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Proceeding Paper

Potential Hypoglycemic Secondary Metabolites from *Argyreia nervosa* (Burm. f.) Bojer Influencing Human Gut Health †

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Abstract: In the last few decades, natural products from plants have developed immense importance in human health due to their therapeutic multi-functionality. They have also been reported to enhance human gut health, which is another important factor in overall human health. Diabetes Mellitus Type 2 (DM 2) is now a global concern with 6.28% of the world's population affected by it. Many hypoglycemic drugs currently available in the market are either directly or indirectly based on a number of plant secondary metabolites. In the current study, we aimed to find out the hypoglycemic secondary metabolites from a leaf methanolic extract of *Argyreia nervosa* (Burm. f.) Bojer (Family: Convolvulaceae). In the in vitro experiment, this extract showed good inhibitory activity against Porcine Pancreatic Alpha-amylase (PPA) with IC_{50} value of 1.1 mg/mL. The presence of Quercetin and Ursolic acid was identified in the leaf methanolic extract using HPTLC, HPLC, and MS analysis. The calculated IC_{50} values against PPA, for standard Quercetin and Ursolic acid, were 16.5 µg/mL and 13.2 µg/mL, respectively. The in silico studies used both of these compounds as ligands against PPA (PDB ID: 1OSE) in AutoDock 4.2.6. Significant binding energies of −9.89 kcal/mol and −8.96 kcal/mol were seen for Quercetin and Ursolic acid, respectively, while Acarbose (the drug used as the positive control) had a binding energy of −12.48 kcal/mol. Both Quercetin and Ursolic acid strongly interacted with the pivotal amino acid residues such asGlu233, Asp197, and Asp300, which are present at the active site of PPA, thus upholding our in vitro experimental results. Both the compounds have exhibited beneficial effects on human gut health in DM 2 and related complications. The docking results of them with a few intestinal markers that are significant in gut health will also be discussed.

Keywords: *Argyreia nervosa*; Diabetes Mellitus Type 2; Quercetin; Ursolic acid; human gut health; molecular docking

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1. Introduction

Plants have impacted modern medicine by supplying several phytochemicals with therapeutic multi-functionality. In recent years, mankind has faced various disease outbreaks, in which plant-based medicines have proven their supremacy. Today, among the different life-style diseases, Diabetes mellitus Type 2 (DM 2) has received the attention of scientists and researchers worldwide. It is becoming a global concern as about 6.28% of the world's population is affected by it [1]. DM 2 is a metabolic disorder that is characterized by hyperglycemia due to escalating defects in insulin secretion on the background of insulin resistance. Presently, there are a number of synthetic medicines available to treat this condition. However, these medicines have some side effects on human health. Thus, the hunt for new plant-based anti-hyperglycemic molecules is still on.

One of the most common strategies to treat DM 2 is to inhibit the isoforms of the α-amylase enzyme. This enzyme plays a crucial role in the digestion of food and catalyzes the hydrolysis of starch, increasing blood sugar levels. The association between human pancreatic α-amylase and post-prandial hyperglycemia has been published previously. The delayed starch digestion caused by α-amylase inhibition reduces the rate of absorption of glucose into the blood [2]. Apart from the usual DM 2 complications, recent reports have shown that the human gut microbiota also is negatively affected due to DM 2. Almost 90% of the adult human gut microbiota consists of either Bacteroidetes or Fermicutes. In DM 2 patients, the ratio of Bacteroidetes to Fermicutes decreased and there was a significant drop in the abundance of Bifidobacteria [3].

Plant secondary metabolites belonging to different classes such as phenols, flavonoids, terpenes, glycosides, alkaloids, and saponins have been reported for their α-amylase inhibitory potential. Among them, flavonoids have been extensively studied for their positive effect on the gut microbiota of DM 2 patients [4]. *Argyreia nervosa* (Burm. f.) Bojer is a medicinal vine that has been utilized for many years due to its therapeutic properties [5]. Our own group has shown that the flavonoids from *A. nervosa*, namely, Vitexin, Myricetin, Isoquercetin, and Rutin, have strong α-amylase inhibitory potential [6]. However, none of the earlier studies have reported the effect of the secondary metabolites of *A. nervosa* on human gut microbiota.

