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Flavonoids from *Argyreia nervosa* (Burm.f) Bojer: A Ready Arsenal against Pests as Well as Diabetes †

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Abstract: Diabetes mellitus -Type 2 (DM 2) has currently become one of the most challenging non-infectious diseases to treat. Enzymes such as alpha-amylase and alpha-glucosidase involved in carbohydrate metabolism are useful targets to treat the disease. Plants produce an immense variety of flavonoids with diverse biological activities. They are involved in interactions with other plants, animals and microbes. They can act as anti-microbial toxins or as anti- or pro-oxidants. We aimed to find out if flavonoids from leaf extracts of *Argyreia nervosa* (Burm.f) Bojer (Family: Convolvulaceae) could exhibit alpha-amylase inhibitory activity in vitro and in silico. The leaf flavonoids were extracted in chloroform by routine protocols and their profiling was carried out using LC-MS technique. The chloroform extract was also tested for its inhibitory activity against porcine pancreatic alpha-amylase enzyme in vitro, where it showed excellent inhibition. The molecular docking study was done only for flavonoids from LC-MS compound list using AutoDock 4.2.6. The compounds were docked against porcine pancreatic alpha-amylase (PDB ID: **1OSE**). This structure already contained a bound molecule of 'Acarbose' (a prescribed drug, an amylase inhibitor and positive control in our in vitro experiments). Out of these, top 4 flavonoids, Vitexin (Apigenin 8-C Glucoside, a flavone), Rutin (a flavonol), Myricetin (a flavonol) and Isoquercetin (a flavonol), showed the highest binding energies of −12.4 kcal/mol, −15.04 kcal/mol, −10.71 kcal/mol and −11.89 kcal/mol respectively. 'Acarbose' had binding energy of −11.48 kcal/mol. Thus all the 4 secondary metabolites showed comparable or higher binding energies than Acarbose. The ligands interacting with the amino acid residues ASP197, GLU233, TRP59 of Amylase protein seemed to show an excellent inhibitory effect among the 15 secondary metabolites studied. This is also proven by our experimental data which will be discussed in detail.

Keywords: Diabetes mellitus; alpha-amylase; flavonoids; in silico; *Argyreia nervosa*

1. Introduction

Diabetes mellitus (DM), a disorder that affects about 422 million people worldwide, is fast becoming a global concern [1]. Type 2 diabetes (DM 2) is the most common variant of DM and usually starts at later life (over the age of 40) and mainly in obese individuals. In this type, the insulin producing cells in the pancreas produce insulin, but the output is inadequate for the body's need or there is a defect in liberation or/and action of insulin, commonly referred to as 'insulin resistance'. Despite the introduction of several oral non-insulin drugs to treat DM 2, patient compliance remains poor due to severe side effects. Hence search for new molecules with reduced side effects is still continuing.

The conventional strategies of treating DM 2 include stimulation of endogenous insulin secretion, enhancing insulin action at the target site, use of oral hypoglycemic agents and inhibition of dietary starch digestion by glycosidases and alpha-amylases. Partial inhibition of carbohydrate digestion is one of the modes of action to reduce the blood glucose level. Pancreatic alpha-amylase (E.C. 3.2.1.1) plays a key role in digestion of food and catalyses hydrolysis of starch to produce oligosaccharides. These oligosaccharides are further digested by other enzymes to produce glucose which later on enters the blood-stream leading to post-prandial hyperglycemia. The correlation between the post-prandial hyperglycemia and activity of human pancreatic alpha-amylase has been reported previously. Inhibition of alpha-amylase results in delayed starch digestion and further reduces the rate of absorption of glucose into the blood [2].

Natural products are widely used for many human ailments due to lesser side effects on humans. Among these secondary metabolites, flavonoids are the common group of polyphenols that are consumed by humans regularly in their diet. Flavonoids are mainly divided into anthocyanins and anthoxanthins. Anthocyanins occur in colourful flowers and fruits while anthoxanthins are colorless compounds which are further categorized into flavones, flavans, flavonols, flavanols, isoflavones, and their gylcosides [3]. Flavonoids act as plants chemical defence compound [4].

