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**Purification, Characterization and Molecular docking studies of analogous alpha
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Abstract:	<p>Background - A large number of protein inhibitors are found in higher plants, cereals and legumes. These inhibitors are helpful in the prevention as well as medical treatment of metabolic syndromes such as type 2 diabetes. Basically, the diabetes mellitus is a chronic disorder that occurs either due to inadequate insulin secretions or when body fails to utilize the produced insulin. The alpha amylase inhibitors are termed as starch blockers. They catalyse hydrolysis of α-(1,4)-D-glycosidic linkages of starch and other glucose polymer. They play a significant role in inhibition of the activity of salivary and pancreatic amylase in vitro and in vivo. Method -The present study was based on the purification, characterization and molecular docking studies of the alpha amylase inhibitor isolated from the kidney bean sample. The seed sample was collected from G.B. Pant Nagar University. The crude extract was prepared, the in-vitro studies and heat stability were determined. Following it, purification was carried out using ammonium sulphate precipitation and size exclusion chromatography was done. Later, characterization and molecular docking studies of the purified sample was done after obtaining GC-MS results. Result - The in-vitro analysis was done and noted that the inhibitory activity was $96.5 \pm 0.84\%$ post size exclusion chromatography. The molecular weight was about 54kDa. The molecular docking studies revealed that there were interactions between the ligand molecules (the constituents that were selected from the GC-MS chromatogram peaks based on the height and area) and the human pancreatic alpha amylase (1HNY). The hydrogen bonding as affinity binding capacity was also been observed in each of the ligand-protein interaction. Conclusion - From the tests, it has been elucidated that the alpha amylase inhibitor isolated from the sample has a great potential to serve as an anti-diabetic drug. However, further in-vivo studies are required to be applied to the diseased animals in order to check the potential effects and to explore the optimization and development of the anti-diabetic drug.</p>

Purification, Characterization and Molecular docking studies of analogous alpha amylase inhibitor compounds

Abstract

Background - A large number of protein inhibitors are found in higher plants, cereals and legumes. These inhibitors are helpful in the prevention as well as medical treatment of metabolic syndromes such as type 2 diabetes. Basically, the diabetes mellitus is a chronic disorder that occurs either due to inadequate insulin secretions or when body fails to utilize the produced insulin. The alpha amylase inhibitors are termed as starch blockers. They catalyze the hydrolysis of α -(1,4)-D-glycosidic linkages of starch and other glucose polymer. They play a significant role in inhibition of the activity of salivary and pancreatic amylase in vitro and in vivo. **Method** -The present study was based on the purification, characterization and molecular docking studies of the alpha amylase inhibitor isolated from the kidney bean sample. The seed sample was collected from G.B. Pant Nagar University. The crude extract was prepared, the in-vitro studies and heat stability were determined. Following it, purification was carried out using ammonium sulphate precipitation and size exclusion chromatography was done. Later, characterization and molecular docking studies of the purified sample was done after obtaining GC-MS results. **Result** - The in-vitro analysis was done and noted that the inhibitory activity was $96.5 \pm 0.84\%$ post size exclusion chromatography. The molecular weight was about 54kDa. The molecular docking studies revealed that there were interactions between the ligand molecules (the constituents that were selected from the GC-MS chromatogram peaks based on the height and area) and the human pancreatic alpha amylase (IHNY). The hydrogen bonding as affinity binding capacity was also been observed in each of the ligand-protein interaction. **Conclusion** – From the tests, it has been elucidated that the alpha amylase inhibitor isolated from the sample has a great potential to serve as an anti-diabetic drug. However, in order to check the potential effects and to explore the optimization and development of the anti-diabetic drug the in-vivo studies can also be done further.

Keywords- Alpha amylase inhibitor, diabetes, GC-MS analysis and molecular docking.

1. Introduction

The diabetes mellitus is a chronic disorder that give birth to the number of ailments that ultimately affects the body. Basically, DM is caused either due to inadequate insulin secretions or when body fails to utilize the produced insulin. It initiates with the signs and symptoms that include polyuria (a condition causing increased urination), glycosuria (presence of reducing sugars in the urine), excessive hunger and fatigue (Bhosale *et al.*, 2011). There are various different subclassifications of the diabetes such a diabetes mellitus type I and type II, gestational and neonatal diabetes and maturity-onset diabetes of the young (MODY). Above all, the type II diabetes mellitus is more prevalent in all the nations as compared to the other types. As per world health organisation (WHO), the type II diabetes contributes about 85% in developed countries (Brayer *et al.*, 2000). For the treatment of diabetes various therapeutic drugs and diets are prescribed to reduce the post prandial hyperglycaemia. The drugs belongs to the classes of thiazolidinediones (TZDs), sulfonylureas, DPP-4 inhibitors and SGLT2 inhibitors. Despite the advantage of reducing blood glucose levels they have adverse effects on the body. Thus, an

