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Research Article

GC-MS SCRUTINY OF PHYTOCONSTITUENTS, *IN VITRO* EVALUATION OF ANTIHYPERGLYCEMIC, ANTIADIPOGENIC ACTIVITIES, AND CYTOTOXIC EFFECT USING 3T3 L1 ADIPOCYTE CELL LINE AND MOLECULAR DOCKING STUDIES OF *PREMNA CORYMBOSA*

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ABSTRACT

Objective: The present study was intended to list out the phytochemical multiples and to investigate the antihyperglycemic effect of *Premna corymbosa* using *in vitro* assays and *in silico* molecular docking methods.

Methods: The phytochemical multiples of methanol proportion of *P. corymbosa* leaves were appraised by Gas Chromatography-Mass Spectrometry (GC-MS) scrutiny to illustrate the attendance of phytochemical composites. Moreover, the *in vitro* antihyperglycemic, antiadipogenic activities, and cytotoxic effects of the extract were elucidated using a 3T3 L1 adipocyte cell line. Mode of action of phytochemical composites in methanol leaf extract of *P. corymbosa* was probed by Western blotting with IRS1, IRS2, mTOR, and glucose transporter type 4 (GLUT 4) receptors. At present, to probe the consequence of the aboriginal drugs, it is necessary to perform *in silico* docking on the diabetic receptor which could be useful for the progress of enhanced formulation for the psychoanalysis of diabetes.

Results: The GC-MS scrutiny depicted the being there of thirty-five phytochemical multipart. Amid the thirty-five multipart's recognized, focal composites were Phytol, acetate (RT-16.78), n-Hexadecanoic acid (RT-18.16), Phytol (RT-19.51), 9,12,15-octadecatrienoic acid (Z,Z,Z) (RT-19.85), octadecanoic acid (RT-20.04), and Bis (2-ethylhexyl) phthalate (RT-23.09). The results of the glucose conception assay, adipocyte differentiation assay, and MTT assay showed potent *in vitro* antihyperglycemic activity with methanol leaf extract of *P. corymbosa* in 3T3l1Cell line. The results attained from western blotting revealed good antihyperglycemic activity of *P. corymbosa.* The *in silico* molecular docking results illustrated that the selected herbal lead compound is an effective target against the receptors. The compound showed favorable interactions with the amino acid residues thereby substantiating their proven efficacy as an antihyperglycemic compound.

Conclusion: The outcome of the current study substantiates the antihyperglycemic prospective of the methanol leaf extract of *P. corymbosa* on the hyperglycemic causal agents and its activity against diabetes by a molecular approach.

Keywords: Antihyperglycemic activity, *Premna corymbosa,* Phytochemicals, 3T3 L1 adipocyte Cell line, *In silico* molecular docking.

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INTRODUCTION

Diabetes mellitus is an endemic metabolic anarchy. Diabetes is a metabolic chaos that arises, as the pancreas does not fabricate ample insulin or when the carcass cannot efficiently employ the insulin it generates. Blood glucose echelon and carcass influence have been frequently gauged to scrutinize the glycemic manage mechanism. Health-giving vegetation is a pricey bequest from temperament to human being. Medicinal plants play an indispensable function in the improvement of incontrovertible therapeutic agents. It is anticipated that above 1000 foliage species are exercised in opposition to diabetes as folk pills. The pervasiveness of diabetes universally is at the moment anticipated to be something like 366 million, far afield from the 285 million expected by the World Health Organization for 2010 from transnational figures congregated in 2008 [1]. In topical decades, cram on phytochemical component of therapeutic foliage and its pharmacological tricks have perceived ample interest [2-5]. Phytochemicals are the inferior metabolites existing to a lesser degree in superior foliage and they encompass alkaloids, steroids, flavonoids, terpenoids, tannins, and scores of others [6]. The phytochemical cram of curative flora is indispensable to resolve the attendance of dynamic ideology in them. The curative usefulness of health-giving plant life epitomizes owed to the excellence and amount of chemical composites. In excess of 1 200 kinds of foliage have been scrutinized for bustle on the origin of ethno-medicinal uses [7]. The energetic multipart of curative vegetation plays an earth-shattering task in the supervision of diabetes mellitus more than ever in embryonic nations. The phytochemical module is the rudimentary cause for the establishment of copious pharmaceutical industries and makeshift drug formulations [8]. Gas chromatography is a significant, frequently inimitable contrivance in phytochemical psychiatry even at a negligible amount of foliage chemical multipart. GC-MS is a faultless modus operandi for qualitative and quantitative psychotherapy for explosive and semi-volatile complex [9].

3T3L1 cells represent a good replica for the uptake of glucose because they have been hugely used to explicate the technique in the uptake of glucose in muscle cells and also have whole insulin signaling pathways and articulate insulin-sensitive glucose transporter type 4 (GLUT 4) transporters [10-12]. 3T3L-1 fibroblasts are antecedent cells utilized as a mock-up to study adipogenesis [13]. Adipogenesis is a multipart and synchronized procedure that commences with the upregulation of two premature transcription factors, cytosine-cytosine-adenosine-adenosine-thymidine/enhancer-binding protein beta (C/EBP) and C/EBP, which then stimulates the expression of PPARγ, the master gene in adipogenesis, which boosts

the expression of proteins connected with the adipocyte phenotype. Glucose transporters are primarily accountable for the uptake of glucose by insulin-mediated cells, as the insulin level decreases and glucose transporters budge athwart the membrane into intracellular pools for the intention of storage and this procedure is cast-off [14,15]. A vital feature of supporting whole-body glucose homeostasis is insulin's ability to agitate glucose uptake into muscle and adipose tissue [16]. GLUT4 is the most widespread glucose transporter ascertained in insulin-responsive tissues together with skeletal muscle and adipose tissue [17,18].

Molecular docking is a technique drawn on to establish the emplacement of the ligand to the receptor (protein) to figure a steady composite. Molecular docking also facilitates researchers to competently probe the activities of ligands in the binding site of the target protein, receptor, or enzyme with a substantial extent of precision [19]. The major target of this investigation is to establish the finest probable pose of the ligand with the smallest binding energy [20]. The communication connecting the ligand and the enzyme might foretell the enzyme inhibition or activation activity since it is understood that the natal bustle of the ligand is linked with the binding likeness of the ligand to the enzyme [21,22]. The ligands were docked one at a time to get together their lone binding affinities (kcal/mol). The exploit of computational-based molecular docking techniques and meta-analysis in drug discovery has urbanized swiftly in the growth procedure so that it becomes a more effectual and well-organized technique to exploit [23,24].

Premna corymbosa (Family: Verbenaceae), called kulamani (Tamil), is a small tree. Leaves are utilized to mend weakness of limbs, to assuage headache, and to treat diabetes [25,26]. Qualitative and quantitative scrutiny is awfully indispensable for recognizing and quantifying the energetic ideology present in the therapeutic flora which is noteworthy for health-giving deeds and drug grounding. In view of the facet, the current cram was pre-meditated to scrutinize the phytochemical composites of *P. corymbosa* by GC-MS psychiatry. In the current research work, *in vitro* studies were conducted on the glucose utilization of 3T3L-1 cell lines and *in silico* molecular docking interactions with models designed to activate particular antidiabetic targets to examine the antidiabetic efficacy and mechanism of action. The results obtained in the present study clearly demonstrate that *P. corymbosa* extract enhances glucose uptake under *in vitro* conditions.

METHODS

Plant collection and extraction

Leaves of *P. corymbosa* collected from Kelambakkam forest located at Chennai. The leaves were cleansed with water to get rid of dust and sand, shade-dried at room temperature, and powdered. The powdered leaves were subject to consecutive extraction with the solvent methanol using a soxhlet extractor [27]. The extorts were desiccated in a vacuum pump at 40°C. The desiccated crude extracts were stockpiled in a refrigerator for further use.

