CHAPTER 8

ANTI-DIABETIC POTENTIAL OF GEDUNIN USING ENZYME KINETICS AND IN SILICO STUDY

8.1 Introduction

Medicinal plants are essential tools for treating human illnesses. There have been compelling traditional norms of restorative care, such as Chinese and Unani, born and practised more on the eastern landmass over the previous 2500 years. These conventions are still prospering, as around 80% of the general public in developing countries rely on these prescription frameworks for their critical healthcare needs**(Sen *et al.*, 2015).** These plants contain compounds that can be used as therapeutic precursors in drug production. Many studies have been conducted on various medicinal herbs, and it has been discovered that they have distinct effects on the nervous, circulatory, respiratory, digestive, and urinary systems, as well as on the sexual organs, skin, eyesight, hearing, and taste **(Sen et *al.*, 2015).** Diabetes mellitus is a metabolic condition defined by a loss of glucose homeostasis due to carbohydrate, lipid, and protein digestion caused by faults in insulin synthesis, emission, and action. Organs, such as the kidneys, liver, eyes, nerves, heart, and veins, may suffer long-term damage. Some of these organs can cause death in case of organ dysfunction. However, recently, there has been a dramatic increase in the number of physically inactive, obese, or have type 2 diabetes **(Aleksandrov *et al.*, 2010)**. The fact suggests that obesity and physical inactivity are likely to be the primary causes of the rising diabetes burden in affluent countries. Diet is one of the most critical factors for various diseases,

including diabetes **(Refardt et *al.*, 2020).**

The bioactive compounds that make neem very popular are salannin, Nimbin, meliantriaol, tetraterpenoid, triterpenoid, flavonoids, gedunin, saponin, tannins, quercetin gallic acid, limonoids, azadirachtin, and all of these belong to the limonoids, sterols, stigmasterol classes, **(Gupta *et al.,* 2017).** These bioactive compounds, such as seeds, leaves, fruits, and root extracts, are found in various parts of the neem, including molecular mechanisms, cellular mechanisms, programmed cell death, autophagy, DNA repair, inhibitory effects, anti-inflammatory, detoxifying, anti-metastatic, immune surveillance, and anti-angiogenic effects and so forth **(Hao *et al.,* 2014).**

Triterpenoid, a bioactive compound, was heavily oxidized and isolated in 1968. The secondary metabolite of neem took 17 years to predict its structure **(Hao *et al.,* 2014).** Tetraterpenoids such as gedunin can be extracted from neem fruit or oil, and it has been shown to act as an anticancer agent, antimalarial, and insecticide due to the variety of chemical surfaces inhibiting Hsp90 proteins. Each offers regioselectivity.

Azadirachta indica seeds are high in fatty acids, which account for roughly half of the seed weight, such as oleic acid, stearic acid, and palmitic acid **(Hao *et al.,* 2014),** and its oil includes certain essential amino acids. Many researchers believe that fatty acids in neem seeds have medicinal properties that can cure skin disorders.

One of the primary chemical components of *Azadirachta indica* is gedunin (a tetranortriterpenoid) from *Azadirachta indica* seeds. Recent research has demonstrated that gedunin can suppress the growth of cancer cells, including prostate, breast, pancreatic, ovarian, and colon growth. The Hsp90 (heat shock protein 90) inhibitor has also been reported **(Hao *et al.,* 2014)**. Moreover, recent

silica research has reported the pharmacological similarity of gedunine for β-catenin chain A in cancer stem cells **(Braga *et al.*, 2020).**

Gedunin is a multi-target compound that modulates pathogen-associated molecular patterns (PAMPs) and triggers an anti-inflammatory reaction via heme oxygenase and IL-10 production, which increases the therapeutic efficiency of gedunin **(Liao *et al.*, 2019).**

Efforts in this article are directed toward studying the activity of gedunin in inhibiting alpha-amylase and alpha-glucosidase enzymes.

Alpha-amylases are starch hydrolyzing enzymes that produce several products upon hydrolyses, such as dextrins and small polymers of glucose units, leading to high blood glucose levels, as in the case of type 2 diabetes mellitus **(Liao *et al.*, 2019).** Inhibitors of this enzyme maintain postprandial glucose levels by delaying digestion and absorption of intestinal sugars. Standard treatment for type 2 diabetes can be done by reducing insulin demand in the body, inhibiting the digestion of carbohydrates such as alpha-amylase inhibition, or increasing insulin reaction at target sites. α-Glucosidase (EC 3.2.1.20), maltase,

glucoinvertase, glucosidosucrase, maltase-glucoamylase, alpha-glucopyranosidase, glucosidoinvertase, alpha-glucopyranosidase, glucosidoinvertase, alpha-glucoside hydrolase, alpha-1In contrast, beta-glucosidase is a carbohydrate-digesting enzyme **(Gonzalez *et al.*, 2019)**. Alpha-glucosidase is a digestive enzyme that converts starch and disaccharides to glucose. Maltase, a maltose-cleaving enzyme, is functionally comparable **(Zhang *et al.*, 2020).** According to WHO, Diabetes caused an estimated 1.6 million deaths worldwide in 2016. According to a national diabetes report in 2017, diabetes became the 7th leading cause of death in the United States. Diabetes is a

significant cause of blindness, kidney failure, heart attack, stroke, and lower-limb amputation. The neem plant is traditionally cultivated, and its root extracts have been used for decades in northeast (Sikkim) India to treat diabetes. We focused on gedunin as a contemporary source of lead for the development of novel alpha-amylase and alpha-glucosidase *inhibitors*. Herbal drugs have low risks of side effects, are expensive, and show valuable results in due course of time compared to conventional drugs.

Reducing postprandial hyperglycemia by blocking carbohydrate-hydrolyzing enzymes in the gastrointestinal tract is a potential method for diabetes treatment, particularly in type 2 diabetes **(Zhang *et al.*, 2020).** -Amylase degrades long starch chains, whereas -glucosidase degrades oligosaccharides and disaccharides **(Zhang *et al.*, 2020).** Inhibitors of these enzymes reduce carbohydrate digestion, extending the total digestion time, producing a decrease in glucose absorption, and as a result, blunting postprandial plasma glucose levels.

Several anti-diabetic drugs are on the market, such as acarbose, inhibit-amylase, and -glucosidase. Acarbose is an oligosaccharide of microbial origin (Actinoplanes) that inhibits brush-border enzymes, such as glucoamylase, dextrinase, maltase, sucrase, and pancreatic -amylase *in vitro* and in vivo **(Brandt *et al.,* 2008).** Because of intramolecular nitrogen, acarbose binds to the carbohydrate-binding site of the -glucosidase enzyme with an affinity 104–105 times greater than a typical substrate. Because the C–N connection in the acarbose unit cannot be broken, the enzymatic process comes to a halt **(Wang *et al.,* 2021).** While acarbose and other comparable medicines effectively lower blood glucose levels, long-term usage is frequently accompanied by adverse side effects **(Zhang *et al.,* 2020).** Therefore, there is a need for natural -glucosidase and -amylase

inhibitors with no adverse or undesired side effects. Herbal extracts have long been used as inhibitory agents against -glucosidase and -amylase **(Brandt *et al.,* 2008),** which are often rich in polyphenolics and may reduce postprandial hyperglycemia via their significant antioxidant and enzymatic inhibitory actions.

