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Cloning and molecular modeling of free fatty acid receptor GPCR 43 with dietary flavonoids as novel ligands

*Arooma Ihtsham[1], Rida Hayat[1], Fariha Khan[1,] **

[1]Dept. of Biosciences, Functional Genomics Laboratory,
COMSATS University Islamabad,
45550 Islamabad, Pakistan

**Corresponding author: farihakhan2031@gmail.com*

Abstract

G-protein couple receptors (GPCRs) are considered as the largest membrane protein family involved in the regulation of body homeostasis in health and disease. GPCR43 or FFA2 (free fatty acid receptor 2) is implicated in diabetes. Efficient methods are needed to express GPCRs for structural studies. Small GPCR fragments consisting of 1-2 transmembrane domains are routinely used in NMR studies. In the present study, the first three transmembrane segments 1-3 of GPCR43 (GPCR43-TM1-3) were cloned and expressed with expression enhancement tag, AT4 and His tag at the C and N termini respectively into pET23b(+). The plant compounds, flavonoids, with reported beneficial effects in diabetes mellitus type 2 (T2DM) were subjected to docking against the target, GPCR43. Our results revealed that the ligands exhibited better binding interaction to GPCR43. Diosmin was predicted to be the best ligand with good binding affinity than the other ligands. Hence, we concluded that Diosmin may become a potential drug candidate for T2DM via GPCR43 pathway. However, studies are warranted to confirm its efficacy in animal models of T2DM.

Keywords: Cloning; Diabetes type 2; Diosmin; Flavonoids; G-protein coupled receptors (GPCRs).

1. Introduction

G-protein coupled receptors (GPCRs) generally regarded as seven transmembrane (7TM) receptors, is the largest family of the cell surface receptors with more than eight hundred GPCRs identified in the human genome. GPCRs based on sequence homology and functional similarities have been categorized into A-F classes (Fredriksson *et al.,* 2003). Class A rhodopsin like receptors is the largest family with about 80 % GPCRs belonging to this class, contains many hormones, neurotransmitters and light receptors representing interesting targets for drug discovery (Hu *et al.,* 2017). GPCR43 or FFAR2, a member of class A GPCR, is highly expressed in the adipose tissue, gastrointestinal tract (GI), and immune cells (Kimura *et al.,* 2014). GPCR43 reportedly functions in regulation of glucose metabolism by acting at the beta cells and adipose tissues as well as mitigates inflammation (Villa *et al.,* 2017; Xu *et al.,* 2019). Several reports show that the activation of GPCR43 by short chain fatty acids regulates insulin secretion besides protective effects on β

cells in response to insulin resistance highlighting the significance of GPCR43 in T2DM and obesity (Tolhurst *et al.,* 2012; Forbes *et al.,* 2015; Priyadarshini *et al.,* 2015; McNelis *et al.,* 2015; Tang *et al.,* 2015). For instance, Gαq/11-biased agonists of GPCR43 with 100-fold increased potency were identified causing an increase in glucose stimulated insulin secretion (GSIS) and growth of beta cells (Villa *et al.,* 2017). Recently, exercise induced improvement in T2DM was hindered by inhibiting GPCR43 in mice models (Yang *et al.,* 2020).

Diabetes mellitus is considered among the oldest diseases of humankind. A chronic condition with estimated 415 million people worldwide and the number of patients is increasing steadily. More than 90% of diabetics have T2DM which results in microvascular and macrovascular complications resulting in physical deficiencies as well as financial losses with an enormous burden on health systems (Olokoba *et al.,* 2012; Chatterjee *et al.*, 2017). No treatments have been found yet. The disease is managed by adopting healthy lifestyle, exercise and medications including antihyperglycaemic, antihypertensive agents, cholesterol lowering and antiplatelet drugs (Stumvoll *et al.,* 2005).