Toll-like receptors (TLRs) are the pattern recognition receptors present in the immune cells and intestinal epithelial cells of humans. They play a pivotal role in initiating the inflammatory response toward a broad spectrum of microbial components and controlled activation of both innate and adaptive immunity. Scientific reports have shown an increase in the activity and expression of TLR2 in both type-I and type-II diabetes [7]. It is reported that the microbial consortium present in the intestine is one of the important factors regulating the TLR activation. However, it is also well studied that dietary habits modulate the gut microbiota composition [8].

In the present study, we identified two important pancreatic α-amylase (PPA) inhibitory secondary metabolites, namely, Quercetin and Ursolic acid from *A. nervosa* that also impact the human gut microbiota. The identification of secondary metabolites was achieved using HPLC and ESI/MS analysis and the PPA inhibitory capacity was demonstrated by in vitro quantitative DNSA assay. Further, the results were validated by the molecular docking studies using the crucial proteins involved in the food digestion (PPA) and human gut microbiota stability (TLR2).

2. Materials and Methods

2.1. Collection and Identification

The leaves of *Argyreia nervosa* (Burm. f.) Bojer were collected from Savitribai Phule Pune University campus (Latitude 18.549120° N and Longitude 73.828120° E). The collected specimen was deposited and identified at the Botanical Survey of India, Western Regional Centre, Pune-7 with voucher number (BSI/WRC/IDEN.CER./2020/94).

2.2. Preparation of Leaf Extracts

The hot extraction method was followed using Soxhlet apparatus to prepare the leaf extracts. A sequential extraction using six solvents with increasing polarity viz. pet ether, chloroform, ethyl acetate, acetone, and water was carried out. These extracts were concentrated using a rotary evaporator and stored at 4 °C for further use.

2.3. Preliminary Phytochemical Analysis

The preliminary phytochemical tests were performed in triplicates to check the presence of different phytochemicals [9].

2.4. Qualitative Starch-Iodide Assay

The preliminary check of the PPA inhibition potential of the leaf extracts was carried out by performing the method proposed by Zinjarde et al. [10] and detailed in Kulkarni and Kamble [6].

2.5. HPTLC Analysis

2.5.1. Preparation of Standard Stock Solutions

The standard solutions of Quercetin (Sigma-Aldrich, St. Louis, MO, USA, Catalogue No. Q4951) and Ursolic acid (Sigma-Aldrich, USA, Catalogue No. U6753) were prepared at a concentration of 1 mg/mL each in absolute methanol. Ultra-sonication ensured acomplete dissolution.

2.5.2. Sample Preparation

The dry methanol leaf extract of *A. nervosa* was accurately weighed to be 5 mg and re-dissolved in 1 mL methanol. This sample was sonicated and filtered through a 0.45 µm membrane filter prior to application on a TLC plate.

2.5.3. Instrumentation and Experimental Conditions

The chromatography was performed according to the method reported with slight modifications [11]. The HPTLC system utilized was created by CAMAG (CAMAG, Muttenz, Switzerland). The mobile phases Toluene: Ethyl acetate: Formic acid (5:4:1 *v/v/v*) and Toluene: Acetone: Formic acid (7.8:2.2:0.15 *v/v/v*) were used for the separation of Quercetin and Ursolic acid, respectively. The obtained data were analyzed using WinCATS software (Version 1.4.5) by measuring the peak areas.