8.2. Experimental

8.2.1 Molecular Docking

Gedunin with PubChem ID: 12004512 was docked with the target enzyme α-amylase PDB id 3BAI and α-glucosidase enzyme with PDB id 5KZX. The structures of the enzymes and ligands were obtained from the Protein Data Bank, RCSB database, and National Centre for Biotechnology Information, PubChem database (https://pubchem.ncbi.nlm.nih.gov/). The I.C.M. molsoft software was used for docking studies of α-amylase and glucosidase **(Ding *et al.,* 2020).**

Docking with Molsoft was achieved by converting the enzyme PDB file into an ICM object, which included the addition of hydrogen bonds, assignment of atom types, and charges from the residue templates. ICM small-molecule docking was performed: (a) Setup docking project: (1) Set project name, (2) Setup the receptor, (3) Review and adjust binding site, (4) Make receptor maps, and (b) start docking simulation. The ICM stochastic global optimization algorithm attempts to find the global minimum of the energy function that includes five grid potentials describing the interaction of the flexible ligand with the receptor and the internal conformational energy of the ligand **(Morris *et al.,* 2008)**. All the inhibitors were compared to the best binding free energy (minimum) obtained from the runs.

8.2.2 Simulation

Molecular dynamics is a method used to explore the structure of a compound/solid using classical mechanics and generating a trajectory. Gromacs, which use the concept of a periodic boundary to form boxes/grids and groups to show action, has been used for MD. After the arrangement of different information records using the OPLS force field (conf.gro for organizes, Topol.top for topology, and the fundamental parameter file, grompp.mdp), **(Gipson *et al.*, 2008),** the essential strides in utilizing GROMACS are to run the pre-processor grompp and run the principle energy minimization program mdrun. Reproduction might be achieved by ensuring multiple steps utilizing designated checkpoint files.

8.2.3 Alpha-amylase inhibition assay

A total of 500jtL of extract (2-12mg/ml) was placed in a tube, and 500 jtL of 0.02M sodium phosphate buffer (pH 6.9) containing a-amylase solution (2U/mL) was added. This solution was pre-incubated at 25ºC for 10 min. Then, 250 jtL of 0.5% starch solution in 0.02M sodium phosphate buffer (pH 6.9) was added at timed intervals and then incubated25ºC for 10min. Terminated the reaction by adding 500 jtL of dinitrosalicylic acid (DNS) reagent. The tubes were then incubated in boiling water for 5min and cooled to room temperature. Diluted the reaction mixture with 5mL of distilled water, and the absorbance was measured at 540 nm using a spectrophotometer **(Alqahtani *et al.*, 2019).** Prepared a control using the same procedure but replacing the extract with distilled water. Calculated the a-amylase inhibitory activity as percentage inhibition **(Braga *et al.*, 2020):**

$$Inhibition\% = \frac{ABcontrol - ABextract}{ABcontrol} * 100$$

8.2.3.1 Mode of inhibition

The extract with the lowest IC_{50} concentration was used for the inhibition assay. As methanol extracts had the lowest IC_{50} values, the Michaelis-Menten and Lineweaver-Burk equations were used to calculate the mode of inhibition of alpha-amylase. This IC_{50} concentration (mg/ml) of methanol extracts gave results for enzyme kinetics studies. Organized two reactions with the former having 250µl of plant extract pre-incubated with an alpha-amylase enzyme(2U/ml) for 10 min @ 25°C and later having 250µl of buffer in place of extract rest remaining same. A soluble starch solution of 250µl in increasing concentration (0.1-5mg/ml) was added as a substrate to both reactions. The reaction mixture was incubated for 10 min. The enzymatic reaction was stopped using 1 ml DNS reagent and allowed to boil for 5 min. The amount of reducing sugars was estimated spectrophotometrically using a standard maltose curve and was converted into reaction velocities. The kinetic constants Km and Vmax were calculated from the slope and intercept values in the double reciprocal plots. The product (reducing sugars) concentration was determined using a standard maltose curve and expressed as millimoles of equivalent**s (Alqahtani *et al.*, 2019).**

8.2.4 Alpha Glucosidase Inhibition assay

Glucosidase activity was determined using a slightly modified version of the **(Tadera *et al.*, 2020)** technique. The test was performed by measuring the transformation of the substrate pNPG into -D-glucose and p-nitrophenol by -

glucosidase at 405 nm. In a test tube, the enzyme (0.05 U/mL) dissolved in 100 mM phosphate buffer (pH 6.8) was pre-incubated with gedunin (0–200 M) for 5 min at 37 °C. pNPG (600 M) was added in steps and incubated with the reaction mixture at 37 °C for 30 min. The enzymatic reaction was monitored spectrophotometrically by measuring the absorbance at 405 nm **(Zhang *et al.*, 2021).** The results are related to the slope measured between 5 and 20 min. The test results were unaffected by the amount of DMSO used. Acarbose (0–2000 μM) was used as the positive control.

8.2.4.1 Mode of Inhibition

The extract with the lowest $_{IC50}$ concentration was used for the inhibition assay. As the methanol extract had the lowest $_{IC50}$ value, the Michaelis-Menten and Lineweaver-Burk equations were used to calculate the mode of inhibition of alpha-glucosidase.

The enzyme (0.05 U/mL) was pre-incubated with gedunin for 5 min at 37 °C in 100 mM phosphate buffer (pH 6.8). pNPG was added in three steps (300, 600, and 1200 M) and incubated with the reaction mixture for 30 min at 37 °C.