GC-MS psychotherapy

GC-MS is the best technique to identify the bioactive constituents of long-chain hydrocarbons, alcohols, acids, esters, alkaloids, steroids, and amino and nitrogen compounds. The phytochemicals were analyzed by GC-MS Agilent 5975-C Series instrument employing the electron impact mode (ionizing potential-70 eV) and a capillary column (DB-5 ms Agilent) (length 30 m × diameter 0.25 mm, film thickness 0.25 μm) packed with 5% phenyl dimethyl silicone) and the ion source temperature was monitored at 200°C. Further, the GC-MS settings were indicated as the initial column temperature was set at 70°C and kept hold for 2 min; the temperature was increased to 300°C at a rate of 10°C/min for 9 min and placed in isothermal condition for 2 min. The column oven temperature was maintained at 70°C. Helium was used as carrier gas with 99.9995% purity. Samples were injected at a temperature of about 250°C with a split ratio of 10:1 with a flow rate of helium 1.51 mL/min, mass scan (m/z): 45–1000, total MS running time: 36 min [28].

Credentials of module

Reading on mass spectrum GC-MS was performed with the database of the National Institute of Standards and Technology (NIST). The spectrum of the anonymous module was weighed against the spectrum of the branded module stockpiled in the NIST library. The name, molecular weight, and structure of the composites of the ordeal supplies were determined.

Determination of antihyperglycemic activity in 3T3 L1 cell line

Preparation of media and reagents required for cell culture

Chemicals

All cell culture solutions and supplements were purchased from Life Technologies Inc., USA. Insulin, 3-isobutyl-1-methyl xanthine, dexamethasone, Sigma (UK). Antibodies such as insulin receptor β, insulin receptor substrate 1 and 2 (IRS1-2), mTOR, and GLUT4 were from Cell Signaling Technology. ECL Western Blotting Detection Kit from Amersham. All the other chemicals and organic solvents used were of the highest analytical grade.

Preparation of DMEM for cell culturing

The powdered media was dissolved in 900 mL of autoclaved distilled water in a conical flask under sterile conditions. Sodium bicarbonate of 3.7 g was added and stirred until it completely dissolved and the pH of the medium was adjusted to 7.2 by adding 3 mL of 1N HCl. Following these antibiotics such as penicillin 120U/mL, streptomycin 75 μg/mL, gentamycin 160 μg/mL, and amphoterincin B 3 μg/mL were added. Finally, 10% FCS was added and the medium was sterilized using a 0.2-micron filter under pressure and stored at 4°C [29].

Preparation of saline: Trypsin versene (STV)

The following solutions were prepared

- 10× Saline: 8 g NaCl, 0.4 g KCl, 1.0 g D-Glucose, and 0.35 g $NaHCO_3$ were dissolved in 100 mL water. 10× saline was filter sterilized and stored at 4°C.

Versene

- 1 g of EDTA was weighed and added to 90 mL of distilled water. The solution was completely dissolved by adding 5N NaOH in drops thereafter filter was sterilized and stored at 4°C.

For STV preparation

- 100 mg of trypsin, 10 mL of 10× saline, and 2.5 mL of versene were added and were made up to 100 ml using double distilled water. It was then filtered sterilized and stored at 4°C.

Cell passaging

3T3L1 adipocyte cell lines were grown on polystyrene-coated flasks with DMEM as the growth medium. The cell line was passaged after attainment of confluency, i.e., after every 2 days. Passaging was performed in a laminar flow hood as explained below. The culture medium was removed completely and the traces of the medium were removed by washing with STV after which 2.5 mL STV was added to the flask and incubated at 37°C for a few minutes until the cells started detaching from the surface. After complete detachment, STV action was neutralized using DMEM-containing serum. The cells were pelleted by centrifugation at 15,000 rpm for 3 min and the supernatant was discarded. The cell pellet was resuspended in fresh medium and seeded into flask or plates according to the requirement and incubated at 37°C.

Differentiation of 3T3L1 pre-adipocytes to adipocytes

3T3L1 pre-adipocytes were cultured in DMEM WITH 10% FCS. On reach of confluency, the cells were subjected to a differentiation medium (combination of 0.5 mmol of IBMX, 0.25 μmol/l of DEX, and 1 mg/l of DMEM medium with 10% FCS) to differentiate into adipocytes. Three days after induction, the differentiated medium was replaced with a medium containing 1 mg/mL insulin alone. The medium was

subsequently replaced again with fresh culture medium (DMEM with 10% FBS) after 2 days by monitoring the formation of multinucleation in cells, the extent of differentiation was measured [30].

Cryopreservation of cell culture

For cryopreservation, the cell pellet was resuspended in a cryopreservation medium comprising of 10% DMSO in serum, transferred to cryovial, and frozen at respective temperatures starting from 4°C, −20°C, −80°C and gradually stored in liquid nitrogen.

Measurement of glucose uptake using 6-NBDG

Glucose uptake assays were performed to seed 10^4 3t3-l1 pre-adipocytes in black 96 well tissue culture plates: culture in DMEM 10% calf serum, induce adipogenesis by treatment with 0.5 mM 3-isobutyl-1-methyl-xanthine, 2 µg/mL dexamethasone, and 1 µg/mL insulin was added after 48 h the culture in the presence of 1 µg/mL to maintain up to 7 days. After 1 hour, change the culture media to serum-free, low-glucose DMEM (1 g/L glucose, 200 µL total volume per well). Incubate for 30 minutes with serum-free, low-glucose DMEM containing 20 µM 6-NBDG, then wash the cells three times with phosphate-buffered saline (PBS). Lyse the adipocytes with 70 µL of 0.1 M potassium phosphate buffer (pH 10) containing 1% Triton X-100 for 10 minutes in the dark. Add 30 µL of DMSO and homogenize by pipetting. Immediately measure the fluorescence using a micro-plate reader (466-540 nm). All the assays were performed in triplicates for concordancy. Results were expressed as % glucose uptake with respect to solvent control based on CPM values. Rosiglitazone (50 µm) was used as a positive control. % of glucose uptake is calculated by the formula [31].

$$\%\ of\ glucose\ uptake = \frac{(OD\ control - OD\ test)}{OD\ control} \times 100$$

Cytotoxicity assay using MTT

MTT assay is a calorimetric method used to measure cell viability. Yellow MTT (3-(4,5-Dimethlthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in the mitochondria of living cells. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore, conversion can be directly related to the number of viable (living) cells. Different concentration of extracts was prepared 100 ng, 10 ng, 1 ng, and 1 µg. The cells were grown in 96 well plates and treated with different concentrations of the extracts. After 24 h of incubation, cells are treated with MTT reagent and incubated for 2–4 h. The reaction is terminated by aspiration of the media, the formed formazan crystals were dissolved in DMSO and the absorbance was read at 595nm which is directly proportional to cell viability. Based on cell viability, the % cytotoxicity of the extracts was calculated [32].

$$\%\ of\ cytotoxicity = \frac{(OD\ control - OD\ test)}{OD\ control} \times 100$$

Antiadipogenic assay

Nile Red is a fluorescent dye used extensively to study intracellular lipid accumulation. 3T3L1 pre-adipocytes (10,000 cells/well) were seeded in 96-well plates. After 80% confluence, logarithmic doses of extracts (1 ng to 100 µg) were added along with the differentiation medium. Cells were washed with 200 µL of 1× PBS (pH 7.4) and 5 µL of Nile Red reagent was added. Working Nile red reagent was prepared by adding 2 µL of stock Nile Red (1 mg/mL) in 50% DMSO and 50% 1× PBS. After 10 min of incubation, % inhibition of lipid accumulation was measured using excitation/emission wavelength at 485/572 nm [33].

