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Insulinomimetic activity of two new gallotannins from the fruits of *Capparis moonii*

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ABSTRACT

Bioassay guided fractionation of the hydro-alcoholic extract of the fruits of *Capparis moonii*, led to the isolation of two new chebulinic acid derivatives. The compounds **1** and **2** displayed significant glucose uptake effect of 223% and 219% over the control at the 10 ng/ml and 100 ng/ml concentration, respectively. The increased glucose uptake effects of the compounds were associated with significant IR and IRS-1 phosphorylation, GLUT4 and PI3-kinase mRNA expression in the L6 cells.

1. Introduction

Type 2 diabetes mellitus is a metabolic syndrome, characterized by fasting and post-prandial hyperglycemia and is constantly on the rise.[1] Despite considerable progress in the management of diabetes mellitus with synthetic drugs, the search for indigenous natural antidiabetic agents is still an area, left largely uninvestigated. Natural products have played a key role in the discovery of antidiabetic drugs being derived from a natural product or a natural product lead. Plant derived antidiabetics continue to remain as the primary source for novel therapeutics.[2–4] Based on the above facts, using the bioassay guided fractionation approach, we made an attempt to isolate the bioactive compounds of *Capparis moonii,* that were chiefly responsible for the antidiabetic activity. *Capparis moonii* (L.) Wigh (Rudanti) is a tree frequently found in the Konkan regions and grows vigorously in hot semi-arid conditions.[5] Fruits are subglobose or ovoid and comprise of many seeds and are generally large in size.[6] They are reported to be used in the treatment of inflammation, as a stomachic, adaptogen and against tuberculosis, and the later, was confirmed to possess tuberculostatic and also antibacterial properties.[7,8] The hydrolyzable tannins isolated from numerous plants find wide use as tanning agents[5] and have recently attracted attention due to their numerous biological activities and their implications in potential benefits to human health.[9–12] In this paper, we report on the structure elucidation of two new hydrolyzable gallotannins, (**1** and **2**) from the fruits of *Capparis moonii,* together with their significant effect on glucose uptake, IR-β phosphorylation, IRS-1 phosphorylation, GLUT4 and PI3-kinase mRNA expression in the L6 cells. The new compounds were isolated using bioassay guided fractionation technique and characterized using IR, MS, 1D and 2D NMR spectroscopic techniques. This is the first report of gallotannins from the fruits of *Capparis moonii.*

2. Results and discussion

Bioassay guided fractionation of the fruits of *Capparis moonii*, led to the identification of an active butanol fraction. Reverse phase column chromatography of this active fraction yielded two bioactive gallotannins (**1** and **2**). Compound **1** was isolated as colorless crystals. Positive-ion ESI-MS showed a pseudomolecular ion peak at *m/z* 975 $[M+H]^+$ which corresponded to the molecular formula, $C_{41}H_{34}O_{28}$. The IR spectra of the compound exhibited typical bands at 1705 cm^{-1} (C=O) for a carboxylic acid group, 1760 cm^{-1} (C=O) for a δ lactone of the chebuloyl moiety, and a hydroxyl at 3450 cm^{-1} (OH), further supported by ^{13}C NMR resonances at δ_C 172.9 and δ_C 172.1, respectively. The presence of at least three galloyl moieties was inferred from the observation of characteristic three proton singlets (6.93, 7.02, and 7.05), 18 sp^2 carbon signals (DEPT-90 spectrum), corresponding to 24 carbons and three ester