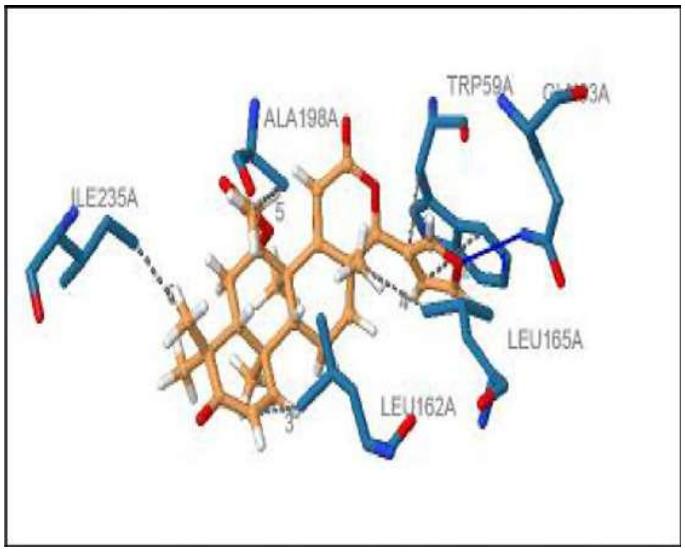
The nonlinear regression Michaelis–Menten enzyme kinetics and the corresponding Lineweaver–Burk double reciprocal plots for each concentration of the inhibitor and substrate were used to investigate the type of inhibition (competitive, uncompetitive, non-competitive, or mixed) of the tested gedunin. The amount of reducing sugars was estimated spectrophotometrically using a standard maltose curve and was converted into reaction velocities. The kinetic constants Km and Vmax were calculated from the slope and intercept values in the double reciprocal plots. The product (reducing sugars) concentration was

determined using a standard maltose curve and expressed as millimoles of equivalents **(Alqahtani *et al.*, 2019).**

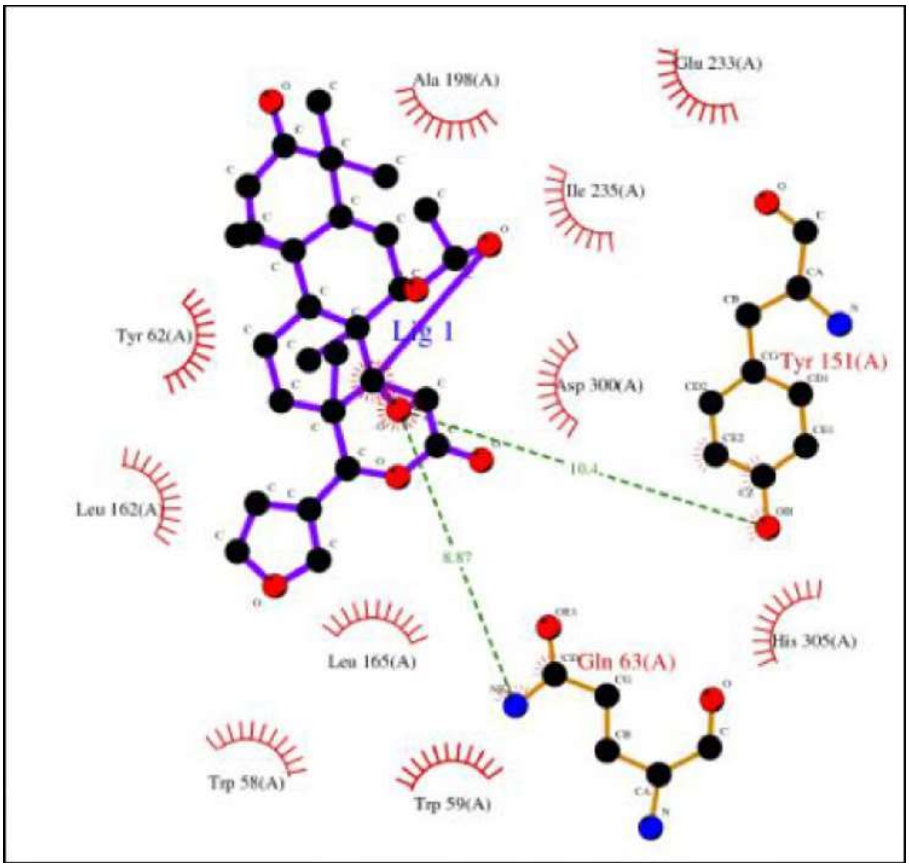
8.3. Results and Discussion

8.3.1 Molecular Docking

The structures of the enzymes and ligands were obtained from the Protein Data Bank, RCSB database, and National Centre for Biotechnology Information, PubChem database (https://pubchem.ncbi.nlm.nih.gov/), respectively. The active site of the enzyme obtained from the FT server showed amino acids TRP A 329, ASP A 357, ILE A 358, ILE A 396, ASP A 398 for alpha-amylase, and ASP 323 ILE 125 ILE129 TRP 256 for alpha-glucosidase. The best binding pose was chosen by considering the lower binding energy among the poses with ICM scores of 55,42, and 30, among which a 55 icm score was chosen for alpha-amylase, whereas for alpha-glucosidase, an ICM score of 25 was selected as the best pose among the score of 16,20,25. Images of the best pose were captured **(Figure 8.3., 8.4.).** These docking results relate to our *in vitro* results of non-competitive inhibition in the case of alpha-glucosidase, as the docking took place in the allosteric site of the alpha-glucosidase enzyme and mixed in the case of alpha-amylase since docking took place inside the active site and the allosteric site of both alpha-amylase enzymes. **(Figure 8.1, 8.2) (Table 8.1, 8.2)** depicts the interactions between the enzyme and gedunin, such as hydrogen bonds, salt bridges, and hydrophobic interactions.



(a)



(b)

Figure 8.1. (a) alpha-amylase interaction with ligand gedunin where orange represents ligand (b) alpha-amylase interaction ligplot where blue represents ligand.

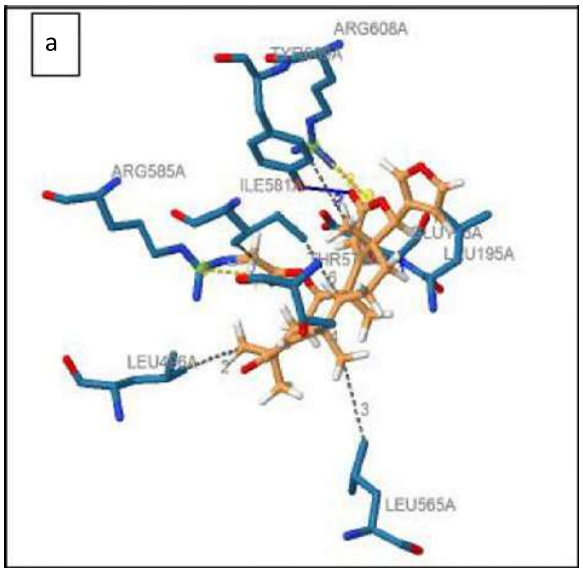
Table 8.1. alpha-amylase interaction with the ligand gedunin.