alternative approach to tackle with this problem is by using of plant-based diets and drugs (Demir *et al.*, 2019). As per World health Organization, it has been reported that in case of diabetic patients the consumption of more leguminous food is beneficial due to low glycemic index. Additionally, they also contain various bioactive compounds and alpha amylase inhibitor that aids in inhibiting the activity of alpha amylase. Thus, reducing the blood glucose level.

Basically, the alpha amylase inhibitor has been used for generation of drugs that are curative in case of diabetes such as voglibose, miglitol (Dilip *et al.*, 2006). It works on the principle of the breaking down of large complex carbohydrates starch into smaller starch molecules. Primarily, these inhibitors cleave the α (1 \rightarrow 4) glycosidic linkage present in starch by the process of the endohydrolysis. In the present study, in-vitro evaluation, purification and characterization of the alpha amylase inhibitor that has been extracted from the sample was done.

Material and Methodology

2.1 Sample Collection and Analysis- *Phaseolus vulgaris* seed sample was collected from G.B Pant Nagar University of Agricultural and Technology, Pantnagar, Uttarakhand. The collected sample was stored in airtight container.

1.2 Methodology

2.2.1 Preparation of crude extract – The crude extract was prepared using 100mg of seed sample in extraction buffer (10mM Tris -HCl, 500mM NaCl, 1% 2-Mercaptoethanol, 0.1% Triton- X-100, 2mM PMSF). The seeds were grinded into fine powder and about 0.1gm of sample was weighed and dissolved in 100 ml of extraction buffer. The contents were homogenized using pestle and motor and transferred into falcon tubes, followed by centrifugation at 10,000 rpm at 4°C for 15 mins. The supernatant was collected and utilized for further experimentations (Iguti *et al.*, 1991).

1.2.2 In-vitro assessment of alpha amylase inhibitory activity - The analysis of alpha amylase inhibitory activity was done by using soluble starch solution (0.4ml, 1% w/v) prepared in 80mM phosphate buffer (pH 6.9), porcine pancreatic alpha amylase (0.2ml, 0.05% w/v) in 20mM acetate buffer (pH- 4.5) was added and then incubated for 15mins at 37°C. Then, the reaction was stopped by addition of 0.8ml dinitrosalicylic acid reagent. The contents were heated in boiling water for about 5 mins and then cooled immediately. The optical density was measured at 540nm. Alpha-amylase inhibitory activity was determined according to equation shown below:

$$\text{Inhibitory activity (\%)} = \left[\frac{M_o - M_i}{M_o} \right] \times 100$$

Where, M_o and M_i are amount of maltose (mg/ml) produced in absence and presence of inhibitor respectively, under the same conditions (Ishimoto *et al.*, 1989).

1.2.3 Purification of α -amylase inhibitor - The crude protein extract was taken and subjected to ammonium sulphate precipitation (30-90% saturation) at 4°C. The precipitate was then dissolved in 10mM Tris-HCl and was dialyzed against buffer in batches. Then, the size exclusion chromatography was performed using Sephadex G-50 column (Singh *et al.*,

2021).

1.2.4 Characterization of the purified alpha amylase inhibitor

Heat stability of alpha-amylase inhibitor - For determination of optimum temperature for activity of the enzyme, the assay was carried out by taking 0.5 ml of the enzyme extract. The reaction mixture was incubated for 10 to 15 min at 40°C to 100°C, followed by the addition of 1.0 ml of dinitrosalicylic acid reagent (DNS reagent). The optical density was measured at 540 nm. (Qian *et al.*, 1994).

1.2.4.1 **Molecular weight determination** - The purified sample was characterized to determine molecular weight of purified alpha amylase inhibitor. SDS-PAGE was done as per the following protocol (Marshall *et al.*, 1975). Initially, gels were prepared using acrylamide and bisacrylamide following it 1.875 M Tris- HCl (pH 8.8) separating gel and 0.6 M Tris-HCl (pH 6.8) were used. Then, the gels were cast. The sample was mixed with sample buffer (1M Tris HCl (pH=6.8), SDS, beta-mercaptoethanol, glycerol and 1%) before loading. The sample was loaded along with ladder. The gel was run at 120-140V for about 1.5- 2 hours in electrophoretic buffer containing. Then, the gels were stained for 45-50 mins in a solution containing Coomassie brilliant blue following it, the gel was kept in **destaining** solution (glacial acetic acid and methanol) for overnight (Moreno *et al.*, 1990).

