RESEARCH ARTICLE

Glucagon Like Peptide (Glp – 1) Receptor: A Promising Therapeutic Target for Screening of Herbal Antidiabetic Compounds

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Abstract

Background: Diabetes mellitus and its associated complications have become endemic to developing countries and major threat to health economy of developed nations. Although there are a number of allopathic medicines available for its treatment, they present a lot of challenges due to their adverse effects. Hence, a need for a target-specific herbal alternative is required. **Aim:** This study explores glucagon-like peptide-1 (GLP-1) protein as a possible therapeutic target for various antidiabetic compounds. **Methods:** Albino Wistar rats were used as subjects to screen the differentially expressed proteins in type 2 diabetes-induced rats and identify and characterize novel targets for screening of herbal antidiabetic compounds. Widely used and well-established single dose (40 mg/kg) STZ i.p. acute model was used to induce the hyperglycemia. Glucagon-like Peptide-1Receptor was identified in rat's serum. Plant-based anti-hyperglycemic compounds were docked at various phases of GLP-1 receptor to identify the most potent compound having high antidiabetic efficacy. **Results:** Ten different herbal compounds were docked against GLP-1 for antidiabetic efficacy using research collaborator for structural bioinformatics - protein data bank. It was found that Myricetin a flavonoid extracted from the leaves of *Myrica rubra* bonds to the GLP/receptor using least of energy and hence is best of ligand among all available options. **Conclusion:** Myricetin does have the best of antidiabetic potential out of all 10 screened herbal drugs, as depicted in the docking studies. GLP-1 receptor has shown the promising results as a possible target for antidiabetic drug screening.

Key words: Glucagon-like peptide-1, molecular docking, proteomics, type 2 diabetes mellitus

INTRODUCTION

Proteomics has opened the new window in the field of drug targeting. Changes in protein expression due to stimulus or conditioning can be measured in a systematic manner and can be used for elucidating the mechanism of cell function, signaling for disease diagnosis, and receptor identification to target the therapeutics.

Proteomic technology also deals with a wide variety of biomarker discovery and drug development. Altered protein expression due to disease offers the basis for detection of biomarkers and drug targets through analyzing the protein expression profiles with the help of proteins present in body fluids including serum, spinal fluid, and urine. Markers are used widely for screening, diagnosis, staging, prognosis, and detection of recurrent disease.[1] The information gained through new diagnostic techniques today in the field of proteomics can be directly used to develop drug treatment strategies.

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Diabetes mellitus (DM) is a chronic metabolic disorder with towering prevalence throughout the world. Economic burden of this insidious disorder has gradually increased overdeveloped and developing countries. With the estimation of increased diabetic patients, the possible number would be 552 million over 2030 has reported by IDF.[2] Instead of its individual alteration in body functions, DM is the hallmarks of several micro and macrovascular disorders. Several finding suggests that impaired insulin action can significantly interrupt the level of lipid profile, leads to dyslipidemia which becomes a detrimental cause of vascular diseases.[3-6] Increase oxidative stress is another significant risk factor which drives DM-associated micro and macrovascular complications. Indeed, several latest reports reveal that DM significantly increase oxidative stress and vascular diseases.[7-10] The right selection of animal models provides the intense possibility of exploration, characterization of disease pathophysiology and target identification for novel therapeutic agents.[10]

The drug molecule is triggered after the perfectly binding to the receptor protein. This type of protein-ligand interaction is comparable to the lock, and key mechanism, in which the lock encodes the protein and the key is an ensemble with the ligand. The major driving force for binding appears to be hydrophobic interaction whose specificity is, however, controlled by hydrogen bonding interactions.

This paper reports the systematically monitoring of the changes in protein expression with the progression of Type 2 Diabetes in Wistar rat model. It also identifies and characterizes a novel protein Glucagon-like Peptide-1Receptor (GLP-1R) which can be a prospective drug target and evaluates the efficacy of various antidiabetic compounds by molecular docking studies.