Recently scientists have proved that polyphenolic compounds possess significant hypoglycemic properties. They act via different mode of actions such as inhibition of carbohydrate digestion, insulin stimulation from pancreatic beta cells, activation of insulin receptors, intestinal glucose absorption, modulation of glucose release from liver and modulation of intracellular signalling pathways and gene expression [5]. The phenols and flavonoids further inhibit the key enzymes in carbohydrate metabolism such as pancreatic alpha amylase and intestinal alpha glucosidase [6]. HPLC analysis methanolic extract of *Synsepalum dulcificum* showed that it contained rutin, quercitrin, isoquercitrin, kaempferol and few other secondary metabolites [7]. The flavonoid rich extract showed antidiabetic potential in type 2 diabetic rats [8].

Tadera et al. studied the inhibitory effect of few flavonoids against yeast and rat small intestinal alpha glucosidase and porcine pancreatic alpha amylase. This study showed that the flavonoids myricetin, quercetin and luteolin were potent porcine pancreatic alpha amylase inhibitors with the IC_{50} value less than 500 μM [9].

In recent years, in-silico methodologies have become an important aspect of drug discovery process due to their time saving and cost effective nature. These computer aided drug design (CADD) approaches are advantageous for reducing the use of animals for in vivo experiments. Many in-silico studies have been proposed to prove the potency of natural products to inhibit the alpha amylase and alpha glucosidase enzymes.

Piparo et.al reported that the structural arrangement of the flavonoids is very important to inhibit the alpha amylase enzyme. This computational ligand docking study suggested that the inhibitory potentiality of flavonoids depends on the conjugated π-system formation which stabilizes the interaction with active pocket and hydrogen bonds present in between the catalytic residues of binding sites and hydroxyl groups of polyphenol ligand. They reported that the flavonols such as myricetin, kaempferol, rhamnetin, isorhamnetin, quercetin, quercetagetin and fisetin showed higher docking scores suggesting the strong binding and inhibition of salivary alpha amylase. While for flavanols, flavanones, and isoflavones docking scores were lowest suggesting their inability to inhibit salivary alpha amylase [10].

Argyreia nervosa (Berm.f.) Bojer. is a well known medicinal plant which has many therapeutic properties like anti-inflammatory, anti-microbial, anti-viral, anti-ulcer, hepatoprotective, analgesic, immunomodulatory and anti-hyperglycemic [11]. The silver nano-particles synthesized using the aqueous leaf extract of *A. nervosa* exhibited the alpha-amylase and alpha-glucosidase inhibitory activity with increasing concentration [12]. The ethanolic root extract of *A. nervosa* proved effective in controlling hyperglycemia in normal and diabetic rats [13]. Latha et.al demonstrated that the dose dependent reduction in blood glucose level in normal and alloxan-induced diabetic rats was achieved when treated with alcoholic extract of *A. nervosa* [14]. Although this plant has been screened for its

secondary metabolite content, very few studies have reported the hypoglycemic activity and none have studied the mechanism of action of any of these metabolites.

The current study was carried out to identify and validate the alpha amylase inhibitory flavonoids from *Argyreia nervosa* (Berm.f.) Bojer. The identification of the flavonoids from chloroform leaf extract was done by using LC-MS technique and the inhibitory activity was demonstrated using preliminary in vitro starch iodide assay. Further, the results were validated by the molecular docking studies and highly promising compounds have been shortlisted.

2. Materials and Methods

2.1. Collection of Plant Material

The fresh and healthy leaves of *Argyriea nervosa* (Berm.f.) Bojer were collected from Savitribai Phule Pune University campus (Latitude 18.549120° N and Longitude 73.828120° E). The authentication of plant material was done by Botanical Survey of India, Western Regional Centre, Pune-7. (BSI/WRC/IDEN.CER./2020/94)

2.2. Preparation of Plant Extracts

The fresh collected leaves were washed under running tap water, dried and then chopped into small pieces. Using muslin cloth, this sample was wrapped. The hot extraction i.e., Soxhlet extraction method was used. Sequential extraction was carried out using 6 solvents with increasing polarity.The extracts were concentrated using rotary evaporator and stored at room temperature. 50 g of fresh leaves were used for extraction. The yield of Pet ether, Chloroform, Ethyl acetate, Acetone, Methanol and Water extracts was 0.19%, 0.33%, 3.5%, 3.24%, 5.74% and 5.90% respectively.

2.3. Preliminary Phytochemical Analysis

The extracts obtained were subjected to preliminary phytochemical analysis using standard procedures given by Trease & Evans (2002) to determine the presence of different phytochemicals [15].

