

IDENTIFYING ANTI-DIABETIC POTENTIAL OF ALPHA-AMYLASE INHIBITORS IN *OCIMUM BASILICUM* USING *IN VITRO* AND *IN SILICO* APPROACHES

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Abstract

Type 2 Diabetes (T2D), a chronic metabolic disorder and the most common type of diabetes, is caused by reduced insulin secretion or insulin resistance in the body, leading to ineffective glucose uptake by the cells and eventually resulting in hyperglycemia. Metformin, sulfonylureas, and glitazones are the currently available commercial drugs used to treat T2D. These drugs either reduce the blood glucose level or elevate the insulin produced. However, the high cost, unavailability, and various side effects occurring from the use of these drugs have resulted in people looking for healthier and cost-effective ways to treat this disorder, including the use of plant extracts. This study highlights the alpha-amylase inhibition properties of *Ocimum basilicum* (basil) extracts through *in vitro* qualitative and quantitative inhibition assays. It also focuses on *in silico* approaches such as molecular docking and molecular dynamics simulation to determine the strength of the alpha-amylase inhibition. *In vitro* study revealed 1:20 diluted ethanolic, methanolic, and aqueous extracts of *O. basilicum* strongly inhibited salivary amylase. *In silico* analysis revealed Gamma Sitosterol, a compound present in relative abundance in *O. basilicum*, could be one of the phytochemicals responsible for this anti-diabetic property of *O. basilicum*. Thus, Gamma Sitosterol can be used as a potential therapeutic for T2D alongside other measures such as physical exercise and diet because the findings in this paper, although pertaining to human salivary amylase, can be extrapolated to human pancreatic amylase as salivary amylase and pancreatic amylase are known isoenzymes and share ~97% sequence homology.

Keywords: Diabetes; GC-MS; Molecular docking; Molecular dynamics simulation; Starch diffusion assay; T2D

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Introduction

Diabetic cases have been rising for quite some time now, with 8.9% of the Indian population and 8.8% of the world's population being affected (Mohan and Pradeepa, 2021). This is only heightened by the fact that people now lead a more sedentary and unhealthier lifestyle.

T2D, or diabetes mellitus or non-insulin dependent diabetes, is the most common type of diabetes wherein the human body becomes resistant to the action of insulin. This disorder is marked by high blood pressure, increased hunger and thirst, unexplained loss of weight, and frequent urination. Complications of T2D range from oxidative stress (Brownlee, 2001) to diabetic retinopathy, cardiovascular disorders, kidney failure, and even stroke (Mahler and Adler, 1999; Wu *et al.*, 2014).

Current classes of drugs used to treat T2D include biguanides (Metformin), sodium-glucose cotransporter (SGLT2) inhibitors (Canagliflozin), GLP antagonists (Liraglutide), sulfonylureas (Glipizide), dipeptidyl peptidase 4 (DPP-IV) inhibitors (Sitagliptin and Vildagliptin), insulin (Humulin R, Levemir) and thiazolidinediones (Rosiglitazone) (Chaudhury *et al.*, 2017; Missoun, 2018; Tran *et al.*, 2020). Side effects from these drugs range from low to prominent risk of hypoglycemia and pancreatitis, vitamin B12 deficiency, bone fractures, and bladder cancer. (Chaudhury *et al.*, 2017)

India is known to have traditionally used various indigenous plants such as turmeric (*Cucurma longa*), garlic (*Allium sativum*), sweet orange (*Citrus sinensis*), and basil (*Ocimum sanctum* or *Ocimum tenuiflorum*) to manage and treat diabetes (Tundis *et al.*, 2010; Arumugam *et al.*, 2013; Salehi *et al.*, 2019). Another species from the *Ocimum* genus, *Ocimum basilicum* (basil), has been previously used to inhibit albumin glycation, treat hyperlipidemia, and as an anti-inflammatory and anti-microbial activity agent (Bower *et al.*, 2015; Liu *et al.*, 2017). This study explores its anti-diabetic nature.

An *in silico* study reported that *O. basilicum* should inhibit pancreatic α -amylase in the small intestine, thereby controlling blood sugar levels (Noor *et al.*, 2019). *In vivo* studies indicated that *O. basilicum* strongly inhibited the activity of α -amylase, α -glucosidase and aldose reductase (Saraswat *et al.*, 2008; Cazzola *et al.*, 2010; El-Beshbishy and Bahashwan, 2011).

