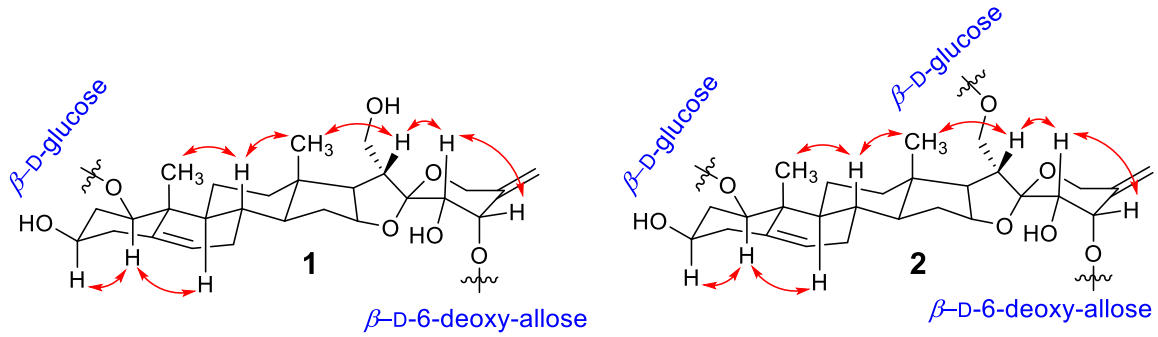

Fig. 3 The key NOE (↔) correlations of compounds **1** and **2**.