2.4. Preliminary Starch-Iodide Assay

The method reported by Ponnusamy et.al (2011) was used to perform alpha-amylase inhibition assay [16]. Alpha-amylase enzyme from porcine pancreas (PPA) (Sigma-Aldrich, USA, Catalogue No. A6255) was used for the assay based on colour reaction produced by the starch iodine complex (starch-iodide assay). The assay mixture consisted of phosphate buffer (pH 6.9 containing 6.7 mM sodium chloride), 0.02 units of PPA enzyme and 20 µL of plant extract in DMSO (1 mg/mL *w*/*v*). The assay mixture was incubated at 37 °C for 15 min. Then soluble starch (1%, *w*/*v*) was added to each assay and reincubated at 37 °C for 15 min. 20 µL of 0.2 M Tris solution was used to stop the enzymatic reaction after the incubation period and finally 20 µL of Iodine reagent was added. The color change of the reaction mixture was observed. The positive drug control (SC) was a synthetic, known PPA inhibitor, acarbose (Glucobay tablets, Bayer). A dark-blue colour indicated the presence of starch; implicating inhibition of amylase enzyme by the plant extract. A yellow colour indicated the absence of starch implicating fully active amylase enzyme that degraded the starch and hence absence of amylase inhibitory activity in the plant extract. A brownish colour indicated partially degraded starch in the reaction mixture.

2.5. Determination of Total Flavonoid Content

For determination of total flavonoid content, aluminium chloride colorimetric method was followed with slight modifications [17]. The stock solution of 5 mg/mL of chloroform extract was used. The sample (0–200 µL) was mixed with 1.25 mL distilled water and 75 µL 5% $NaNO_2$ solution prior to 5 min incubation period. 150 µL of 10% $AlCl_3$ was added and again the test solution was incubated for 6 min. Then 500 µL of 1 M NaOH and 275 µL distilled water was added to the test

solution. The absorbance was read at 510 nm using UV-VIS spectrophotometer (UV-1800, SHIMADZU, Japan). The total flavonoid content was calculated using calibration curve. The results were expressed as a quercetin (Sigma, St. Louis, MO, USA) equivalent in μg/mg of sample.

2.6. Column Chromatography

The column chromatography was performed to separate the compounds from the chloroform leaf extracts showing promising alpha amylase inhibitory activity in the preliminary starch iodide assay. 5 mg of concentrated extract was dissolved in 1 mL of chloroform and passed through the 20 cm long silica gel (60–120 mesh size, SDFCL, India) column with 20 mL chloroform, methanol and water each, successively. The fractions were collected and again checked for PPA inhibitory activity. The fraction showing positive result was carried forward for the LC-MS analysis.

2.7. LC-MS Analysis

The MS acquisitions were performed in both positive and negative electrospray ionization mode. The capillary voltage, cone voltage, fragmentor voltage were 4 kV, 45 V and 170 V, respectively. The gas temperature was set at 325 °C. Data was acquired at scan rate of 3 Hz in mass range 100–100 *m*/*z*. Data was further analyzed with Mass Hunter qualitative software and METLIN database.

The system used was Agilent 1260 binary LC System and 6540 ultra-high definition accurate mass QTOF LC/MS system for liquid chromatography and mass spectrometry respectively. A 1 μL aliquot of sample solution was injected into Agilent Zorbax Extend C18 RRHT column (50 × 2.1 mm, 1.8 μm) with the flow rate of 0.3 mL/min and run time of 30 min. The mobile phase consisted of H_2O-0.1% formic acid (A) and Acetonitrile (B), and the gradient elution procedures were performed as follows: 0–5 min (95% A: 5% B), 5–18 min (5% A: 95% B), 18–27 min (5% A: 95% B), 27–27.10 min (95% A: 5% B) and 27.10–30 min (95% A: 5% B).

2.8. Molecular Docking Studies

2.8.1. Softwares and Tools

Protein Data Bank (PDB), PubChem, Open Babel, Discovery Studio Visualizer, AutoDock Version 4.2.6, MGL Tools, CastP.

2.8.2. Ligand Preparation

The secondary metabolites detected in LC-MS analysis were used as the ligands for docking studies. Their 3D structures were obtained from PubChem database (https://pubchem.ncbi.nlm.nih.gov/) in SDF format. Open Bable software (https://github.com/openbabel/openbabel/releases/tag/openbabel-3-1-1) was used to convert this SDF format to PDB format of ligand.