This study hypothesizes that *Ocimum basilicum*, a herb commonly used worldwide, can be used as a potential therapeutic for treating T2D on the basis that two other species in the same genus

are known to reduce blood glucose levels and attempt to focus on the inhibitory effect of *O. basilicum* extracts on α -amylase, a key enzyme involved in T2D, using *in vitro* and *in silico* approaches, thus proving the plant's anti-diabetic nature.

Materials and Methods

Collection and Extraction

1 gram of dried *O. basilicum* leaves was macerated in distilled water, 70% ethanol, and 70% methanol to make aqueous, ethanolic, and methanolic extracts, respectively. The solutions were left overnight for extraction and subject to evaporation the next day. The residue was collected and stored at 4°C for future use. A portion of the residue was also used to make dilutions of 1:10 and 1:20 for each of the aforementioned extracts.

Phytochemical Analysis

Phytochemical analysis provides insights into the significant phytochemicals present in plants. Thus, *O. basilicum* was qualitatively screened to determine the presence of bioactive compounds (Gul *et al.*, 2017; Tiji *et al.*, 2021).

Extraction of α -amylase from Human Saliva

α -amylase was extracted from human saliva using a method similar to that of amylase extraction from grains (Pertin *et al.*, 1966; Silano *et al.*, 1975). Saliva was collected in a beaker to which calcium chloride (CaCl_2) solution was added. This solution was incubated at 37°C for 3 hours and then centrifuged for 20 minutes at 8000 rpm and 4°C. The obtained supernatant was stored at 4°C. Dilutions of 1:10 and 1:20 were also prepared with 0.05M phosphate buffer.

Determination of Activity and Specific Activity of α -amylase from Human Saliva

The DNS (dinitro salicylic acid) method (Bernfeld, 1955; Mehrabadi and Bandani, 2009) was used to construct a standard maltose curve, and Lowry's assay (Lowry *et al.*, 1951) was used to construct a standard protein curve. These curves were then used to determine the activity and specific activity of salivary α -amylase, respectively.

$$\text{Activity} = \frac{\mu\text{mol of Maltose} \times \text{Dilution Factor}}{\text{Incubation Time} \times \text{Volume of Enzyme}}$$

$$\text{where, Volume of Enzyme} = \frac{\text{Total Volume}}{\text{Dilution Factor}} \quad (\text{Bernfeld, 1955})$$

Specific activity = $\frac{\text{Total Activity}}{\text{Total Protein Content}}$ (Bernfeld, 1955)

The unit of enzyme activity was taken as $\mu\text{mol}/\text{min}$ ml and that of specific activity was taken as $\mu\text{mol}/\text{mg min}$.

For this study, α -amylase activity and specific activity was calculated as follows:

Incubation time = $10+10+5=25$ minutes

For 1:10 diluted salivary amylase,
Amount of maltose from graph = $2.572 \mu\text{mol}$
Activity = $(2.572 \times 10) / (25 \times 1) = 1.0288 \mu\text{mol}/\text{min}$ ml.
Specific activity = $1.0288 / 107.9048 = 0.009 \mu\text{mol}/\text{mg min}$

For 1:20 diluted salivary amylase,
Amount of maltose from graph = $2.316 \mu\text{mol}$
Activity = $(2.316 \times 20) / (25 \times 1) = 1.8528 \mu\text{mol}/\text{min}$ ml.
Specific activity = $1.8528 / 28.8571 = 0.064 \mu\text{mol}/\text{mg min}$

Inhibition Assays

Quantitative Amylase Inhibition Assay

DNS method (Bernfeld, 1955; Mehrabadi and Bandani, 2009) was used to determine the concentration of mercuric chloride (HgCl_2), a known inhibitor of α -amylase, and that of *O. basilicum* extracts that exhibit the best percentage of inhibition against amylase to be used for qualitative assay. Concentrations of HgCl_2 ranging from 10^{-2}M to 10^{-6}M and 1:10 and 1:20

dilutions each of aqueous, ethanolic, and methanolic *O. basilicum* extracts were used.

1:20 dilution of amylase was observed to have a higher activity and specific activity (Table 1) and thus, this dilution was used for the inhibition assays.

