4.1 Introduction

Understanding the mechanism of action with respect to any candidate drug molecule is a necessary prerequisite before it can be forwarded for the further stages in the long process of drug discovery and development. Several pathways have been reported to be involved in the pathogenesis of insulin resistance and T2DM depending upon the various tissue types. Since, about 70-75% of total glucose is stored in the muscle tissue, occurrence of insulin resistance in these types of cells increases the risk of developing T2DM[1]. In the muscle cells, the downregulation of insulin receptor gene is considered to be one of the major reasons for the development of insulin resistance where activated PKCθ plays an important role[2]. Furthermore, low grade inflammation of muscle cell is also found to be a risk factor[3]. Apart from PKCθ, other PKC isoforms like PKCε also plays a pivotal role in the development of insulin resistance in the muscle cells[4].The present study reveals that the active fraction of the *L. aspera* ethanolic extract (LAE) could significantly restore the phosphorylation of AKT and thereby induced the glucose uptake in the FFA treated L6 myotubes. In this chapter, we try to investigate the underlying mechanism of the LAE in the reversal of FFA induced inhibition of insulin action and glucose uptake. In the present study, we demonstrate the unexplored mechanism of action of the LAE to overcome the damage of insulin action in the FFA treated L6 myotubes. The *in vitro* result was also further validated in *in vivo* experimental diabetic mice model.

4.2 Materials and Methods

4.2.1 Chemicals and Reagents

All the cell culture materials were obtained from Gibco-BRL, Life Technologies Inc., Gaithersburg, MD 20884-9980, USA and Sigma Aldrich, St. Louis, MO, USA. [^{3}H] 2- deoxyglucose (Specific activity: 12.0 Ci/mmol) was purchased from GE Healthcare Bioscience Ltd., Kowloon, HK. Antibodies used viz. anti-pIRβ, anti-pIRS1, anti-pPI3K,anti-pPDK1, anti-pAKT1/2/3, anti-pGLUT4, anti-PKCθ, anti-NFκBp65, anti-IKKα/β, anti-IL6, anti-TNFα, anti-IL1β, anti-TLR4, anti-Fetuin A, and some corresponding non-phosphorylated as well as anti- β actin and anti- α tubulin were purchased from Santa Cruz Biotechnology Inc., USA. Alkaline phosphatase conjugated anti mouse, anti rabbit and anti goat

secondary antibodies were also purchased from Santa Cruz Biotechnology Inc., USA. All the other chemicals were purchased from Sigma Aldrich, St. Louis, MO, USA, Himedia, Mumbai, India, Thermo Scientific, Waltham, MA, USA and BioBharati Life Science, Kolkata, India. Both the analytical and HPLC grade solvents were purchased from Merck, Mumbai, India

4.2.2 Cell culture and treatment

L6 skeletal muscle cell line and RAW264.7 murine macrophage cell line were procured from theNational Centre for Cell Science (NCCS), Pune, India and cultured in culture flask containing DMEM media supplemented with 2 mM L-glutamine, 1mM sodium pyruvate, 10% FBS and penicillin (100 units/ml), streptomycin (10 µg/ml) and allowed to incubate at 37^{o}C in a humidified 5% CO_2 environment. The confluent cells were treated without (control) or with 0.75 mM palmitate (lipid containing media was prepared by conjugation of free fatty acid with bovine serum albumin (BSA) as described by Dey et al., 2005)[5] or with the bioactive compounds along with palmitate and was incubated for 6 hrs. Initially the cells were treated with the LAE and incubated for 2 hrs followed by 4 hrs incubation with palmitate. After termination of the incubations, L6 cells were pelleted by centrifugation at 2000 rpm for 5 mins. The pellet were resuspended in NP-40 lysis buffer [1% NP-40, 20mM HEPES(pH 7.4), 2 mM EDTA, 100 mM NaF, 10mM sodium pyrophosphate, 1mM sodium orthovanadate, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 µg/ml pepstatin and 1mM PMSF] and sonicated on ice for 10 mins. The cell lysates were centrifuged at 13000 rpm for 10 mins at 4ºC. The supernatant was collected and the protein concentrations were determined by the method of Lowry et al, 1951[6]. After the termination of incubation of the RAW264.7 macrophage cells, total RNA were extractedfor further study.

4.2.3 Preparation of high fat diet (HFD) model

The albino BALB/c mouse model was used for the animal study following the guidelines approved by the Visva-Bharati (A Central University) Animal Ethics Committee. Nine disease free healthy mice were segregated into three groups with 3 mice in each group as control, HFD and HFD+ LAE respectively. Animalsof each group were allowed to live normally under 12 hrs light/dark cycles at

temperature 23±2 ºC with *ad libitum* access to food and water. The HFD mice were fed high fatty diet food containing 32.5% lard, 32.5% corn oil, 20% sucrose and 15% protein, whereas the control group of mice were fed with standard diet containing 57.3% carbohydrate, 18.1% protein and 4.5% fat. After the generation of the HFD mice, a group of mice was treated with the LAE active fraction through oral gavage.

4.2.4 Collection of blood plasma

The blood of the experimental mice was collected form the orbital sinus under ether anaesthesia for the biochemical, haematological and molecular study. An aliquot of whole blood was centrifuged immediately at 2000 rpm for 20 mins at 4ºC. The supernatant containing blood plasma was collected and used for further study.

4.2.5 Biochemical and haematological analysis from the blood and serum isolated from the treated and control mice

The biochemical parameters, including cholesterol, triglycerides, low density lipoprotein (LDL) cholesterol, very low density lipoprotein (VLDL) cholesterol, alanine aminotransferase (ALT or SGPT), aspartate aminotransferase (AST or SGOT) and alkaline phosphatase (ALP), blood urea, serum creatinin, bilirubin, blood urea nitrogen (BUN) were estimated using commercial kits. The haematological parameters were also analysed for all the 3 groups of mice.

4.2.6 Isolation of skeletal muscle tissue

Skeletal muscle was isolated from the hamstring region of the experimental mice. The muscle tissue was dissected out, washed thoroughly and subjected to digestion in DMEM in presence of trypsin (0.02%) and collagenase (0.5%) for 30 mins at 37 ºC in presence of 95% O_2 /5% CO_2. The dispersed skeletal muscles were pelleted by brief centrifugation at 500xg for 5 mins followed by washing and processed for further study.

4.2.7 Isolation of adipocyte

The abdominal adipocyte was also isolated from the control and experimental mice. Briefly, the abdominal fat pads were removed, minced, and digested using 3.3 mg/ml type II collagenase and 5.5 mM glucose, 5% (w/v) fatty acid (FA) free BSA containing Hanks buffered saline solution (HBSS) at 37 °C for 1 hr. The digested primary adipose cells were then passed through a tissue sieve and centrifuged at 500xg for 5 mins. The pellet containing adipocytes were then washed extensively using Dulbecco's modified Eagle's medium containing 2% fetal bovine serum (FBS) by centrifugation. The supernatant was discarded and the pellete containing adipocyte was stored in 4ºC for further study.

4.2.8 Preparation of cell lysate:

The isolated skeletal muscle tissue from the control and treated mice were resuspended in lysis buffer (1% NP-40, 20 mM HEPES of pH 7.4, 2 mM EDTA, 100 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1μg/ml leupeptin, 1μg/ml aprotinin, 1μg/ml pepstatin and 1 mM PMSF) and sonicated on ice for 10 mins. The cell lysates were centrifuged for 10 mins at 10,000x g and the supernatant was subjected to determine the protein concentration following the method described by Lowry et al., 1951[6].

4.2.9 SDS-PAGE and Immunoblot

The control and the treated cell lysates were prepared according to the protocol describes previously. Initially, 60 μg of protein were resolved on 10% SDS-PAGE and transferred to PVDF membranes of pore size 0.45 μm (Millipore) using transfer buffer, pH8.0 for 1.5 hrs. Membranes were blocked with 5% non-fat dried milk in TBST (Tris buffered saline+ Tween 20) buffer for 2 hrs and incubated with different primary antibodies generated in goat, rabbit and mice for 4 hrs. The membrane bound primary antibodies were visualised using the corresponding secondary antibodies tagged with alkaline phosphatase for 2 hrs and were developed with the corresponding substrates, 5-bromo 4-chloro 3-indolyl phosphate/ nitro blue tetrazolium (BCIP/NBT). Image of the bands were observed using gel documentation apparatus (Gel Doc XR+ Imager,BioRad, USA). Band intensities were quantified by available GelQuant.NET software v 1.6.8.

4.2.10 cDNA synthesis

Total RNA was extracted from the control and the treated macrophagecells using TRI reagent (Sigma Aldrich) and reverse transcription was performed using RevertAid cDNA synthesis kit, Fermentas Life Sciences, Hanover, MD, USA and Super Reverse Transcriptase MulV Kit, BioBharati Life Science, India according to the manufacturer's protocol.

4.2.11 Semi quantitative RT-PCR

The semiquantitative RT-PCR was performed to check the relative expression of the mRNA of the target genes. The amplification of the cDNA of the respective mRNA was performed using *Taq* DNA Polymerase plus kit, BioBharati Life Science, India with a little modification of the manufacturer's protocol and DyNAzyme polymerase of Thermo Scientific, Waltham, MA, USA.

4.2.12 Statistical analysis

All data were derived from at least two or three independent experiments and statistical analyses were conducted using the GraphPad PRISM v 6.07 software. The one tailed p-value was calculated to analyse the significant differences. Means were compared by a post hoc multiple range test. All values were means±SEM. A level of $p<0.05$ was considered significant.

