



ISSN (E): 2320-3862
ISSN (P): 2394-0530
<https://www.plantsjournal.com>
JMPS 2022; 10(6): 49-56
© 2022 JMPS
Received: 14-09-2022
Accepted: 19-10-2022

Nasrin Akter Shova
Department of Biotechnology
and Genetic Engineering,
University of Development
Alternative, Lalmatia, Dhaka-
1207, Bangladesh

Md Abdus Samad Azad
Department of Biotechnology
and Genetic Engineering,
University of Development
Alternative, Lalmatia, Dhaka-
1207, Bangladesh

MM Maidul Islam
Department of Biotechnology
and Genetic Engineering,
University of Development
Alternative, Lalmatia, Dhaka-
1207, Bangladesh

Rownak Jahan
Department of Biotechnology
and Genetic Engineering,
University of Development
Alternative, Lalmatia, Dhaka-
1207, Bangladesh

Mohammed Rahmatullah
Department of Biotechnology
and Genetic Engineering,
University of Development
Alternative, Lalmatia, Dhaka-
1207, Bangladesh

Corresponding Author:
Mohammed Rahmatullah
Department of Biotechnology
and Genetic Engineering,
University of Development
Alternative, Lalmatia, Dhaka-
1207, Bangladesh

Oral glucose tolerance test with methanol extract of *Hibiscus sabdariffa* L. calyces and *in silico* alpha-amylase inhibition studies with selected calyce phytochemicals

Nasrin Akter Shova, Md Abdus Samad Azad, MM Maidul Islam, Rownak Jahan and Mohammed Rahmatullah

DOI: <https://doi.org/10.22271/plants.2022.v10.i6a.1490>

Abstract

Background: Diabetes mellitus (DM) is an endocrinological disorder characterized by increased blood glucose, which in turn leads to increased oxidative stress and development of other complicated disorders. The disorder is caused either by non-production of insulin by pancreatic β -cells or development of insulin resistance. In recent years, diabetes is increasing rapidly. Although high blood glucose (hyperglycemia) is controllable through anti-diabetic drugs and/or insulin injection, rural people in Bangladesh prefer plant-based traditional medicines dispensed by traditional medicinal practitioners.

Methods and findings: The present study objective was to conduct oral glucose tolerance test (OGTT) in glucose-challenged mice with methanol extract of *Hibiscus sabdariffa* calyces (MEHS); the calyces are widely used in Bangladesh for controlling hyperglycemia. At doses of 100, 200 and 400 mg per kg body weight, MEHS reduced blood glucose level by 55.8, 62.0 and 62.77%, respectively compared to control mice, which were untreated. Glibenclamide, by comparison, reduced blood glucose level by 61% at a dose of 10 mg per kg body weight. Furthermore, *in silico* molecular docking studies showed that a number of phytochemicals present in *Hibiscus sabdariffa* calyces have predicted low binding energies for human α -amylase (PDB number: 2QV4) with the potential for inhibiting hyperglycemia.

Conclusions: (MEHS) reduced blood glucose by nearly 63% at a dose of 400 mg/kg, which was comparable to that of glibenclamide at a dose of 10 mg/kg. *In silico* studies further indicated that a number of phytochemicals of the calyces of *Hibiscus sabdariffa* merits further evaluation towards new anti-diabetic drug discovery.

Keywords: Phytotherapy, OGTT, *Hibiscus sabdariffa*, calyces, Bangladesh

Introduction

Diabetes mellitus (DM) is a progressively debilitating disorder characterized primarily by elevated concentrations of blood glucose caused through non-production of insulin by pancreatic β -cells (Type 1) or development of resistance to insulin (Type 2). During initial stages, DM may cause polyuria, polydipsia, and polyphagia^[1]. Elevated oxidative stress caused by hyperglycemia increases pro-inflammatory pathways, which can lead to irreversible disorders with poor prospects of cure like diabetic neuropathy, diabetic nephropathy, diabetic retinopathy, and heart disorders, as well as foot ulcers, which can even lead to amputation of foot^[2-7]. Type 2 DM patients (45-88%) also develop oral mucosal disorders^[8].

