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Design and synthesis of lupeol analogues and their glucose uptake stimulatory effect in L6 skeletal muscle cells ☆

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ABSTRACT

Structure modifications of lupeol at the isopropylene moiety have been described via allylic oxidation using selenium dioxide. The antidiabetic efficacy of lupeol analogues were evaluated in vitro as glucose uptake stimulatory effect in L6 skeletal muscle cells. From all tested compounds, **2**, **3**, **4b** and **6b** showed significant stimulation of glucose uptake with respective percent stimulation of 173.1 (p <0.001), 114.1 (p <0.001), 98.3 (p <0.001) and 107.3 (p <0.001) at 10 μM concentration. Stimulation of glucose uptake by these compounds is associated with enhanced translocation of glucose transporter 4 (GLUT4) and activation of IRS-1/PI3-K/AKT-dependent signaling pathway in L6 cells. Structure–activity relationship analysis of these analogues demonstrated that the integrity of α,β-unsaturated carbonyl and acetyl moieties were important in the retention of glucose uptake stimulatory effect. It is therefore proposed that naturally occurring lupeol and their analogues might reduce blood glucose, at least in part, through stimulating glucose utilization by skeletal muscles.

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Despite the immense technological advancement, management of diabetes mellitus without any side effects is still a challenge to the medical science. This global pandemic is driven by type 2 diabetes mellitus[1] (T2DM), a chronic and progressive metabolic disorder affecting approximately 4% population worldwide and is expected to increase to 5.4% in 2025.[2] The 2011 WHO data show that T2DM affects >346 million patients worldwide.[3] It is characterized by abnormalities in carbohydrate, and lipid metabolisms associated with insulin resistance and decreased pancreatic β-cell function which causes hyperglycaemia as well as secondary complications, such as hyperlipidemia, hyperinsulinemia, hypertension, and atherosclerosis.[4] Currently, there is no absolute cure for diabetes. T2DM is initially managed by regular exercise and dietary modification. Nowadays, it is managed by therapeutic agents alone or in combination with others or with insulin. Oral hypoglycemic agents, such as sulfonylureas, metformin, glucosidase inhibitors, troglitazone, etc. are using as the main means for T2DM but two most popular agents now in use are the thiazolidinediones (TZDs) and the biguanides.[5] The TZDs are widely used but having undesirable side effects (weight gain, fluid retention and heart failure etc.).

The biguanide metformin does not cause weight gain but mainly acts in liver rather than muscle and thus on its own is not a complete therapy,[6] and in addition, they are not suitable for use during pregnancy.[7] Therefore, the searches for the safer and effective new therapies that can treat T2DM on a long term basis without causing adverse events are highly desirable. As a part of our interest in search of bioactive natural product derived analogues,[8] we have taken a targeted approach to investigate the analogues of active triterpenoid molecule for the management of T2DM.

Triterpenoid skeleton is readily available source of rigidity, chirality and lipophilicity combined in a single aliphatic system which not only possesses a high number of natural stereogenic centers but also can be easily functionalized at two positions C-3 and C-20, respectively. Lupane-type triterpenoids having four cyclohexane rings, all in the chair conformation and one cyclopentane ring in envelope conformation are the building blocks of the triterpenoids.[9] Most of the compounds possess a hydroxylic group at carbon C-3 and showed different substitution (Ring A) patterns.[10] In the last two decades, hundreds of publications have been presented the broad spectrum of biological activities of triterpenoids, such as anticancer,[11] anti-inflammatory,[12] antioxidatives,[13]

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hepatoprotective,[14] antidyslipidemic,[15] antimalarial[16] and anti-HIV.[17] Therefore, they are regarded as an important chemical pool for further pharmaceutical development. Structural modifications based on these skeleton, have been extensively explored to find out more potent modified pentacyclic triterpenes as preventive and therapeutic agents.[18]

The fundamental triterpenoid lupeol (**1**, Fig. 1) is accessible, abundant, and valuable bioactive lupane type skeleton. The unique structure and characteristic biological properties of **1** attracted many research groups, and a number of synthetic reports on the structural modification have been appeared.[19] However, on the basis of literature survey, modification in isopropylene functionality in lupeol is not very much actively explored.[20] In fact, developing versatile methods for the modification of isopropylene functionality opens the door to an inextinguishable and structurally diverse variety of lupeol for biological relevance. Therefore, herein, we wish to report the design, synthesis and biological evaluation of lupeol analogues of a new class, exhibiting an in vitro glucose uptake stimulatory effect in clonal skeletal muscle cells. The structure of known and new compounds **8**–**12** and **13** were elucidated on the basis of NMR as well as mass spectrometry methods and their purity was determined by HPLC analysis for cellular assay.