The percentage of inhibition was determined using the simple percentage formula:

$$\text{Percentage of inhibition} = \left(\frac{x-y}{x} \right) \times 100$$

where, x = O.D of positive control and y = O.D of the test

O.D value is reciprocal to the inhibition. Therefore, the higher the O.D values, the lower the inhibition. Higher O.D reveals that the plant extract is unable to inhibit amylase and a higher amount of maltose is released. Lower O.D reveals higher inhibitory activity of the plant extract.

The percentage inhibition of HgCl_2 dilutions has been reported (Table 2) and plotted (Figure 1) and that of *O. basilicum* extracts has also been reported (Table 3). 10^{-2}M HgCl_2 dilution showed the best inhibition while 1:20 dilution of ethanolic, methanolic, and aqueous *O. basilicum* extracts showed a percentage inhibition greater than 50%. These were thus used in further experimentation.

The comparison of the percentage inhibition of the controls and aforementioned extracts has been plotted in Figure 2. Since 10^{-2} diluted HgCl_2 gave maximum inhibition, it was considered as 100% inhibition.

Table 1. Phytochemical analysis

Phytochemicals	Test	Observation	Inference
Alkaloids	Wagner's test	Reddish brown precipitate	Negative
Terpenoids	Copper acetate test	Emerald green precipitate	Positive
Phenols	Ferric chloride test	Greenish-black, blue-black, red or purple precipitate	Positive
Flavonoids	Alkaline reagent test	Yellow precipitate is observed which disappears after adding diluted acid	Positive
Steroids	Liebermann-Buchard's test	Violet-blue ring	Positive
Triterpenes	Salkowski's test	Golden yellow precipitate	Positive
Saponins	Froth test	Persistence of foam for 10 minutes	Negative

Table 2. Activity and Specific Activity of 1:10 and 1:20 dilutions of Amylase

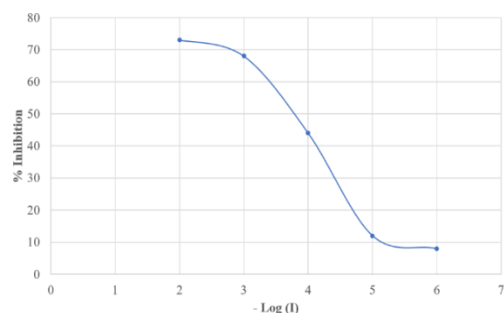
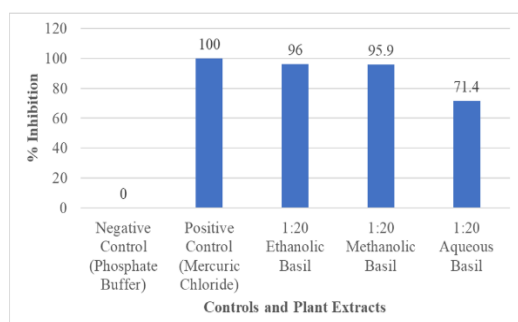
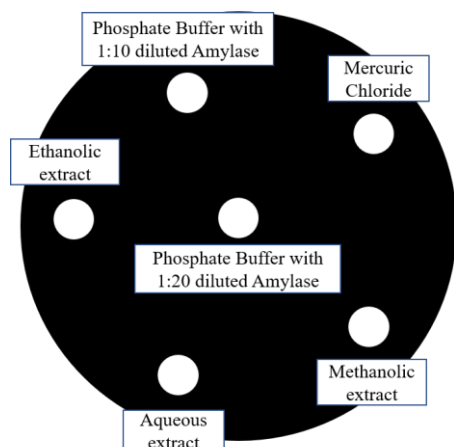
Dilution	Activity	Specific Activity
1:10 Amylase	$1.0288 \mu\text{mol}/\text{min ml}$	$0.009 \mu\text{mol}/\text{mg min}$
1:20 Amylase	$1.8528 \mu\text{mol}/\text{min ml}$	$0.064 \mu\text{mol}/\text{mg min}$

Table 3. Percentage inhibition of different concentrations of mercuric chloride

O.D of positive control (x)	O.D of HgCl_2 concentration (y)	Percentage of inhibition	-Log(I)
1	0.27	73	2
1	0.32	68	3
1	0.56	44	4
1	0.88	12	5
1	0.92	8	6