Bangladesh is no exception to the rising prevalence of diabetes as is happening throughout most of the world. A study conducted in 2018 found that in a representative population of men and women in Bangladesh 25 years or above, a fifth of them were hypertensive, which percentage increased with age and body mass index^[9]. Patients with hypertension commonly exhibit resistance to insulin and show greater risk of developing DM^[10]. Both hypertension and DM have common mechanisms in the form of elevated oxidative stress and inflammatory conditions. According to a World Health Organization report in 2016, around 8% of the Bangladesh population had diabetes^[11]. A survey conducted with 7,535 individuals concluded that in the 50-54 age group DM was present in 33.3% of the people in Bangladesh^[12].

Another survey conducted from 2012 till 2015 in a rural area of Bangladesh among adults of

31 or more years of age found that one in ten persons had diabetes [13].

According to the Diabetic Association of Bangladesh, on average a diabetic patient needs Bangladesh Taka (BDT) 2000 per month for diabetes treatment (BDT 101.3 = 1 US \$ currently). As reported in the Daily Star newspaper of Bangladesh on November 14 2021, high treatment costs and limited accessibility to treatment is increasing the diabetes burden in Bangladesh. Adding to the above are adverse effects of anti-diabetic drugs, forgetfulness of the mainly less literate rural people in taking appropriate dosages on time and in the prescribed amounts, and reluctance to visit doctors and take drugs or insulin injections. As a result people are more prone to visit traditional medicinal practitioners, who use various plants to lessen hyperglycemic conditions in diabetic patients [14].

Anti-diabetic plants, considered beneficial in lowering elevated glucose in blood or alleviating morbidities induced by DM, have been reported from many regions in the world [15-17]. These plants can prove useful in the treatment of diabetes in several ways. They are quite easily available to both traditional medicinal practitioners and diabetic patients; they are affordable; and they can form a medicinal plant base for the scientist/researcher in quest for lead compounds or new anti-diabetic drugs. From these view points and the fact that Bangladesh is a developing country with low income but with a rich variety of medicinal plant species, we had been for over the last ten years conducting ethnomedicinal surveys [18-38]. Our surveys were followed by analyzing phytochemicals and performing a number of pharmacological activity studies on these plants [39-46]. Foremost among these studies were oral glucose tolerance test (OGTT) for determination of

antihyperglycemic activity, which can form a basis for further studies on the anti-diabetic potential of any plant species.

Hibiscus sabdariffa L. (Malvaceae) is a plant widely available in Bangladesh; its calyces are considered highly medicinal. It is known as roselle in English; in Bangladesh, the Bengali name is chalta. The plant grows 7-8 feet tall and its flowers have a conspicuous calyx at the base, which turns bright red and fleshy with the maturation of the fruit. A very recent review has summarized the phytochemicals reported thus far to be present in roselle calyces and has come to the conclusion that roselle has the potential of being a promising anti-diabetic plant after also reviewing the anti-diabetic activity experiments done with calyces and other parts of roselle plant [47 and pertinent references therein]. In the present study, we wanted to evaluate whether methanol extract of *Hibiscus sabdariffa* calyces can improve oral glucose tolerance in mice. Since inhibition of α -amylase can control postprandial hyperglycemia [48], a second objective was to perform *in silico* studies on the binding of a few randomly selected calyces phytochemicals to human pancreatic α -amylase.

Methods

Collection of plant material

Calyces of *Hibiscus sabdariffa* were collected from Bandarban, a district in the Chittagong Hill Tracts, Bangladesh. A trained botanist at the University of Development Alternative identified the calyces (Figure 1). Calyces were cut into thin strips and air-dried outside direct sunlight for 96 hours.



Fig 1: Fresh calyces of *Hibiscus sabdariffa* L.

Preparation of methanolic extract of *Hibiscus sabdariffa* calyces (MEHS)

The air-dried calyces were powdered and 45.8g of the powder was extracted with three volumes of methanol (1:3, w/v) at ambient temperatures for 48h with frequent stirring. The extract (MEHS) was evaporated to dryness at 40 °C and kept as small aliquots at -20 °C till necessary. The final weight of MEHS was 4.5g. Tween 20 was used to prepare a suspension of the extract before it was administered to mice in oral glucose tolerance test (OGTT).