2.6. HPLC Analysis

The HPLC analysis was carried out on Waters 2998 HPLC system (Milford, MA, USA) coupled with a photodiode array detector at the Central Instrumentation Facility of S. P. Pune University. The eluted fractions were monitored at 254 nm and were collected at different time points. These collected fractions were checked for their PPA inhibitory potential as mentioned in Section 2.4 and the positive fractions were carried forward for ESI/MS analysis to detect the active compounds.

2.7. ESI-MS Analysis

The electrospray ionization mass spectral measurements were performed on a Bruker Nano-Advance UHPLC LC-MS-MS with a TOF analyzer at the Central Instrumentation Facility of S. P. Pune University. The dry methanolic extract was redissolved in HPLC grade methanol and filtered through 24 µM filter before being injected into the mass spectrometer.

2.8. Quantitative 3,5-Dinitrosalicyclic Acid (DNSA) Assay

To quantify the PPA inhibition, a widely used DNSA assay was performed according to the method proposed by Miller in 1959 [12], and the calculations were performed using the formulae detailed by Kamble et al. [13].

2.9. Statistical Data Analysis

Each experiment was performed in triplicates. The presented data are expressed as mean ± SEM (Standard Error of Mean). The ANOVA was performed for the statistical analysis using PAST version 4.11. The p values ≤ 0.05 were considered as significant.

2.10. In Silico Docking Studies

2.10.1. Protein Preparation

The 3D crystal structures of PPA (PDB ID: 1OSE) and TLR-2 (PDB ID: 5D3I) were retrieved from the Protein Data Bank. Prior to docking, the protein was prepared by removing the pre-existing water and ligand molecules using Discovery Studio Visualizer

v20.1.0.19295. The polar hydrogen atoms and Kollman charges were added using AutoDock 4.2.6 software [14]. This processed protein was subsequently saved as PDBQT file and used for docking.

2.10.2. Ligand Preparation

The 3-D structures of the ligand molecules were obtained from the PubChem database in SDF format. Using Discovery Studio Visualizer, these ligands were converted to PDB format.

2.10.3. Docking Studies

The prepared ligands were docked on the receptor proteins PPA and TLR-2 using AutoDock 4.2.6. For PPA, a grid box with spacing 0.375A°centered at (45 A° × 40 A° × 44 A°) was generated at the center of the co-crystallized ligand (Acarbose, positive control) [13]. For TLR-2, the grid box with spacing 0.375 A° centered at (100 A° × 90 A° × 90 A°) was generated around the binding pocket detected with the help of a CASTp online server [15]. The Lamarckian Genetic Algorithm was used for searching the best conformer of ligand molecules. In the docking process, a total of 10 conformers were considered for each compound. Of these 10 conformers, the one with the lowest binding energy and inhibition constant was regarded as having the highest affinity toward PPA and TLR2. Discovery Studio Visualizer was used to visualize the docked conformations of the interacting ligand molecules with selected proteins.

3. Results

3.1. Preliminary Phytochemical Analysis

Although the phytochemical analysis was performed for all the prepared extracts, here, we present the data related to methanol extract as they showed good PPA inhibition. The preliminary phytochemical analysis of the methanol leaf extract of *A. nervosa* showed the presence of phenols, flavonoids, terpenes, and alkaloids.

3.2. Qualitative Starch-Iodide Assay

The qualitative starch-iodide assay was performed and analyzed on the basis of the color of the reaction, which is related to the starch-iodine complex formation. The deep blue color of the reaction suggested that the PPA enzyme was inhibited by the plant secondary metabolites present in the extract keeping the starch undigested. The yellow color of the reaction indicated a complete digestion of the starch by the fully active PPA enzyme. The brownish color of the reaction suggested the partial degradation of the starch. In this assay, chloroform, acetone, and methanol extracts showed good inhibitory activity. However, the methanol extract was carried forward as the deep blue color of the inhibition persisted even after 24 h.