1.2.4.2 **GC-MS analysis of analogous alpha amylase inhibitor compounds** – In this case, the 25gm of seed sample was homogenised properly in 150ml of methanol and it was kept for 5 days. Then, the solution was filtered using Whatman filter paper. After filtration, the sample was centrifuged at 15000 rpm for 15mins. The supernatant was collected and stored in cooling conditions (Mosca *et al.*, 2008). Then, the GC-MS analysis was done in order to find out the chemical constituents that are present in the purified extract. The SH-Rxi-5Sil MS column (30 metre, 0.25mmID, 0.25um df) was used for the analysis. During the experimentation the helium gas was used as a carrier gas and 1ml/min of the flow rate was maintained. The temperature of 280°C was set for the injection transfer and for the ion source whereas the ionization energy was 70 electron volts. At last, the data was collected (Mubarak *et al.*, 2005).

1.2.4.3 **Molecular docking** – The molecular modeling software, Autodock-vina was used to study molecular docking. PDB or proteins 1HNY alpha-amylase was selected and extracted from the protein data bank. The structure of this molecule was downloaded in 3D conformation in the sdf format that was converted into pdb using pymol software. Then, the pdbqt file was prepared for both ligand as well as the protein molecule using autodock. The molecular docking was performed using autodock vina tool and the results were analyzed in discovery studio to see the ligand and protein interactions (Huang *et al.*, 2010).

b. Results and Discussion

3.1 Purification and alpha amylase inhibitory activity- The Chromatography fractions were

collected at a constant flow rate of $100\mu\text{l}/\text{min}$. The in-vitro enzymatic inhibitory activity was carried out and it has been found that the crude extract of the sample *Phaseolus vulgaris* shows $60.3 \pm 0.49\%$ inhibition, whereas after ammonium sulphate precipitation it shows the maximum inhibitory activity of $86.8 \pm 0.97\%$ and protein content was 10.5% . After purification via sephadex G-50 column the inhibitory activity was $96.5 \pm 0.84\%$ while protein content reduced to 7.96% . It has been noted that the inhibitory activity was increasing after each step of the purification. Thus, the specific activity was also rising as it was 4.0 in case of crude extract but rise to 12.12 after purification.

3.2 Heat stability- In this case, the sample was incubated at different temperatures ranging from 40°C - 100°C for 10 -15 mins. After incubation, the optical density of sample was measured at 540nm . It was observed that the sample has shown the good enzymatic activity at temperature ranging from 40°C - 70°C but above this temperature range the alpha amylase inhibitor starts degrading and the enzymatic activity started declining.

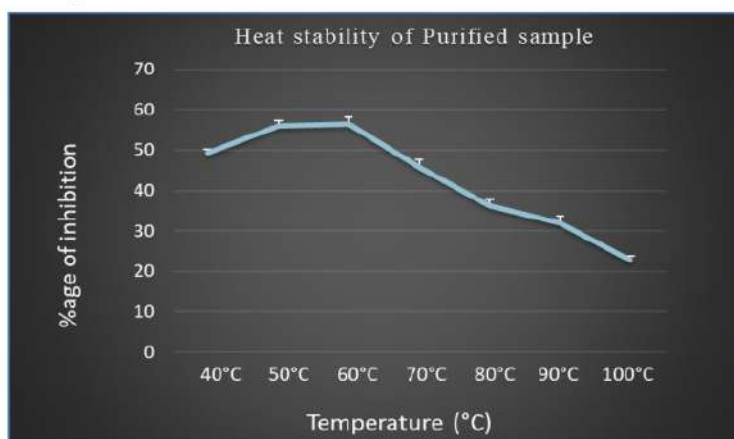


Fig.1 The graph represents the effect of temperature on the in-vitro alpha amylase inhibitory activity of the purified sample

3.3 Characterization of purified alpha amylase inhibitor

3.3.1 Molecular weight determination - The purified fraction after size exclusion chromatography was loaded in SDS-PAGE gel in order to determine the molecular weight. Both Native-Page as well as SDS-PAGE characterization has been done. The figure 3, represents the Native- Page of the purified sample (Pant Anupama) a single band has been observed corresponding to the molecular weight of 54KDa. While, the figure 4 shows the bands as two subunits of 22KDa and 28KDa this might be due to the denaturation of the pure amylase inhibitor into two subunits.

