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A Study on the Inhibitory potential of DPP-IV Enzyme by apigenin through *in silico* and *in vivo* approaches

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Abstract

Dipeptidyl peptidase-IV (DPP-IV) inhibitors are incretin enhancers used in the treatment of diabetes. Due to their undesirable side effects, development of new agents preferably from natural sources with less adverse effects is needed. Apigenin (API), a well known insulin-secretagogue and insulin-mimetic agent is abundantly present in fruits, nuts and plant derived beverages. The present study investigates the DPP-IV inhibitory potential and antidiabetic effects of API using various approaches. Through in silico study, we showed that API has binding efficacy at Glu206 in the active site of DPP-IV. Plasma glucose and insulin levels were decreased when API (1.5 mg/kg b.w. for every alternate for last 30 days) was administered to high fat, high fructose diet (HFFD) fed rats. Further, the activity of DPP-IV in plasma and hippocampal homogenate was inhibited more in the API- treated group than in the sitagliptin (STG)- treated group. API has a strong inhibitory effect towards DPP-IV enzyme in vivo and in silico and therefore can be a promising compound for type 2 diabetes (T2D) treatment.

Keywords: Apigenin, Docking, DPP-IV inhibition, HFFD, Insulin resistance.

Introduction

Incretins are hormones which play a vital role in glucose homeostasis by pancreatic and extrapancreatic glucoregulatory effects. Dipeptidyl peptidase-IV (DPP-IV) is a protease which rapidly inactivates the incretin hormones like glucagon like peptidase-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP)[1]. Both hormones have very short half-lives (approximately 2 min) due to the rapid degradation by DPP-IV enzyme[2]. DPP-IV inhibitors, gliptins act as incretin enhancers through the inhibition of DPP-IV enzyme[3]. DPP-IV resistant incretin analogues/mimetics are therefore used in type 2 diabetes (T2D) treatment.

Insulin resistance is a pathophysioljhogical state that begins with excess weight gain and obesity and is believed to be the major underlying factor for the development of T2D[4]. Numerous studies have observed peripheral and neuronal insulin resistance associated with cognitive disturbances in diet-induced resistant rats[5]. Previous studies suggest that hippocampus is sensitive to changes in diet and high-calorie diet can impair hippocampal synaptic plasticity and neurogenesis thereby reducing learning and memory[6]. Findings from our laboratory have firmly established that high fat, fructose diet (HFFD) feeding produces adverse changes in skeletal muscle, liver and heart[7,8].

Protein-ligand docking study is a tool to identify the binding modes of protein-ligand interaction. Docking studies are employed in drug development because of its high accuracy. Computational docking analyses have been commonly used for designing inhibitors[9], screening of potential inhibitors[10], and explaining the differences in activity of drugs with different structures[11].

Apigenin (API, 4',5',7'-trihydroxy flavone) is a non-mutagenic, non-toxic flavone shown to have antidiabetic, anti-inflammatory, anti-oxidative and anti-carcinogenic properties[12-14]. API is shown to be neuroprotective improving the blood-brain barrier integrity and learning and memory and to offer neurovascular protection in mice injected with Aβ25–35[15]. Our present study is to focus on targeting DPP-IV enzyme through API as an incretin enhancer and to analyze its antidiabetic potential and DPP-IV inhibitory property by *in silico* and *in vivo* approaches.

Material and Methods

Chemicals: API and sitagliptin (STG) (Januvia) were purchased from Cayman Chemical Company, USA and Merck Pvt. Ltd., USA respectively. Gly-pro-p-nitroanilide (Gly-pro-*p*NA) was obtained from Sigma–Aldrich, USA. Glucose and insulin assay kits were purchased from Agappe diagnostics Pvt. Ltd., Kerala, India and Accubind, Monobind Inc., CA, USA respectively. Chemicals and reagents used in the study were acquired from Himedia Laboratories Pvt. Ltd., Mumbai, India or Sisco Research Laboratories Pvt. Ltd., Mumbai, India.

***In silico:* Molecular modeling:** The DPP-IV inhibitory potential of API was assessed by molecular docking with DPP-IV crystal structure. The docking score of API was compared

with those of the derivatives of API (apigetrin, isorhoifolin, isovitexin, rhoifolin, vicenin, vitexin) using GLIDE software (Maestro, version 8.5, Schrödinger, LLC, 2008).