HYDROPHOBIC INTERACTIONS

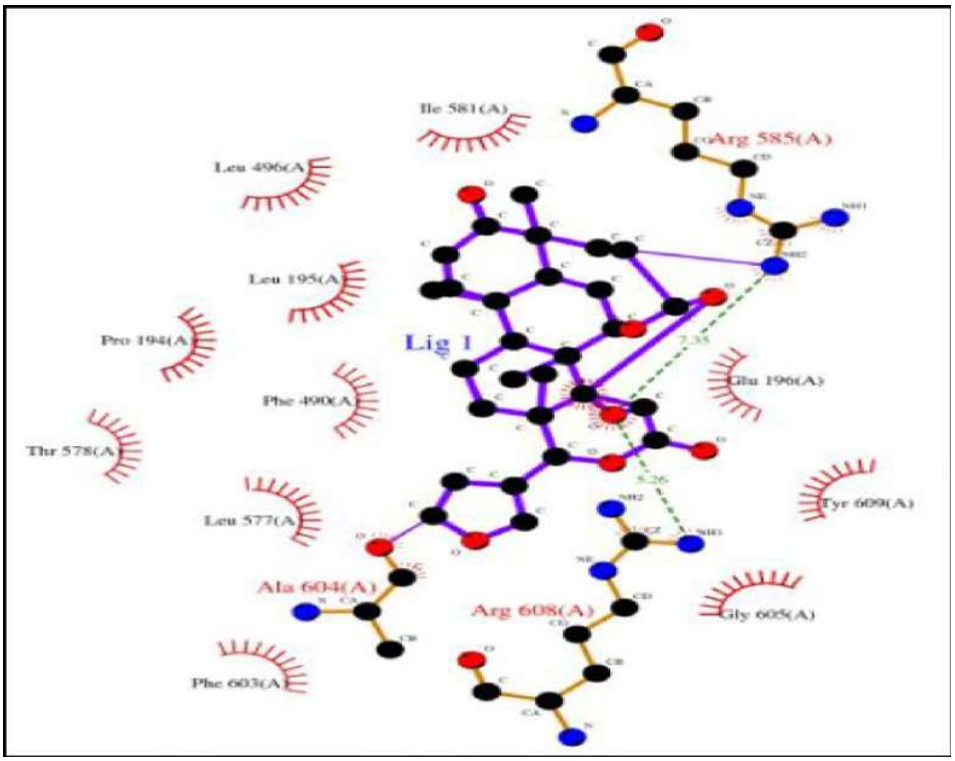
INDEX	RESIDUE	AA	DISTANCE	LIGAND ATOM	PROTEIN ATOM
1	59 A	TRP	3.72	3986	484
2	59A	TRP	3.74	3992	490
3	162 A	LEU	2.87	3988	1289
4	162 A	LEU	3.91	3981	1309
5	198 A	ALA	3.68	3995	1572
6	235 A	ILE	3.29	3986	1876

HYDROGEN BONDS

INDEX	RESIDUE	AA	DISTANCE H-A	DISTANCE D-A	DONAR ANGLE
1	63 A	GLN	2.45	2.84	103.59



(a)



(b)

Figure 8.2. (a) alpha-glucosidase interaction with ligand gedunin where orange represents ligand (b) alpha-glucosidase interaction ligplot where blue represents ligand.

Table 8.2. alpha-glucosidase interaction with the ligand gedunin.

HYDROPHOBIC INTERACTIONS

INDEX	RESIDUE	AA	DISTANCE	LIGAND ATOM	PROTEIN ATOM
1	195 A	LEU	3.35	6677	868
2	496 A	LEU	3.69	6680	3194
3	565 A	LEU	3.96	6675	3732
4	578 A	THR	3.56	6676	3841
5	581 A	ILE	3.32	6686	3862
6	609 A	TYR	3.47	6672	4080

HYDROGEN BONDS

INDEX	RESIDUE	AA	DISTANCE H-A	DISTANCE D-A	DONAR ANGLE
1	196 A	GLU	1.93	2.75	138.69
2	609 A	TYR	2.53	3.21	128.98

SALT BRIDGES

INDEX	RESIDUE	AA	DISTANCE	LIGAND GROUP	LIGAND ATOMS
1	585 A	ARG	4.23	CARBOXYLATE	6654,6658
2	608 A	ARG	4.14	CARBOXYLATE	6653,6655

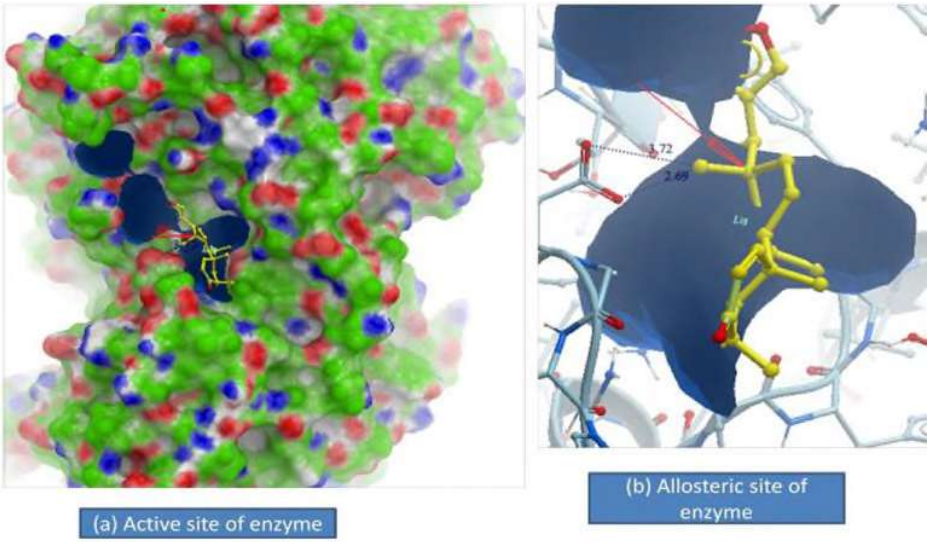


Figure 8.3. Best binding pose of gedunin inside alpha amylase pocket (a) gedunin inside the active site of enzyme (b) gedunin inside the allosteric site of the enzyme.

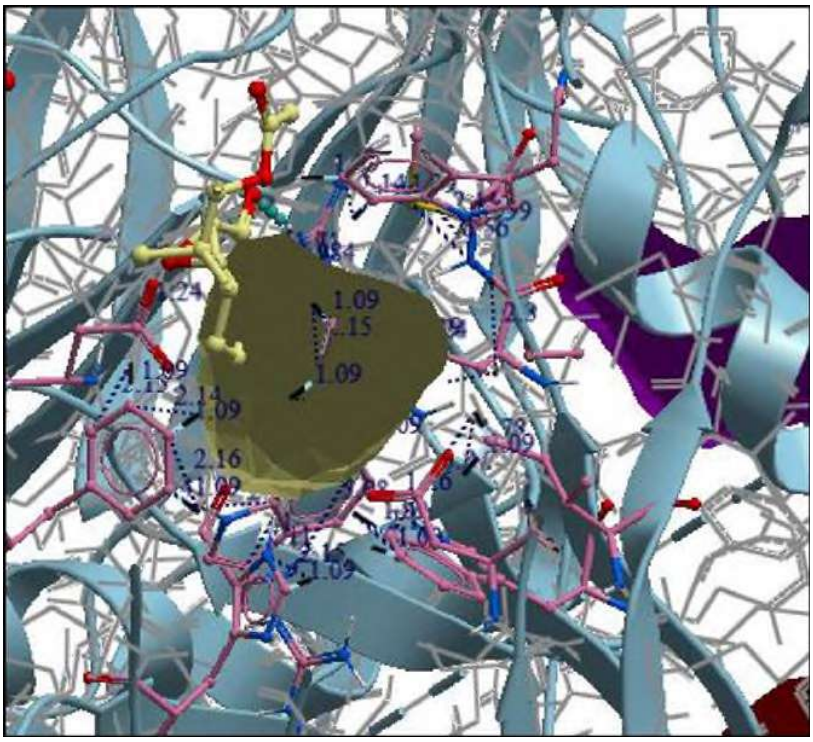


Figure 8.4. Best binding pose of gedunin (yellow) inside alpha Glucosidase pocket.