Western blot analysis

Preparation of cell lysate

The cells after their respective treatments were scraped using PBS and were pelleted at 5000 rpm for 5 min at 4°C. The cells were re-suspended in 100 µL of lysis buffer (1% IGEPAL, 150 mM NaCl, 50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 21 mg/mL aprotinin, 10 mg/mL leupeptin) and incubated for 45 min at 4°C. Subsequently, the samples were centrifuged at 14000 g at 4°C for 10 min and supernatants were collected [34]. The protein content of the supernatant was estimated by Bradford's method.

Protein estimation of the obtained cell lysate

Protein estimations were performed using Bradford's method [35].

Preparation of Bradford reagent

20 mg of Coomassie brilliant blue G-250 + 10 ml of methanol + 20 ml H_3PO_4. The final volume was made up to 200 ml using distilled water and filtered. This solution appears pale-straw in color and is stored in the dark.

Procedure for protein estimation

The standard, bovine serum albumin (BSA) was taken at concentrations of 20 µg, 40 µg, 60 µg, 80 µg, and 100 µg (i.e., from 1 mg/mL stock of BSA, 20, 40, 60, 80, and 100 µL). 5 µL of cell lysate samples were added to each well and the volume was made up to 200 µL with 0.15 M NaCl. 200 µL of NaCl served as blank. The samples were incubated with 1 mL of Bradford's reagent for 5 min in dark. The absorbance was read at 595 nm. The readings from protein standards were used to plot a standard graph and to estimate the value of the cell lysate samples.

Total protein was estimated using this method

Equal quantities of protein samples were loaded for carrying out western blot analysis [36].

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis

Buffers, reagents, and gel composition for SDS-PAGE were as follows

Monomer solution

29% Acrylamide + 1% N,N'-methylene bisacrylamide ~ 30% acrylamide.

Separating gel buffer

1.5M Tris HCl at pH 8.3.

Stacking gel buffer

- 1 M Tris HCl at pH 6.8
- 10% SDS

Polymerization enhancer

- Ammonium per sulfate
- 140 mg/mL

Monomer and polymerization inducer

N, N, N', N'-Tetramethylenediamine (TEMED)

Reagents and gel composition for SDS-PAGE

Reagents used	Separating Gel (10%)	Stacking Gel (5%)
Double distilled water	4 mL	2.7 mL
30% Acrylamide	3.3 mL	0.67 mL
Tris	2.5 mL (1.5M, pH 8.3)	0.5 mL (1M, pH 6.8)
10% SDS	0.1 mL	0.04 mL
APS	0.004 mL	0.004 mL
TEMED	0.004 mL	0.004 mL

Sample loading buffer

- 10% (w/v) SDS
- 10% (v/v) 2-β mercaptoethanol
- 50% sucrose
- 0.025% Bromophenol blue in 0.25× stacking gel buffer.

Electrophoresis buffer used for running the gel

- 0.025 M Tris HCl
- 0.25M glycine
- 0.1% SDS pH 8.3.

Polyacrylamide gel was casted using the above-mentioned solutions and run at 15 mA for approximately 75 min.

Western blotting

Buffers used for Western blotting:

Transfer buffer

- 25 mM Tris
- 192 mM Glycine
- 20% Methanol, 0.1% SDS.

PBS (pH 7.2)

- NaCl 80 g/l
- KCl 2 g/l
- KH_2PO_4 2.4 g/l
- Na_2HPO_4 4.4 g/l
- 0.1 % Tween 20 (only for PBS Tween preparation).

Ponceau S

0.5 g Ponceau in 1 mL glacial acetic acid was made to 100 mL.

Substrate buffer (for 25 mL)

- 1 M Tris (pH 9.5): 2.5 mL
- 4 M NaCl: 0.625 mL
- 1 M $MgCl_{2.}$ 0.125 mL

Development buffer

- Nitro blue tetrazolium (NBT): 0.5 g in 10 mL of 70% dimethyl formamide (DMF)
- 5 Bromo-4-Chloro-3-Indolyl Phosphate (BCIP): 0.5 g in 7 mL of 10 % DMF
- Developing substrate: NBT (66 µL) + BCIP (33 µL) and made up to 10 mL with substrate buffer.

After electrophoresis, the gel was washed in a transfer buffer. Nitrocellulose membrane and Whatman filter papers of 8 × 6 cm size were incubated in transfer buffer before transfer. The membrane was placed on the filter papers and the gel was carefully layered over it. Air bubbles should be avoided between the layers during the transfer. The transfer was set up at 120 mA for 90 min at 20 mV. After transfer, the membrane was stained using ponceau S to visualize the marker lane. The membrane was then washed thrice with PBS and 5% skimmed milk was added for blocking overnight at 4°C. The blocking reagent was washed with PBS and PBS tween alternatively, 3 times, each for 3–4 min. The blot was then blocked using 1% BSA in PBS with the primary antibody (1:2000 of anti-*IRS/IRS1/GLUT4/mTOR/*actin antibody). Then, the blot was rocked gently at room temperature for 1½ h, after which, the blot was washed again with PBS, 3 times, each for 3–4 min. Later, the blot was incubated with appropriate secondary antibody (HRP conjugate) prepared in 1% BSA in PBS for 1 h at room temperature. Then, the blot was washed with TPBS and PBS tween alternatively, 3 times, each for 3–4 min, and the blot was developed with equal parts of the Peroxide Solution and the Luminol Enhancer Solution (ECL-Amersham ECL Western Blotting Detection Kit) to cover the membrane, incubate the membrane for 1 minute at room temperature, and place in a plastic sheet protector or clear plastic wrap. Working in a dark room with a safe light, place the covered membrane in a film cassette with the protein side facing up. Place X-ray film on top of the membrane and expose for 1 minute. Develop the X-ray film using appropriate developing and fixing solutions.

RESULTS AND DISCUSSION

GC-MS psychotherapy

The phytochemical composites present in the methanol extort of *P. corymbosa* was branded by means of GC-MS psychotherapy (Fig. 1). As a result of construing these composites, it is established that *P. corymbosa* own diverse health-giving relevance. The dynamic ideology with their retention time (RT), molecular formula (MF), molecular weight, and concentration (%) in extort was accessible. Utterly thirty-five composites branded from the methanol extort of the *P. corymbosa* are offered in Table 1. In the midst of the thirty-five composites recognized, focal composites were phytol, acetate (RT-16.78), n-hexadecanoic acid (RT-18.16), phytol (RT-19.51), 9,12,15-octadecatrienoic acid (Z,Z,Z) (RT-19.85), octadecanoic acid (RT-20.04), and bis (2-ethylhexyl) phthalate(RT-23.09).

Amid the documented phytochemicals, some composites were high flying due to its natal significance. Hexadecanoic acid ethyl ester, squalene possesses the prospective of antioxidant bustle [37,38]. Not long squalene also encompasses chemopreventive motion in opposition to colon carcinogenesis [39,40]. Phytol is acknowledged to be an acyclic diterpene alcohol that is a herald for vitamins E and K [41]. 9, 12, octadecadienoic acid (Z, Z)-boast the potential of anti-inflammatory and antiarthritic as descripted by prior investigators [42]. Octadecanoic acid composite owns the potential of antioxidant, antimicrobial, hypocholesterolemic, antiarthritic, and anti-inflammatory [43]. Vitamin E plays a prominent role in neurological functions [44]. Vitamin E furthermore defends lipids and thwarts the oxidation of polyunsaturated fatty acids [45]. The ligand gamma sitosterol with four dissimilar target proteins illustrates it as a good quality molecule that harbors fine with diverse aspirations allied to diabetes mellitus, accordingly gamma sitosterol can be mulled over for mounting into a forceful antidiabetic medication [46].