Preparation of proteins and ligand: Docking was performed using GLIDE software[16]. DPP-IV (PDB: 1X70) was retrieved from the data base. The crystal structures were cleaned by deleting the ligand and cofactor. The protein structures were pre-processed, refined and the geometries were optimized with OPLS-2005 force field using the standard protocol in GLIDE software. The structure of ligands (apigetrin, isorhoifolin, isovitexin, rhoifolin, vicenin, vitexin and API) were manipulated and adjusted with LigPrep 2.5 (Schrödinger LLC, Portland, USA;).

First, all the protonation states at pH 2.0 – 7.0 were generated using Epik software[17] and for the generation of ligand binding site for metalloproteins the "Add metal binding site" was selected. Tautomers if any were generated at the respective pH. All combinations were generated to determine chiralities and to maintain stereochemistry. The geometries of the ligand are minimized using Schrodinger's Optimization Potential for Ligand Simulation (OPLS-2005) force field.

Receptor Grid Generation: The shape of API and its derivatives, and their binding properties with DPP-IV enzyme were represented by the different sets of fields on a grid. Previous information reported in the literature on crystal structure and docking and the Q-Site finder software were used.

Ligand Docking: The protein-ligand docking was performed in Glide XP extra precision mode[18]. The ligand structure was kept flexible while the protein structure was rigid. The best docked pose was saved. The results of the best poses were analyzed using the G score and Dock score and number of H-bonds. Post docking calculation like the estimation of binding energy of the ligands with receptor was performed by automated Multi ligand Biomolecular Association with Energetics (MBAE).
The ligand binding energy was calculated using:

Ligand Binding energy, $\Delta E = E_{complex} - E_{ligand} - E_{protein}$
The negative binding score indicates higher affinity of the ligand to the protein.

Maintenance of animals: Male albino Wistar rats of body weight 100 to 120 g were purchased from Pranav Agro Industries Ltd., Bangalore, India and maintained in polypropylene cages under hygienic conditions with a standard light (12 h light/ 12 h dark cycle) and temperature (22-24°C). The animals were given free access to standard pellet and water *ad libitum* and maintained in the Central Animal House, Rajah Muthiah Medical College and Hospital (RMMC and H), Annamalai Nagar. The experimental procedures were approved by the Institutional Animal Ethics Committee of Animal Care (IAEC), Annamalai University (No.160/1999/ CPCSEA/1101).

Composition of HFFD: The HFFD prepared in the laboratory had the following composition (g/100 g): fructose 45.0, ground nut oil 10.0, beef tallow 10.0, casein 22.5, DL-methionine 0.3, wheat bran 5.5, vitamin mixture 1.2 and mineral mixture 5.5. The standard diet, commercially obtained from Pranav Agro Industries Ltd., Bangalore, India contained 60 % (w/w) starch, 22.08 % (w/w) protein and 4.38 % (w/w) fat, normal chow provided 382.61 cal/100 g while HFFD provided 471.25 cal/100 g.

Animal Groups: The animals were grouped into five with six animals in each group (Group I- Control, Group II- HFFD, Group III- HFFD + API, Group IV- HFFD + STG, Group V- Control + API). The experimental duration was 60 days. For treatment, 1.0 ml of API dissolved in 0.1 % dimethyl sulfoxide (DMSO) was injected intraperitoneally (i.p.) at a dose of 1.5 mg/kg b.w. every alternate day for last 30 days of the experimental period[19]. For the comparative study, 1.0 ml of STG (30 mg/kg b.w.) dissolved in 0.9 % saline[20] was given orally for the last 30 days of the experimental duration.

Sample preparation: At the end of 60 days, the animals were denied food overnight and sacrificed the next day by cervical dislocation. Blood was collected by sino-ocular puncture. Hippocampus was removed and immediately cleaned with ice-cold saline (0.89 % sodium chloride). Tissue sample was homogenized and plasma was collected for the assay of DPP-IV enzyme, glucose and insulin.

***In vivo* DPP-IV inhibition assay:** DPP-IV activity in plasma and hippocampal homogenate was measured according to the method of Kreisel et al.. A total volume of 0.20 ml assay mixture contained 0.1 M Tris-HCl buffer (pH 8) and 2×10^{-3}M of Gly-Pro-*p*-NA, a chromogenic substrate. After incubation for 30 min at 37°C, the reaction was stopped by adding 0.80 ml of sodium acetate (1M, pH 4.5). The amount of p-nitroaniline released was arrived using the molar extinction co-efficient of pNA (9600 $M^{-1}\cdot L\cdot cm^{-1}$) and the absorbance of the samples was measured in a UV-Vis spectrophotometer at 405 nm. Activity of the enzyme was expressed in unit per litre (U/L) for plasma and unit per mg protein (U/mg protein) for homogenate.