MATERIALS AND METHODS

Animal models

Animals

Animal protocol was approved by Institutional Animal Ethical Committee as per the guidelines of CPCSEA. (CPCSEA\AIP\2013\004) New Delhi, India. Adult Wistar albino rats (200–220 g/either sex) were obtained from the departmental animal house and retained at normal laboratory conditions (25 ± 1°C temperature; 45–55% relative humidity and normal day/night cycle) and supply food/water *ad libitum*.

Study design

Animals were distributed in two groups containing six rats in each group. One group received low dose STZ (40 mg/kg/*i.p*/once) as compared to a normal control group where animals received placebo. Animals were allowed to develop DM for 4 weeks.

After 4 weeks, animals were screened for mortality rate, glycated hemoglobin (HbA1c) level, insulin level, blood glucose level (fasting blood glucose and postprandial glucose level), C-peptide level, and lipid profile.

STZ was procured from Sigma-Aldrich Ltd, St Louis, Mo, USA. Diagnostic kits were acquired from commercially available sources. Instead of these all chemicals employed in the current study were of analytical grade.

Induction and assessment of DM

Freshly prepared citrate buffer having pH 4.5 was used as a vehicle for dissolving of STZ (40 mg/kg/i.p/once) and administered to rats for inducing experimental DM. After 72 h of STZ administration serum, glucose level was evaluated by GOD-POD method. Rats having glucose level of more than 180 mg/dl were considered as diabetic. Animals were fasted overnight for measuring fasting glucose level; whereas postprandial blood glucose was measured in animals having pre-treatment of glucose solution (2 g/kg/p.o) before 120 min of analysis.[11] Diabetic rats were allowed to induce diabetes-associated metabolic changes for 4 weeks after the administration of STZ.

Measurement of HbA1c, serum insulin, and C-peptide

Level of HbA1c and serum insulin was measured by commercially available ELISA kit based on the Sandwich-based assay, whereas C-peptide was measured by ELISA kit based on the competition-based assay.

Lipid profile (total cholesterol [TC], triglyceride [TG], high-density lipoprotein [HDL], and low-density lipoprotein [LDL])

Serum lipid profile including TC, HDL, LDL, and TG was measured using the commercially available kit as appraised by Sharma *et al*. 2017.[12]

Beta cell destruction

The structural abnormalities of pancreas were evaluated for beta cells destruction. With the completion of the study protocol, all animals form each category were sacrificed. The isolated pancreas was immediately fixed in 10% formalin solution and embedded in paraffin wax. Sectioned get stained with hematoxylin/eosin and evaluated under an inverted microscope.

Statistical analysis

All values have expressed in mean ± standard deviation (SD). Statistical analysis executes using one-way ANOVA followed by Tukey's multiple comparison *post hoc* test (Sigma Plot

version 11.0, from Systat Software, Inc., San Jose California USA). *P* value having a measurement <0.05 has reflected the statistical significant.

Proteomics studies for drug target identification

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out using bis-acrylamide gel (12% Resolving and 5% staking). 10% APS and TEMED were added for polymerization. Samples were prepared in loading buffer (0.625M Tris + 2% SDS + 10% Glycerol + 5% β-mercaptoethanol + 0.001% bromophenol blue) and heating the mixture at boiling water. The sample was loaded into the wells and run using electrophoresis unit set at 100V using Tris-Glycine buffer (1.44% Glycine + 0.3% Tris-base + 0.1% SDS) for 4 h at 100 volts. The gel was immersed in 5 volumes of staining solution (40% Methanol + 10% Acetic acid + 50% Dist. Water + 0.1%Coomasie blue) for 4 h at room temperature.

After 4–5 h, the gel was placed in destaining solution (40% methanol+10% acetic acid +50% distilled water) after staining to visualize the protein bands.

Preparation of albumin free sample

Column equilibrations

Transfer 0.5 ml of the suspended medium slurry to a spin column centrifuge the spin column in the collection tube at 3,000 rpm for 5–10 s to remove the storage solution add 0.3 ml equilibration buffer to the medium in the spin column and centrifuge at 3,000 rpm for 5–10 s. Repeat the step 3 twice using the same collection tubes.