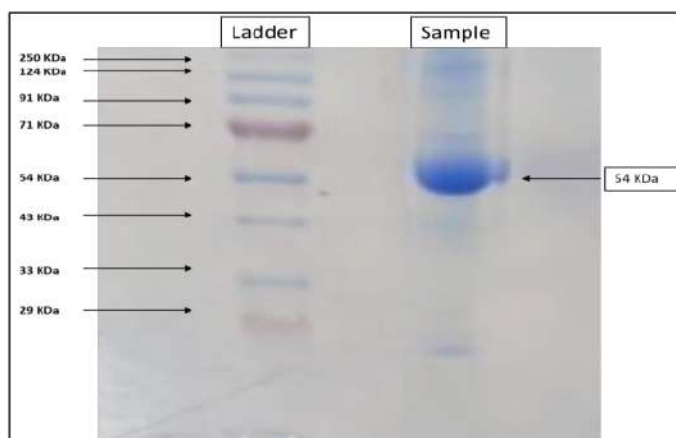


Fig.2 Native-Page of the purified sample was carried out, the image shows the protein ladder and the bands of the sample at 54KDa.

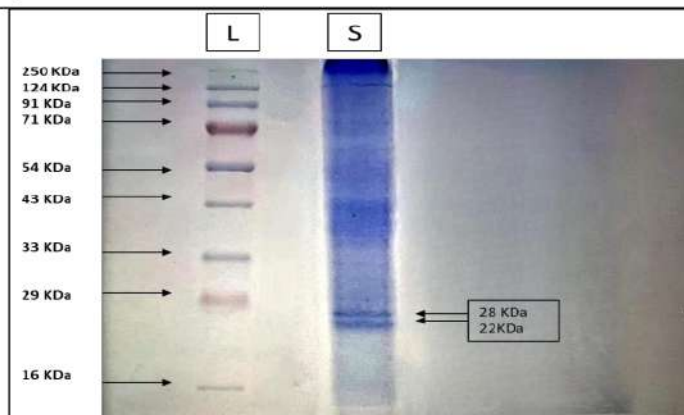
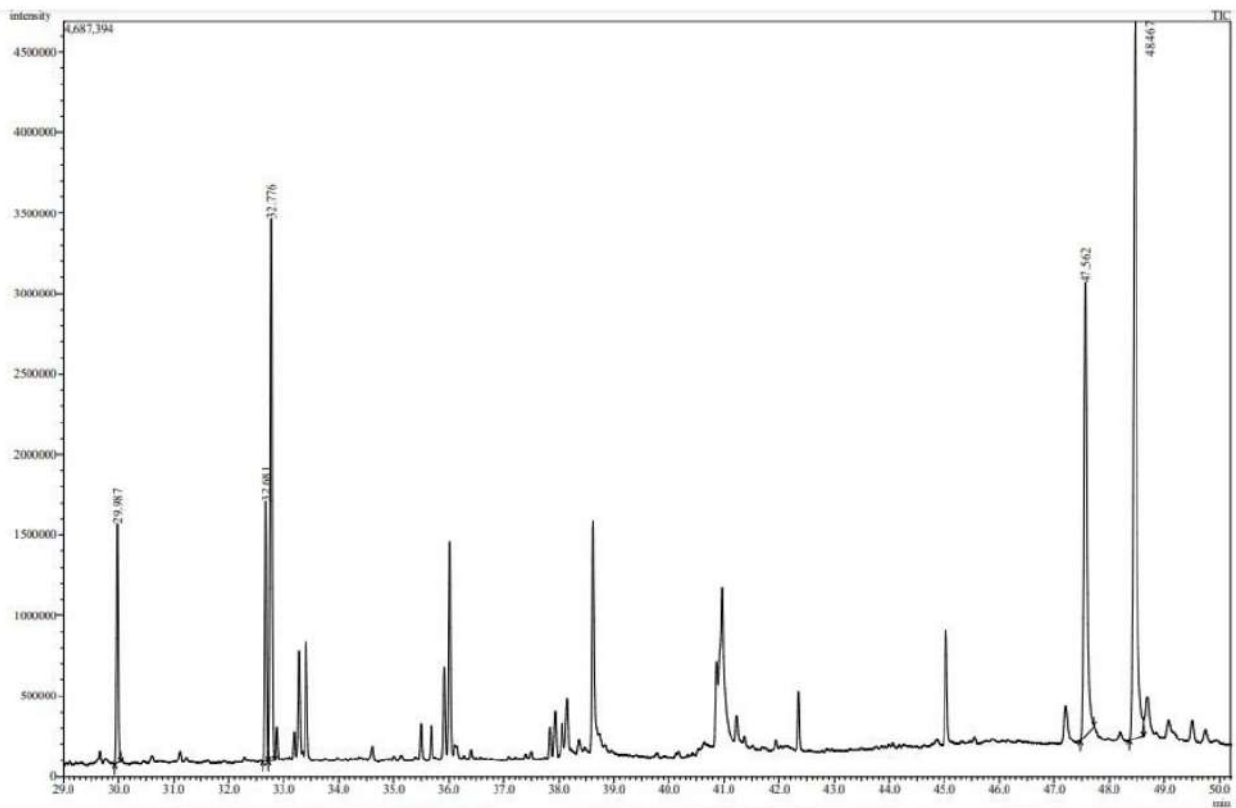