ANTIHYPERGLYCEMIC ACTIVITY OF METHANOL LEAF EXTRACT OF *P. CORYMBOSA* ON 3T3-L1 CELL LINES

MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

The capability of the cells to survive a toxic insult has been the basis of most cytotoxicity assays. It depends both on the number of viable

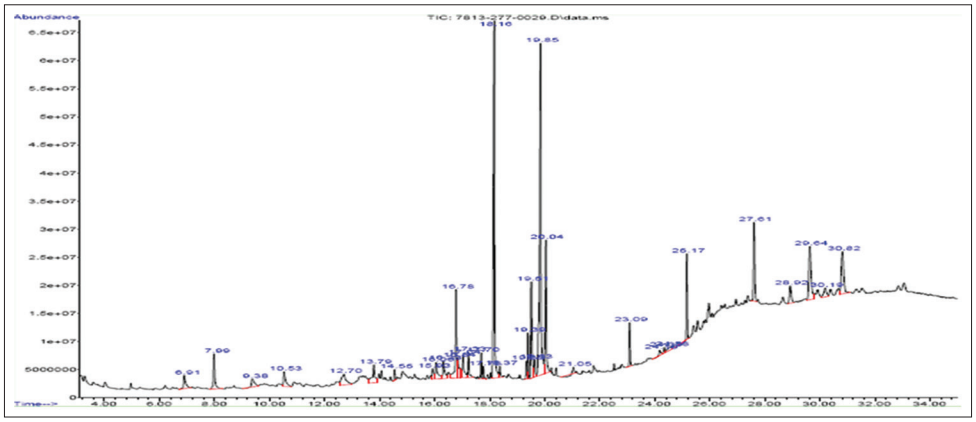

Fig. 1: GCMS Chromatogram of Premna corymbosa

Table 1: List of branded phytochemicals from methanol leaf extract of *Premna corymbosa* by GC-MS analysis

S. No	RT	Name of the compound	Molecular formula	Molecular weight	Peak area %
1	6.91	5-methyl-5-oxazolecarboxamide, 4-methyl-	$C_5H6N_2O_2$	126	1.22
2	7.98	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	C6H8O4	144	2.21
3	9.38	Benzofuran	C_8H6O	118	1.05
4	10.54	2-Methoxy-4-Vinylphenol	$C_9H_{10}O_2$	150	1.42
5	12.70	S(-)-Cathinone	$C9H_{11}NO$	149	2.31
6	13.79	2,3,5,6-tetrafluoroanisole	$C_7H_4F_4O$	180	2.07
7	14.55	Urea	CH_4N_2O	60	0.35
8	15.93	4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol	$C_{10}H_{12}O_3$	180	0.85
9	16.06	Tetradecanoic acid	$C_{14}H_{28}O_2$	228	1.61
10	16.33	Ethanamine	C_2H_7N	45	1.34
11	16.78	Phytol, acetate	$C_{22}H_{42}O_2$	338	4.79
12	16.84	D-galactose	$C6H_{12}O6$	180	1.15
13	17.03	Galactose	$C6H_{12}O6$	180	1.58
14	17.23	9-octadecyne	$C_{18}H_{34}$	250	1.28
15	17.70	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	1.00
16	17.78	Benzenepropanoic acid	$C_9H_{10}O_2$	150	0.47
17	18.16	n-Hexadecanoic acid	$C_16H_{32}O_2$	256	
18	18.37	Hexadecanoic acid, ethyl ester	$C_{18}H_36O_2$	284	0.56
19	19.34	9,12-Octadecadienoic acid (Z, Z)	$C_{18}H_{32}O_2$	280	0.61
20	19.40	9,12,15-octadecatrienoic acid, methyl ester, (Z, Z, Z)-	$C_{19}H_{32}O_2$	292	1.75
21	19.51	Phytol	$C_{20}H_{40}O$	296	5.53
22	19.63	Methyl stearate	$C_{19}H_{38}O_2$	298	0.86
23	19.85	9,12,15-octadecatrienoic acid, ethyl ester, (Z, Z, Z)-	$C_{20}H_{34}O_2$	306	19.83
24	20.04	Octadecanoic acid	$C_{18}H_36O_2$	284	6.43
25	21.05	1,6-diaminohexane-N, N, N', N'-tetraacetic acid	$C_{14}H_{24}N_2O_8$	348	0.04
26	23.09	Bis (2-ethylhexyl) phthalate	$C6H_4(C_8H_{17}CO)_2$	390	1.64
27	24.19	bicyclo 4.2.0 octa-1 3 5-triene	C_8H_8	104	0.37
28	24.35	Bacchotricuneatin c	$C_{20}H_{22}O_5$	342	0.33
29	24.58	2-Methyl-Z, Z-3,13-octadecadienol	$C_{19}H_36O$	280	0.50
30	25.16	Squalene	$C_{30}H_{50}$	410	2.73
31	27.62	Vitamin E	$C_{29}H_{50}O_2$	430	4.25
32	28.92	(R)-(-)-14-Methyl-8-hexadecyn-1-ol	C17H32O	252	1.48
33	29.63	Gamma-Sitosterol	$C_{29}H_{50}O$	415	5.30
34	30.19	cyclohexene, 4-(4ethylcyclohexyl) -1-pentyl-cyclodocosane	$C_{19}H_{34}$	262	1.10
35	30.81	cyclohexene, 4-(4ethylcyclohexyl) -1-pentyl-longifolenaldehyde	$C_{15}H_{24}O$	220	4.57

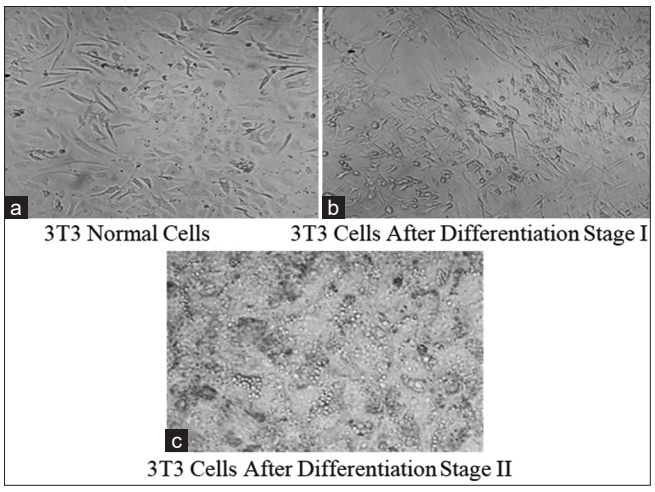

Fig. 2: Various growth stages of 3T3-L1 cell lines. (a) Is the confluent cell stage, it illustrates the adhesion of all cells in a culture disk. (b) Is differentiated cell stage, it occurred when cells convert to specific cells. (c) Is the inhibited cell stage, it comes when P. corymbosa extract was poured on 3T3-L1 adipocytes during differentiated cell stage

cells and on the mitochondrial activity of cells. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay is based on the supposition that dead cells or their products do not reduce tetrazolium. Tetrazolium salts are reduced only by metabolically active cells. Thus, MTT can be reduced to a blue-colored formazan by mitochondrial enzyme succinate dehydrogenase. The amount of formazan produced is directly proportional to the number of active cells [47]. In the present study, the methanol leaf extract of *P. corymbosa* was screened using MTT for its cytotoxicity against three 3T3 L1 cell lines at different concentrations to determine the IC50 value. The cytotoxicity of the methanol leaf extract of *P. corymbosa* was found to be dose dependent as represented in Table 2. The methanol leaf extract of *P. corymbosa* did not confer any significant lethality to the healthy 3T3 cell lines confirming the safe nature of the extract which is revealed in Fig. 2. The MTT assay of the methanol leaf extract of *P. corymbosa* in 3T3-L1cell line confirmed that there was no toxicity effect for the extract from 10 ng to 100 µg concentrations shown in Fig. 3, thereby confirming the safe nature of the extract. The outcome of the present study substantiates that extract can be safely consumed. The extracts were then subjected to scrutinize for its glucose uptake. On the basis of the cytotoxicity assay, the dose was decided for glucose uptake using a cell line. The present study confirmed that the methanol leaf extract of *P. corymbosa* does not cause any adverse effects and hence could be considered non-toxic and safe.

