Proceeding Paper

α-Amylase Inhibitory Secondary Metabolites from Artemisia pallens Wall ex DC—Biochemical and Docking Studies †

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Abstract: Diabetes Mellitus Type-2 (DM-2) has become a challenging disease worldwide as many young adults are also getting affected by it due to sedentary lifestyle and wrong diets. Multiple studies have shown that control over α-amylase enzyme in gut could be a better approach to treat DM-2. The secondary metabolites produced by the plants have various biological properties and many are used as drugs. In the current study, we isolated secondary metabolites from acetone leaf and bud extracts of Artemisia pallens Wall ex DC (Family: Asteraceae) and tested them for their porcine pancreatic α-amylase (PPA) inhibitory activity in vitro and in silico. This extract exhibited good PPA inhibition, with IC50 value of 388.05 µg/mL. The IC50 value of Acarbose (a known pancreatic α-amylase inhibitor drug/positive control) was 9.71 µg/mL. Various secondary metabolites detected from acetone leaf and bud extract by LC-MS analysis, were used for the molecular docking studies using AutoDock 4.2.6. The co-crystallised structure of PPA and Acarbose was retrieved from Protein Data Bank (PDB ID: 1OSE). The binding energies of few metabolites were (kcal/mol): Isoquercetin (−1.57), Cryptochlorogenic Acid (−1.17), Cirsilineol (−10.24), Kaempferide (−9.99), Fustin (−9.86), 6-Demetroxycapillarisin (−9.82), Piperine (−9.45), Ergometrine (−9.43), Apigenin (−9.38) & Artemisinin (−9.27). Acarbose had a binding energy of −17.58 kcal/mol. All the metabolites looked highly promising as α-amylase inhibitors and most of them interacted with PPA via hydrogen bonding with crucial amino acid residues: Asp197, Asp300 & Glu233. Thus the acetone extract of A. pallens leaf and buds can potentially inhibit PPA (strong amino acid sequence similarity with human pancreatic α-amylase) and hence extrapolation of these inhibitory results could be valid for human pancreatic α-amylase as well.

Keywords: diabetes mellitus; alpha-amylase; in silico docking; Artemisia pallens Wall ex DC

1. Introduction

Diabetes Mellitus Type-2 (DM2) is a lifestyle induced metabolic disease. Prolonged high blood sugar level (chronic hyperglycaemia), dyslipidaemia and impaired protein metabolism are the usual symptoms of this disease which leads to liver, pancreas, eyes, nerves and kidney damage. The worldwide survey by WHO [1], showed that globally 422 million people are affected by DM and it may soon become a leading cause of death by 2030. According to International Diabetes Federation, 72.9 million people in India are suffering from DM [2]. These patients show impaired insulin production, secretion, activity of insulin receptor or overall metabolism [3]. Many therapeutic treatments of DM2 are in use and they trigger the endogenous insulin secretion as well as stimulate the action of insulin at target organ or tissue. An additional target is to correct the imbalance of a number of metabolic enzymes in Diabetic patients. Pancreatic alpha amylase plays a key role in breakdown of starch and produces simple oligosaccharides. These simple oligosaccharides pass through the small intestine where these are acted upon by further digestive
enzymes and degraded into glucose which is absorbed into the bloodstream leading to post-prandial hyperglycemia. If phytochemicals are able to inhibit such digestive enzymes, the degradation of dietary starch to simple monomeric glucose is slowed down, preventing the harmful post-prandial glucose surge [4].

Synthetic drugs are widely used for treatment of DM but they have negative side effects on humans. Hence search for new anti-hyperglycemic molecules of plant origin is still on. Such phytochemicals are envisaged to have negligible side effects [5]. *Artemisia* is a large and economically important genus containing more than 500 species. Most of the species are perennial herbs with a few annual or biannual exceptions [6]. This genus came into limelight when Prof. Tu Youyou was awarded the Nobel Prize in Physiology or Medicine (2015) for conclusively showing that Artemisinin is the effective secondary metabolite from *Artemisia annua* L. exhibiting strong anti-malarial activity against *Plasmodium* spp [7]. In traditional herbal systems of medicine, this genus is also being used to treat diabetes for many years [8]. Nofal et al. observed that aqueous and ethanolic extracts of *A. judaica* significantly reduced blood glucose levels in diabetic rats but had no effect on normal rats [9]. Anaya-Eugenio et al. isolated Eupatilin and Salvinine from *A. ludoviciana* and fully revealed their hypoglycemic and anti-hyperglycemic effects along with enzyme binding studies [10]. In Japan, *A. campestris* has been used as a folk medicine in liver and kidney complications. The investigation done by Aniya et al. proved that the aqueous extract of *A. campestris* possessed strong antioxidant and hepatoprotective activity [11]. One of the few reports about *A. dracunculoides* indicated the presence of coumarins [12]. Their anti-diabetic activity has been reported [13]. Apart from the alpha-amylases and alpha-glucosidases, other enzymes such as aldose reductases also play crucial role in glucose metabolism. The ethanolic extract of *A. dracunculus* exhibited excellent aldose reductase inhibitory activity. At the concentration of 3.75 μg/mL of extract, about 40% enzyme activity was retarded. It was quite comparable to the Quercitrin (a known aldose reductase inhibitor) which retarded the enzyme activity by 54% at the same concentration [14].

