Original Article

Biological evaluation of (3β)-STIGMAST-5-EN-3-OL as potent anti-diabetic agent in regulating glucose transport using *in vitro* model

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ABSTRACT

Aim: Insulin resistance is characterized by alterations in insulin signaling components thereby resulting in reduced glucose uptake. The mechanistic role of (3β)-stigmast-5-en-3-ol in augmenting glucose uptake to overcome insulin resistance is deciphered in this study.

Main methods: L6 myotubes, rat skeletal muscle model have been used to check the effect of (3β)-stigmast-5-en-3-ol, a plant phytosterol isolated from the ethyl acetate extract of *Adathoda vasica* on glucose transport. The influence of (3β)-stigmast-5-en-3-ol on various cellular targets of insulin signaling cascade has been evaluated using inhibitors on glucose uptake as well as gene and protein expression to unravel the mechanistic action in triggering glucose uptake.

Results: (3β)-stigmast-5-en-3-ol promoted glucose uptake in a dose dependent manner under insulin resistant condition. As assessed by inhibitor studies using Genistein (IRTK inhibitor) and Wortmannin (PI3K inhibitor), gene expression and protein expression studies using specific primers and antibodies, an activation of IR-β, IRS-1, PI3K, AKT/PKB, PKC by both the crude and (3β)-stigmast-5-en-3-ol were observed. This suggested that (3β)-stigmast-5-en-3-ol induced glucose uptake functions through the PI3K dependent pathway in L6 myotubes. Both, the crude and (3β)-stigmast-5-en-3-ol activates GLUT 4 transport (evident from increased mRNA levels and redistribution of GLUT4 from intracellular membrane to plasma membrane through translocation studies). Confocal microscopy revealed a substantial increase in redistribution of FITC tagged GLUT4 throughout the cells.

Conclusion: Our results emphasize the insulin-like effect of (3β)-stigmast-5-en-3-ol in stimulating glucose transport *in vitro* and provide evidence in its beneficial role possessing antidiabetic property apart from its existing cholesterol lowering efficacy.

1. Introduction

The main pathophysiological defect of type 2 diabetes is insulin resistance, a decrease in cellular response to insulin. This includes impairment in the insulin signaling pathway leading to a failure of the insulin stimulated glucose uptake in targeted tissues like muscle and fat. In muscle cells, this is due to the inability of the insulin to stimulate the translocation of GLUT4 (Glucose transporter 4) to plasma membrane [1]. An effective glucose uptake mechanism occurs when insulin binds to its tyrosine kinase receptor, leading to tyrosine phosphorylation of insulin receptor substrate (IRS)-1 protein followed by the activation of Src homology domain protein like Phosphatidyl inositide kinase (PI3K). The downstream targets of PI3K include AKT/PKB (Protein Kinase B) and PKC (Protein Kinase C), whose activation/phosphorylation triggers the translocation of GLUT4 from intracellular pool to the plasma membrane [2,3]. Natural products provide a wide range of therapeutics for many diseases and infections. Traditional knowledge driven drug development owes safe drug discovery, and facilitates the creation of new chemical entities [4].

Adathoda vasica (L.) Nees (family Acanthaceae) is a well-known, traditionally used plant in Ayurveda and Unani medicine [5]. Many reports emphasize the medicinal value of *A. vasica* and its usage for treating diseases such as bronchitis, asthma, fever, jaundice, vomiting, leucoderma, tumors and diabetes [6]. The antitussive effect [7] and the antioxidant property [8] of this plant are established. World Health Organisation has reported *A. vasica* as a recommended medicinal plant with therapeutic utility and non-toxic, with greater usage in primary health care in both adults and children [5]. The ethanolic extracts from the leaves of *A. vasica* showed hypoglycemic activity after oral administration in rats and rabbits [9]. The major constituents of *A. vasica* are essential oil, steroids and quinazoline alkaloids like vasicine, vasicinone, vasicinolone,

deoxyvasicine, vasicol, peganine, 2′-hydroxy-4-glucosyl-oxychal-cone, kaempferol and (3β)-stigmast-5-en-3-ol [5].

(3β)-stigmast-5-en-3-ol is a plant phytosterol found commonly in many plants. Other common names of (3β)-stigmast-5-en-3-ol includes Betasitosterol, (3β)-stigmast-5-en-3-ol, 22:23-dihydro-stigmasterol, alpha-dihydrofucosterol, cinchol, cupreol, rhamnol, quebrachol and (3β)-stigmast-5-en-3-ol. The anti-proliferative effect of (3β)-stigmast-5-en-3-ol, has been investigated using *in vitro* models [10] and also has been documented for its cholesterol lowering effect [11]. Hwang and co-workers [12] reported on the enhancement of glucose uptake by betasitosterol through LKB1-mediated AMPK activation in *in vitro* model. However its mechanistic role in triggering insulin signaling cascade for augmenting glucose transport needs exploration. Against this background, the present study aims at finding the mechanistic action of (3β)-stigmast-5-en-3-ol on the insulin signaling cascade using L6 myotubes, a well established skeletal muscle model for evaluating the anti-diabetic potential.

2. Materials and methods

2.1. Chemicals and reagents

Unless otherwise stated, materials were obtained from Sigma–Aldrich, St. Louis. All cell culture solutions and supplements were purchased from Life Technologies Inc. (Gaithersburg, MD, USA). 2-Deoxy D-[1-^{3}H] glucose and hybond C membrane were obtained from Amersham Pharmacia Biotech, (Buckinghamshire, UK). DMEM, Trizol reagent and MMLV reverse transcriptase, dNTP, Taq polymerase and synthesized primers were obtained from GIB-CO BRL, (Carlsbad, CA, USA). Wortmannin, Mowiol, anti-GLUT4 and anti-actin antibodies were obtained from Calbiochem (Darmstadt, Germany). Rosiglitazone was a kind gift from Dr. Reddy's Laboratories, Hyderabad. Primers were synthesized from GIBCO BRL (Carlsbad, CA, USA). IR, Phospho-IR, IRS-1, phospho-IRS-1 antibodies were procured from Santa cruz. Anti-phospho AKT and p85 PI3K were obtained from BD Pharmingen (San Diego, CA, USA) Alkaline phosphatase-conjugated secondary antibodies, Horse radish peroxidise-conjugated secondary antibodies and anti-goat IgG-FITC were obtained from Santa Cruz Biotechnology, USA. All other HPLC and analytical grade solvents and silica gel required for column purification were obtained from SISCO Research Laboratories, India.