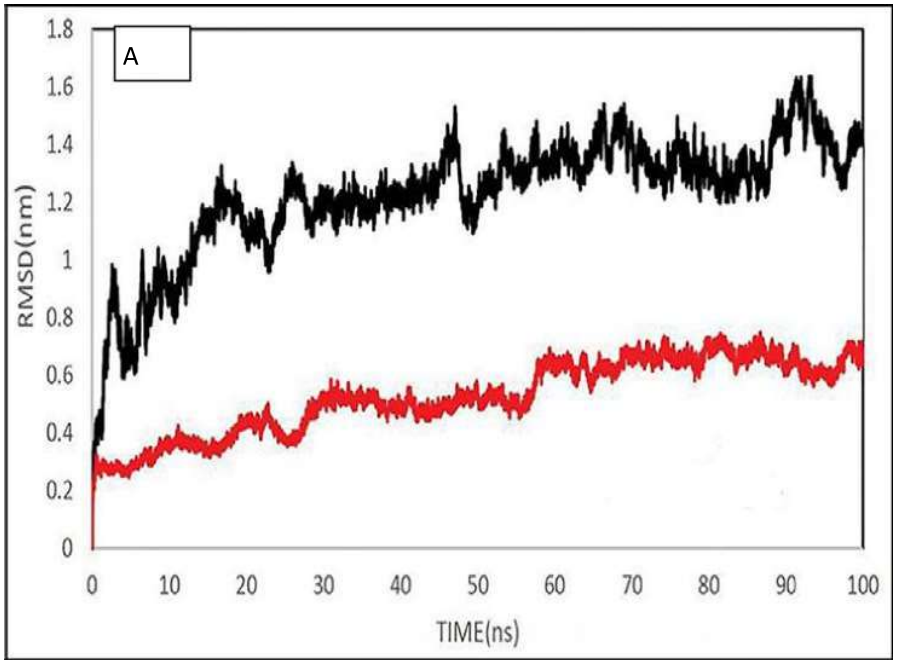
8.3.2 Simulation

Molecular dynamics (MD) is a method used to study the structure of compounds/solids using classical mechanics and to create a trajectory. Gromacs were used for MD, which uses the concept of a periodic boundary to create boxes/grids and groups to show the action.

Gromacs 2020 was utilized for MD, which uses a periodic boundary to build boxes/grids and groups to depict motion. Following the information arrangement, the topology was created using the opls force field for the protein and the Gaff force field for the ligand. After adding 4Na for neutralization, steric conflicts were eliminated. The steepest descent technique minimized the energy until the maximum force fell below 1000KJ/mol/nm. Subsequently, the system was

solvated using a TIP3 water model. The complexes were all subjected to a 100 ns MD simulation.

The radius of gyration (RMSF) and radius of gyration (RG) were determined. The solvent-accessible surface area was determined using post-MD simulation analysis (SASA). Principal component analysis (PCA) determines the direction and magnitude of the dominant movements. For The last 15ns, GBSA (generalized born surface area) was calculated. Gromacs tools were used to compute the number of hydrogen bonds**. (Hub *et al.,* 2020)** Simulation studies show that (a) gedunin-amylase has minimum RMSD (root mean square density), which implies tight binding between ligand and receptor, whereas (b) gedunin has less close binding evident by nonoverlapping RMSD (root mean square density) in case of alpha-glucosidase. **(Figure 8.5., 8.6.)**