Albumin depletion

Add the serum sample (50–100 µl) to the top of the packed column bed and incubate at room temperature for 10 min. Centrifuge the spin column and collection tube at 3,000 rpm for 20 s. Reapply the eluate in the collection tube to the top of the medium bed. Incubate for 5 min centrifuge the spin column in the same collection tube as before for 20 s. Wash the remaining unbounded proteins from the spin column by adding 200 µl of equilibration buffer to the top of the medium bed, centrifuge for 20 s. The albumin depleted sample was stored at −20°C for long-term storage.

2D Electrophoresis

After separation, identification of proteins in samples has been performed by displacement in two dimensions oriented at right angles to one another.

First, the proteins were separated on the basis of their isoelectric points. This allows the proteins to resolve independently on the basis of IEF and molecular weights over a larger area, increasing the resolution of each component. This is followed by separation on the basis of molecular weight. The specific sport of interest was eluted and trypsinized, and then matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) analysis has been performed to determine the mass of peptides. Before IEF focusing the protein sample was diluted in rehydration buffer (8 M Urea+ 4% CHAPS + 40mM Tris+0.2% Biolyte3/10) (Ampholyte freshly added just before rehydration)+ tributylphosphine (TBP) added to the final concentration 1X (200 mM stock to 2 mM) such that 125ul will have 50–100 mg protein for 7 cm IPG Strip. The sample was loaded the suitable volume. The IPG strips were placed side down slowly from one end to avoid air bauble then the IPG stripes were overlayed with 1.5 ml of mineral oil and left in the rehydration tray overnight at RT. To dehydrate the IPG strips remove the cover from the rehydration tray and blot the tip of the strip on a piece of filter paper to drain mineral oil. Place the focusing tray into the PROTEAN IEF cell and place the equilibration buffer-1(6 M Urea+ 0.375 M Tris [pH 8.8] + 2% SDS + 20% Glycerol + 2% DTT) for 10 min and shake. Change the equilibration buffer-2 (6 M Urea + 0.375 M Tris [pH 8.8] + 2% SDS + 20% Glycerol+ 2.5% Iodoacetamide [w/v]) for 10 min and continue shaking. Perform vertical SDS-PAGE at 150 volts. Stain the gel and destain and observe the protein profile.

MALDI-TOF- mass spectrometry (MS)

Before tryptic digestion, samples were reduced and alkylated, and the proteins were denatured in urea+ Bis-tris propane, pH 8.5 reduced with TBP. Alkylation was performed with 15 mM iodoacetamide. To prepare samples for enzymatic digestion, 50 mM ammonium bicarbonate was added as diluents to obtain a final concentration of 1 M urea. Aliquots of approximately 100 µg proteins were digested with 2 µg trypsin pre-dissolved in 1 mM HCl, for 12–18 h at 37°C. Guanidination was performed following the instructions provided with the ProteoMass Guanidination Kit. Samples, both before or after guanidination, were combined with an equal volume of cyano-4-hydroxycinnamic acid solution and spotted onto a MALDI target. MALDI-MS data were acquired using a Kratos Axima-CFR+ mass spectrometer in positive ion reflectron mode.

Molecular docking studies

Ligand and protein receptor preparation

Ten herbal compounds exhibited antidiabetic properties were curated from the literature. Maestro workspace was used to sketch the 2D structure of the compounds. LigPrep2.8 module was used for generating low energy conformations of ligands16. Similarly, the protein receptor was retrieved from the Protein Data Bank directly in the Protein Preparation Wizard of Maestro9.6 Environment (ref). Molecular weight, octanol/water partition coefficient, brain/blood partition

coefficient, Caco-2 cell permeability, HERG K + channel's IC50 values, Human oral absorption, drug-likeliness (Lipinski's rule of five), and TPSA like properties were calculated. iGEMDOCK9 software was used to perform docking studies with our identified protein GLP-1R receptor. Ligands were docked flexibly, and conformation generation was limited to variation around asymmetric torsion bonds. Only low energy conformations were kept. The best ligand was chosen according to their Docking Score and Glide Score which are calculated using the standard formula followed by iGEMDOCK9.