2.8.3. Protein Preparation

The crystal structure of PPA (PDB ID: 1OSE) was retrieved from Protein Data Bank (https://www.rcsb.org/) in PDB format [18]. This retrieved structure was complexed with water molecules, other atoms and ligands which increases its resolution. The protein was prepared using Discovery Studio software where water molecules, other heteroatoms and previously co-crystallized ligands were removed so as to free up the active sites on protein molecule. Using AutoDock 4.2.6, the polar hydrogen atoms and Kollman charges were added on protein to achieve a well protonated state. This prepared protein was saved as a .pdbqt format file prior to docking [19].

2.8.4. Binding Pocket Identification

The binding pocket is a cavity on the surface or in the interior of a protein where the ligand molecule can fit while interacting with the protein. The online software CASTp (http://sts.bioe.uic.edu/castp/) was used to identify the binding pocket of PPA. [20].

2.8.5. Docking Studies Using AutoDock

The prepared ligands were docked on the receptor PPA protein (both in PDBQT file format) using AutoDock 4.2.6. A grid box with spacing 0.375 A° centered at (72 A° × 60 A° × 65 A°) was set using the knowledge of active sites present on the receptor protein. For docking, the receptor protein was set as a rigid molecule and the ligand was set as a flexible molecule.

2.8.6. Protein-Ligand Interaction

The software Discovery Studio Visualizer (https://discover.3ds.com/discovery-studio-visualizer-download) was used to visualize the protein-ligand interactions [19] in 2-D and 3-D formats (3-D Data not shown). The pdb file containing the docked conformation was investigated for the presence of intermolecular interactions and their strengths which includes hydrogen bonds and Van der Waal interactions.

2.8.7. Lipinski Rule for Ligands

The selected ligand molecules for docking studies were screened for Lipinski's rule of five [21]. This rule states that the drug molecule should not violate more than one among the five rules enlisted below:

(1) Molecular mass <500 Da. (2) Hydrogen bond donors <5 (3) Hydrogen bond acceptors <10 (4) High lipophilicity (Log p < 5) (5) Molar refractivity between 40 and 130

The Supercomputing Facility for Bioinformatics & Computational Biology, IIT Delhi (http://www.scfbio iitd.res.in/software/drugdesign/lipinski.jsp) was used to check the Lipinski's rule for the selected metabolites where the PDB structures of the metabolites were uploaded to their online server [22].

3. Results

3.1. Preliminary Phytochemical Analysis

Although the phytochemical analysis was performed for all the extracts, here we are presenting data only related to chloroform extract as this extract showed the best PPA inhibition. The preliminary phytochemical analysis of the chloroform extract of leaves of *A. nervosa* majorly showed the presence of phenols, flavonoids, terpenes and quinines.

3.2. Preliminary Starch-Iodide Assay

In this assay, among the six prepared extracts pet ether, chloroform and ethyl acetate extracts showed the positive results (Figure 1). But only the cholroform extract was carried forward for further analysis.

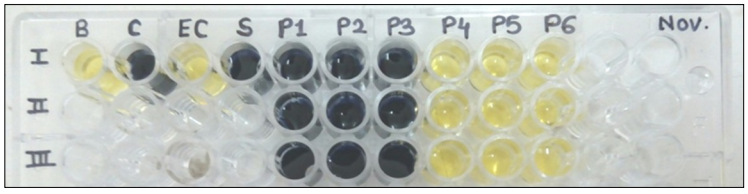

Figure 1. PPA Inhibitory Activity of Various Plant Extracts Using Qualitative Starch Iodide Assay. B: Blank, C: Control, EC: Enzyme Control, SC: Substrate (drug) Control, P1: Pet Ether, P2: Chloroform, P3: Ethyl Acetate, P4: Acetone, P5: Methanol, P6: Water.

3.3. Determination of Total Flavonoid Content

Total flavonoid content of chloroform leaf extract calculated using the calibration curve was 378.83 ± 0.76 µg/mg sample.

3.4. Column Chromatography

The fourth fraction eluted with chloroform exhibited positive result for starch-iodide assay and it was carried forward for LC-MS analysis.

3.5. LC-MS Analysis

The LC-MS analysis revealed the presence of total 20 secondary metabolites belonging to different classes (Unpublished data). Among these, 4 compounds belonging to class flavonoid were selected for in-silico study.

