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# *In vitro*, *in silico* and Pharmaco-toxicological Efficiencies of some Triazole Derivatives on Inhibition of Digestive Enzyme Alpha-amylase

**Elif Ayazoglu Demir**[1*]
https://orcid.org/0000-0001-7188-2176

**Ahmet Yasar**[4]
https://orcid.org/0000-0002-5487-1536

**Ahmet Colak**[2]
https://orcid.org/0000-0003-2291-8800

**Olcay Bekircan**[2]
https://orcid.org/0000-0002-9609-8959

**Selcen Celik Uzuner**[3]
https://orcid.org/0000-0002-9558-7048

**Aylin Kabahasanoglu**[2]
https://orcid.org/0000-0002-5178-1594

[1]Karadeniz Technical University, Macka Vocational School, Department of Chemistry, Chemistry Processing Technologies, Trabzon, Turkey; [2]Karadeniz Technical University, Faculty of Science, Department of Chemistry, Trabzon, Turkey; [3]Karadeniz Technical University, Faculty of Science, Department of Molecular Biology and Genetics, Trabzon, Turkey; [4]Karadeniz Technical University, Faculty of Pharmacy, Department of Analytical Chemistry, Trabzon, Turkey.

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*Correspondence: eliffayaz@gmail.com; Tel.: +90 462-3777672 (E. A. D.).

**HIGHLIGHTS**

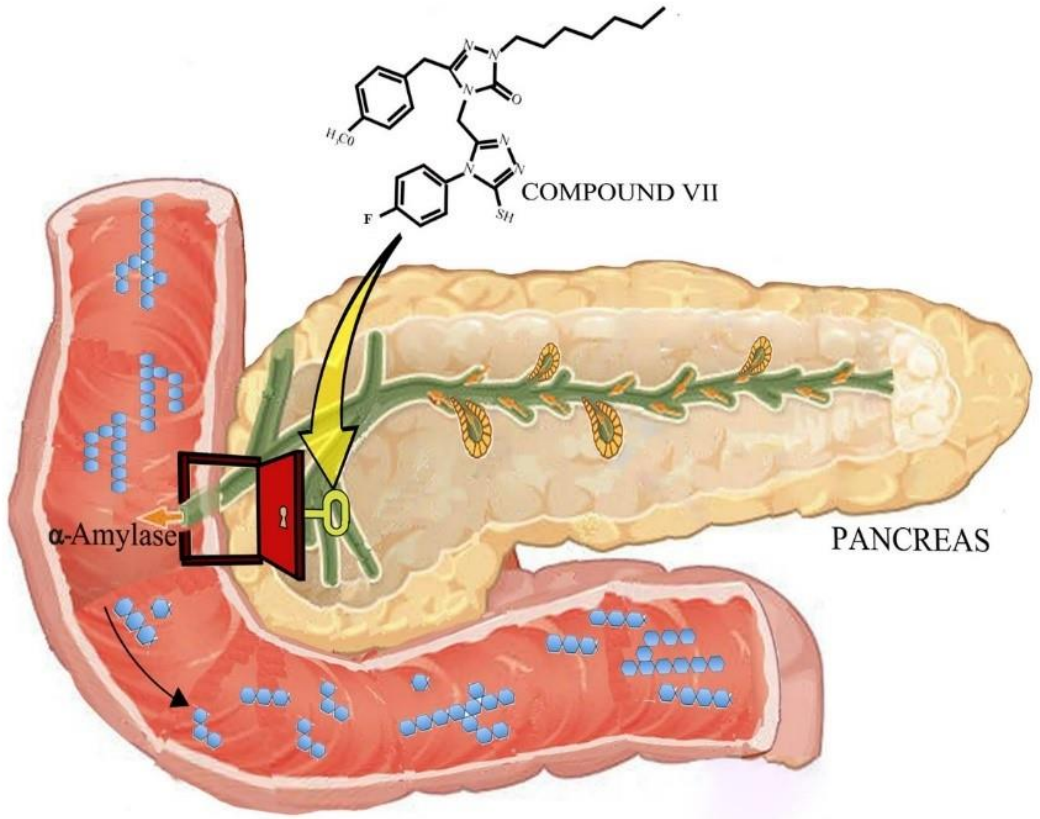
- T2DM is increasing rapidly in the world and this brings a constant search for drugs.
- Compound VII showed the strongest inhibition on α-amylase activity with low $IC_{50}$ value.
- Compound VII, amylase inhibition activity was found to be strong on AR42J cells.
- Compound VII may play role an alternative and non-cytotoxic α-amylase inhibitor for T2DM.