2.2. Cell culture of L6 myotubes

L6, a differentiating monolayer myoblast culture (obtained from ATCC-CRL-1458) was maintained in DMEM (Dulbecco's modified Eagle's medium) with 10% FBS and supplemented with penicillin (120 units/mL), streptomycin (75 μg/mL), gentamycin (160 μg/mL) and amphotericin B (3 μg/mL) in a 5% CO_2 environment. For differentiation, the L6 cells were transferred to DMEM with 2% FBS for 4 days, post-confluence. The extent of differentiation was established by observing the multinucleation of cells. Once differentiated, the cells were incubated 24 h with high glucose medium (the above mentioned DMEM medium with a supplement of 25 mM/L glucose) for the cells to acquire an insulin resistant state [13,14].

2.3. Measurement of 2-deoxy-D-[1-^{3}H] glucose uptake

L6 myoblasts grown in 24-well plates (BD Falcon) were serum starved for 5 h and were incubated with the plant extracts and compound for 24 h and subjected to glucose uptake assay as reported [15]. Glucose uptake values were corrected for non-specific

uptake in the presence of 10 μM cytochalasin B, (5–8% of total uptake). All the assays were performed in duplicates and repeated three times, for concordance. Results have been expressed as a percentage of glucose uptake with respect to the solvent control. Rosiglitazone (50 μM) was used as the positive control.

For inhibitor studies, L6 myotubes were treated with Genistein −50 μM [16] and Wortmannin −100 nM [17], 30 min prior to the incubation with the plant extracts and compound followed by the glucose uptake assay.

2.4. Extraction of A. vasica ethyl acetate leaf extract and isolation of (3β)-stigmast-5-en-3-ol

The leaves of *A. vasica* after authentication were dried, powdered and sequentially extracted using organic solvents including hexane, dichloromethane, ethyl acetate and methanol by cold maceration as previously reported [18]. All the extracts were checked for glucose uptake efficacy. The ethyl acetate extract showed maximum glucose uptake activity compared to the other extracts, and was selected for purification. A Column of silica gel 100–200 mesh size was packed with hexane. The sample (active ethyl acetate extract) was loaded as dried slurry of silica gel (1:20 by ratio). The column was eluted with a gradual increase in polarity, using ethyl acetate. The eluted fractions were tested for glucose uptake activity. The active fraction was purified by additional column chromatography and its purity established by HPLC. The structure of the active compound was determined by ^{1}H, ^{13}C, DEPT 90, DEPT 135, COSY and ESI-MS. The compound was structurally characterized as (3β)-stigmast-5-en-3-ol with elemental composition and mass of $C_{29}H_{50}O$ and 414 respectively.

2.5. Reverse transcriptase-polymerase chain reaction

RT-PCR was carried out as described previously [19]. The isolated mRNA were converted to cDNA by reverse transcription and subjected to PCR using specific primers as follows: IRTK, F: 5′ ATC TGG ATC CCC CTG TAA ACT GTC 3′; R: 5′ ATG TGG GTG TAG GGG ATG TGT TCA 3′ (Genbank accession number NM_017071.1), GLUT 4, F: 5′ CGG GAC GTG GAG CTG GCC GAG GAG 3′; R: 5′ CCC CCT CAG CAG CGA GTG A 3′ [20], PI3K p85α regulatory subunit, F: 5′ TGA CGC TTT CAA ACG CTA TC 3′; R : 5′ CAG AGA GTA CTC TTG CAT TC 3′ [21], PKC α , F: 5′ ACC TGA AGC TGG ACA ACG 3′; R: 5′ GCT ACT CCT ACG GTA TTT 3′(Genbank accession number NM 001105713.1) and GAPDH, F: 5′ CCA CCC ATG GCA AAT TCC ATG GCA 3′; R: 5′ TCT AGA CGG CAG GTC AGG TCC ACC 3′ [22]. PCR products were resolved in agarose gels, stained with ethidium bromide and photographed. Rosiglitazone and Insulin were used at 50 μM and 100 nM respectively to determine the efficacy. The expression levels were quantitated by scanning on a gel documentation and analysis system (Chemi Imager 4400, Alpha Innotech Corporation).

2.6. Immunoprecipitation

L6. myotubes were treated with optimum concentrations of *A. vasica* ethyl acetate extract and (3β)-stigmast-5-en-3-ol for indicated time periods after serum starvation for 8 h. The cell lysates were prepared as reported [23]. The lysates were immunoprecipitated using Protein A Sepharose beads with specific phosphorylated forms of anti-IRβ and anti-IRS-1 [24]. The immunoprecipitated lysates were then transferred onto nitrocellulose membrane.

2.7. Western blot

The immunoblots were probed with the respective primary antibody (IR-β, IRS-1, p85PI3K, pAKT and AKT), followed by specific secondary antibody and visualized using a chromogenic substrate. The density of the protein bands were quantitated by scanning the blots on a gel documentation and analysis system (Chemi Imager 4400, Alpha Innotech Corporation).

2.8. Subcellular membrane fractionation

Subcellular membrane fractions were obtained using the differential ultra-centrifugation method, as described previously [15], with minor modifications. Briefly, L6 myotubes after treatment with plant extract and compound were washed and resuspended in buffer I (250 mM/L Sucrose, 5 mM NaN_3, 20 mM HEPES, 200 μM/L PMSF, 1 μM/L Pepstatin, 1 μM/L Aprotinin and 2 mM/L EGTA, pH 7.4). Cell lysate was homogenized using 20 strokes of a Dounce homogenizer (0.5 cycles, 10 pulses; 2 min each and lag time of 1 min for each pulse). The cell homogenates were centrifuged at 750g for 5 min at 4 °C to remove cell debris. The Plasma membrane (PM) fraction was obtained by centrifugation of the resulting supernatant at 30,000g for 40 min at 4 °C. The resultant pellet was resuspended in Buffer I to constitute the PM fraction. Supernatant was removed and centrifuged at 100,000g for 75 min at 4 °C to generate the cytosol fraction from the obtained pellet. The light microsome (LM) pellet resuspended in buffer I and PM fraction were assayed for soluble protein content by Bradford's assay. 5′ nucleosidase and NADPH cyt-c reductase were used as the marker enzymes for PM and LM respectively. The membrane fractions were subjected to electrophoresis on 10% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with anti-GLUT4 antibody.