RESULTS

Evaluation of mortality, serum glucose level

All these parameters were analyzed after completion of experimental protocol (4 weeks). Indeed, there was no mortality found in any group up to 4-week duration [Table 1]. Moreover, measurable multiplication ($P < 0.05$) in serum glucose level was reported in STZ treated as compared to normal control group. The increase in fasting and postprandial glucose level was almost double at 4th week in diabetic control animals as compared to normal control which clearly confirmed the induction of DM [Figure 1a and 1b, respectively].

Assessment of HbA1c

HbA1c test is the routine test for diabetic people. The increased level of HbA1c level indicates the presence of diabetes. The severity of hyperglycemia has been confirmed by significant ($p< 0.05$) increased level of HbA1c in diabetic control animals as compared to normal control [Figure 1c]. The total increase in HbA1c in diabetic control animals was almost double to normal control.

Assessment of C-peptide and insulin

The C-peptide and insulin are released simultaneously by the splitting of proinsulin in equivalent amounts. Indeed, the half-life of insulin is comparatively small as compared to C-peptide, and thus the C-peptide level gives more precise information of total release of insulin from the pancreas. A significant reduction ($P < 0.05$) in C-peptide and insulin level was observed in diabetic animals as compared to normal control animal [Figure 1d and 1e, respectively] that indicates the destruction of the pancreas or limited supply of both hormones.

Assessment of lipid profile

The state of dyslipidemia has represented by increased level of TC, LDL, and TG whereas decreased level of HDL. The similar observation has found in present investigation where at the end of protocol significant ($P < 0.05$) increase in TC, LDL, TG, and reduced level of HDL was measured in all animal of the diabetic group as compared to normal control group as shown in Table 1. These observations confirm the association of dyslipidemia with a diabetic condition.

Histological assessment of pancreas

Animal of normal control group has shown the normal architecture of islets of Langerhans in histological evaluation which was surrounded by acinar cells of the exocrine portion of the pancreas. Moreover, the pancreatic structure was divided by intact intralobular and interlobular connective tissue septa.[13,14] Whereas, in diabetic groups, the pancreas cells (acinar cells) were swelled and small vacuoles were present in the exocrine part. Whereas, the presence of islets of Langerhans was entirely lost or shrunk in the diabetic animal as compared to normal control [Figure 2a and b].

Proteomics studies for target identification

1D and 2D electrophoresis

1D and 2D electrophoresis were done for serum samples of diabetic as well as control rats for the identification of receptors for drug targeting. We have observed many differentially expressed proteins in 1D protein profiling [Figure 3a]. Differential upregulation and downregulation were observed in the proteins of control as well as STZ-treated animals [Figure 3b]. We have taken few proteins to be validated for prospective drug target.

Table 1: Evaluation of mortality rate and various lipid parameters including total cholesterol TC, LDL, HDL, and triglyceride level in diabetic animals as compared to normal control group

Sr. No.	Assessed parameters	Normal control	Diabetes control
1	Mortality rate (%)	0	0
2	Total cholesterol (mg/dl)	57.87±9.8	198.56±9.82*
3	TG (mg/dl)	64.82±2.64	252.51±4.96*
4	HDL (mg/dl)	33.62±2.67	28.91±3.26*
5	LDL (mg/dl)	14.3±1.81	43.23±3.19*

All data are expressed as Mean±SD, value of significance has represented by *P<0.05 as compared with normal. SD: Standard deviation, TC: Total cholesterol, LDL: Low-density lipoprotein, HDL: High-density lipoprotein AD