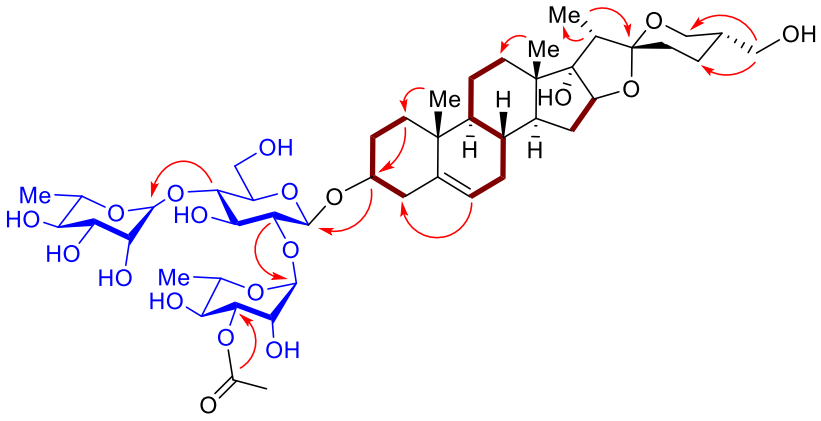

Fig. 4 Main key HMBC (→) and COSY (▬) correlations of compounds **3**

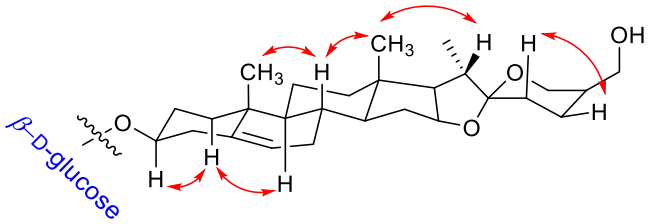

Fig. 5 The key NOE (↔) correlations of compounds **3**

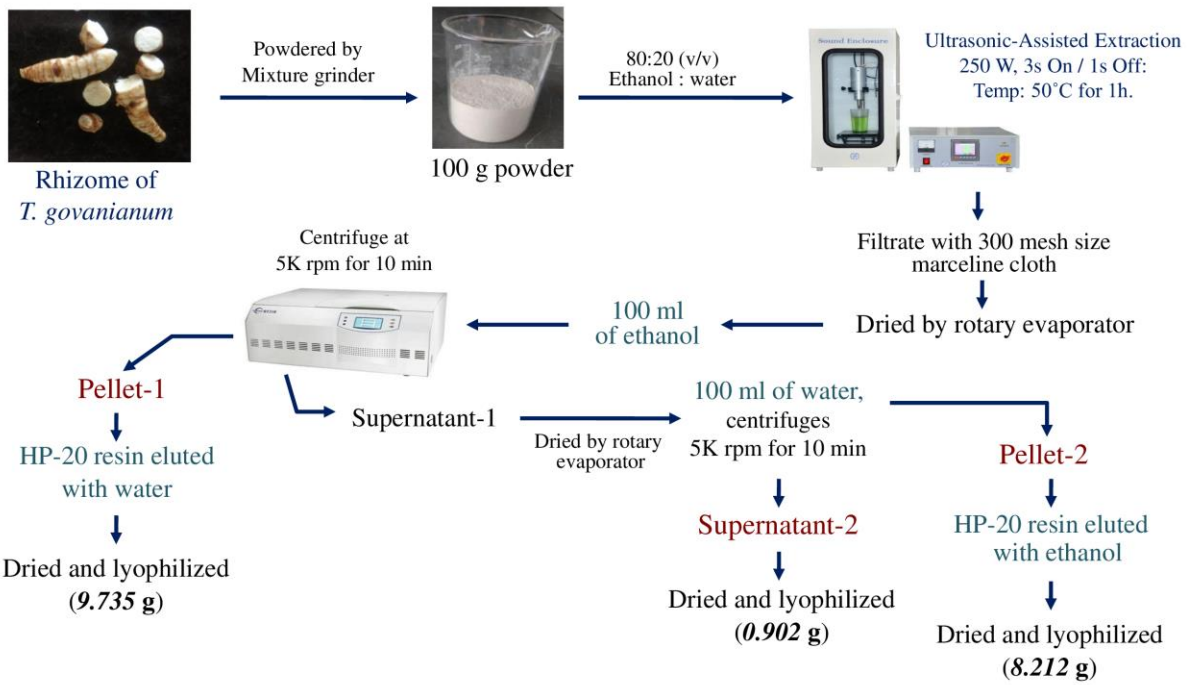

Fig. 6 The graphical representation of the extraction and enrichment protocol for total steroidal saponins from the rhizomes of *T. govanianum*.

