

Vitexin isolated from *Acanthus ilicifolius* L. leaf enhances GLUT-4 translocation in experimental diabetic rats

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Abstract

Acanthus ilicifolius L. leaf is extensively used in the Indian and Chinese medicine systems to treat diabetes mellitus. In this study, the antidiabetic effect of vitexin isolated from *A.ilicifolius* leaf extract and their effect on GLUT-4 translocation and PPAR- γ expression was evaluated high-fat diet-streptozotocin (HFD-STZ) induced rats. *In vitro* antidiabetic effect of vitexin was investigated through glucose uptake activity in L6 (rat skeletal muscle) cell lines. Vitexin (10 and 20 mg/kg BW) was administered orally to HFD-STZ-induced diabetic rats for 48 days. The effect of vitexin on body weight, fasting blood glucose, serum insulin, total protein, urea, creatinine, and liver enzymes was examined. GLUT-4 translocation and PPAR- γ expression were studied in the skeletal muscle and adipocytes of experimental rats. The interaction of vitexin with GLUT-4 and PPAR- γ was validated by molecular docking analysis. Vitexin significantly lowered the blood glucose and also normalized other biochemical parameters. Further, the treatment with vitexin up-regulates the mRNA expression of GLUT-4 and PPAR- γ in diabetic rats. *In silico* analysis also supports the promising interactions between vitexin and target proteins. These results explained that vitexin up-regulates the mRNA expression of GLUT-4 and PPAR- γ and enhanced the translocation of GLUT-4 which maintains glucose homeostasis. Thus vitexin can be a gold promising drug for type II diabetes mellitus in the future.

1. Introduction

Type II diabetes mellitus (DM) is a major metabolic disorder, affecting approximately 5–6% of the global population annually and becoming a serious health issue Kifle ZD and Enyew EF (2020). Type II DM is diagnosed by the elevated level of glucose in the blood for a prolonged time which might be due to reduced uptake of glucose and metabolism by peripheral tissues such as skeletal tissue and adipocytes. Insulin, a pancreatic hormone, enhances blood glucose uptake into peripheral tissues, thereby regulating glucose homeostasis (Juvekar and Bandawane 2009; Ashok Kumar 2012). The rate-limiting stage in carbohydrate metabolism is glucose absorption, which in mammals is aided by glucose transporters (GLUTs) (Maughana 2009). GLUT-4 is the major glucose transporter mainly expressed in adipocytes and skeletal muscles. The binding of insulin to its receptors in the cell membrane stimulates various intracellular proteins for the transport of GLUT-4 from the storage vesicles to the plasma membrane for further metabolism (Zaid et al. 2008). Since type II diabetes is characterized by insulin resistance, a defect in GLUT-4 translocation affects glucose uptake and consequently results in a higher level of blood glucose (Sameer et al. 2006). Currently, available synthetic drugs for upregulating GLUT-4 transportation are reported for their side effects (Sangeetha et al. 2013). Many researchers isolated the compounds from medicinal plants for various ailments and have shown a high therapeutic efficacy than chemically-synthesized drugs. Therefore, there is a need to search for compounds in antidiabetic medicinal plants that can facilitate the GLUT-4 translocation and is beneficial for managing type II DM (Nazarian et al. 2006).

Acanthus ilicifolius L. (Acanthaceae) is a medicinal plant from the mangrove region but normally grows in normal salinity. The plant is commonly known as sea holy or Holly mangrove which has been

employed in traditional Indian and Chinese medicine to cure different ailments (Amritpal et al. 2009; Babu et al. 2001). The *A. ilicifolius* plant is referred to as Sahachara in Ayurveda and is used to cure rheumatism, asthma, and coughs in India. The entire plant is used to make tonic, expectorant, and stimulant medications (Saranya et al. 2015). Seaside residents frequently use boiled root in milk to treat weakness (Dey et al. 2012). The leaf is used to prepare formulations in Ayurveda to treat diabetes and snake bite (Mastaller et al. 1997). The leaf is frequently used in Malaysia to cure rheumatism, neuralgia, and wounds from poison arrows. People who live close to mangroves practice chewing plant leaves to ward off snake bites Ganesh and Vennila (2010). The leaf juice is used to treat hepatic ailments and prevent alopecia (Chakraborty et al. 2007). Different parts of the plant have been proven for their pharmacological activities such as anti-inflammatory (Mani Senthil Kumar et al. 2008), antioxidant (Asha et al. 2012), antinociceptive and analgesic activity (Wang et al. 2013), anticancer activity (Poorna et al. 2011), anti-leishmanial activity (Kapil et al. 1994), hepatoprotective activity (Babu et al. 2001), antiasthmatic activity (Ahmed et al. 2005), antimicrobial activity (Ravikumar et al. 2012), osteoblastic activity (Van Kiem et al., 2008), antilipidemic activity and activity against Alzheimer's disease (Burg et al. 2013), antiulcer activity (Nizamuddin et al. 2011). 2-benzoxazolinone Murty and Kamat (1984), isoxazoline (DSouza et al. 1997), coumaric acid derivatives acancifoliuside (Kanchanapoom et al. 2001); vitexin (Wu 2003) and alpha- amyryl (Van Kiem et al. 2008) are some of the compounds reported from this plant.

In our preliminary studies, the methanolic leaf extract of *A. ilicifolius* significantly enhanced glucose uptake activity in L6 cell lines and experimental rats than other prepared extracts. Hence, the same extract was chosen for further isolation and identification of an antidiabetic compound that enhances glucose uptake through GLUT-4 translocation. The present study explored apigenin-8-C-beta-D-glucopyranoside, known as vitexin, the bioactive compound present in the methanolic extract by bioactive-guided separation. Vitexin was isolated from various medicinal plants and proven for their pharmacological activities such as anti-inflammatory, antioxidant, antinociceptive, and anti-depressant (Miao He 2016). However, no research has reported vitexin for its glucose uptake activity with its underlying mechanism of action. Hence, the current study was focused to evaluate the antidiabetic effect of vitexin isolated from *Acanthus ilicifolius* L. leaf and its effects on GLUT-4 translocation in type II diabetic rats.

2. Materials And Methods

2.1 Plant collection and extraction

Fresh greenish leaves of *A. ilicifolius* were collected from Parangipettai, near Pichavaram forest, Cuddalore district in November 2016 and authenticated by Dr. Kathiresan, Annamalai University, Chidambaram. The leaf of *A. ilicifolius* was washed and shade dried. About 3 kg of the powdered leaf was macerated with methanol for 3 days. The extract was filtered and concentrated at 40–45°C using a rotary vacuum evaporator. The percentage yield of extract was 9.8 g/100 g.

2.2 Isolation and identification of active compound

The methanol extract of *A. ilicifolius* was fractioned under silica gel chromatography (100–200 mesh, 800 gm, 70 cm × 4 cm). The column was eluted with solvents of increasing polarity hexane, chloroform, ethyl acetate, and their mixtures. In total, 84 minor fractions were collected in a conical flask and transferred with proper labeling. The solvents in the separated fractions were recovered by simple distillation. Then all the separated and concentrated fractions were placed in thin layer chromatography (TLC) for isolation and identification of active principle. The concentrated fractions were placed in alumina sheets (0.2 mm) the different ratios of solvents such as petroleum ether, ethyl acetate, and methanol at different ratios were used as mobile phase. The details of the solvent system are:

Petroleum ether (5%): Ethyl acetate (4%): Methanol (1%)

Petroleum ether (4%): Ethyl acetate (4%): Methanol (2%)

Petroleum ether (3%): Ethyl acetate (1%): Methanol (1%)

Petroleum ether (1%): Ethyl acetate (6%): Methanol (3%)

The fractions with similar R_f (Retention factor) values were pooled as major fractions. The major fraction which was identified as an active fraction by glucose uptake activity in L6 cell lines was further purified for identification through spectroscopic analysis. The isolated purified fraction was tested for phenol (brown color obtained in ferric chloride test) and quinine (given deep pink color with alcoholic NaOH). The physical data like color, appearance, melting point, and spectroscopic analysis like GC (Acq Method AUTO GC MS), FTIR (ABB Bomem Inc., Canada), LC-MS (MassLynx 4.1), ^1H NMR, ^{13}C NMR data were compared with the literature. The active compound was identified as apigenin-8-C- beta-D-glucopyranoside (vitexin).

2.3 Cytotoxicity and glucose uptake activity of vitexin

Cytotoxicity and glucose uptake activity of isolated vitexin in L6 cell lines was examined by the standard protocols (Gupta et al. 2009).

2.4 *In silico* molecular docking of isolated compound vitexin

The structures of vitexin and pioglitazone were sketched using Chems sketch. The structures were energy-minimized using ArgusLab. The structures of PPAR- γ (ID: 2XKW) were retrieved from PDB (Berman et al. 2000). The structure of GLUT-4 was modeled using the SWISS-MODEL server based on the sequence obtained from UniProt (Acc.no: P14672) (Schwede 2003). The details of the template and sequence identity of the modeled structure are given below. The protein structure was energy minimized using the GROMOS96 force field using GROMACS 4.5.6 (Berendsen 1995). Modeling of GLUT-4 was done by following specifications.

Template: 17–483 (4ZWC: A); Solute carrier family 2, facilitated glucose transport member 3 Resolution: 2.60 Å

Sequence identity: 60.86

Molecular docking analysis was performed using the PatchDock server (Schneidman-Duhovny 2005). The lowest energy conformation was chosen as the best binding conformation of the ligand with the protein based on the score and atomic contact energy (ACE). The interaction pattern of the ligand with the proteins was investigated and visualized using PyMol (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC).

2.5 *In vivo* studies of isolated vitexin

2.5.1 Acute toxicity test

An acute toxicity test was conducted following OECD guidelines for the inclusion of animals and test doses. The overnight fasted animals were separated into six groups. Each group contained six rats (3 male + 3 female). The experimental rats were administered orally with vitexin (50, 100, 200, 400, and 800 mg/kg BW) by gavage. The normal group animals were administered distilled water. All the experimental animals were monitored for any physical and behavioral changes for the first 1 hour, then next for 4 hours, and again for 24 hours. The physical, behavioral, autonomic, and neurological changes of animals were observed continuously for fourteen days.

2.5.2 Animals and housing

Swiss albino Wister female and male rats were procured from the animal house, Vellore Institute of Technology, Vellore, Tamil Nadu after clearance of animal ethics from the Institutional animal ethical Committee (VIT/IAEC/IX/July 26/18). All rats were maintained in standard polypropylene cages. The cages filled with sawdust bedding and animals were properly housed on an approximate 12-hour light and dark cycle at 23°C to 28°C. Experimental rats were supplied with potable water and a standardized diet ad libitum. All the protocols for animal experiments were conducted as per the guidelines of the OECD.

2.5.3 Experimental induction of diabetes

Except for normal group rats, all other animals were supplied with a high-fat diet (HFD) for 5 weeks (Srinivasan et al. 2005) to induce hyperglycemia. After 5 weeks, except for normal and HFD control rats others were administered with a single intraperitoneal injection of streptozotocin (40 mg/kg BW) dissolved in fresh ice-cold citrate buffer (pH 4.5, 0.1 M). The normal and HFD control rats were administered with citrate buffer alone to equalize the hormonal changes and stress. After 72 hours of STZ administration, the experimental rats were tested for fasting glucose (FBG) by measuring tail vein blood glucose level in a glucose meter (Accucheck - Johnson & Johnson Company). Rats that have shown FBG over 240 mg/dL were considered diabetic and included in further studies.

2.5.4 Experimental design

Wistar rats of both sexes were randomly separated into six groups consisting of six numbers each. Normal HFD control and diabetic control animals received the vehicle alone (Normal saline). Positive control rats received the standard drug Pioglitazone (10 mg/kg BW). The isolated vitexin from *A. ilicifolius* were administrated by oral gavage at doses of 10, 20 mg/kg BW not exceeding 1.5 mL per day. After 48 days of treatment, all experimental animals were sacrificed by the cervical dislocation method under anesthesia.

Group I (Normal control) : Normal rats + normal saline (1.5 mL/kg BW)

Group II (HFD control) : HFD rats + normal saline (1.5 mL/kg BW)

Group III (Diabetic control) : Diabetic rats + normal saline (1.5 mL/kg BW)

Group IV (Experimental I) : Diabetic rats + isolated vitexin (10 mg/kg BW)

Group V (Experimental II) : Diabetic rats + isolated vitexin (20 mg/kg BW)

Group VI (Positive control) : Diabetic rats + pioglitazone (10 mg/kg BW)

2.5.5 Processing of blood samples

The blood samples were collected by cardiac puncture in sterile test tubes and incubated for 20 minutes for coagulation at room temperature. The blood samples were collected in serum tubes and centrifuged at 3000 rpm for 10 minutes to isolate the serum in sterile vials at -80°C for further experiments. The plasma was collected from EDTA-added tubes by centrifuged at 3000 rpm for 10 minutes to separate plasma and stored in sterile vials. The whole blood was utilized to calculate glycated hemoglobin.