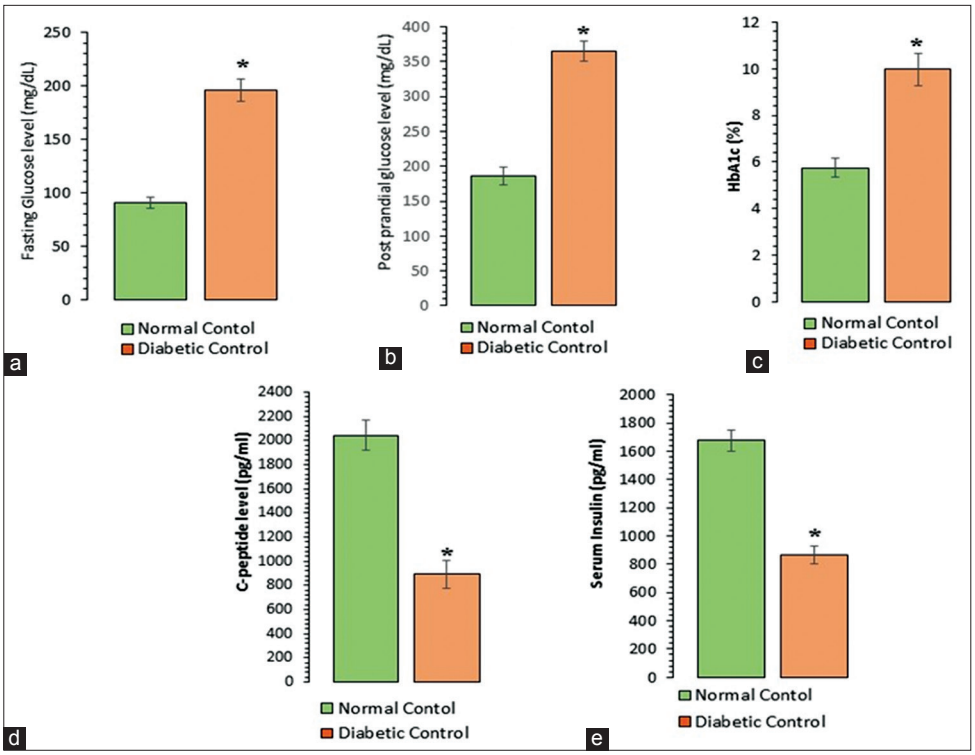

Figure 1: Evaluation of biochemical estimation of glucose level (fasting glucose and postprandial glucose level), HbA1c, C-peptide, and serum insulin for different diabetic animals as compared to normal control group. All data are expressed as Mean ± SD, the value of significance has represented by **P*<0.05 as compared with normal.

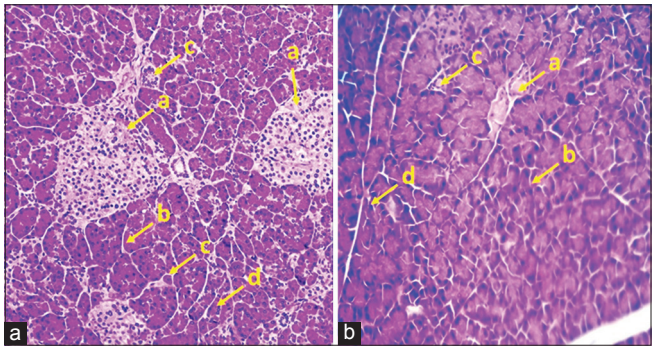

Figure 2: Represents the structural changes in histological observation of rat pancreatic section of normal control and diabetic groups. Normal control [Figure 2a] animal tissue showed normal architecture of islets of Langerhans "a" packed in acinar cells "b." Pancreatic lobules are seen in exocrine components "c" and interlobular connective tissue septa "d" is observed. Whereas, the pancreatic tissue of diabetic animals [Figure 2b] has shown significant damage or entirely loss of islets of Langerhans "a," swelled acinar cells "b," and flattened interlobular ducts "c.'