Table 4. Percentage inhibition of *O. basilicum* extracts

O.D of positive control (x)	O.D of plant extract (y)	Percentage of inhibition	Extract
0.98	0.95	3.06	1:10 methanolic extract
0.98	0.92	6.12	1:10 ethanolic extract
0.98	0.65	33.67	1:10 aqueous extract
0.98	0.04	95.92	1:20 methanolic extract
0.98	0.03	96.94	1:20 ethanolic extract
0.98	0.28	71.43	1:20 aqueous extract

**Figure 1. Percentage inhibition of different concentrations of mercuric chloride****Figure 2. Percentage inhibition of *O. basilicum* extracts compared to Positive Control (Mercuric Chloride) and Negative Control (Phosphate Buffer)****Figure 3. Starch Diffusion Assay Template**

Qualitative Starch Diffusion Assay

The anti-diabetic activity of the α -amylase inhibitors in *O. basilicum* was evaluated using the starch diffusion assay, similar to the agar diffusion method (Heatley, 1944). Petri plates were prepared using 1% agar and 1.5% starch. Phosphate buffer incubated with 1:10 and 1:20 diluted amylase solutions as the negative controls, mercuric chloride as the positive control, and the prepared *O. basilicum* extracts incubated with amylase were added to the punched wells (Figure 3). The petri plates were incubated overnight at 37°C. Iodine was poured over each plate the next day to check for hydrolysis of starch.

A blue-black colour on the addition of iodine indicates the presence of starch. If the starch was hydrolyzed by amylase, then a clear zone can be seen around the well where the starch has been hydrolyzed.

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

To identify the anti-diabetic compounds present in *O. basilicum*, GC-MS was performed which revealed the 47 compounds present in *O. basilicum* (Table 4 and Supplementary Information Figure 1). *In silico* approach was used to further analyse the identified compounds.

In silico Analysis

Protein Selection and Refinement

To identify a suitable template for molecular docking studies, RCSB Protein Data Bank (PDB) (Berman, 2000) was screened. A crystal structure of human salivary amylase with PDB ID 1SMD (Ramasubbu *et al.*, 1996) was chosen. To compare the docking results of human salivary amylase with that of human pancreatic α -amylase, a crystal structure of human pancreatic α -amylase in complex with Acarbose with PDB ID 1XD0 (Li *et al.*, 2004) was chosen.

Ligand Selection and Preparation

Compounds analysis that exhibited anti-diabetic activity and were not a health hazard were manually identified and considered for *in silico*

analysis. PubChem (Kim *et al.*, 2022) and ChEBI (Hastings *et al.*, 2015) were used to retrieve the structures of the identified anti-diabetic compounds in *O. basilicum*. Using the OpenBabel (O'Boyle *et al.*, 2011) console in PyRX 0.9.8 version (Dallakyan and Olson, 2015) software, the retrieved structures were energetically optimized using a universal force field.

Molecular Docking and Molecular Dynamics Simulation

AutoDock Vina (Trott and Olson, 2009) from the PyRx 0.9.8 version (Dallakyan and Olson, 2015) software was used to perform molecular docking studies, with Acarbose, a known anti-diabetic, as the reference ligand. Each lead ligand is docked against the protein backbone and ranked based on the binding affinity between that ligand and the protein. The protein-ligand interactions were analyzed using LigPlot⁺ (Laskowski and Swindells, 2011).

Among the docked ligands, Gamma Sitosterol had a comparable vina score as compared to Acarbose. Thus, the docked complex of Gamma Sitosterol (ligand) and human salivary amylase (protein) was simulated using Desmond Maestro for 100 nanoseconds.

The interactions of Gamma Sitosterol and Acarbose with salivary amylase (Figure 4) and pancreatic amylase (Figure 5) were visualized in 2D using LigPlot⁺. The hydrogen bond interaction and hydrophobic interaction with the residues, as well as the common interacting residues between the two ligands, were tabulated (Table 5).

Molecular dynamics simulation was performed with Gamma Sitosterol and the pancreatic amylase complex using Schrödinger Desmond (Bowers *et al.*, 2006) since Gamma Sitosterol had a Vina score comparable to that of Acarbose.

RMSD, RMSF, and Protein-Ligand Contacts plots were generated to understand the stability of the structure of the apo-protein and the protein-ligand complex, to characterize the local fluctuations that occur in the protein during its interaction with the ligand, and to understand how the protein interacts with the ligand, respectively.

