Journal of Pharmacognosy and Phytochemistry
Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
www.phytojournal.com
JPP 2024; 13(3): 226-235
Received: 26-03-2024
Accepted: 29-04-2024

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Extraction of α glucosidase inhibitory glycoside from the roots of *Clerodendrum phlomidis* L.

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DOI: https://doi.org/10.22271/phyto.2024.v13.i3c.14964

Abstract
Background: Medicinal plants have therapeutic legacy from herbal ancestry as medicine since ancient times for various ailments. *Clerodendrum phlomidis* L. (Family Lamiaceae) is a sage glory bower, worthy shrub of south-east Asian countries with great ethno-therapeutic ponderosity in diverse indigenous cultures and for medication of numerous ailments including hyperglycemia. Having many traditional values isolation of active constituents has been aimed with *in vitro* α glucosidase inhibitory activity from roots of this plant.
Methods: A concentrated methanolic extract of the dried roots of plant with active fractions on subjection to silica gel loaded glass column A & B, packed with chloroform and methanol respectively, gradient flow of solvents leads isolation of phytoconstituents. A volume of 50 µL of each sample solutions and 50 µL of phosphate buffer having 0.1 U/ml, α-glucosidase solution was incubated and 50 µL and 2.5 mM solution of PNPG solution in phosphate buffer was added to each well for re-incubation at 37 °C. Reaction was completed by adding 100 µL of Na_2CO_3 (0.2 M) into each well and absorbance were recorded at 405 nm and compared with the control and standard for enzymatic α-glucosidase activity.
Results: Isolation of six new phytoconstituents characterized as stigmata-5-en-3β-olyl salicylate 2′-octadeca-9 ″,12 ″,15 ″-trienoate (β-sitosterol 3-*O*-salicylate 2′-linolenate, 1), *n*-octadec-9-enoyl *O*-β-*D*-galacturanopyranoside (2), (*Z*)-*n*-dotratriacont-24-enoic acid (3), methoxy arabinopyranosyl- (2→1')-arabinopyranosyl-(2'→1")-arabinopyranosyl-(2"→1"')- arabinopyranosyl-(2"'→1"")- glucopyranoside (4), *n*-docosanyl 6′-methoxy-α-*D*-glucopyraranosyl-(2′→1″)-α-*D*-6″-methoxy-glucopyranosyl-(2″→1‴)-α-*D*-glucopyraranosyl-(6‴→1⁗)- α- *D*-glucopyraranoside (5) and 6-methoxy-α-*D*-glucopyranosyl-(2→1′)-α-*D*- glucopyranosyl-(6′→1″) - α-*D*- glucopyranosyl- (6″→1‴)- α-*D*- glucopyranoside (6).
Conclusion: The compounds 5 and 6 exhibited significant α-glucosidase enzymatic inhibitory potential showing IC_{50} values (mg/ml) 1.52±0.14 and 0.78±0.02, respectively, against standard acarbose IC_{50} 0.55±0.03.

Keywords: *Clerodendrum phlomidis* L., Roots, Phytoconstituents, Isolation, Characterization, α-Glucosidase inhibition

Introduction
Irregular blood sugar is severe threat to global society and occurring due to change of life styles, food habits and stresses coined as diabetes. It is a complex metabolic disorder and also associated with several other complications. People dying due to diabetes are much higher than caused by diseases like cancer [1]. It is escalating rapidly in all socioeconomic groups of most countries, and the dominance of the illness will grow nearly 380 million populations by 2025 [2]. Of the total count, approximate 90% people suffer from type 2 diabetes [3]. Blood glucose is the main source of energy available in the food materials controlled by insulin hormone, produced in the pancreas, helps transformation of glucose to energy in the cells. The relative deficits in insulin regulation resulted in to improper balances in metabolism of carbohydrates [4]. Fast uptake of glucose needed in postprandial hyperglycemia, intestine and α-glucosidase plays significant contribution to hydrolysis to starch and other oligosaccharides [5]. Inhibition of this enzyme efficiently manages the postprandial emersion of blood sugar level. Therefore, a vital tactic for regulation of α-glucosidase enzyme activity to monitor the postprandial elevated sugar level [6]. Molecules such as acarbose have been tested clinically to regulate sugar levels more specifically for the postprandial conditions in type 2 diabetes cases [7] with few non anticipated side effects of gastrointestinal tract [8]. Therefore, effective inhibitors from natural molecules are required as a remedy for managing upcoming improper sugar level and adverse effects. In this sequence *Clerodendrum phlomidis* L., (syn. *C. multiflorum* Burm, family Lamiaceae), is a traditional medicinal plant distributed all over the world including India [9] and commonly known as arni, agnimantha and wind-killer.

It is used to treat various health disorders including hyperglycemia [10-13], syphilis, typhoid, cancer, jaundice and hypertension [14]. It is an ingredient of the Ayurvedic bitter tonic, Dashmula, taken orally to relieve fatigue after child birth [11], as a hepatoprotective[15] and Immunomodulatory [16]. In previous study some phytoconstituents have been reported [17] but first time isolation of new α-glucosidase inhibitory compounds have been targeted to validate its traditional therapeutic claims with safer alternative for the further researchers and cumulate essential data to develop more effective anti-diabetic agents to fulfill future expectations.

Materials and Methods

Chemicals and instruments

Infra Red (IR) spectra were recorded using KBr pellets on Jasco FT/IR-5000 Spectrometer instrument (FTS 135, Hong Kong). Ultra Violet spectra were determined on Lambda Bio 20 Spectrophotometer (Perkin Elmer, Schwerzenbach, Switzerland) in methanol as a solvent. The $^{[1]}$ H NMR (400 MHz) and $^{[13]}$C carbon NMR (100 MHz) spectra were scanned with Bruker ARX-Spectrometer (Rheinstetten, Germany), using $CDCl_3$ and DMSO-d_6 solvents and TMS (Fluka analytical, Sigma-Aldrich, Netherland) as an internal standard. The coupling constants (J values) are expressed in Hertz (Hz). Synapt-mass-spectrometric data was recorded with Q-TOF-ESI (Waters Corp., UK) with +ESI technique by affecting electron impact ionization at 70 eV on a Jeol SX-102 mass spectrometer equipped with direct inlet prob system. The *m/z* values of the more intense peaks are mentioned and the figures in bracket attached to each *m/z* values indicated relative intensities with respect to the base peak. Isolation operation was performed with glass column having silica gel (Qualigens, Mumbai, India, 60-120 mesh). The solvents used were purchased from Merck Specialties (E. Merck, Pvt. Ltd. New Delhi, India). Precoated TLC plates (silica gel 60 F_{254}, Merck, Darmstadt, Germany) were used for purification. Spots on TLC plates were visualized by exposing to iodine vapors and UV radiations (254 and 366 nm) and spraying with anisaldehyde-sulfuric acid solution. For enzymatic activity PNPG, α-glucosidase (EC 3.2.1.20) and acarbose were acquired from Sigma-Aldrich chemical company (St. Louis, USA) and micro-plate reader (Spectra Max M2/M2^e, Molecular device Co., Sunnyvale, CA, USA).