This approach relies on the allylic oxidation procedure that ensures the introduction of formyl functionality at C-30 position for subsequent derivatization on lupeol scaffold which was isolated from *Bombax ceiba* stem bark in our laboratory[21] and structure was confirmed on the basis of NMR data reported in the literature.[22] As depicted in scheme 1, SeO_2 was chosen for the preparation of allylic alcohol via allylic oxidation of isopropylene moiety, which can be subsequently converted into α,β-unsaturated aldehyde. Treatment of compound **1** with easily accessible SeO_2 in reflux ethanol furnished 30-formyl lupeol (**2**) in excellent yield. The NMR spectrum of **2** showed α,β-unsaturated carbonyl moiety, which was confirmed by long bond HMBC correlations between the two singlet at δ 6.30 (1H, br s, H-29a), 5.92 (1H, br s, H-29b) and one multiplet at δ 2.73 (1H, m, H-19) with the carbonyl carbon at δ 195.0 (CHO).

This oxidation product having α,β-unsaturated functionality may be considered as reactive motifs which are suitable for further derivatization and thus acting as amenable building blocks of molecules. Treatment of **2** with Ac_2O/pyridine yielded the corresponding acetate **3**, while esterification of **2** with different carboxylic acid afforded compounds **4a**–**4c** in the presence of Ac_2O/pyridine. Compound **3** was mixed with 10% KOH in ethanol, followed by addition of hydrated hydroxyl amine at room temperature for 4 h to give **5** in quantitative yield. Reaction of **5** with different carboxylic acids and DCC (1.1 equiv.) in dry CH_2Cl_2, catalyzed by DMAP gave respective esters **6a**–**6c** in 70–80% yield. Compounds **6a**, **6b** and **6c** were obtained as mixture of ester derivatives when we used 1.0 equiv of carboxylic acid. In contrast excess amount of acids quantitatively afforded esterified compounds at both positions as major one. Minor esters were not isolated. To obtain new derivatives with modifications at C-30 position, the set of transformations presented in Scheme 2 was applied. In order to cyclise the C-29 and C-30 positions, lupeol was first acetylated at C-3 position as in **3** and the obtained product was then reduced into **7** with sodium borohydride in ethanol at room temperature. The allylic oxidation of the hydroxyl group of 30-hydroxy, compound **7** with allyl bromide in dry CH_2Cl_2, first led to a compound **10** which was subsequently converted to an appropriate compound **11** with the use of Grubbs II generation catalyst[23] in the presence of additive $Ti(O^iPr)_4$.

Taking under consideration the above mentioned result of Grubbs approach, the propargylic oxidation[23] of **7** with propargyl bromide in dry CH_2Cl_2 was also performed which was subsequently converted to an appropriate compound **9**. Further, some additional derivatives were synthesized via iron catalyzed cross coupling reaction of alcohol and terminal alkene following the reaction sequence shown in scheme 2. From known literature protocol[24], C-20–C-29 double bond of compound **2** can be cross coupled with benzyl alcohol in dichloro ethane catalyzed by $FeCl_3$ (10 mol %) at reflux temperature for two hours and the compound with β-hydroxy ketone functionality, is obtained as one of the major product. It is also known, that the coupling of ketone and hydroxyl groups of compound **12** with the use of $NH_2OH{\cdot}HCl$ in ethanol at room temperature for 1 h, leads to a compound **13** with isooxazoline ring containing an additional aromatic moiety.

All the compounds were evaluated for glucose uptake stimulatory effect in L6 myotubes. As shown in Table 1, from the tested compounds **2**, **3**, **4b** and **6b** showed significant stimulation of glucose uptake with respective percent stimulation of 173.1, 114.1, 98.3 and 107.3 at 10 μM concentration. Rosiglitazone was used as positive control, which showed 112.4% percent stimulation of glucose uptake at 10 μM concentration. Remaining compounds showed little or no stimulatory effect on glucose uptake in L6 myotubes.

Further examination indicated a concentration dependent stimulation of glucose uptake upon treatment with **2**, **3**, **4b**, and **6b** in skeletal muscle cells (Fig. 2). It is further assessed whether the ability of these compounds to stimulate glucose uptake was mediated by potentiation of insulin action or activation of cellular processes independent of the hormone. Cells were treated with **2**, **3**, **4b**, and **6b** (10 μM) for 16 h with final three hour in serum-deprived medium and a subset of cells was stimulated with insulin (100 nM) for 20 min before the measurement of glucose uptake.