**Abstract:** Obesity is one of the main health problems associated with a range of diseases. Genetic disposition is related to the risk for obesity but external conditions such lifestyle also increase the incidence. Current COVID-19 pandemic conditions around the globe have been reported to increase the cases of Type-2 diabetes mellitus (T2DM) due to prolonged sedentary life. Among the various treatment modalities, applications of α-amylase inhibitors are commonly used worldwide. Commercially available anti-diabetic drugs are potent inhibitors of α-amylase that reduce postprandial hyperglycemia. In this study, α-amylase inhibition efficiencies of some 1,2,4-triazole derivatives were evaluated. Furthermore, it has been attempted to determine the possible inhibition mechanism of the strongest inhibitor compound among the 8 candidate molecules for α-amylase. Compound VII showed the strongest inhibition on α-amylase activity with low $IC_{50}$ value (150 µM). An inhibitory kinetic analysis on α-amylase activity by Compound VII was found to be reversible and uncompetitive. Furthermore, molecular docking studies with this molecule showed that it could bind to the catalytic site of the enzyme by performing weak interactions with Ser56, Tyr59, Tyr62, Asp176, Asp274 and Leu142 residues. Cytotoxic potential of Compound VII on amylase overexpressing AR42J

pancreatic cancer cells was also performed using trypan blue staining and the compound at the highest dose 10 µM was found to be cytotoxic, but effective for alpha amylase inhibition at non-cytotoxic doses. The results showed *in vitro* effect of Compound VII on alpha-amylase inhibition in cells. Here, we suggest an alternative and non-cytotoxic α-amylase inhibitor for T2DM.

**Keywords:** α-Amylase inhibition; 1,2,4-triazole; Docking; Cytotoxicity; T2DM.

**GRAPHICAL ABSTRACT**




## INTRODUCTION

Diabetes mellitus (DM) is one of the most common diseases in the world and the incidence increases during COVID-19 pandemic conditions [1]. It is estimated that 451 million adults around the world are diabetic and this disease will reach 693 million people by 2025 [2]. DM is a metabolic disorder of the endocrine system that caused by abnormal plasma blood glucose levels occurred with some complications including hyperglycemia, polyphagia, polydipsia, and polyuria. DM is concomitantly accompanied by various diseases such as neuropathy, nephropathy, angiopathy, cardiovascular diseases and retinopathy [3, 4]. Diabetes is a complex disease occurred by intrinsic and extrinsic conditions, and mainly classified as Type-1 and 2. Type-1 is more based on genetic background, but Type-2 is more related to personal eating habits and lifestyle. Type-2 diabet mellitus (T2DM) is the most common type of diabetes, accounting for 90-95% of cases [4]. One of the ways to control T2DM disease is to control hyperglycaemia after eating. Stabilization of blood glucose for diabetic patients is important, since it prevents the complexity relationship between hyperglycaemia and diabetes [3]. Amylase inhibition slows carbohydrate digestion and thus causes prolongation of overall time for carbohydrate digestion. As a result, the rate of glucose absorption in the metabolism decreases so that the postprandial plasma reduces glucose uptake [4].

Recent COVID-19 pandemic conditions experienced all over the world negatively affect people's lives in many ways. In addition, within the scope of the measures taken and compulsory to be followed in the world, many people have to live under heavy stress [5]. This situation has forced both a healthy and regular diet and a sedentary life. It is predicted that T2DM will be one of the global concerns at the end of the pandemic [6].

α-Amylases, α-1,4-glucan-4-hydrolase (EC 3.2.1.1) have activities on conversion of oligosaccharides and disaccharides into monosaccharides which can be easily absorbed. This suggests that amylases are

one of the key enzymes of the digestive system so that being a target for drug design studies [3]. Amylase inhibitors have been a prominent focus of attention due to their treatment potential for HIV infection, metastatic cancers, diseases with lysosomal storage abnormalities and specially diabetes [7-9]. The range of relevant diseases conditions suggests that α-amylase appears to be a target enzyme in the design of suitable drug molecules for therapies of diabetes, obesity and hyperlipidemia [3]. The inhibition of amylase may have profound effects on the mechanisms in controlling digestive system and glycoprotein release and may alter the process. Currently, different amylase inhibitors such as voglibose, acarbose and miglitol used as anti-HIV, anti-cancer, and anti-diabetic agents [10]. However, some side effects such as hepatotoxicity, diarrhea, stomach gas, and abdominal distension are observed in the use of these drugs [11]. Hence, it is important to alleviate the adverse effects resulting in serious disruptive impacts on the metabolism, with the application of anti-diabetic drugs or the discovery of new drugs.

Heterocyclic compounds are very important nuclei for drug discovery because they have a wide variety of binding interaction potentials [12]. These compounds are used as important building blocks in such many natural products, pharmaceuticals, and functional materials [3, 8, 13]. Triazole structures carry nitrogen atoms in their ring systems and act as key structural units in many pharmaceutical preparations [13]. The 1,2,4-triazole core is one of the most prominent heterocyclic structures and undoubtedly found a lot of natural products and bioactive molecules [7]. Triazole compounds, however, are promising heterocycles in the pharmaceutical industry. These are the most studied clinical entities in single and/or fused structures with different biologically active heterocyclic compounds [12]. The most remarkable isomers are 1*H*-[1,2,4] -triazoles which form a part of some biologically active pharmaceutical products [14]. It has been reported that heterocyclic compounds containing triazole derivatives have antimicrobial [15], antiviral [16], diuretic [12], anti-inflammatory [17], anticancer [18]. Also in addition COVID-19 associated is used as anti-fungal [19]. In the literature, triazoles have been reported as potent α-amylase inhibitors to control blood sugar levels in the diabetes mellitus [3, 20]. It has been reported that several triazole compounds exhibit a reversible and non-reversible inhibition on α-amylase [3, 21].