2.9. Immunofluorescence analysis and confocal microscopy

L6 myotubes (60% of differentiated cells) grown on coverslips, after treatment with the crude and pure at indicated time points were washed with 1X PBS. The cells were fixed with 3.7% formaldehyde in PBS for 10 min at room temperature, and permeabilized in PBS containing 0.01% Triton-X for 10 min. The cells were then blocked with 3% FBS in PBS for 10 min and incubated with anti-goat GLUT 4 antibody (1:250 dilution, sc-1607) in 3% FBS in PBS for 1 h at 4 °C. This was followed by incubation with 1:100 dilution of donkey anti-goat IgG-FITC (sc-2024) for 1 h at 4 °C. All coverslips were washed with PBS thrice during each incubation step and finally mounted on slide with Mowiol for confocal microscopy. Fluorescence was measured with Leica TCS SP2 inverted spectral confocal microscope (Germany) equipped with 60× objective with excitation and emission of 468 nm and 543 nm (for FITC) respectively.

2.10. Statistical analysis

Mean ± SEM were represented. Statistically significant differences between groups were analysed using Student's t-test. The statistical significance was evaluated using one way ANOVA to determine differences among groups and Tukey's multiple comparison tests to determine significant differences between groups. These were applied using SPSS version 11.0 (SPSS, Cary, NC, USA) and the criterion for statistical significance was $P < 0.05$.

3. Results

3.1. (3β)-stigmast-5-en-3-ol stimulates glucose uptake in L6 myotubes

To examine the effect of ethyl acetate extract and its bioactive compound, (3β)-stigmast-5-en-3-ol on glucose transport , L6 myotubes were treated with varying concentrations of the extract and the compound at three different time points (12, 24 and 36 h). A dose- and time-dependent increase in glucose uptake activity was observed on treatment with the crude extract as well as the purified compound. At 24 h, maximal effect was achieved which sustained even at 36 h (data not shown for 12 and 36 h). The ethyl acetate extract and (3β)-stigmast-5-en-3-ol significantly stimulated basal glucose uptake and the optimal dose with maximum activity was found to be 1 μg/mL (102.9% of vehicle control, $p < 0.05$) and 100 ng/ml (113.6% of vehicle control, $p < 0.05$) respectively (Fig. 1), and these concentrations were used for further studies.

3.2. Activation of IR-β, IRS-1, PI3K, AKT/PKB and PKC by (3β)-stigmast-5-en-3-ol in L6 myotubes

To determine whether ethyl acetate extract and (3β)-stigmast-5-en-3-ol are capable of modulating activation of insulin receptor, gene and protein expression studies were performed. The ethyl acetate extract and (3β)-stigmast-5-en-3-ol increased the mRNA levels of Insulin receptor from 6 h onwards, and a significant increase in expression was observed at 18 h ($p < 0.05$) as shown in Fig. 2a and b. In addition, the expression of phospho-specific IR-β and IRS-1 were also checked. Our results revealed the increase of
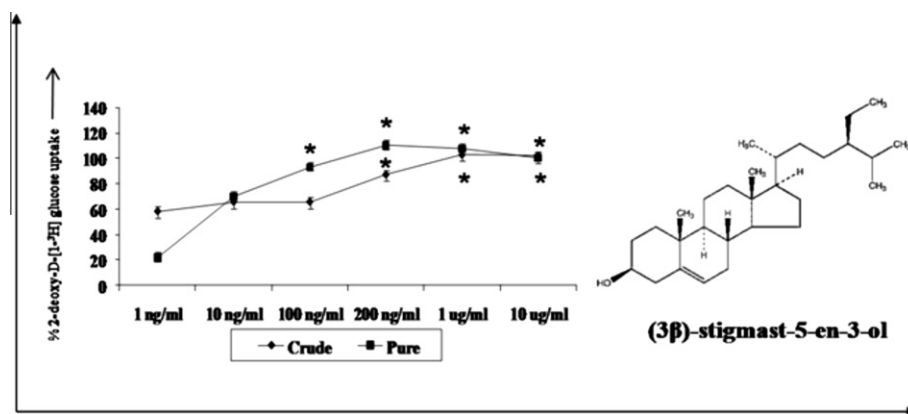