Collection and of plant material

The *Clerodendrum phlomidis* L. (CP) roots were obtained from the Herbal Garden, Jamia Hamdard, New Delhi and authenticated by taxonomist Prof. M. P. Sharma, Department of Botany, Faculty of Science, Jamia Hamdard. A voucher specimen of the drug sample was preserved in the Department of Pharmacognosy and Phytochemistry, School of Pharmaceutical Education and Research, Jamia Hamdard, New Delhi, with a reference no. PRL-JH/06/2011.

Preparation of crude extract and isolation

The dried and pulverized CP roots (3.5 kg) were extracted exhaustively with methanol (6L) in a Soxhlet apparatus. The dried dark brown viscous residue (210 g, yield 6.0%) was subjected to fractionation with *n*-hexane (3 × 500 ml, 5.0 g), chloroform (3 × 500 ml, 21.0 g) and equal ratio of chloroform-methanol (3 × 500 ml, 85.0 g) for assessment of activity. Fractions with significant activity was dissolved in suitable amount of chloroform (column A) and methanol (column B), and adsorbed over silica gel for column by mechanical process on a steam bath to obtain respective slurries. The air dried slurries was loaded over silica gel stationary phase, stabilized and saturated previously with chloroform to indigenously made glass columns of diameter 7 cm and height of 95 cm. The mobile phase was eluted with chloroform and combination of chloroform-methanol as 99:1, 3:1, 13:7 and 1:1 *v/v*, ratio to alienate the crucial compounds 1-6. Detailed layout of methodology was depicted in graphical abstract (Figure 1).

In vitro enzymatic α-glucosidase activity

The inhibitory activity of α-glucosidase was evaluated as mentioned[18-19] with trivial change as, a volume of 50 µL of sample solutions (extract, CP-5 and CP-6) and 50 µL of phosphate buffer (0.1 M, pH 6.8) having 0.1 U/ml, α-glucosidase solution and was incubated at 37 °C in 96 well plates for 10 min. After pre-incubation, 50 µL and 2.5 mM solution of PNPG solution in phosphate buffer was added to each well and incubated at 37 °C for next 20 min followed by reaction was completed by adding 100 µL of Na_2CO_3 (0.2 M) into each well, and absorbance readings (A) were recorded at 405 nm with micro-plate analyzer and compared with the control which had 50 µL of buffer solution in place of the extract and compounds (CP-5 and CP-6). The% inhibition was calculated by given expression and IC_{50} value was analyzed with respect to reference compound.

Inhibition (%) = (A control - A sample)/A control ×100.

Results

Isolation of phytoconstituents

β-Sitosterol 3-*O*-salicylate 2′-linolenate (1)

Chloroform elution, a yellow semi solid mass (1), recrystallized with acetone-methanol, 1:1, *v/v*), 4 g, 0.24% yield, R_f 0.6 (chloroform). UV λmax (MeOH); 203, 274 nm; IR υ_{max} (KBr): 2927, 2854, 1729, 1643, 1525, 1445, 1379, 1273, 1189, 1078, 889, 721 cm^{-1}; ^{1}H NMR ($CDCl_3$): Table 1; $^{[13]}$ C NMR ($CDCl_3$): Table-1; ESI MS *m/z* (rel. int): 794 $[M]^+$ ($C_{54}H_{82}O_4$) (2.2), 517 (3.8), 413 (4.7), 381 (51.6), 261 (4.7).

Oleiyl *D*-galacturanopyranoside (2)

Chloroform elution, yellowish sticky semisolid mass (**2**), purified from chloroform-methanol, 1:1, *v/v*, 01 g, 0.06% yielded, R_f 0.2 (chloroform). UV λ_{max} (MeOH): 207 nm (log ε 4.3); IR υ_{max} (KBr): 3416, 3310, 2927, 2853, 1721, 1698, 1635, 1445, 1381, 1274, 1192, 1078, 970, 721 cm^{-1}; ^{1}H NMR ($CDCl_3$): Table-2: $^{[13]}$ C NMR ($CDCl_3$): Table-3; ESI MS *m/z* (rel. int.): 458 $[M]^+$ ($C_{24}H_{42}O_8$) (11.3), 282 (43.1), 265 (8.5), 193 (3.8).

(*Z*)- *n*- Dotriacont-24- enoic acid (3)

Chloroform elution with methanol, 99:1, *v/v*, yellowish sticky mass (**3**), purified by preparative TLC using acetone-methanol (1:1, *v/v*) 01 g, 0.06% yield, R_f 0.8 (chloroform); UV λmax (MeOH): 205 nm (log ε 5.1); IR υ_{max} (KBr): 3285, 2923, 2853, 1709, 1642, 1461, 1282, 942, 725 cm^{-1}; ^{1}H NMR ($CDCl_3$): Table-2; $^{[13]}$ C NMR ($CDCl_3$): Table-3 ESI MS *m/z* (rel. int.): 478 $[M]^+$ ($C_{32}H_{62}O_2$) (34.1), 353 (28.2), 379 (23.1), 125 (14.5).

Methoxy tetraarabinosyl glucoside (4)

Chloroform-methanol elution at 3:1, *v/v*, a dark red semisolid mass (4), purified by preparative TLC with chloroform-methanol, 2:3 *v/v*, yielded 08 g, 0.48% yield, R_f: 0.75 in chloroform-methanol, 1:1, *v/v*; UV λ_{max} (MeOH): 210 nm (log ε 3.2). The IR υ_{max} (KBr): 3515, 3456, 3370, 3285, 2939, 2842, 1656, 1425, 1360, 1066 cm^{-1}; ^{1}H NMR (DMSO-d_6): Table-2; $^{[13]}$ C NMR (DMSO-d_6): Table-3; ESI MS *m/z* (rel. int.): 722

$[M]^+$ ($C_{27}H_{46}O_{22}$) (1.8), 295 (13.5), 279 (10.1), 179 (3.2), 163 (5.4), 147 (18.3).

Behenyl dimethoxytetraglucoside (5)

Chloroform-methanol elution with 13:7, *v/v*, a dark red semisolid stuff (5); purified by preparative TLC with chloroform-methanol, 1:1 *v/v*, 04 g, 0.24% yield, R_f : 0.5 in chloroform-methanol, 1:1, *v/v*. UV λ_{max} (MeOH): 211 nm (log ε 3.1). The IR υ_{max} (KBr): 3510, 3413, 3257, 2974, 2842, 1722, 1632, 1456, 1365, 1180, 1047, 877, 720 cm^{-1}; ^1H NMR (DMSO-d_6): Table 2; $^{[13]}$ C NMR (DMSO-d_6): Table-3; ESI MS *m/z* (rel. int.): 1016 $[M]^+$ ($C_{48}H_{88}O_{22}$) (1.3), 677 (34.1), 515 (58.5), 501 (1.9), 342 (9.8), 339 (62.6), 179 (7.2).