As shown in Figure 3, insulin alone caused 2.2-fold (p <0.001) stimulation of glucose uptake in L6 myotubes over basal state. Pretreatment with **2**, **3**, **4b** or **6b** (16 h) caused further increase in insulin signal with respect to insulin treated control, but the gain in transport was lower than that calculated from the independent effect of insulin and test compounds (Fig. 3). These observations suggest that both inputs might stimulate glucose uptake by different sub cellular pathways, but some elements common to the action of both agents may be finite.

In skeletal muscle, stimulation of glucose uptake is mostly attributed to increased translocation and redistribution of the GLUT4 to the plasma membrane, where they facilitate the entry of glucose inside the cell.[25] Thus, the effect of **2**, **3**, **4b**, and **6b** on GLUT4 translocation was determined in L6-GLUT4*myc* cells. Similar to glucose uptake, treatment with **2**, **3**, **4b** or **6b** significantly enhanced GLUT4*myc* translocation to cell surface at 10 μM concentration (Fig. 4).

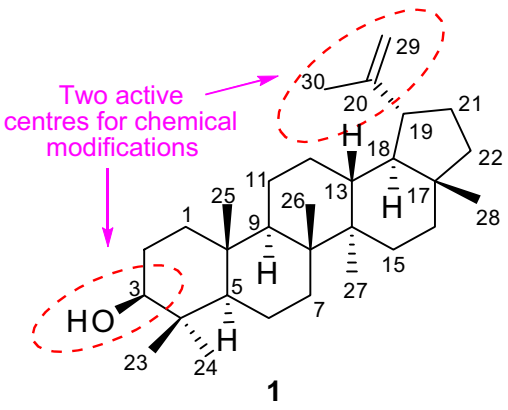
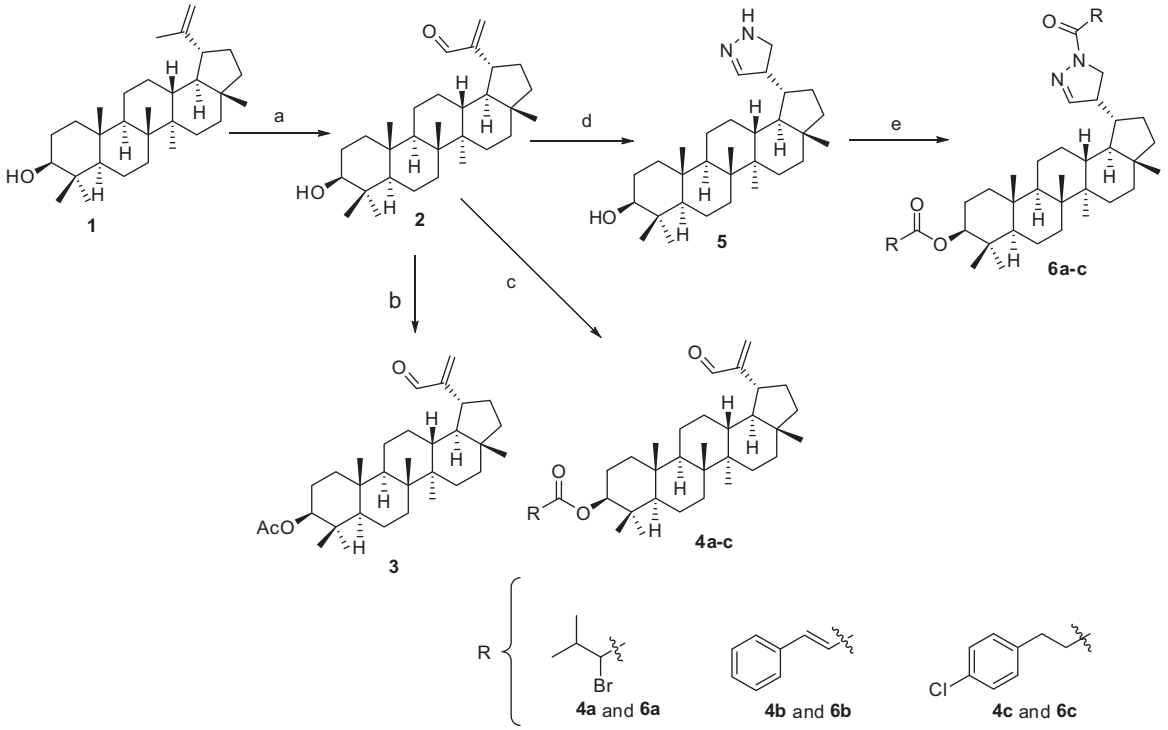
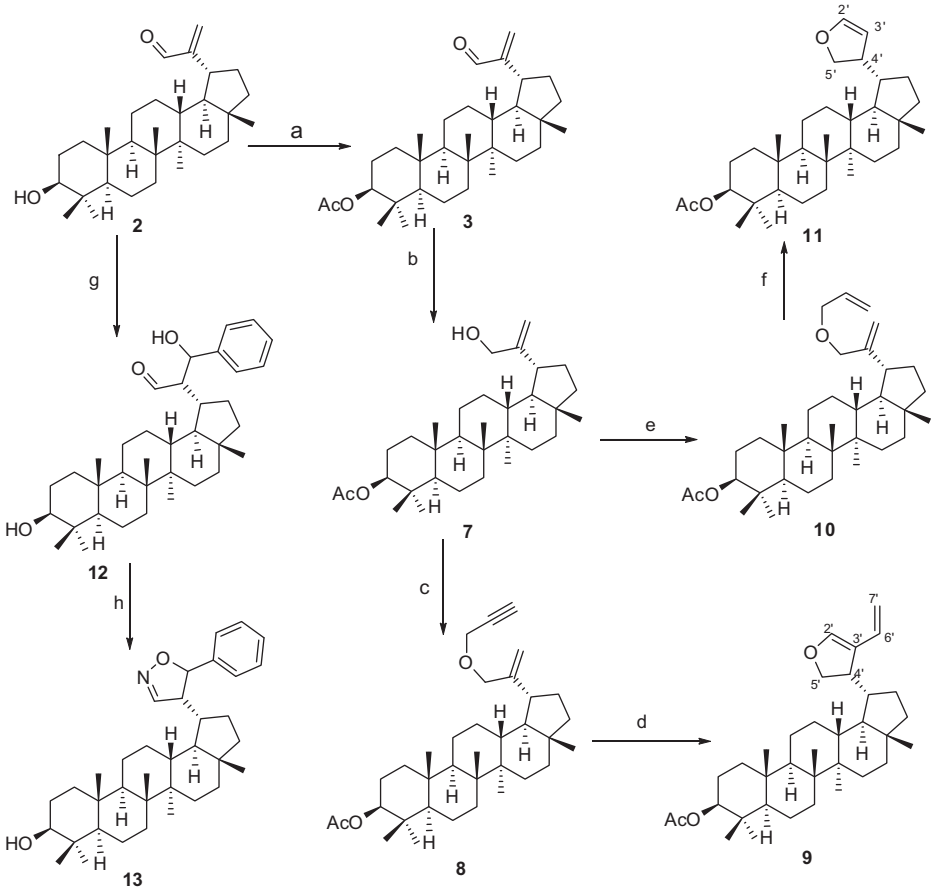