Glucose conception assay

Medicinal plants augment the glucose uptake by GLUT4 translocation and were attested by the *in vitro* glucose model. The 3T3 cell lines are the best characterized cellular model origin to study glucose uptake and GLUT4 translocation. 3T3-L1 cells are an excellent experimental model to rapidly screen the effects of crude drugs on glucose uptake [48]. The use of NBDG by the research community to monitor glucose uptake has steadily increased. Hence, in this study, 3T3 L1 cell lines are used to determine the glucose uptake activity of methanol leaf extract of *P. corymbosa* and the results are

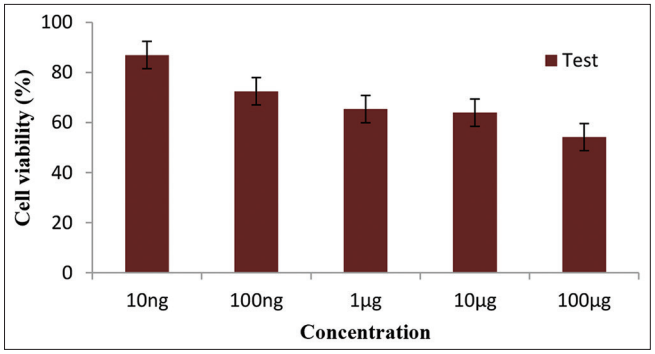

Fig. 3: Graphical representation of MTT assay

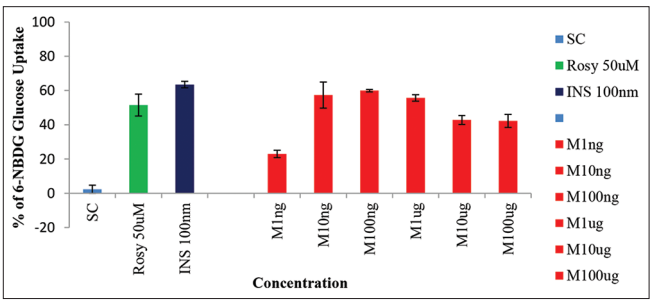

Fig. 4: Graphical representation of glucose conception assay

shown in Fig. 4. The glucose utilization in 3T3 L1 cell lines showed that the methanol leaf extract of *P. corymbosa* was found to be prominent over control. The 3T3 L1 cell lines enhance the glucose uptake by 59.90±0.711 at 100 ng concentration. These results were compared with insulin and rosiglitazone, which were used as the standard antidiabetic drugs. Insulin at a concentration of 100 nm and rosiglitazone at a concentration of 50 uM were found to enhance the glucose uptake over control. The major glucose transporter found in skeletal muscle and adipose tissue is GLUT-4, which is trans-located from an intracellular membrane storage site to the plasma membrane. The glucose uptake rate in 3T3-L1 cells revealed the fact that the extract showed an increase in glucose uptake rate in dose-dependent manner comparable to insulin. The outcome of the present study confirmed that the methanol leaf extract of *P. corymbosa* enhances glucose uptake under *in vitro* conditions. This may be due to the presence of phytoconstituents in the leaves of *P. corymbosa* or due to its effect on the receptors on the cell membrane.

Antiadipogenic Assay

To determine the antiadipogenic activity of the test extract, an *in vitro* adiposity differentiation assay was conducted using 3T3-L1 cell lines. In normal condition, 3T3-L1 pre-adipocyte cells have fibroblastic phenotype. When this cell was treated with differentiation media, they accumulated lipid droplets inside the cell and achieved adipocyte phenotype. 3T3-L1 cells are known to differentiate into adipocytes under the appropriate conditions [49] and have been useful as a model for adipose cells, which are one of the major sites of lipid and glucose metabolism. The methanol leaf extract of *P. corymbosa* caused significant inhibition of adipocyte cells and inhibition was more in differentiated cells as compared to non-differentiated cells (Fig. 5). This finding confirmed the antiadipogenic effect of *P. corymbosa* extract as illustrated in Table 4 and Fig. 6.

Western blot analysis

Methanol leaf extract of *P. corymbosa* activates multiple signaling pathways, but the activated signaling pathways necessary for increased glucose uptake and the antiadipogenic mechanism are not well understood. And hence further analysis carried out through and it

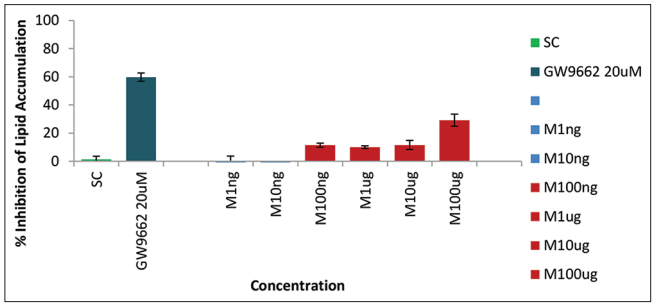

Fig. 5: Graphical representation of antiadipogenic assay

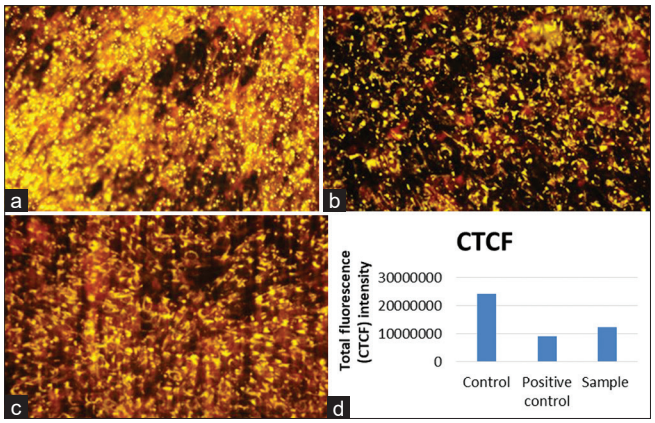

Fig. 6: Fluorescent-stained intracellular lipid droplet images. (a) Control, (b) Positive control, (c) Test Sample, (d) Graphical representation showing the intensity of lipid accumulation

demonstrated that after 24th h (Fig. 7), it was upregulated by mTOR and induced glucose uptake and antiadipogenic activity during adipocyte differentiation through GLUT 4 expression.

Effect of methanol leaf extract of *P. corymbosa* on *in silico* molecular docking

Pharmacophore modeling is generally related to docking studies in which the first step aligns the ligand into a flexible macromolecule and then estimates the tightness of the interaction by assigning scores. Glide is used to perform automated docking with full acyclic ligand flexibility, partial cyclic ligand flexibility, and partial protein flexibility in the neighborhood of the protein active site [50]. The result is analyzed based on the docking score, binding modes, and molecular interactions with 9,12,15-octadecatrienoic acid (Fig. 8). Molecular docking continues to hold great promise in the field of computer-based drug design, which screens small molecules by orienting and scoring them in the binding site of a protein. The *in silico* molecular docking analysis of the selected 9,12,15-octadecatrienoic acid from methanol leaf extract of *P. corymbosa* and the receptors IRS1 (Fig. 9), nuclear factor kappa B (NFκB) (Fig. 10), phosphatidylinositol-3 kinase (Fig. 11), peroxisome (Fig. 13) proliferator-activated receptor gamma (PPAR-γ) (Fig. 12), and protein kinase C which are involved in the regulation of insulin resistance in diabetes has been performed. The inhibiting susceptibility of the compounds was evaluated using their genetic optimization for ligand docking (GOLD) scores generated by the GOLD software. The best docking solutions GOLD score for each compound was considered. The GOLD software resulted in identifying the best compound that interacts with the receptor. The results were evaluated based on the binding compatibility, i.e., docked energy in kcal/mol (fitness).