Fig. 1. Comparative analysis of *Adathoda vasica* ethyl acetate extract and (3β)-stigmast-5-en-3-ol on 2-deoxy-D-[1-³H] glucose uptake. Dose response analysis at 24 h and the structure of (3β)-stigmast-5-en-3-ol were displayed. The results were expressed as percentage glucose uptake with respect to the vehicle control. The positive control, Rosiglitazone (50 μM) showed 132.2% uptake. The values are mean of ±S.E., $n = 3$ in duplicates. (*), $P < 0.05$ as compared with vehicle control group.
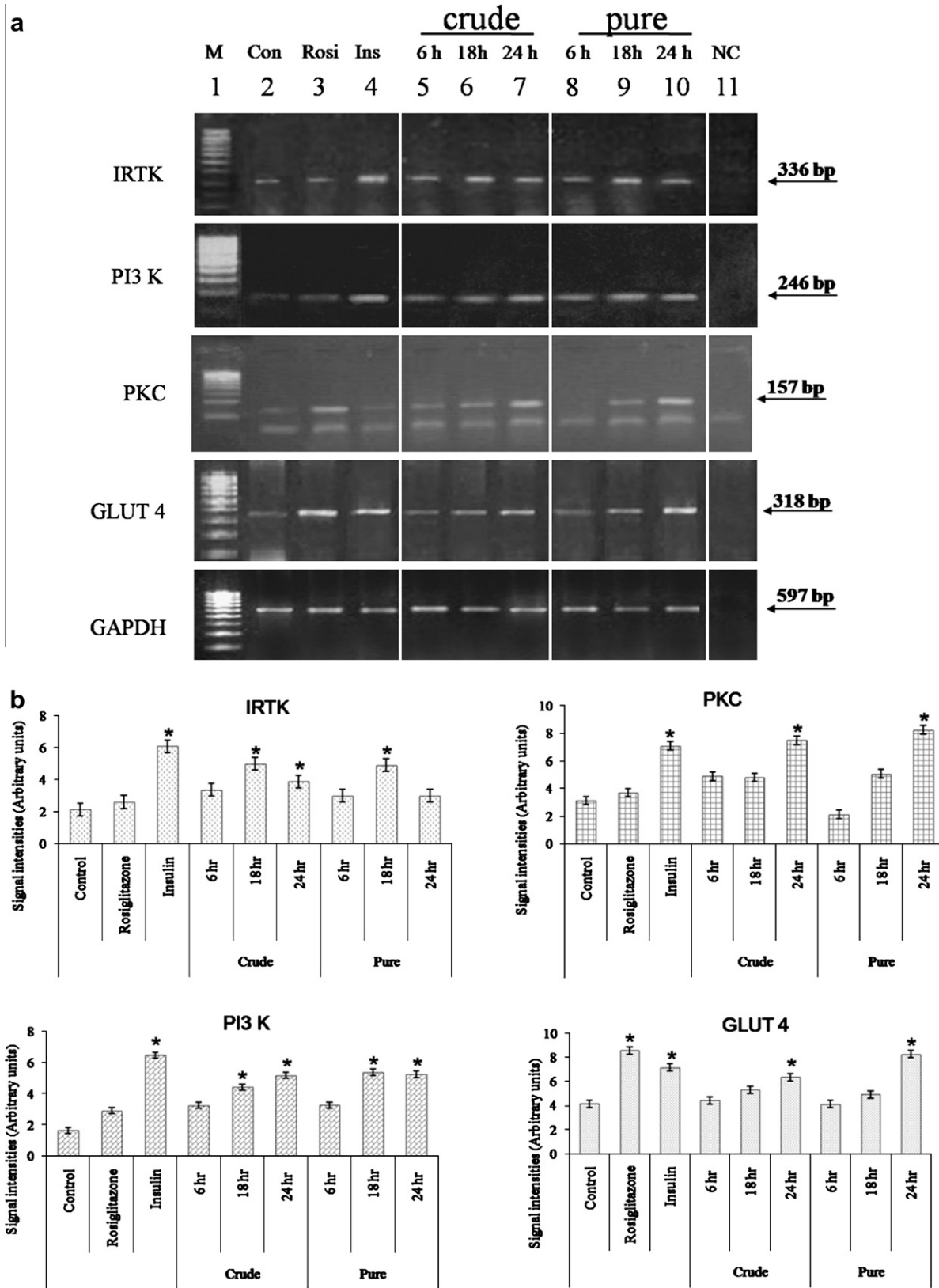
Fig. 2. (a) Effect of *Adathoda vasica* ethyl acetate extract and (3β)-stigmast-5-en-3-ol on IRTK, PI3K, PKC and GLUT4 mRNA expression. L6 myotubes were incubated with Insulin (100 nM), Rosiglitazone (50 μM), crude (1 μg/mL) or pure (100 ng/mL) at indicated time points for each marker. Lane 1 denotes the 100 bp marker, Lanes 2–4 indicates untreated cells (control), Rosiglitazone and Insulin respectively. Lanes 5–7 and 8–10 indicates 6 h, 18 h and 24 h of *Adathoda vasica* crude treated cells and (3β)-stigmast-5-en-3-ol treated cells respectively. Lane 11 shows the PCR negative control. GAPDH transcripts were used as the internal control. Insulin serves as a positive control for IRTK, PI3K, PKC and GLUT4 expressions. Rosiglitazone serves as a positive control for GLUT4 and negative control for IRTK, PI3K and PKC expressions. All the samples were run on the same gel and the dividing lines are for better understanding of the data. (b) Semi-quantitative analysis of IRTK, PI3K and GLUT4 mRNA transcripts upon treatment with *Adathoda vasica* ethyl acetate extract and (3β)-stigmast-5-en-3-ol. The signaling intensities of IRTK, PI3K, PKC and GLUT4 transcripts were quantified using densitometric scanning. The signaling intensities were quantified arbitrarily. Bars represent mean of ±S.E., $n = 3$ and a representative agarose gel is shown here. (*), $P < 0.05$ as compared with untreated control group.

IR-β and IRS-1 tyrosine phosphorylation upon treatment with ethyl acetate extract and (3β)-stigmast-5-en-3-ol, as shown in Fig. 3a. A representative densitometric scanning for semi-quantitative analysis is shown in Fig. 3b. Taken together, these data suggested that the ethyl acetate extract and (3β)-stigmast-5-en-3-ol activated IR-β and IRS-1 similar to the positive control, insulin.

To examine whether (3β)-stigmast-5-en-3-ol stimulates glucose transport in a PI3K dependent manner, RT-PCR analysis and phospho-detect p85 PI3K were employed. As shown in Fig. 2a, treatment with crude and (3β)-stigmast-5-en-3-ol resulted in a time dependent increase in mRNA levels of PI3K. This data was substantiated by enhancement in the levels of p85 PI3K on treatment with the same (Fig. 4a).

To assess the effect of (3β)-stigmast-5-en-3-ol on PKB/AKT activation, the phosphorylation pattern was also studied. Both the crude and (3β)-stigmast-5-en-3-ol augmented the levels of phospho-AKT as shown in Fig. 4a. The next proximal kinase, PKCα was also analysed using RT-PCR. The crude as well as (3β)-stigmast-5-en-3-ol were able to significantly increase the mRNA levels at 24 h ($p < 0.05$) as indicated in Fig. 2. Collectively, our data demonstrates that the crude extract and (3β)-stigmast-5-en-3-ol activates the insulin receptor, insulin receptor substrate-1, followed by the activation of the downstream kinases like PI3K, AKT and PKC α, which accounts for their anti-diabetic potential.

3.3. Translocation of GLUT4 from light microsomes to plasma membrane by (3β)-stigmast-5-en-3-ol

To check whether the increase in glucose uptake by (3β)-stigmast-5-en-3-ol is attributable to the translocation of GLUT4 from light microsomes (LM) to plasma membrane (PM), the translocation pattern was studied qualitatively, using confocal microscopy and semi-quantitatively determined by measuring the amount of GLUT4 in LM and PM using immunoblot. At earlier time points (Insulin-5 min, 6 h treatments with Rosiglitazone, A. vasica crude extract and (3β)-stigmast-5-en-3-ol), lack of detectable fluorescence was noticed suggesting low levels of GLUT4. At 12 h, the presence of GLUT4 is localized near the nucleus, which exhibits enhanced fluorescence upon treatment with crude extract and (3β)-stigmast-5-en-3-ol. At 24 h, ethyl acetate extract, (3β)-stigmast-5-en-3-ol, Rosiglitazone and insulin (15 min) showed increased fluorescence through out the cell (Fig. 5).

Fig. 6a explains the translocation pattern of GLUT4 protein in response to crude and (3β)-stigmast-5-en-3-ol from LM to PM. Our results revealed that (3β)-stigmast-5-en-3-ol significantly augmented GLUT4 protein in PM content ($p < 0.01$) like the ethyl acetate crude extract. The translocation pattern was comparable with that of the positive control, insulin. Semi-quantitative analysis is illustrated in Fig. 6b.