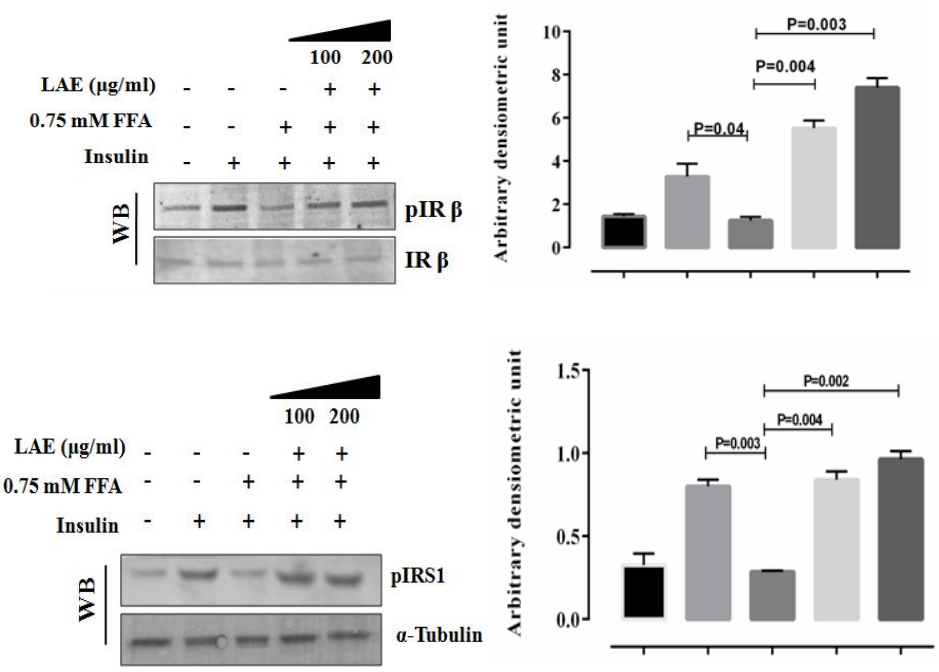
4.2.13 *Insilico* docking study

Molecular Docking studies were carried out using Molegro Virtual Docker (MVD) 5.0 (MolegroApS, Aarhus, Denmark). The scoring functions of the ligands and the H-bonds formed with the amino acids were used in the prediction of the binding affinities, different binding modes and orientation of the compounds in the active site(s) of TLR4 (PDBID: 2Z65). The water molecules were removed in the study. The cavity detection algorithm in MVD was used for optimizing the potential binding site(s), i.e., it makes it possible to define an approximate location of the most likely interaction sites. A set of 100 runs was given for each docking study using 2000 interactions. Both the Rerank score and the MolDock score were calculated. The best fit score was obtained on the basis of the MolDock score[7].

4.3 Results

4.3.1 Active fraction of *L. aspera* (LAE) reverses palmitate mediated alteration of insulin sensitivity

The insulin signalling cascade that ultimately leads to the uptake of glucose in the glucose storage cells is well characterized. Binding of insulin to the α subunit on the extracellular domain of insulin receptor leads to autophosphorylation of tyrosine kinase in β- subunit of insulin receptor and thus triggers the signalling pathway. To investigate whether the impaired insulin signalling by palmitate could be restored by the LAE, we incubated L6 myotubes with palmitate in the presence or absence of the LAE active fraction. Insulin signalling was augmented by insulin from upstream to downstream through the phosphorylation of IRβ, IRS1, PI3K, PDK1, AKT and GLUT4. Addition of palmitate significantly attenuates this stimulation (Fig 4.1). From the band intensities of non phosphorylated IRβ, AKT and GLUT4, it is confirmed that neither insulin nor palmitate could alter the levels of these proteins. The decrease of phosphorylation of IRβ, IRS1, PI3K, PDK1, AKT and GLUT4 by FFA has been reported earlier[8,9,10].Addition of LAE restored the phosphorylation of these proteins significantly in a dose dependent manner with p values less than 0.05 (Fig. 4.1)



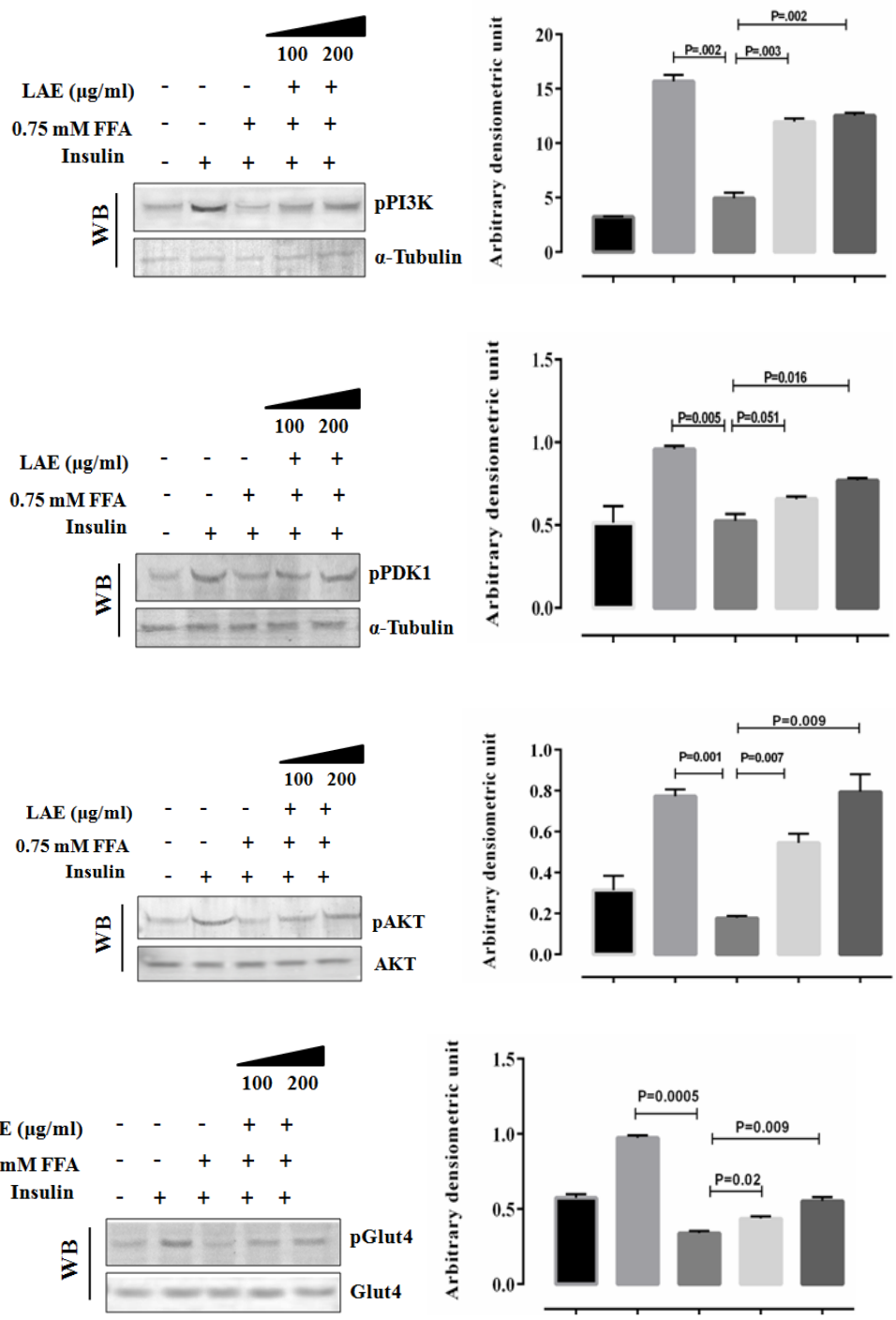


Figure 4.1:Palmitate inhibition of insulin signalling pathway was prevented by LAE. L6 myotubes were treated for 4 hrs with plamitate or palmitate along with various conc. of *L. aspera* active fraction (LAE) followed by 30 mins incubation with insulin. Western blot was performed with anti- pIRβ, anti-pIRS1, anti-pPI3K, anti-pAKT, anti-pGlut4. Some non phospho specific antibodies, anti-IRβ, anti-AKT, anti-Glut4 and anti-β actin were used as the loading control. Densiometric analysis carried out by using the GelQuant.NET software v 1.6.8. Each value is the Mean ± SEM of two individual experiments and the p values were calculated using GraphPad PRISM v 6.07

4.3.2 LAE inhibits palmitate induced phosphorylation of PKCθ in L6 skeletal muscle cells

Several studies linked PKC translocation with defective insulin stimulated glucose metabolism suggesting the association between PKC activation and insulin resistance in the skeletal muscle cells. The novel PKC (nPKC) isoforms, mainly PKCθ and PKCε were found to be more with elevated level of lipid intermediates including DAG[5, 11]. Thus, the skeletal muscle isolated from the HFD fed rats exhibited an increase in translocation of PKCθ and PKCε in conjugation with the elevated lipid content along with diminished glucose disposal[12,13]. Previous studies demonstrated that, the elevated FFA activates PKCθ resulting in induced insulin resistance in the rat skeletal muscle cells[14]. It has also been reported that the elevated FFA induced PKCθ activation is one of the responsible factors for the ser/thr phosphorylation of IRS1 protein present in the insulin signalling pathway and impairs it in muscle cells. To observe the affect of LAE on the palmitate induced PKCθ phosphorylation, western blot analysis was performed using specific phospho-PKCθ antibody. Alpha- tubulin was used as the loading control. Western blot analysis demonstrates that enhanced phosphorylation of PKCθ (pPKCθ) in the palmitate treated cells were significantly reduced by the treatment with LAE (Fig. 4.2).