IRS has been suggested as a molecular target of free fatty acids for insulin resistance [51,52]. IRS-1 plays an important role in adipocyte

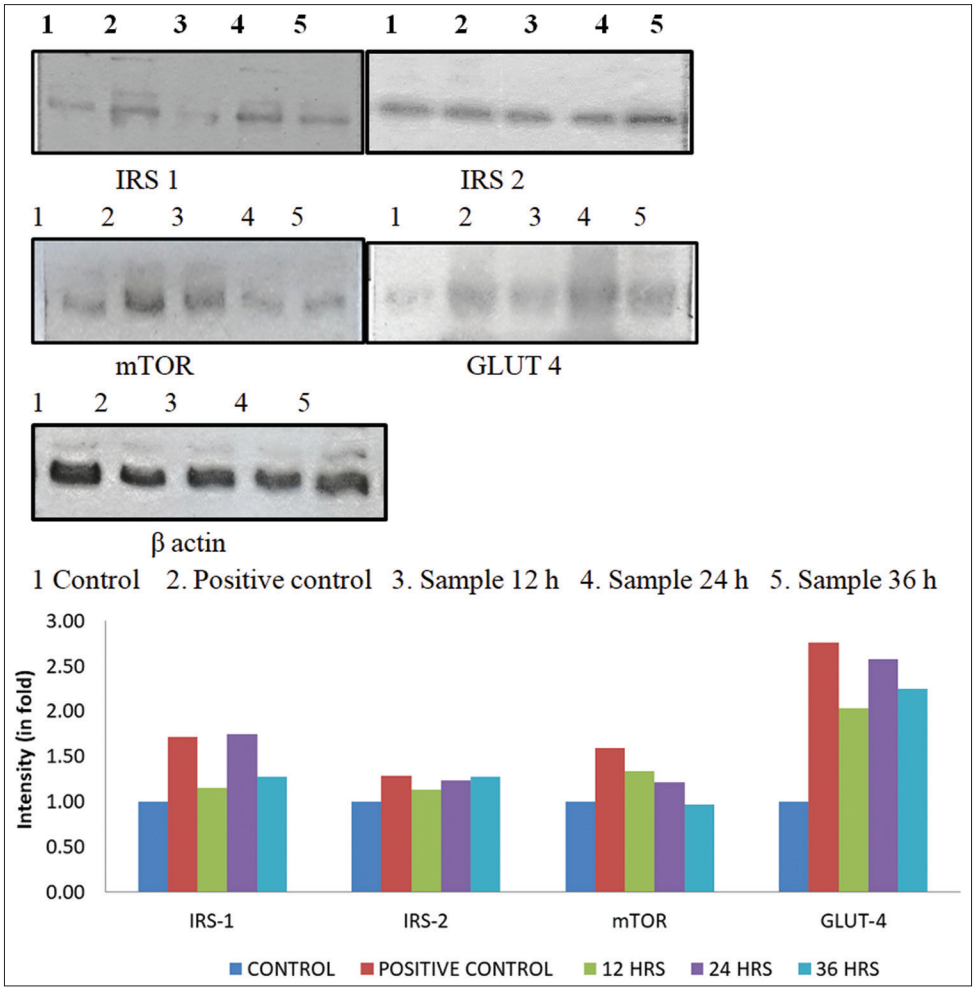

Fig. 7: Western blotting

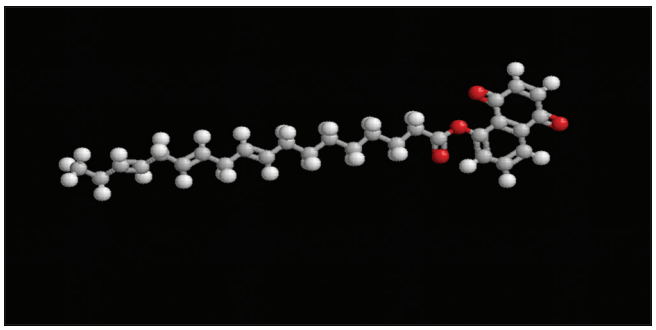

Fig. 8: Three-dimensional structure of 9, 12, 15-octadecatrienoic acid

Fig. 9: Crystal Structure of IRS1

differentiation and the metabolic actions of insulin. PI3 kinase activity increased during adipocyte differentiation and reached a maximum at 8 days after induction, a result consistent with observations in adipocyte cell lines 3T3-L1 [53]. It is consistent with the hypothesis that one of the roles of IRS-1 and IRS-2 in adipocyte differentiation is upregulation of mRNA expression of PPARγ. On the other hand, the fact that PPARγ is unable to completely rescue the defective adipocyte differentiation suggests that IRS-1 and IRS-2 may stimulate adipocyte differentiation through distinct mechanisms in addition to induction of PPARγ. PPARγ and C/EBP family proteins are expressed at specific times during adipogenesis. Several gene-targeting studies have demonstrated the biological significance of PPAR γ and C/EBP family proteins in adipogenic differentiation [54-59]. Fasshauer *et al.* [60] showed that IRS-2, rather than IRS-1, is critical for insulin-stimulated GLUT4 translocation and glucose uptake in adipocytes. In 3T3-L1 cells treated with the adipogenesis induction cocktail, C/EBPb and C/EBPd are expressed in an early phase followed by the induction of C/EBPa and PPAR γ [61,62]. Since PPAR γ is known to be induced through the expression of C/EBPb and C/EBPd [62], our results proved that PPARγ works as a downstream regulator of adipocyte differentiation. A drug molecule is triggered when the binding of a small molecule to the receptor protein is perfectly done. Such protein-ligand interaction is comparable to the lock-and-key principle, in which the lock encodes the protein and the key is ensembled with the ligand. The major