3.6. Molecular Docking Studies

3.6.1. Ligand Preparation

The 3D ligand structures (Figure 3) were retrieved from PubChem database in SDF format and converted to PDB format using Open Bable software. The structures were saved in PDBQT format after energy minimizing step using MGL tools.

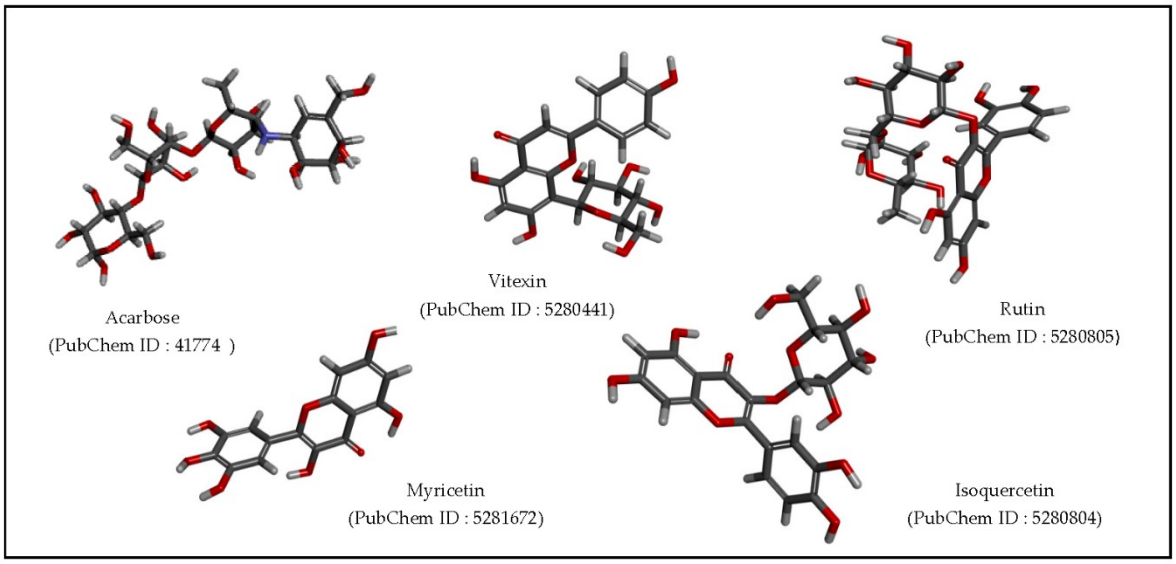

Figure 2. The 3D ligand structures used in docking studies for targeting alpha-amylase enzyme.

3.6.2. Protein Preparation

The crystal structure of PPA (PDB ID: 1OSE, Figure 3) was retrieved from Protein Data Bank. The appropriate procedure was followed to prepare protein structure suitable for docking.

Figure 3. Prepared porcine pancreatic alpha amylase protein structure.

3.6.3. Binding Pocket Identification

The binding pocket was identified by using CASTp online software. The PPA has following amino acids in its binding pocket: TRP58, TRP59, TYR62, GLN63, HIS101, TYR151, LEU162, VAL163, LEU165, ARG195, ASP197, ALA198, LYS200, HIS201, GLU233, ILE235, ASN298, HIS299, ASP300, HIS305 and GLY306 (Figure 4).

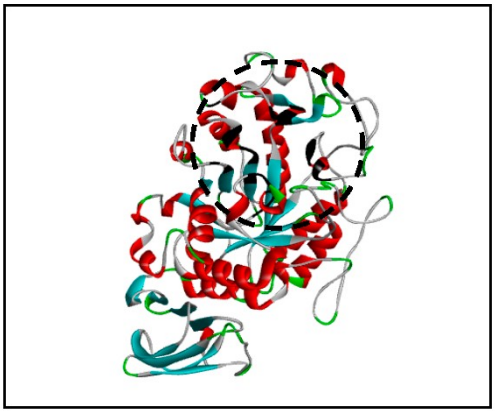

Figure 4. PPA binding pocket. (Amino acid residues shown in black colour inside dotted circle).

3.6.4. Docking Studies Using AutoDock

The previously mentioned ligands were docked on receptor protein i.e PPA using AutoDock Version 4.2.6. The docking score is represented as binding energy in kcal/mol. All four ligands showed better or comparable binding capacity with respect to the drug Acarbose (Figure 5). Acarbose showed binding energy of −11.48 kcal/mol whereas other four ligands that are vitexin, rutin, myricetin and isoquercetin showed binding energies of −12.4 kcal/mol, −15.04 kcal/mol, −10.71 kcal/mol and −11.89 kcal/mol respectively.