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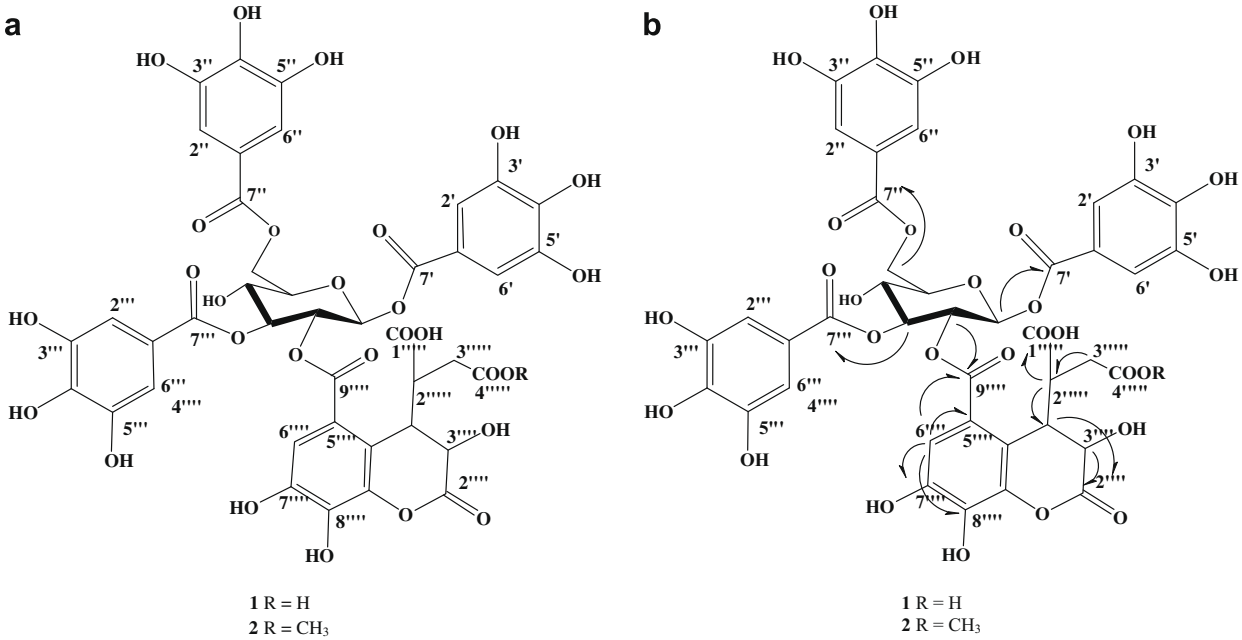
Table 1
NMR spectral assignments in MeOH-d_4 for gallotannin **1**

C/H atom	^{1}H[f]	COSY	^{13}C[e]	HMBC
Sugar G-1	6.35, *d* (4.5)	H-2	91.7	C-5′, COO
G-2	5.25, *d* (4.5)		72.3	C-1′
G-3	6.08, *d* (3.5)	H-4	62.0	COO
G-4	4.99, *d* (2.8)		68.4	
G-5	4.58, *m*		75.4	
G-6	4.58, *m*		64.2	
	4.44, *m*			
Galloyl A 1′			118.6	
2′/6′	6.93, *s*		108.6	C-1′, COO, C-3′/5′, C-4′
3′/5′			145.6[a]	
4′			139.7[b]	
COO			165.3	
Galloyl B 1″			117.4[c]	
2″/6″	7.05, *s*		108.6	C-1″, COO, C3″/5″
3″/5″			145.5[a]	
4″			139.4[b]	
COO			163.8	
Galloyl C 1‴			117.5[c]	
2‴/6‴	7.02, *s*		109.0/108.9	C-1‴, COO, C3/5, C-4‴
3‴/5‴			145.7[a]	
4‴			139.2[b]	
COO			163.8	
Chebuloyl CO			169.3	
3⁗	4.84, *d* (7.3)		65.0	C-4⁗
4⁗	4.78, *d* (8.0)		40.0	C-2⁗, C-3⁗, C-8a, C-6⁗
4a			116.4	
5⁗			114.8	
6⁗	7.41, *s*		115.7	COO, C-7⁗⁗, C-8a, C-5⁗⁗
7⁗			145.8[a]	
8⁗			139.5[b]	
8a			140.5	
1⁗⁗-COOH			172.9[d]	
2⁗⁗	3.68, *m*	H-3′	38.3	2⁗⁗-CO
3⁗⁗	2.05, *m*		29.5	
4⁗⁗-COOH			172.1[d]	
9⁗CO			164.2	

[a,b,c,d] Signals within a column are interchangeable and are represented correctly.
[e] Assignments were confirmed by HSQC (^{13}C–^{1}H heteronuclear coupling experiment).
[f] Figures in parentheses = *J* values in Hz.