*Artemisia pallens* is a small aromatic herb with excellent essential oil content. Few scientists have reported its anti-hyperglycemic activity. Hence there is still scope to explore this species for its therapeutic properties. In 1996, Subramoniam et al. reported the significant dose dependent blood glucose lowering effect of methanol extract of aerial plant parts of *A. pallens* [15]. But no active compounds were described. The present study was done to explore the anti-hyperglycemic potential of *A. pallens*. The crude acetone extract of leaf and buds showed α-amylase inhibitory activity in primary starch-iodide assay. This was then quantified as IC₅₀ value with DNSA assay. The secondary metabolites from the acetone crude extract were identified by the LC-MS technique. These results of amylase inhibition were then cross confirmed with *in silico* docking studies.

2. Methods

2.1. Collection of Plant Material

The plant material of *Artemisia pallens* Wall. ex DC. was collected from Jejuri, district Pune, Maharashtra (Latitude 18.292450° N and Longitude 74.144680° E). The plant was identified and authenticated by Botanical Survey of India, Western regional centre, Pune-7. (Voucher number: BSI/WEC/Iden.Cer./2021/1603210007747).

2.2. Preparation of Plant Extracts

Fresh leaf and buds were cleaned and used for extraction. The extracts were prepared by hot extraction method i.e., by using Soxhlet apparatus (Borosil, India). For extraction, 50 g of cleaned fresh leaf and buds were weighed and wrapped in a muslin cloth. The sequential extraction was carried out with 250 mL of each of six different solvents with ascending polarity such as pet ether, chloroform, ethyl acetate, acetone, methanol and water. Three consecutive hot extraction cycles run for each solvent. Each of the solvent ex-
tracts were collected separately, filtered and then all the extracts were dried under reduced pressure with the help of the rotary vacuum evaporator (Buchi, Germany) to yield crude powder extracts for further experiments. These concentrated extracts were then stored at 4 °C until further use.

2.3. Qualitative Phytochemical Analysis of Extracts

The phytochemical screening of six different extracts were carried out by standard methods reported by Trease and Evans for the presence of alkaloids, tannins, saponins, glycosides, steroids, flavonoids, phenols, terpenoids and quinines [16].

2.4. α-Amylase Inhibition Assay

The standard assay reported by Sudha et al. was referred to perform the α-amylase inhibition assay [17]. For primary screening of inhibitor from extracts pure α-amylase enzyme from porcine pancreas (PPA) (Sigma-Aldrich Catalogue No. A6255) was used. The assay was based on colour reaction produced by starch iodine complex, also known as starch-iodide assay. The assay mixture consisted of phosphate buffer (pH 6.9 containing 6.7 mM sodium chloride), 0.2 units of porcine pancreatic amylase (PPA) solution & 20 μL of plant extract in DMSO (1 mg/mL w/v). The reaction mixture was incubated at 37 °C for 15 min. Then soluble starch (1%, w/v) was added to each reaction well & again incubated at 37 °C for 15 min. 20 μL of 0.2 M Tris solution was used to stop the enzymatic reaction & then 20 μL of Iodine reagent was added. The colour change of the reaction mixture was observed. The positive drug/substrate control (SC) was a synthetic, known PPA inhibitor, Acarbose (Glucobay tablets, Bayer AG).