Plants contain a tremendous wealth of bioactive compounds with healthcare benefits including terpenes, flavonoids, alkaloids and sterols (Kris-Etherton *et al.,* 2002). Many flavonoids such as disosmin, rutin, catechin and EGCG have been reported for therapeutic effects in diabetes (Nagao *et al.,* 2009; Gothai *et al.,* 2016; Eng *et al.,* 2018; Gosslau *et al.,* 2019; Unuofin & Lebelo*,* 2020).

It is estimated that almost one third of the drugs act via GPCRs. Structure elucidation is a key area for the drug designing which requires cloning and expression studies. The current work is focused on GPCR43 as a T2DM target. First three transmembrane segments of GPCR43 (GPCR43-TM1-3) were cloned and expressed in *E. coli.* GPCR43 was modeled and docking studies were carried out by using plant-based compounds with therapeutic effects on T2DM.

2. Materials and Methods

2.1 Plasmid construction and cloning

The expression vector pET23(b+) containing strong T7 promoter with IPTG inducible expression system was used to design expression construct. The bacterial codon-optimized gene for full-length GPCR43 was synthesized and cloned into pET-23b(+) vector (Gene Universal, USA) with N-terminal expression enhancement AT-rich gene tag, AT4 (AAATATTATAAA) (Pandey *et al.,* 2014). The plasmid construct hence obtained had an N-terminal AT4 tag and a hexahistidine-tag (6xHis-tag) at the C-terminus. First three transmembrane segments of GPCR43 (GPCR43-TM1-3) were expressed by subcloning the cDNA encoding transmembrane segments (315bp) into pET-23b (+) using NdeI and XhoI restriction sites. Forward (ATACTACATATGAAATATTATAAAATGCTGCCTGACTGGAAGTCC) and reverse primers (ACTAGCCTCGAGTTAATGGTGATGGTGATGGTGAATGCTAATGCCGGCCAGCAG) were designed to amplify GPCR43-TM1-3.

2.2 Protein expression

E coli strains have been reported to produce high protein yield. Recombinant proteins were expressed using IPTG inducer. Briefly, a few colonies were inoculated in 5 mL LB media with chloramphenicol (34 µg/mL) and ampicillin (100 µg/mL) antibiotics. The cultures were incubated overnight at 37℃. The next day cultures were shifted to 15 mL media with antibiotics and incubated for almost 3-4 hours until the optical density (OD_{600}) reached 0.5-0.7. Cultures were induced with IPTG and 1 mL culture was taken as un-induced (centrifuged at 10,000 rpm for 2 minutes). After induction, the cells were grown for 3 h at 37 °C and harvested by centrifugation (10000 rpm at 4 °C for 10 minutes). Protein expression was observed on 12 % SDS-PAGE (Schägger, 2006). Western blotting was performed by transferring the gel onto PVDF membrane and blot was detected by using anti-His antibody (Invitrogen).

2.3 Structure prediction and docking

The full-length sequence of GPCR43 (Uniprot ID: O15552) protein was used as query in I-TASSER to predict three-dimensional structure model. Images of the model were generated using Chimera. The predicted models were refined by Galaxy and ModRefiner and further validated by SAVES server which is composed of several online servers such as Procheck, ERRAT, Verify3D, whatcheck and Ramachandran plot. The binding pockets were identified by CASTp.
Four active compounds of plant origin, catechin, diosmin, rutin and EGCG, reportedly involved in T2DM were selected. Molecular docking was performed by PyRx, a software for computational drug discovery. In AutoDock4, 50 conformations were analyzed for GPCR43 and Ligands interactions and best conformation was selected on the basis of binding energy, number of hydrogen bonds and maximum number of residues. Further, the best selected conformations were studied through LigPlus.

3. Results

3.1 Cloning and expression of GPCR43-TM1-3 in *E.coli*

GPCR43-TM1-3 was successfully cloned with small tags, N-terminal AT4 tag and C-terminal His tag into pET23b(+) (Figure. 1a-d). The cloned insert was confirmed by PCR amplification with gene specific primers by using the recombinant plasmid (pET23b(+)-GPCR43-TM1-3) as template (Figure 1d). Expression of recombinant GPCR43-TM1-3 (12kDa) was induced with 1mM IPTG in *E. coli* BL21 (DE3) which was confirmed by western blotting (Figure 2).