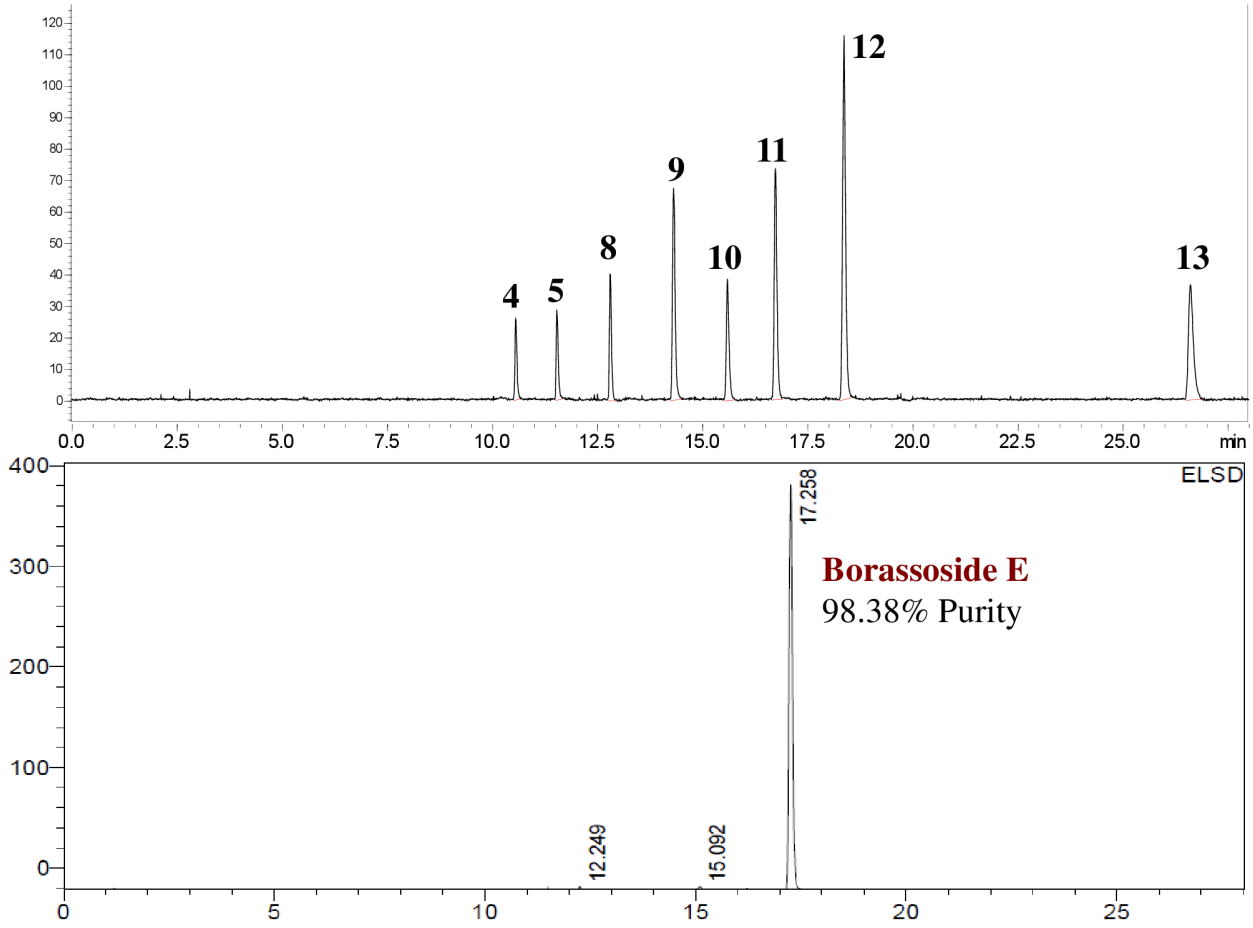

Fig. 7 UPLC-ELSD spectrum of steroidal saponins from *T. govanianum* and gram scale isolated borassoside E.

[govanoside B (**4**), protodioscin (**5**), pennogenin-tetraglycoside (**8**), pennogenin-diglycoside (**9**), borassoside E (**10**), borassoside D (**11**), pregna-chacotrioside (**12**), and diosgenin (**13**)]