Measurement of glucose, insulin and insulin sensitivity indices: Glucose and insulin in plasma were assayed using kits and insulin sensitivity indices like homeostatic model assessment (HOMA) and quantitative insulin sensitivity check index (QUICKI)[22] were calculated as shown below.

HOMA= [Insulin (µU/ml) × glucose (mM)]/22.5
QUICKI = 1/[log(insulin µU/ml) × log (glucose mg/dL)]

Statistical analysis: The data obtained was evaluated for statistical significance was done by one way analysis of variance (ANOVA) followed by Tukey's Multiple Range Test for multiple comparisons of groups. SPSS statistical software (version 16.0; SPSS, Chicago, IL, USA) was used. A value of $P< 0.05$ was considered significant for all the cases.

Results and Discussion

***In silico study*:** Molecular docking was performed to find the interaction between API and its derivatives with DPP-IV enzyme using Schrödinger software. Docking score, glide score and hydrogen bond interaction were analyzed. The binding affinity of API and its derivatives (apigetrin, isorhoifolin, isovitexin, rhoifolin, vicenin and vitexin) with DPP-IV enzyme are tabulated in table-1 while figure-1 depicts the pictorial representation of the same. The docking score and the formation of hydrogen bonds of the docked proteins are also tabulated in Table 1. Of all these compounds tested API binds at Glu206, Tyr662, Arg358 and Phe357 of DPP-IV enzyme (figures-2a and 2b).

***In vivo* DPP-IV inhibition study:** Based on the binding efficiency of API with DPP-IV enzyme, we analyzed the in vivo DPP-IV inhibition potential of API. The results showed that API administrated animals significantly greater inhibition of DPP-IV by API than STG both in plasma and hippocampal homogenate (table-3).

Glucose, insulin and insulin sensitivity*:* Table 2 shows the plasma glucose and insulin levels in experimental rats. Compared to all other groups HFFD-fed group, registered a rise in glucose and insulin levels in plasma, HOMA and QUICKI were also significantly altered in HFFD group when compared to control suggesting insulin resistance in HFFD group. API administration significantly decreased glucose and insulin levels and improved insulin sensitivity.

Discussion: DPP-IV is a proteolytic enzyme that specifically inactivates proteins that have an amino acid sequence with proline or alanine at the N-terminal penultimate position[23]. DPP-IV has three different binding pockets/active sites namely S1, S2 and S3. The S1 active site (Ser630, Asn710, and His740) is composed of side chains of catalytic triad involved in strong hydrophobic interactions[24] and the S2 active site (Glu205, Glu206 and Tyr662) is present near the cavity of DPP-IV. The S3 active site (Ser209, Arg358, and Phe357) allows larger groups outside the pocket and the inside position favors smaller groups[25]. DPP-4 inhibitors interact with the S2 pocket at Glu205 and Glu206 by forming salt bridges. This interaction plays an important role in inhibiting the enzyme[26]. Interestingly, docking analysis showed that API could dock into S2 and S3 pockets with residues Glu206, Tyr662, Arg358 and Phe357 (Figures 2a and 2b) at the active sites of DPP-IV. A study conducted by Fan et al., (2013)[27] reported that resveratrol, luteolin, API and flavones had low *Ki* values to inhibit DPP-IV activity and thereby they have high affinity to the active site of DPP-IV. API derivatives, apigetrin binds at S2 pocket, isorhoifolin and isovitexin at S3 pocket and rhoifolin, vicenin and vitexin had no DPP-IV inhibitory effect. We presume that the binding of API with the enzyme leads to conformational changes in DPP-IV. In the present docking study, API showed lower binding energy than its derivatives indicating more potent inhibitory effect of API.

Assay of DPP-IV enzyme *in vivo* showed significant inhibition by API and STG, but API has better efficacy in rat plasma (38% and 25% inhibition by API and STG respectively) (table-3) and also in hippocampal homogenate (24% and 16% inhibition by API and STG respectively) (table-3). The differences in percentage inhibition of enzyme show that API has more inhibitory potential than STG in HFFD-fed insulin resistant rats.