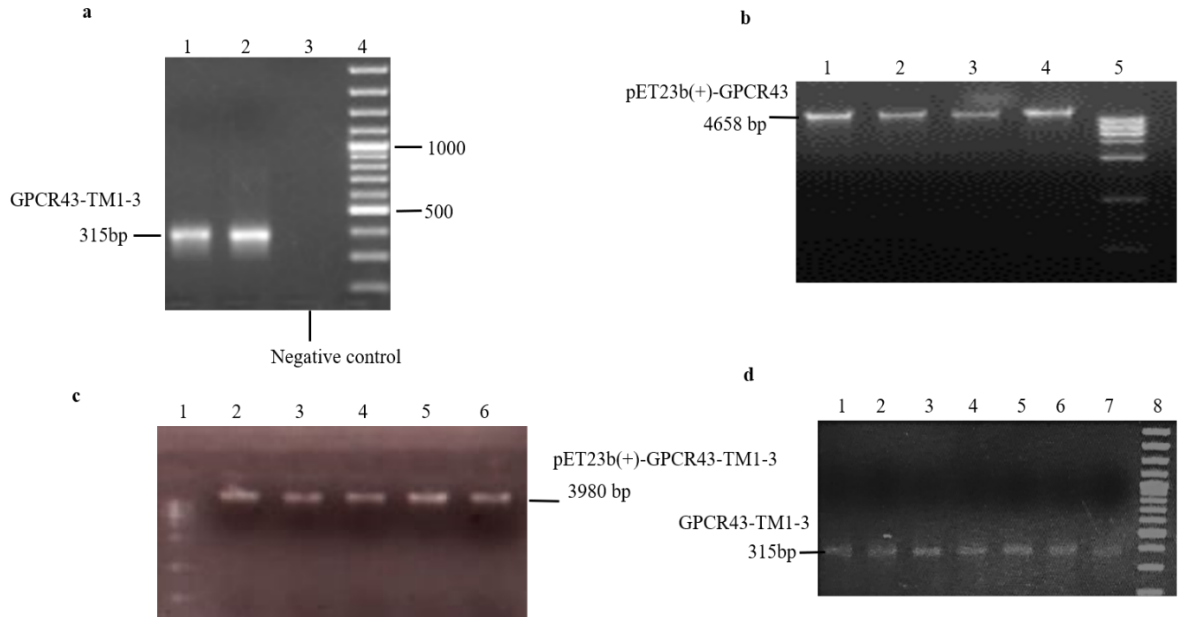

Fig. 1. cDNA Cloning of the first three transmembrane segments of GPCR 43 (GPCR43-TM1-3) in pET23b(+). (a) PCR amplification of cDNA encoding GPCR43-TM1-3. Lane 1-2, PCR products showing 315bp of GPCR43-TM1-3, Lane3, Negative control, Lane 4, 100bp DNA marker. (b) Transformation of *E. coli* with the designed vector construct, pET23b(+)-GPCR43 which contains full length cDNA of GPCR43 and plasmid isolation. Lane 1-4, pET23b(+)-GPCR43, Lane 5, 1 Kb DNA marker. (c) Subcloning the cDNA encoding transmembrane segments (315 bp) into pET-23b (+) using NdeI and XhoI pET23b(+)-GPCR43 yielded 3980bp of recombinant pET23b(+)-GPCR43-TM1-3. Lane 1, DNA marker, Lane 2-6, Extracted pET23b(+)-GPCR43-TM1-3. (d) Confirmation of the insert by using pET23b(+)-GPCR43-TM1-3 as DNA template in PCR. Lane 1-7, PCR amplification of the insert of 315bp, Lane 8, DNA marker.