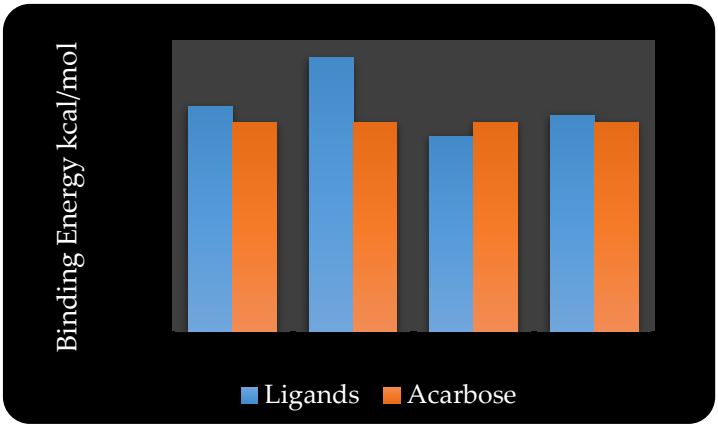

Figure 5. The comparative graph of binding energy of ligands and Acarbose.

3.6.5. Protein-Ligand Interaction

The protein-ligand interactions were studied using Discovery Studio Visualizer. The interactions of ligands with the amino acid residues of the target protein are shown in Table 1. The 2-D structures of protein-ligand interaction are shown in Figure 6. The 2-D structures depicted all the intermolecular interactions such as conventional hydrogen bonds, pi-interactions, Van der Waals interactions, carbon hydrogen bonds as well as the unfavourable ones.

Table 1. The binding energy of ligands and acarbose with PPA.

Sr. No	PubChem ID	Compound Name	Binding Affinity kcal/mol	No. of H bonds formed	Interacting Residues with Their Bond Length A°
1	41774	Acarbose	−11.48	7	GLY 306-1.95 A°, ILE 235-2.77 A°, TYR 151-2.19 A°, TYR 151-2.28 A°, TYR 151-2.34 A°, ASP 197-2.07 A°, ASP197-2.17 A°.
2	5280441	Vitexin	−12.4	6	ASP 197-2.00 A°, ASP 197-2.21 A°, ASP 300-2.12 A°, ASP 300-2.19 A°, HIS 299-2.76 A°, GLU 233-2.03 A°.
3	5280805	Rutin	−15.04	6	TRP 59-2.63 A°, TRP 59-2.06 A°, GLU 233-2.00 A°, ASP 197-1.67 A°, HIS 101-2.70 A°, LYS 200-3.20 A°.
4	5281672	Myricetin	−10.71	4	TYR 62-2.37 A°, ASP 197-1.74 A°, ASP 197-1.83 A°, ASP 300-2.24 A°.
5	5280804	Isoquercetin	−11.89	5	GLY 306-2.97 A°, ASP 197-2.18 A°, GLU 233-2.13 A°, GLU 233-1.94 A°, TRP 59-2.14 A°.