Figure 1. Basic skeleton of starting compound (lupeol), showing the chemically active centers.



Scheme 1. Reagents and conditions: (a) SeO_2 (1.1 equiv), ethanol, reflux, 48 h (b) Ac_2O (1.1 ca.), pyridine, dry CH_2Cl_2, rt, 1 h (c) DCC (1.1 equiv), DMAP (cat.), RCOOH (1.0 equiv), dry CH_2Cl_2, rt, 6–8 h (d) $NH_2NH_2.H_2O$ (1.1 equiv), 10% KOH, ethanol, rt, 4 h (e) DMAP (cat.), RCOOH (1.0 equiv), dry CH_2Cl_2, rt, 6–8 h.



Scheme 2. Reagents and conditions: (a) Ac_2O (1.1 ca.), pyridine, dry CH_2Cl_2, rt, 1 h (b) $NaBH_4$, ethanol, rt, 4 h (c) Propargyl bromide, dry CH_2Cl_2, K_2CO_3, , 0 °C to rt, 4 h (d) Grubbs II (10 mol %), dry CH_2Cl_2, $Ti(O^iPr)_4$, 35 °C, 48 h (e) Allyl bromide, dry CH_2Cl_2, K_2CO_3, 0 °C to rt, 2 h (f) Grubbs II (10 mol %), dry CH_2Cl_2, $Ti(O^iPr)_4$, 35 °C, 48 h (g) $FeCl_3$ (10 mol %), benzyl alcohol, dry DCE, 70 °C, 2 h (h) NH_2OH·HCl, ethanol, rt, 1 h.