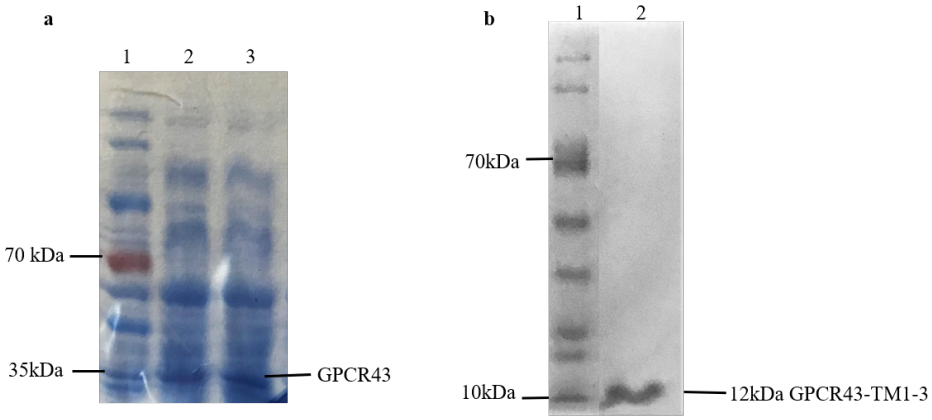

Fig. 2. Protein expression of recombinant GPCR43 in *E. coli* BL21(DE3) pLysS strain. (a). Lane 1, Pre-stained protein marker, Lane 2, uninduced sample, Lane 3, Induced protein sample. (b) Expression of GPCR43-TM1-3 by western blotting. Labe 1, Pre-stained protein marker, Lane 2, 12kDa fragment corresponds to GPCR43-TM1-3.

3.2 Molecular Docking Analysis

The best GPCR43 model was selected on the basis of C score and RMSD value. Molecular docking was carried out to understand the molecular interactions between the active sites of protein target and the natural products (catechin, diosmin, rutin and EGCG) (Table 1, Figure 3-Figure 6).

Table 1. Docking studies of GPCR43 with plant-based ligands scores.

Ligand	Binding Scores	Active Residues
Catechin	-7.3	Pro3, Lys6, Ser7, Ile10, Lys65, Glu68, Ala69, Ala70, Ser71, Asn72, Phe73, Pro252, Arg255, Ser256, Val259
Diosmin	-9.5	Pro3, Asp4, Ser7, Lys65, Ala69, Asn72, Phe73, Thr153, Glu154
Rutin	-8.4	Pro3, Ala69, Asn72, Phe73, Pro252, Ser256
EGCG	-7.5	Lys6, Ser7, Ala69, Asn72, Pro252, Arg255

CASTp calculated the active site, pocket area, and binding pocket volume of the protein. The residues which occupy the active site for GPCR43 are PRO3, LYS6, SER7, ILE10, TYR14, LYS65, LEU62, ALA69, ALA70, ASN72, TRP75, PHE73, CYS82, THR85, SER86, PHE89, TYR90, PRO252 and ARG255. (Figure 3). The docking pose was ranked by energy, the more negative the value, the better the interactions. The best interaction was nominated based on binding affinity values (Table 1). Analysis with Lig+ indicated the hydrogen bonds and hydrophobic interactions of both ligand and receptor, binding energy and binding residues (Figure 3b, 4b, 5b, 6b).