2.5.6 Estimation of biochemical parameters

The blood collected in serum tubes was incubated for complete coagulation. The clotted blood was centrifuged at 3000 rpm for 12 minutes and serum samples were aspirated and stored at -80°C until further experiments. The fasting serum insulin (FSI) concentration was estimated by a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Roche Diagnostics, Germany). Homeostasis of model assessment insulin resistance (HOMA-IR) was determined by the following formula,

$$\text{HOMA-IR} = \text{Fasting serum insulin} \times \text{Fasting blood glucose} / 22.5$$

Glycosylated hemoglobin (HbA1c %) was estimated in EDTA blood samples by the commercial test kit from Crest Biosystems, Goa, India. The levels of serum total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL), urea, creatinine, alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total protein (TP), chloride and calcium were determined spectrophotometrically according to the instructions of commercially available ready to use kits from Span diagnostics ltd, Surat, Gujarat, India. Very low-density lipoproteins (VLDL) and low-density lipoproteins (LDL) were determined using the Friedewald formula (Mohammed et al. 2016),

$$\text{VLDL} = \text{TG} / 5$$

$$\text{LDL} = \text{TC} - (\text{HDL} + \text{VLDL})$$

2.5.7 Gene Expression by quantitative polymerase chain reaction

2.5.7.1 Total RNA extraction

RNA extraction was done by using RNA iso plus from TAKARA. The 100 mg of adipose and muscle tissue sample was taken from -80°C . The tissues were homogenized by an appropriate volume of RNA iso plus using a micro pestle. The homogenized sample was incubated for five minutes at room temperature and centrifuged at 12000 rpm at 4°C for 5 minutes. Then, 0.2 mL of chloroform was mixed with the supernatant and a milky white layer was formed. The sample was incubated at room temperature for 5 minutes and then centrifuged at 12000 rpm for 15 minutes at 4°C . Then an equal volume of isopropanol was mixed with the clear aqueous phase to precipitate RNA and spun at $12000\times g$ for 10 minutes at 4°C . An equal volume of 75% ethanol was mixed into the solution to wash it thoroughly and to remove salt precipitates. The solution was centrifuged at 7500 rpm at 4°C for 5 minutes. Then the supernatant was removed and the final pellet was air-dried. Finally, the pellet was dissolved in DEPC-treated water or RNase-free water. The RNA quantity was quantified using UV-spectrometer and the ratio was 1.7–2.1.

2.5.7.2 cDNA synthesis using iScript real time

The first strand of cDNA was synthesized from 1000 ng of total RNA by the real-time method by utilizing iScript's first cDNA synthesis kit from BIO-RAD according to the manufacturer's instructions.

2.5.7.3 Real-time polymerase chain reaction

The expression of gene levels was quantified by real-time polymerase chain reaction (RT-PCR) by using SYBER green dye from applied biosystems life technologies and the method used to analyze the gene expression was the $2^{-\Delta\Delta\text{Ct}}$ method. Beta-actin acts as a housekeeping gene. This real-time assay was done by using different PCR conditions and protocols. For one reaction 10 μL of the reaction mix, 0.5 μL of 10 Pico molar diluted forward and reverse primer, 3 μL of water, and 1 μL of 200 ng cDNA. The target genes were quantified using 0.2 mL strips. The amplification of the target genes was Roche light cycler-nano. NTC acts as a negative control. The defined program of this real-time PCR was initial denaturation of 95°C for 10 minutes, 40 cycles of 3-step amplification consisting of denaturation – 95°C for 15 sec, annealing 60°C for 1 minute and extension of 72°C for 30 sec, and the melting curve was analyzed from 600C to 950C for 15 sec. The expression of each target gene has its own (ct) amplification rate value at the threshold level where the final transcribed products of the PCR start to sense the fluorescence. Primer sequences are as follows.

PPAR gamma - F 5' GCCTAAGTTTGAGTTTGCTGTG 3'

PPAR gamma -R 5' GCGGTCTCCACTGAGAATAATG 3'

Beta actin F 5' GTGGCTGAGGACTTTGATTGT 3'

Beta actin R 5' GTGAGGGACTTCCTGTAACCA 3'

GLUT-4 F 5' GTGCCTATGTATGTGGGAGAAA 3'

GLUT-4 R 5' GCAACCAGAATGCCAATGAC 3'

The annealing temperature for all the primers was – 60°C.

2.5.8 Statistical analysis

All the data are expressed as Mean $\pm$ SEM and were determined by one-way analysis of variance (ANOVA), followed by Student's t-test and Tukey's multiple comparison tests by using Graph pad Prism version 5.0, and the values of $p < 0.05$ were considered as significant statistically. Microsoft Excel 2007 was used to plot the graphs.

3. Results

3.1 Separation and *in vitro* glucose uptake activity of fractions

The minor fractions are pooled as major fractions and the results of the percentage of glucose uptake activity of each fraction were listed in Table 1. Among all the 22 major fractions 14, 15, 16, 17 and 18 fractions exhibited higher glucose uptake activity in L6 cell lines 22.93 ± 0.67 , 27.46 ± 0.62 , $72.99 \pm 0.62^{***}$, 34.91 ± 0.62 and 21.08 ± 0.62 respectively. Major fraction 16 showed a higher percentage of glucose uptake ($72.99 \pm 0.62^{***}$) in L6 cell lines when compared to other major fractions. Hence this fraction is further purified in column chromatography and analyzed spectroscopically for the identification of antidiabetic molecules present in it.

Table 1
Glucose uptake activity of fractions in L6 cell lines

Minor fraction	Major fraction	Rf value	% of Glucose Uptake in L6 Cell Lines
1-3	1	0	OD values were less than the control
4-5	2	0.84, 0.61	OD values were less than the control
5-11	3	0.65, 0.43	OD values were less than the control
12-16	4	0.89	0.91 ± 0.62
17-20	5	0	3.24 ± 1.09
21-23	6	0.80, 0.69	2.82 ± 0.27
24-25	7	0.74	5.34 ± 2.98
26-27	8	0.77	6.43 ± 2.04
28-32	9	0.88, 0.78, 0.43	5.81 ± 2.92
33-36	10	0.82, 0.39	10.39 ± 0.63
37-41	11	0.87	8.94 ± 0.76
42-47	12	0.81	12.91 ± 0.69
48-51	13	0.23	16.13 ± 0.92
52-54	14	0.84	22.93 ± 0.67
55-56	15	0.76	27.46 ± 0.62
57-59	16	0.47	$72.99 \pm 0.62^{***}$
60-61	17	0.29	34.91 ± 0.62
62-65	18	0.34	21.08 ± 0.62
66-68	19	0.21	8.91 ± 0.62
69-70	20	0.82	5.16 ± 0.86
71-73	21	0.4	10.18 ± 2.62

Minor fraction	Major fraction	Rf value	% of Glucose Uptake in L6 Cell Lines
74–84	22	0	7.95 ± 3.67

3.2 Identification of active compound

The compound was identified based on spectroscopic analysis and the pieces of evidence are represented below. The active principle from the methanolic extract of *A. ilicifolius* was identified as vitexin (apigenin-8-C-beta-D-glucopyranoside) (Fig. 1). The purified fraction showed a single peak at the retention time of 5.68 minutes in LC (Fig. 2) and 26.14 minutes in GC (Fig. 3). This confirms the presence of a single compound in the isolated and purified fraction. 5,7-dihydroxy-2-(4-hydroxyphenyl)-8-((3R,4R,5S,6R)-3,4,5-trihydroxy-6 (hydroxymethyl) tetrahydro-2H-pyran-2-yl)-4H-chromen-4-one: yellow colour needles: ¹H NMR (500 MHz, DMSO-*d*₆): δ 3.25 (1H, s, glc-6a), 3.37 (1H, s, glc-6b), 3.39 (1H, s, glc-1), 3.53 (1H, t, glc-5), 3.76 (1H, dd, J = 5.3, 5.3 Hz, glc-4), 3.83 (1H, t, glc-3), 4.61 (1H, t, glc-2), 4.71 (1H, s), 4.69 (1H, s), 5.00-5.028 (2H, m), 6.28 (1H, s), 6.79 (1H, s), 6.89 (2H, d, J = 8.25 Hz), 8.04 (2H, d, J = 8.25 Hz), 10.35 (1H, s), 10.84 (1H, s), 13.17 (1H, s) (Fig. 4). ¹³C NMR (125 MHz, DMSO-*d*₆): δ 61.75, 71.00, 71.31, 73.84, 79.12, 82.30, 98.61, 102.91, 104.52, 105.06, 116.28, 116.51, 12.08, 129.01, 129.43, 156.47, 160.86, 161.59, 163.01, 164.42, 182.56 (Fig. 5). The melting point is 265°C. The NMR was consistent with the literature on apigenin-8-C-beta-D-glucopyranoside (vitexin). The spectra obtained from 2D COSY and HSQC also confirm that the compound isolated from MLEA is vitexin. The counterplots of HSQC proved the bonding between carbon and oxygen which correlates with the structure of vitexin. 2D COSY further confirms the number of carbons present in the isolated compound which is identical to the flavones compound, vitexin (Fig. 6 and Fig. 7). Further, it exhibited the molecular ion peak at 436.82 *m/z* which is corresponding to C₂₁H₂₀O₁₀ (Fig. 8). IR (KBr) ν Max cm⁻¹: 3367 (aliphatic hydroxyl), 3217 (aromatic hydroxyl), 2908 (aliphatic), 1649, 1564 (aromatic C = H), 1176, 1037 (aromatic C = O) (Fig. 9).

3.3 Glucose uptake activity of isolated vitexin in L6 cell lines

Vitexin isolated from methanolic leaf extract of *A. ilicifolius* increases the glucose uptake in L6 cell lines even at the lower concentration. The results were statistically significant (*p* < 0.005) when compared with the standard drug pioglitazone. The results are given in Fig. 10.

3.4 *In silico* molecular docking of isolated compound vitexin

To evaluate the antidiabetic effect of vitexin molecular docking analysis was performed against its molecular targets. The docking results are compared with that of the standard drug pioglitazone to check the efficiency of vitexin (Table 2). The compounds were found to interact with their respective targets by forming hydrogen bonds. The GLUT-4-vitexin complex (Fig. 11) and GLUT-4-pioglitazone complex

(Fig. 12) had an ACE score of 147.60 and – 296.08, respectively. The PPAR-γ-vitexin complex (Fig. 13) and PPAR-γ-pioglitazone complex (Fig. 14) had an ACE score of -212.35 and – 140.49, respectively.

Table 2
Docking Score and Binding Energy of the Compounds with the Proteins

Protein-Ligand Complex	Score of the Best Conformation	ACE
GLUT-4-vitexin	5122	-147.60
GLUT-4-pioglitazone	5176	-296.08
PPAR-γ-vitexin	4432	-212.35
PPAR-γ-pioglitazone	5020	-140.49

ACE-Atomic contact energy

3.5 Acute toxicity of vitexin

The oral acute toxicity of vitexin was tested with several dose levels in experimental animals for 14 days. No signs and symptoms of acute toxicity were noticed in any one of the experimental groups of treated rats. None of the rats showed changes in normal behavior or death throughout the 14 days of the experimental period. No significant changes were observed in the weight of the liver, kidney, and heart when compared with the zero doses (administered with distilled water) of rats. Hence, the minimum lethal dose (LD50) could not be calculated. Since the acute toxicity study of vitexin resulted in a high margin of safety and the absence of mortality at a single dose of up to 800 mg/kg BW, further *in vivo* studies were undertaken. No behavioral changes were recorded between the 1/10th, and 1/20th doses, and was considered a safe dose that was followed for this experiment. Therefore, doses of 10 mg/kg BW and 20 mg/kg BW were fixed as test doses for further studies.

3.6 Effect of vitexin on body weight

The effects of vitexin on body weight in normal, HFD, and diabetic control animals are given in Table 3. HFD animals demonstrated a statistically significant ($p < 0.05$) increase in body weight at end of the treatment due to the accumulation of fat in adipose tissue. Besides, HFD-fed diabetic control rats have also explained a significant elevation in body weight compared to the normal control rats but slightly less than HFD control rats. HFD-fed STZ-induced diabetic control rats treated with vitexin at 10 and 20 mg/kg BW for 48 days demonstrated a statistically significant reduction in body weight to a normal level.