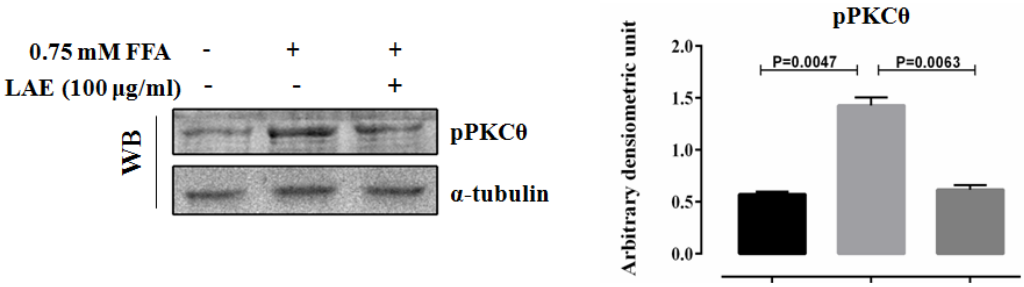


Figure 4.2: LAE reduced thePalmitate induced activation of PKCθ. L6 myotubes were treated for 4 hrs with plamitate or palmitate along with various concentrations of the LAE or without any of them (control). Cell lysates of all treatment conditions were isolated and subjected to SDS-PAGE followed by immunoblotting using anti-pPKCθ antibody. Alpha-tubulin was used as the loading control. Densitometric analysis was carried out by using the GelQuant.NET software v 1.6.8. Each value is the Mean ± SEM of two individual experiments and the p values were calculated using the GraphPad PRISM v 6.07

4.3.3 Palmitate induced overexpression of pro-inflammatory cytokines was attenuated by the LAE through downregulation of the NFκBp65 phosphorylation

Western blot was also performed to determine the content of proinflammatory cytokines in the culture media. The result obtained reveals that by the treatment of FFA, the content of proinflammatory cytokines, *viz.*, TNFα, IL6 that were released from the cells to the media was more with significant p value 0.036 and 0.018 respectively than the control which could be lowered significantly by the treatment with the LAE with p value 0.07 and 0.013 in case of TNFα and IL6 respectively. (Fig. 4.3)

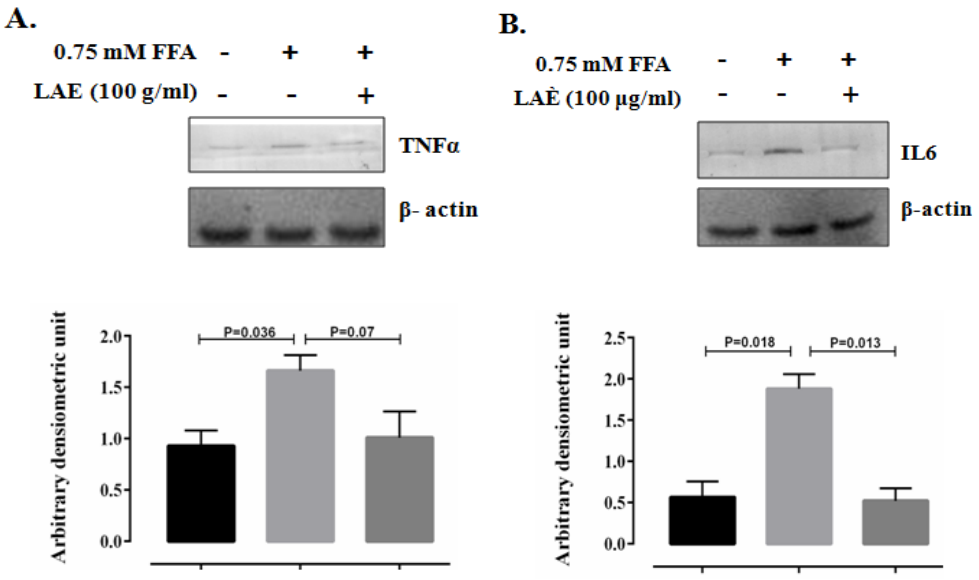


Figure 4.3:Palmitate induced proinflammatory cytokines level was suppressed by LAE. L6 myotubes were treated for 4 hrs with plamitate or palmitate along with various concentrations of the LAEor without any of them (control). Culture media was collected from the cultured samples and lyophilized followed by immunoblot analysis using anti-TNFα and anti-IL 6 antibody. Alpha-tubulin was used as the loading control. Densitometric analysis was carried out by using the GelQuant softwarev 1.6.8. Each value is the Mean ± SEM of two individual experimentsand the p values were calculated using the GraphPad PRISM v 6.07

NF-κB is known to play a critical role in implementing the pathogenesis in insulin resistance and type 2 diabetes [15, 16, 17, 18, 19]. Activation of NF-κB by phosphorylation in presence of FFA leads to the overproduction of pro-inflammatory cytokines in both adipocyte and muscle cells[20,21,22]. The elevated levels of pro-inflammatory cytokines are the key factors in the development of

insulin resistance and the related metabolic syndromes[23]. To evaluate the effect of the LAE on NFκBp65 activation, L6 myotubes were treated with palmitate and palmitate along with the LAE or without any of them followed by western blot analysis using anti- pNFκBp65 antibody. Alpha tubulin was used as the loading control. From the western blots and the densitometric analysis it was found that palmitate induced NFκBp65 phosphorylation is four fold more than the control ($p < 0.023$). On the other hand, cells treated with LAE, the level of NFκBp65 phosphorylation was reduced significantly (approximately four fold) than the palmitate treated cells ($p < 0.022$). (Fig. 4.4)

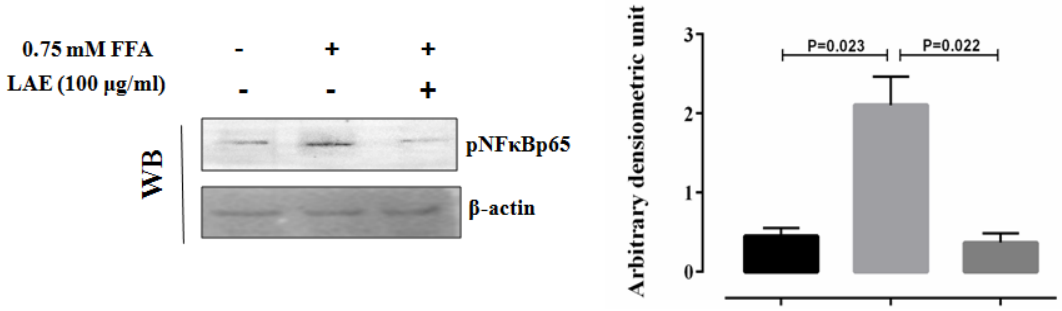


Figure 4.4: LAE reduced thePalmitate induced activation of NFκBp65. L6 myotubes were treated for 4 hrs with plamitate or palmitate along with various concentrations of the LAE or without any of them (control). Immunoblot analysis was performed using anti-pNFκBp65 antibody. Alpha-tubulin was used as the loading control. Densitometric analysis carried out by using the GelQuant.NET software v 1.6.8. Each value is the Mean ± SEM of two individual experiments and the p values were calculated using GraphPad PRISM v 6.07

4.3.4 High fat diet induced T2DM mice model

High fatty diet (HFD) intake for a prolonged period (30days) increased the body weight of mice to more than 24% in comparison to the control mice. Mice with average body weight of 25 gm were used for the study. After 30days of feeding with the standard diet (SD), the body weight of the mice remained almost the same at 26 gm whereas in the high fed diet (HFD) fed mice the body was become 31gm. After 30days, when the body weight of the HFD mice became 31gm, a group of HFD mice were fed the LAE active fraction orally (60 mg/kg body weight) for 15 days on regular basis. After 15 days the body weight of the LAE treated HFD mice was noticed to be32.3 gm suggesting the efficacy of the LAE treatment towards reducing HFD induced gain of body weight (Fig. 4.5A).

The body weight of the HFD fed mice without the LAE became 35 gm with 40% increases in comparison to the control.

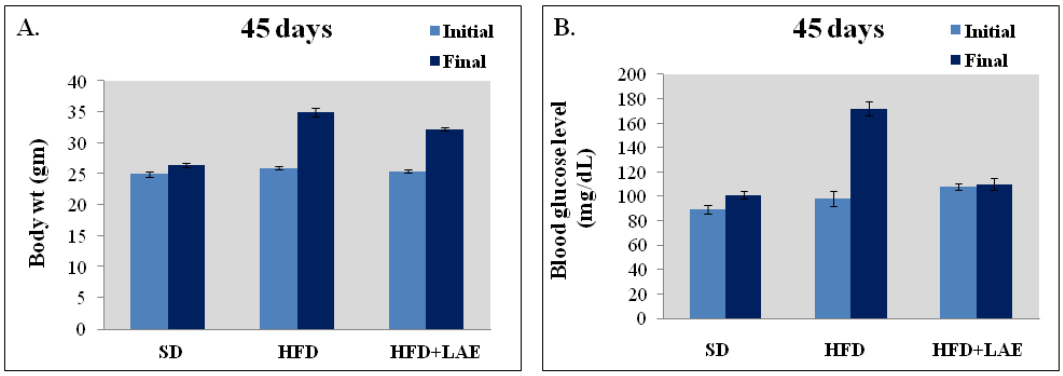


Figure 4.5: LAE effects on HFD-fed diabetic mice. (A) Body weight of SD or HFD fed mice; (B) Blood glucose levels of mice in response to the LAE administration for 15 days in the HFD fed mice.