Chemicals and drugs

Glucose and glibenclamide were purchased from Square Pharmaceuticals Ltd., in Dhaka, Bangladesh. Glucometer and

strips were obtained from Lazz Pharma, Bangladesh.

Mice

Swiss albino mice of both sexes, (12-15g weight) were used in this study. The purchasing of mice was done from International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR, B). Mice were acclimatized for 72h prior to experiments. During this 72h, they were kept in a room where the temperature was maintained at 25°C and given standard food (mice chow) and water freely. The Institutional Animal Ethical Committee of University of Development Alternative, Dhaka, Bangladesh approved the study following guidelines by European Union for animal experiments.

Oral glucose tolerance test (OGTT)

We followed the previously described procedure of Joy and Kuttan with some minor modifications for OGTT [49]. The mice were divided into five groups of five mice each. Group 1 received vehicle (1% Tween 20 in water, 10 ml/kg body weight) and served as control, Group 2 received standard drug (glibenclamide, at a dose of 10 mg/kg body weight). Groups 3-5 received, respectively, MEHS at doses of 100, 200 and 400 mg per kg body weight. Oral administration by gavage was followed to deliver extract, vehicle, and drug to mice. Control and experimental mice received the same amount of Tween 20. After a period of 1h [50, 51], all mice were administered 2g glucose/kg body weight. Heart puncturing, as described earlier [50, 51], was followed to collect blood samples 2h following administration of glucose. Blood glucose levels were measured with a glucometer. The percent lowering of blood glucose levels were calculated as described below. Percent lowering of blood glucose level = $(1 - W_e/W_c) \times 100$, where W_e and W_c represents the blood glucose concentration in glibenclamide or MEHS administered mice (Groups 2-5), and control mice (Group 1), respectively.

Precautions were taken that the mice did not suffer unnecessarily during the the experiment. We tried collecting blood by puncturing tail veins of mice, but the blood obtained in this manner, in our hands, was too little to measure with glucometer strips.

Statistical analysis

Values are expressed as mean \pm standard error of mean (SEM). For comparing purposes in the statistical sense, Independent Sample t-test was carried out. Statistical significance (marked by *) was considered to be indicated by a p value < 0.05 in all cases, as previously described [52].

Molecular docking studies with AutoDock Vina (Blind Docking Methodology)

Receptor Preparation

We took human pancreatic alpha-amylase complexed with nitrite and acarbose (an inhibitor of alpha-amylase) as our target receptor. We took X-ray crystallographic structure of the protein from protein data bank (pdb) holding the pdb ID-2QV4. Water molecules were removed from the protein structure and polar hydrogen atoms added with the aid of Pymol software [53]. Protein molecule was then saved in pdb format. Docking studies were carried out with both the 2QV4 (PDB ID) structure with acarbose bound and 2QV4 from which acarbose has been removed.

Docking parameter

The generated grid box covered the whole 2QV4 human

pancreatic alpha amylase protein molecule. Grid box parameter for the protein is given below.

The center was at X: 18.66, Y: 50.02, Z: 17.79 and the dimensions of the grid box were, X: 82.16, Y: 132.90 and Z: 91.91 (unit of the dimensions, Å) for 2QV4 (with acarbose). The center was at X: 19.17, Y: 46.67, Z: 18.98 and the dimensions of the grid box were, X: 83.01, Y: 127.52, and Z: 81.32 (unit of the dimensions, Å) for 2QV4 (without acarbose).

Ligand Preparation

Ligand molecules were downloaded from Pubchem [54] as sdf format. They were optimized with the force field type MMFF94 using Openable software and saved in pdbqt format.