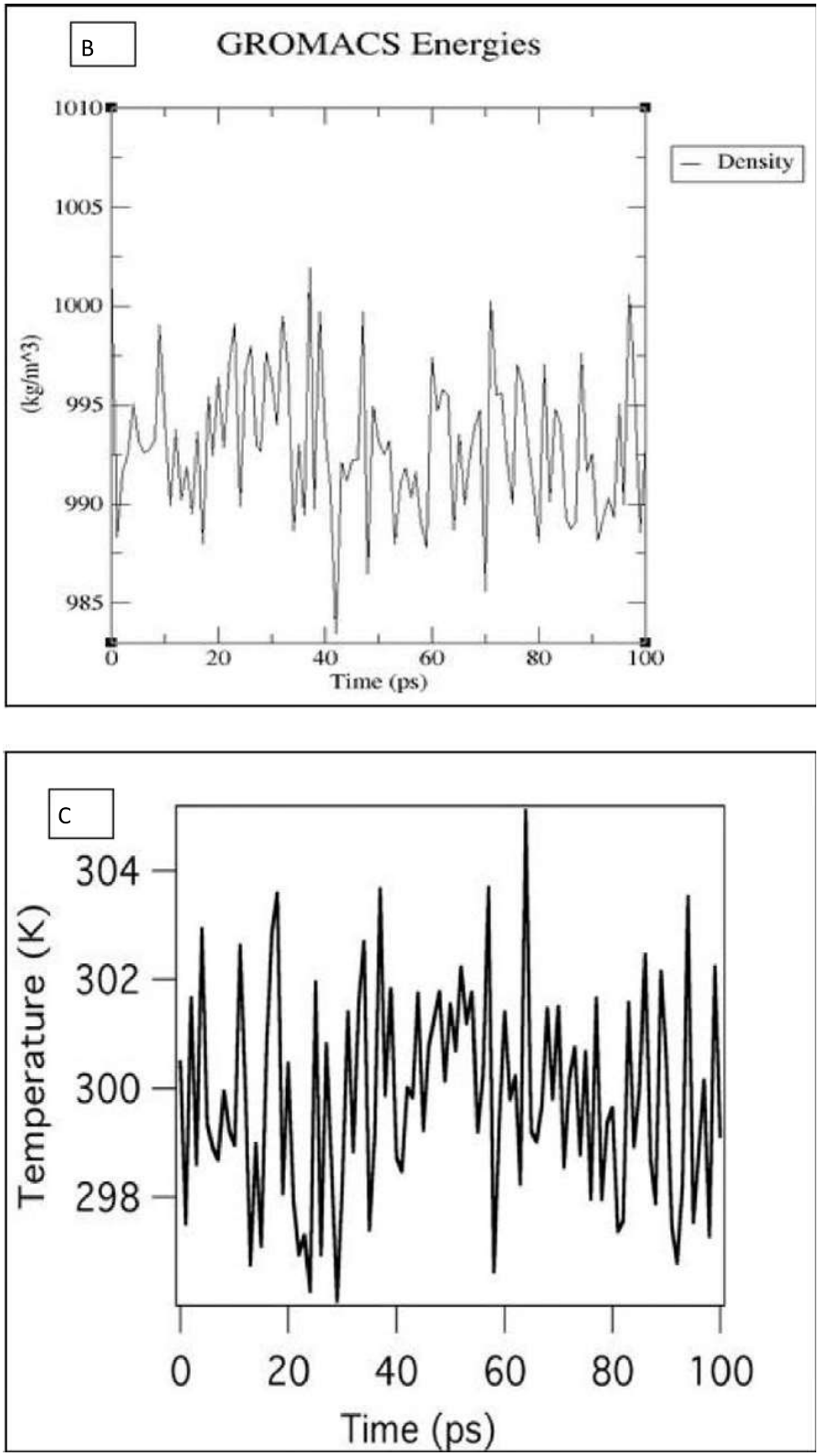
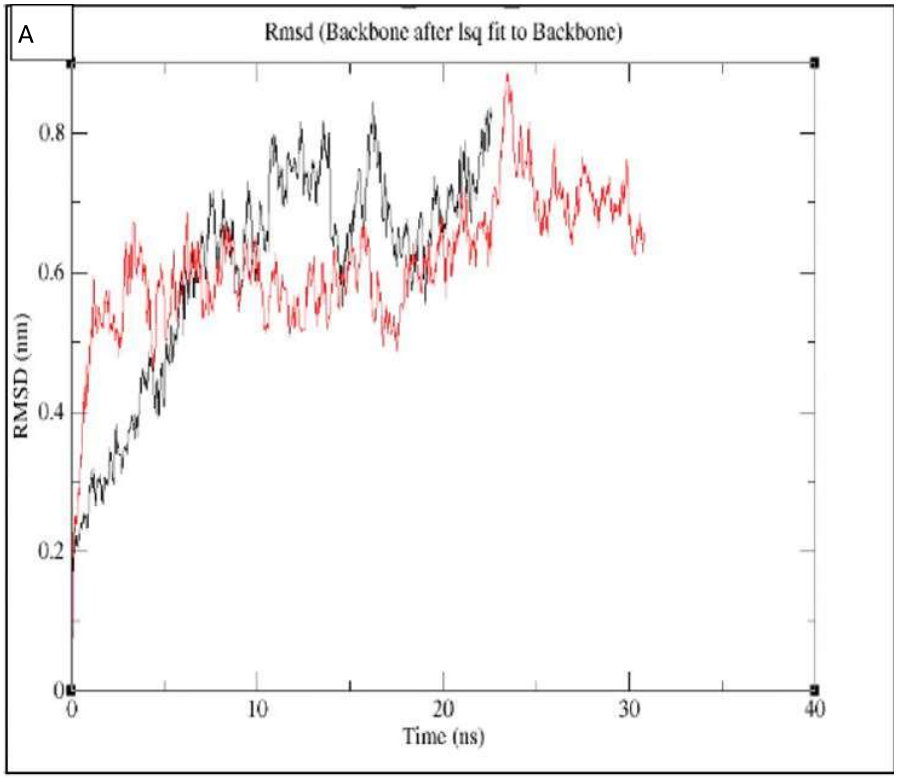
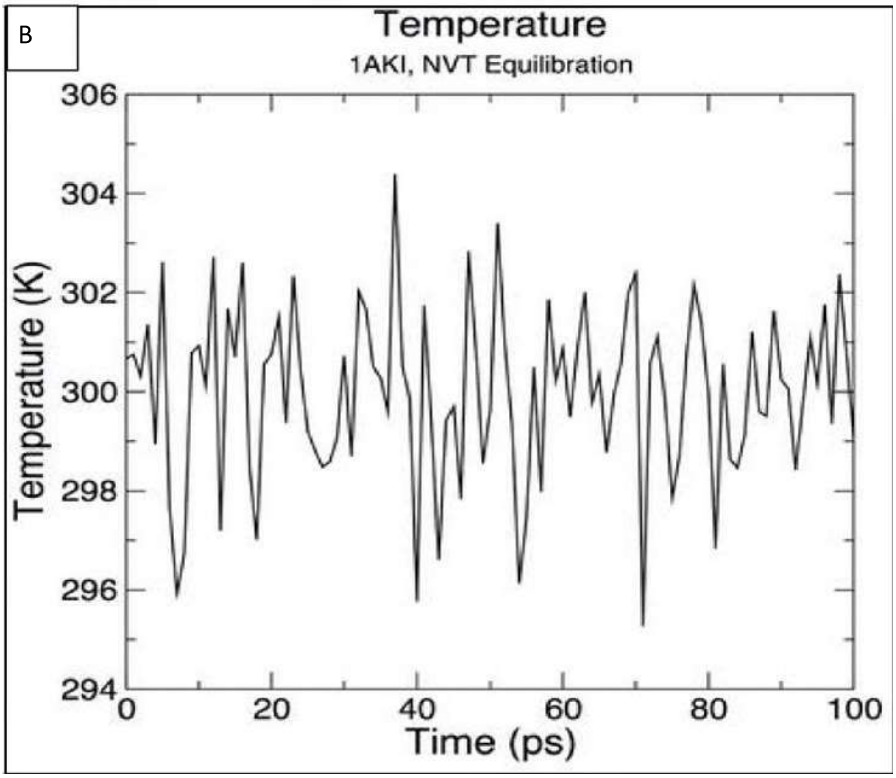


Figure 8.5. Simulation graphs showing (A) rmsd of alpha-glucosidase-gedunin complex, (B) density of alpha-glucosidase-gedunin complex, (C) temperature of the alpha glucosidase-gedunin complex.