Fig.3 SDS-Page of the purified sample was carried out, the image shows the protein ladder and the bands of the sample showing two subunits of 28 KDa and 22 KDa.

3.3.2 GC-MS analysis and Molecular docking - The purified methanol extract of the *Phaseolus vulgaris* has shown twenty-five peaks from time ranging between twenty-nine to forty-nine minutes. The identities of these compounds in terms of the retention time, area and height percentage have been studied and it has been observed that five compounds have covered maximum area and height in terms of the percentage (figure 4). They are namely, hexadecanoic acid-methyl ester which has 3.92% and 5.46% of area and height respectively. After this, the 9,12, Octadecanoic acid (Z, Z)-methyl ester has covered 4.27% of area and 5.93% of height. The maximum peak height and area is covered by gamma sitosterol i.e., 22.64% of area and 16.34% of height. Whereas, the second highest in number was stigmasterol with area and height percentage of 12.54 and 10.33. In case of the 9,12,15, Octadecanoic acid, methyl ester the third highest percentage of the area and height has been covered i.e. area 9.97% and height 12.35%.



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Fig.4 Chromatogram of methanolic extract of *Phaseolus vulgaris* along with the table below describing each peak name, area and height.

Peak#	Name	R.Time	Area	Area %	Height	Height %
1	Hexadecanoic acid, methyl ester	29.987	3282061	3.92	1487003	5.46
2	9,12-Octadecadienoic acid (Z, Z)-, methyl ester	32.681	3576152	4.27	1614437	5.93
3	9,12,15-Octadecatrienoic acid, methyl ester	32.776	8357197	9.97	3363820	12.35
4	Stigmasterol	47.562	10510315	12.54	2813135	10.33
5	Gamma-Sitosterol	48.467	18968903	22.64	4450414	16.34

Table. 1 Table describing each peak name, area and height formed from methnolic extract of *Phaseolus vulgaris*

In addition to the study height as well as area percentages of the peaks it has also been studied that these 5 constituents have some specific characteristics in terms of the pharmacological properties. According to already present reports (Mukherjee *et al.*, 2006), the octadecanoic acid exhibits anti-diabetic properties same as alpha-glucosidase inhibitors. Whereas, as per the research done by (Renganatha *et al.*, 2021) the in-silico study has revealed that the hexadecenoic acid possess the properties of the potential alpha amylase inhibitor. It has been elucidated by Adewole *et al.* (2022) that the stigmasterol has a potential mechanism in order to act against alpha amylase and alpha glucosidase enzymes, thus it can be a great therapeutic component that can be utilized in lowering the blood glucose level. Therefore, these components can be widely used as a curative for the treatment of the diabetes mellitus. Furthermore, the molecular docking studies has been done that reveals the interactions between the ligand molecules (the constituents that were selected from the GC-MS chromatogram peaks based on the height and area) and the human pancreatic alpha amylase (1HNY). The hydrogen bonding has been observed in each of the ligand-protein interaction. Also, the affinity binding has also been calculated. The details are as follows. The figure 5 represents the molecular docking between the ligand Hexadecanoic acid, methyl ester and the protein namely 1HNY. It has been observed that the there were total nine conformations of the binding between the ligand and the protein but of the nine the one that has shown maximum hydrogen bonding was mode 2 which shows 2 hydrogen bonds with affinity of -6.5 kcal/mol. In case of the docking between the ligand 9,12, Octadecanoic acid (Z, Z)-methyl ester and 1HNY (figure 6) out of the nine conformations of the binding between the ligand and the protein the one that has shown maximum hydrogen bonding was mode 2 which shows 3 hydrogen bonds with affinity of -7.4 kcal/mol. Likewise, in case of the 9,12,15, Octadecanoic acid, methyl ester and 1HNY binding (figure 7) the total hydrogen bonds noticed was 1 with affinity value -6.0 kcal/mol. The total 2 hydrogen bonds with affinity -8.7 kcal/mol and -9.5 kcal/mol was observed in both Stigmasterol (figure 8) and gamma sterol (figure 9) respectively. In each figure, the hydrogen bonding is represented as pink-white-green color which depicts the donor and the acceptor sites between the binding molecules.