The HFFD-fed rat form an excellent model to study obesity and insulin resistance as the diet simulates ‘‘Western type’’ high-calorie diet[28]. Consumption of high fat with high quantities of fructose promotes weight gain, obesity, insulin resistance and subsequently T2D[29]. HFFD feeding impairs insulin signaling and glucose uptake and utilization with 15 days of feeding[7]. In our present study, rats fed with high calorie diet for 60 days displayed altered HOMA and QUICKI values, insulin resistance, hyperglycemia and hyperinsulinemia. These findings are consistent with our earlier reports[5,30]. Since DPP-IV inhibitors (STG, vildagliptin and saxagliptin) have several side effects like headache, nausea, weight gain, swelling low blood sugar levels especially when taken in excess[31], API which is non-toxic[12] can be a good therapeutic agent for T2D.

Table-1
Docking score, glide score and H-bond score of API and its derivative with DPP4 enzyme.

Name of the compound and structure	Amino acid residues of showing hydrogen bond with flavonoids	Docking score	Glide score	Hydrogen bond score
Apigetrin	Lys554, Asp545, Gln553, Tyr547, Glu205, Trp627,Gly628	-8.2	-8.2	-4.5
Isorhoifolin	Ser630, Gln553, Tyr547, Val207, Arg358, Trp629	-10.8	-10.8	-7.4
Isovitexin	Gln553, Arg358, Val207,Tyr547, Lys554, Ser552	-7.8	-7.8	-4.8
Rhoifolin	Tyr666, Tyr456, Arg429, Tyr547, Tyr585	-8.5	-8.5	-4.1
Vicenin	Arg125, Hie126	-9.1	-9.1	-6.2
Vitexin	Gln553, Tyr547, Arg125, Ser552, Ser630, Lys554	-7.1	-7.1	-3.5
API	Glu206, Tyr662, Phe357, Arg358, Val207	-2.8	-2.8	-2.9

Table-2
Plasma glucose, insulin, HOMA and QUICKI values of experimental animals.

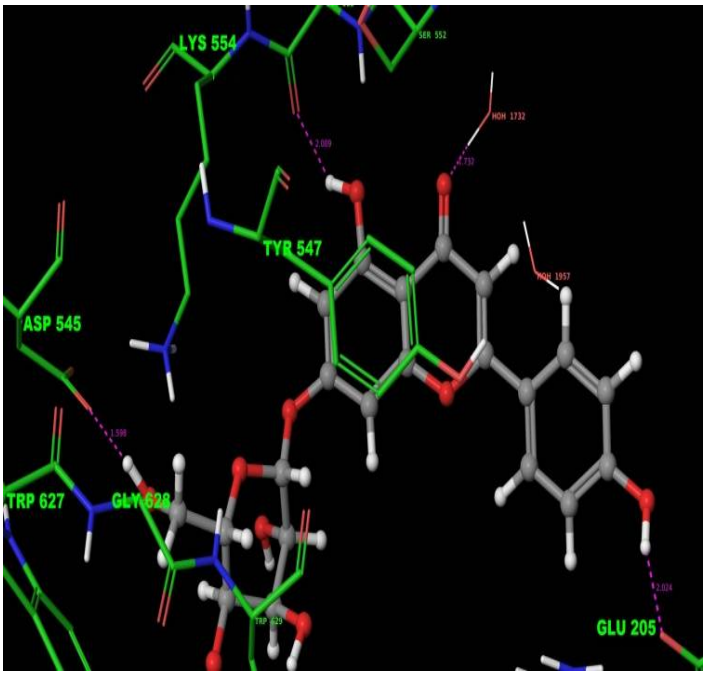
PARAMETERS	CON	HFFD	HFFD + API	HFFD + STG	CON + API
Glucose(mg/dL)	100.90±3.02	158.44±12.67[a]	116.04±4.64[a,b]	134.19±6.70[a,b,c]	94.38±2.83
Insulin (µU/mL)	16.8±0.50	31.0±2.48[a]	23.3±0.93[a,b]	26.7±1.33[a,b,c]	16.5±0.49
HOMA	0.70±0.02	2.02±0.16[a]	1.12±0.05[a,b]	1.47±0.08[a,b,c]	0.64±0.02
QUICKI	0.41±0.01	0.31±0.02[a]	0.35±0.02[a,b]	0.33±0.02[a,b,c]	0.42±0.01