3.4. Inhibition of Insulin receptor and PI3K blocks the glucose uptake mechanism

To confirm whether (3β)-stigmast-5-en-3-ol enhances glucose transport the same way as insulin, the involvement of insulin receptor and PI3K was further checked using inhibitor studies. As shown in Fig. 7a, pretreatment with genistein (specific inhibitor for Insulin receptor tyrosine kinase [16]) followed by incubation with ethyl acetate extract or (3β)-stigmast-5-en-3-ol resulted in decrease in glucose uptake up to $67.20 \pm 7\%$ and $49.68 \pm 3\%$ respectively. Likewise, pretreatment with wortmannin (a specific PI3K inhibitor [17]) and subsequent incubation with ethyl acetate ex-
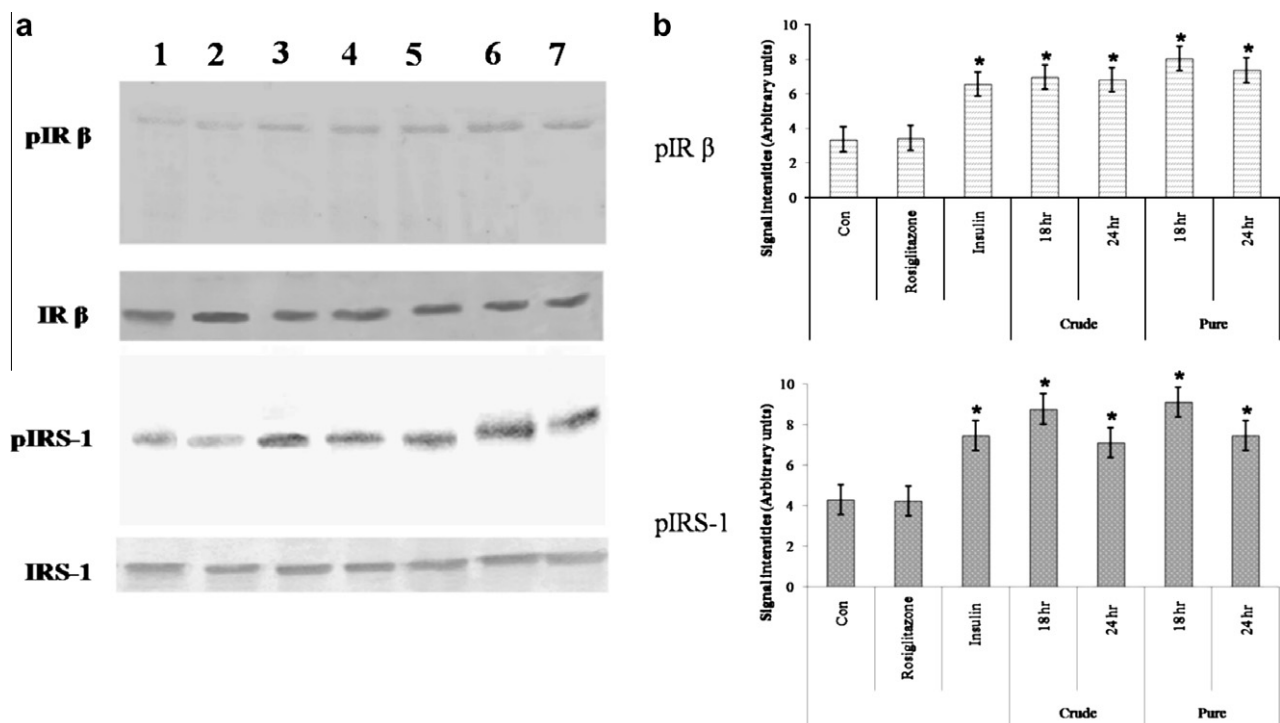