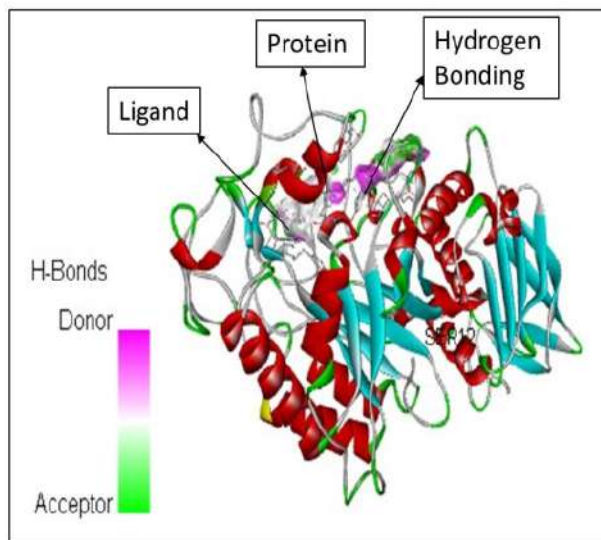


Fig.5 The molecular docking between the ligand Hexadecanoic acid, methyl ester and the protein namely 1HNY

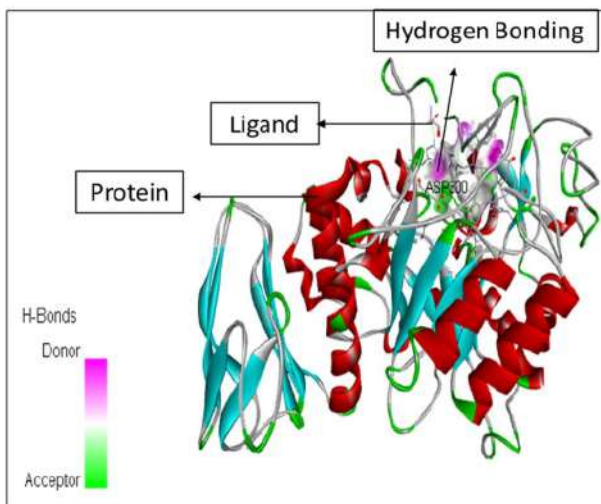


Fig.6 The figure represents the molecular docking between the ligand 9,12-Octadecanoic acid (Z,Z)-methyl ester and 1HNY.

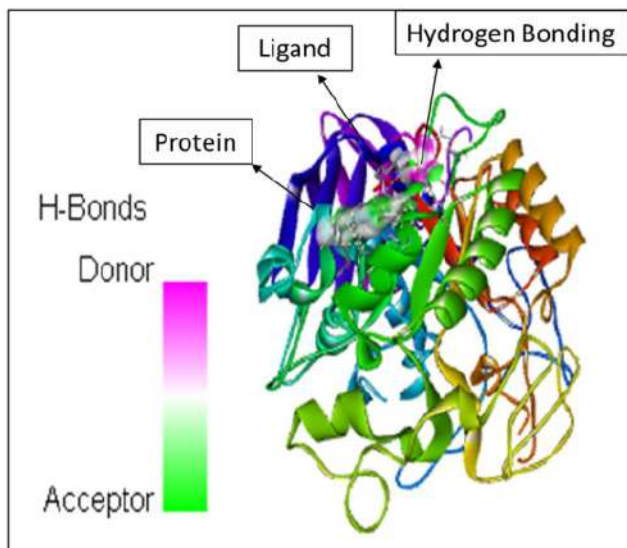


Fig.7 The molecular docking between 9,12,15, Octadecanoic acid, methyl ester and IHNY