A
Rmsd (Backbone after lsq fit to Backbone)
RMSD (nm)
0
0.2
0.4
0.6
0.8
0
10
20
30
40
Time (ns)


B
Temperature
1AKI, NVT Equilibration
Temperature (K)
294
296
298
300
302
304
306
0
20
40
60
80
100
Time (ps)

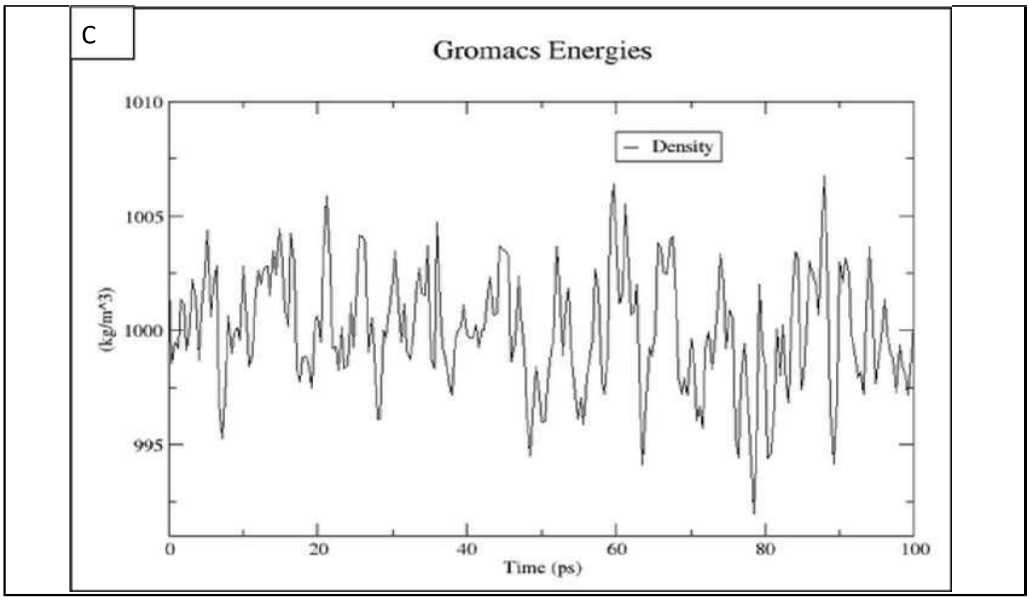


Figure 8.6. Simulation graphs showing (A) rmsd of alpha-amylase-gedunin complex, (B) density of alpha-amylase-gedunin complex, (C) temperature of alpha-amylase -gedunin complex.

8.3.3 Alpha Amylase and alpha-glucosidase inhibition

The mode of inhibition was determined by the Lineweaver-Burk plot, which revealed that the inhibition type was mixed for alpha-amylase and noncompetitive for alpha-glucosidase with N value of 9. **(Figure 8.7., 8.8.).** Experiments were conducted using miglitol and voglibose as well, which are standard alpha-glucosidase enzyme inhibitors with N value of 9 and showing competitive inhibition **(Figure 8.9, 8.10).** Gedunin has 65.4 mg/ml IC50 for alpha-amylase and 50 mg/ml for alpha-glucosidase. The standard deviation for alpha-glucosidase was 0.89, and for alpha-amylase, it was 0.923. Reaction velocities were calculated using a maltose standard at different concentrations of substrate **(Alqahtani *et al.,* 2019).** Several animal experiments have demonstrated that acarbose can delay the breakdown of sucrose and glucose. However, starch is less efficient than polyphenolic-enhanced crude natural plant extracts **(Gin *et al.,* 1999).** lowering blood sugar levels, and Ishige's diphlorethohydroxycarmalol substance was obtained by **Heo *et al***. Many tannins, which interfere with gastrointestinal function, are abundant in medicinal plant extracts. Another clinical investigation determined whether the decrease in blood glucose level was caused by decreased food intake in mice treated with the ethanolic extract of S. Cumini and tannic acid. In another study conducted by Oliveira et al., P. Ramiflora extract consumption was found to help reduce food intake, body weight, and blood sugar levels. As a result, it was determined that phenolic compounds found in its extract are efficient for lowering weight and blood sugar levels and are hence linked to weight loss with dietary restriction and alpha-amylase inhibition **(Maddi *et al.,* 2014)** Activity.

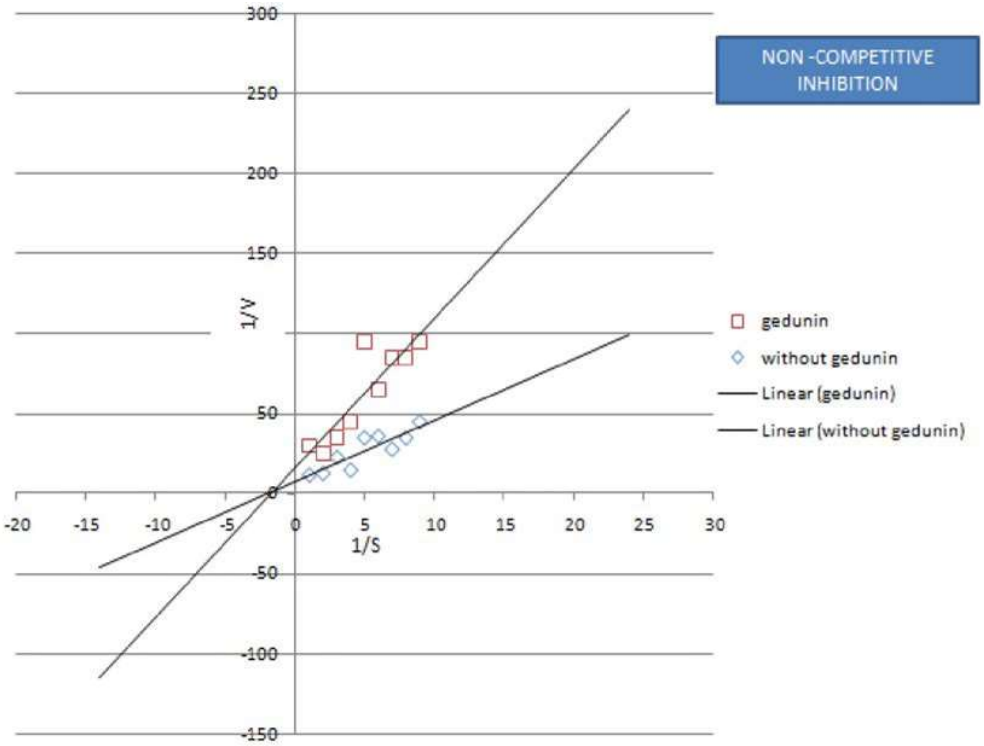


Figure 8.7. Non-competitive mode of inhibition of the alpha-glucosidase enzyme.