Docking

We have done here blind docking method for screening phytochemicals, which can be potential inhibitors. The grid box in Autodock Vina was generated aiming to cover up the whole protein molecule with or without bound acarbose. We have used exhaustiveness '16' for better ligand and protein binding. AutoDock Vina tool shows a total nine docked poses for each ligand; among them pose 1 is the best pose, which has the highest binding affinity. We have saved pose 1 in pdb format by using Pymol for further analysis. To be noted is that acarbose was removed to see whether there were any changes in the amino acid interactions of alpha-amylase with the screened phytochemicals. The region where the ligand binds effectively with protein molecule can be found in blind docking. In Auto Dock Vina [55] a total of nine docked poses for each ligand are provided; among them pose 1 is the best pose, which shows the highest binding affinity. Pose 1 was saved in pdb format by using Pymol for further analysis. 2D diagram and ligand-protein amino acid interactions were depicted from Discovery Studio Software [56].

Results and Discussion

OGTT results

Even at the lowest dose of 100 mg/kg body weight, MEHS lowered blood glucose levels by 55.83%. Further increases in doses of MEHS only slightly increased the percent lowering of blood glucose. At doses of 200 and 400 mg/kg, MEHS lowered blood glucose by 62.04 and 62.77%, respectively. Glibenclamide (a standard blood glucose lowering drug), when administered to mice at a dose of 10 mg/kg reduced blood glucose by 60.94%. The results are shown in Table 1 and suggests that even low doses of MESH are able to lower blood glucose in a significant manner.

Table 1: Effect of MEHS on blood glucose level following 120 minutes of glucose loading in hyperglycemic mice.

Treatment	Dose administered (mg/kg body weight)	Level of glucose in blood (mmol/l)	% reduction of blood glucose level
Control	10 ml	6.85 \pm 0.08	-
Glibenclamide	10 mg	2.68 \pm 0.13	60.94*
(MEHS)	100 mg	3.03 \pm 0.36	55.83*
(MEHS)	200 mg	2.60 \pm 0.23	62.04*
(MEHS)	400 mg	2.55 \pm 0.10	62.77*

All administrations were done orally. Values are represented as mean \pm SEM, (n=5); * $p < 0.05$; significant compared to hyperglycemic control animals.

Molecular docking results

Gossypetine, hibiscetine, delphinidin, and quercetin have been reported to be present in *Hibiscus sabdariffa* calyces [57]. It was of interest to see through molecular docking studies whether these four phytochemicals can bind to human pancreatic alpha-amylase, inhibition of which can reportedly

lower the level of post-prandial hyperglycemia by controlling breakdown of starch [58]. Human pancreatic alpha-amylase (PDB ID 2QV4) with bound or removed acarbose was used for molecular docking studies with the four phytochemicals. The predicted binding energies of the phytochemicals with alpha-amylase are shown in Table 2.

Table 2: Predicted binding energies of *Hibiscus sabdariffa* calyce phytochemicals with alpha-amylase

Phytochemical	Predicted Binding Energy ($\Delta G = -\text{kcal/mol}$) 2QV4 with bound acarbose	Predicted Binding Energy ($\Delta G = -\text{kcal/mol}$) 2QV4 without bound acarbose
Delphinidin	-7.2	-8.7
Gossypetin	-7.5	-9.0
Hibiscetin	-7.5	-8.7
Quercetin	-7.9	-9.1

Interestingly, all four phytochemicals that were screened for predicted binding energy studies with human pancreatic alpha-amylase (PDB ID 2QV4) showed lower predicted binding energies, that is, greater affinity for alpha amylase from which acarbose has been removed. This is not surprising since acarbose is an inhibitor of alpha amylase [59], and if the screened phytochemicals also are inhibitors of alpha amylase, then their binding affinities will potentially increase following removal of acarbose, and their binding to amino acid residues of 2QV4 will potentially be similar to acarbose. The catalytic residues of alpha amylase are Glu233 and Asp197, and another inhibitor like acarbose, namely montbretin A, binds to these two catalytic site amino acids besides other amino acid residues like Ile235, His201, His305, Lys200, Glu240, His101, Gln63, Asn298, Asp356, Arg159, and Asp300 [60]. The amino acid residues non-bonding interactions of the four phytochemicals with 2QV4 (bound and removed acarbose) are shown in Table 3.