Methoxy -*α*-*D*-tetraglucopyranoside (6)

Chloroform-methanol elution with 1:1, *v/v*, a pale yellow semisolid mass (**6**), purified with preparative TLC, chloroform-methanol, 1:1 *v/v*, 1.98 g, 0.12% yield, R_f : 0.2 (chloroform-methanol, 1:1 *v/v*); UV λ_{max} (MeOH): 208 nm (log ε 3.1); IR υ_{max} (KBr): 3490, 3427, 3371, 3286, 2927, 1617, 1390, 1048, 850 cm^{-1}; ^1H NMR (DMSO-d_6): Table 2; $^{[13]}$ C NMR (DMSO-d_6): Table-3. ESI MS *m/z* (rel. int.): 680 $[M]^+$ ($C_{25}H_{44}O_{21}$) (60.3), 503 (18.1), 341 (11.2), 193 (15.6), 179 (8.2).

Structure elucidation and identification

Compound 1 was obtained as a yellow semisolid mass from chloroform eluants. It showed a UV absorption at 274 nm for aromaticity and characteristic IR absorption bands for ester group (1729 cm^{-1}), unsaturation (1643 cm^{-1}), aromatic ring (1525, 1078 cm^{-1}) and long aliphatic chain (721 cm^{-1}). On the basis of mass and ^{13}C NMR spectral data the molecular ion peak of 1 was determined at *m/z* 794 $[M]^+$ consistent with the molecular formula of an aromatic ester, $C_{54}H_{82}O_4$. The ion peaks arising at *m/z* 413 [CO-O′ fission, $C_{29}H_{49}O]^+$, 517 $[C_{29}H_{49}O\text{-}C_7H_4O]^+$ and 261 [M-533, CO″-O′ fission, $CH_3(CH_2CH{=}CH)_3(CH_2)_7CO]^+$ suggested that salicylic acid is esterified with sterol ring and linolenic acid. The ^1H NMR spectrum of 1 displayed two one-protons doublet at δ 7.53 (*J*=8.7 Hz) and 7.13 (*J*=8.5 Hz) and two one-proton multiplets at δ 7.34 and δ 5.96 each accounted to aromatic H-3′, H-6′, H-4′ and H-5′ protons, respectively. Seven one-proton multiplets at δ 5.78 (H-12″), 5.34 (H-6), 5.31 (H-13″), 5.19 (H-10″), 5.01 (H-15″), 4.94 (H-9″), 4.90 (H-16″) were ascribed to the vinylic protons. A one-proton broad multiplet at δ 4.26 with half width of 18.5 Hz was attributed to *α*-oriented oxymethine H-3 proton. Two three-proton broad singlets at δ 1.01 and 0.67 were assigned to tertiary C-19 and C-18 methyl protons, respectively. Three doublets at δ 0.96 (*J*=6.1 Hz), 0.86 (*J*=6.3 Hz) and 0.84 (*J*=6.5 Hz) and two triplets at 0.79 (*J*=6.1 Hz) and 0.81 (6.2), each integrating for three protons each, were associated correspondingly with the secondary C-21, C-26 and C-27 and primary C-29 and C-18″ methyl protons. The remaining methylene and methine protons resonated between δ 2.75- 1.12. The ^{13}C NMR spectrum of 1 showed signals for aromatic and vinylic carbons between δ 156.16-109.58, oxymethine carbon at δ 71.75 (C-3), ester carbons at δ 174.15 (C-1) and 173.81 (C-1) and methyl carbons in the range of δ 20.83 - 14.14. Acid hydrolysis of 1 yielded linolenic acid, β-sitosterol and salicylic acid, co-TLC comparable.[20] On the basis of spectral data analysis and chemical reactions, the structure of 1 has been formulated as stigmata-5-en-3β-olyl salicylate 2′- octadeca-9″, 12″, 15″-trienoate (β-sitosterol 3-*O*-salicylate 2′-linolenate), and a new β-sitosterol salicylate ester (Fig. 3).

Compound 2, designated as oleiyl *D*-galacturanopyranoside, was obtained as a yellowish semisolid mass from chloroform-methanol (1:1, *v/v*) eluants. It responded positively to tests of glycosides and showed IR absorption bands for hydroxy1 groups (3416 cm^{-1}), ester function (1721 cm^{-1}), carboxylic group (3310, 1698 cm^{-1}), unsaturation (1635 cm^{-1}) and long aliphatic chain (721 cm^{-1}). Its molecular weight was established at *m/z* 458 on the basis of mass and ^{13}C NMR spectra consistent with the molecular formula of a fatty acid hexoside, $C_{24}H_{42}O_8$. The ion peaks arising at *m/z* 265 [C-O fission, $CH_3(CH_2)_7CH{=}CH(CH_2)_7CO]^+$, 281 [O-$C_{1'}$ fission, $CH_3(CH_2)_7CH{=}CH(CH_2)_7COO]^+$ and M-281 [193, $C_6H_9O_7]^+$ suggested that oleic acid was esterified with the hexanoic acid. The ^1H NMR spectrum of 2 showed two one-proton multiplet at δ 5.27 and δ 5.13 assigned to vinylic H-9 and H-10 protons and a one-proton doublet at δ 4.94 (*J*=7.3 Hz) ascribed to anomeric H-1′. The other sugar protons resonated between δ 4.68- 4.28. A two-proton triplet at δ 2.23 (*J*=9.3 Hz) and two multiplets at δ 2.13 and 1.95 integrating for two protons each were ascribed to methylene H_2-2 protons adjacent to ester group and to H_2-8 and H_2-11 protons, nearby the vinylic carbons, respectively. The other methylene protons were resonated between δ 1.67-1.18. A three-proton triplet at δ 0.85 (*J*=6.2 Hz) was accounted to C-18 primary methyl protons. The $^{[13]}$ C NMR spectrum of 2 displayed signals for ester carbon at 172.16 (C-1), vinylic carbons at δ 130.02 (C-9) and 127.92 (C-10), anomeric carbon at δ 108.31 (C-1′), carboxylic group δ 179.03 (C-6′), other sugar carbons between δ 71.79 - 62.10, methylene carbons in the range of δ 41.02- 22.68 and methyl carbon at δ 14.15 (C-18). Acid hydrolysis of 2 yielded oleic acid, m.p. 13-14 °C, R_f 0.34 (glacial acetic acid, 85%) and β-*D*-galacturonic acid, m.p. 159 °C, R_f 0.17 (*n*-butanol- pyridine - water, 3:2:1.5). On the basis of spectral data analysis and chemical reactions, the structure of 2 has been formulated as *n*-octadec-9-enoyl *O*-β-*D*-galacturanopyranoside, a new fatty acid glycoside (Fig. 3).