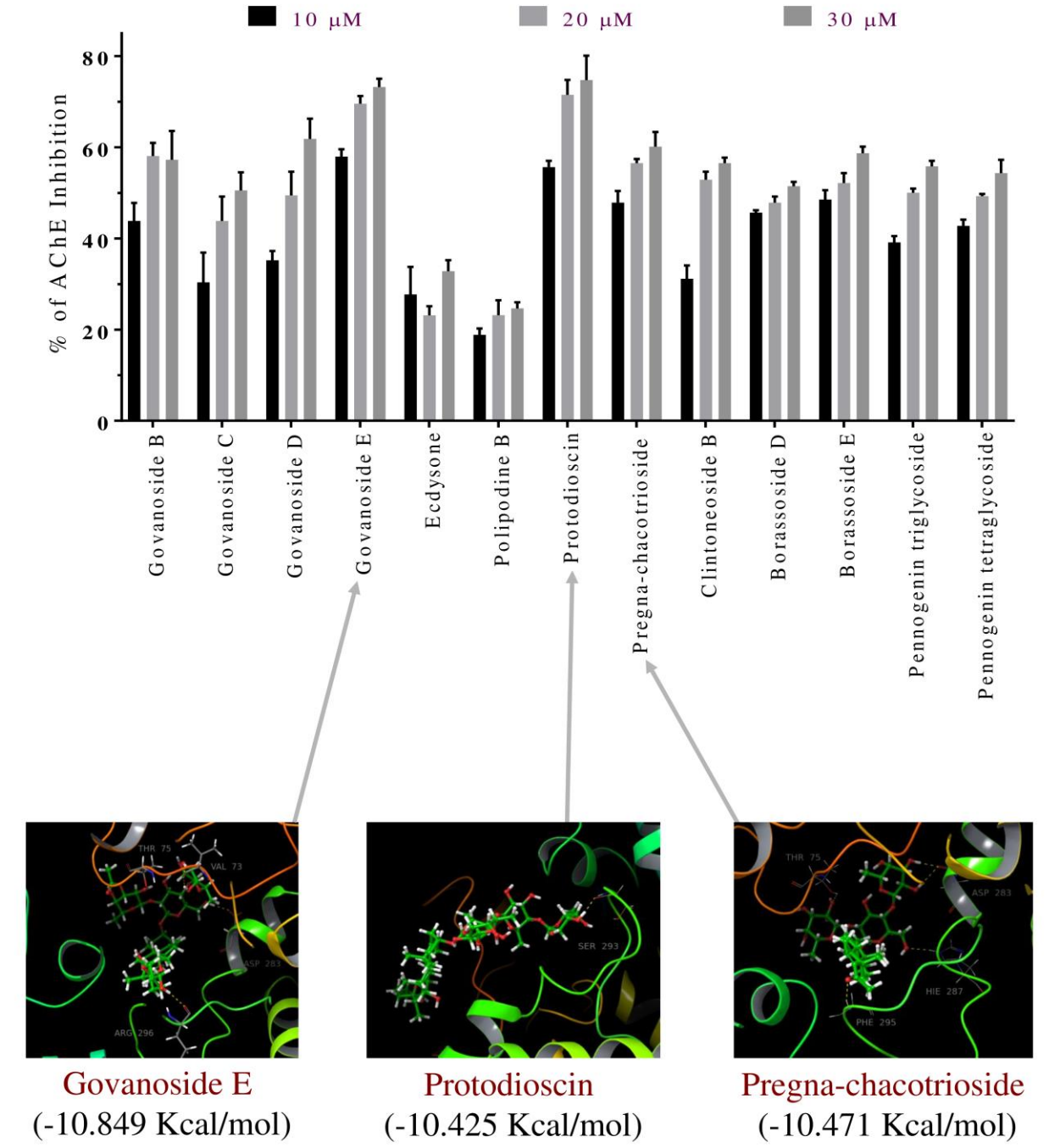

Fig. 8 Graphical representation of antagonistic effects of isolated pure compounds on AChE activity with putative interaction of best-docked compounds.

Tables

Table 1 ^{1}H (600 MHz) and ^{13}C-NMR (150 MHz) spectral data of aglycone portions for compound **1-3** (Compound **1-2** in CD_3OD and compound **3** in pyridine d5), δ in ppm, J in Hz.