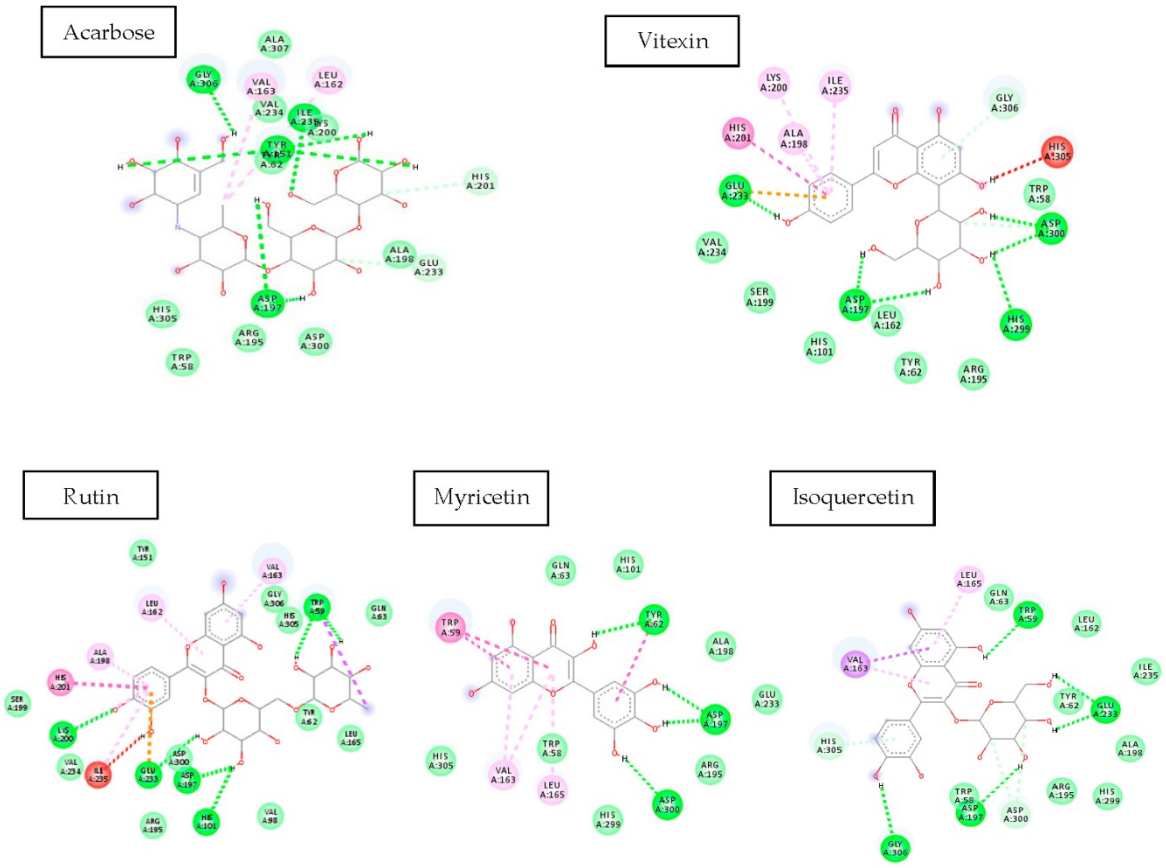

Figure 6. 2-D representation of different types of intermolecular protein-ligand interactions. Fluorescent Green: Conventinal H Bond, Light Green: Van der Waal interactions, Light Pink, Dark Pink, Purple, Orange: Pi-interactions, Red: Unfavourable Donor-Donor.

3.7. Lipinski Rule for Ligands

The ligands used in docking studies were also screened for the Lipinskie Rule of Five. The results showed that vitexin and myricetin violated just one rule. Isoquercetin and rutin violated 2 and 3 rules respectively (Table 2).

Table 2. Physicochemical parameters of ligands screened for Lipinski Rule.

Sr. No	Ligand	Molecular Weight g/mol	Hydrogen Bond Donor	Hydrogen Bond Acceptor	LogP	Molar Refractivity	Rules Satisfied
1	Vitexin	432.4	7	10	−0.065500	103.534050	4/5
2	Rutin	610.5	10	16	−1.878800	137.495483	2/5
3	Myricetin	318.23	6	8	1.716500	75.715279	4/5
4	Isoquercetin	464.4	8	12	−0.730600	106.273842	3/5

4. Discussion

Plants produce a variety of organic compounds as a result of their metabolic processes. These secondary metabolites play a key role in the plant's own survival as they are involved in interactions with other plants, animals and microbes. Flavonoids, a group of plant secondary metabolites, have been studied by researchers to a great extent as they possess useful properties such as anti-cholinesterase activity, anti-inflammatory activity, radical scavenging activity and many others. They also play key role as phytoalexins, signal molecules, allopathic compounds, detoxifying agents and antimocrobial defensive compounds. These flavonoids are also reported for their insecticidal and

antimicrobial property as they interfere with nucleic acids and proteins. Therefore they are important compounds in developing pesticides in agriculture industry [23].

In the present study, four compounds belonging to class flavonoids have been studied for their porcine pancreatic alpha amylase (PPA) inhibition potential. These were isolated from the PPA inhibitory chloroform extract of the leaf of *A. nervosa*. The phytochemical analysis of this extract showed the presence of phenols, flavonoids, terpenes and quinones. Out of them, flavonoids were present in abundance as the total flavonoid content was 378.83 ± 0.76 μg/mg sample.

Column chromatography of this chloroform extract was performed to get rid of the chlorophyll present and to separate metabolites. Among the fractions eluted, the 4th fraction eluted with chloroform exhibited significant alpha amylase inhibitory activity. The LC-MS analysis of this fraction showed presence of different secondary metabolites. Only a part of the data is presented here.