Table 1
The glucose uptake stimulation of luepol derivatives with isopropylene moiety modifications

S. No.	Compound No.	Concentration (μM)	% Glucose uptake stimulation (IC_{50})
1	Control	10	00
2	Rosiglitazone	10	112.4 (4.8 μM)
3	**1**	10	26.8
4	**2**	10	173.1 (4.2 μM)
5	**3**	10	114.1 (5.2 μM)
6	**4a**	10	3.1
7	**4b**	10	98.3 (6.2 μM)
8	**4c**	10	0.5
9	**5**	10	16.1
10	**6a**	10	8.4
11	**6b**	10	107.3 (4.9 μM)
12	**6c**	10	2.8
13	**7**	10	22.2
14	**8**	10	19.2
15	**9**	10	2.1
16	**10**	10	8.5
17	**11**	10	3.6
18	**12**	10	6.4
19	**13**	10	13.2

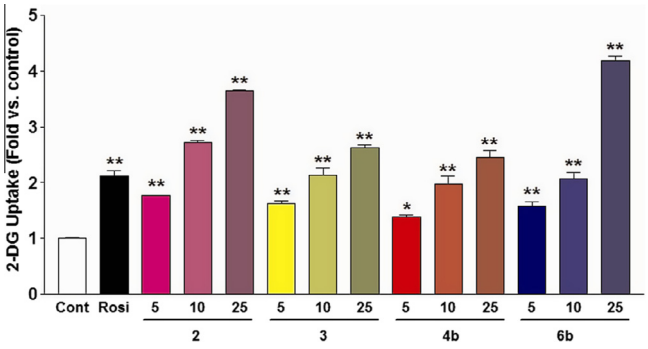


Figure 2. Dose-dependent effect of compounds (**2**, **3**, **4b** and **6b**) on glucose uptake in L6 myotubes. Cells were incubated with vehicle (Cont, DMSO), rosiglitazone (Rosi, 10 μM) or different concentrations of test compounds (5, 10, 25 μM) for 16 h and glucose uptake was as described. Results are expressed as fold stimulation over control. Results shown are mean ± SE of three independent experiment, each performed in triplicate. $^{*}p$ <0.05, $^{**}p$ <0.01, relative to control.

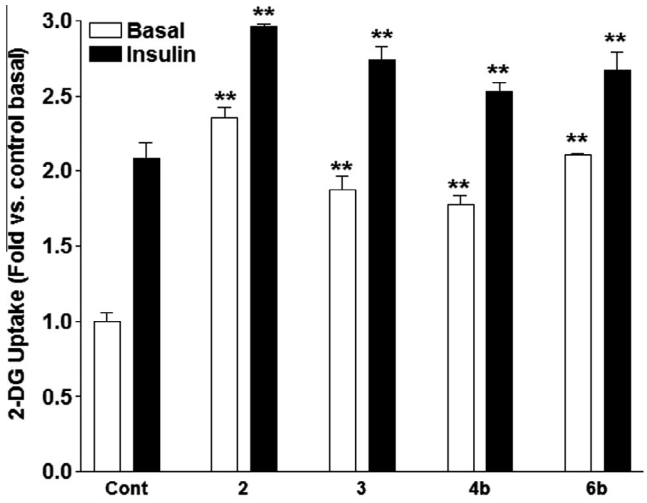


Figure 3. Effect of compounds (**2**, **3**, **4b** and **6b**) on insulin stimulated glucose uptake in L6 myotubes. Cells were treated with test compounds (10 μM) for 16 h with final three hour in serum free medium and a subset of cells was stimulated with insulin (100 nM) for 20 min before the measurement of glucose uptake. Results are expressed as fold stimulation over control basal. Results shown are mean ± SE of three independent experiment, each performed in triplicate. $^{**}p$ <0.01, relative to respective control condition.

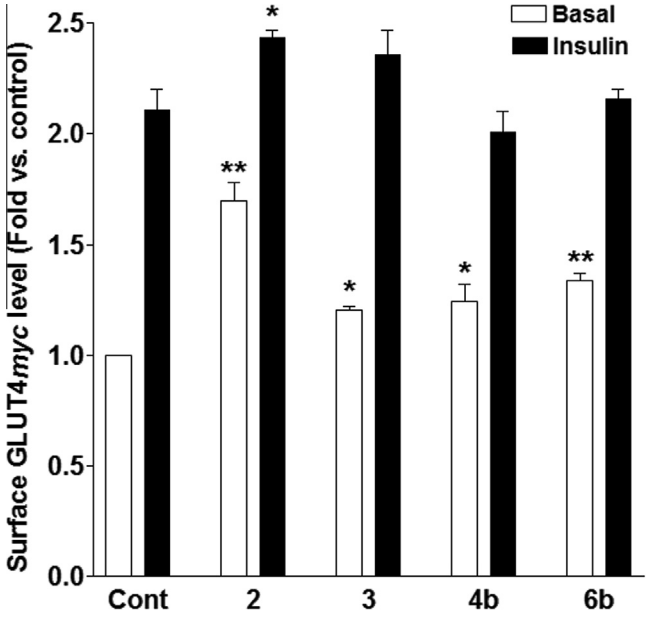


Figure 4. Effect of compounds (**2**, **3**, **4b** and **6b**) on GLUT4 translocation in L6-GLUT4*myc* myotubes. Cells were treated with test compounds (10 μM) for 16 h with final three hour in serum free medium and a subset of cells was stimulated with insulin (100 nM) for 20 min before the measurement of surface level of GLUT4*myc*. Results are expressed as fold stimulation over control basal. Results shown are mean ± SE of three independent experiments, each performed in triplicate. $^{*}p$ <0.05, $^{**}p$ <0.01, relative to respective control condition.