Fig. 3. (a) Effect of Adathoda vasica ethyl acetate extract and (3β)-stigmast-5-en-3-ol on protein expression of pIR-β and pIRS-1. L6 myotubes were incubated with Insulin (100 nM), Rosiglitazone (50 μM), crude (1 μg/mL) or pure (100 ng/mL) at indicated time points for each marker. Whole cell lysates were immunoprecipitated using Protein A sepharose beads with phospho-specific antibodies followed by western blot as mentioned in the methods section. Lanes 1–3 indicates untreated cells (control), Rosiglitazone and Insulin treated cells. Lanes 4 & 5 and 6 & 7 indicate 18 h and 24 h of Adathoda vasica ethyl acetate extract treated cells and (3β)-stigmast-5-en-3-ol treated cells respectively. IR-β and IRS-1 protein expression were shown as immunoblot. (b) Semi-quantitative analysis of pIR-β, pIRS-1 protein expression upon treatment with Adathoda vasica ethyl acetate extract and (3β)-stigmast-5-en-3-ol. The signaling intensities were quantified arbitrarily. Bars represent mean of ±S.E., n = 3 and a representative blot is depicted here. (*), $P < 0.05$ as compared with untreated control group.
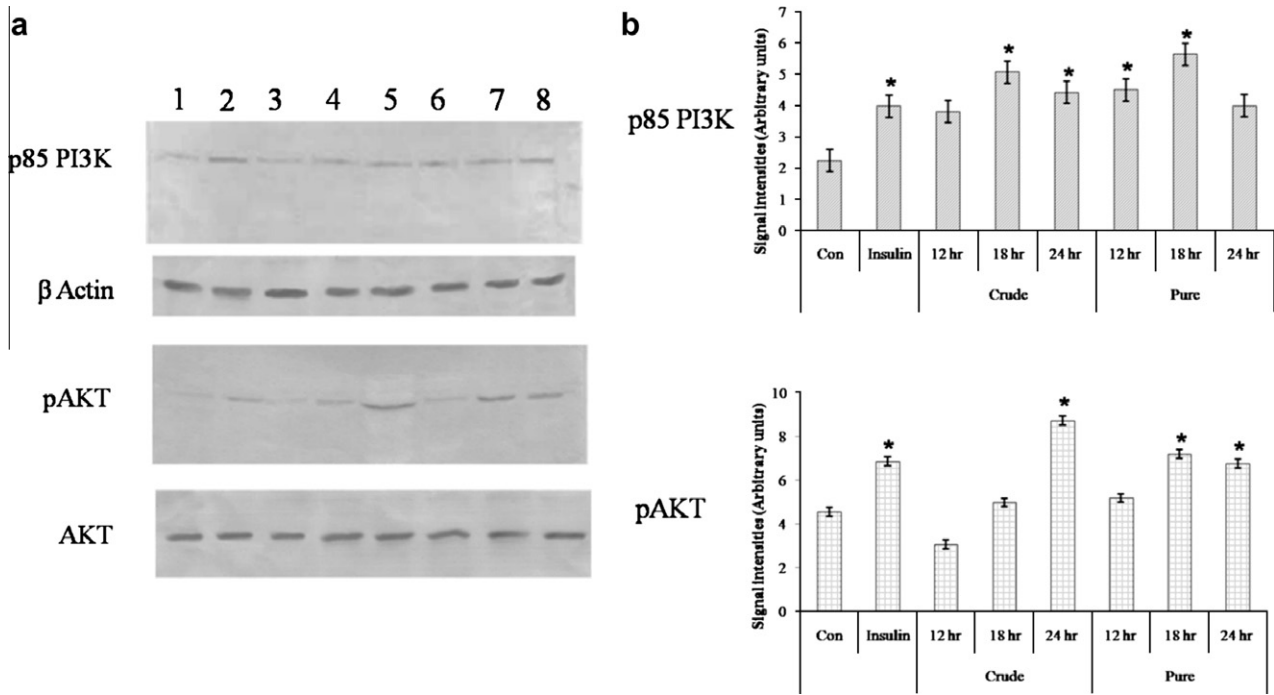
Fig. 4. (a) Effect of *Adathoda vasica* ethyl acetate extract and (3β)-stigmast-5-en-3-ol on protein expression of p85 PI3K and pAKT. L6 myotubes were incubated with Insulin (100 nM), Rosiglitazone (50 μM), crude (1 μg/mL) or pure (100 ng/mL) at indicated time points for each marker. Whole cell lysates were immunoprecipitated using Protein A sepharose beads with phospho-specific antibodies followed by western blot for pAKT protein expression whereas for p85 PI3K whole cell lysates were used for western blot. Lane 1 indicates untreated cells (control) and Lane 2 indicates Insulin treated cells. Lanes 3–8 indicate 12 h,18 h and 24 h of *Adathoda vasica* ethyl acetate extract treated cells and (3β)-stigmast-5-en-3-ol treated cells respectively. β-actin and AKT served as loading control for protein expression of p85 PI3K and pAKT respectively. (b) The signaling intensities of p85 PI3K, pAKT protein expression upon treatment with *Adathoda vasica* ethyl acetate extract and (3β)-stigmast-5-en-3-ol were quantified arbitrarily. Bars represent mean of ±S.E., n = 3 and a representative blot is depicted here. (*), $P < 0.05$ as compared with untreated control group.
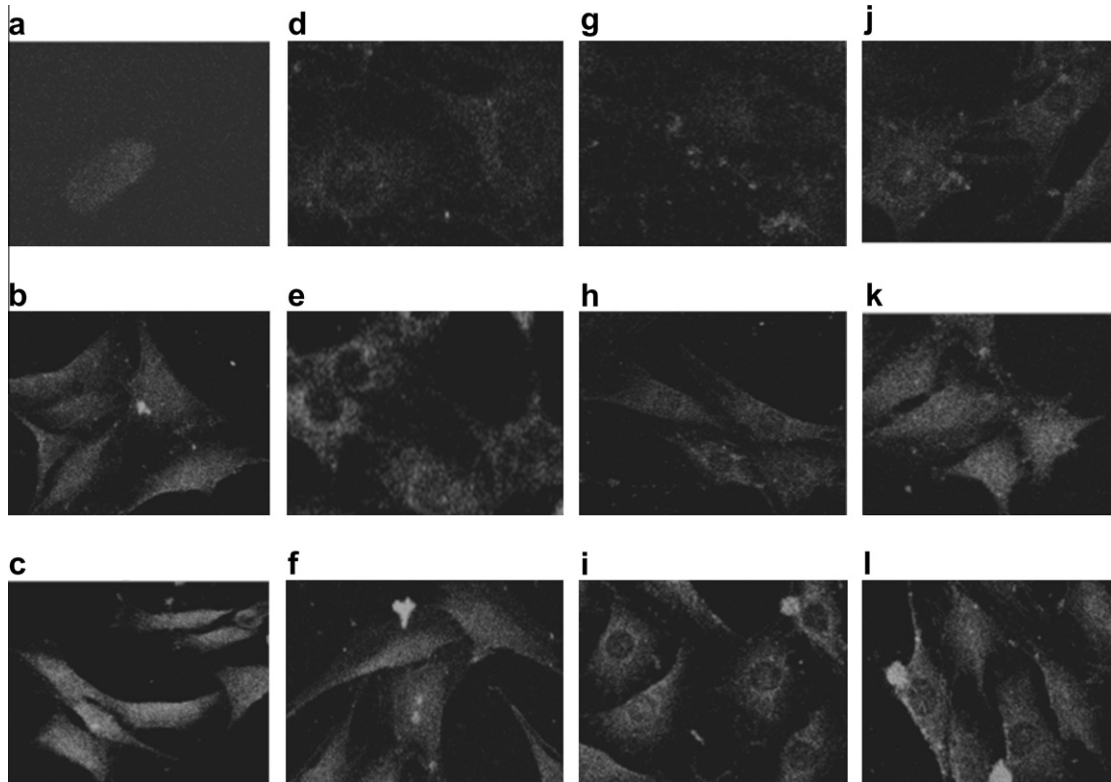

Fig. 5. Confocal immunofluorescence microscopy of FITC tagged GLUT4 in skeletal muscle cells. L6 cells (treated) were fixed onto coverslips using 3.7% formaldehyde and permeabilized with 0.01% Triton X-100. The cells were then labeled with anti-goat GLUT 4 primary antibody and after subsequent washing with 1X PBS, fluorescence stained with donkey anti-goat IgG-FITC secondary antibody. The coverslips after thorough washing were mounted on the slides using Mowiol for confocal microscopy. Panel (a) refers to untreated cells and panel (b and c) indicates the fluorescence pattern of the cells treated with Insulin for 5 min and 15 min respectively. (d–f), (g–i) and (j–l) represents the cells treated with 6 h, 12 h and 24 h of Rosiglitazone, *Adathoda vasica* ethyl acetate extract and (3β)-stigmast-5-en-3-ol respectively. 600× magnification.
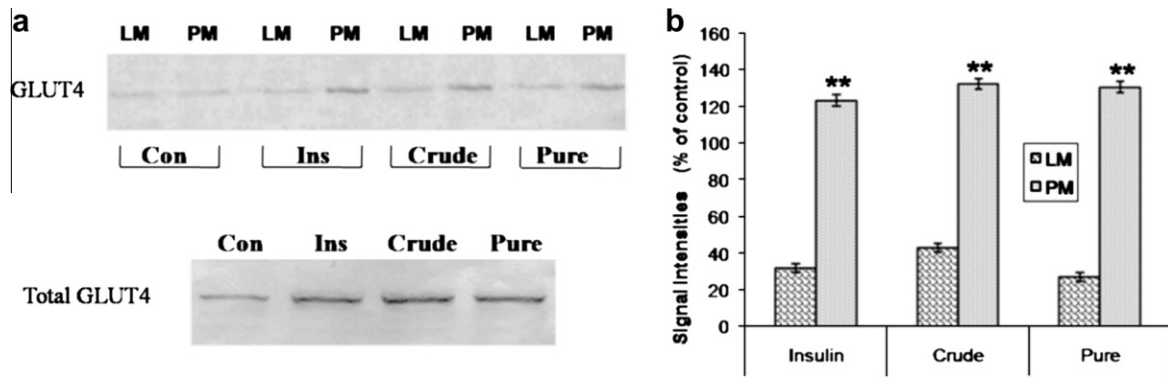
Fig. 6. (a) GLUT4 translocation pattern of the cells treated with *Adathoda vasica* ethyl acetate extract and (3β)-stigmast-5-en-3-ol using subcellular membrane fractions. Serum-starved L6 myotubes were treated with Insulin for 15 min, crude and pure for 24 h, followed by sub-cellular membrane fractionation as mentioned in the methods section. The resultant LM and PM fractions were immunoblotted with anti-GLUT4 antibody and the translocation pattern analyzed. Total GLUT4 content were also shown. (b) Representative densitometry analysis of GLUT4 translocation. The signaling intensities of GLUT4 protein expression in LM & PM were detected and quantitated using densitometric scanning. The signaling intensities were quantified arbitrarily and the data was expressed as percentage over control. Bars represent mean of ±SE., $n = 3$ and a representative blot is depicted here. (**), $P < 0.01$ as compared with untreated control group.