Along with the body weight, blood glucose levels of the mice were also checked periodically. At the beginning, all the mice showed the average blood glucose level at 93.5 mg/dl. After continuous feeding of the HFD for 30 days, the blood glucose level of the mice reached up to 141 mg/dl. The blood glucose level remained almost same in the normal diet fed control mice having the value of 103 mg/dl. After 15 days of the LAE treatment, the blood glucose level of HFD+LAE mice was reduced to 110 mg/dl where the HFD mice attained the blood glucose level at 172 mg/dl due to continuous supply of high fatty diet (Fig. 4.5B). This observation suggested the blood glucose modulating role of the LAE in the experimental animals.

4.3.5 Biochemical parameters of animals treated with HFD and HFD+LAE

Effect of the LAE active fraction was investigated on various biochemical parameters in SD, HFD and HFD+LAE fed mice. From the results it was found that, in the HFD mice the amount of cholesterol, triglycerides and low density lipoprotein (LDL) cholesterol was significantly higher than that in the SD fed mice whereas the same biochemical parameters were found to be lower in the HFD+LAE mice which are comparable to the control mice. Other parameters, *viz.*, low density lipoprotein (LDL) cholesterol and very low density lipoprotein (VLDL) cholesterol were less than that in the SD fed control mice and these remained same in the HFD+LAE mice (Table 4.1).

Table 4.1: Effect of the LAE active fraction on bio-chemical parameters

Parameters	Control (n=3)	HFD (n=3)	HFD+LAE (n=3)
Cholesterol (mg %)	99.0±6.57	109.0±5.37	103.0±4.62
Triglycerides (mg %)	95.0±5.24	112.0±10.50	106.0±8.07
HDL Cholesterol (mg/dL)	46.0±3.21	49.0±2.05	50.0±3.94
LDL Cholesterol (mg/dL)	32.0±5.13	41.0±4.25[a]	30.6±2.81[b]
VLDL Cholesterol (mg/dL)	21.0±2.03	19.0±4.19	22.4±1.17

[a] represents p value < 0.05 *w.r.t.* SD mice, [b] represents p value < 0.05 *w.r.t.* HFD mice

4.3.6 Determination of sub acute toxicity of the LAE

All the animals were carefully observed for the development of any toxic signs or symptoms after 15 days of the LAE oral treatment. Oral administrations of the LAE did not produce any significant change in the weight of the major organs when compared to that in the control SD mice (Table 4.2).

Table 4.2: Effect of LAE active fraction on organ weight (in gm)

Organ	SD (n=3)	HFD (n=3)	HFD +LAE (n=3)
Liver	1.67±0.14	1.88±0.23	1.56±0.20
Lung	0.22±0.04	0.23±0.04	0.24±0.03
Kidney	0.46±0.09	0.52±0.14	0.42±0.16
Heart	0.17±0.02	0.22±0.06	0.17±0.03
Spleen	0.13±0.01	0.14±0.03	0.10±0.05

[a] represents p value < 0.05 *w.r.t.* SD mice, [b] represents p value < 0.05 *w.r.t.* HFD mice

To evaluate the effect of the LAE active fraction on blood cells, the different haematological parameters of the 3 different types of mice, *viz.*, SD, HFD

and HFD+LAE were checked. From the result, it has been found that there is no significant change in the different haematological parameters in the 3 groups of mice. Table 4.3 shows the values of different parameters in all the cases.

Table 4.3: Effect of LAE active fraction on haematological parameters

Parameters		**SD (n=3)**	**HFD (n=3)**	**HFD+ LAE (n=3)**
Haemoglobin (%)		13.0±1.06	13.6±3.29	13.7±1.19
Leucocyte Count/ mm^3		4800±3.14	7300±7.9[a]	6200±6.63[b]
Differential Leucocyte Count (%)	Neutrophil	25±0.8	38±1.6[a]	36±1.9[b]
	Lymphocytes	73±2.01	56±4.19[a]	59±2.06
	Monocytes	1±0.039	3±0.13[a]	3±0.302
	Eosinophils	1±0.072	3±0.57	2±0.66
	Basophils	00	00	00
Erythrocyte Count/ mm^3		4.50±1.71	4.70±0.92	4.74±1.66

[a] represents p value < 0.05 *w.r.t.* SD mice, [b] represents p value < 0.05 *w.r.t.* HFD mice

Estimation of the alanine aminotransferase (ALT or SGPT), aspartate aminotransferase (AST or SGOT) and alkaline phosphatase (ALP) is one of the most widely used means of measuring hepatocellular injury. From the study it was found that high fatty diet increased the level of these 3 enzymes in the blood of HFD mice than in the normal fed control mice while the administration of the LAE to the HFD mice suppressed the induced level of these enzymes in the blood of HFD+LAE mice. The values of the enzymes present in the blood of the 3 different groups of mice are given in the table 4.4.

Table 4.4: Level of liver enzymes present in the blood of 3 different groups of mice

Parameters	SD (n=3)	HFD (n=3)	HFD+ LAE (n=3)
SGPT (IU/L)	36±1.43	178±3.07[a]	159±0.76[b]
SGOT (IU/L)	38±3.01	172±5.3[a]	166±2.6[b]
ALP (IU/L)	62±1.18	256±2.03[a]	138±0.53[b]

[a] represents p value < 0.05 *w.r.t.* SD mice, [b] represents p value < 0.05 *w.r.t.* HFD mice

To asses the effect of the LAE in kidney function the level of blood urea, serum creatinin, bilirubin, blood urea nitrogen (BUN) were estimated. It was found that the levels of all these factors were significantly more (p value < 0.05) in case of the HFD mice whereas these remained under control after 15 days of oral administration of the LAE active fraction in a group of the HFD mice (Table 4.5)

Table 4.5: Levels of different parameters for the measure of kidney function.

Parameters	SD (n=3)	HFD (n=3)	HFD+ LAE (n=3)
Urea (mg/dL)	35±0.34	60±1.08[a]	45±0.67[b]
Creatinin (mg/dL)	0.10±0.04	0.36±0.03[a]	0.18±0.01[b]
Bilirubin (mg/dL)	0.15±0.04	0.38±0.06[a]	0.24±0.03[b]
BUN (mg/dL)	16.33±0.83	28±0.71[a]	21±1.04[b]

[a] represents p value < 0.05 *w.r.t.* SD mice, [b] represents p value < 0.05 *w.r.t.* HFD mice

4.3.7 Feeding with LAE restores phosphorylation of proteins involved in the insulin signalling pathway in the HFD induced T2DM mice model.

The *in vitro* study showed that, treatment with the LAE in FFA induced L6 cells restores phosphorylation of factors involved in the insulin signalling pathway. To confirm the result *in vivo*, a group of HFD fed mice were treated with the LAE for 15 days as described in the last paragraph. The muscle tissue homogenate was

isolated from the three experimental groups of mice. From the immunoblot analysis of the muscle homogenate it was observed that the phosphorylation of the major proteins, e.g., PI3K, PDK1, AKT and GLUT4 were reduced in the HFD fed mice suggesting impaired insulin signalling. However, 15 days of LAE treatment to the HFD mice restored the phosphorylation of the insulin signalling proteins pointing at the role of the LAE in the modulation of the insulin signalling pathway (Fig. 4.6).