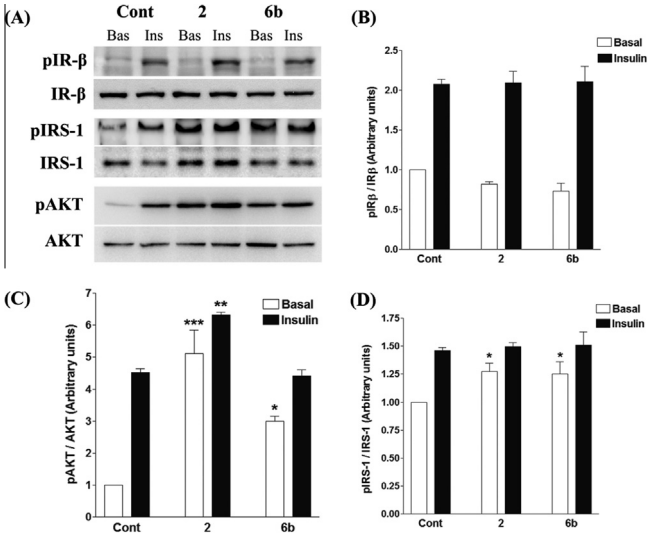


Figure 5. Effect of compounds (**2** and **6b**) on insulin signaling pathway in L6 myotubes. Cells were treated with test compounds (10 μM) for 16 h with final three hour in serum free medium and a subset of cells was stimulated with insulin (100 nM) for 20 min. Cells were lysed and equal amount of protein was analyzed for phosphorylation of the major components of insulin signaling pathway by western blotting (A) and densitiometric quantification of pIRß/IRß (B), pAKT/AKT (C) and pIRS-1/IRS-1 (D). Results of three independent experiments are presented as mean ± SEM. $^{*}p$ <0.05, $^{**}p$ <0.01, relative to respective control condition.

In skeletal muscle cells, insulin exerts its effect by binding to its receptor on cell surface, resulting in the activation of the insulin receptor (IR) tyrosine kinase followed by signaling through insulin receptor substrate (IRS) proteins, Phosphatidylinositol-3-kinase (PI-3-K) and the Ser/Thr kinase AKT.[26] Since, our compounds stimulated glucose uptake independently of insulin, we investigate the effect of most active compounds (**2** and **6b**) on activation of the

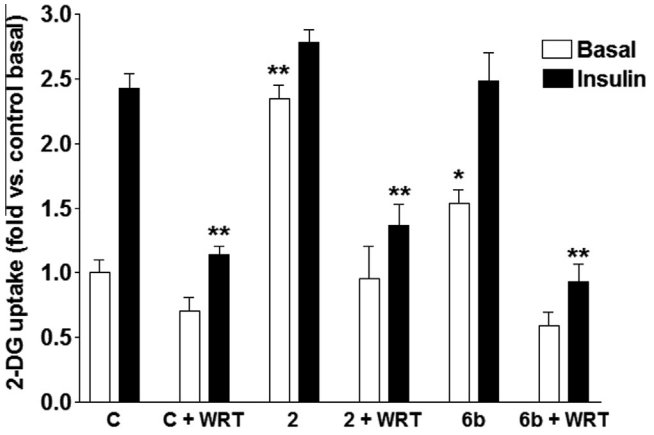


Figure 6. Effect of wortmannin on insulin- and compounds (**2** and **6b**)-induced glucose uptake in L6 myotubes. Cells were treated with test compounds (10 μM) for 16 h without or with wortmannin (50 nM) followed by insulin treatment and determination of glucose uptake and described earlier. Results are expressed as fold stimulation over control basal. $^{*}p$ <0.05, $^{**}p$ <0.001 relative to respective control condition.