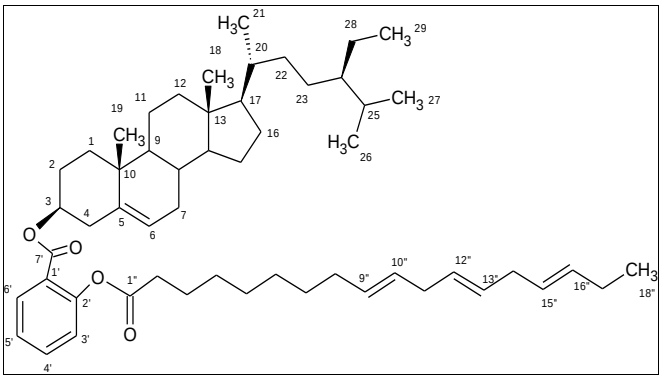
Compound 3 was obtained as a pale yellow sticky mass from chloroform-methanol (99:1, *v/v*) eluants. It yielded effervescence with sodium bicarbonate and showed distinctive IR absorption bands for a carboxylic group (3285, 1709 cm^{-1}), unsaturation (1642 cm^{-1}) and long aliphatic chain (725 cm^{-1}). Its mass spectrum showed a molecular ion peak at *m/z* 478 $[M]^+$ consistent with the molecular formula of an unsaturated fatty acid, $C_{32}H_{62}O_2$. The ion fragments generating at *m/z* 125 [C_{23} - C_{24} fission, $CH_3(CH_2)_6CH{=}CH]^+$, 353 [M-125; $(CH_2)_{22}COOH]^+$ and 379 [C_{25} - C_{26} fission, $CH{=}CH(CH_2)_{22}COOH]^+$ suggested the location of the vinylic linkage at C-24. The ^1H NMR spectrum of 3 exhibited two one-proton multiplets at δ 5.15 ($w_{1/2}$=10.2) and 5.10 ($w_{1/2}$=9.6) each assigned to cis-oriented vinylic H-24 and H-25 protons, respectively. A two- proton triplet at δ 2.09 (*J*=7.5 Hz) was ascribed to methylene H_2-2 protons adjacent to the carboxylic function, other methylene protons from δ 1.80 to 1.01 and a three-proton triplet at δ 0.63 (*J*= 5.7 Hz) accounted for the terminal C-32 primary methyl protons. The ^{13}C NMR spectrum of 3 exhibited signals for the carboxylic carbon at δ 179.21 (C-1), vinylic carbons at δ 129.94 (C-24) and 127.87 (C-25), methylene carbons between δ 34.02- 22.63 and methyl carbon at δ 14.06 (C-32). On the basis of the spectroscopic studies, the structure of 3 was fully established and determined as (*Z*)-*n*-dotratriacont-24-enoic acid. This is a new fatty acid reported from this plant (Fig. 3).

Compound 4, designated as methoxy tetraarbinosyl glucoside, and was obtained as a dark red semisolid mass from chloroform-methanol (3:1, *v/v*) eluants. It gave positive tests

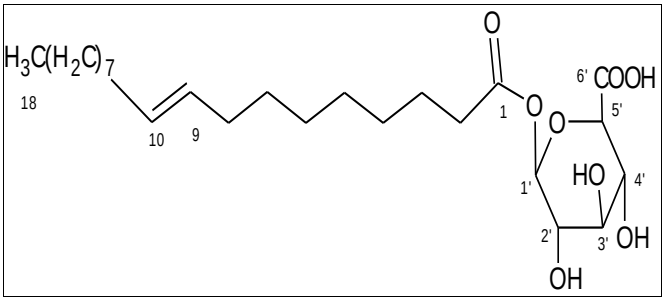
for glycosides and displayed characteristic IR absorption bands for hydroxyl groups (3515, 3456, 3370, 3285 cm^{-1}). On the basis of mass and [13] C NMR spectral data the molecular ion peak of 4 has been determined at *m/z* 722 $[M]^+$ corresponding to a molecular formula of a methoxy tetraarbinosyl glycoside, $C_{27}H_{46}O_{22}$. The ion fragments generating at *m/z* 147 ($C_6H_{11}O_4$), 163 ($C_6H_{11}O_5$), 179 [M-543, $C_6H_{11}O_6$], 279 ($C_6H_{11}O_5$-$C_5H_8O_4$) and *m/z* 295 ($C_6H_{11}O_5$-$C_5H_8O_5$) suggested that methoxy tetraarabinosyl was linked with a glucose unit. The ^1H NMR spectrum of 4 exhibited five one-proton doublets at δ 5.22 (*J*=*7*.3 Hz), 4.99 (*J*=*7*.1 Hz), 4.93 (*J*=*7*.5 Hz), 4.68 (*J*=*7*.8 Hz) and 4.29 (*J*=*7*.5 Hz) anomeric H-1, H-1', H-1", H-1'" and H-1"" protons, respectively. The oxymethine sugar protons appeared between δ 4.10- 3.40. Two two-proton multiplets at δ 3.35 and δ 3.32 were accounted to oxymethylene H_2-5 and H_2-5' protons, and a four-proton multiplet at δ 3.26 were due to methylene H_2-5" and H_2-5'" protons, respectively. A three-proton broad singlet was ascribed to methoxy protons. A two-proton double doublet at δ 3.01 (*J*=*7*.8, 8.4 Hz) was assigned to hydroxymethylene H_2-5"" protons. The ^{13}C NMR spectrum of 4 exhibited signals for the anomeric carbons at δ 104.64 (C-1), 102.25 (C-1'), 98.42 (C-1"), 97.07 (C-1'") and 92.49 (C-1""), other sugar protons appeared between δ 83.07- 61.44, and methoxy carbon signal at δ 50.03. The presence of ^1H NMR signals in the downfield region at δ 4.03 (H-2), 3.98 (H-2'), 3.92 (H-2") and 3.73 (H-4'") and carbon signals at δ 83.07, 82.06, 81.46 and 76.42 suggested the attachment of sugar units with (2→1'), (2'→1"), (2"→1'") and (4'"→1"") linkages. Acid hydrolysis of 4 produced β-*D*-arabinose, R_f 0.42 (*n*-butanol-pyridine-water, 3:1:1) and D-glucose, R_f 0.26 (*n*-butanol-acetic acid - water, 4:1:5). On the basis of the above mentioned discussion the structure of 4 has been elucidated as methoxy arabinopyranosyl- (2→1')-arabinopyranosyl-(2'→1")-arabinopyranosyl-(2"→1'")- arabinopyranosyl-(2'"→1"")-glucopyranoside. This is a new pentaglycoside isolated from this plant (Fig. 3).