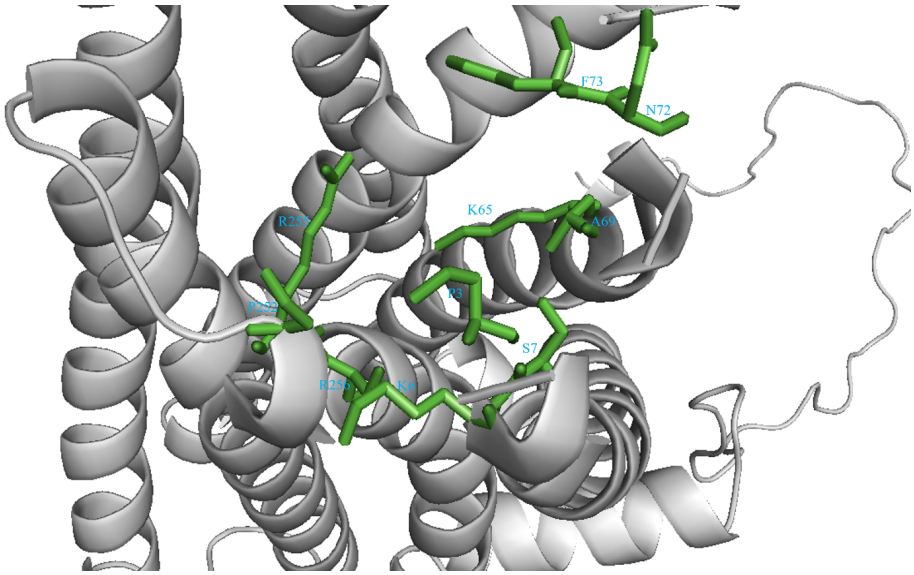

Fig. 3. Binding pocket of GPCR43**.** The amino acids involved in the binding pocket were predicted by CASTp. The side chains of the important residues are highlighted.

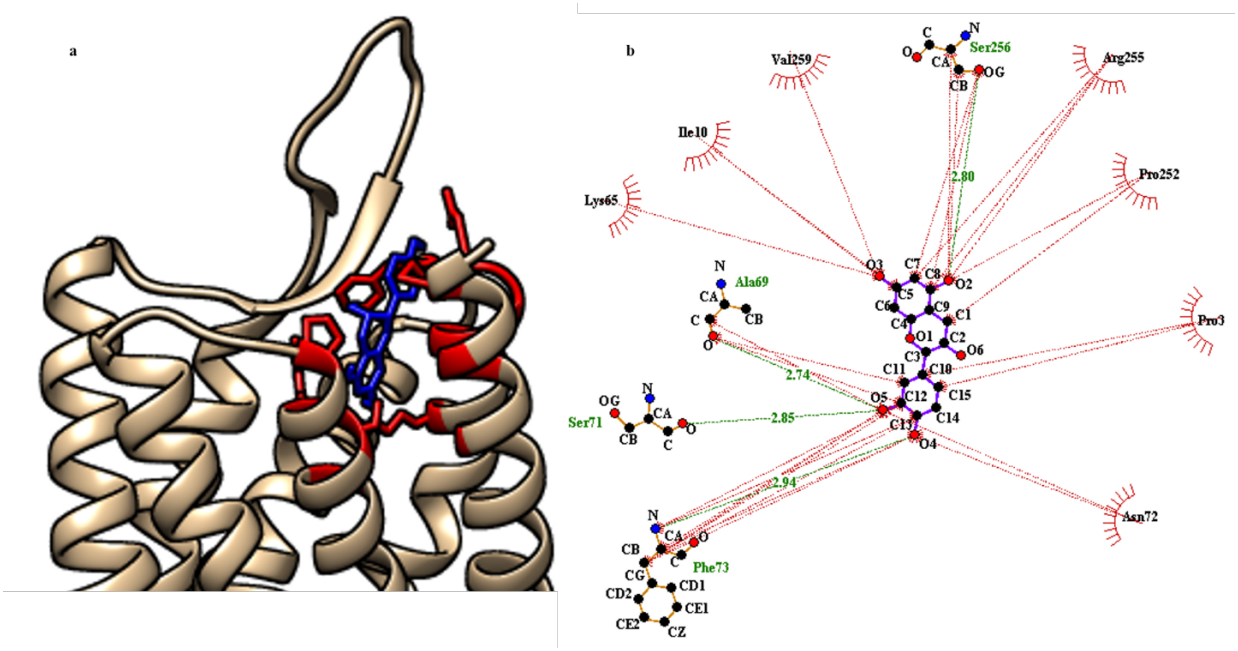

Fig. 4. Docking of GPCR43 with catechin. (a) GPCR43 with catechin docked in the active site. (b) Interactions between GPCR43 and catechin, the residues in the binding sites are highlighted. Visualization of interactions between receptor (brown edges) with ligand (purple edges) in LigPlot along with hydrophobic interactions (red lashes) and hydrogen bond (green).