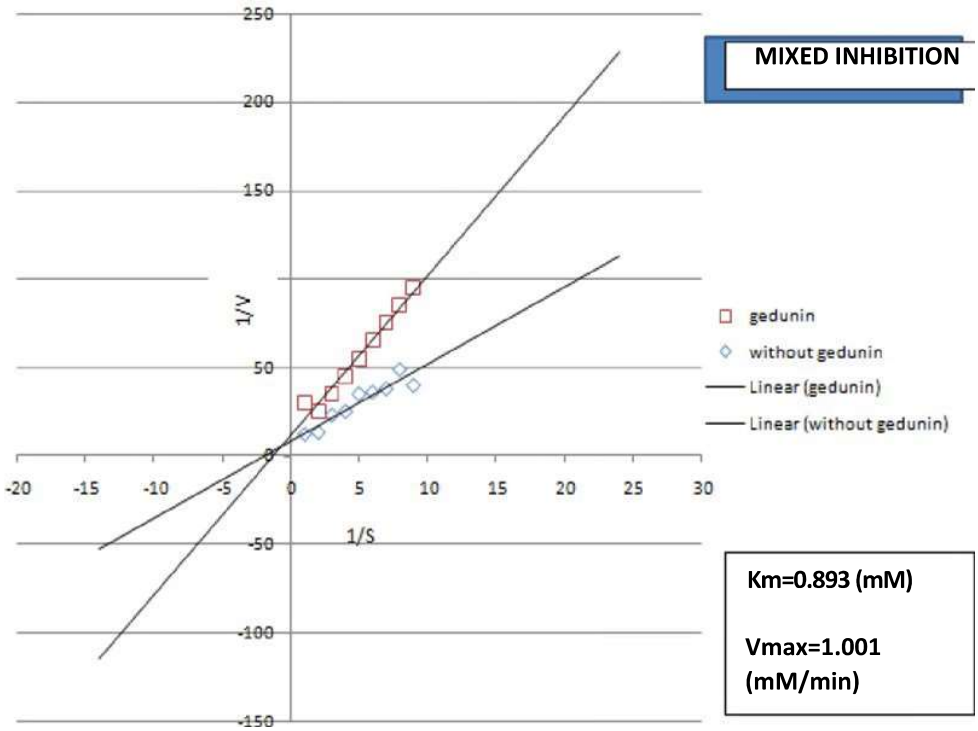


Figure 8.8. Mixed mode of inhibition of alpha-amylase enzyme.

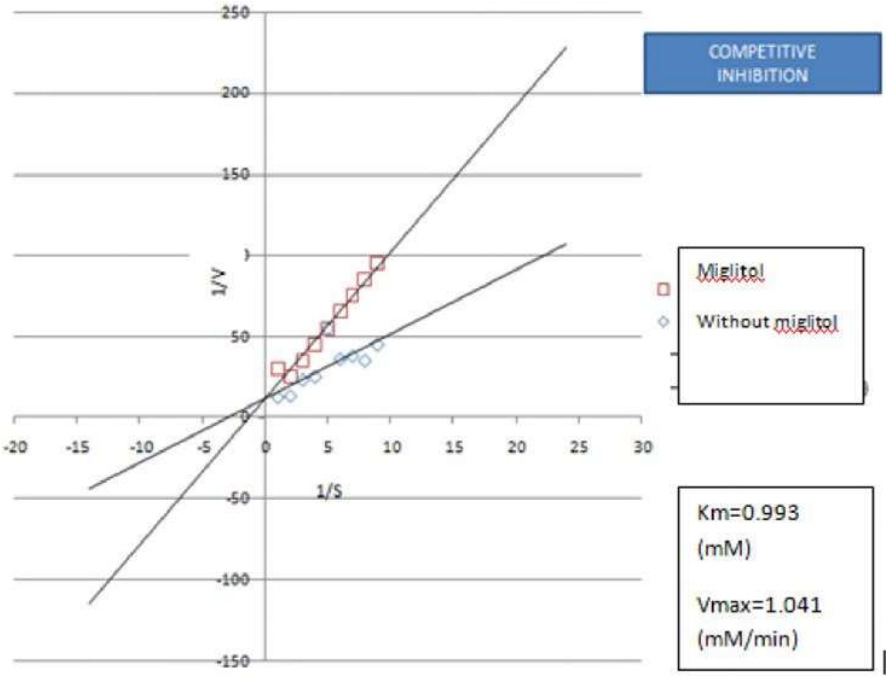


Figure 8.9. Competitive mode of inhibition of alpha-glucosidase enzyme inhibitor Miglitol.

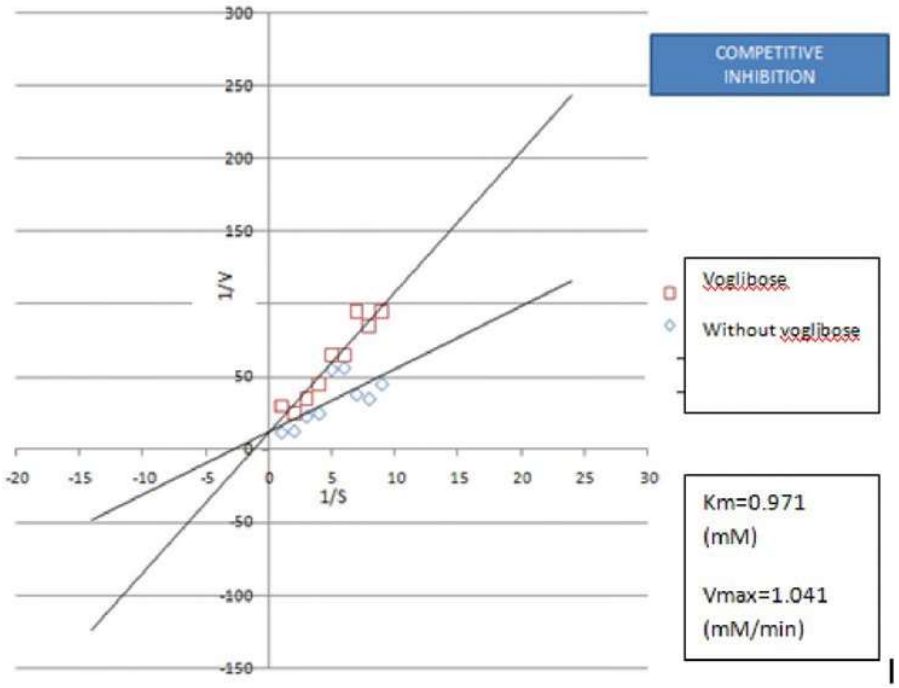


Figure 8.10. Competitive mode of inhibition of alpha-glucosidase enzyme inhibitor Voglibose.

8.4. Conclusion

Approximately 25% of prescription drugs come from plant extracts in today's world. A systematic study on the *Azadirachta indica* (neem) plant was conducted to evaluate its antidiabetic potential via inhibiting alpha-amylase and alpha-glucosidase enzymes. It is a known enzyme that is involved in carbohydrate metabolism. *Azadirachta indica* (neem) is used in ancient scriptures as a multipurpose plant with therapeutic value. The enzyme assay showed non-competitive and mixed inhibition of the enzyme, which makes way for further investigation of the active constituents and, thus, a new herbal medicine for diabetes. *In vitro* studies have shown that gedunin binds to enzymes at allosteric sites to form hydrogen bonds, salt bridges, and hydrophilic interactions. Many synthetic drugs are available to treat diabetes; however, these have numerous side effects. Using herbal remedies such as *Azadirachta indica* could be an alternative therapy for diabetes.