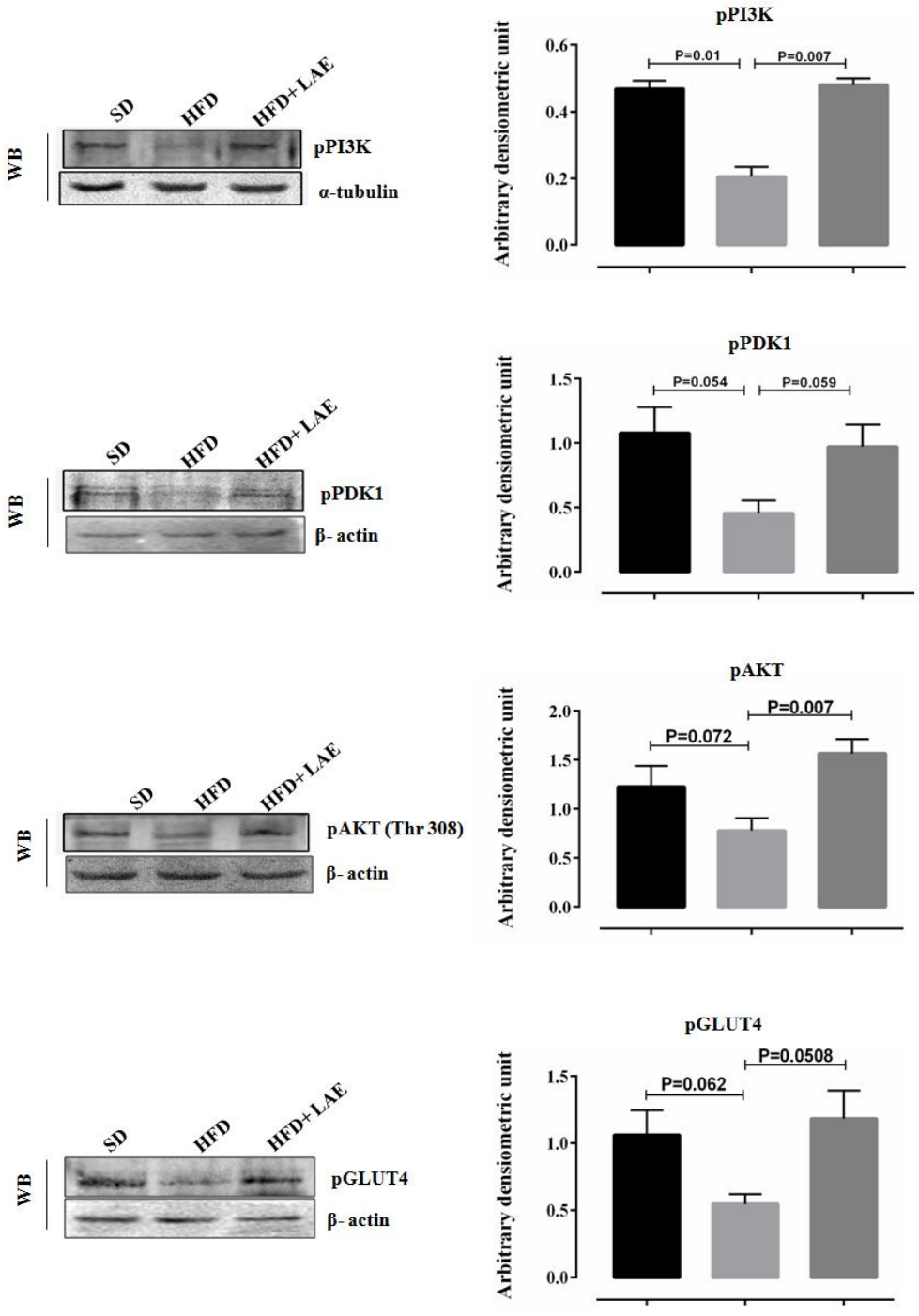


Figure 4.6: HFD induced impairment of insulin signalling pathway was restored by LAE in mice model. Muscle tissue was isolated from SD or HFD or HFD + LAE mice. Western blot analysis was performed from tissue homogenates of each group of mice using anti-PI3K, anti-PDK1, anti-AKT (Thr 308) and anti-GLUT4 antibody. Alpha-tubulin was used as the internal loading control. Densitometric analysis was carried out by using the GelQuant.NET software v 1.6.8. Each value is the Mean ± SEM of two individual experiments and the p values were calculated using GraphPad PRISM v 6.07.

4.3.8 LAE attenuates palmitate induced activation of PKCθ and NFκBin muscle isolated from HFD induced T2DM mice model

From the *in vitro* experiments it was found that the LAE could reduce the phosphorylation of kinase independent PKCθ induced by the action of FFA and hence it ameliorated the FFA induced suppression of protein phosphorylation involved in the insulin signalling pathway. Since the LAE augmented phosphorylation of the insulin signalling proteins in the HFD mice, western bolt analysis was performed to check the activity of the LAE in suppression of PKCθ phosphorylation. From the western blot analysis it was observed that the PKCθ phosphorylation was significantly more (approximately two fold) in the HFD mice Fifteen days of LAE treatment could reduce the PKCθ activation in HFD mice.

From the immunoblot analysis it was also observed that along with PKCθ phosphorylation, the LAE significantly reduced the HFD induced phosphorylation of IKKα/β and NFκBp65 in the experimental mice (Fig. 4.7). This result corroborates nicely with the *in vitro* observations.

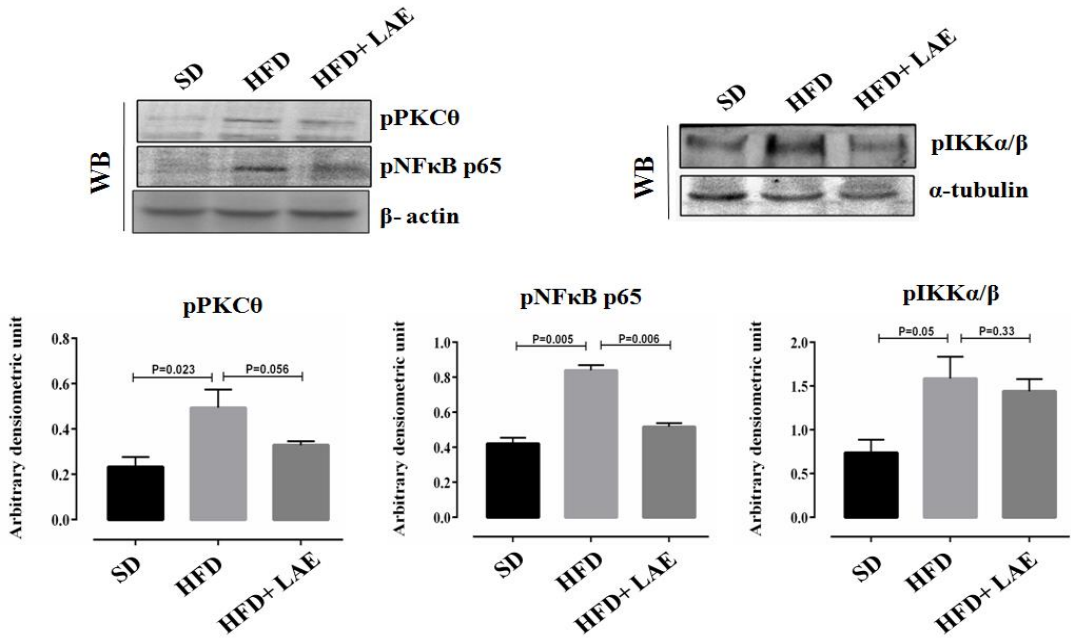


Figure 4.7: LAE attenuated palmitate induced activation of PKCθ and NFκB in HFD mice. Muscle tissue was isolated from SD or HFD or HFD + LAE mice followed by tissue homogenization. Western blot analysis was performed from tissue homogenates of each group of mice using anti-PKCθ, anti NFκB p65 and anti-IKKα/β antibody where α-tubulin was used as the internal loading control. Densitometric analysis was carried out by using the GelQuant.NET software v 1.6.8. Each value is the Mean ± SEM of two individual experiments and the p values were calculated using the GraphPad PRISM v 6.07.

4.3.9 HFD induced overexpression of proinflammatory mediators were reduced by LAE in the adipocyte of the HFD induced diabetic mice model

Adipose tissue is one of the target sites of insulin stimulated glucose storage in the body. Several reports suggest that, elevated level of FFA alters the normal adipocyte function resulting in the development of insulin resistance. Along with the macrophage, adipocyte is also responsible for FFA induced overproduction of proinflammatory cytokines *via* NFκB pathway in association with Toll Like Receptor 4 (TLR4) and Fetuin-A (FetA)[24]. It is well established that, the elevated level of FFA is also responsible for the overexpression of FetA and TLR4 and multiple proinflammatory cytokines leading to inflammation during insulin resistance in the body.

The *in vitro* results showed the overproduction of proinflammatory cytokines in the FFA treated muscle cells were downregulated due to the LAE treatment. To confirm this observation, the effects of the LAE in alteration of FFA

induced overexpression of these proteins in adipocytes isolated from experimental mice model were studied. From the immunoblot analysis of the tissue homogenate of the adipocyte isolated from the HFD fed mice, significant elevation of IL6, TLR4 and FetA was observed (Fig. 4.8). In contrast, there was significant downregulation of these proteins isolated from mice fed with the LAE (Fig. 4.8).