3.3. HPTLC Analysis

Through HPTLC analysis, it was observed that the methanolic leaf extract of *A. nervosa* possessed two important plant secondary metabolites, namely, Quercetin and Ursolic acid. Quercetin was separated and detected on the TLC plate (Figure 1A,B) and Ursolic acid was detected and separated on the TLC plate (Figure 2A,B).

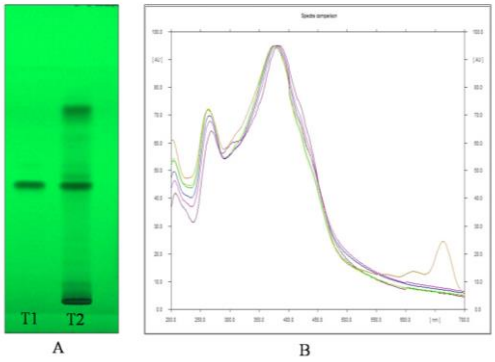


Figure 1. (**A**) Image of HPTLC separation of leaf methanolic extract of *A. nervosa*. Track T1 represents Quercetin standard and track T2 represents leaf methanolic extract of *A. nervosa* with matching band of quercetin with Rf value of 0.48. (**B**) Matching identity of Quercetin in standard (Shown in different colours for different standard concentration) and sample track (Orange coloured) by comparing UV-VIS overlay spectra at 380 nm.

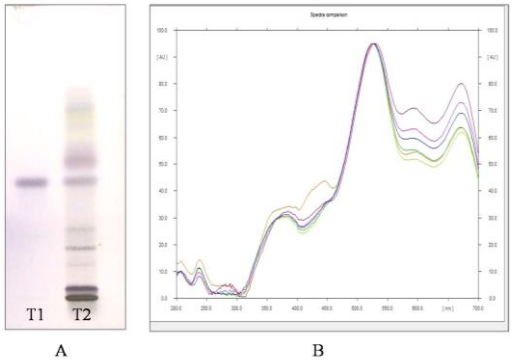


Figure 2. (**A**) Image of HPTLC separation of leaf methanolic extract of *A. nervosa* after derivatization with anisaldehyde-sulphuric acid reagent. Track T1 represents Ursolic acid standard and track T2 represents leaf methanolic extract of *A. nervosa* with matching band of Ursolic acid with Rf value 0.57. (**B**) Matching the identity of Ursolic acid in standard (Shown in different colours for different standard concentration) and sample track (Orange coloured) by comparing UV-VIS overlay spectra at 540 nm.

3.4. HPLC Analysis

The crude methanolic leaf extract was subjected to the HPLC analysis. The peaks of this sample were collected at three different time points, namely, at 5 min, 6–12 min (5 components), and 14 min (Figure 3). These fractions were subjected to starch-iodide assay. Among these, the fraction collected at 14 min showed strong PPA inhibitory activity. Hence, this fraction was further analyzed using ESI mass spectrometry.

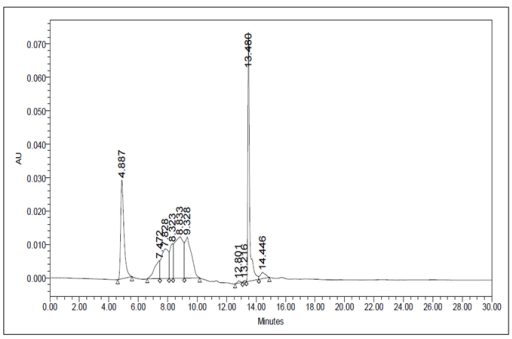


Figure 3. HPLC chromatogram of *A. nervosa* leaf methanol extract.

3.5. ESI-MS Analysis

The ElectroSpray Ionization-Mass Spectrometry (ESI-MS) analysis indicated the presence of two abundant peaks of molar mass 301.1400 and 467.1931, which are assigned to Quercetin and Ursolic acid, respectively (Figure 4).