Table 3
Effect of Vitexin on Body Weight

Groups	Body weight in grams	
	Initial	Final
Normal control	201.70 ± 4.432	241.20 ± 1.046
HFD	234.50 ± 6.125*	399.50 ± 5.731*#
Diabetic control (HFD + STZ)	230.50 ± 5.590*	354.80 ± 2.626*
D + Vitexin (10 mg/kg BW)	221.70 ± 2.629	272.70 ± 4.410*#
D + Vitexin (20 mg/kg BW)	232.20 ± 5.828*	265.00 ± 3.697*#
D + Pioglitazone (10 mg/kg BW)	230.80 ± 5.480*	307.30 ± 3.461*#

D-Diabetic, HFD-High fat diet. Values are the Mean ± SEM of six rats in each group. Values with superscript '*' statistically differ from normal control rats ($p \leq 0.05$) and '#' statistically differ from diabetic control rats ($p < 0.05$) (One way ANOVA followed by Tukey's multiple comparison test)

3.7 Effect of Vitexin on fasting blood glucose

The elevation in the level of fasting blood glucose (FBG) was observed in diabetic control, but treatment with vitexin (10 and 20 mg/kg BW) for 48 days significantly ($p < 0.05$) reduced FBG when compared with diabetic control rats. The HFD-fed control group also showed increased FBG levels but not as much as diabetic rats (Table 4). Diabetic rats treated with vitexin (10 and 20 mg/kg BW) explained a significant decrease in the FBG level compared to diabetic control rats.

Table 4
Effect of Vitexin on fasting blood glucose

Groups	Fasting Blood Glucose (mg/dL)	
	Initial	Final
Normal control	89.83 ± 3.673	94.00 ± 5.663
HFD	136.20 ± 5.474*	174.20 ± 3.167*#
Diabetic control (HFD + STZ)	256.50 ± 2.997*	308.80 ± 6.896*
D + Vitexin (10 mg/kg BW)	260.20 ± 3.525*	116.20 ± 3.591*#
D + Vitexin (20 mg/kg BW)	254.20 ± 2.654*	114.50 ± 2.432*#
D + Pioglitazone (10 mg/kg BW)	257.00 ± 2.338*	116.70 ± 2.275*#

D-Diabetic, HFD-High fat diet. Values are the Mean ± SEM of six rats in each group. Values with superscript '*' statistically differ from normal control rats ($p \leq 0.05$) and '#' statistically differ from

diabetic control rats ($p < 0.05$) (One way ANOVA followed by Tukey's multiple comparison test)

3.8 Effect of vitexin on insulin, HOMA- IR, and HbA1c

The result of the effect of vitexin on insulin, HOMA- IR, and glycated hemoglobin levels were listed in Table 5. The increased level of insulin, HOMA-IR, and HbA1c were noted in diabetic control rats when compared to normal control group rats. Oral administration of vitexin (10 and 20 mg/kg BW) for 48 days significantly reduced these levels to the normal range. The results were statistically significant $p < 0.05$ when compared to untreated diabetic rats.

Table 5
Effect of vitexin on insulin, HOMA- IR, and HbA1c

Groups	Insulin ($\mu\text{U/mL}$)	HOMA-IR	HbA1c (%)
Normal control	19.23 $\pm$ 1.02	3.53	5.01 $\pm$ 1.14
HFD	28.52 $\pm$ 2.19*	12.24	8.93 $\pm$ 1.95*
Diabetic control	22.12 $\pm$ 0.98*	16.84	11.82 $\pm$ 0.23*
D + Vitexin (10 mg/kg BW)	11.28 $\pm$ 0.63#	5.23	7.11 $\pm$ 1.56#
D + Vitexin (20 mg/kg BW)	13.19 $\pm$ 0.92#	4.81	6.18 $\pm$ 1.09#
D + Pioglitazone (10 mg/kg BW)	12.24 $\pm$ 0.86*#	5.47	6.45 $\pm$ 1.45#
D - Diabetic, HFD - High-fat diet. HOMA – IR – Homeostatic model assessment – Insulin resistance. Values are the Mean $\pm$ SEM of six rats in each group. Values with superscript '*' statistically differ from normal control rats ($p \leq 0.05$) and '#' statistically differ from diabetic control rats ($p < 0.05$) (One way ANOVA followed by Tukey's multiple comparison test)			

3.9 Effect of vitexin on liver function markers

The liver function marker enzymes such as ALT, AST, and ALP levels in serum are depicted in Table 6. There was a significant increase ($p \leq 0.05$) in the concentration of these hepatic enzymes in the untreated diabetic rats observed when compared to normal group rats. Administration with vitexin (10 and 20 mg/kg BW) reduced the concentration of these enzymes to a normal level.

Table 6
Effect of vitexin on liver enzymes

Groups	ALT (U/L)	AST (U/L)	ALP (U/L)
Normal control	26.00 ± 1.12	29.83 ± 1.83	89.50 ± 2.65
HFD	30.00 ± 0.57#	43.67 ± 1.58#	124.00 ± 4.69*#
Diabetic control (HFD + STZ)	44.00 ± 1.48*	60.00 ± 1.39*#	172.20 ± 2.86*
D + vitexin (10 mg/kg BW)	30.33 ± 2.07#	33.67 ± 1.38#	134.50 ± 4.21*#
D + vitexin (20 mg/kg BW)	25.67 ± 1.08#	30.50 ± 1.82#	88.00 ± 2.26#
D + pioglitazone (10 mg/kg BW)	26.50 ± 1.17#	33.83 ± 1.53#	100.50 ± 5.33#

D - Diabetic, HFD - High-fat diet. ALT - Alanine transaminase, AST – Aspartate transaminase, ALP - Alkaline phosphatase. Values are the Mean ± SEM of six rats in each group. Values with superscript ‘*’ statistically differ from normal control rats ($p \leq 0.05$) and ‘#’ statistically differ from diabetic control rats ($p < 0.05$) (One way ANOVA followed by Tukey’s multiple comparison test)

3.10 Effect of vitexin on renal function markers

In diabetic control rats, significant elevation of urea and creatinine levels, and a decrease in total proteins, albumin, and globulin levels were observed (Table 7). Treatment by vitexin at 10 and 20 mg/kg BW for 48 days explained a significant ($p \leq 0.05$) decrease in the levels of urea and creatinine as well as a significant ($p \leq 0.005$) elevation in total protein compared to diabetic control rats. The pioglitazone (10 mg/kg BW) treated group rats also significantly ($p \leq 0.005$) decreased the urea and creatinine compared to untreated diabetic rats but not increased the total protein significantly compared to normal and the diabetic rats treated with vitexin 10 and 20 mg/kg BW.

Table 7
Effect of vitexin on renal function

Groups	Urea (mg/dL)	Creatinine (mg/dL)	Total Protein (mg/dL)	Albumin (mg/dL)	Globulin (mg/dL)
Normal control	18.17 ± 1.69	0.71 ± 0.076	7.30 ± 0.170	5.23 ± 0.050	2.07 ± 0.060
HFD	27.46 ± 1.02*	1.32 ± 0.151*	6.12 ± 0.080#	4.34 ± 0.230*	1.78 ± 0.930
Diabetic control (HFD + STZ)	53.16 ± 2.32*	1.83 ± 0.135*	5.43 ± 0.230*	3.21 ± 0.310	2.22 ± 0.140*
D + vitexin (10 mg/kg BW)	31.74 ± 1.05*#	1.28 ± 0.049*	6.31 ± 0.130#	4.22 ± 0.910#	2.09 ± 0.060#
D + vitexin (20 mg/kg BW)	22.20 ± 2.30*#	0.77 ± 0.170#	7.78 ± 0.190#	5.46 ± 0.130#	2.32 ± 0.560#
D + pioglitazone (10 mg/kg BW)	22.42 ± 2.37*#	0.58 ± 0.088#	5.86 ± 0.100*	4.82 ± 0.100	1.04 ± 0.340*

D - Diabetic, HFD - High-fat diet. Values are expressed as the Mean ± SEM for six rats in each group. Data with superscript ‘*’ significantly differ from the normal control group ($p \leq 0.05$) and ‘#’ significantly differs from the diabetic control group ($p < 0.05$) (One Way ANOVA followed by Tukey’s multiple comparison test).

3.11 Effect of vitexin on GLUT-4 expression

Figure 15 and Fig. 16 show the GLUT-4 mRNA expression level of normal control, diabetic control, and treated rats in adipose tissue and skeletal muscle respectively. The GLUT-4 mRNA expression level was significantly ($p \leq 0.005$) improved in vitexin (20 mg/kg BW) treated diabetic rats than in HFD and diabetic control rats.

3.12 Effect of vitexin on PPAR-γ expression

Figure 17 shows the PPAR-γ mRNA expression level of normal control, diabetic control, and treated rats in adipose tissue. There was a significant ($p \leq 0.005$) importance in PPAR-γ mRNA expression level of vitexin (20 mg/kg BW) treated diabetic rats than HFD and diabetic control rats.

4. Discussion

Diabetes mellitus (DM) is one of the most challenging multifactorial disorders affecting approximately 347 million people globally. DM is characterized by hyperglycemia emerging as a result of reduced

glucose uptake, and the metabolism of dietary nutrients such as carbohydrates, protein, and fat due to a defect in the secretion and action of insulin both Juvekar and Bandawane (2009). Following the advancement of macro and microvascular problems such as retinopathy, nephropathy, neuropathy, and coronary heart diseases for an extended period of time, these altered metabolic processes become chronic (Ashok Kumar 2012). Many modern drugs currently available are reported for their side effects and reduction in the quality of life. In contrast, phytomedicines are highly expected for their therapeutic efficacy without harmful effects. The current study was designed to explore the possible mode of action and margin of safety of one such compound present in *A. ilicifolius* in HFD-STZ diabetic rats.

Bioactive guided fractionation of methanolic extract of *A. ilicifolius* explored the antidiabetic molecule. Based on the one-dimensional and 2-dimensional nuclear magnetic resonance, the obtained compound was characterized and identified as vitexin, an apigenin flavone molecule, whose occurrence has never been reported from this extract. The L6 cell line isolated from rat skeletal muscle cells was chosen to investigate the glucose uptake activity of vitexin because 70% of glucose uptake occurs through the peripheral tissues. Vitexin proves its non-toxic nature and significantly enhances the glucose uptake activity in L6 cell lines. Since the glucose uptake was highly regulated by transporter proteins, *in silico* and gene expression study was carried out with special reference to its GLUT-4 translocation.

GLUT-4 showed a better binding preference for pioglitazone; however, PPAR- γ showed a higher preference for vitexin at the same active site. By comparing the binding energy observed, vitexin shows a higher preference towards PPAR- γ . From the *in silico* studies, it was understood that vitexin could act as a ligand for the activation of GLUT-4. The binding of the vitexin may induce the function of these proteins which are deregulated in the diseased condition. Further, to understand the beneficial effects of vitexin in diabetic conditions an extensive *in vivo* study was undertaken.

Due to the intake of HFD, the body weight of the rats increased which results in obesity and insulin resistance which are the characteristic of type II DM. In the untreated group, HFD enhances the storage of excess fat in adipose tissue whereas treatment with vitexin (10 and 20 mg/kg BW) effectively reduced the weight by promoting glycogen storage in the liver. In contrast to this, the standard drug pioglitazone fails to reduce body weight which is one of the remarkable side effects of the drug. But vitexin maintains the body weight more or less to the normal level and prevents harmful effects on the body.

After 3 days of STZ induction, an increased level of blood glucose was noticed due to insulin deficiency or resistance in all STZ-treated groups. The HFD rats also showed elevation in FBG after 48 days which indicates the prediabetic stage is influenced by diet. The administration of vitexin enhanced the uptake of glucose and utilization by the peripheral tissue thereby they reduced the glucose level in fasting serum. It seems that vitexin acquired antihyperglycemic activity fairly because of its capability to enhance peripheral uptake of glucose by glucose transporters.

Because of the HFD feeds, there was a compensatory insulin production in HFD and untreated diabetic control rats which results in hyperinsulinemia. Besides, the normal level of insulin in treated rats is due to enhanced sensitivity to insulin and good glycemic control. HOMA-IR is the calculated factor used to

measure insulin resistance. The increased HOMA-IR in diabetic and HFD rats indicates increased insulin resistance. The treatment with vitexin effectively (20 mg/kg BW) reduced insulin resistance. Vitexin treatment significantly reduced glucose to the normal level and it may be due to possible extra-pancreatic mechanism(s) such as glucose uptake through glucose transporters in adipose tissues and skeletal muscles.

In contrast to single glucose measurement, which only offers a result that is accurate at the moment a blood sample is taken, glycosylated hemoglobin is a reliable index that indicates the average blood glucose across the lifetime of RBCs. (Weng et al. 2014). The elevation in HbA1c explains the poor glycemic control in the diabetic control group. High risks for the advancement of comorbidities such as retinopathy, nephropathy, and neuropathy are predicted by elevated HbA1c values Calisti and Tognetti (2005). Administration of vitexin significantly decreased the increased level of glycosylated hemoglobin in diabetic rats by which validating its efficiency in long-term glycemic control of diabetes mellitus and its associated complications.