carbonyls (δ_C 165.3, 163.8 and 163.8) according to the ^{1}H and ^{13}C NMR spectra, respectively. Comparison of the NMR data (see Table 1) with the COSY spectrum, revealed the sugar to be glucose (COSY cross peaks between H-1 and H-2/H-3 and H-4 of the sugar), with an anomeric carbon δ_C 91.7 and a methylene carbon δ_C 64.2 bound to δ_H 4.58 (1H, m), respectively. Application of COSY, HSQC, and HMBC experiments, further established that all the three galloyl moieties were linked to G-1, G-3, and G-6 of glucose, through ester linkages corresponding from C-1 of the galloyl units (Fig. 1). In addition, the NMR spectrum also displayed signals characteristic of a chebuloyl moiety, a lactone carbonyl at δ_C 169.3, a one proton singlet at δ_H 7.41, that displayed (HMBC cross peaks) ^3J correlations with an ester carbonyl (δ_C 164.2), a ^4J correlation with a quaternary carbon at δ_C 140.5 and a ^2J correlation to the carbon bearing hydroxyl at δ_C 145.8. Furthermore, an oxymethine that appeared as a doublet at δ_H 4.84, *d*, 7.3 Hz, (δ_C 65.0) was coupled to a saturated methine, δ_H 4.78, *d*, 8.0 Hz, (δ_C 40.0) that displayed ^2J and ^3J couplings with the lactone carbonyl (δ_C 169.3) and the quaternary carbon (δ_C 116.4), respectively. In addition to these, the ^{13}C NMR spectrum showed signals for a methylene carbon (δ_C 29.5), a saturated methine (δ_C 38.3) and two carboxyl carbons (δ_C 172.1 and 172.9). The saturated methine resonating at δ_H 3.68 showed a ^2J correlation with the sp^3 carbon at δ_C 29.5 and ^2J and ^3J coupling to the first carboxylic moiety at δ_C 172.9 (C-1⁗⁗) and the second carboxylic carbonyl at δ_C 172.1, supporting that it was linked to the pyrone ring of the chebuloyl moiety at C-4⁗. On the basis of the above evidence, compound **1** was identified as 1,3,6-tri-*O*-galloyl-2-chebuloyl-β-D-glucopyranoside, a new gallotannin.

Compound **2** was isolated as pale yellow crystals. Positive-ion ESI-MS displayed a molecular ion peak at *m*/*z* 989 $[M+H]^+$ which corresponded to the molecular formula $C_{42}H_{36}O_{28}$. The IR spectra exhibited absorption bands for carbonyl group at 1735 cm^{-1} (C=O) characteristic for an ester linkage, 1705 cm^{-1} (C=O) for a carboxylic acid group, 1760 cm^{-1} (C=O) for a lactone of the chebuloyl moiety and a hydroxyl at 3450 cm^{-1} (OH). A close inspection of the NMR spectrum (see Table 2) of **2** and on comparison with the data obtained from **1**, confirmed **2** to be a 1,3,6-tri-*O*-galloyl-2-chebuloyl ester-β-D-glucopyranoside, a new gallotannin which was esterified at C-4⁗of the chebuloyl moiety.



1 R = H
2 R = CH_3

Figure 1. Key HMBC correlations of **1** and **2**.

Table 2
NMR spectral assignments in MeOH-d_4 for gallotannin **2**

C/H atom	^{1}H[g]	COSY	^{13}C[f]	HMBC
Sugar G-1	6.35, *d* (2.5)	H-2	92.8	C-5′, COO
G-2	5.25, *s*		71.6	C-1′
G-3	6.08, *s*	H-4	62.0	COO
G-4	4.68, *s*		67.1	
G-5	4.55, *m*		75.9	
G-6	4.52, *m*		65.2	
	4.71, *m*			
	4.44, *m*			
Galloyl A 1′			117.4[a]	
2′/6′	6.91, *s*		110.2[b]	C-1′, COO, C-3′/5′, C-4′
3′/5′			146.4[c]	
4′			140.1[d]	
COO			167.8	
Galloyl B 1″			120.0	
2″/6″	7.05, *s*		110.4[b]	C-1″, COO, C3″/5″, C-4″
3″/5″			146.5[c]	
4″			140.5[d]	
COO			166.1[e]	
Galloyl C 1‴			120.7	
2‴/6‴	7.02, s		110.7[b]	C-1‴, COO, C3‴/5‴, C-4‴
3‴/5‴			146.7[c]	
4‴			140.8[d]	
COO			166.0[e]	
Chebuloyl CO			170.6	
3⁗	3.80, *m*		69.5	C-4⁗
4⁗	4.71, *d* (7.1)		40.0	C-2⁗, C-3⁗, C-8a, C-6⁗
4a			116.0	
5⁗			119.1	
6⁗	7.40, *s*		117.6[a]	COO, C-7⁗⁗, C-8a, C-5⁗⁗
7⁗			146.6[c]	
8⁗			140.4[d]	
8a			140.9[d]	
1⁗⁗-COOH			174.5	
2⁗⁗	3.68, *m*	H-3′	39.9	2-CO
3⁗⁗	2.15, *m*		30.7	
4⁗⁗-COOH			173.5	
–OCH_3	3.50, *s*		52.4	
9⁗CO			166.2[e]	