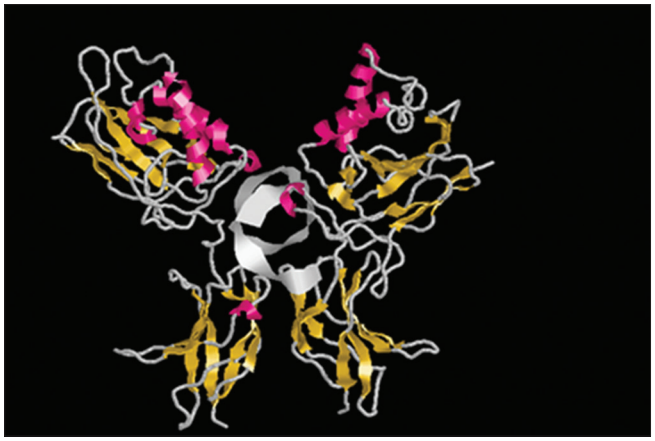

Fig. 10: Crystal Structure of NFκB

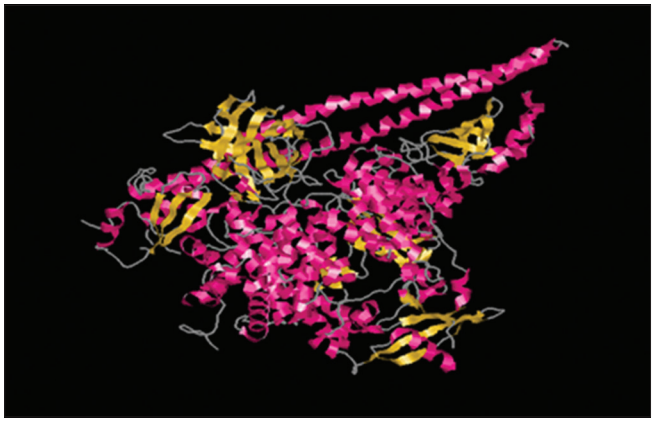

Fig 11: Crystal Structure of PI3 Kinase

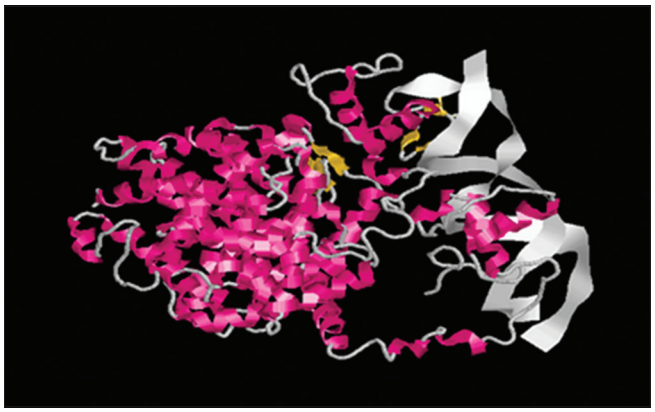

Fig. 12: Crystal Structure of PPAR γ

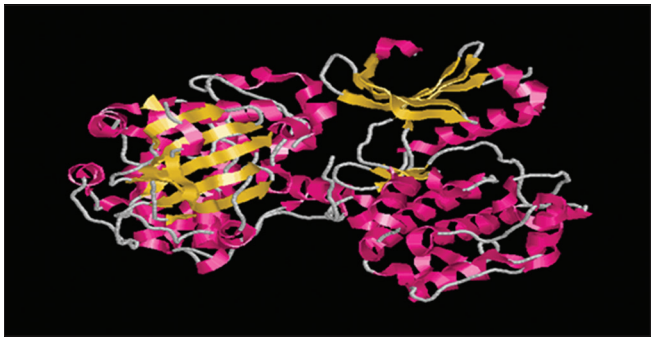

Fig. 13: Crystal Structure of Protein Kinase C

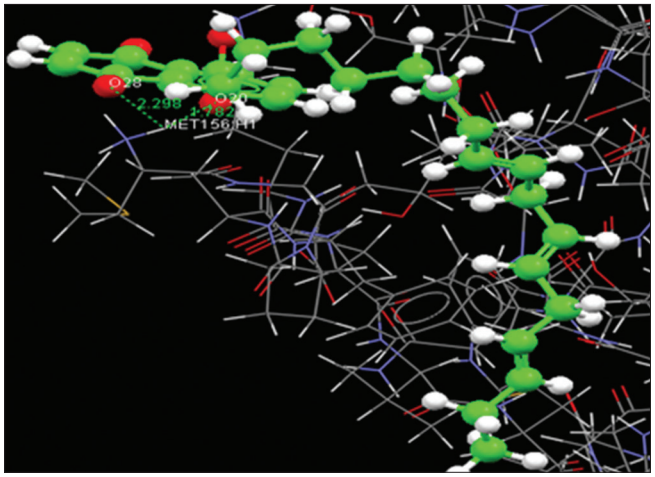

Fig. 14: IRS1 in complex with 9, 12, 15-octadecatrienoic acid

Table 2: Effect of methanol extract of *Premna corymbosa* on MTT assay

Concentration	Cell viability (%)
10 ng	86.92
100 ng	72.46
1 µg	65.35
10 µg	63.91
100 µg	54.16

Table 3: Effect of Methanol extract of *Premna corymbosa* on glucose conception assay in 3T3-L1 cell line

Concentration	% Glucose uptake
Control	2.37
Rosy 50 uM (Std)	51.51±6.41
INS 100 nm (Std)	63.42±1.90
M1 ng	22.99±2.13
M10 ng	57.38±7.59
M100 ng	59.90±0.71
M1 ug	55.70±1.90
M10 ug	42.78±2.61
M100 ug	42.28±3.80

Results are expressed as Mean±SEM; n=6

Table 4: Effect of methanol leaf extract of *Premna corymbosa* on antiadipogenic assay in 3T3-L1 cell line

Concentration	% Inhibition of lipid accumulation
Control	2.17
GW9662 20uM (Std)	59.70±2.95
M1 ng	−5.08±8.80
M10 ng	−4.42±0.16
M100 ng	11.41±1.47
M1 ug	10.01±0.94
M10 ug	11.48±3.21
M100 ug	29.15±4.35

Results are expressed as Mean±SEM; n=6

driving force for binding appears to be hydrophobic interaction whose specificity is however controlled by hydrogen bonding interactions [63]. Hence, phytocompounds from methanol leaf extract of *P. corymbosa* were selected and further investigated for its binding efficiency to evaluate the best-fit molecule using GOLD (genetic optimization of ligand docking). The receptors bound ligand was docked deeply within the binding pocket region forming the interactions. The outcome of our study showed that the active compound 9, 12, 15-octadecatrienoic acid (Fig. 15) binds with the receptor PPAR-γ with the highest GOLD score of 34.89 (Table 5)