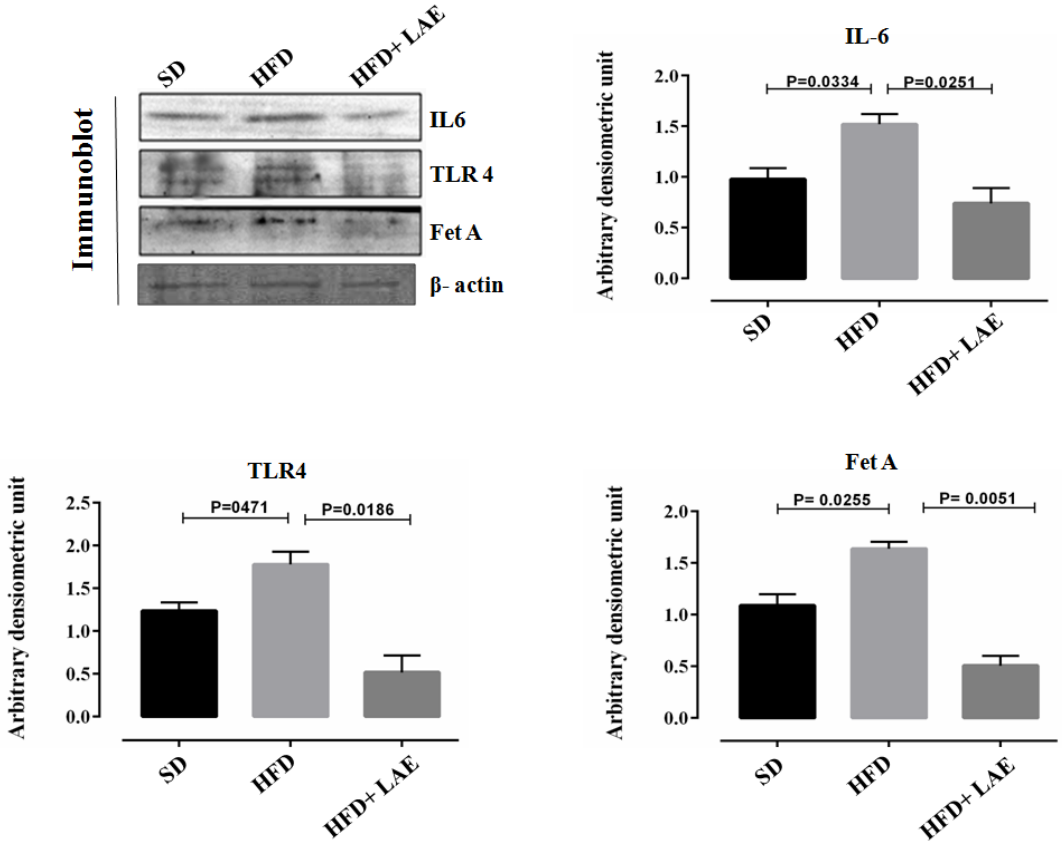


Figure 4.8: HFD induced overexpression of proinflammatory mediators were reduced by LAE in mice model. Adipose tissue was isolated from SD or HFD or HFD + LAE mice followed by tissue homogenization. Western blot analysis was performed from tissue homogenates of each group of mice using anti-IL-6, anti-TLR4, anti-FetuinA antibody where α-tubulin was used as the internal loading control. Densitometric analysis was carried out by using the GelQuant software v 1.6.8. Each value is the Mean ± SEM of two individual experiments and the p values were calculated using the GraphPad PRISM v 6.07

4.3.10 LAE reduces expression of proinflammatory cytokines in the serum of HFD induced T2DM models

To confirm the effect of the LAE in suppressing proinflammatory cytokines production in the HFD mice, blood plasma was collected from the experimental sets of mice. Immunoblot analysis revealed that in the HFD fed mice the proinflammatory cytokines were overexpressed in the plasma. However, treatment

with the LAE reduced the cytokines suggesting the role of LAE in attenuation of the HFD induced overexpression of proinflammatory cytokines (Fig. 4.9).

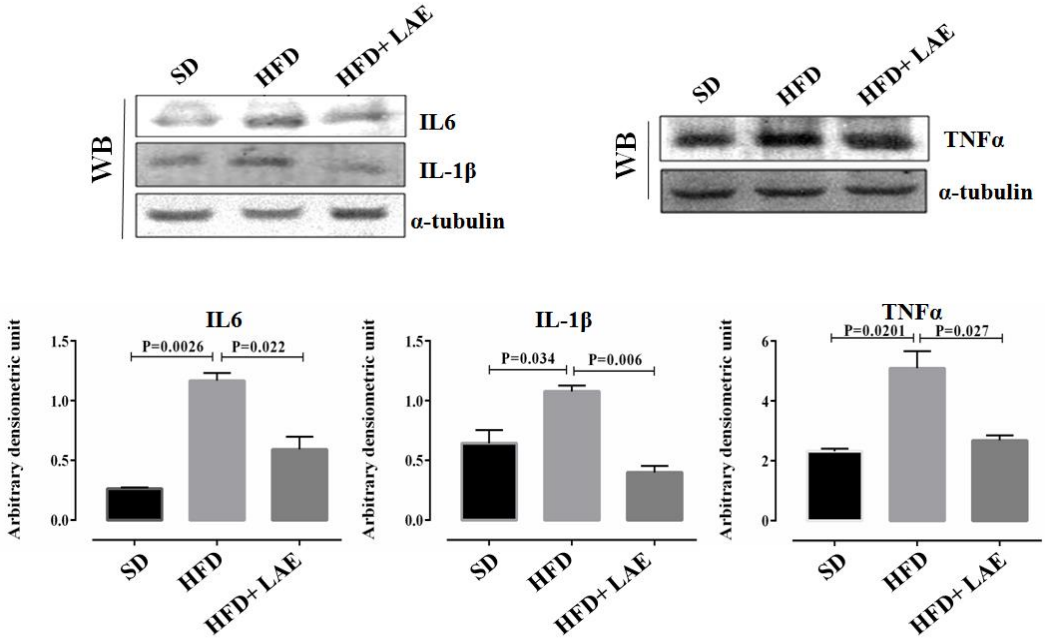


Figure 4.9: LAE reduced the expression of proinflammatory cytokines in the serum of HFD mice. Blood serum of SD or HFD or HFD + LAE mice were collected and performed immunoblot analysis using anti-IL6, anti-IL-1β,anti- TNFα and anti- α-tubulin. Densitometric analysis was carried out by using the GelQuant.NET software v 1.6.8. Each value is the Mean ± SEM of two individual experiments and the p values were calculated using the GraphPad PRISM v 6.07

4.3.11 LAE reduces FFA induced overexpression of proinflammatory cytokines in RAW264.7 macrophage cells

Macrophages are the emerging as key players in the pathogenesis of insulin resistance. It is well established that the cross-talk between the inflammatory macrophages and the adipocytes as well as the muscle cells are involved in the insulin resistance in peripheral tissues [25] .Interestingly, obesity leads to a macrophage phenotypic switch from an anti-inflammatory M2 polarization state to a pro-inflammatory M1 polarization state in the adipose tissue and contributes to insulin resistance[26]. Moreover, lipid oversupply to the muscle cells leads to the polarization of resident skeletal muscle macrophages[3]which eventually overexpresses the proinflammatory cytokine genes[27]. To find out the possible effect of the LAE active fraction on macrophage polarization, RT-PCR was performed to investigate the gene expression profile of the M1 markers TNFα, IL6, MCP1,

iNOS using gene specific primers. The RT-PCR analysis revealed that in the presence of palmitate the expression of TNFα is 1.5 fold more than that in the control whereas in the IL6, MCP1 and iNOS the increase were 1.4, 1.2 and 2.4 folds more than the control. In contrast, with the pretreatment of cells with the LAE fraction, the level of these M1 specific genes were found to be significantly less than that in the palmitate treated cells in concentration dependent manner (Fig. 4.10 A, B, C and D).

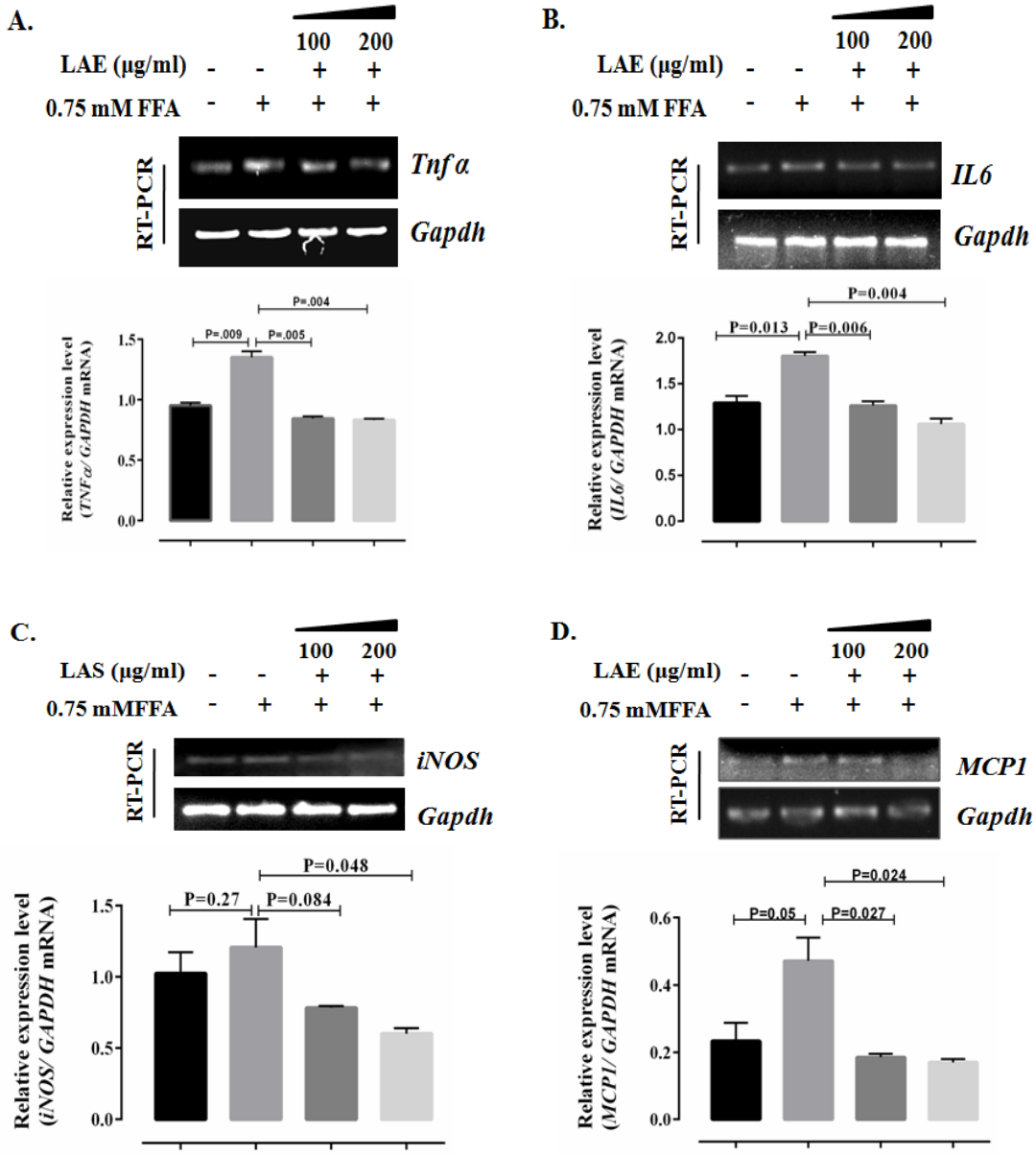


Figure 4.10: LAE reduced the FFA induced overexpression of proinflammatory cytokines in macrophage. RAW264.7 murine macrophage cells were treated for 4 hrs with plamitate or palmitate along with various concentrations of LAE or without any of them (control) and were subjected for RT-PCR analysis using gene specific primers of (A)

Tnf-α, (B) IL-6, (C)iNOS and (D) MCP1genes. A housekeeping gene Gapdh was also amplified simultaneously to serve as internal control. Relative expression level of each gene was calculated using the GelQuant.NET software v 1.6.8. Each value is the Mean ± SEM of two individual experiments and the p values were calculated using the GraphPad PRISM v 6.07.