2.5. 3,5-Dinitrosalicylic Acid (DNSA) Assay

The inhibition assay was executed by using method described by Miller [18] with slight modifications. The total assay mixture composed of 500 μL of 0.02 M sodium phosphate buffer (pH 6.9 containing 6 mM sodium chloride), 0.2 units of PPA solution and extract of concentration 2.5 mg/mL (25, 50, 75, 100 and 125 μL). All the reactants were incubated at 37 °C for 10 min. After this pre-incubation, 500 μL of 1% (w/v) starch solution in the above buffer was added to each tube and incubated further at 37 °C for 15 min. The reaction was terminated with addition of 1.0 mL DNSA reagent, placed in boiling water bath for 5 min, cooled to room temperature, diluted and the absorbance was measured at 540 nm. The control PPA at 0.2 U.mL⁻¹ represented 100% enzyme activity and did not contain any plant extract. To eliminate the absorbance produced by plant extract, appropriate extract controls with the extract in the reaction mixture except for the enzyme were also included. The known PPA inhibitor Acarbose was used a positive control. The other quantifiers were calculated as follows:

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\text{% Relative PPA activity} = \left(\frac{\text{enzyme activity of test}}{\text{enzyme activity of control}}\right) \times 100
\]

\[
\text{% inhibition in the PPA activity} = (100 - \text{% relative enzyme activity})
\]

2.6. Determination of Total Flavonoid Content of Acetone Extract

For determination of total flavonoid content, aluminium chloride colorimetric method was followed with slight modifications [19]. The stock solution of 5 mg/mL of Acetone extract was used. The stock solution of acetone leaf extract (25–125 μL) was mixed with 1.25 mL distilled water and 75 μL 5% NaNO₂ solution prior to 5 min incubation period. 150 μL of 10% AlCl₃ 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.
2.7. Determination of Total Phenol Content of Acetone Extract

Total phenol content was determined by the Folin-Ciocalteu method with slight modifications [19]. A 5 mg/mL stock solution of Acetone extract was prepared. From this sample, 10–50 µL was then mixed with 0.5 mL of a 10 fold diluted Folin-Ciocalteu reagent (HiMedia, India) and 1 mL of 7.5% sodium carbonate. The tubes were covered with parafilm and allowed to stand for 30 min at room temperature. Then the absorbance was read at 760 nm spectrophotometrically (UV-1800, SHIMADZU, Japan). The total phenolic content was calculated using calibration curve. The concentration of phenolic content was expressed as Gallic Acid (Sigma, USA) equivalent in µg/mg of sample.

2.8. Determination of Total Terpenoid Content of Acetone Extract

Total terpenoid content was determined according to Wei et al. with slight modifications [20]. A 5 mg/mL stock solution of Acetone extract was prepared and a sample from this (10–50 µL) was mixed with 500 µL of 5% (W/V) vanillin-acetic solution and 300 µL sulfuric acid. This mix was incubated at 70 °C for 30 min. Then the mixed solution was cooled and diluted to 3.25 mL with acetic acid. Then the absorbance was read at 573 nm spectrophotometrically (UV-1800, SHIMADZU, Japan). The blank consisted of all the reagents and solvents without sample solution. The concentration of Terpenoid content was determined using the standard Ursolic Acid (Sigma, USA) calibration curve.

2.9. LC-MS/MS Analysis of Acetone Extract

LC-MS/MS method was developed and used for identification of secondary metabolites present in crude acetone extracts of leaf and buds of A. pallens in this study. All MS acquisitions were performed in the positive electrospray ionization mode. The capillary voltage, cone voltage, fragmentor voltage were 4 kV, 45V and 170V, respectively. The gas temperature was set at 325 °C. Data was acquired at scan rate of 3Hz in mass range of 100–1000 m/z. Further data was analyzed with Mass Hunter qualitative software and METLIN database.

LC-MS/MS experiments were performed on Agilent 1260 binary LC System (Agilent, USA). Agilent Zorbax Extend C18 RRHT column (50 x 2.1 mm, 1.8 µm) was used. The mobile phase was composed of (A) Water + 0.1% Formic acid and (B) Acetonitrile. A gradient programme was set as: 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). The flow rate of the mobile phase was 0.30 mL/min. The injection volume was 1 µL. Compounds were characterized by their MS, MS/MS spectra, as well as UV spectra.

2.10. Molecular Docking Studies

2.10.1. Softwares and Tools

Protein Data Bank (PDB), PubChem, Discovery Studio Visualizer, AutoDock Version 4.2.6.

2.10.2. Ligand Preparation

The secondary metabolites detected in LC-MS analysis were used as the ligands for docking studies. Their 3-D structures were obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov/) database in SDF format. This SDF format was converted into PDB format using Discovery Studio Visualizer software.

2.10.3. Protein Preparation

The crystal structure of PPA (PDB ID: 1OSE) was obtained from protein data bank (www.rcsb.org) and further prepared for docking by removing water molecule and ligand molecule which co-crystallized at protein structure and active site. Polar hydrogen atom
and Kollman charges were added to protein by using AutoDock Version 4.2.6 programme. This complex was saved as a pdbqt format file for docking.