	Compound **1**		Compound **2**		Compound **3**	
Position	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	84.7	3.50 (m)[a]	84.8	3.49 (m)[a]	38.0	0.99 (m)[a], 1.78 (m)[a]
2	37.4	1.73 (m)[a], 2.08 (m)[a]	37.6	1.73 (m)[a], 2.09 (m)[a]	30.6	1.30 (m)[a], 2.08 (m)[a]
3	69.1	3.37 (m)[a]	69.1	3.36 (m)[a]	78.5	4.24 (m)[a]
4	43.4	2.21 (m)[a], 2.23 (m)[a]	43.35	2.19 (m)[a], 2.23 (m)[a]	39.3	2.07 (m)[a], 2.87 (m)[a]
5	139.4	-	139.4	-	141.1	-
6	126.0	5.56 (d, J = 5.8)	126.1	5.55 (d, J = 5.7)	122.4	5.36 (d, J = 4.6)
7	32.6	1.54 (m)[a], 1.99 (m)[a]	32.6	1.54 (m)[a], 1.97 (m)[a]	32.9	1.54 (m)[a], 1.87 (m)[a]
8	34.1	1.56 (m)[a]	34.0	1.55 (m)[a]	32.7	1.56 (m)[a]
9	51.0	1.36 (m)[a]	51.0	1.34 (m)[a]	50.7	1.00 (m)[a]
10	43.3	-	43.30	-	37.6	-
11	24.7	1.41 (m)[a], 2.45 (m)[a]	24.8	1.39 (m)[a], 2.46 (m)[a]	21.4	1.56 (m)[a], 1.58 (m)[a]
12	40.9	1.19 (m)[a], 1.69 (m)[a]	40.9	1.17 (m)[a], 1.75 (m)[a]	32.5	1.53 (m)[a], 2.17 (m)[a]
13	41.7	-	41.7	-	45.6	-
14	57.9	1.21 (m)[a]	57.9	1.20 (m)[a]	53.4	2.10 (m)[a]
15	33.1	1.45 (m)[a], 1.99 (m)[a]	33.0	1.40 (m)[a], 1.93 (m)[a]	32.3	1.54 (m)[a], 2.22 (m)[a]
16	84.4	4.53 (m)[a]	84.2	4.50 (m)[a]	90.5	4.51 (m)[a]
17	58.7	1.84 (m)[a]	58.7	1.81 (m)[a]	90.6	-
18	17.1	0.93 s	17.1	0.92 s	17.6	0.95 s
19	15.3	1.09 s	15.3	1.08 s	19.9	1.08 s
20	46.5	2.72 (m)[a]	44.2	2.90 (m)[a]	45.3	2.31 (m)[a]
21	62.8	3.52 (m)[a] 3.67 (m)[a]	70.8	3.47 (m)[a], 3.98 (m)[a]	10.3	1.26 (d, J = 7.1)
22	111.9	-	111.6	-	110.7	-
23	72.1	3.74 (m)[a]	71.7	3.82 (d, J = 4.0)	32.3	1.79 (m)[a], 2.20 (m)[a]
24	83.3	4.29 (d, J = 3.9)	83.4	4.27 (d, J = 3.9)	24.0	1.82 (m)[a], 1.89 (m)[a]
25	144.4	-	144.4	-	39.5	2.79 (m)[a]
26	62.1	4.46 (d, J =11.8), 3.72 (m)[a]	62.1	4.45 (d, J = 11.8) 3.68 (m)[a]	64.4	3.93 (m)[a], 4.10 (m)[a]
27	114.0	4.99 (s), 5.10 (s)	114.1	4.96 s, 5.07 s	64.8	3.68 (m)[a] , 3.75 (m)[a]

m[a] : multiplet overlapped signals

Table 2 ^{1}H (600 MHz) and ^{13}C-NMR (150 MHz) spectral data of glycone portions for compound **1-3** (Compound **1-2** in CD_3OD and compound **3** in pyridine d5), δ in ppm, J in Hz.