The liver is the metabolic organ in the body, maintaining and controlling lipid balance (Thilakarathna et al. 2012). The liver function test is assessed by measuring the enzymes AST, ALT, and ALP in serum. These enzymes are regarded as reliable hepatic biomarker enzymes, which are present in the cytoplasm of hepatocytes under normal physiological conditions. Elevation of serum ALT, AST, and ALP indicates the impaired liver function observed in STZ-induced diabetic rats. This is due to the possibility that diabetic rats may develop hepatocellular necrosis changes, which point to both the hepatotoxic effects of STZ and oxidative stress. (Chafiaa Mehenni et al. 2016) also reported that significantly enhanced levels of ALT, AST, and ALP activities in diabetic control rats are due to the hepatotoxic effect of STZ. In addition to this, some plant extracts used for the control of various diseases frequently lead to subsequent hepatic toxicity as the management persists for a longer period. In the current study, liver function was estimated to ensure whether vitexin is safe to be employed for the management of diabetes and dyslipidemia for a longer period. The abnormal level of total protein, albumin, and globulin are common in hepatotoxicity due to intracellular damage and irregular metabolism of proteins. Treatment with vitexin caused a decrease in the ALT, AST, and ALP and increased levels of proteins to the normal level, which can improve the damage caused by oxidative stress or STZ and thereby show its hepatoprotective effect in diabetic rats.

The kidney is crucial in the removal of metabolic waste products from blood circulation, such as urea and creatinine, and it also regulates electrolyte levels, which help to maintain physiological homeostasis (Patel 2014). Elevations in urea, creatinine, and reduction in protein levels are remarkable biomarkers of kidney dysfunction. The elevation was due to altered protein metabolism under diabetic conditions. Urea, the excretory substance produced through the metabolism of protein and creatinine is formed endogenously which indicates the glomerular filtration rate (Perrone et al. 1992). An increase in urea and creatinine, in addition, to a decrease in total proteins, demonstrated the dysfunction of kidneys in diabetic control rats. In the current study, it is proved that administration of vitexin in diabetic rats prevented kidney damage by decreasing the levels of serum urea and creatinine and restoring the total

proteins. From these results, it is understood that vitexin exerted a protective role against kidney dysfunction in treated diabetic rats.

Maintaining a normal level of electrolytes is important for many biological functions. Under diabetic conditions, the levels of sodium, potassium, calcium, and chloride were reduced due to abnormal metabolism. The present results demonstrate that the treatment with vitexin restores electrolytes which helps to alleviate complications due to diabetes.

Glucose uptake is the rate-limiting step in glucose metabolism. Glucose transporters facilitate glucose uptake across the cell membrane. Since glucose is a polar molecule, the metabolism of glucose mainly depends on glucose transporters Rhodes and White (2002). GLUT-4 is the important glucose transporter present predominantly in adipocytes, skeletal muscle, and heart muscle cells that maintain the whole body's glucose homeostasis. In normal conditions, insulin binds with insulin receptors and phosphorylates the cascade proteins which trigger GLUT-4 translocation from cytosol vesicles to the plasma membrane for glucose uptake and utilization Rakhshandehroo et al. (2010). In diabetic conditions, because of the absence of an insulin signaling mechanism due to insulin resistance, glucose absorbed from the diet does not enter the cells for further metabolism and hence there is an elevation in glucose level. Insulin resistance is the main characteristic of type II DM mainly promoted by a high-fat diet which disturbs the metabolism of glucose and lipids by down-regulating the transcription proteins such as GLUT-4 and PPAR- γ .

In the current study decreased level of GLUT-4 mRNA expression in skeletal muscle and adipose tissues in HFD-STZ-induced diabetic rats was observed. Hence, the activation of GLUT-4 plays a crucial role in insulin signaling and is considered one of the important targets for the management of type II DM. Several studies reported that the phytoconstituents isolated from medicinal plants can enhance the mRNA expression of GLUT-4 for glucose disposal. Treatment by vitexin at 20 mg/kg BW improved the expression level of GLUT-4 mRNA and showed the increased utilization of glucose in skeletal muscle by promoting the transportation of GLUT-4 from intracellular vesicles to the cellular membrane.

PPAR- γ predominantly found in adipocytes is a ligand-dependent transcription factor that governs the genes involved in lipid metabolism. The activation of PPAR- γ improves the fatty acid storage and oxidation of adipocytes Kershaw and Flier (2004). Thus, PPAR- γ is crucial in maintaining energy homeostasis by binding with specific ligands and has been considered a target for the management of type II DM. The binding of ligands with PPAR- γ activates the transcription factors that promote adipogenesis in adipocytes and sensitizes the genes of insulin signaling. PPAR- γ activation also enhances the expression of genes that trigger the metabolism of glucose in the adipocytes including GLUT-4 Gandhi et al. (2013).

In this study, it is proved that during the 48 days of treatment with flavonoids ligand vitexin, the mRNA expression of PPAR- γ in adipocytes of diabetic rats was improved significantly. Therefore, these results explained that treatment by vitexin in HFD-STZ induced diabetic rats' up-regulated PPAR- γ expression and explained a significant effect on preventing the resistance to insulin. Furthermore, this study shows down-

regulation of PPAR- γ expression in the adipose tissue of diabetic control rats although treatment with vitexin significantly up-regulated expression of PPAR- γ . Many phytoconstituents isolated from medicinal plants act as a natural ligand for PPAR- γ and enhance mRNA expression (Liang et al. 2003). Vitexin is a flavonoid molecule that improved PPAR- γ expression in adipocytes as well as a decrease in weight in treated diabetic rats. Vitexin enhances the activation of PPAR- γ which in turn up-regulates the GLUT-4 expression and thereby enhances the glucose uptake in adipocytes. Thus, vitexin enhances the mRNA expression of PPAR- γ and GLUT-4 in type II diabetic rats.

5. Conclusion

The present study provides evidence for vitexin as an antidiabetic agent. *In vitro* glucose uptake assay explains that it enhances the uptake of glucose by skeletal cells, which is a major defect in type II diabetes. A long-term study of vitexin revealed its significant effect on blood glucose, insulin, lipid profiles, hepatic enzymes, and other body profiles in HFD-STZ-induced diabetic rats. In addition, treatment with vitexin up-regulates the mRNA expression of PPAR- γ in adipocytes and thereby facilitates the transportation of glucose by the activation of GLUT-4 in peripheral tissues. Hence, vitexin can become a promising new-generation antidiabetic drug for insulin resistance-related type II diabetic patients.

Abbreviations

A. ilicifolius: *Acanthus ilicifolius*, ALT: Alanine transaminase, ALP: Alkaline phosphatase, AST: Aspartate aminotransferase, ANOVA: Analysis of variance, ACE: Atomic contact energy, BW: Body weight, ^{13}C NMR: Carbon-13 nuclear magnetic resonance, cDNA: Complementary DNA, DM: Diabetes mellitus, DEPC: Diethyl pyrocarbonate, DMSO: Dimethyl sulfoxide, ELISA: Enzyme-linked immunosorbent assay, EDTA: Ethylene diamine tetraacetic acid, FBG: Fasting blood glucose, FSI: Fasting serum insulin, FTIR: Fourier Transform Infrared, GC: Gas Chromatography, GLUT-4: Glucose transporter protein type-4, HbA1c: Glycated haemoglobin, HSQC: Heteronuclear Single Quantum Coherence, HDL: High density lipoprotein cholesterol, HFD: High Fat Diet, HFD-STZ: High Fat Diet-Streptozotocin, HOMA-IR: Homeostasis of model assessment insulin resistance, ^1H NMR: Hydrogen-1 nuclear magnetic resonance, IR: Infrared, λ_{max} : Lambda Max, LD₅₀: lethal dose, 50%, LC-MS: Liquid Chromatography-mass spectrometry, LDL: Low density lipoproteins, NTC: Negative Temperature Coefficient, NMR: Nuclear Magnetic Resonance, OECD: Organisation for Economic Co-operation and Development, PPAR- γ : Peroxisome proliferator-activated receptor gamma, PCR: Polymerase Chain Reaction, PDB: Protein Data Bank, RBC: Red Blood Cell, RT-PCR: Real time polymerase chain reaction, R_f: Retention factor, RNA: Ribo Nucleic Acid, RNase: Ribonuclease, NaOH: Sodium hydroxide, STZ: Streptozotocin, TLC: Thin layer chromatography, TC: Total cholesterol, TP: total protein, TG: Triglycerides, 2D COSY: Two-dimensional correlation spectroscopy, and VLDL: Very low density lipoproteins.

Declarations

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ETHICAL APPROVAL

The study was conducted after obtaining the approval from Institutional Animal Ethical Committee (VIT/IEAC/9th/july26th/18). All the protocols on animal experiments were performed as per the guidelines of OECD.

COMPETING INTERESTS

The authors wish to declare that there are no competing interests.

AUTHOR CONTRIBUTIONS

Study conception and design, material preparation, data collection, analysis, and writing of the first draft of the manuscript [Gayathri GA]. Manuscript writing, revision, and edition [Kavya P]. Manuscript revision [Ashwini D, Eshika Chakraborty, Idris Adewale Ahmed]. Research work design and supervision, supervision of manuscript writing, revision, editing, and approval of the final version of the manuscript [Gayathri M]. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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AVAILABILITY OF DATA AND MATERIALS

Available.