It can be seen from Table 3 that none of the four phytochemicals of *Hibiscus sabdariffa* evaluated in the present study (delphinidin, gossypetin, hibiscetin, and quercetin) bound to either of the two catalytic site amino acid

residues of 2QV4 (when bound to acarbose), namely Glu233 and Asp197, which is consistent with previous studies that these two catalytic site amino acid residues would be bound to acarbose, thus preventing other ligands to bind to the same catalytic site amino acid residues. However, a different scenario was observed when binding studies were carried out with 2QV4 from which acarbose was removed. With the exception of gossypetin, the other phytochemicals bound to one or both amino acid residues of the catalytic site, Glu233 and Asp197. Gossypetin did not interact in a favorable way with Glu233 or Asp197, but did interact with Asp300, an amino acid residue reportedly involved in interaction with the alpha amylase inhibitor montbretin A.

The 2D interactions of the phytochemical delphinidin with 2QV4 (acarbose bound) and 2QV4 (acarbose taken off) are shown in Figures 2 and 3, respectively. The 2D interactions of the phytochemical quercetin with 2QV4 (acarbose bound) and 2QV4 (acarbose taken off) are shown in Figures 4 and 5, respectively. The 2D interactions show similar trend of amino acid residues interacting with the phytochemicals as given in Table 3.

Table 3: Non-bonding interactions of phytochemicals with amino acid residues of 2QV4 (with bound acarbose). Catalytic site amino acids of alpha amylase are depicted in bold

Compound	2QV4 (with bound acarbose)		2QV4 (without bound acarbose)	
	Interacting amino acid residue	Type of bonding	Interacting amino acid residue	Type of bonding
Delphinidin				
	ARG252	Conventional Hydrogen Bond	GLN63	Conventional Hydrogen Bond
	ARG252	Conventional Hydrogen Bond	ARG195	Conventional Hydrogen Bond
	PRO332	Conventional Hydrogen Bond	GLU233	Conventional Hydrogen Bond
	PRO4	Pi-Alkyl	ASP197	Pi-Anion
	PRO4	Pi-Alkyl	TRP59	Pi-Pi Stacked
			TRP59	Pi-Pi Stacked
			TRP59	Pi-Pi Stacked
			TRP59	Pi-Pi Stacked
			TYR62	Pi-Pi Stacked
			LEU165	Pi-Alkyl
Gossypetin				
	PRO332	Conventional Hydrogen Bond	GLN63	Conventional Hydrogen Bond
	GLY334	Conventional Hydrogen Bond	HIS299	Conventional Hydrogen Bond
	PHE335	Carbon Hydrogen Bond	ASP300	Conventional Hydrogen Bond
	ASP402	Pi-Anion	TYR62	Conventional Hydrogen Bond
	PRO4	Pi-Alkyl	TRP59	Pi-Pi Stacked
	PRO4	Pi-Alkyl	TRP59	Pi-Pi Stacked
			TRP59	Pi-Pi Stacked
			TRP59	Pi-Pi Stacked
			TYR62	Pi-Pi Stacked
Hibiscetin				
	ARG346	Conventional Hydrogen Bond	GLN63	Conventional Hydrogen Bond
	ASP353	Conventional Hydrogen Bond	ARG195	Conventional Hydrogen Bond
	ILE312	Conventional Hydrogen Bond	GLU233	Conventional Hydrogen Bond
	ILE312	Conventional Hydrogen Bond	ASP300	Conventional Hydrogen Bond
	GLN302 ARG303	Amide-Pi Stacked	TYR62	Conventional Hydrogen Bond
			ASP197	Pi-Anion
			TRP59	Pi-Pi Stacked
			TRP59	Pi-Pi Stacked
			TRP59	Pi-Pi Stacked
			TRP59	Pi-Pi Stacked

			TYR62	Pi-Pi Stacked
Quercetin				
	LEU69	Conventional Hydrogen Bond	GLN63	Conventional Hydrogen Bond
	HIS185	Conventional Hydrogen Bond	HIS299	Conventional Hydrogen Bond
	VAL129	Conventional Hydrogen Bond	ASP197	Conventional Hydrogen Bond
	LYS178	Conventional Hydrogen Bond	ASP197	Conventional Hydrogen Bond
	TYR67	Conventional Hydrogen Bond	TYR62	Conventional Hydrogen Bond
	GLU76	Conventional Hydrogen Bond	HIS305	Carbon Hydrogen Bond
	TYR67	Amide-Pi Stacked	TRP59	Pi-Pi Stacked
	LYS68	Pi-Alkyl	TRP59	Pi-Pi Stacked
	LYS68	Pi-Alkyl	TRP59	Pi-Pi Stacked