The four flavonoids, vitexin, rutin, myricetin and isoquercetin were docked onto the PPA enzyme structure because had shown better or comparable PPA inhibitory results to acarbose, the positive control used in our experiments. The mechanism of action of flavonoids suggests that the binding of these flavonoids depends on their structure and the bonding pattern with the receptor molecule. The conjugated pi-system formation is important for the stabilization of the interaction with the binding pocket. In our study it was observed that all the four flavonoids formed pi-interactions with active site amino acid residues (Figure 6). These pi-interactions were pi-anion, pi-alkyl, pi-pi T-shaped, pi-pi stacked, pi-sigma and pi-donor hydrogen bond. When acarbose was studied for its pi-interactions, it was observed that no pi-interaction was formed by acarbose with target protein. These results provided the insight about the higher binding energy of flavonoids than acarbose. The pi-interactions formed in between the flavonoid and target protein strengthened the binding and resulted in significant docking score.

In molecular docking studies, it was observed that the amino acid residues ASP197, GLU233 and TRP59 were common for the four flavonoids from *A. nervosa* showing significant binding energies. In literature, it is reported that the PPA binding pocket possesses these three amino acid residues as a part of the active site where the inhibitor or the ligand actually binds [24]. Also the other amino acid residues such as ASP300, HIS101, HIS299 and LYS200 are part of the active site of the receptor.

Like other glycosidases, active site carboxylic acid is used to catalyse the hydrolytic reaction by alpha amylase. The amino acid residues ASP197, GLU233 and ASP300 were described as the catalytic residues of the active site [25]. It was suggested that the ASP197 acts as a nucleophile which attacks sugar anomeric centre to form a covalently bound reaction intermediate. During this process, the substrate's reducing end is cleaved off the sugar skeleton. Afterwards to break the covalent bond formed between ASP197 and substrate, water molecules attack the anomeric centre. In both the processes GLU233 and ASP300 act as acid/base catalysts independently or cumulatively. The molecular docking results of the four studied flavonoids showed that the above mentioned catalytic amino acid residues were involved in the hydrogen bond formation in between the ligand and the target protein which suggests that each of the ligand is inhibiting the protein efficiently.

When the results of four flavonoids were compared to the result of acarbose (Table 1), they demonstrated that our ligand molecules formed strong interaction with catalytic residues ASP197 and GLU233 or ASP300 to inhibit the alpha amylase enzyme. This also explained the strong PPA inhibition that we had observed in our biochemical assays. In the current study, all the four flavonoids studied were interacting with these essential catalytic residues better than acarbose and these can be developed as potent alpha amylase inhibitors. It will also be interesting to see if these flavonoids show better PPA inhibition singly or synergistically.

The flavonoids were also checked for Lipinski's rule of five. Myricetin and vitexin showed promising results as they violated just one rule of Lipnski. Though some parameters were not followed by the flavonoids studied they can be considered as the strong candidates in the drug designing process against hyperglycemia.

5. Conclusions

The aim of the present study was to find out the porcine pancreatic alpha amylase inhibitory secondary metabolites from *Argyreia nervosa* (Burm.f) Bojer. The chloroform leaf extract of *A. nervosa* was richer in flavonoids as compared to phenols. This extract also demonstrated very strong alpha amylase inhibitory activity biochemically. Out of diverse secondary metabolites identified with LC-MS-MS from chloroform extract, vitexin, rutin, myricetin and isoquercetin showed significant alpha amylase inhibitory activity in silico as well, with rutin showing highest binding energy. ASP197 was the essential amino residue involved in the intermolecular interactions. Also the conjugated pi-system formation was important for stabilizing the interaction. These are the critical aspects in binding of flavonoids to the target protein PPA. This work provides important insights towards drug discovery process for controlling diabetes mellitus.

Author Contributions: A.A.K. established the work flow, designed the experiments, standardized the experimental protocols, contributed in results analysis and manuscript writing. A.D.K. performed the in vitro and in silico experiments and contributed in manuscript writing. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

DM 2	Diabetes mellitus Type 2
LC-MS	Liquid Chromatography-Mass Spectrometry
HPLC	High Performance Liquid Chromatography
CADD	Computer Aided Drug Design
PPA	Porcine Pancreatic Amylase
DMSO	Dimethyl Sulfoxide
SC	Substrate Control

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