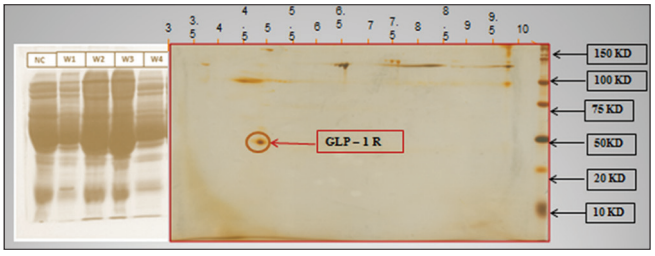

Figure 3: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis Analysis. (a) 1Delectrophoresis showing differentially expressed proteins NC: Normal Control, W1: After 1 week of STZ induction, W2: After 2 weeks of STZ induction, W3: After 3 weeks of STZ induction, and W4: After 4 weeks of STZ induction. (b) 2Delectrophoresis with PD quest analysis showing differentially expressed protein spots.

MALDI-TOF-MS/MS

One of the differentially expressed proteins (GLP-1R) was further characterized by MALDI-TOF-MS/MS. Data were analyzed using Mascot search engine. NCBIprot database was used to search protein sequences and confirmation the genus (Rattus) [Figure 4a and b]. Swiss Prot database was used to search protein sequences and confirmation the genus (Rattus) [Figure 4 a and b]. We have also extracted 3D structure of GLP-1R from PDB database. Peptide sequence was searched in Mascot search engine, and GLP-1R was found.

(Molecular Mass- 52,877Da) [Figure 4a and b]. Database: Swiss Prot 2018_05 (557491 sequences; 199978344 residues); Taxonomy: Rattus (8039 sequences); Timestamp: June 22, 2018 at 06:05:48 GMT; Top score: 93 for GLP1R_RAT, Glucagon-like peptide-1receptor OS-Rattus norvegicus; OX= 10116, GN=Glp 1r, PE=2, SV=1, Protein sequence coverage: 23%; and Protein scoring >52 are significant ($P < 0.05$).

Docking studies

Among 10 ligands, Myricetin showed highest binding energy (−119.933). It was observed that DB02375 (Myricetin) has the best efficacy for the GLP1-R [Table 2]. 3D structure of GLP1-R and best-docked image of GLP1-R with Myricetin has been showed in Figure 5a and b.

DISCUSSION

Type 2 DM has become a popular lifestyle disorder with its associated complications and multi-organ effects. The treatment cost of it's associated macrovascular and microvascular complications are a lot more than the treatment of diabetes alone. Furthermore, the risk of cardiovascular diseases increases at a higher rate. Our studies in animal model development using STZ are in accordance with the earlier report.[10]

The burgeoning field of proteomics plays a powerful and relevant role in the discovery of biomarkers and therapeutic targets, which are biometric measurements that convey information about the biological condition of the diseased subjects and can be compared with the control subjects. Biomarkers have changed the manner in which we diagnose disease, monitor the effect of therapy, classify disease, detect toxicity, and develop new drugs.

In the present study, protein profiles were compared between control and T2DM serum using a proteomic approach. 2D profile of the samples was performed, and one novel protein GLP-1R of molecular weight 52.891KDa was identified and characterized by MALDI-TOF-MS. Takahashi *et al*., in 2017,[15,16] reported proteomic analysis in serum for prediabetes in an animal model; however, the present study reports the identification of GLP-1R in type 2 diabetes model which can be used as the drug target.

Even after the availability of a number of synthetic drugs, the requirement of herbal compounds still holds a promising value due to least side effects. GLP-1R has been identified as a potential target for diabetes treatment.[16] It regulates the blood glucose concentrations by stimulating insulin secretion, reducing gastric emptying rate and increasing the feeling of fullness. Direct administration of insulin leads to severe hypoglycemia in the majority of cases. Hence, administration of GLP-1R is a wise choice of treatment line. Despite such favorable physiological and pharmacological properties, GLP-1R cannot be used as a regular treatment due to its pharmacokinetic properties. A lot of efforts have been made to increase its lifetime (3–5 min). Another approach is to identify its analogs working in the same direction