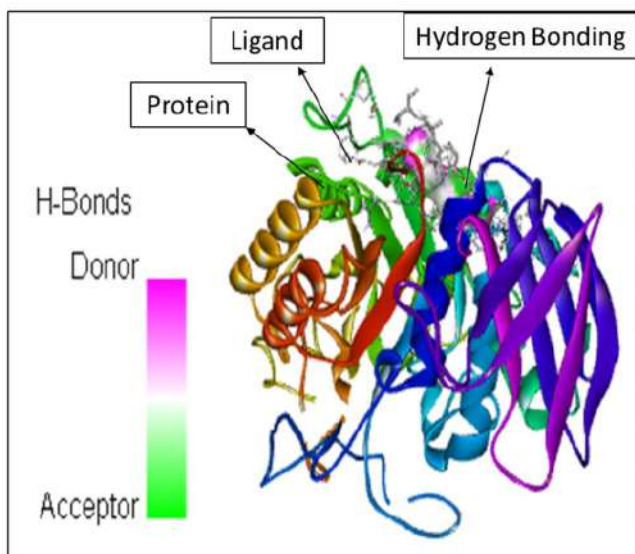


Fig.8 The molecular docking between the ligand stigmasterol and the protein IHNY.

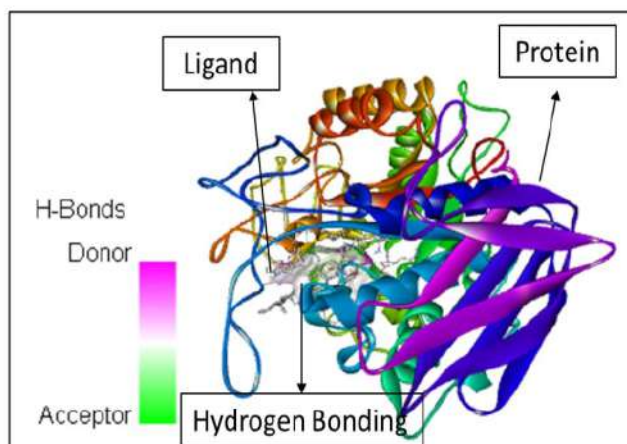


Fig.9 The molecular docking between the ligand gamma-sterol and the protein 1HNY.

Discussion

The alpha amylase hydrolyzes the starch molecule to yield low molecular weight dextrin's and sugars. Therefore, causing in rise in blood glucose level. The inhibition action of α -amylase is carried out by α -amylase inhibitors. They catalyse the hydrolysis of α -(1,4)-D-glycosidic linkages of starch and other glucose polymer. Furthermore, they affect the process of the carbohydrate absorption in the small intestine by inhibiting the activity of the carbohydrate hydrolysing enzymes (Remah et al., 2019). Thus, they are very helpful in management of the postprandial hyperglycaemia (PPHG) in diabetic patients. They also play a significant role in inhibition of the activity of salivary and pancreatic amylase in vitro and in vivo (Narkhede et al., 2011).

Currently, the research is focused on the fabrication of anti-diabetic drug that has a great potential in lowering blood glucose level with least or no side effects. In order to attain the aim, in this study the alpha amylase inhibitor and its analogues were isolated and purified from the sample of the kidney beans (*Phaseolus vulgaris*). The sample was collected and stored in air tight container. Then, the in-vitro analysis of the sample was done against porcine pancreatic amylase and around $60.3 \pm 0.49\%$ inhibitory activity of crude extract was observed. It has been previously reported by Marshall and Lauda (1975) that the percentage of inhibition tends to increase after purification as compare to crude extract. A similar trend was noticed in this study that after purification via ammonium sulphate and sephadex G-50 column the percentage of inhibition reached to $96.5 \pm 0.84\%$. The results were compared with already published data by (Tamil et al., 2010) that the *Phyllanthus amarus* has shown 75.32% alpha amylase inhibitory activity that is quite less than the inhibition percentage obtained in current study.

Similar study has been reported by (Imad et al., 2008) the in-vitro analysis of the Jordanian flora samples the study reveals that out of 35 species only 4 were able to show inhibitory activity but even

their percentage of inhibition was low in comparison to one reported in the present study. The characterization and molecular docking has been done in addition to this the components revealed after the GC-MS analysis possess great hydrogen bonding.

The characterization of the purified extract was done and it was found that a single band of 54KDa was observed in case of Native- Page while two subunits of 28 and 22 KDa were noticed in case of SDS-Page that might be due to denaturation of the pure alpha amylase inhibitor into two subunits. The obtained results were compared with the already published data that shows the molecular weight of the alpha amylase inhibitor was 56KDa (Singh *et al.*, 2021). Further, the GC-MS analysis has been carried out and it has been observed that the components like hexadecenoic acid and octadecanoic acid have previously been discovered to exhibit a variety of pharmacological properties (Remah *et al.*, 2019). The hexadecenoic acid act as an alpha amylase inhibitor whereas octadecanoic acid exhibits properties same as of alpha-glucosidase inhibitor. Also, the docking studies reveals that there is presence of hydrogen bonding between the protein and the ligand molecules. This shows that the inhibitor molecules attack at the binding site of the alpha amylase in order to slow down its process of starch hydrolysis (Ghosh *et al.*, 2014). Therefore, it can be concluded that the alpha amylase inhibitor isolated from the sample has a putative potential of being as alternative herbal medicine against human pancreatic amylase to prevent the spiking of postprandial blood glucose levels in the body.