tract or (3β)-stigmast-5-en-3-ol resulted in a marked decrease in glucose uptake of up to 50.67 ± 3% and 41.35 ± 6% respectively, as shown in Fig. 7b.

4. Discussion

Skeletal muscle is one of the key insulin targeted tissue in maintaining whole body glucose homeostasis, through the stimulation of glucose uptake mediated by GLUT4 translocation [25]. L6 myotubes is a well established skeletal muscle model for studying glu-

Fig. 7. Glucose uptake patterns of *Adathoda vasica* ethyl acetate extract and (3β)-stigmast-5-en-3-ol in presence of Genistein-IRTK inhibitor (a) and Wortmannin-PI3K inhibitor (b). L6 myotubes were treated with Genistein – 50 μM (a) and Wortmannin – 100 nM (b), 30 min prior to the incubation with the crude (1 μg/mL), pure (100 ng/mL) or insulin (100 nM), followed by the 2-deoxy-D-[1-³H] glucose uptake assay. The results were expressed as percentage of glucose uptake with respect to the vehicle control. The values are mean of ±S.E., $n = 3$ in duplicates. (*), $P < 0.05$ as compared with vehicle control group.

cose uptake process [23] and hence used for the present study. Plant derived natural compounds have established a platform for developing new drug synthesis with fewer side effects [26].

Our study demonstrates the anti-diabetic effect of the ethyl acetate extract and (3β)-stigmast-5-en-3-ol of *A. vasica in vitro*. Both the ethyl acetate extract and the (3β)-stigmast-5-en-3-ol were able to stimulate basal glucose uptake in differentiated L6 cells (Fig. 1). Basal but not insulin-mediated glucose uptake was checked, to ensure that (3β)-stigmast-5-en-3-ol behaves like insulin-mimetic compound, and not insulin-sensitizer. Previous reports on the ethanolic extracts from the leaves of *A. vasica* showed hypoglycemic activity after oral administration in rats and rabbits [9]. Since glucose uptake is the primary requisite for maintaining glucose homeostasis, this study could better explain the mechanism behind the hypoglycemic activity of the leaves of *A. vasica*. Furthermore, recent studies on (3β)-stigmast-5-en-3-ol have shown an increase in glucose uptake of around 1.4 fold [12] in L6 myotubes. Our study showed the increase in glucose uptake of up to 1.2 fold at a much lower concentration. In comparison with the crude extract, (3β)-stigmast-5-en-3-ol exhibited a ten-fold enrichment of glucose uptake activity (the optimum concentration of crude extract and (3β)-stigmast-5-en-3-ol was 1 μg/mL and 100 ng/mL respectively).

To unravel the molecular mechanism of ethyl acetate extract and (3β)-stigmast-5-en-3-ol in enhancing glucose transport, their effect on insulin signaling cascade was examined. Jung and co-workers [27] have proven that triterpenoids can act as insulin-mimetics by activating IR-β, IRS-1 and GLUT4 translocation. Similarly, our study was designed to prove whether (3β)-stigmast-5-en-3-ol could serve as a potent insulin-mimetic compound in insulin resistant conditions. Alteration in the level of IR or defects in its signal transduction pathway have been found in diabetic patients associated with decreased levels of IR-β, IRS-1 and PI3K [28,29]. Both the ethyl acetate extract and (3β)-stigmast-5-en-3-ol were able to restore IR-β and IRS-1 tyrosine phosphorylation in L6 myotubes (Fig. 3a). In the present study, the activation of glucose uptake was strongly inhibited by genistein upon treatment with the ethyl acetate extract and (3β)-stigmast-5-en-3-ol, suggesting that glucose transport is dependent on the involvement of the insulin receptor.

Many reports emphasize that PI3K plays a major role in insulin signaling pathway, and regulates insulin-mediated glucose transport [30]. Pretreatment with specific PI3K inhibitor (wortmannin) resulted in a decline in the glucose uptake activity of the ethyl acetate extract and (3β)-stigmast-5-en-3-ol, suggesting the
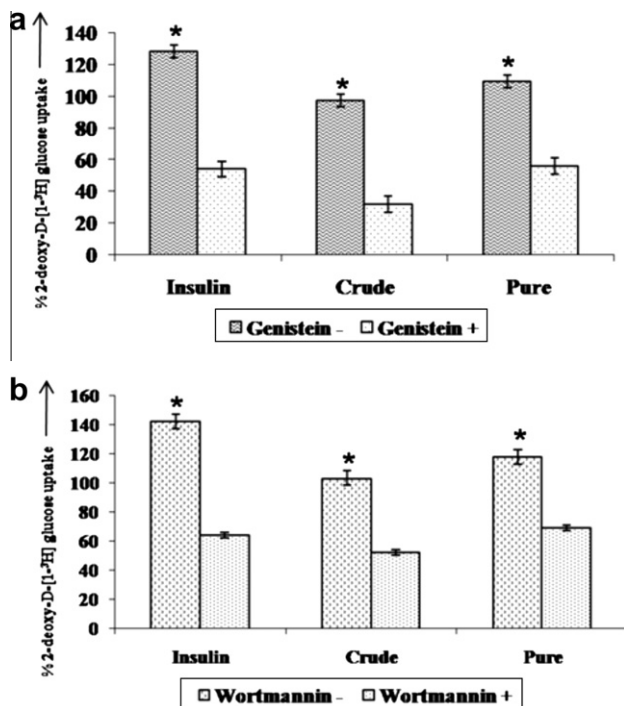
involvement of PI3K in enhancing glucose transport. To substanti-ate the above finding, gene level and protein level expressions were also examined. The enhancement of PI3K expression (mRNA as well as p85 PI3K) on treatment with ethyl acetate extract and (3β)-stigmast-5-en-3-ol proves the involvement of PI3K in insulin signaling cascade.