Results and Discussion

O. basilicum tested positive for the presence of seven bioactive compounds - terpenoids, phenols, flavonoids, steroids, and triterpenes, and negative for two - alkaloids and saponins (Table 6).

The qualitative inhibition assay revealed the 1:20 diluted ethanolic and methanolic *O. basilicum* extracts to have strongly inhibited the activity of

amylase as no clear zones were observed around them (Figure 6), while the 1:20 diluted aqueous extract inhibited amylase better than the negative controls of phosphate buffer incubated with 1:10 and 1:20 diluted amylase solutions. Thus, it can be inferred that these extracts exhibit anti-diabetic activity by acting as amylase inhibitors.



Figure 4. *O. basilicum* Starch Diffusion Assay Plate

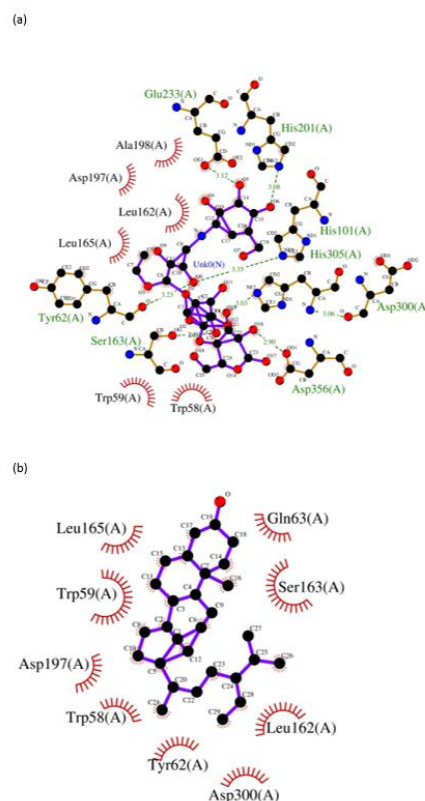


Figure 5. 2D representation of (a) Acarbose and (b) Gamma Sitosterol in salivary amylase on LigPlot⁺