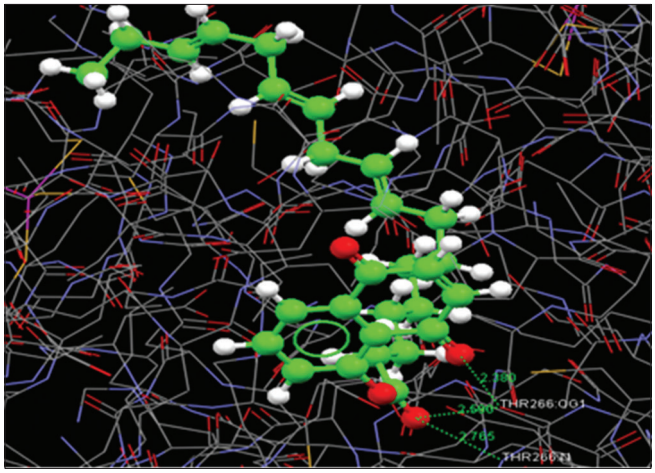

Fig. 15: PPAR γ in complex with 9, 12, 15-octadecatrienoic acid

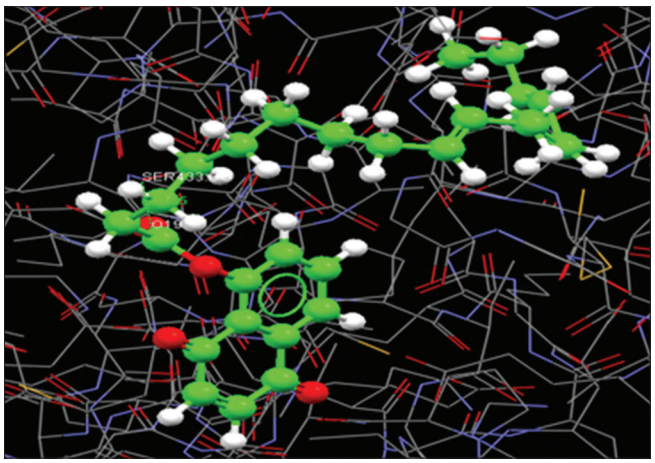

Fig. 16: Protein Kinase C in complex with 9, 12, 15-octadecatrienoic acid

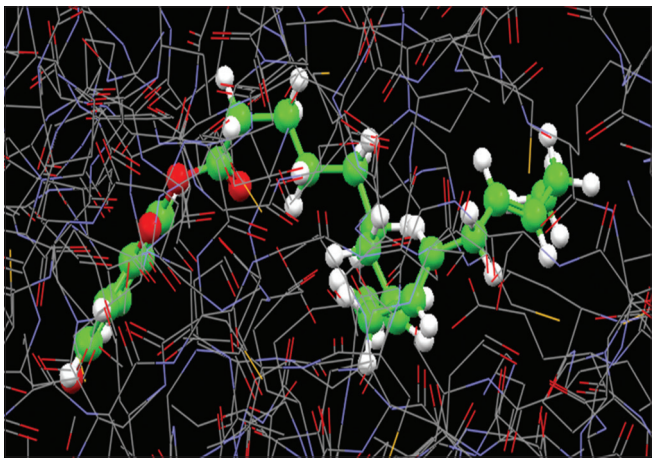

Fig. 17: PI3 KINASE in complex with 9, 12, 15-octadecatrienoic acid

comparatively to the active compound (Fig. 17) and binds with the PI3 kinase receptor with positive GOLD score of 48.66 (Table 5). The active compound 9, 12, 15-octadecatrienoic acid (Fig. 16) was found to bind with protein kinase C with a score of 33.59 (Table 5) and it (Fig. 18) binds with NFκB with a score of 41.03 (Table 5) but it is seen that 9, 12, 15-octadecatrienoic acid (Fig. 14) binds with IRS 1 with a score of 24.05 (Table 5).

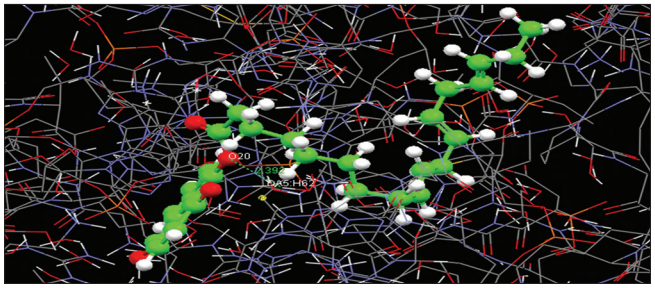

Fig. 18: NFκB in complex with 9, 12, 15-octadecatrienoic acid

Table 5: Docking results of 9, 12, 15-octadecatrienoic acid from the methanol leaf extract of *Premna corymbosa* with receptors IRS1, PPARγ, protein kinase C, PI3 kinsase, and NFκB

Name of protein	Atom in protein	Atom in ligand	H-bond distance	Score
IRS 1	MET156, H1	O20	1.1782	24.05
	MET156, H1	O28	2.298	
PPAR -γ	THR266:OG1	O19	2.59	34.89
	THR266:OG1	O28	2.38	
	THR266:N	O19	2.765	
Protein Kinase C	SER433:OG	O19	2.755	33.59
PI 3 Kinase	NO H BONDS			48.66
NFκB		O20	2.392	41.029

From the analysis of the H-bond formations between the selected active compound (9, 12, 15-octadecatrienoic acid) and the receptors (IRS1, PPAR-γ, protein kinase C, and NFκB), the active compound forms one H-bond with PKC receptor whereas it forms three H-bonds with PPAR-γ and two H-bonds with IRS 1 receptors, respectively (Table 5). The results revealed that the selected herbal lead compound from methanol leaf extract of *P. corymbosa* is an effective target against the receptors. The compound showed favorable interactions with the amino acid residues thereby substantiating its proven efficacy as an anti-diabetic compound. Methanol leaf extract of *P. corymbosa* contains large quantities of 9, 12, 15-octadecatrienoic acid and this compound may induce the expression of IRS1 followed by induction of PPAR **γ** through PKC and NFκB. From the analysis, it is evident that 9, 12, 15-octadecatrienoic acid exhibits a commendable antihyperglycemic property. The results indicate that molecular modeling is a valuable tool for predicting the biological activity of phytochemical constituents. The analysis of the docking result allowed us to know the efficiency and efficacy of the bioactive compound of *P. corymbosa* 9, 12, 15-octadecatrienoic acid to regulate antiadipogenic, antihyperglycemic, and antioxidant activity in diabetes mellitus. The 9, 12, 15-octadecatrienoic acid compound is isolated and found to dock onto the active sites of the various proteins (IRS-1, NFκB, PPARγ, protein kinase, and PI3 kinase) which are known to be involved in the mechanism of antihyperglycemia with antioxidant properties against diabetes mellitus. The 3-D structures of the potent drug targets are retrieved from PDB and their binding sites are determined. The docking interactions between the binding site amino acids of receptor proteins and ligand molecules are presented in Table 5.

Previous work has also shown that a correlation does exist between binding affinity and dock scores [64]. The results of docking determined the optimal orientation of the docked compound exactly to these active sites. Almost 9, 12, 15-octadecatrienoic acid compounds can bind to the amino acid residues in the activation loop of IRS1, PPARγ, protein kinase C, and NFκB but not in the PI3 kinase forming hydrogen bonds. It is suggested that the 9, 12, 15-octadecatrienoic acid compound can form a good complex at the ligand binding domain of the protein and bring about its activation. From the docking simulations, it is observed that this 9, 12, 15-octadecatrienoic acid compound is able to bind to the active sites effectively. From the docking studies, it is proved that 9, 12, 15-octadecatrienoic acid is suggested to upregulate IRS-1 which

depends on increased expression of PPAR-γ and NFκB. Cytosolic kinases like Protein Kinase C have been shown to modulate PPAR-γ and NFκB participate in signal transduction from antioxidant and antihyperglycemic.

CONCLUSION

At present, phytochemical exploration bestows a boulevard for embracing future exploration and improvement tricks for amplifying the offered pharmacological sciences furthermore, cleansing and exploiting time-honored familiarity for the wellbeing of the civilization. The GCMS psychotherapy of the methanol extorts of *P. corymbosa* unveils the attendance of phytochemical composites of the type acids, esters, alcohols, ethers, and so on. As a consequence, the curative vegetation *P. corymbosa* is ascertained to have momentous phytochemicals. The existence of such dumbfound phytochemicals perhaps ascribed to the curative distinctiveness of this plant *P. corymbosa.* The elevations of the crest point toward the relative concentration of the module subsist in the plant extort. Nonetheless, the segregation of individual phytochemical components and subjecting it to natal bustle will certainly bestow prolific upshots. Further investigation is in evolution for the segregation of individual phytochemical components which may act as stencils for new-fangled drug molecules. The outcome from the *in vitro* glucose conception, adipocyte differentiation, and MTT assay in 3T3 L1 adipocyte cell line studies substantiated the potent antihyperglycemic, antiadipogenic activity and also suggested an affirmative sign in the cytotoxic effect of the methanol leaf extract of *P. corymbosa*. The results acquired from western blotting revealed an excellent antihyperglycemic prospective of *P. corymbosa. In silico* molecular docking analysis was performed to learn the binding affinity of the active compounds 9, 12, 15-octadecatrienoic acids. The outcome of the study thereby corroborates the antihyperglycemic efficacy of the 9, 12, 15-octadecatrienoic acids of *P. corymbosa* through good binding scores.

The current study is the first and the foremost of its kind in describing the antihyperglycemic effect of methanol leaf extract of *P. corymbosa* in the management of diabetes in 3T3 L1 adipocyte cells by molecular approach (i.e.) by predicting the binding-conformation of small molecule ligands to the appropriate target binding site.

AUTHORS' CONTRIBUTIONS

Experimental design, guidance, supervision, and review work for the research were done by Dr. R. Senthilkumar. Experimental work, development and interpretation of result, and writing of this manuscript were done by Dr. S. Radhika. Both authors read and approved the final manuscript.

CONFLICTS OF INTERESTS

None.

FUNDING

None.

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