Statements and Declarations

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Compliance with Ethical Standards – There is no ethical issue concerning this article.

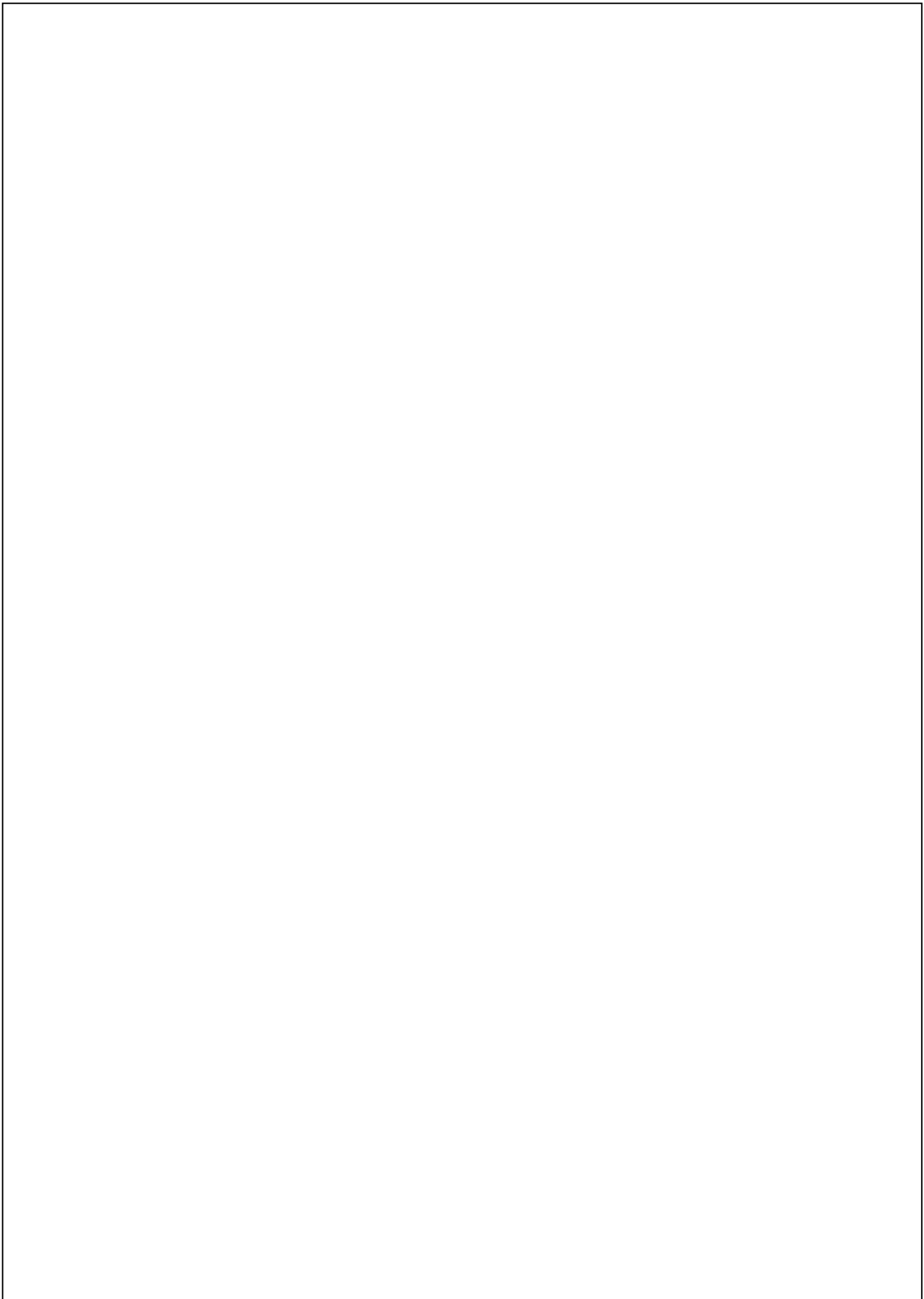
Conflict of Interest – The authors declared that they have no conflict of interest.

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