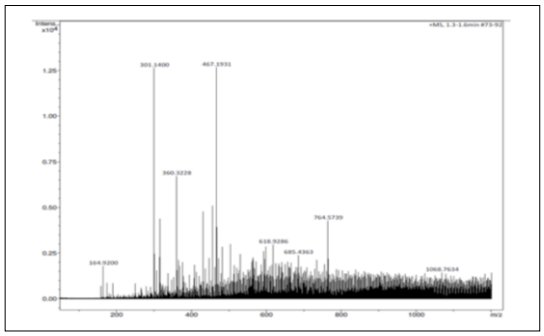


Figure 4. ESI-Mass spectrum of the methanolic extract fraction eluted at 14 min usingHPLC.

3.6. Quantitative 3,5-Dinitrosalicyclic Acid (DNSA) Assay

As the methanol extract showed good results for the qualitative starch-iodide assay, to quantify the result, we performed a DNSA assay. To demonstrate that this inhibitory activity was due to the presence of the secondary metabolites Quercetin and Ursolic acid, a separate DNSA assay was also performed for the standards alone. The percent inhibition of the PPA increased linearly as the concentration of the methanol extract increased. The calculated IC_{50} value of the leaf methanol extract was 1.1 mg/mL. The individual IC_{50} values for Quercetin and Ursolic acid were 16.5 µg/mL and 13.2 µg/mL, respectively.

3.7. In Silico Docking Studies

3.7.1. With PPA

The results of molecular docking using AutoDock 4.2.6 are shown in Table 1, thus indicating the docking scores and interaction details for the identified compounds: Quercetin and Ursolic acid bound with PPA. The binding energies of the ligand molecules is presented in kcal/mol. These studies were performed in comparison with the positive control Acarbose. The interactions between the protein-ligand complexes were analyzed by Discovery Studio Visualizer. The crucial amino acids Glu233, Asp197, and Asp300 in α-amylase were found to be involved in bond formation between the protein and ligand molecule underpinning the theory of α-amylase inhibition.

Table 1. Binding energies of the ligands and Acarbose with PPA. (The residues shown in bold are catalytic residues present at active site of PPA).

Sr. No.	Compound Name	Binding Energy Kcal/mol	Interaction	Amino Acid Residues
1	Quercetin	−9.89	3 H-Bonds	**Asp197**, Tyr62, Gln63
2	Ursolic acid	−8.96	1 H-Bond	**Asp300**
			Van der Waal	**Glu233**, Arg195, **Asp197**, His299, Trp58, His305, Gly164, Gln 63
3	Acarbose	−12.48	9 H-Bonds	His305, His101, Lys200, Tyr151, His201, Glu233, **Asp300**, His299, Trp59
			Van der Waal	Val163, Ile235, Leu162, Gly306, Ala198, Arg195, Trp58, **Asp197**, Leu165, Gln63

3.7.2. With TLR-2

Quercetin interacted with TLR2 via five hydrogen bonds through amino acid residues VAL303, THR330, PHE295, GLY293, and TYR326 while Ursolic acid bound to TLR2 via a single amino acid residue, ASP327 (Figure 5). The binding energies were −11.55 kcal/mol

and −11.85 kcal/mol for Quercetin and Ursolic acid, respectively. From the docking analysis, it can be concluded that the flavanol Quercetin is a strong candidate for the inhibition of TLR2 compared to the terpenoid Ursolic acid, as it interacted with TLR2 via a greater number of hydrogen bonds.