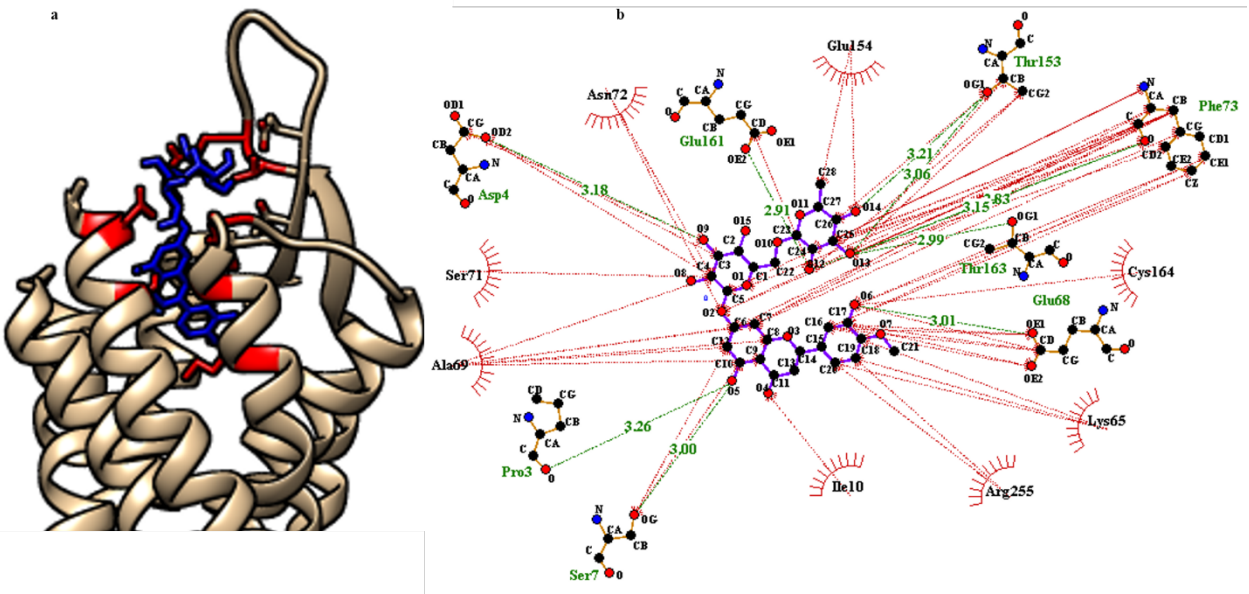

Fig. 5. Docking of GPCR43 with diosmin. (a) A docking pose of GPCR43 with diosmin. (b) Interactions between GPCR43 and diosmin, the residues in the binding sites are highlighted.