2.10.4. Docking Studies

For molecular docking studies, the site where Acarbose got bound to the 1OSE, was selected as the PPA active site and a grid box was set at the centre of the co-crystallized ligand with dimension of 45 Å × 40 Å × 44 Å (X: 37.909; Y: 38.049; Z: -1.869) with grid spacing of 0.375 Å.

2.10.5. Protein-Ligand Interactions

Discovery studio software (https://discover.3ds.com/discovery-studiovisualizer-download) was used to visualize the docked conformations of ligand -protein complex. The lowest binding energy showed by the ligand molecules indicated the highest binding affinity for PPA.

3. Results

3.1. Qualitative Phytochemical Analysis of A. pallens Leaf and Buds Extracts

Qualitative phytochemical analysis was done for all the six extracts. In the present work, we are presenting data related to acetone extract, as it showed the best PPA inhibition. Acetone extract showed presence of tannins, flavonoids, phenols, terpenes and quinines.

3.2. Determination of Total Phytochemicals Content

The total Flavonoid, Phenol and Terpenoid content calculated by using calibration curves of respective standards was 275.5 ± 0.01 µg/mg, 9.1 ± 0.004 µg/mg and 68.5 ± 0.01 µg/mg dry weight of leaf and bud acetone extract respectively (Detailed data not shown).

3.3. LC-MS/MS Analysis of Leaf and Bud Acetone Extract

The identification of secondary metabolites in leaf and bud acetone extract was carried out by LC-MS/MS. This revealed the presence of total twenty four secondary metabolites belonging to different classes. Among these, ten compounds were selected for in-silico study. These would be discussed in detail.

3.4. Qualitative Starch-Iodide Assay for PPA Inhibition

Qualitative screening (colour reaction) of PPA inhibition was performed based on starch-iodine complex formation. In this assay, among the six prepared extracts, petroleum ether, chloroform, ethyl acetate, acetone and methanol extracts showed PPA inhibition (Figure 1). The blue colour of starch-iodide complex due to acetone extract was persistent even after 24 h. Therefore acetone extract was carried forward for further analysis.
3.5. Quantitative DNSA Assay for PPA Inhibition

The crude acetone extract of *A. pallens* showed persistent inhibition of PPA qualitatively. The DNSA assay was performed to measure the PPA inhibition quantitatively. Dose dependant percent inhibition activity of PPA was observed due to acetone leaf and bud extract. PPA inhibition increased as the concentration of the acetone extract increased. The calculated IC₅₀ value for plant extract was 338.05 µg/mL. The increasing concentrations of plant extract as 62.5 µg/mL, 125 µg/mL, 187.5 µg/mL, 250 µg/mL & 312.5 µg/mL showed 28.36%, 35.05%, 38.93%, 43.45% & 46.19% inhibition activity respectively (Figure 2).

3.6. Molecular Docking Studies

Ligand Preparation

The 3D ligand structures of ten compounds were obtained from PubChem database in SDF format and converted to PDB format using Discovery Studio software (Figure 3). The structures were saved in PDBQT format.
3.7. Protein Preparation

The crystal structure of PPA (PDB ID: 1OSE) was obtained from Protein Data Bank. The protein structure prepared by using AutoDock 4.2.6 program (Figure 4).

Figure 3. The 3D Ligand Structures of Ten Secondary Metabolites from Acetone Leaf and bud Extract of *A. pallens* Used in Docking Studies for Targeting PPA. (Acarbose is the drug/positive control).
3.8. Docking Studies by Using AutoDock 4.2.6

The results are shown in Table 1 indicating the docking scores for ten selected ligands bound with PPA. The binding energies of ligands with PPA enzyme are represented as kcal/mol. Docking studies were done in comparison with positive control Acarbose.

Table 1. The Binding Energy of Ligands and Acarbose with PPA.

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<th>No</th>
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<th>Compound Name</th>
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4. Discussion

Since last few decades, secondary metabolites have been investigated for their therapeu tic properties. They have proved effective against many diseases such as cancer, arthritis, malaria, Alzheimer’s and diabetes [21–24].

In this study, ten compounds belonging to different secondary metabolite classes were studied for their PPA inhibitory activity. These were isolated from crude acetone extract of flower leaf and buds of *A. pallens*. A previous study of methanol extract of aerial parts of *A. pallens* reported total phenolic and flavonoid content as 127.16 µg/mg and 13.57 µg/mg respectively [25]. In our study, the total phenol, flavonoid and terpene content of the acetone extract of leaf and buds was 9.1 ± 0.004 µg/mg, 275.5 ± 0.01 µg/mg and 68.5 ± 0.01 µg/mg respectively; that was quite different from the previous study.