[a,b,c,d,e] Signals within a column are interchangeable and are represented correctly.
[f] Assignments were confirmed by HSQC (^{13}C–^{1}H heteronuclear coupling experiment).
[g] Figures in parentheses = *J* values in Hz.

2.1. Effect of compounds on glucose uptake in L6 myotubes

To examine the effect of the bioactive molecules on glucose transport, L6 myotubes were treated with varying concentrations (1 pg/ml–1 μg/ml) of compound **1** and **2** at 24 h. A dose-dependent increase in glucose uptake was observed for both the compounds. Compound 1 at 10 ng/ml produced a maximal glucose uptake of 223% over the control cells (Fig. 2) and under similar conditions positive control, Rosiglitazone at 50 μM exhibited 240% uptake. The optimum concentration showing maximal glucose uptake for compound **2** was found to be 100 ng/ml, where, 219% uptake was observed over the control cells and Rosiglitazone exhibited 203% uptake at 50 μM under similar conditions. 2-Deoxyglucose was used as a marker in the glucose uptake test. Increased glucose uptake in L6 cells attributed to the antidiabetic potential of the compounds invitro.

2.2. Effect of compounds on PI3-kinase and Glut4 mRNA expression in L6 cells

To unravel the molecular mechanism of the compounds in augmenting glucose transport, their effect on the major markers regulating insulin signaling cascade were examined. Incubation of L6 cells with optimized concentrations of both the compounds (**1** and **2**) for 18 h resulted in an enhanced expression of PI3-kinase (Fig. 3) and Glut 4 mRNA expression (Fig. 4) in L6 cells. The expression of PI3-kinase was significantly increased upon treatment with compound **1** (10 ng/ml) and compound **2** (100 ng/ml). The similar expression pattern was detected with insulin which served as the positive control for PI3- kinase expression, Rosiglitazone had no effect on expression of PI3-kinase, hence, thereby served as negative control. The expression of GLUT4 was significantly unregulated upon treatment with compound **1** and **2** which was comparable with both the positive controls, Rosiglitazone (50 μM) and insulin (100 nM). Significant expression of PI3-kinase and Glut 4 mRNA expression partially explained, that these compounds exert glucose transport through PI3-kinase mediated insulin signaling pathway.

2.3. Effect of compounds on IR-β and IRS-1 phosphorylation in L6 cells

To unravel further the effects of compounds on early steps of insulin signaling pathway, the compounds were examined through measuring IR & IRS phosphorylated protein levels. Incubation of L6 cells with optimized concentrations of both the compounds (**1** and **2**) for 18 h, significantly enhanced the insulin receptor beta subunit (Fig. 5) and IRS-1 phosphorylation (Fig. 6). Significant phosphorylation of IR and IRS-1 protein depicts the insulinomimetic effects of the compounds and their possible antidiabetic mechanism via insulin signaling pathway.

3. Conclusion

Two new gallotannins were isolated and their antidiabetic activities were evaluated. They appeared to be primarily acting through stimulation of insulin signaling pathway, by major down signaling events on the insulin pathways (i.e., IR beta subunit and IRS-1 phosphorylation, PI3K and GLUT4 mRNA expression in L6 cells). The gallotannins may be regarded as potential candidates for development of new antidiabetic drugs.