Compound 5, named as behenyl dimethoxytetraglycoside, was obtained as a red sticky semisolid mass from chloroform: methanol (13:7, *v/v*) eluants. It responded glycosidic tests positively and showed IR absorption bands for hydroxyl groups (3510, 3413, 3257 cm^{-1}), ester group (1722 cm^{-1}), and long chain aliphatic hydrocarbon (720 cm^{-1}). On the basis of its mass and ^{13}C NMR spectrum its molecular ion peak was determined as *m/z* 1016 in accordance with the molecular formula of an acyl tetraglycoside $C_{48}H_{88}O_{22}$. The ion peak generated at *m/z* 339 $[CH_3\ (CH_2)_{20}CO]^+$ and 667 $[M\text{-}339]^+$ suggested that behenic acid was esterified with a dimethoxy tetraglycoside unit. The ion fragments produced at *m/z* 179 $[C_6H_{11}O_6]^+$, 342 $[C_6H_{11}O_6\text{-}C_6H_{11}O_4]^+$, 515 [M-501, $CH_3(CH_2)_{20}CO$- $C_6H_9O_4$-OMe]$^+$ indicated that a C_6 sugar unit was linked in the tetraglycoside chain and the methoxy sugar units were located nearby the acyl unit. The ^1H NMR spectrum of 5 exhibited four one-proton doublets at δ 5.18 (*J*=3.6 Hz), 4.91 (*J*=2.8 Hz), 4.88 (*J*=3.4 Hz) and 4.80 (*J*=4.3 Hz) assigned to α-oriented anomeric H-1′, H-1″, H-1‴ and H-1⁗ protons, respectively. The other sugar protons appeared as multiplets from δ 4.26 to 3.34 as one-proton doublets at δ 3.29 (*J*=6.9 Hz, H_2-6'a), 3.26 (*J*=6.9 Hz, H_2-6'b), 3.24 (*J*=6.9 Hz, H_2-6″a), 3.22 (*J*=6.9 Hz, H_2-6″b), 3.19 (*J*=5.7 Hz, H_2-6'"a), and 3.16 (*J*=4.8 Hz, H_2-6'"b) and as a two-proton double doublet at δ 3.07 (*J*=10.8, 11.1 Hz, H_2-6""). Two three-proton broad singlets δ 3.15 and 3.11 were ascribed to the methoxy protons. A two-proton triplet at δ 2.69 (*J*=9.3 Hz) was attributed to methylene H_2-2 protons adjacent to the ester group. The other methylene protons resonated as two-proton multiplets at δ 2.15, 1.78 and 1.31 and as broad singlets at δ 1.29 (12H) and 1.20 (20H). A three-proton triplet at δ 0.86 (*J*=6.8 Hz) was accounted to terminal C-22 primary methyl protons. The ^{13}C NMR spectrum of 5 displayed signals for ester carbon at δ 174.18 (C-1), anomeric carbons at δ 104.40 (C-1′), 102.41 (C-1″), 100.07 (C-1‴) and 98.63 (C-1⁗), other sugar carbons between δ 82.37- 61.33, methoxy carbons at δ 56.09 and 55.77, methyl carbon at δ 14.35 (C-22) and methylene carbons from δ 49.06 to 22.53. The presence of oxymethine H-2′ signal at δ 4.26 and H-2″ at δ 4.20 in the deshielded region of ^1H NMR spectrum and C-2′ at δ 82.37 and C-2″ at δ 81.48 in the ^{13}C NMR spectrum suggested (2′→1″), and (2″→1‴) linkage of two sugar units. The existence of oxymethylene protons in the downfield region between δ 3.29 - 3.16 and carbon signals at δ 63.32 (C-6′), 63.51 (C-6″) and 63.75 (C-6‴) indicated location of methoxy groups at C-6′ and C-6″ and attachment of terminal sugar unit at C-6‴. Acid hydrolysis of 5 yielded behenic acid, m. p. 79-80 ºC and *D*-glucose, R_f 0.26 (*n*-butanol- acetic acid - water, 4: 1: 5). On the basis of above discussion the structure of 5 has been established as *n*-docosanyl 6′-methoxy-α-*D*-glucopyraranosyl-(2′→1″)-α-*D*-6″-methoxy-glucopyranosyl-(2″→1‴)-α-*D*-glucopyraranosyl-(6‴→1⁗)- α- *D*- glucopyraranoside. It is a new acyl tetraglycoside reported from *C. phlomidis* roots (Fig. 3).

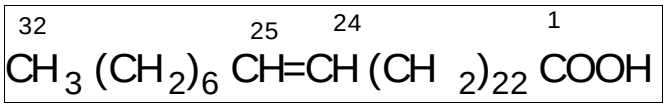
Compound 6, named methoxy-α-*D*-tetraglucoside, was obtained as a pale yellow mass from chloroform- methanol (1:1, *v/v*) eluants. It gave positive tests for carbohydrates and displayed characteristic IR absorption bands for hydroxyl groups at 3490, 3427, 3371 and 3286 cm^{-1}. The mass spectrum of 6 showed a molecular ion peak at *m/z* 680 corresponding to a molecular formula of a tetrasaccharide, $C_{25}H_{44}O_{21}$. The ion fragments arising at *m/z* 179 $[C_6H_{11}O_6]^+$, 341 $[C_6H_{11}O_6\text{-}C_6H_{10}O_5]^+$, 503 $[C_6H_{11}O_6\text{-}C_6H_{10}O_5\text{-}C_6H_{10}O_5]^+$ and 193 [M - 487, $C_7H_{13}O_6]^+$ indicated that three glucose units are attached to a methoxy glucose unit. The ^1H NMR spectrum of 6 exhibited four one-proton doublets at δ 5.65 (*J*=3.7 Hz), 5.38 (*J* = 3.2 Hz), 5.19 (*J*=4.2 Hz) and 4.70 (*J*= 4.2 Hz) assigned to α-oriented anomeric H-1, H-1′, H-1″ and H-1‴ protons, respectively. The other sugar carbinol protons appeared between δ 4.61-3.60. A three-proton broad singlet at δ 3.90 was assigned to the methoxy protons. The oxygmethylene protons resonated as doublets from δ 3.56 to 3.08. The ^{13}C NMR spectrum of 6 displayed signals for anomeric carbons at δ 104.36 (C-1), 101.67 (C-1′), 98.89 (C-1″) and 92.15 (C-1‴) and other sugar carbons between δ 82.52 to δ 60.98. The existence of the oxymethine H-2 proton at δ 4.61 in the ^1H NMR spectrum and ^{13}C carbon signal at δ 82.52 (C-2) in the deshielded region suggested (2→1′) linkage of the first two sugar units. The presence of oxymethylene H_2-6′and H_2-6″ signals in the downfield region at δ 3.42 (2H, d, *J*=9.2 Hz, H_2-6′), 3.26 (2H, d, *J*=9.2 Hz, H_2-6″) and respective carbon signals at δ 62.68 (C-6′) and 62.63 (C-6″) indicated (6′→1″) and (6″→1‴) linkages of the last sugar units. Acid hydrolysis of 6 yielded *D*-glucose, R_f 0.26 (*n*-butanol- acetic acid - water, 4: 1: 5). On the basis of spectral data analysis and chemical reactions, the structure of 6 was formulated as 6-methoxy-α-*D*-glucopyranosyl-(2→1′)-α-*D*- glucopyranosyl-(6′→1″) - α-*D*-glucopyranosyl- (6″→1‴)- α-*D*- glucopyranoside. It is a new tetraglucoside isolated from the *C. phlomidis* roots for the first time (Fig. 3).