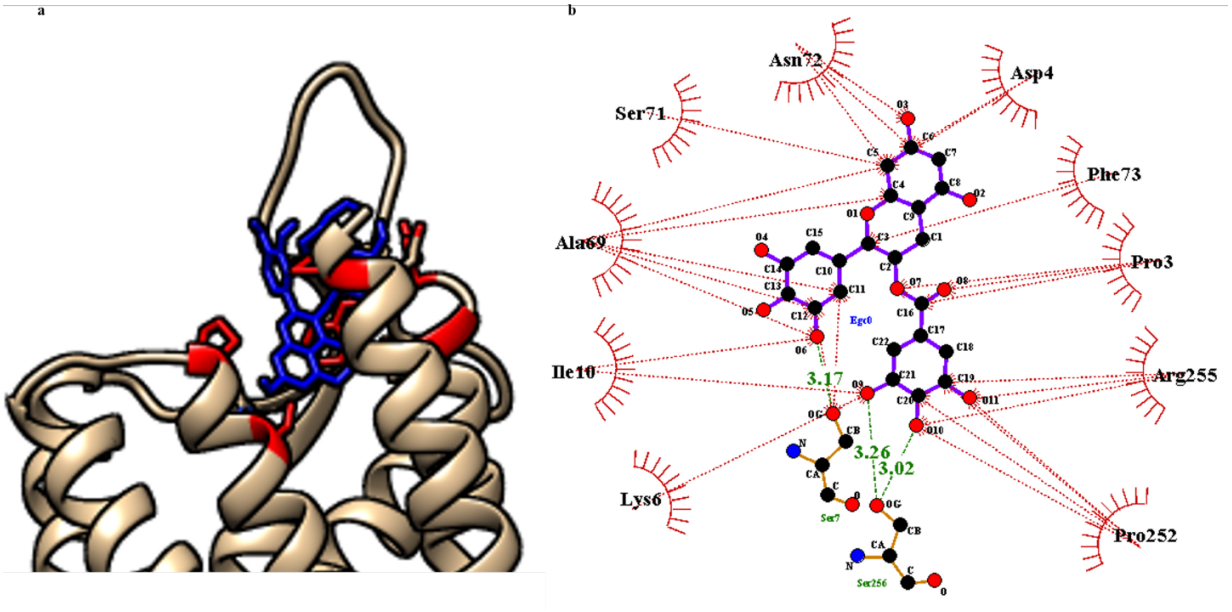

Fig. 6. Docking of GPCR43 with rutin. (a) GPCR43 with rutin docked in the active site. (b) Interactions between GPCR43 and rutin, the residues in the binding sites are highlighted.

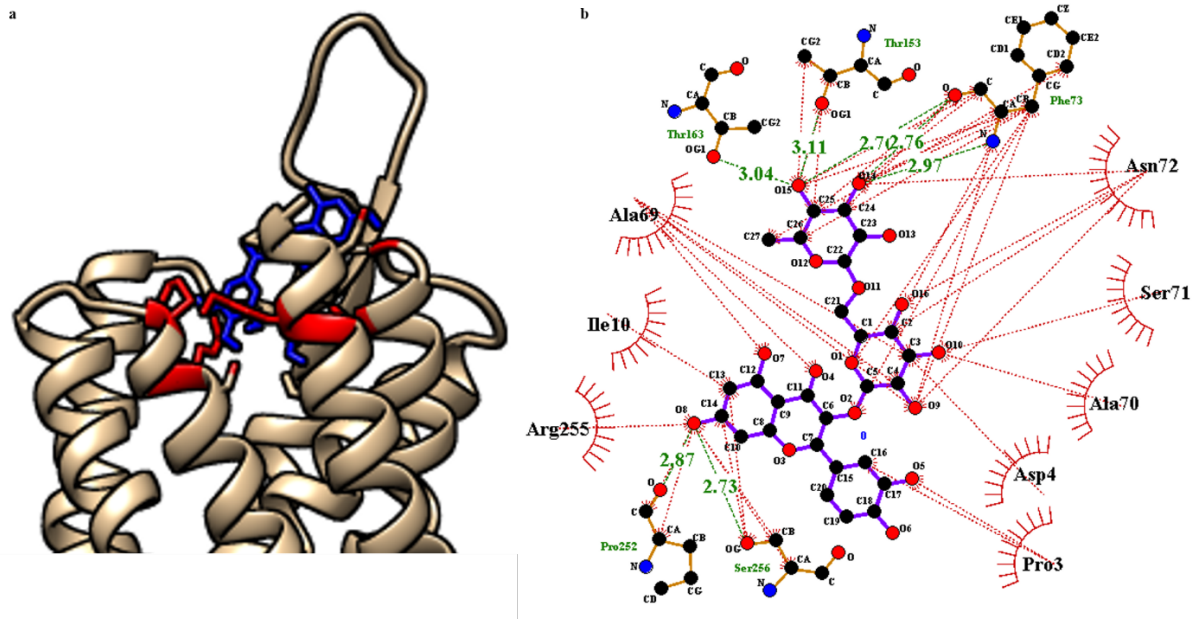

Fig. 7. Docking of GPCR43 with EGCG. (a) A docking pose of GPCR43 with EGCG docked in the active site. (b) Interactions between GPCR43 and EGCG, the residues in the binding sites are highlighted.