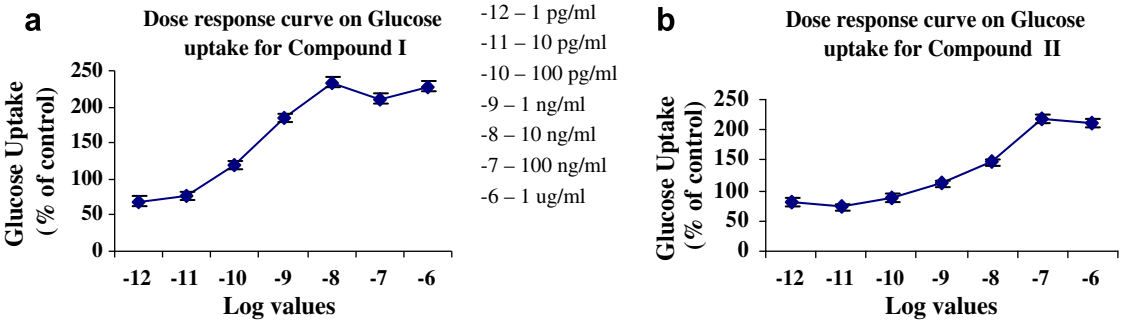


Figure 2. Dose–response curve showing 2-deoxy glucose uptake by compounds.

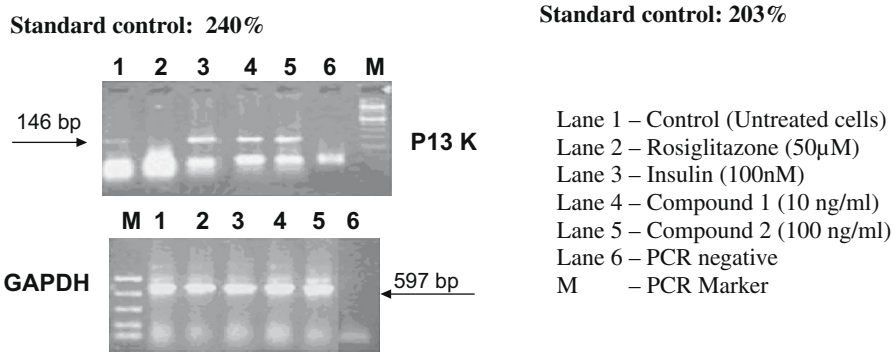


Figure 3. Effect of compounds on PI3-kinase mRNA expression at 18 h.

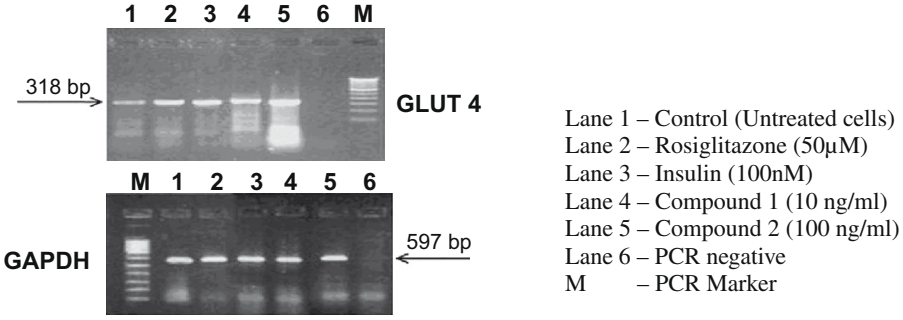


Figure 4. Effect of compounds on glut 4 mRNA expression at 18 h.

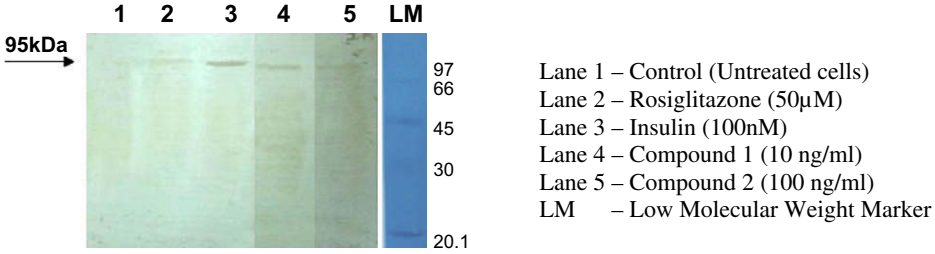


Figure 5. Effect of the compounds on Insulin receptor β phosphorylation in L6 cells at 18 h.