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Figures

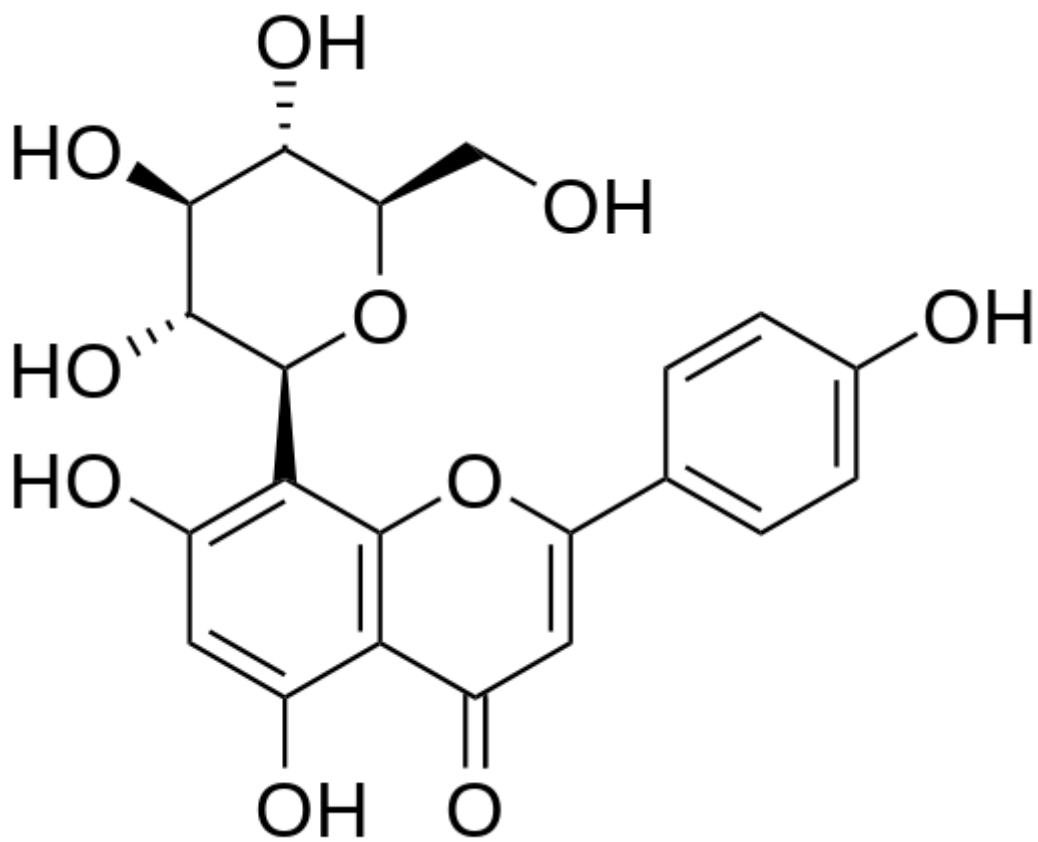


Figure 1

Structure of vitexin

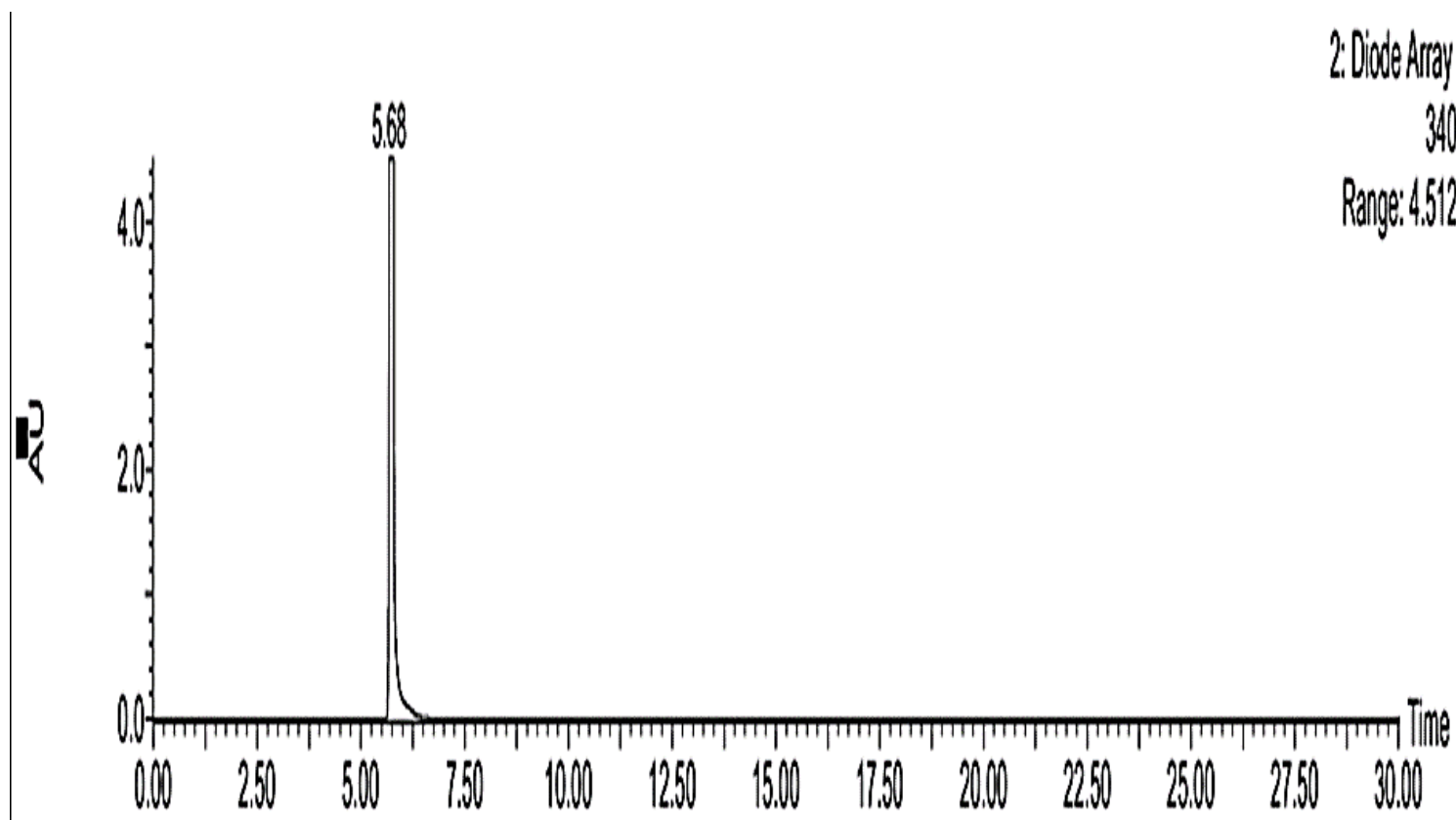


Figure 2

Liquid Chromatogram of Vitexin

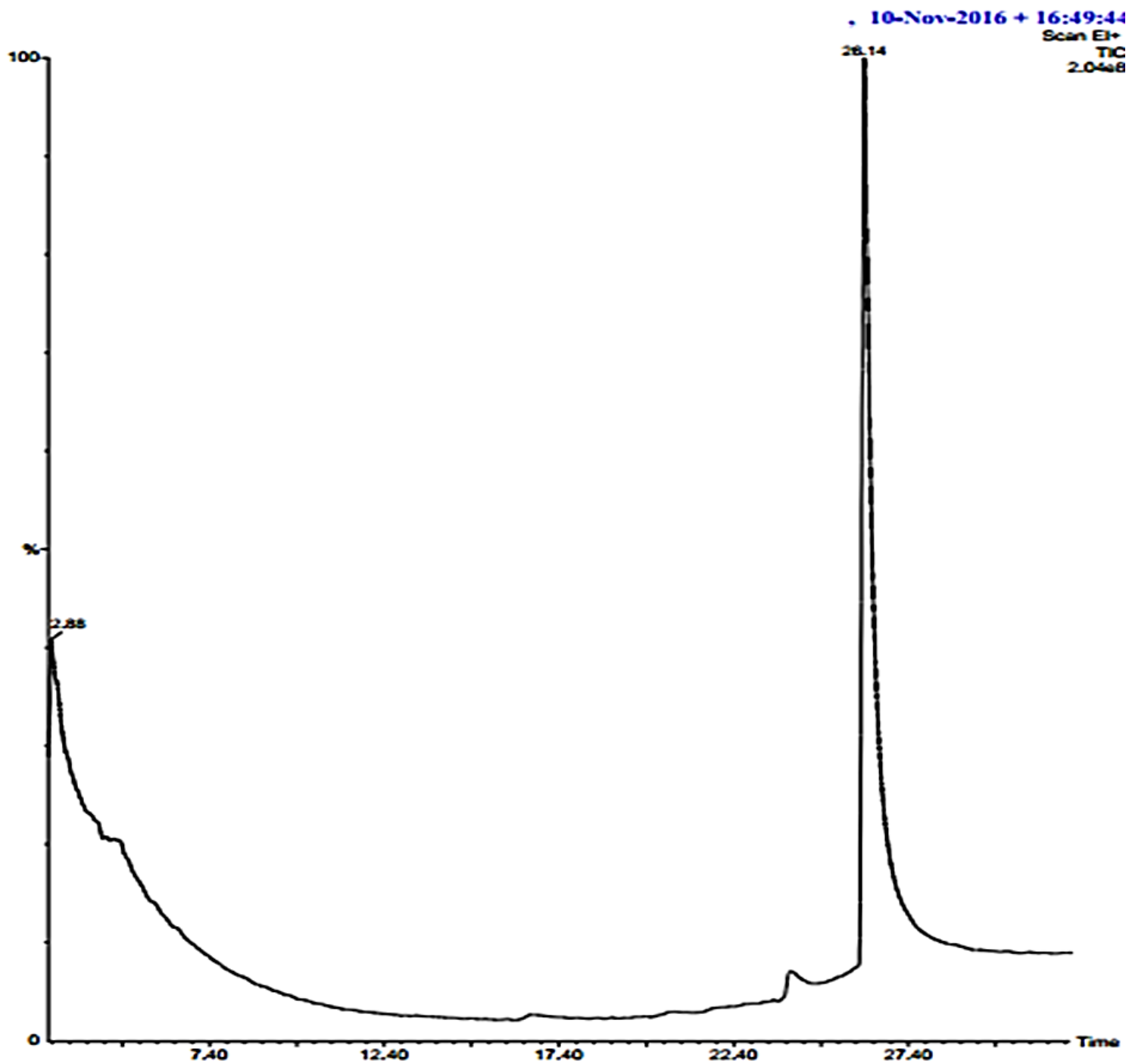


Figure 3

Gas Chromatogram of Vitexin

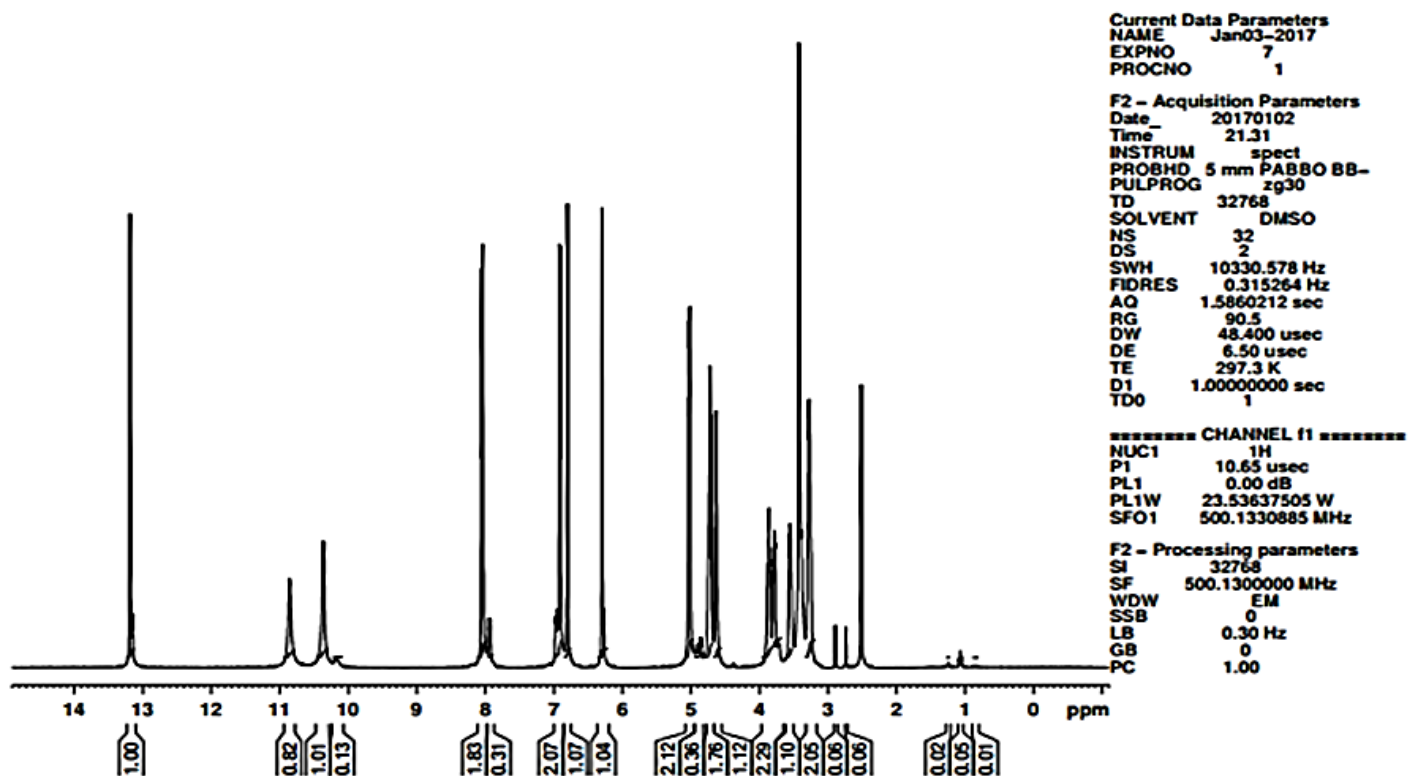