	Compound **1**			Compound **2**			Compound **3**	
Position	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
	1-*O*-*β*-D-Glucose			1-*O*-*β*-D-Glucose			3-*O*-*β*-D-Glucose	
1'	100.3	4.38 (t, *J* = 7.8)	1'	100.3	4.40 (d, *J* = 7.6)	1'	100.4	5.01 (d, *J* = 7.6)
2'	76.9	3.51 (m)[a]	2'	77.1	3.49 (m)[a]	2'	78.7	4.24 (m)[a]
3'	88.9	3.65 (m)[a]	3'	88.6	3.65 (m)[a]	3'	78.2	3.87 (m)[a]
4'	70.7	3.25 (m)[a]	4'	70.5	3.25 (m)[a]	4'	78.9	4.43 (m)[a]
5'	77.4	3.27 (m)[a]	5'	77.3	3.27 (m)[a]	5'	77.4	3.67 (m)[a]
6'	63.5	3.60 (m)[a] 3.91(m)[a]	6'	63.5	3.63 (m)[a] 3.88 (m)[a]	6'	61.7	4.09 (m)[a] 4.24 (m)[a]
	2'-*O*-*α*-L-Rhamnose			2'-O-*α*-L-Rhamnose			2'-*O*-*α*-L-Rhamnose	
1"	101.6	5.41 br s	1"	101.5	5.38 br s	1"	102.5	6.38 br s
2"	72.2	3.94 (m)[a]	2"	71.6	4.09 (m)[a]	2"	70.3	5.07 (m)[a]
3"	71.9	3.66 (m)[a]	3"	80.32	3.67 (m)[a]	3"	77.2	5.93 (dd, *J* = 9.7, 2.7)
4"	74.1	3.38 (m)[a]	4"	72.8	3.47 (m)[a]	4"	71.1	4.62 (m)[a]
5"	69.8	4.09 (m)[a]	5"	69.7	4.12 (m)[a]	5"	70.2	5.10 (m)[a]
6"	18.6	1.25 (m)[a]	6"	18.7	1.25 (d, *J* = 6.1)	6"	19.1	1.80 (d, *J* = 6.0)
	3'-*O*-*β*-D-Xylose			3"-*O*-*β*-D-Apiose			3"-*O*-acetyl-	
1"'	105.3	4.38 (t, *J* = 7.8)	1"'	111.9	5.19 (d, *J* = 2.9)	1"'	171.5	-
2"'	74.8	3.26 (m)[a]	2"'	78.0	3.99 (m)[a]	2"'	21.6	1.91 s
3"'	78.0	3.34 (m)[a]	3"'	65.4	3.61 s		4'-*O*-*α*-L-Rhamnose	
4"'	70.8	3.54 (m)[a]	4"'	80.30	-	1""	103.3	5.89 br s
5"'	67.0	3.28(m)[a] 3.94 (m)[a]	5"'	75.01	3.78 (d, *J* = 9.7) 4.08 (d, *J* = 9.7)	2""	73.0	4.70 (m)[a]
	24-*O*-6-deoxy-*β*-D-Allose*			3'-*O*-*β*-D-Xylose		3""	73.2	4.57 (m)[a]
1""	103.3	4.74 (d, *J* = 8.2)	1""	105.2	4.40 (d, *J* = 7.6)	4""	74.4	4.38 (m)[a]
2""	69.9	3.67 (dd, *J*=8.3, 3.3)	2""	74.8	3.23 (m)[a]	5""	70.8	4.97 (m)[a]
3""	73.3	3.99 (t, *J* = 3.4)	3""	77.9	3.33 (m)[a]	6""	19.0	1.66 (d, *J* = 6.0)
4""	73.5	3.42 (m)[a]	4""	70.8	3.45 (m)[a]			
5""	70.2	3.90 (m)[a]	5""	67.0	3.26 (m)[a] 3.92 (m)[a]			
6""	16.1	1.12 (d, *J* = 6.6)			21-*O*-*β*-D-Glucose			
			1""	105.4	4.20 (d, *J* = 7.3)			
			2""	75.04	3.48 (m)[a]			
			3""	72.4	3.49 (m)[a]			
			4""	70.2	3.80 (m)[a]			
			5""	76.7	3.49 (m)[a]			
			6""	62.5	3.67 (m)[a] 3.75 (m)[a]			
					24-*O*-*β*-D-6-deoxy-Allose*			
			1"""	103.2	4.74 (d, *J* = 8.3)			
			2"""	69.9	3.70 (dd, *J*=8.2, 3.3)			
			3"""	73.3	3.98 (t, *J*=3.3)			
			4"""	73.5	3.40 (m)[a]			
			5"""	70.3	3.90 (m)[a])			
			6"""	16.1	1.12 (d, *J* = 6.5)			

m[a] multiplet overlapped signals, * protons value multiplicity were assigned from 1D TOCSY spectrum.

Table 3 Estimation of total steroidal saponins content

Sample	Quantity (g)	Steroidal saponin yield (%)	Recovery (%)
Pellet-1	9.735	5.1 ± 1.23	0.062
Supernatant-2	0.902	10.6 ± 1.17	0.012
Pellet-2	8.212	88.3 ± 2.59	91.7

Table 4 Results of XP-docking using Glide, steroidal saponins isolated from *T. govanianum* were docked against acetylcholinesterase.

Ligand	Docking Score (kcal/mol)	Ligand	Docking Score (kcal/mol)
Donepezil	-8.083	*β*-Ecdysone	-9.057
Govanoside E	-10.849	Borassoside E	-7.669
Pregna-chacotrioside	-10.471	20-hydroxy- *β*-Ecdysone	-6.910
Protodioscin	-10.425	Pennogenin diglycoside	-6.636
Pennogenin tetraglycoside	-9.404	Borassoside D	-4.804
Clintoneoside B	-9.197	Pennogenin triglycoside	-4.550

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