Table 5. Compounds in *O. basilicum* identified by GC-MS analysis

Peak #	Retention Time	Area	Area %	Name of compound
1	3.225	96620	0.40	2-Butenal,2-ethenyl-
2	3.733	58326	0.24	Oxime-,methoxy-phenyl-
3	3.951	128570	0.54	Pyrrolidine-2,4-dione
4	4.761	193904	0.81	Tetraethyl silicate
5	5.067	77293	0.32	Cyclopropanecarboxylic acid, 3-formyl-2,2-dimethyl-, ethyl ester
6	5.363	102014	0.43	Cyclopropanecarboxylic acid, 3-formyl-2,2-dimethyl-, ethyl ester
7	5.539	118778	0.50	Eucalyptol
8	6.394	4615260	19.24	1,6-Octadien-3-ol,3,7-dimethyl
9	7.192	209319	0.87	Bicyclo[2.2.1]heptan-2-one,1,7,7-trimethyl-,(1R)
10	7.865	132923	0.55	Estragole
11	9.354	690076	2.88	2-Propenoic acid, 3-phenyl, methyl ester
12	9.513	100059	0.42	2-Methoxy-4-vinylphenol
13	10.147	55628	0.23	2,7-Octadiene-1,6-diol,2,6-dimethyl-
14	10.417	2789773	11.63	2-Propenoic acid, 3-phenyl, methyl ester
15	10.976	85219	0.36	Caryophyllene
16	11.149	58205	0.24	Azulene, 1,2,3,4,5,6,7,8-octahydro-1,4-dimethyl-7-(1-methylethenyl)-,[1S-(1.alpha,4.alpha)
17	11.743	86819	0.36	1,6-Cyclodecadiene,1-methyl-5-methylene-9-(1-methylethyl)-,[s-(E,E)]=
18	11.945	105311	0.44	.gamma.-Elemene
19	12.127	286634	1.20	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)
20	12.350	57915	0.24	3,3,5,5-Tetramethylcyclohexyl isopropylphosphonofluoride
21	13.019	199321	0.83	Diethyl Phthalate
22	13.370	108803	0.45	Cubanol
23	13.642	840871	3.51	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)
24	13.816	58573	0.24	Viridiflorol
25	15.561	387818	1.62	3,7,11,15-Tetramethyl-hexadecen-1-ol
26	15.815	74217	0.31	3,7,11,15-Tetramethyl-hexadecen-1-ol
27	16.008	112865	0.47	3,7,11,15-Tetramethyl-hexadecen-1-ol
28	16.457	76396	0.32	Acetamide, 2-(diethylamino)-N-(2,6-dimethylphenyl)-
29	16.796	100217	0.42	1-(+)-Ascorbic acid 2,6-dihexadecanoate
30	17.105	67930	0.28	Hexadecanoic acid, ethyl ester
31	17.352	73207	0.31	3-Carene
32	17.606	72985	0.30	3-Carene
33	18.281	4013291	16.73	Phytol
34	18.597	491121	2.05	Bicyclo[3.1.0]hexan-2-ol, 2-methyl-5-(1-methylethyl)-, (1.alpha,2.alpha,5.alpha)-
35	18.838	454801	1.90	1,5-Dimethyl-1-vinyl-4-hexenyl butyrate
36	19.914	109367	0.46	2-Propenoic acid, 3-phenyl-, methyl ester
37	20.202	713920	2.98	2-Propenoic acid, 3-phenyl-, methyl ester
38	20.376	878011	3.66	2-Propenoic acid, 3-phenyl-, methyl ester
39	22.004	325103	1.36	Benzyl-diethyl-(2,6-xylyl-carbamoylmethyl)-ammonium benzoate
40	22.284	57396	0.24	Azulene, 1,2,3,4,5,6,7,8-octahydro-1,4-dimethyl-7-(1-methylethenyl)-,[1S-(1.alpha,4.alpha)
41	23.973	516887	2.15	2,6,10,14,18,22-Tetracosahexaene,2,6,10,15,19,23-hexamethyl-,(all E)-
42	26.708	201799	0.84	.alpha.-Tocopherol-.beta.-D-mannoside
43	28.078	370597	1.54	Campesterol
44	28.455	477572	1.99	Stigmasterol \$\$ Stigmasta-5,22-dien-3-ol,(3.beta.,22E)
45	29.251	1844005	7.69	.gamma.-Sitosterol
46	30.722	1239038	5.16	4,4,6a,6b,8a,11,12,14b-Octamethyl-1,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a,14,14a,14b-oc
47	32.304	74396	0.31	Silicic acid, diethyl bis(trimethylsilyl) ester

Table 6. Vina results of the lowest energy conformer of the ligands docked against human salivary amylase where rmsd/ub = RMSD upper bound, rmsd/lb = RMSD lower bound

Ligand	Vina score (kcal/mol)	rmsd/ub (Å)	rmsd/lb (Å)
Acarbose	-8.4	0	0
Gamma Sitosterol	-8.0	0	0
Caryophyllene	-7.6	0	0
Gamma Cadinene	-7.1	0	0
Sabinene hydrate	-5.4	0	0
Estragole	-5.3	0	0

Table 7. Vina results of the lowest energy conformer of the ligands docked against human pancreatic amylase where rmsd/ub = RMSD upper bound, rmsd/lb = RMSD lower bound

Ligand	Vina score (kcal/mol)	rmsd/ub (Å)	rmsd/lb (Å)
Acarbose	-9.7	0	0
Gamma Sitosterol	-9.5	0	0
Caryophyllene	-7.3	0	0
Gamma Cadinene	-6.5	0	0
Sabinene hydrate	-5.7	0	0
Estragole	-5.6	0	0

Table 8. Protein-Ligand Interactions

Ligand / PubChem CID	Protein Backbone / PDB ID	Hydrogen Bond Interaction	Hydrophobic Interaction	Common Interacting Residues
Gamma Sitosterol / 457801	Human Salivary Amylase / 1SMD	-	Gln63, Ser163, Leu162, Asp300, Tyr62, Trp58, Asp197, Trp59, Leu165	Ser163, Leu162, Asp300, Tyr62, Trp58, Asp197, Trp59, Leu165
Acarbose / 444254		Asp356, His305, Glu233, His201, Ser163, Asp300, Tyr62, His101	Leu162, Trp58, Ala198, Asp197, Trp59, Leu165	
Gamma Sitosterol / 457801	Human Pancreatic Amylase / 1XD0	-	Gln63, Thr163, Glu233, Leu162, His201, Asp300, Tyr62, His101, Trp58, Asp197, Trp59, Leu165, His305	His201, Glu233, Asp300, Asp197, His101, Tyr62, Gln63, Leu165, Thr163, His305, Leu162
Acarbose / 444254		Lys200, Glu233, Asp197, His101, Thr163, Tyr151	Val234, Ala198, Ile235, Asp300, Tyr62, Gln63, Leu165, Gly306, His305, Leu162, His201	