Figure 4

¹H NMR spectra of Vitexin

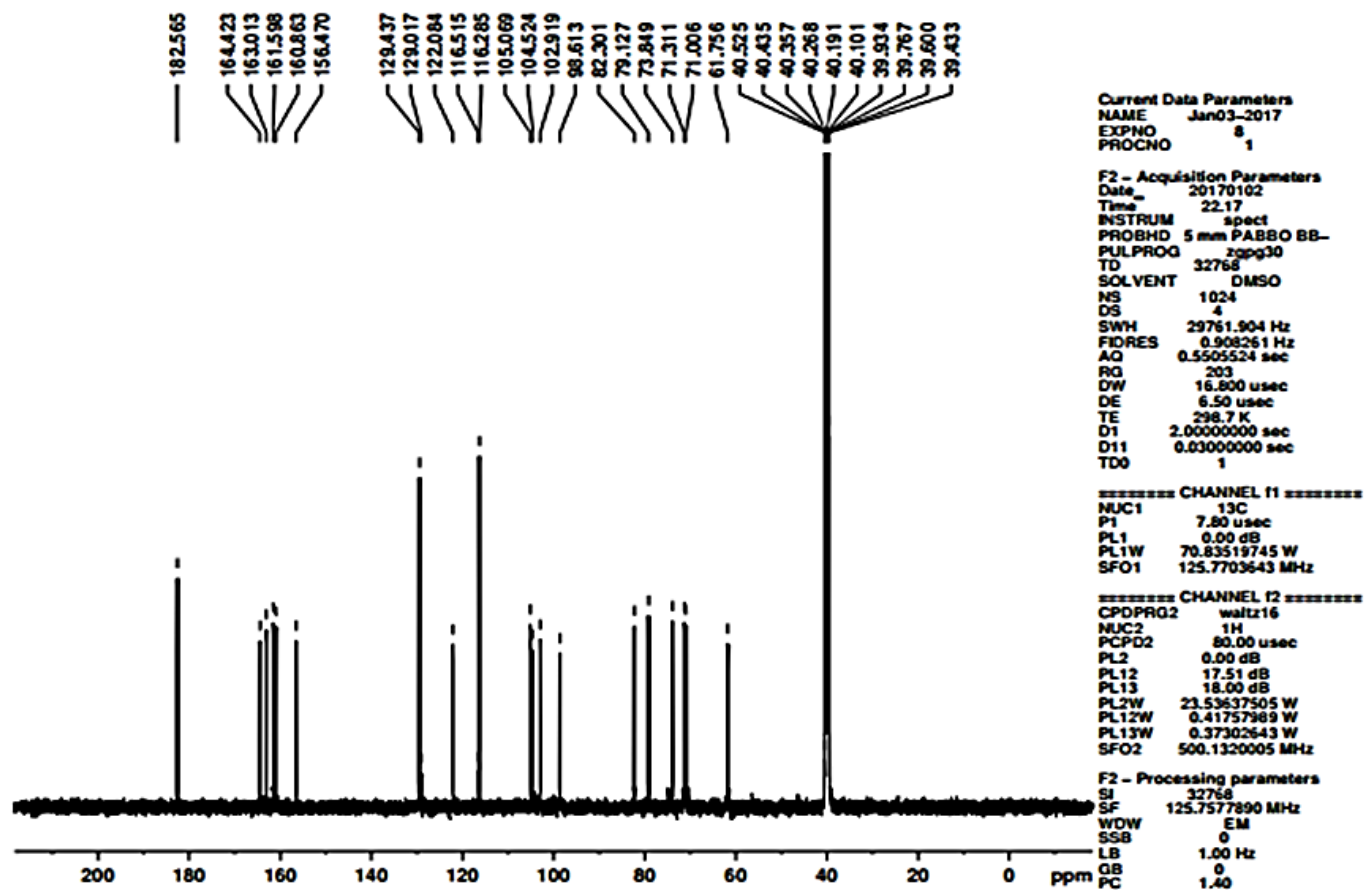


Figure 5

¹³C NMR spectra of Vitexin

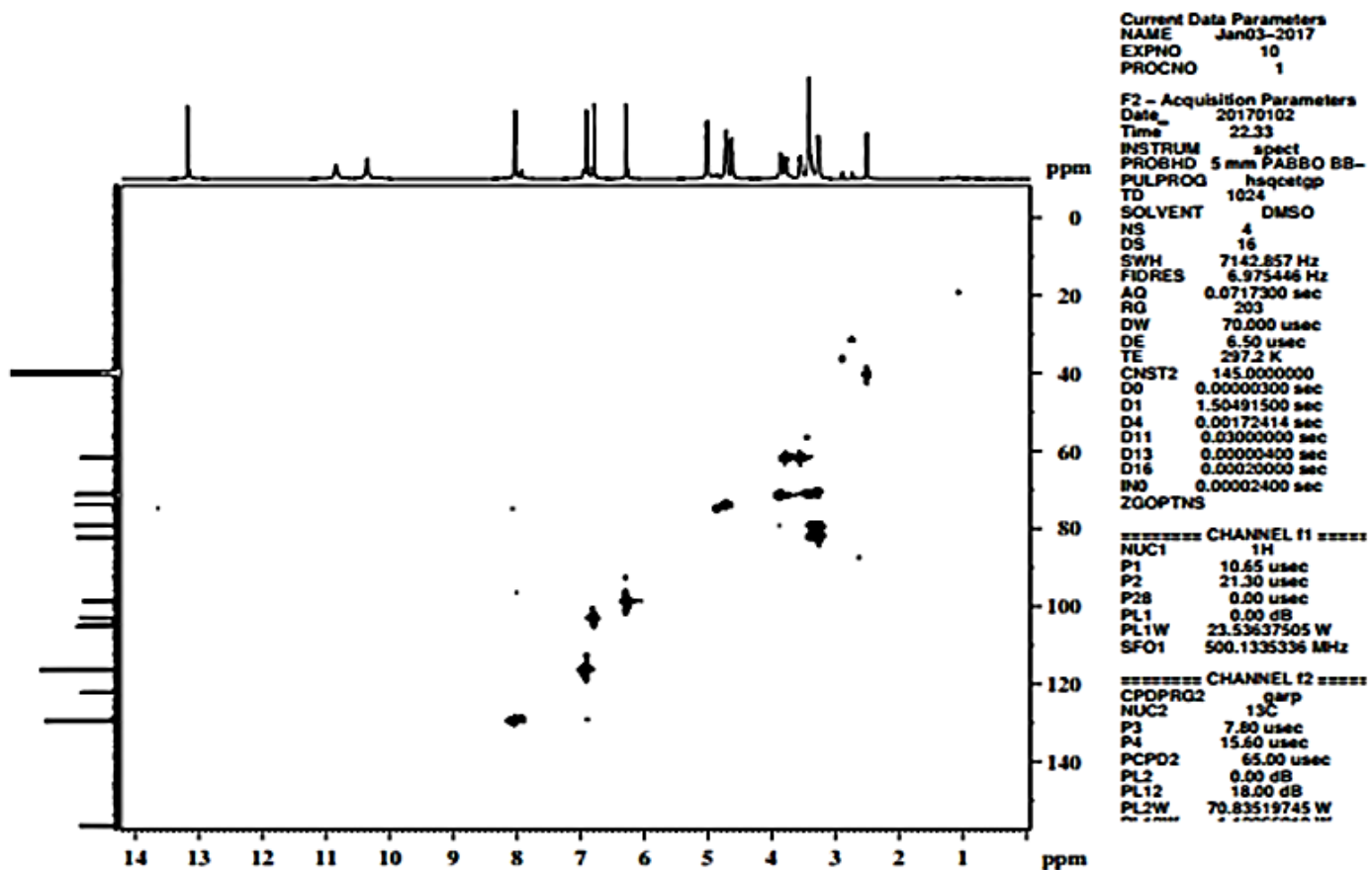


Figure 6

2D HSQC spectra of Vitexin

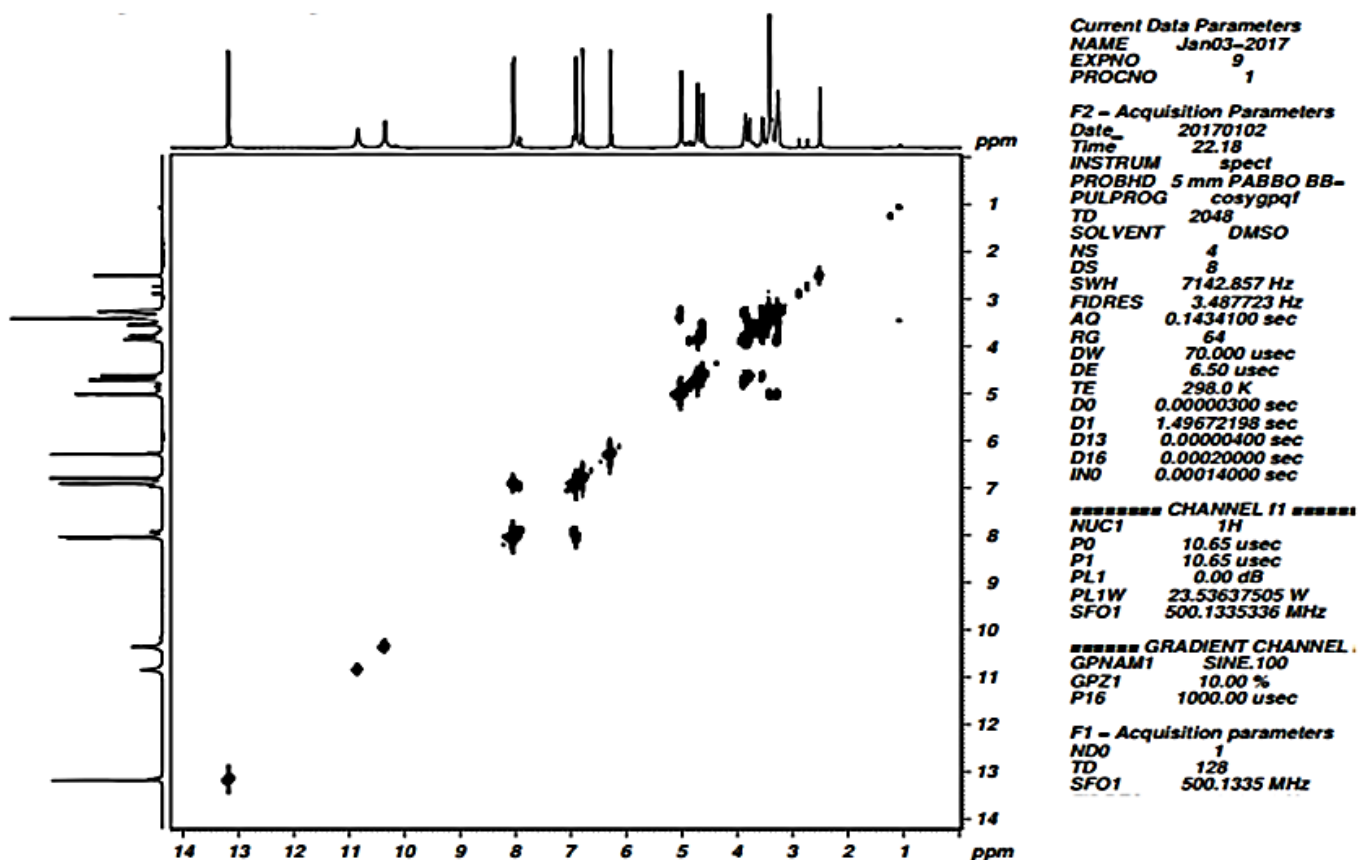


Figure 7

2D COSY of Vitexin

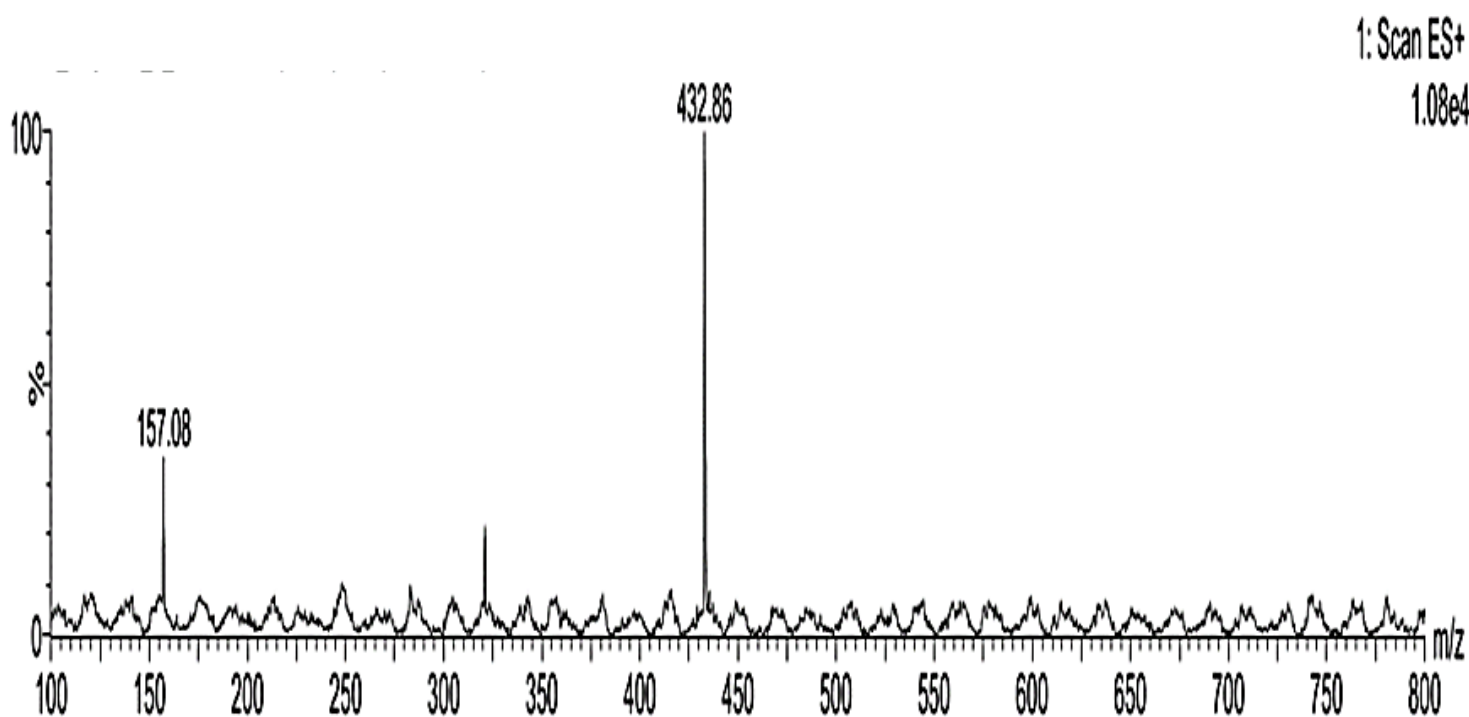


Figure 8

Mass spectra of Vitexin