4. Experimental

4.1. General

IR spectra were recorded on Perkin–Elmer 100 FT-IR spectrometer. ^{1}H and ^{13}C NMR, DEPT-90 & 135, ^{1}H-^{1}H COSY, ^{13}C–^{1}H HSQC and ^{13}C–^{1}H HMBC spectra were recorded in methanol-d_4 and DMSO-d_6 on Avance Bruker DRX (400 MHz) spectrometer. Chemical shifts are expressed in a δ (ppm) scale with tetramethylsilane as an internal standard and coupling constants values J in Hz. ESI-MS spectra were recorded on a MS-MSD Quattro micro spectrophotometer in positive ion mode (Multi mode ionization).HPLC-PDA analysis were performed on WATERS 2496 (separations module) attached with WATERS 2996 photodiode array detector (PDA), whereas semi-preparative HPLC were performed on WATERS 699 (separations module) attached with WATERS 2487 dual wavelength UV detector using ODS-C_{18} column (150 mm × 4.6 mm, 5 μ, Purospher® Star or equivalent) for analytical and ODS-C_{18} (250 mm × 21.2 mm, 15 μ, Hichrom or equivalent) for semi-preparative HPLC using mobile phase acetonitrile in 0.1% aqueous formic acid. Column chromatography was carried out using RP-18 ODS Silica Gel (Merck, Germany) with mobile phase (Miliq-water: methanol). Thin Layer Chromatography was performed on Silica RP-18 F_{254} pre-coated aluminum plates (Merck, Germany). Spots were visualized by UV illumination in UV chamber (Camag, Switzerland) and sprayed with 2% ethanolic ferric chloride and heated. All cell culture solutions and supplements were purchased from Life Technologies (Rockville, MD, USA). 2-Deoxy-D-[1-3*H*]-glucose and Hybond C membranes were obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK). Trizolb reagent, AMLV reverse transcriptase, dNTP, and Taq polymerase were obtained from Gibco-BRL (Grand Island, NY, USA) and New England Biolabs (Hertz, UK). Insulin was obtained from Sigma–Aldrich. Medicinal plants were procured and authenticated from the Herbal Department, Ranbaxy Research Laboratories (Gurgaon, Haryana, India). The thin layer chromatograph (TLC) plates were obtained from Merck (60 F254 grade; Darmstadt, Germany). Rosiglitazone was a kind gift from Dr Reddy's Laboratories (Hyderabad, India). Primers were synthesized by Gibco-BRL. All chemicals and reagents used were purchased from Sigma Chemical Company, Ltd.

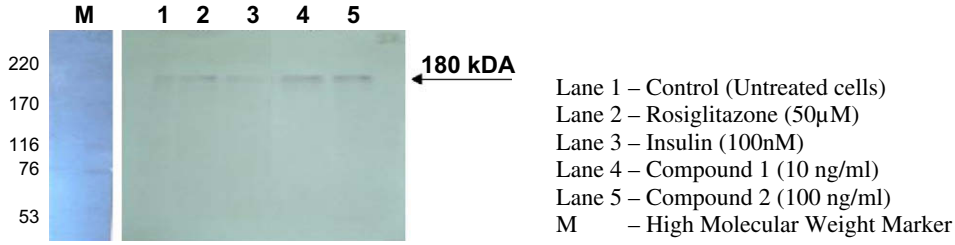


Figure 6. Effect of the compounds on IRS-1 phosphorylation in L6 cells at 18 h.

4.2. Plant material

The fruits of *Capparis moonii* were collected from South region of India, in June 2006. A voucher specimen (SSH/CM/SI/06/06) has been deposited at the Herbarium, Herbal drug Research, Ranbaxy Research Labs, Research and Development-II, Gurgaon, Haryana, India.