Besides being reported as an anti-diabetic plant with the potential to lower blood glucose [47], *Hibiscus sabdariffa* has other beneficial effects in diabetes-induced disorders. Aqueous extract of flowers has reportedly been shown to ameliorate diabetic nephropathy via oxidative status regulation in diabetic kidneys, improving high glucose-caused

osmotic diuresis in renal proximal convoluted tubules located in the renal cortex, and up-regulating transcription mediated by Akt/Bad/14-3-3 γ and NF- κ B [61]. Overall, the evidences point to the plant playing a role in control of DM and towards discovery of new anti-diabetic drugs.

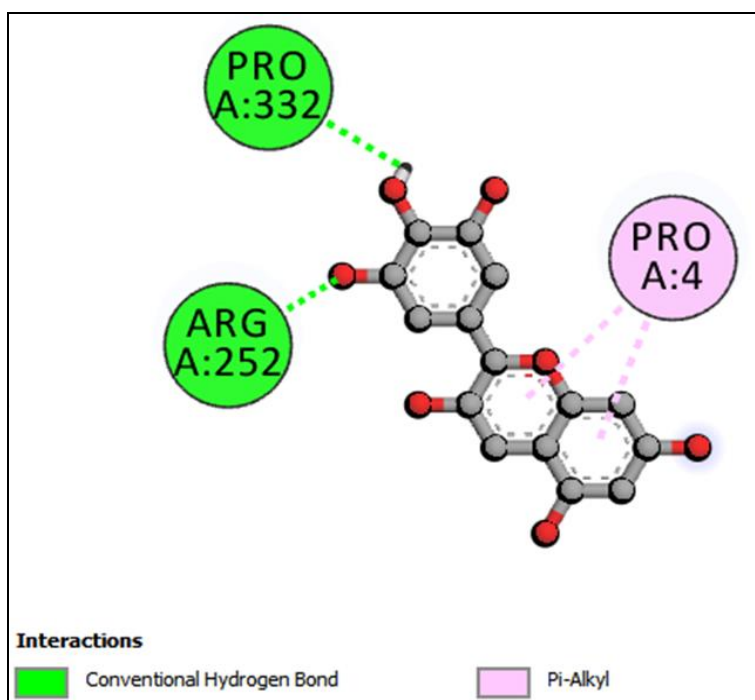


Fig 2: 2D interaction of delphinidin with 2QV4 (acarbose-bound).

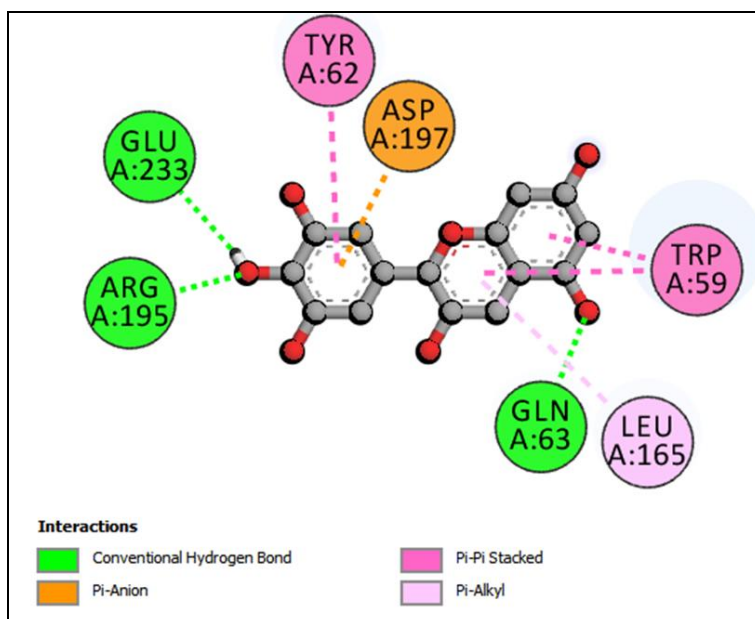


Fig 3: 2D interaction of delphinidin with 2QV4 (acarbose-not bound).