Values are means ± SD of 6 rats from each group. CON- Control rats; HFFD - High fat-high fructose diet-fed rats; HFFD + API - High fat-high fructose diet-fed rats treated with API; HFFD + STG - High fat-high fructose diet-fed rats treated with STG; CON + API- Control rats treated with API.
a -Significant as compared to CON ($P < 0.05$; ANOVA followed by Tukey's test)
b -Significant as compared to HFFD ($P < 0.05$; ANOVA followed by Tukey's test)
c -Significant as compared to HFFD+API ($P < 0.05$; ANOVA followed by Tukey's test)

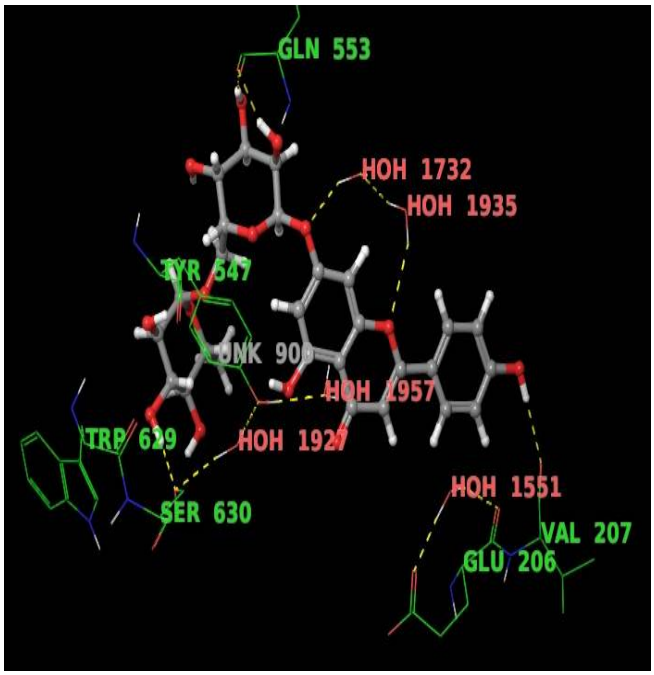
Table-3
***In vivo* DPP-IV enzyme activity in plasma and hippocampal homogenate of experimental animals**

PARAMETERS	CON	HFFD	HFFD + API	HFFD + STG	CON + API
Plasma (U/L)	294.79±16.1	424.32±31.7[a]	263.81±20.5[a,b]	319.97±22.3[a,b,c]	107.25±7.5[d]
Hippocampus (U/mg protein × 10^{-5})	3.4±0.08	5.6±0.35[a]	4.26±0.23[a,b]	4.72±0.31[a,b,c]	3.7±0.12[d]

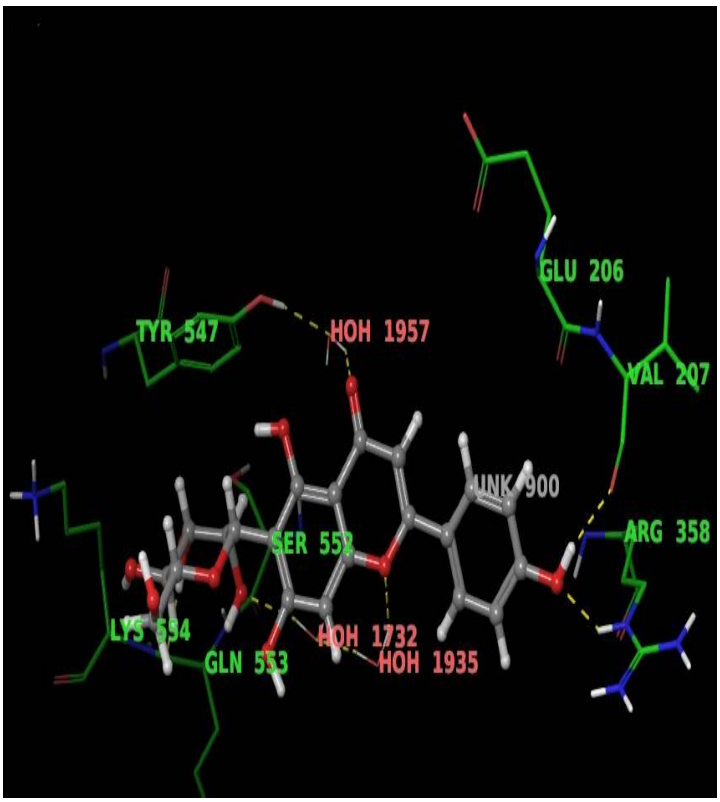
Values are means ± SD of 6 rats from each group. CON- Control rat; HFFD, High fat-high fructose diet-fed rat; HFFD + API, High fat-high fructose diet-fed rat treated with API; HFFD + STG, High fat-high fructose diet-fed rat treated with STG; CON + API- Control rat treated with API.
a -Significant as compared to CON ($P < 0.05$; ANOVA followed by Tukey's test)
b -Significant as compared to HFFD ($P < 0.05$; ANOVA followed by Tukey's test)
c -Significant as compared to HFFD+API ($P < 0.05$; ANOVA followed by Tukey's test)
d -Significant as compared to CON ($P < 0.05$; ANOVA followed by Tukey's test)