4.3. Extraction and isolation

The dried and coarsely powdered fruits (2.1 kg) of *Capparis moonii* were macerated with 50% aqueous methanol (2.5 L × 4) for a period of about 16–20 h each. The combined filtrate was concentrated under reduced pressure to afford a dark brown residue (229.5 g). This residue was then suspended in H_2O (1 L) and sequentially partitioned with chloroform (500 ml × 4) and *n*-butanol (500 ml × 4). The combined chloroform and *n*-butanol layer was passed through bed of anhydrous sodium sulfate separately and concentrated to yield 4.41 g of chloroform fraction and 90 g of *n*-butanol fraction. A part of *n*-butanol fraction was subjected to reverse phase column chromatography using ODS-C_{18} Silica Gel (100 g). Step gradient elution of *n*-butanol fraction with 5%, 10%, 15% up to 80% methanol in miliq-water yielded 20 fractions of volume 100 ml each (dark violet color with 2% ethanolic ferric chloride; positive test for tannins) furnished a pale yellow brown precipitate upon cooling. This partially pure precipitate was sonicated in a mixture of miliq-water and methanol (7:3, 5 ml). The solution thus obtained was filtered through 0.45 μ (millipore nylon membrane, Merck) filter and injected into the Prep-HPLC ($C_{18,}$ 250 mm × 21.2 mm, 15 μ, Hichrom or equivalent) using a step gradient, 5−50% acetonitrile in 0.1% aqueous formic acid with a run time of 58 min. Fraction of the volume 50 ml each were collected and subjected to reverse phase analytical HPLC ($C_{18,}$ 150 mm × 4.6 mm, 5 μ, Purospher® Star or equivalent) using a linear gradient, 2−90% acetonitrile in 0.1% aqueous formic acid with a run time of over 35 min, flow rate 1.0 ml/min, detection at 278 nm for purity determination. Enriched fractions containing compound 1 and compound 2 on the basis of their retention time were pooled separately, evaporated under reduced pressure and crystallized from miliq-water/methanol (6:4) by keeping at room temperature for overnight. Compound **1** was obtained as pale yellow crystals (yield: 0.610 g), whereas, compound **2** was obtained as colorless crystals (0.012 g).

4.3.1. 1,3,6-Tri-*O*-galloyl-2-chebuloyl-β-D-glucopyranoside 1

Pale yellow crystals, IR (KBr) ν_{max} 3450, 2905, 1750, 1735, 1705, 1650, 1530, 1220, 758 cm^{-1}. See Table 1 for ^1H NMR and ^{13}C NMR including COSY and HMBC. LC–ESI-MS (Positive-ion mode) *m*/*z* [974 $[M]^+$ and 975 $[M+H]^+$].

4.3.2. 1,3,6-Tri-*O*-galloyl-2-chebuloyl ester-β-D-glucopyranoside 2

Colorless crystals, IR (KBr) ν_{max} 3450, 2905, 1750, 1705, 1650, 1530, 1220, 758 cm^{-1}. See Table 2 for ^1H NMR and ^{13}C NMR including COSY and HMBC. LC–ESI-MS (Positive-ion mode) *m*/*z* $[M+H]^+$ 989.

4.4. Bioassays

4.4.1. Cell culture

L6 cells (CRL-1458; American Type Culture Collection, Bethesda, MD, USA), were maintained in Dulbecco′s modified Eagle′s medium (DMEM) with 10% fetal bovine serum (FBS), penicillin (120 units/ml), streptomycin (75 lg/ml), gentamycin (160 lg/ml) and amphotericin B (3 lg/ml), in a 5% CO_2 environment. For differentiation, L6 cells were transferred to DMEM with 2% FBS for 4 days after they had reached confluence. The extent of differentiation was established by observing the number of multinucleate cells. In the present experiment, 90% of myoblasts fused into myotubes.