It was also observed that the diameter of the clear zone around the phosphate buffer incubated with the 1:10 diluted amylase solution was larger than that of the phosphate buffer incubated with the 1:20 diluted amylase solution, suggesting that the 1:20 diluted amylase solution is more optimal.

Among the 47 compounds present in *O. basilicum*, all the compounds were manually verified for their anti-diabetic activity as biological activity proof and from this literature search, five compounds, known for their anti-diabetic effects and which are not hazardous to health, were considered for molecular docking studies. The five compounds are Caryophyllene, Estragole, Sabinene hydrate (i.e., Bicyclo[3.1.0]hexan-2-ol, 2-methyl-5-

(1-methylethyl)-, (1.alpha,2.alpha,5.alpha)-, γ -Cadinene (i.e., Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)) and γ -Sitosterol. Vina results reveal Gamma Sitosterol to have a binding affinity closer to that of Acarbose in both human salivary amylase and human pancreatic amylase (Tables 7 and 8 respectively) among the considered five ligands.

Gamma Sitosterol interacts with 13 residues when docked in pancreatic amylase and 9 residues when docked in salivary amylase. It should be noted that Gamma Sitosterol interacts hydrophobically with the same nine residues in human pancreatic amylase that it interacts with in human salivary amylase. Moreover, the Vina score for Gamma

Sitosterol and Acarbose is comparable in both salivary and pancreatic amylase (Tables 7 and 8 respectively). In fact, the Vina score for these ligands are better when docked against pancreatic amylase compared to salivary amylase. Additionally, Gamma Sitosterol and Acarbose have common interacting residues in both the protein backbones (Table 5). Thus, this study strongly supports the inhibition of both salivary and pancreatic amylase activity, even though all *in vitro* experiments were performed with salivary amylase.

In the RMSD plot (Figure 7), it can be observed that between ~5 ns to ~30 ns, ~40 ns to ~50 ns, and ~80 ns to ~90 ns, the protein-ligand complex undergoes high structural changes compared to the C α of the APO form and the ligand is not stably bound in the binding pocket of the protein. However, C α of the APO form has higher RMSD values compared to the protein-ligand complex. Thus, it can be inferred that the protein-ligand complex is relatively more stable than the APO form alone.

The peaks in the RMSF plot reveal the protein areas that fluctuate the most during the simulation. Here, it can be seen that the C α of the residues between ~120 to ~380 and those between ~440 to ~480 fluctuate more compared to C α of other

residues in the RMSF of the protein backbone (Figure 8). The 350th protein residue approximately fluctuates the most, followed by the residues at the 150th and 380th index approximately. The RMSF of Gamma Sitosterol fit on protein shows that atoms at the 21st, 27th, and 29th indices fluctuate the most during the simulation (Figure 9).

The generated protein-ligand contacts plot by MD (molecular dynamics) simulation (Figure 10) shows hydrophobic interactions, hydrogen bonds and water bridges. Tyr59 interacted with the ligand for the longest time at 0.8, followed by Asn105 at approximately 0.58. Asn105 interacted with the ligand via hydrogen bonds and water bridges, with hydrogen bonding interactions lasting longer than water bridges. It is important to note that all the residues interacting hydrophobically depicted in the 2D interaction of salivary amylase and Gamma Sitosterol on LigPlot⁺, with the exception of Ser163, also interact hydrophobically with the ligand during the MD simulation. Ser163 formed water bridges with the ligand during the simulation for an extremely short time duration. One must also consider that LigPlot⁺ does not depict water bridges and hence, it is not possible to know if Ser163 formed water bridges in the 2D protein-ligand interaction plot as well.