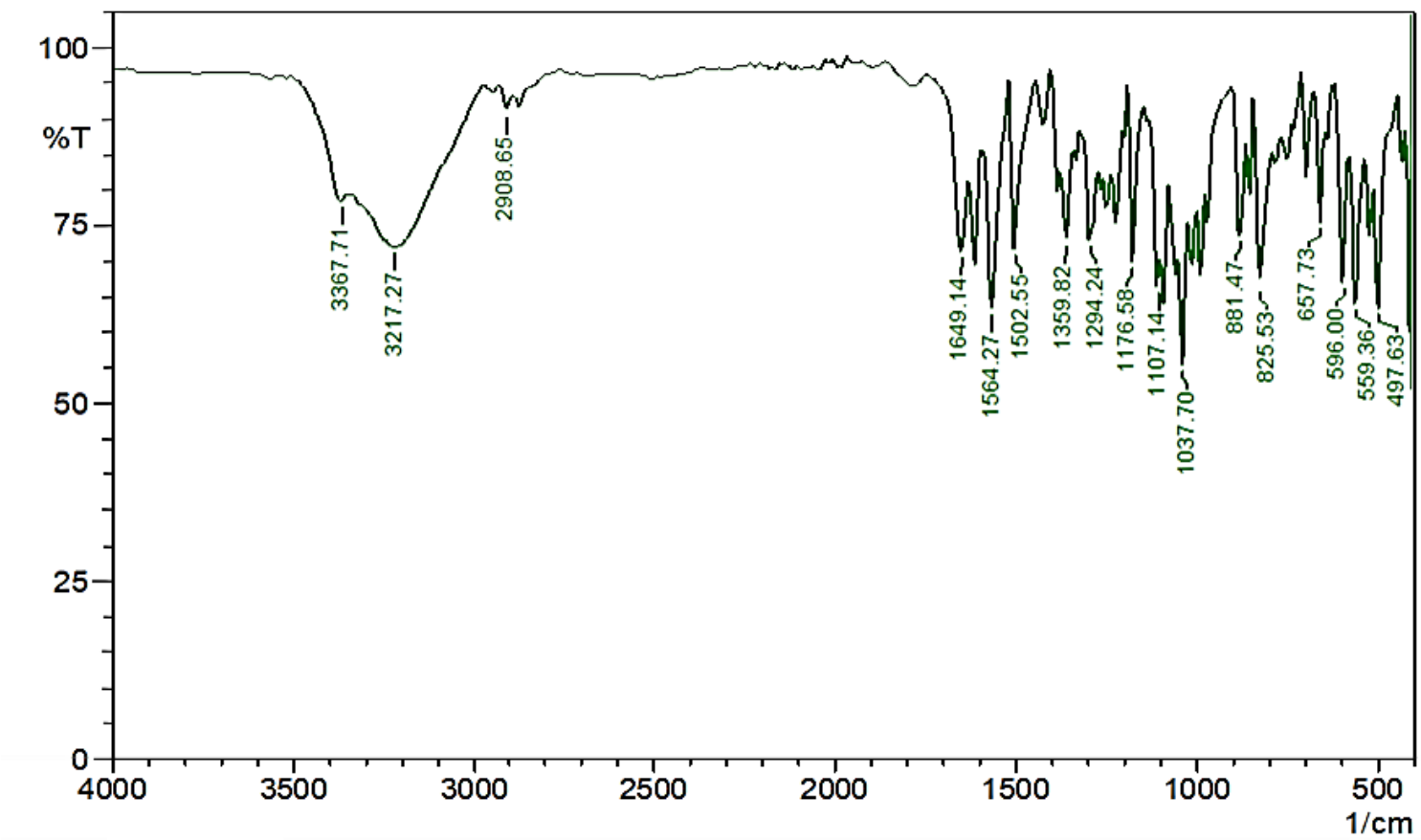


Figure 9

FTIR spectra of Vitexin

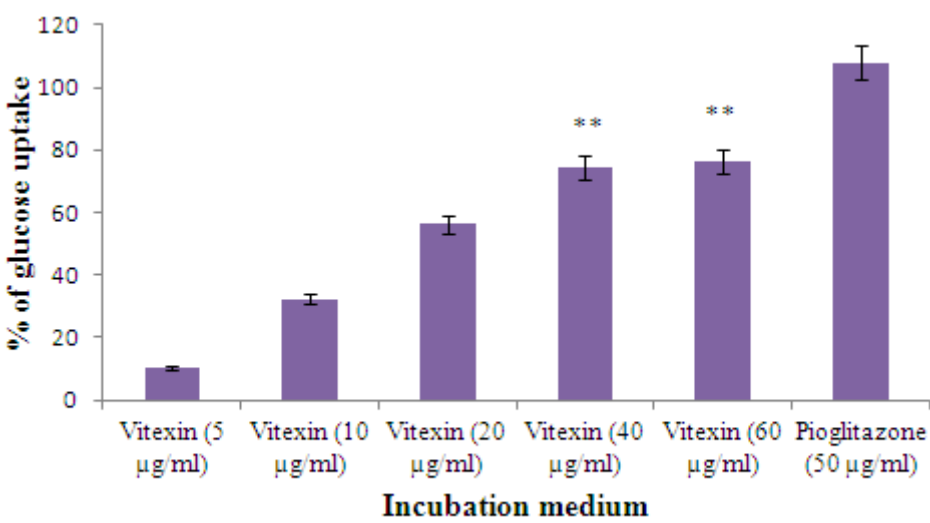


Figure 10

Glucose uptake activity of isolated vitexin in L6 cell lines

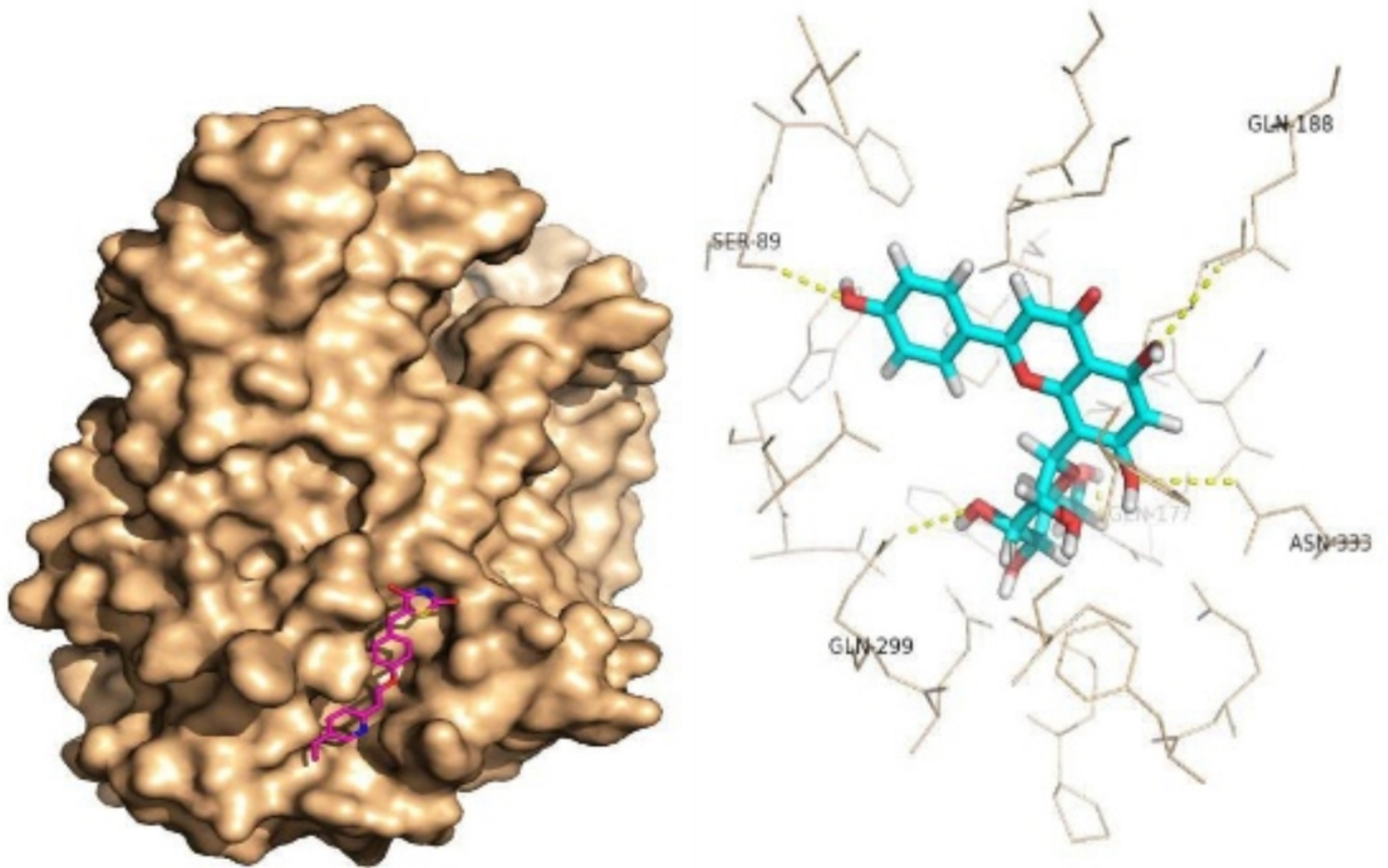


Figure 11

GLUT-4-vitexin complex

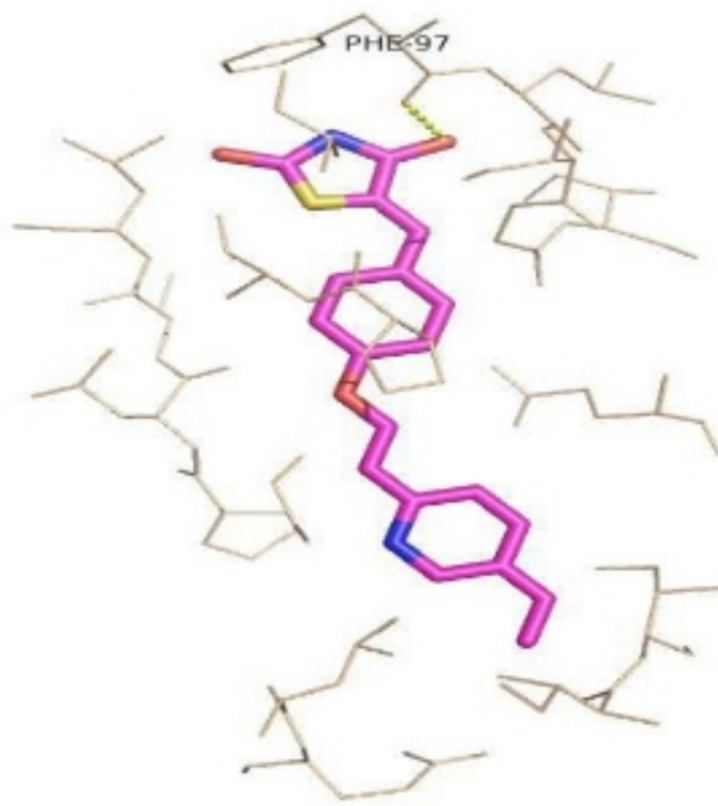
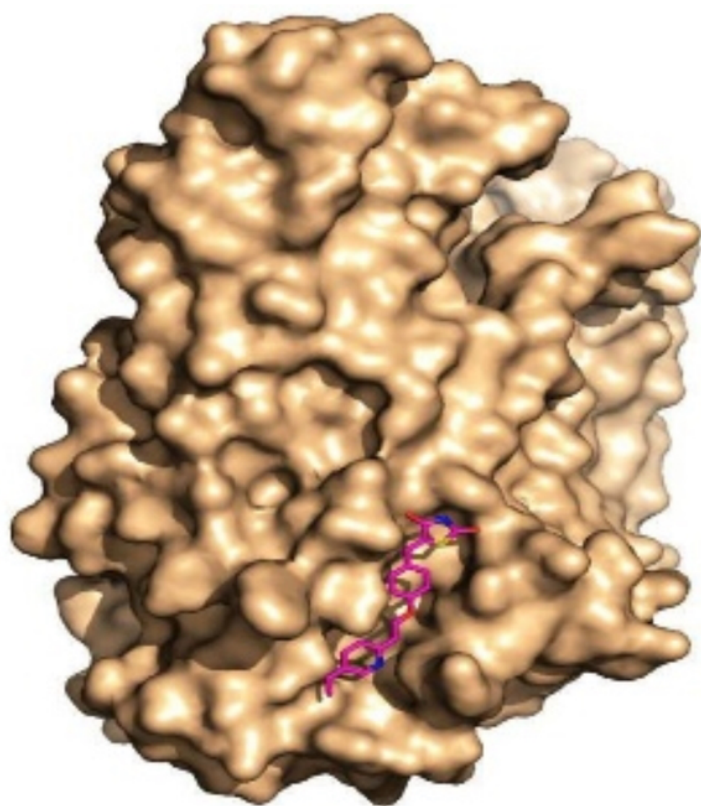