4.4.2. Measurement of 2-deoxy-D-[1-3*H*]-glucose

Glucose uptake was determined in L6 myoblast cells grown in 24-well plates (BD Falcon, San Diego, CA, USA) as described previously.8 Briefly, serum starved (5 h) differentiated myotubes were incubated with plant extracts for 24 h and then stimulated with insulin (100 nmol/L) for 20 min. After incubation with insulin, cells were rinsed once with HEPES-buffered Krebs′–Ringer phosphate solution (composition [in mmol/L]: NaCl 118; KCl 5; $CaCl_2$ 1.3; $MgSO_4$ 1.2; KH_2PO_4 1.2; HEPES 30, pH 7.4) and were then incubated for 15 min in HEPES-buffered solution containing 18.5 kBq/ml 2-deoxy-D-[1-3*H*]-glucose. Cells were washed three times with ice-cold HEPES buffer solution and lysed in 0.1% sodium dodecyl sulfate (SDS). The radioactivity incorporated into the cells was measured using liquid scintillation counter. Glucose uptake was corrected for non-specific uptake in the presence of 10 mol/L cytochalasin B (5–10% of total uptake). All assays were performed in duplicate and repeated three times for concordance. Results are expressed as percentage glucose uptake with respect to solvent control (there was a <5% difference between control [untreated cells] and solvent control [cells treated with 2 lL dimethyl sulfoxide]). Rosiglitazone (50 μmol) was used as a positive control.

4.4.3. Measurement of Glut4 (glucose transporter-4) and PI3-kinase (phosphatidylinositol 3-kinase) mRNA expression by reverse transcription-polymerase chain reaction

Reverse transcription–polymerase chain reaction (RTPCR) was performed as described previously.9 Briefly, L6 myotubes, after incubation with the plant extracts, were lysed in total RNA isolation reagent trizol. The isolated mRNA was converted to cDNA and subjected to PCR using specific primers, as follows: GLUT4 sense 5′-CGG GAC GTG GAG CTG GCC GAG GAG-3′ and antisense 5′-CCC CCT CAG CAG CGA GTG A-3′ (318 bp); PI3-K sense 5′-CAA AGC CGA GAA CCT ATT GC-3′ and antisense 5′-GGT GGC AGT CTT GTT GAT GA-3′ (146 bp); and GAPDH sense 5′-CCA CCC ATG GCA AAT TCC ATG GCA-3′ and antisense 5′-TCT AGA CGG CAG GTC AGG TCC ACC-3′ (597 bp). The PCR was performed using a minicycler (MJ Research, Waltham, MA, USA) with 95 °C for 5 min for initial denaturation, followed by 95 °C for 1 min for denaturation. The annealing temperature was maintained for 1 min at 55 °C for GLUT4 and GAPDH or 63 °C for PI3-K. Extension was performed

at 72 °C for 1 min. This entire process was repeated for 35 cycles, followed by a final extension at 72 °C for 10 min. The PCR products were run on 1.5% agarose gels, stained with ethidium bromide, and photographed. All PCR products were consistent with the predicted sizes.

4.4.4. Measurement of IR (Insulin Receptor) beta and IRS-1 (Insulin Receptor Substrate 1) phosphorylation by immunoprecipitation

L6 cells were seeded in 12-well plates and allowed to differentiate for 4 days in medium containing 2% serum. Cells were treated with the compounds **1** and **2** at the concentration of 10 & 100 ng/ml, respectively, for 24 h and with insulin (100 nM) for 15 minutes. Thereafter, these were washed once with ice-cold PBS (phosphate buffered saline) and lysed in 1 ml of lysis buffer (50 mM Hepes, 150 mM NaCl, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 1 mM sodium orthovanadate, 50 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1% Triton X-100 pH 7.4). The lysates were centrifuged, and the supernatants incubated with 50 μl of protein A-Sepharose beads that had been coated with monoclonal anti-IR/IRS-1 antibody. The immunoprecipitates were washed three times with 500 μl lysis buffer and then analyzed by SDS–polyacrylamide gel electrophoresis and immunoblotting.

4.4.5. Western blotting

Immunoprecipitates obtained in above procedure were boiled in Laemmli SDS sample buffer, resolved by SDS–PAGE, and transferred to a PVDF (polyvinylidene fluoride) membrane. The membrane was blocked in TBST (25 mM Tris–HCl, pH 8.0, 125 mm NaCl, 0.1% Tween 20) containing 5% skimmed milk for 1 h and then incubated with anti-phosphotyrosine (primary) antibody for 1 h at room temperature. The blot was washed extensively with TBST and further incubated with secondary antibody conjugated to HRP (horseradish peroxidase). After further washing with TBST, the blots were developed using enhanced chemiluminescence (ECL kit).

4.4.6. Statistical analysis

All data are expressed as the mean ± SEM. Differences between mean values were analyzed using analysis of variance; the level of significance was set at $P < 0.05$.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.04.032.

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