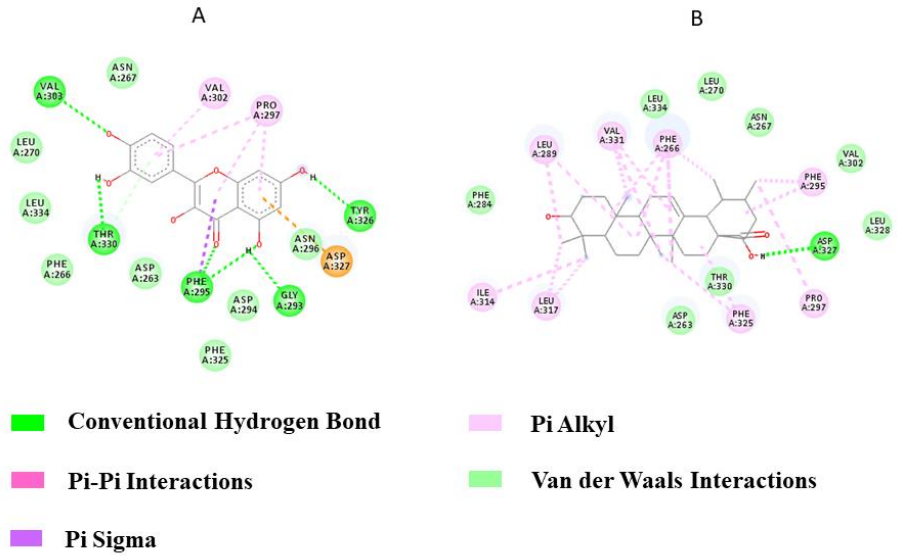


Figure 5. 2D representation of TLR2 Protein-Ligand interactions: (**A**) Quercetin, (**B**) Ursolic acid.

4. Discussion

Plant-derived natural products have been known for their diverse and versatile therapeutic properties. These have been explored for their efficacy against many diseases such as cancer, arthritis, malaria, Alzheimer's, and diabetes [16].

In the present study, the leaf methanolic extract of *A. nervosa* was explored for its α-amylase inhibitory activity. It showed the presence of major classes of secondary metabolites viz. phenols, flavonoids, terpenes, and alkaloids. In the qualitative starch-iodide assay, the methanol extract showed persistent PPA inhibition. To find out the active PPA inhibitory molecules in the methanolic extract, HPTLC and HPLC analyses were performed. This unveiled the presence of two important secondary metabolites, namely, Quercetin and Ursolic acid. The presence of these secondary metabolites was validated using the ESI-MS analysis.

Quercetin, a well-known flavonoid, is known for its α-amylase inhibitory activity. A study performed in rat pancreas reported that Quercetin inhibited the α-amylase with an IC_{50} value of 0.061 µM [17]. Ursolic acid, a terpenoid, is known for its strong α-amylase inhibitory property. The in vitro study carried out by Wang et.al proved that Ursolic acid possessed α-amylase inhibitory potency with an IC_{50} value of 0.482 ± 0.12 mg/mL [18]. We found the IC_{50} values of Quercetin and Ursolic acid to be 16.5 µg/mL and 13.2 µg/mL (Section 3.6).

The molecular docking analysis showed that both Quercetin and Ursolic acid have a binding affinity toward PPA, with binding energies of −9.89 kcal/mol and −8.96 kcal/mol, respectively. The positive control, Acarbose, had a binding energy of −12.48 kcal/mol. Thus, both the secondary metabolites have exceptionally good PPA inhibition potency. The molecular docking and molecular dynamics studies of Quercetin and Ursolic acid from *Morinda citrifolia* fruits against human pancreatic α-amylase demonstrated that both phytochemicals were good inhibitors with binding energies of −2.16 kcal/mol and −8.58 kcal/mol, respectively [19].

It needs to be noted that, although both Quercetin and Ursolic acid have shown strong PPA inhibitory potential with low IC_{50} values individually in our experiments, our methanolic extract containing these two active phytochemicals has shown a high IC_{50}

value, indicating the possibility for the presence of certain additional molecules that are interfering with the PPA inhibition. Thus, the crude extract warrants further purification.

A healthy gut microbiota is an essential aspect of the overall health of the human body. Studies have shown that gut microbiota plays a pivotal role in physiological processes in the body such as the regulation of fat storage, immunity, biological barriers, nutritional absorption, growth, and development [20]. Plant-derived phenols, flavonoids, terpenoids, and alkaloids have beneficial effects on human gut microbiota.