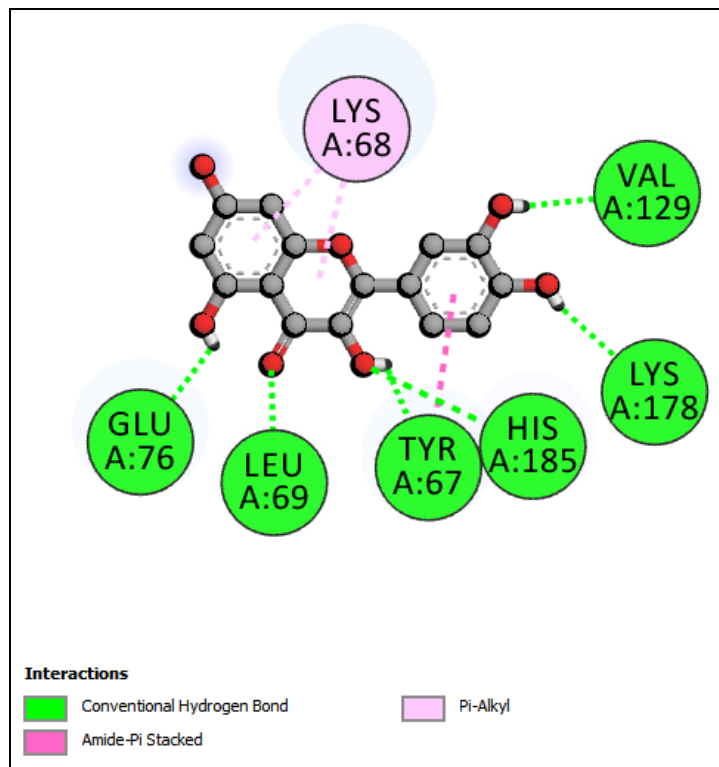


Fig 4: 2D interaction of quercetin with 2QV4 (acarbose-bound).

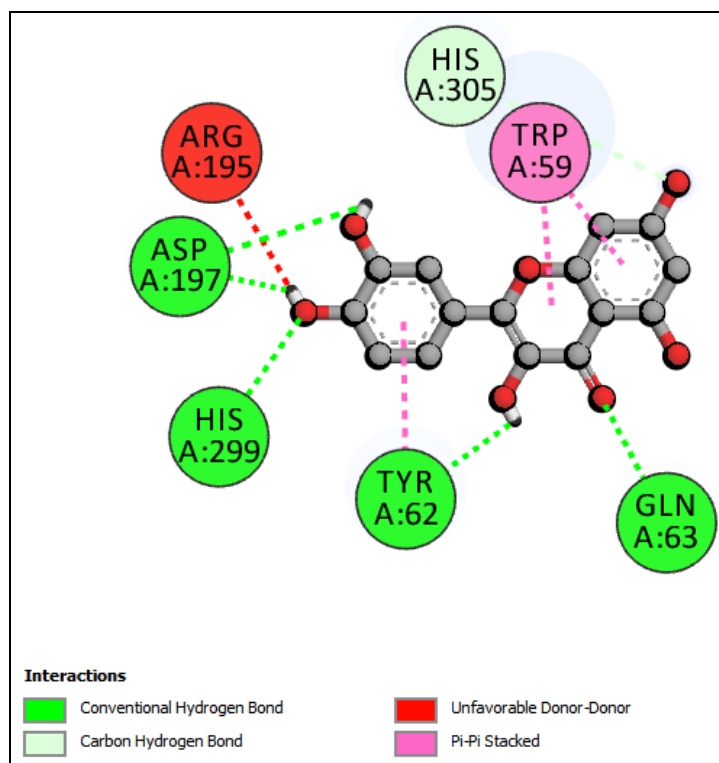


Fig 5: 2D interaction of quercetin with 2QV4 (acarbose-not bound).

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