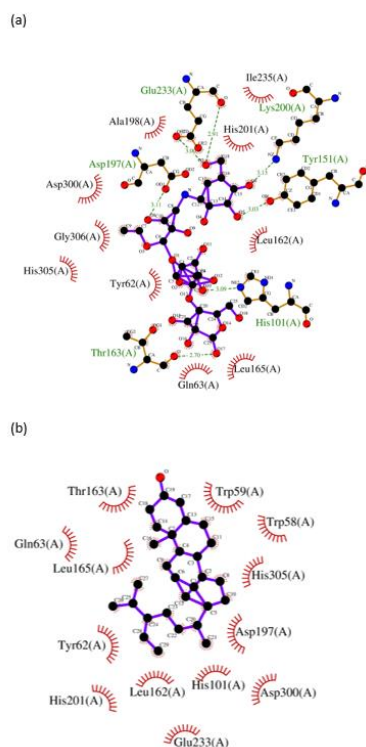


Figure 6. 2D representation of (a) Acarbose and (b) Gamma Sitosterol in pancreatic amylase on LigPlot⁺

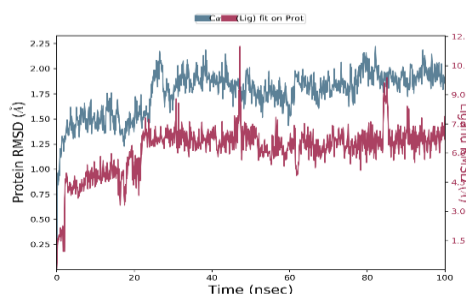


Figure 7. RMSD of Protein-Ligand Complex. The blue peaks indicate the RMSD of the APO protein whereas the maroon peaks indicate that of the protein-ligand complex

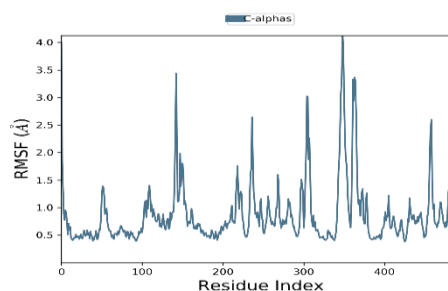


Figure 8. RMSF of APO Protein Backbone

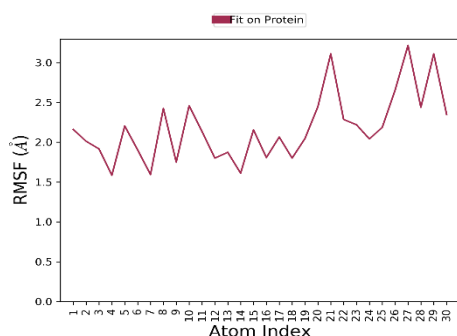


Figure 9. RMSF of Protein-Ligand (Gamma Sitosterol) complex.

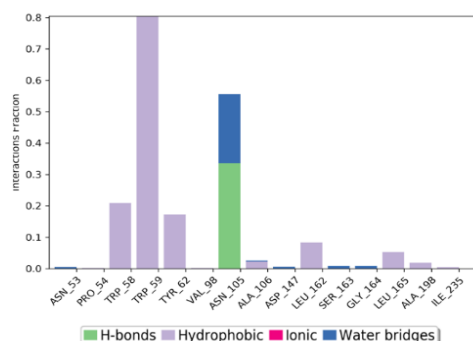


Figure 10. Protein-Ligand Contact Interaction Plot

Conclusions

With T2D increasing progressively worldwide and on the path to becoming an epidemic, it is imperative that an easily available, affordable, safer and healthier alternative be found. As divulged in this paper, 1:20 diluted ethanolic, methanolic and aqueous extracts of *O. basilicum* have shown inhibitory activity against α -amylase *in vitro*. It was found that Gamma Sitosterol, one of the compounds found in relative abundance in *O. basilicum* as revealed through GC-MS analysis, is one of the phytochemicals exhibiting this property through *in silico* analysis. Although human salivary amylase has been used in both *in vitro* and *in silico* studies, the findings can be extrapolated to human pancreatic amylase as salivary amylase and pancreatic amylase are isoenzymes and are ~97% homologous. Therefore, it can be conferred that Gamma Sitosterol can be used as a potential therapeutic for T2D alongside other measures such as physical exercise and diet. However, *in vivo* studies as well as isolation and phytochemical characterisation are necessary to completely validate the findings.

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