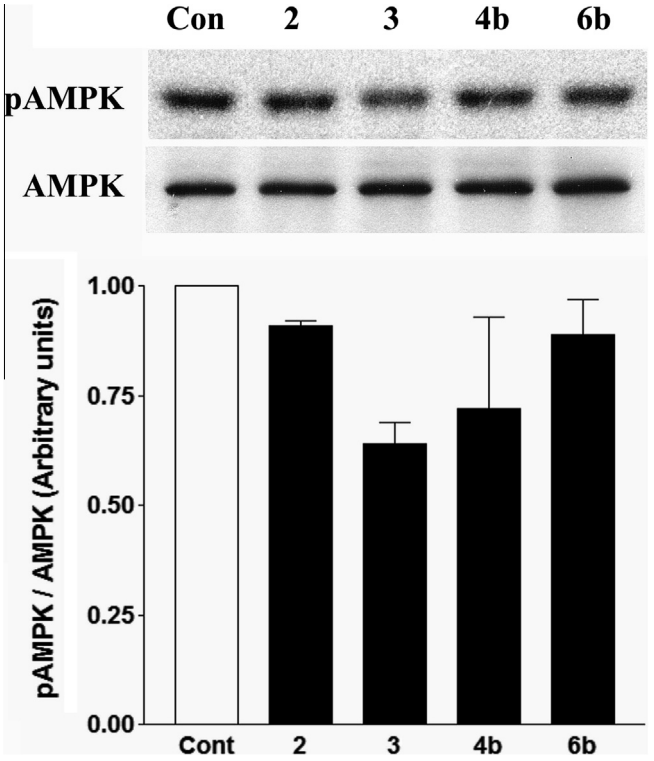


Figure 7. Effect of compounds (**2**, **3**, **4b** and **6b**) on phosphorylation of AMPKα (Thr-172). L6 myotubes were treated with teat compounds (10 μM) for 16 h followed by lysis and western analysis. Shown are representative immunoblots and densitometric quantification of phosphor-AMPKα (Thr-172) relative to total AMPKα. Results of three independent experiments are presented as mean ± SEM.

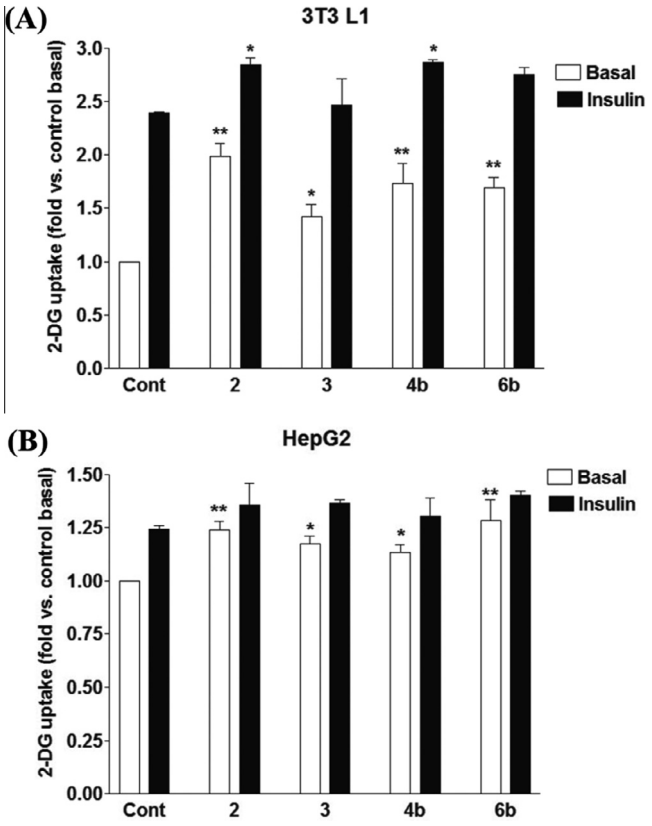


Figure 8. Effect of compounds (**2**, **3**, **4b** and **6b**) on glucose uptake in 3T3 L1 adipocytes (A) and HepG2 hepatic cells (B). Cells were treated with test compounds (10 μM) for 16 h with final three hour in serum free medium and a subset of cells was stimulated with insulin (100 nM) for 20 min before the measurement of glucose uptake. Results are expressed as fold stimulation over control basal. Results shown are mean ± SE of three independent experiment, each performed in triplicate. $^{*}p$ <0.05, $^{**}p$ <0.01, relative to respective control condition.