From the densitometric analysis it was clear that the LAE active fraction could prevent macrophage polarization from M2 to M1 state in presence of palmitate.

4.3.12 Expression of anti inflammatory cytokine genes augments in the LAE treated macrophage cells

Since it is reported that free fatty acid reduces the gene expression of the anti-inflammatory cytokines, this could be one the reasons of macrophage polarization from M2 to M1 state. The expression of some anti-inflammatory genes in presence of palmitate and palmitate along with the LAE were checked. From the RT-PCR analysis it was seen that in presence of palmitate the expressions of some anti-inflammatory genes, *viz.*, IL4, IL13 were downregulated in contrast to the control. However, the expression of these two cytokines was induced when the cells were pretreated with the LAE (Fig. 4.11 A and B). The result suggests that the LAE can modulate the expression of the anti-inflammatory cytokines.

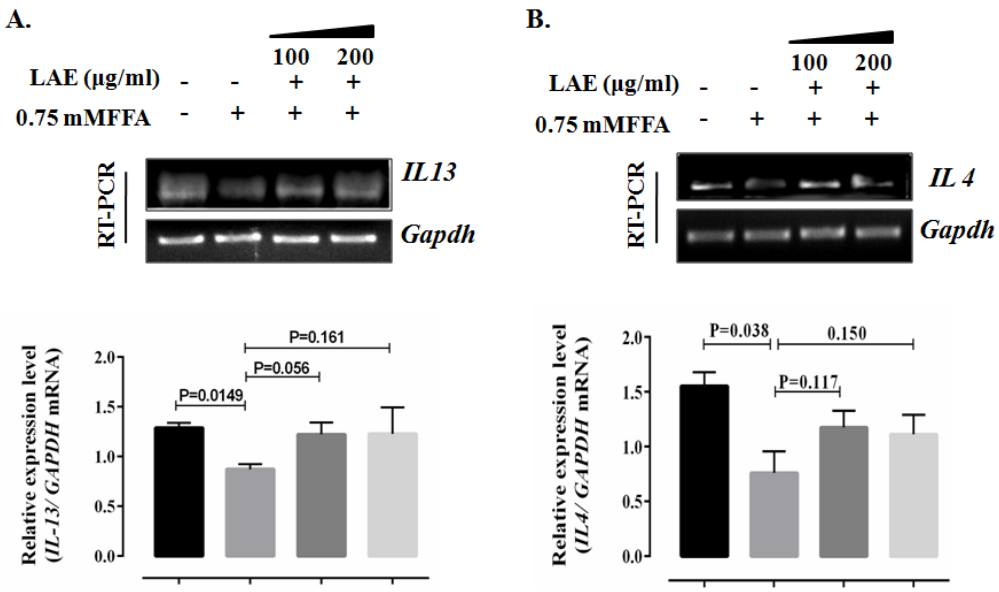


Figure 4.11: LAE induced the gene expression of anti-inflammatory cytokines in presence of FFA in macrophage.RAW264.7 murine macrophage cells were treated for 4 hrs with plamitate or palmitate along with various concentrations LAE or without any of

them (control) and were subjected for RT-PCR analysis using gene specific primers of (A) IL-13 and (B) IL-4 genes. A housekeeping gene Gapdh was also amplified simultaneously to serve as internal control. Relative expression level of each gene was calculated using the GelQuant.NET software v 1.6.8. Each value is the Mean ± SEM of two individual experiments and the p values were calculated using the GraphPad PRISM v 6.07

4.3.13 Active fraction of LAE abrogates palmitate induced overexpression of Tlr4 and Ahsg genes RAW264.7 cell line

Overproduction of proinflammatory cytokines is one of the responsible factors for the development of insulin resistance where the TLR4, a cell surface receptor involved in innate immunity plays an important role. Binding of exogenous and endogenous ligands including FFA to TLR4 initiates the inflammatory pathways in immune as well as in the insulin target cells resulting in the elevated secretion of proinflammatory cytokines which contribute to insulin resistance and its co-morbidities[28]. Hence, the elevated level of FFA increases the risk of developing insulin resistance *via.* the TLR4 pathway. It has been reported that though FFA activates the TLR4 pathway. FFA doesnot bind to TLR4 directly. A liver secretory protein called fetuin-A (FetA) plays as intermediator in binding of FFA to the TLR4 and activates the pathway. In contrast, elevated level of FFA is reported to be the responsible factor for overproduction of FetA from both hepatic as well as adipose cell. Since the LAE showed its activity in suppression of proinflammatory cytokines release, its role in TLR4 and Ahsg gene expression in macrophage cell linewas investigated as Ahsg encodes for FetA. From RT-PCR analysis it was found that in the FFA treated macrophage cells, the expression of both TLR4 and Ahsg increased significantly with p values0.03 and 0.015 respectively (Fig. 4.13 A and B). When the cells were treated with different concentrations of the LAE in presence of FFA, the expression of TLR4 and Ahsg were found to be reduced in comparison to that in the FFA treated macrophage cells with significant p values (Fig. 4.12 A and B).

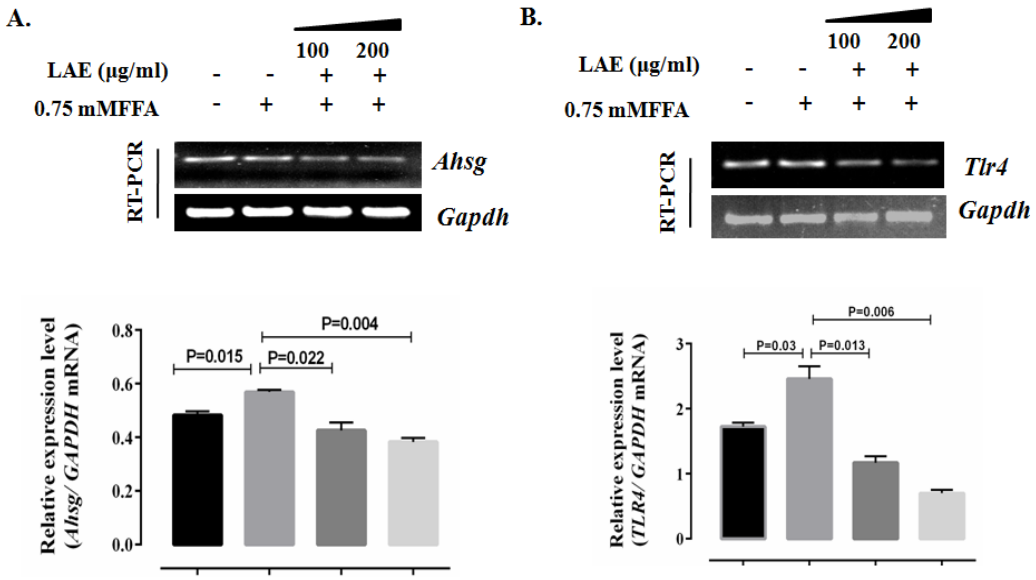


Figure4.12: LAE reduced the FFA induced overexpression of proinflammatory mediators in macrophage. RAW264.7 murine macrophage cells were treated for 4 hrs with plamitate or palmitate along with various concentrations of LAE or without any of them (control) and were subjected for RT-PCR analysis using gene specific primers of (A) Ashg and (B) Tlr4genes. A housekeeping gene Gapdh was also amplified simultaneously to serve as internal control. Relative expression level of each gene was calculated using the GelQuant.NET software v 1.6.8. Each value is the Mean ± SEM of two individual experiments and the P values were calculated using the GraphPad PRISM v 6.07

4.3.14 *In silico* molecular docking study reveals the binding of swietenine with TLR4

To study whether the active compound swietenine could bind to the TLR4 receptor to suppress the expression of the inflammatory genes in the insulin target cells, a molecular docking study was performed. Swietenine was found to dock with TLR4 with high docking efficiency with MolDock score -34.24. The amino acids to which the ligands docked to the target protein TLR4 were Phe 121, Leu 61, Tyr 102, Ile 94 and Ile 80 forming 5 numbers of H-bonds of length 2.64 Å, 3.19 Å, 2.47 Å, 1.87 Å and 2.09 Å (Fig. 4.13).