Figure 12

GLUT-4-pioglitazone complex

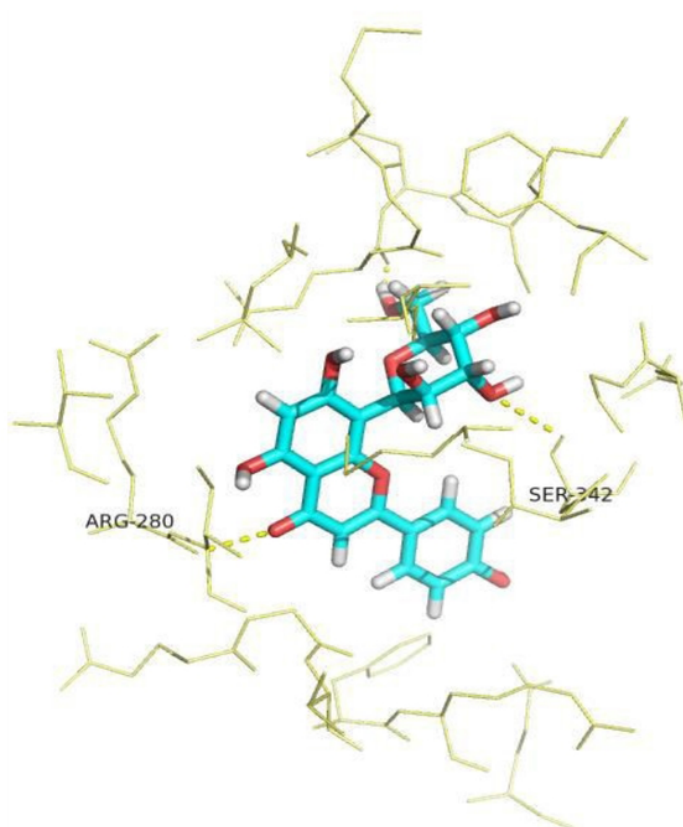
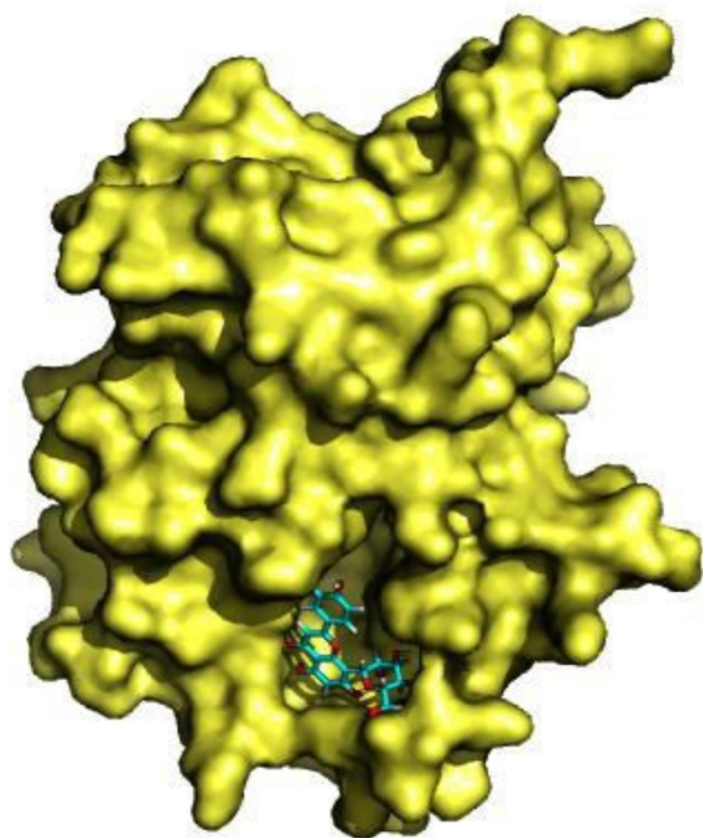


Figure 13

PPAR- γ -vitexin complex

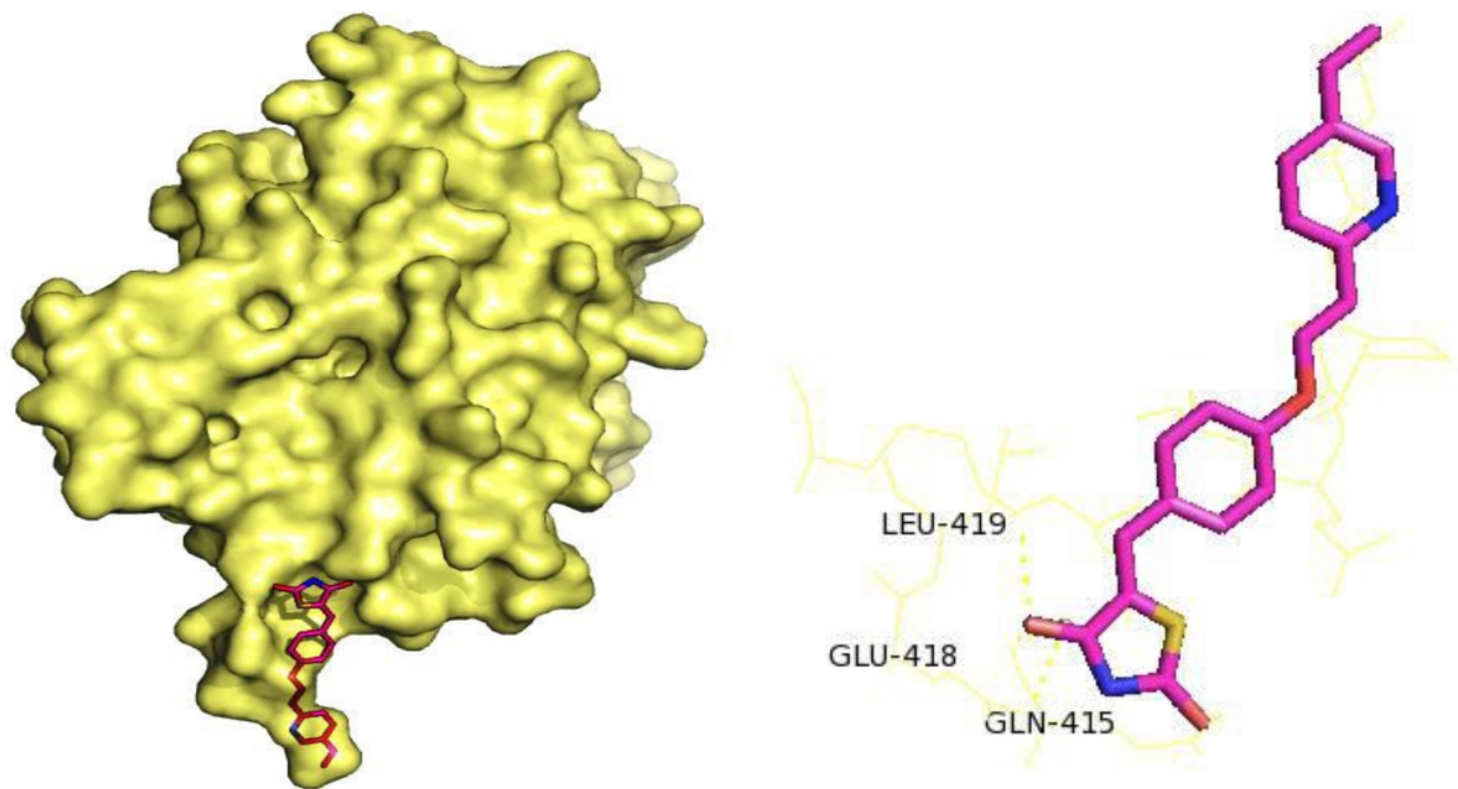


Figure 14

PPAR- γ -pioglitazone complex

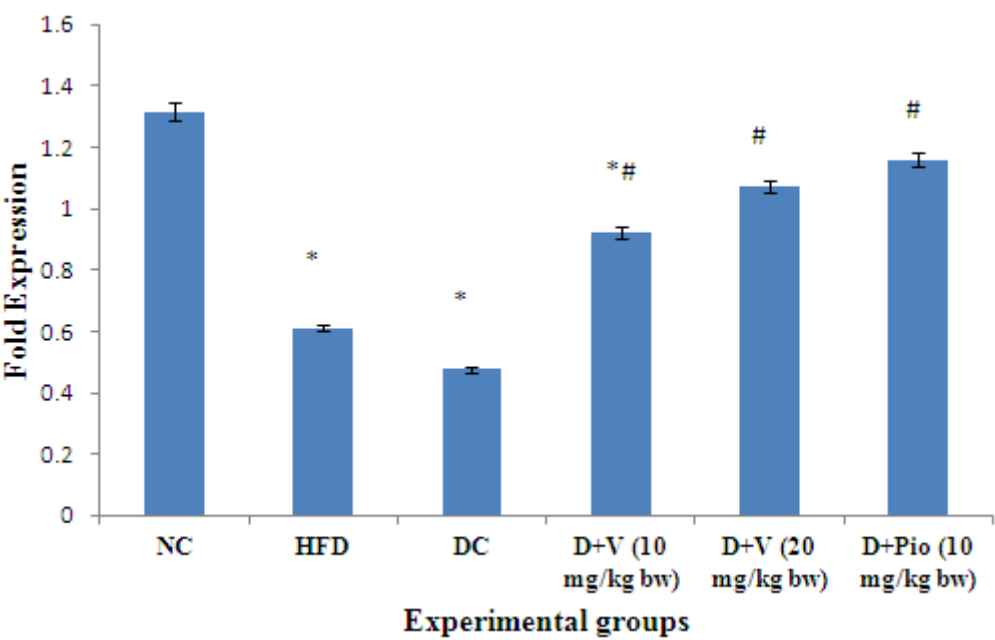


Figure 15

GLUT-4 mRNA expression level of normal control, diabetic control and treated rats in adipose tissue

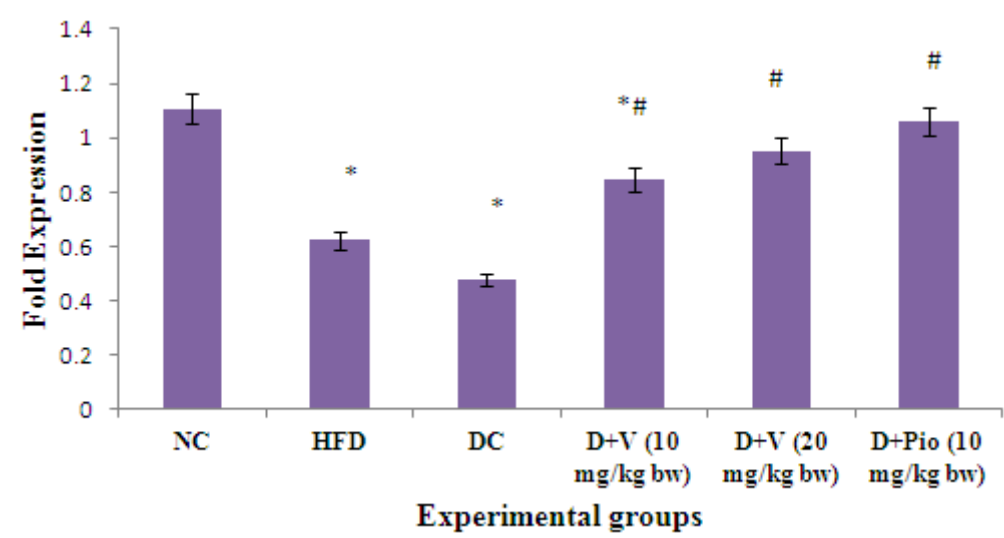


Figure 16

GLUT-4 mRNA expression level of normal control, diabetic control, and treated rats in skeletal muscle

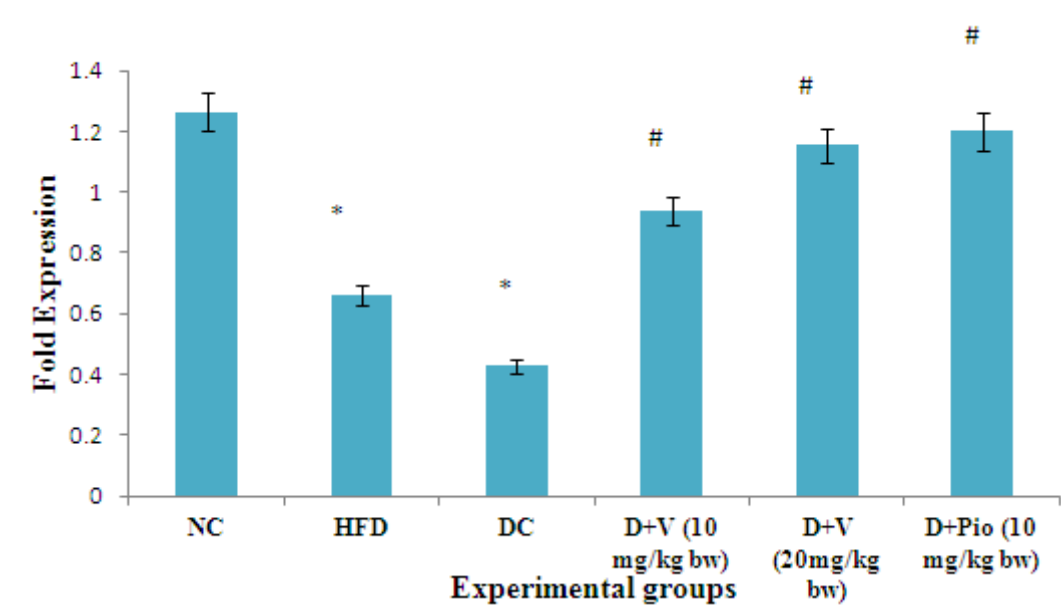


Figure 17

PPAR-γ mRNA expression level of normal control, diabetic control and treated rats in adipose tissue