LC-MS/MS analysis identified the presence of total twenty four major secondary metabolites. Among them, ten compounds showed good affinity and binding score for PPA. These ten compounds were selected for detailed *in-silico* study.

Alpha-amylase uses active site carboxylic acid to catalyse its hydrolytic reaction. Among the amino acid residues present at the active site, Asp197, Glu233 and Asp300 are proven for their importance in catalytic activity. These are completely conserved and essential amino acids for the efficient hydrolysis of oligosaccharides [26]. Rydberg et al. reported that amino acid residue Asp197 is a catalytic nucleophile and Glu233 is a general acid catalyst by using site-directed mutagenesis and kinetic and structure determination techniques [27]. They also suggested that Asp300 plays key role in catalysis of amylase. It is also seen our docking studies that the catalytic amino acid residue Asp197 is involved in interaction with all the ten selected secondary metabolite ligands.

In literature, flavonoid class of compounds are shown to be potent alpha-amylase inhibitors [28] due to their unique structure. The ability of flavonoids to inhibit PPA is based on formation of conjugated π-system which maintains the stability of receptor-ligand complex [29]. In the present study, we observed that in molecular docking study, secondary metabolites belonging to the flavonoid group like, Isoquercetin, Cirsiniliol, Apigenin, Fustin and Kaempferide formed π-interactions as well as hydrogen bonds (Figure 5) with different amino acid residues present at the active site of PPA enzyme, stabilizing the interaction.

![Acarbose and Isoquercetin](image-url)
C. Cryptochlorogenic Acid

D. Cirsilineol

E. Kaempferide

F. Fustin

G. Demetroxycapillarisin

H. Piperine
Few studies have also investigated various alkaloids for their anti-diabetic potential. These alkaloids could inhibit alpha-amylases and alpha-glucosidase enzymes [30,31]. The alkaloid Ergometrine (Figure 5I studied here also showed good binding energy of −9.43 kcal/mol with involvement of Asp197 in hydrogen bonding. Piperine, a well-known alkaloid (Figure 5H interacted with PPA through key amino acids and retarded the enzyme action [32]. Our docking results with Piperine also showed that it interacted with crucial amino acid Asp197 through van der Waals interactions.

Previous studies showed that Chlorogenic Acid, an isomer of Cryptochlorogenic Acid reported here, exhibited good level of PPA inhibition resulting in low post prandial hyperglycemia [33]. In our study too, Cryptochlorogenic Acid (Figure 5C. showed binding energy of −11.17 kcal/mol which could form stable receptor-ligand complex. The terpene class of secondary metabolite have shown good alpha-amylase inhibitory potential [34]. Another terpene, Artemisinin (Figure 5K formed π-interactions and hydrogen bonds while interacting with PPA, suggesting a stable receptor-ligand complex development.

A. dracunculoides contained coumarins [12] and in literature coumarins have been reported for their alpha-amylase inhibitory property. The indentified coumarin: 6-Demethoxycapillarisin interacted via hydrogen bonding (Figure 5G as well as π interaction with PPA in our studies, thus proving earlier studies.

5. Conclusions

A rapid and efficient method involving LC-MS/MS analysis and molecular docking was established and effectively used for screening of α-amylase inhibitors from A. pallens. Twenty four major compounds were identified from acetone extract of A. pallens by LC-
MS profiling and among them ten compounds were found to have good binding affinity for α-amylase as determined by molecular docking. All the ligand molecules interacted with the important catalytic residues Asp197, Glu233 and Asp300 of PPA through hydrogen bonds, van der Waal forces, and pi-pi interactions. The present work reveals a new insight towards the α-amylase inhibitory secondary metabolites of A. pallens which might be useful for application in functional therapeutics for management of DM-2.

**Author Contributions:** A.A.K. established the work flow, designed the experiments, standardized the experimental protocols, contributed in results analysis and manuscript writing. R.P.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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**Abbreviations**

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>DM2</td>
<td>Diabetes mellitus Type-II</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid Chromatography-Mass Spectrometry</td>
</tr>
<tr>
<td>PPA</td>
<td>Porcine Pancreatic Amylase</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>SC</td>
<td>Substrate Control</td>
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<tr>
<td>DNSA</td>
<td>3,5-Dinitrosalicylic acid</td>
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**References**