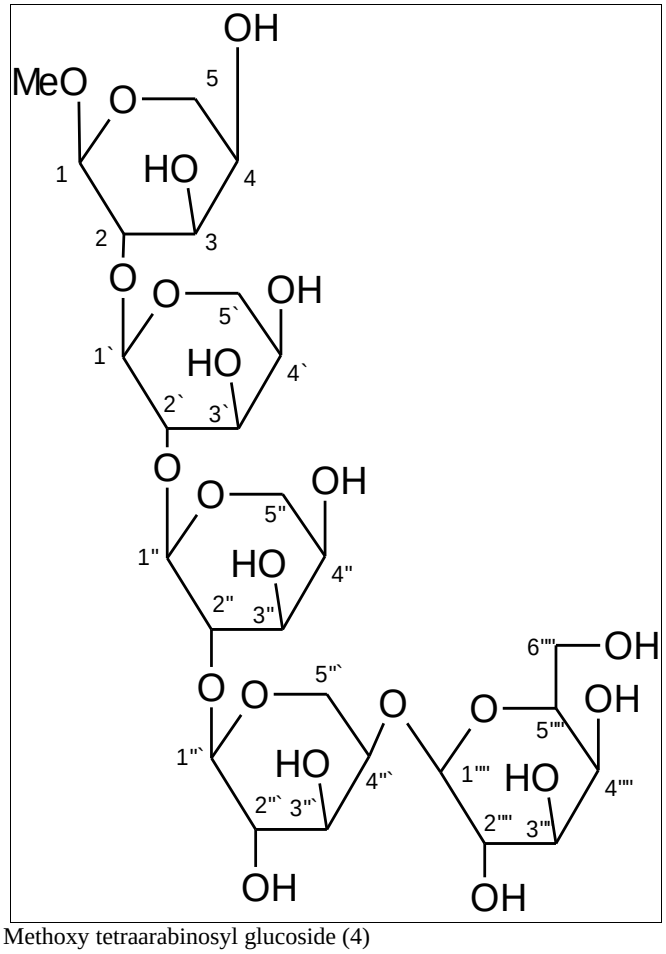
β-Sitosterol 3-*O*-salicylate 2′-linolenate (1)



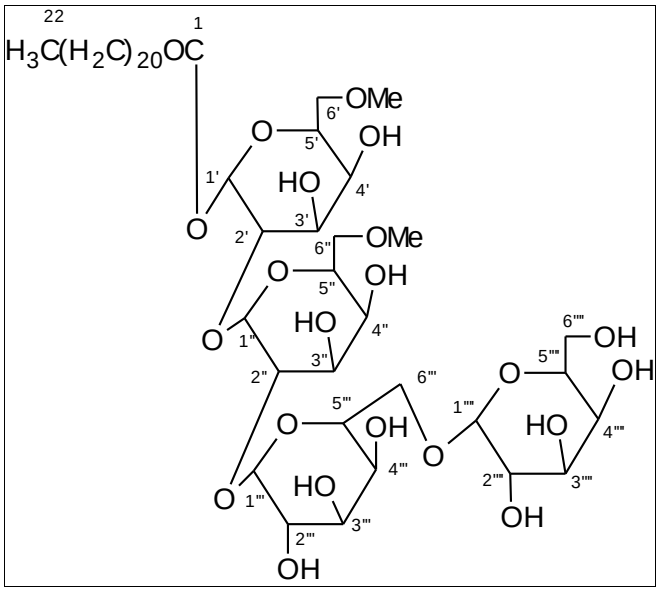
leiyl *D*-galacturanopyranoside (2)



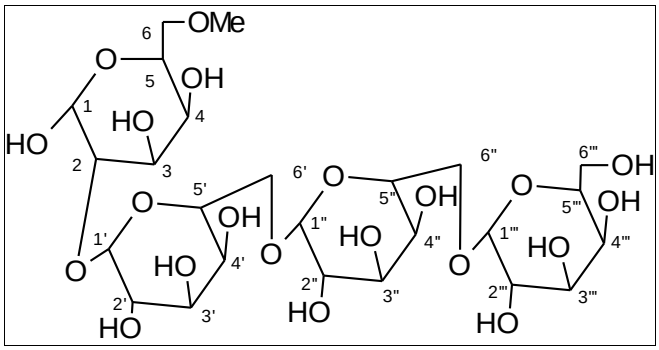
(*Z*)- *n*- Dotriacont-24- enoic acid (3)



Methoxy tetraarabinosyl glucoside (4)



Behenyl dimethoxy tetraglycoside (5)



Methoxy-α-D-tetraglucoside (6)

Fig 1: Chemical constituents of 1-6 isolated from the roots of *Clerodendrum phlomidis.*

A-glucosidase inhibitory enzymatic activity

On the basis of history and ethno-medical claims *in vitro* α-glucosidase inhibitory activity of methnolic root extracts and different fractions of plant, having diverse nature of compounds, were assayed in quest of possibility of compounds and activity (Fig 1). The fractions having optimum sign of activity were subjected to isolation process to get possible leads. Spectral data analysis exhibited role of new compounds predominantly altering the enzymatic activity. Its action again tested against standard acarbose for therapeutic validation. The enzymatic inhibitory activities of compounds 5 and 6 were established with IC_{50} value (mg/ml) 1.52±0.14 and 0.78±0.02 respectively against standard acarbose, IC_{50} 0.55±0.03 (Fig 2). Compound 6 has greater possibility in future as an alternative having closer inhibitory concentration with acarbose.

Discussion

Phytoconstituents from natural resources are foundation of drug development since ancient period and used extensively as a tool for crude information. They provide pure and accurate lead substances for treating various diseases to mankind.[21] Because of long evolutionary selection process, more complex and wider structural diversity [22], as well as activity of natural products including flavonoids, xanthones, triterpenoids, alkaloids, glycosides, alkyl disulfides, amino butyric acid derivatives, guanidine, polysaccharides and peptides have

strong probability in various disorders like hyperglycemia [23], and nervous and musculoskeletal related complications, neuralgia, constipation, appetizer and digestive [27]. Even development of dimethyl biguanide (metformin) was happened due to attention paid on *Galega officinalis*, was source of galegine stablished a backbone for synthesis of most effectively used first line antidiabetic drug again shaped major attention towards herbal drugs for challenges. Almost 2,50,000 higher medicinal plants have reported and less than 1% have been screened in depth for any biological screening. A sensible rationale that medicinal plants with a long account of human use will eventually more trust for novel drug prototypes, systematic and intensive searches for new drugs to treat hyperglycemia seems to be a great utility in future [24]. Natural compounds and their structural analogues also have a complex symbiotic and joint evolutionary history that provides nourishment, therapeutic agent and energy [25], have made a major contribution to pharmaceutical industry as an alternative tool to fill the demands and challenges with sustained technical barriers to screening, isolation, characterization and optimization, which contributed to a time-line in their pursuit [26]. Even in COVID pandemic few traditional medicines again groped for its antiviral and immune-modulatory behavior to minimize global crisis again enticed anticipation for researcher [28]. Therefore plants have considerable magnitude of application in food, fodder and medicines [29].