The plant-based ligands were docked successfully with good docking scores and interactions. The binding residues such as PRO3, LYS6, SER7, ALA69, ASN72, PHE73, PRO252, ARG255, are predominantly involved in interaction with the ligands (Table 1). According to the docking results, Diosmin and rutin were predicted to be the best ligands with binding score of -9.5 and -8.4 respectively.

4. Discussion

GPCRs are the key players in a multitude of physiological processes and hence, regarded as the promising drug targets (Wiseman *et al.,* 2020). The structure-aided drug design requires purified proteins to obtain three dimensional structures. Recombinant protein production lies at the heart of structural biology. Obtaining high quantities of purified membrane proteins is certainly a challenging task for which scientists have been developing protocols for optimization of protein expres sion. Expression of membrane proteins like GPCRs is difficult owing to the big size, poor stability, and low expression. Herein, we report the successful expression of first three transmembrane domains of GPCR43 by using a small expression tag, AT4. AT rich gene tags next to the translation start site were reported to enhance cell free expression previously (Haberstock *et al.,* 2012). AT rich small gene tags were used to successfully express first three transmembrane segments of apelin receptor which could not be expressed without AT tag (Pandey *et al.,* 2014). Fusion protein tags have greatly facilitated the expression and purification of soluble proteins (Esposito & Chatterjee, 2006; Ki & Pack, 2020). Large tags such as glutathione S-transferase (Harper & Speicher ,2011) and maltose-binding protein (Khan *et al.,* 2014) enhance expression and aid in purification but needs to be cleaved off using an appropriate protease before subsequent analysis due to their large sizes. However, protease digestion gets quite difficult as the fusion protein is insoluble under the conditions needed for protease activity due to the high hydrophobicity of membrane proteins. Therefore, using small tags like AT4 which does not require removal can be highly advantageous in membrane proteins especially involving the structural studies by NMR spectroscopy (Zhou *et al.,* 2010).

Structure modeling and docking studies have become instrumental in structure-aided drug discovery (Gschwend *et al.,* 1996; Meng *et al.,* 2011, Pinzi & Rastelli, 2019). The docking and virtual screening methods have provided various ways to target GPCRs for therapy (Evers & Klabunde, 2005; Chen *et al.,* 2007; Michino *et al.,* 2009; Kooistra *et al.,* 2013; Beuming *et al.,* 2015; Kooistra *et al.,* 2016). Plants contain an inexhaustible array of natural products which can be exploited for drug discovery. For instance, 40 percent of the drugs in the market of western countries originate from plants (USDA website). Plants have been widely used for the treatment of DM (Modak *et al.,* 2007; Rizvi & Mishra, 2013; Kooti *et al.,* 2016; Farzaei *et al.,* 2017).

In the present study, the docking of GPCR43 was conducted with the plant-derived ligands reported with beneficial effects in diabetes. Our results indicate that the selected plant-based ligands were capable of binding to the active site of GPCR43 with good binding scores especially diosmin and rutin.

Rutin and diosmin, two polyhydroxyflavone glycosides found in citrus peel and present in the pulp of various citrus species show diverse pharmacological effects in diseases associated with chronic inflammation (Gosslau *et al.,* 2019). Studies provided evidence that rutin was able to reduce serum glucose levels in animal models of T2DM (Niture *et al.,* 2014; Lee *et al.,* 2016; Liang *et al.,* 2018). Diosmin possess antiplatelet activity and is shown to have preventive effects on various diseases such as hyperglycemia, hyperlipidemia, ulcer, chronic venous insufficiency and hemorrhoids (Zaragozá *et al.,* 2021).

Our group plans to conduct the further investigation of these candidate compounds in animal models to be used in the drug discovery of T2DM.

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