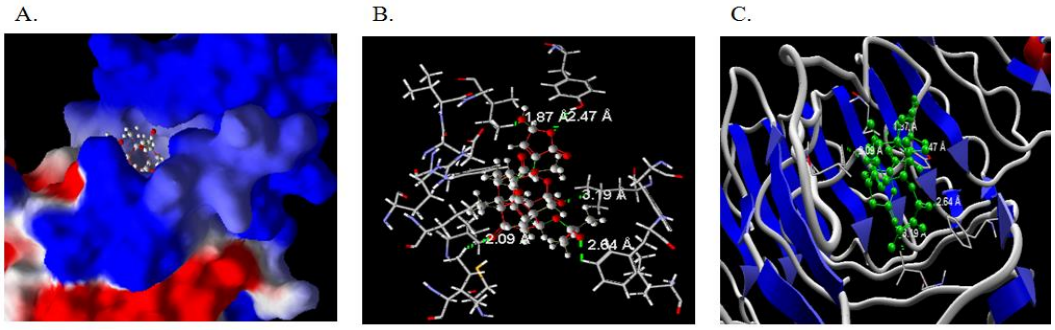


Figure 4.13: Docking study of swietenine with TLR4. (A) Ligand docking within the protein cavity, (B) orientation of the ligand with respect to the target protein and (C) binding of protein- ligand in secondary structure view.

4.4 Discussion

The insulin signalling pathway plays a critical role in glucose homeostasis. Binding of insulin to its receptor leads to the translocation of GLUT4 glucose transporters from the cytoplasm to the membrane of the cells and facilitates movements of glucose molecules inside the cell[29,30].Defects in this signalling cascade caused by the elevated saturated free fatty acid or lipid molecule is a well established fact. This induces the imbalance of glucose homeostasis causing hyperglycemia by developing insulin resistant state to the cells. Active LAE fraction improves the FFA induced suppression of GLUT4 activation resulting in increased glucose uptake by the L6 skeletal muscle cells. On exploring the mechanism, it was observed that LAE could activate the proteins involved in insulin signalling pathway by phoshorylation. It has been established that the FFA induced insulin resistance may be possible in various pathways depending upon the different tissue types. In the muscle cells, FFA activates PKCε and PKCθ resulting in insulin resistance. From the result it was observed that the LAE suppressed the FFA induced PKCθ due to which the LAE could induce the phosphorylation of IRS1 at the tyrosine residue instead of the serine as activated PKCθ is reported to be an inducer of the ser/thr phosphorylaion of IRS1.

Since inflammation is reported to be one of the major inducers of insulin resistance, the levels of some proinflammatory cytokines were investigated by immunoblot analysis. From the result obtained, it was revealed that due to the treatment with the LAE in the FFA treated cells, the level of proinflammatory cytokines were found to be less than that in the FFA treated L6 muscle cells.

To check the effect of the LAE active fraction in the high fatty diet (HFD) induced animal model, the *in vivo* study was performed by generating HFD induced diabetic mice model. Because, appropriate experimental models are essential tools not only for understanding the pathogenesis of the disease, but also for the testing of the various therapeutic agents in the organ systems. The animal of type 2 diabetes can be obtained either spontaneously or can be induced by chemicals or dietary or surgical manipulations and/or by combination thereof[31]. Fat enriched diets have been used for decades to model obesity, dyslipidemia and insulin intolerance in rodents[32]. It has been observed that the disorders achieved by high fat feeding closely resemble the human metabolic syndrome including T2DM[33,34,35,36]. Continuous feeding of high fatty diet for 30 days, a significant blood glucose level was observed in the HFD mice along with the increased body weight. After 30 days of high fatty diet, the HFD group of mice achieved high glucose level confirming the development of hyperglycemic T2DM model. A group of HFD mice was allowed to treat with the LAE for 15 days. Fifteen days of treatment with the LAE, the blood glucose level of the mice was lowered down to normal blood glucose range with the reduction of body weight. This confirmed the presence of hypoglycaemic as well as lipid lowering effect of the LAE. From, the *in vivo* study it was also found that the LAE could induce phosphorylation of proteins involved in the insulin signalling pathway and as the result of that, the presence of insulin secreted from the β cells of mice pancreases could induce glucose uptake in the mice resulting in the blood glucose level in the LAE treated HFD mice. Similar to the results from *in vitro* studies, PKCθ phosphorylation was found to be decreased in case of the LAE treated HFD mice model whereas PKCθ phosphorylation was observed to be high in the HFD induced diabetic mice model.

The release of pronflammatory cytokines from adipocyte is also a responsible factor of adipocyte insulin resistance. Several reports suggest that FFA acts as inducer of adipocyte cytokine secretion. With reference to the results obtained from the *in vitro* study, using L6 skeletal muscle cells, some of the proinflammatory marker proteins involved in insulin resistance were studied using immunoblot analysis of the adipose tissue homogenate isolated from the experimental mice model. From the immunoblot analysis, it was observed that, by the treatment with the LAE, the level of proinflammatory cytokine IL6 was found

to be less along with the other proinflammatory modulators like TLR4 and FetA, etc. In this context, the lower levels of released cytokines present in the serum of the experimental mice confirmed the role of the LAE in dowregulating the level of proinflammatory cytokines in the HFD induced diabetic mice model.

Although the muscle cells produce low grade inflammation, the most interesting part of this mechanism is that the secreted pro-inflammatory cytokines from the muscle cells induce the activation of resident macrophages of the muscle cells. The secreted muscle pro-inflammatory cytokines also help in macrophage infiltration in the muscle of high fat-fed mice with a pro-inflammatory phenotype. The increased number of resident macrophages in the muscle cells can be polarised independently by the effect of saturated fatty acid [37, 38, 39] resulting in insulin resistance in the muscle cells. In the adipose tissue, it was reported that the release of cytokines from the adipose cells influences the production of the proinflammatory cytokines from the resident macrophage of the adipose tissue known as the adipose tissue macrophage (ATM). The release of proinflammatory cytokines from both the adipocyte and the ATM leads to the development of insulin resistance and T2DM. In this condition the ATM is present in its M1proinflammatory state instead of in the M2anti-inflammatory state. The gene expression study of the M1 and M2 specific marker genes of the macrophage cells, revealed that the LAE subdues the polarization of the macrophage from M2 to M1 state in the FFA treated RAW264.7 murine macrophage cells by overexpressing the M2 anti-inflammatory cytokines genes.

TLR4 is reported as a driver of macrophage polarization inducing the proinflammatory cytokines genes expression. The reduced expression of TLR4 in macrophage by treatment of the LAE in presence of FFA in this study confirms the presence of anti-inflammatory property in the active fraction of *L. aspera* ethanolic extract.

Hence from the study, it was concluded that the active fraction of the *L. aspera* is responsible for the suppression of FFA induced overexpression of the proinflammatory cytokines. In such context, the *L. aspera* active fraction reduced the expression of the TLR4 in all the cell types, *viz*., muscle, adipocyte and macrophage. The TLR4 expression was reported to be significantly high in the

insulin resistant patients. In the present study, significantly high expression of TLR4 was observed in case of the FFA treated cells, whereas the LAE could suppress this level. So in case of the muscle cells, since the treatment of the LAE downregulated the expression of TLR4 in presence of FFA, cells could not produce the low grade inflammation in muscle and severe inflammation was not possible by the activation of macrophage present in the muscle tissue. Similarly, in the adipose tissue, due to the lower expression of TLR4 by the action of the LAE, in presence of FFA, adipocytes couldnot produce the elevated level of proinflammatory cytokines. As a result of that macrophage could not be activated or polarized to the proinflammatory state. On the other hand, in case of macrophage itself, the lower expression of TLR4 in presence of FFA reduced the expression of proinflammatory markers by inducing the anti-inflammatory marker genes expression.

Due to the suppression of the proinflammatory cytokines gene expression by the treatment of the LAE, the overall content of proinflammatory cytokines were found to be reduced in the LAE treated HFD mice whereas the level of these proteins were found to be high in the HFD fed mice.

From the *in silico* docking study, the probable active compound present in the active fraction of the LAE ethanolic extract, i.e., swietenine was found to be bind to the TLR4 with high biding affinity. On searching the binding property of swietenine to TLR4 it was found that swietenine binds to the various amino acid residues, *viz.*, phenylalanine 121, leucine 61, tyrosine 102, isoleucine 94 and 80 present in the active site of TLR4 forming 5 numbers of H-bond.

Since, activated PKCθ is responsible for the activation of NFκB in B-cell and T-cell, it could also be possible that by the treatment of the LAE suppression of the activation of PKCθ and hence phosphorylation of NFκBp65 was reduced and finally reduced level of proinflammatory cytokines secretion occurred. Though there is no report on the activated PKCθ induced activation of NFκB in muscle cells, the present study points at more investigation to prove the hypothesis.

It is expected that drugs benefit the recipient, but at the same time, it is a fact that there is no drug which is totally free from harmful effects. Therefore, when any drug is used, it is necessary to consider not only the beneficial effect but

also check the harmful effects. Hence, all the drugs, whether it is chemically synthesised or plant based product have to pass through stringent toxicity testing before approval can be granted for their use in general population. In recent years, there is increasing trend for using alternative system of medicine as they are not only effective but also very safe as compared to the synthetic ones. The claim that natural plant product are safe should be accepted only after plant product passes through the toxicity testing using modern scientific methods. Acute and subacute toxicity studies in animals are of value in predicting, potential toxic effects of a chemical in human being exposed to near fatal doses. In toxicological point of view, the comparison of organs between the different groups of experimental animals has been evaluated to check the toxic effect of the compounds[40,41]. The effect of the compounds on biochemical and haematological parameters was also measured to anticipate the toxic effect of the drugs. The treatment of mice with active fraction of the LAE could not induce any kind of clinical sign of toxicity indicating that it could be used for further study to isolate the active component for the treatment of T2DM.

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