List of figures

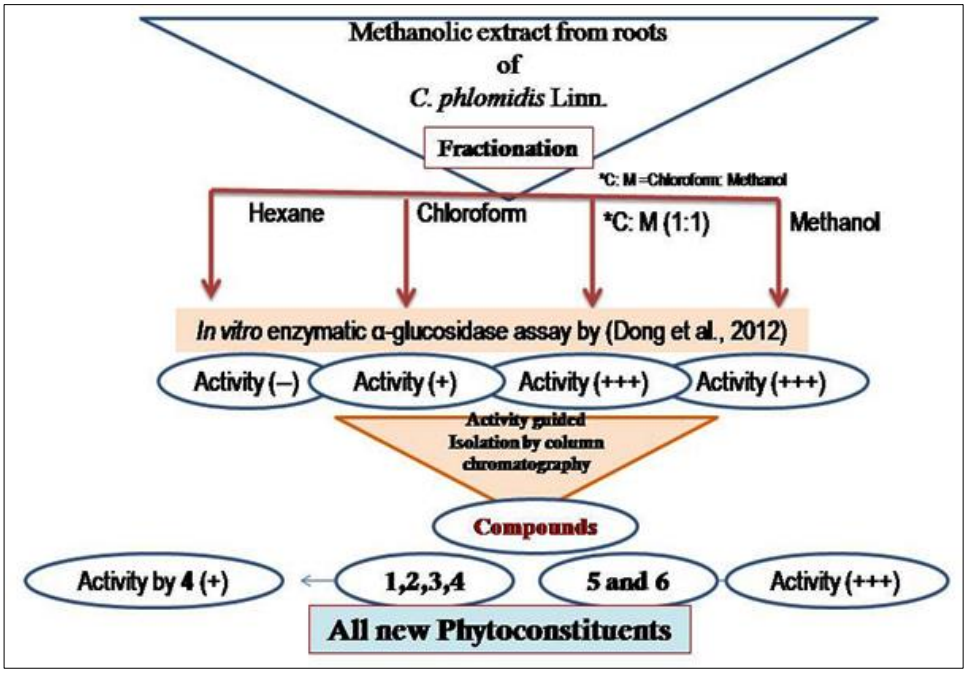


Fig 1: Graphical abstract portraying schematic isolation methodology

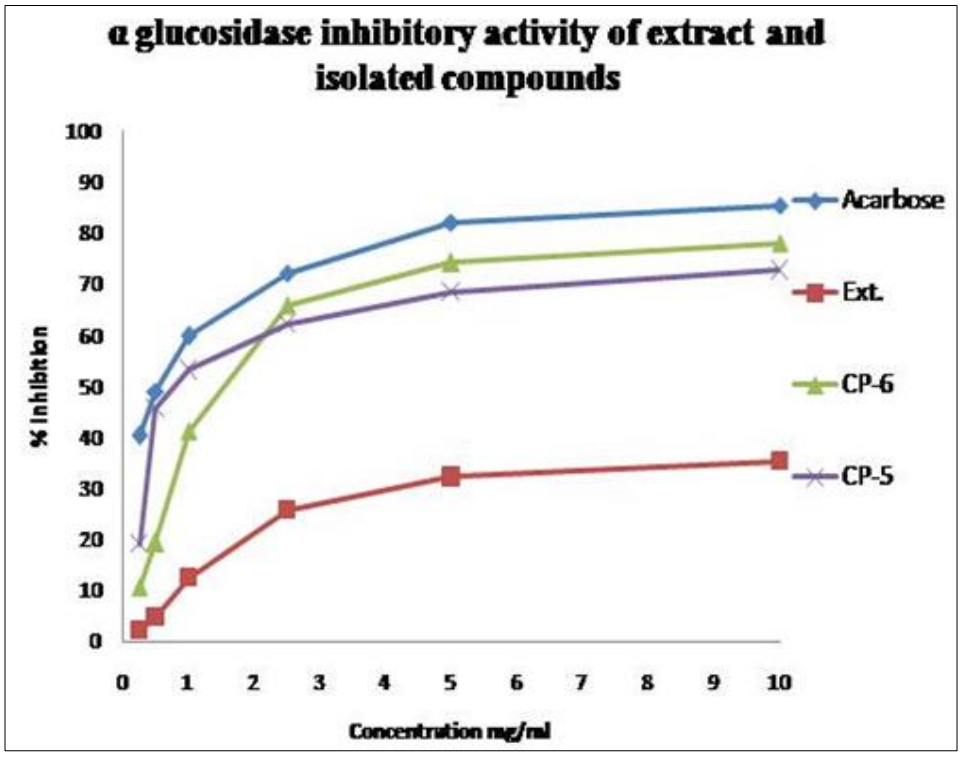


Fig 2: Alfa glucosidase inhibitory activity

List of tables attached

Table 1: ^{13}C NMR and ^{1}H NMR data (chemical shift, δ) of compounds 1.

Position	1		Position	1	
	^{13}C	^{1}H		^{13}C	^{1}H
1	39.69	1.47 m	1'	148.48	--
2	31.45	1.58 m	2'	158.16	--
3	71.75	4.26 brm (18.5)	3'	147.07	7.53 d (8.7)
4	42.25	2.34 m	4'	123.95	7.34 m
5	140.76	--	5'	119.10	5.96 m
6	121.62	5.34 m	6'	137.17	7.13 d (8.5)
7	31.65	2.06 m	7'	174.15	-
8	36.50	1.68 m	1"	173.81	--
9	51.96	1.57 m	2"	34.09	2.53 t
10	37.01	--	3"	31.91	1.64 m
11	24.33	1.53 m	4"	30.21	1.55 m
12	40.15	1.50 m	5"	29.69	1.29 m
13	50.05	--	6"	29.51	1.29 m
14	56.84	1.48 m	7"	29.51	1.32 m
15	25.54	1.61 m	8"	29.49	1.96 m
16	29.14	1.88 m	9"	130.18	4.94 m
17	55.90	1.46 m	10"	130.90	5.19 m
18	12.07	0.67 brs	11"	34.85	2.75 m
19	20.83	1.01 brs	12"	128.05	5.78 m
20	41.17	1.62 m	13"	124.46	5.31 m
21	20.25	0.96 d (6.1)	14"	34.50	2.71 m
22	33.81	1.27 m	15"	114.12	5.01 m
23	27.20	1.16 m	16"	109.58	4.90 m
24	27.21	1.39 m	17"	22.57	1.98 m
25	29.35	1.58 m	18"	14.14	0.81 t (6.2)
26	19.18	0.86 d (6.3)	-	-	-
27	19.41	0.84 d (6.5)	-	-	-
28	24.94	1.12 m			-
29	12.16	0.79 t (6.1)	-	-	-

Table 2: ^{1}H NMR spectral data (chemical shift, δ) of compounds 2-6.