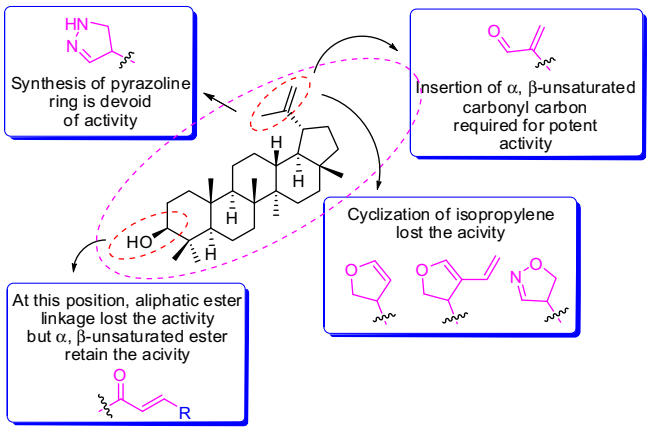


Figure 9. Structure activity relationship.

major components of insulin signaling pathway. As shown in Figure 5, Insulin alone significantly increased the phosphorylation of IR-β, IRS-1and AKT. Treatment with **2** or **6b** significantly stimulated the phosphorylation of IRS-1 and AKT, without affecting IR-β phosphorylation, under basal condition. Furthermore, stimulation of glucose uptake by **2** or **6b** was abolished in presence of wortmannin, which is a specific inhibitor for PI-3-K (Fig. 6). Results indicate the involvement of IRS-1/PI-3-K/AKT-mediated signaling pathway under biological effects of these compounds.

Involvement of PI-3-kinase independent pathways has also been established to stimulate glucose utilization in skeletal muscle cells. Activation of AMPK pathway has been shown to facilitate glucose uptake and GLUT4 translocation. To delineate the possible contribution of AMPK in observed glucose uptake stimulatory effect of **2**, **3**, **4b** and **6b**, we determine effect on activation of AMPK by measuring the phosphorylation of Thr-172, the principle activating phosphorylation site within AMPKα. Treatment with **2**, **3**, **4b** or **6b** had no effect on AMPK activation (Fig. 7). We further

investigated whether these compounds had the ability to stimulate glucose uptake in other insulin sensitive tissues, by evaluating their effect in mouse 3T3L1 adipcytes and human HepG2 hepatic cells. As demonstrated in Figure 8, Treatment with **2**, **3**, **4b** or **6b** significantly stimulate glucose uptake in both adipose and hepatic cells, indicating their ability to enhance overall glucose utilization.

On the basis of analysis of SAR study as in Figure 9, Compounds **2**, containing oxo functionality at C-30 position arose as the main member of these analogues, exhibiting an unusual potency of glucose uptake stimulatory effect. The derivative **3** with a 3-*O*-acetyl group was found to be less active than compound **2** but about 4.5-fold more active in comparison to lupeol. However, introduction of various groups and modification in oxo functionality were associated with sudden loss in potency of glucose uptake stimulation as in case of compounds **4c** (0.5%), **5** (16.1%), **6a** (8.4%) **4a** (3.1%), **6c** (2.8%), **7** (22.2), **8** (19.2%), **9** (2.1%), **10** (8.5%), **11** (3.6%), **12** (6.4%), **13** (13.2%) except the compound **4b** and **6b** which displayed a remarkable ability to stimulate glucose uptake in L6 skeletal muscle cells after administration of 10 μM concentrations, compared with reference compound **1**. Compounds without oxo functionality at C-30 position did not show any remarkable activity. Results are presented in (Figs. 2–8 and Table 1).

In conclusion, a set of isopropylene modified analogues of lupeol were synthesized and tested for anti-diabetic activity in L6 skeletal muscle cells. Among the compounds tested **2**, **3**, **4b** and **6b** containing α,β-unsaturated carbonyl moiety showed the potent in vitro glucose uptake stimulatory effect. This work suggests that lupeol can further provide additional lead compounds on the route to unravel physiological and therapeutic implications of glucose uptake stimulatory effect. In vivo biological evaluation of the title compounds and further derivatization of this triterpenoid scaffold are currently in progress and will be reported in due course.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.04.059.

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21. We have isolated lupeol (**1**) from *n*-Butanol fraction of ethanol extract (200 g) of *Bombax ceiba* stem bark. *n*-Butanol fraction (100 g) was fractionated by gel filtration chromatography over sephadex LH-20 and followed by sephadex G-25 using a H_2O/MeOH as eluent gradient (100:00, 90:10, 75:25, 50:50 and 00:100). It was packed over silica gel (60–120 mesh) bed using $CHCl_3$ and MeOH eluent system with increasing polarity of methanol. This afforded fifteen fractions (F1–F8). Similar fractions were combined into eight major fractions on the basis of TLC profile. First fraction (F1, 25 g) was rechromatographed over silica gel (230–400 mesh, 300 g) using *n*-hexane/ethyl acetate (98:2) as eluent system in isocratic manner. Fractions of 100 ml each were collected. Fraction having same TLC profile, combined together, concentrated which afforded solid material. It was recrystalized in chloroform at room temperature. White microcrystalline solid of **1** was obtained.
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