(3β)-stigmast-5-en-3-ol -induced anti-proliferation in human leukemia cells is mediated by promoting spindle microtubule dynamics through the Bcl-2 and PI3K/Akt signaling pathways [10], which coincides well with the increment in PI3K and pAKT levels upon treatment with (3β)-stigmast-5-en-3-ol in our study. There is a clear picturisation of the signaling events occurring in skeletal muscle in augmenting glucose uptake. To determine the engagement of the other downstream protein kinase, the role of PKCα was examined. The increase in PKCα on treatment with the extract and (3β)-stigmast-5-en-3-ol concurs with the existing liter-ature in bringing about effective glucose transport in rat skeletal muscle cells [31].

The next important target under investigation is GLUT4, which is the major insulin responsive glucose transporter in skeletal mus-cle cells. The gene expression studies revealed the enhancement of GLUT4 levels upon treatment with the ethyl acetate extract and (3β)-stigmast-5-en-3-ol and is responsible for the increment in glucose uptake. Similar findings were reported on the glucose up-take activity of cinnamic acid which is attributed by the increased expression of GLUT4 in L6 myotubes [32]. Insulin resistance is found to be associated with impairment of GLUT4 translocation from internal pool to the plasma membrane in skeletal muscles [1]. Confocal analysis revealed the presence of GLUT4 around the nucleus at 12 h and at 24 h fluorescence was observed throughout the cells. This is due to the complete redistribution of FITC tagged GLUT4 throughout the cytoplasm. A clear picture of GLUT4 translo-cation from the intracellular pool to the plasma membrane could be evidenced if more sensitive cell lines like L6 GLUT4myc myo-blasts or 3T3-L1 adipocytes were used. However, an increase in the intensity of fluorescence was observed, which clearly denotes the increment of GLUT4 in the cells incubated with ethyl acetate extract and (3β)-stigmast-5-en-3-ol. We have also analysed the GLUT4 translocation using sub-cellular membrane fractionation and western blot. The ethyl acetate extract and (3β)-stigmast-5-en-3-ol were able to translocate GLUT4 from the intracellular pool to the plasma membrane on a par with the positive control, insulin. The presence of cycloheximide (a protein synthesis inhibitor) pre-vented the glucose uptake stimulated by crude extract and (3β)-stigmast-5-en-3-ol (data not shown), thereby indicating that the increased glucose uptake is primarily due to the increased GLUT4 protein synthesis only. Another report indicated that the transloca-tion of GLUT4 to the plasma membrane by troglitazone on L6 myo-tubes is responsible for glucose uptake efficacy [15], and well supports our data. Collectively, these results indicate that (3β)-stigmast-5-en-3-ol induced glucose transport is mediated in a PI3K dependent manner on L6 myotubes. Our study is the first to unravel the molecular mechanism of (3β)-stigmast-5-en-3-ol by studying its effect on key targets in insulin signaling (IR-β, IRS-1, PI3K, AKT, PKC & GLUT4), which elucidates the anti-diabetic prop-erty in vitro. (3β)-stigmast-5-en-3-ol was found to be non-toxic in L6 myoblasts which was determined by the lactate dehydrogenase release (data not shown). Moreover, (3β)-stigmast-5-en-3-ol is nei-ther genotoxic nor cytotoxic and recommended safe for therapeu-tic use [33].

5. Conclusion

We have displayed the possible mechanism underlying the anti-diabetic activity of (3β)-stigmast-5-en-3-ol in L6 myotubes (Fig. 8). Our study is the first to demonstrate the activation of IR-β, IRS-1, PI3K, AKT/PKB, PKCα and GLUT4 by (3β)-stigmast-5-en-3-ol in augmenting glucose transport in rat skeletal muscles. Moreover, the restoration of glucose uptake activity by (3β)-stig-mast-5-en-3-ol is evidenced without the stimulation of insulin, suggesting that insulin-like property is a/the mechanism underly-ing the anti-diabetic activity of (3β)-stigmast-5-en-3-ol in vitro. Overall, our results suggest that (3β)-stigmast-5-en-3-ol may have therapeutic effects in improving insulin resistance, in addition to its cholesterol lowering effects.

Fig. 8. Schematic diagram explaining the possible mechanism of action of (3β)-stigmast-5-en-3-ol. (3β)-stigmast-5-en-3-ol exerts its anti-diabetic effect through activation of cellular targets including IR, IRS-1, PI3K, PKC and GLUT4 of insulin pathway resulting in enhanced glucose transport in L6 myotubes.

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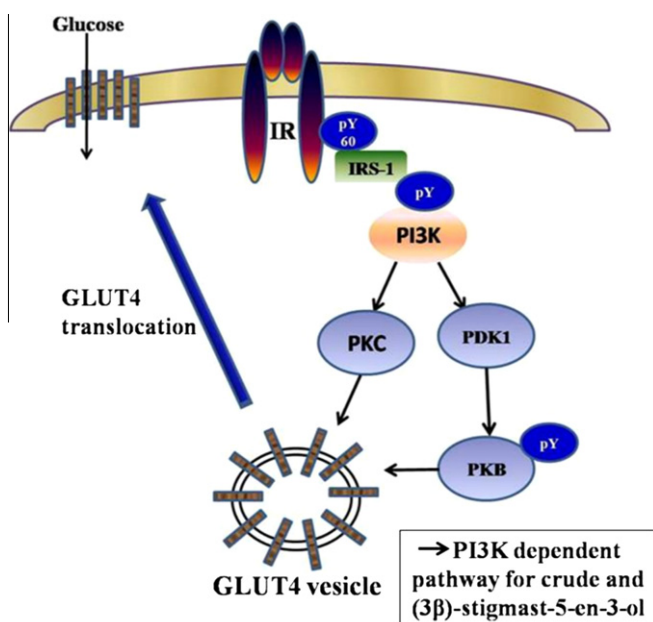
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