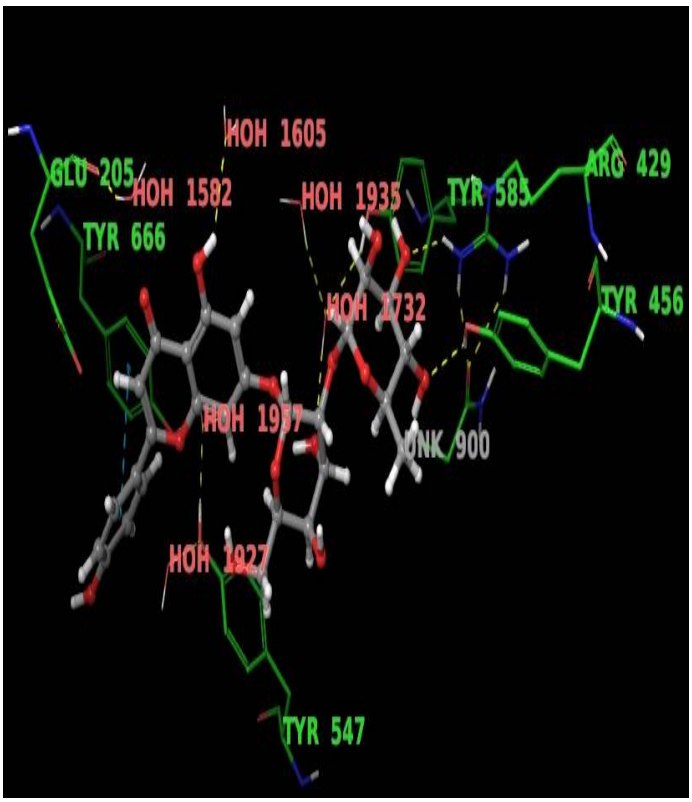
Apigetrin



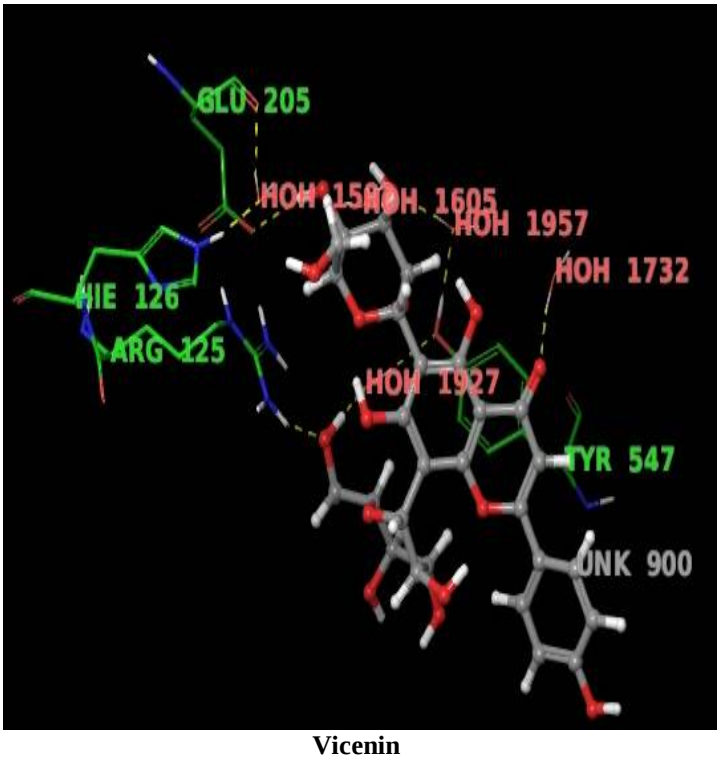
Isorhoifolin



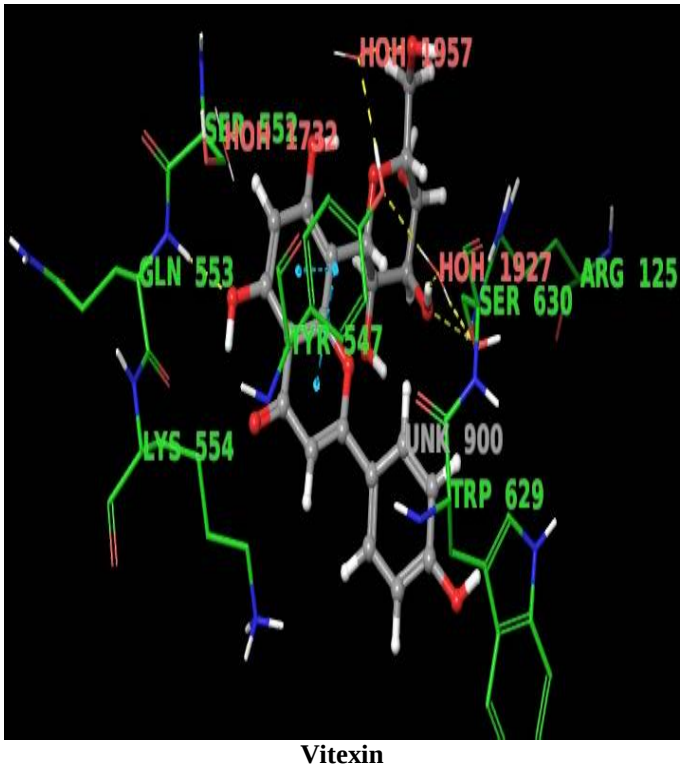
Isovitexin



Rhoifolin

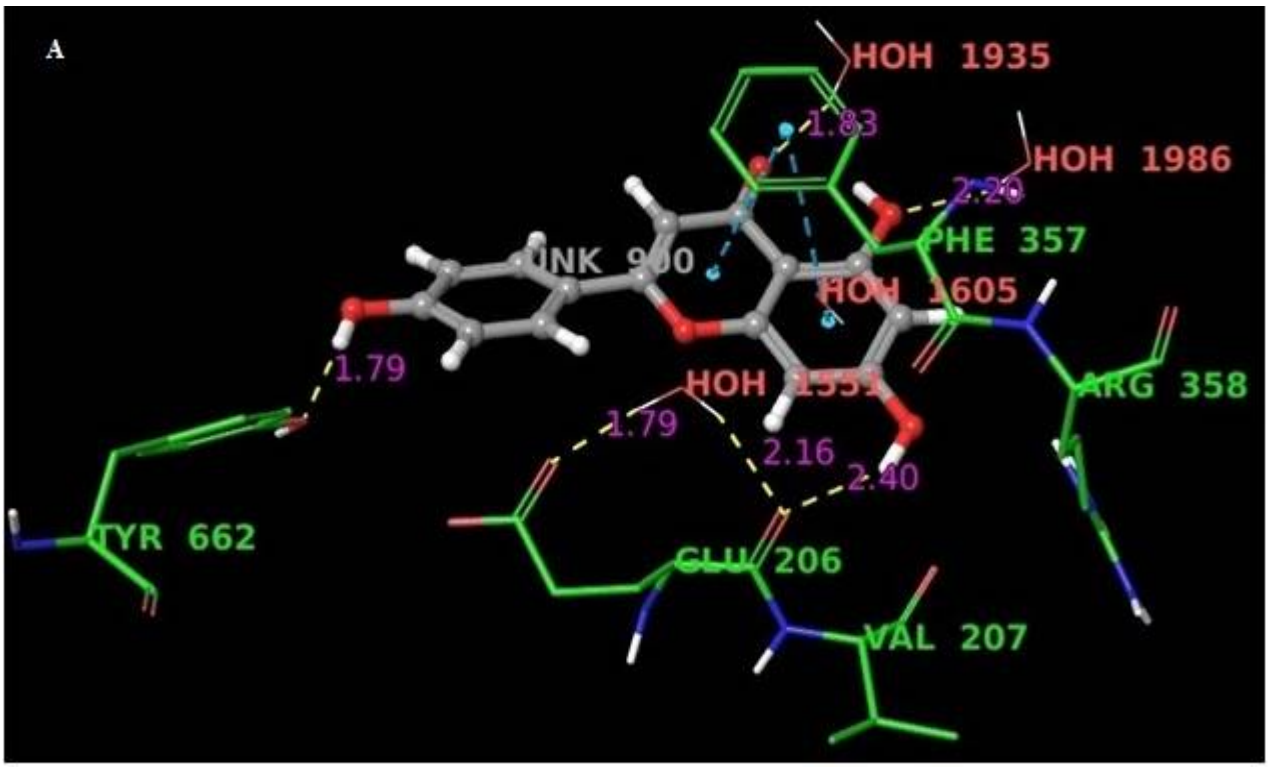


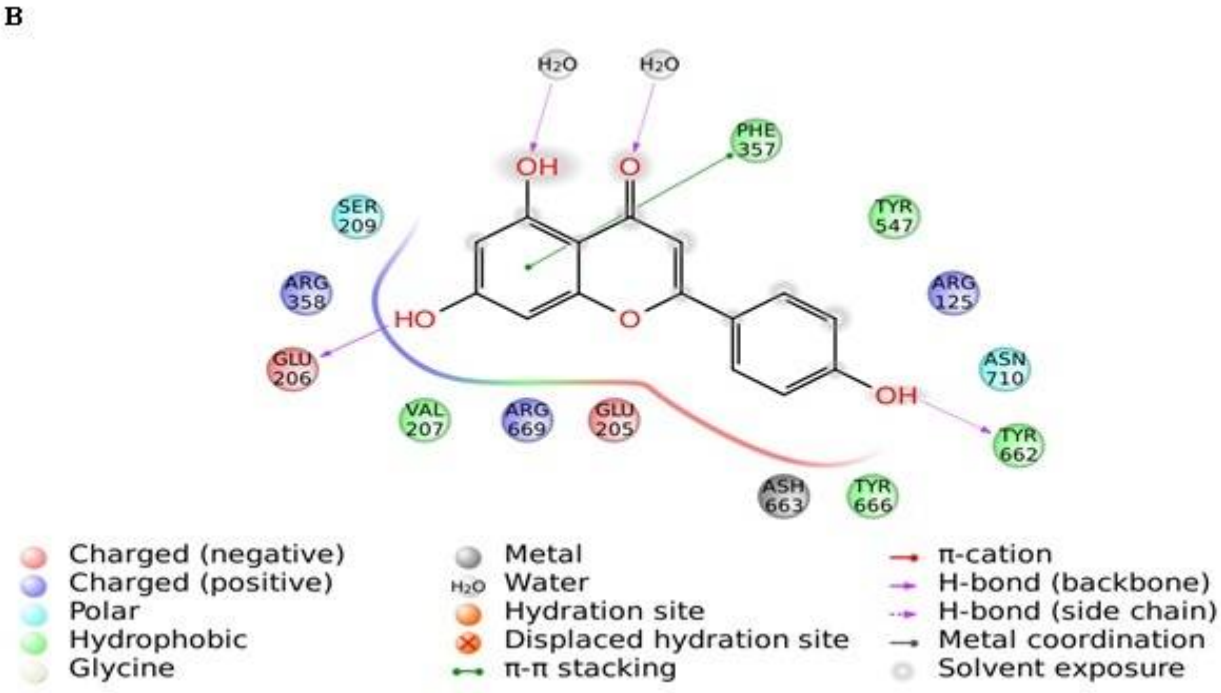
Vicenin



Vitexin

Figure-1
Docking images of API derivatives with DPP-IV enzyme





Figures-2a and 2b

Docking images of API to DPP-IV protein active site formed H-bonds with residues Glu206, Tyr662, Phe357, Arg358

Conclusion

API has a strong inhibitory action on DPP-IV enzyme through *in silico* and *in vivo* studies and thus may be used as DPP-IV inhibitor. API also has a potent antihyperglycemic activity. The results observed in this study are the first step in exploring an incretin enhancer which is specific and efficacious in improving T2D symptoms in humans. Further studies are warranted using this compound.

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