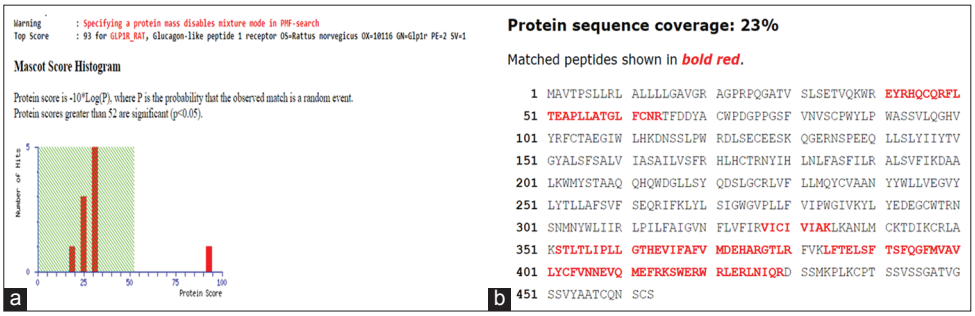

Figure 4: (a and b) Glucagon-like Peptide-1Receptor characterized by matrix-assisted laser desorption ionization time-of-flight

Table 2: Binding energies of ligands bind to GLP1-R

Ligand	Compound	Total Energy	VDW	HBond	Elec	AverConPair
DB01698.pdb	Rutin	−82.4977	−70.5027	−11.995	0	21.32
DB01852-0.pdb	Kaemferol	−113.412	−97.2139	−22.1981	0	8.35417
DB02375-1.pdb	Myricetin	−119.933	−73.2174	−40.7157	0	29.7917
DB02709-0.pdb	Resveratrol	118.128	125.44	−7.31193	0	6.92982
DB04216-1.pdb	Quercetin	−101.807	−81.7449	−20.0624	0	13.1087
DB08995-0.pdb	Diosmin	−116.1	−94.7139	−21.386	0	12.9833
DB11263-0.pdb	Polydatin	−84.5193	−68.0421	−16.4772	0	16.3571
DB11672-1.pdb	Curcumin	−101.38	−94.3798	−7	0	28.8
DB14086-0.pdb	Cianidanol	−102.697	−83.8	−18.8967	0	28.3636
DB00136-1.pdb	Calcitriol	−97.7505	−85.3715	−12.379	0	26.6

GLP1-R: Glucagon-like Peptide-1Receptor

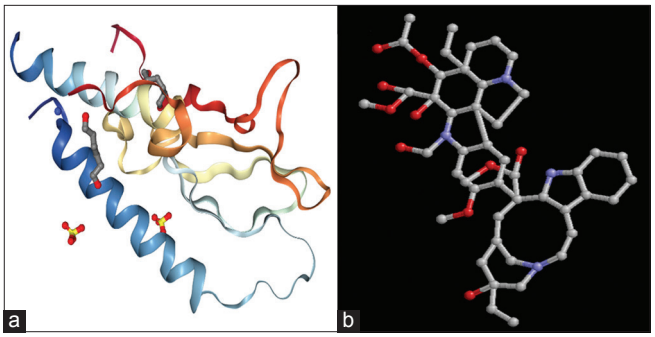

Figure: 5 (a). 3D structure of Glucagon-like Peptide-1 Receptor (GLP1-R) curated from www.rcsb.org (b) best-docked pose of GLP1-R with Myricetin

In this study, 10 different types of herbal compounds have been elucidated through molecular docking mechanisms for best fit as an antidiabetic agent. Research collaborator for structural bioinformatics-protein DataBank has been used to perform the docking studies. Myricetin which is obtained from the leaves of *Myrica rubra* was found to be the best docked among all available herbal compounds. It makes the best ligand with GLP-1 receptor with the lowest energy requirement for binding and giving the best of GlideScore. Hence, Myricetin has the potential of becoming GLP-1R analog, with better pharmacokinetic profile and improved antidiabetic efficacy.

CONCLUSIONS

GLP-1R was identified and validated in the diabetic condition in an acute model of T2DM this can be a potential target for diabetes. GLP-1R has been used to screen the herbal antidiabetic compounds by docking studies, and myricetin has been observed to be the most efficacious drug. These finding presents a therapeutic target as well as a herbal compound which can be used for treating diabetes.

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