Position	2	3	4	5	6
1	-	-	5.22 d (7.3)	--	5.65 d (3.7)
2	2.23 t (9.3)	2.09 t (7.5)	4.03 m	2.69 t (9.3)	4.61 m
3	1.67 m	1.36 m	3.68 m	2.15 m	3.99 m
4	1.18 brs	1.07 brs	3.43 m	1.74 m	3.88 m
5	1.18 brs	1.07 brs	3.35 m	1.31 m	4.51 m
6	1.18 brs	1.07 brs	--	1.29 brs	3.56 d (9.50), 3.49 d (10.1)
7	1.33 m	1.07 brs	--	1.29 brs	--
8	2.13 m	1.07 brs	--	1.29 brs	--
9	5.27 m	1.07 brs	--	1.29 brs	--
10	5.13 m	1.07 brs	--	1.29 brs	--
11	1.95 m	1.07 brs	--	1.29 brs	--
12	1.52 m	1.07 brs	--	1.29 brs	--
13	1.18 brs	1.07 brs	--	1.20 brs	--
14	1.18 brs	1.01 brs	--	1.20 brs	--
15	1.18 brs	1.01 brs	--	1.20 brs	--
16	1.18 brs	1.01 brs	--	1.20 brs	--
17	1.18 brs	1.01 brs	--	1.20 brs	--
18	0.85 t (6.2)	1.01 brs	--	1.20 brs	--
19	--	1.01 brs	--	1.20 brs	--
20	--	1.01 brs	--	1.20 brs	--
21	-	1.07 brs	--	1.20 brs	--
22	--	1.36 m	--	0.86 t (6.8)	--
23	--	1.80 m	--	--	--
24	--	5.15 m ($w_{1/2}$=10.2)	--	--	--
25	--	5.10 m ($w_{1/2}$=9.6)	--	--	--
26	--	1.76 m	--	--	--
27	--	1.07 brs	--	--	--
28	--	1.07 brs	--	--	--
29	--	1.01 brs	--	--	--
30	--	1.01 brs	--	--	--
31	--	1.01 brs	--	--	--

32	--	0.63 t (5.7)	--	--	--
1'	4.94 d (7.3)	--	4.99 d (7.1)	5.18 d (3.6)	5.38 d (3.2)
2'	4.34 m	--	3.98 m	4.26 m	4.23 m
3'	4.28 m	--	3.61 m	3.76 m	3.99 m
4'	4.68 m	--	3.40 m	3.44 m	3.82 m
5'	4.62 m	--	3.32 m	4.16 m	4.51 m
6'		--	--	3.29 d (6.9) 3.26 d (6.9)	3.42 d (9.2)
1"	--	--	4.93 d (7.5)	4.91 d (2.8)	5.19 d (4.2)
2"	--	--	3.92 m	4.20 m	4.23 m
3"	--	--	3.58 m	3.73 m	3.96 m
4"	--	--	3.40 m	3.41 m	3.72 m
5"	--	--	3.26 m	4.13 m	4.43 m
6"	--	--	--	3.24 d (6.9) 3.22 d (6.9)	3.26 d (9.2)
1"'	--	--	4.68 d (7.8)	4.88 d (3.4)	4.70 d (4.2)
2"'	--	--	3.85 m	3.87 m	4.20 m
3"'	--	--	3.55 m	3.63 m	3.96 m
4"'	--	--	3.73 m	3.37 m	3.60 m
5"'	--	--	3.26 m	4.07 m	4.43 m
6"'	--	--	--	3.19 d (5.7) 3.16 d (4.8)	3.15 d (8.1) 3.08 d (8.4)
1""	--	--	4.29 d (7.5)	4.80 d (4.3)	--
2""	--	--	3.80 m	3.82 m	--
3""	--	--	3.50 m	3.55 m	--
4""	--	--	3.39 m	3.34 m	--
5""	--	--	4.10 m	4.03 m	--
6""	--	--	3.01 dd (7.8, 8.4)	3.07 dd (10.8, 11.1)	--
OMe	--		3.13 brs	3.15 brs	3.90 brs
OMe	--	--	--	3.11 brs	--

Coupling constants in Hz are provided in parenthesis.

Table 3: ^{13}C NMR data (chemical shift, δ) of compounds 2-6.

Position	2	3	4	5	6
1	172.16	179.21	104.64	174.18	104.36
2	41.21	34.02	83.07	49.06	82.52
3	24.32	29.63	75.12	31.73	71.38
4	24.72	29.55	70.79	31.03	68.81
5	27.20	29.55	64.53	29.44	77.18
6	29.68	29.55	--	29.43	63.41
7	29.68	29.55	--	29.41	--
8	37.68	29.55	--	29.88	--
9	130.02	29.55	--	29.46	--
10	127.92	29.55	--	29.48	--
11	37.24	29.55	--	29.45	--
12	33.95	29.55	--	29.41	--
13	31.66	29.55	--	29.36	--
14	31.44	29.55	--	29.30	--
15	29.50	29.55	--	29.22	--
16	30.20	29.46	--	29.13	--
17	22.68	29.40	--	28.97	--
18	14.15	29.31	--	29.10	--
19	--	29.26	--	27.08	--
20	--	29.21	--	24.37	--
21	--	29.12	--	22.53	--
22	--	29.03	--	14.35	--
23	--	31.87	--	--	--
24	--	129.94	--	--	--
25	--	127.87	--	--	--
26	--	31.47	--	--	--
27	--	29.55	--	--	--
28	--	27.15	--	--	--
29	--	25.58	--	--	--
30	--	24.67	--	--	--
31	--	22.63	--	--	--
32	--	14.06	-	--	--
1'	108.31	--	102.25	104.40	101.67
2'	70.08	--	82.06	82.37	73.27

3'	64.46	--	73.39	70.98	70.42
4'	62.10	--	70.58	71.15	66.44
5'	71.79	--	63.94	77.33	76.83
6'	179.03	--	--	63.32	62.63
1"	--	--	98.42	102.41	98.89
2"	--	--	81.46	81.48	72.45
3"	--	--	72.52	70.72	70.01
4"	--	--	70.21	69.07	67.04
5"	--	--	63.48	77.19	76.80
6"	--	--	--	63.51	62.63
1"'	--	--	97.07	100.07	92.15
2"'	--	--	76.42	72.97	71.74
3"'	--	--	72.15	70.57	69.23
4"'	--	--	69.48	68.05	65.52
5"'	--	--	63.16	76.23	74.65
6"'	--	--	--	63.75	60.98
1""	--	--	92.49	98.63	--
2""	--	--	75.56	72.90	--
3""	--	--	71.72	70.37	--
4""	--	--	68.05	68.05	--
5""	--	--	76.90	75.76	---
6""	--	--	61.44	61.33	--
OMe	--		50.03	56.09	55.71
OMe	--	--	--	55.77	--

Conclusion

Chromatographic separation with roots of traditional plants yielded six new secondary metabolite along with bioactive leads. These compounds have enhanced the phytochemical profile of *C. phlomidis* and will play important role as chemical markers in quality analysis, identification and authentications. The compounds **5** and **6** exhibited significant α-glucosidase enzymatic inhibitory action which proven traditional claims and may be useful for development of potential lead for type 2 diabetics complications in future as an alternative modality. This study will assuredly provide essential track to researcher for development of molecular mechanisms.

Acknowledgement

The authors express their gratitude to School of Pharmaceutical Education and Research, Jamia Hamdard, New Delhi, India for recording spectral data, analysis, and research and instrumentation facility.

Conflicts of Interest

The authors declare no conflict of interest.

Ethics approval

None to declare. This study did not involve human or animal specimens

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