A study conducted on STZ-induced diabetic peripheral neuropathy (DPN) rat models reported that these rats showed gut dysbiosis compared to control rats, as observed by fecal microbiome16S rRNA gene sequencing. Quercetin was found to be bactericidal against potential pathogenic bacteria such as *Oxalobacter* and *Klebsiella*, that are positively correlated with DPN phenotypes and high ROS production levels. On the other hand, Quercetin enriched the abundance of probiotic genera like *Actinobacteria* that were negatively correlated with DPN phenotypes and ROS production levels [21].

Terpenoids also display anti-inflammatory, anti-viral, anti-cancer, anti-microbial, and anti-diabetic activities. However, so far, very few terpenoids have been investigated for their effects on gut microbiota. In the gut, terpenes are metabolized in the liver as well as biotransformed by intestinal microorganisms such as *Fusobacterium nucleatum*, *Bacteroides fragilis*, and *Peptostreptococcus anaerobius* through the processes of hydrolysis and reduction. It is widely known that about 30% of diabetic people suffer from liver malfunction. Wan et al. reported the repairing effect of Ursolic acid, a tri-terpenoid, on bacterial dysbiosis in liver fibrosis in mice. The potential beneficial bacteria, such as *Firmicutes*, *Lactobacillus*, and *Bifidobacterium* were seen to be increased in the Ursolic acid group [22].

A time-dependent high glucose dose is known to induce a marked increase in Toll-Like Receptors TLR2 and TLR4 mRNA and protein expression in human monocytes. TLR2 and TLR4 mediate inflammation in activated cells via NF-ḱB signaling. Thus, targeting these inflammatory receptors by the action of plant secondary metabolites may be an advantageous strategy to ameliorate the development of DM2 and complications related to it. Especially, TLR2 is known to be responsible for production of several molecules, including ROS and pro-inflammatory cytokines that contribute to the worsening of type II diabetes and related complications. Considering these facts, the down-regulation of TLR2 can be used as a novel approach to treat type II diabetes [23].

5. Conclusions

The intent of the present study was to find out the hypoglycemic secondary metabolites from the leaf methanolic extract of *Argyreia nervosa* (Burm. f.) Bojer. The leaf methanolic extract contained Quercetin and Ursolic acid. They exhibited great α-amylase inhibitory activity in both in vitro and in silico experiments. For the first time, we have shown excellent in silico docking of both these molecules on the active site of TLR-2, a bridging molecule of inflammation and gut microbiota composition. Our current work proposed the multi-functional role of Quercetin and Ursolic acid as α-amylase inhibitors as well as human gut health enhancers. Further experimental studies would be carried out in this regard.

Author Contributions: Conceptualization, A.A.K. (Anjali A. Kulkarni); methodology, A.D.K. and A.A.K. (Anjali A. Kulkarni); software, A.D.K.; validation, A.D.K. and A.A.K. (Anjali A. Kulkarni); formal analysis, A.D.K.; investigation, A.D.K. and A.A.K. (Anjali A. Kulkarni); resources, A.D.K. and A.A.K.(Anjali A. Kulkarni); data curation, A.D.K., A.A.K. (Anupa A. Kumbhar) and A.A.K. (Anjali A. Kulkarni); writing—original draft preparation, A.D.K. and A.A.K. (Anjali A. Kulkarni); writing—review and editing, R.P.K., A.A.K. (Anupa A. Kumbhar), and A.A.K. (Anjali A. Kulkarni); visualization, R.P.K., A.A.K.(Anupa A. Kumbhar) and A.A.K. (Anjali A. Kulkarni); supervision, A.A.K. (Anjali A. Kulkarni); project administration, A.A.K. (Anjali A. Kulkarni); funding acquisition A.D.K. and A.A.K. (Anjali A. Kulkarni). All authors have read and agreed to the published version of the manuscript.

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