Regarding the importance of 1,2,4-triazole compounds for the treatment of T2DM by the inhibition of amylase, in this study, some novel 1,2,4-triazole derivatives were repurposed to be examined in terms of α-amylase inhibition potentials using in glass, *in silico* and *in vitro* approaches.

## MATERIAL AND METHODS

### Chemicals and reagents

α-Amylase enzyme (A6380), 3,5-dinitrosalicylic acid (DNS, D0550), acarbose (J61737), Roswell Park Memorial Institute-1640 Medium (RPMI-1640, Cat# R8758), Dulbecco's Modified Eagle's Medium (DMEM, Cat# D6429), trypan blue solution (Cat# T4049) and other reagents used in the inhibition studies were purchased from Sigma-Aldrich (St. Louis, MO, USA). İn addition fetal bovine serum (FBS,Cat# 10270) and penicillin/streptomycin (Cat# 15140-122) were purchased from Gibco (CA, USA).

### α-Amylase activity assay

α-Amylase activity was determined using with slight modifications of DNS method based on the calculation of the amount of released reducing sugars [1, 22]. Briefly, the reaction mixture containing 630 µL pH 7.00 sodium phosphate buffer (0.05 M), 50 µL of soluble starch solution (0.035%, by mass per volume) and 20 µL of α-amylase solution (25 $ng.mL^{-1}$ in 0.05 M, pH 7.00 sodium phosphate buffer) was incubated at 40 °C for 15 min. The blank solution was free of enzymes. 700 µL of 3,5-dinitrosalicylic acid (DNS) reagent was added to the samples and the reaction mixture was boiled for 10 min, the reactions were then stopped. Subsequently, the reaction mixture was kept at room temperature for 15 min for thermal stabilization. The absorbance was measured at 540 nm compared to blank (Perkin Elmer, Lambda 25). One unit of α-amylase activity was defined as 1 µmol of reducing sugars that was released under optimum reaction conditions in 1 min reaction time at 40 °C [23].

### Optimization of enzyme activity

The variables used in the design of the inhibition studies were primarily optimized for the previously mentioned commercial amylase enzyme. For this, preliminary tests were performed, and activity curves were created to verify the minimum concentration and maximum activity of the amylase enzyme required for the experiments. A series of activity measurements (at enzyme concentration range of 0.09-250 $ng.mL^{-1}$) were

performed to determine suitable enzyme concentration for correctly measuring reaction velocity for α-amylase activity in the presence of constant soluble starch concentration as a substrate, at room temperature and pH 7.0 (50 mM phosphate buffer). The calculated enzyme activities were calculated by plotting the activity versus increasing enzyme concentration and continued in this way for further characterization and inhibition studies [24]. In order to determine the substrate concentration, a series of activity measurements were performed again by preparing reaction mixtures at constant enzyme concentration, temperature and pH in the presence of soluble starch as a substrate in the concentration range of 0.02-0.1%. Lineweaver-Burk graph was drawn to obtain Km and Vmax values [25]. To find the optimum pH value for the enzyme activity, 50 mM acetate buffer (pH 5.0), 50 mM phosphate buffer (pH 6.0-7.0) and 50 mM glycine-NaOH buffer (pH 8.0 -9.0) were used and pH-relative activity (%) graph was created [23]. Optimum temperature of the α-amylase activity was also determined by monitoring enzyme activities at different temperature between 10 °C and 90 °C and temperature-relative activity (%) graph was created [24].

## Biological Activity Methods

### Pretreatments

The stock solutions of organic molecules used in the experiments were prepared in absolute DMSO. Final solvent (DMSO) concentration in the reaction mixture was 1% [26]. Aliquots to be used in inhibition experiments were carried out by diluting in different concentrations in stock solutions of organic molecules performed on the day of the experiment.

### α-Amylase inhibition studies

For inhibition studies, the reaction mixtures containing 20 µL α-amylase enzyme solution, 10 µL inhibitor solution was pre-incubated for 20 min in a water bath at 40 ºC. This studies inhibitor molecules solutions in used were evaluated in different concentrations (Compound I: 0-540 µM; Compound II: 0-9626 µM, Compound III:0-1300 µM; Compound IV: 0-417 µM; Compound V: 0-1 mM; Compound VI:0-0.7 mM; Compound VII: 0-0.67 mM; Compound VII: 0-0,65 mM). Following this procedure, soluble starch solution and 50 mM phosphate buffer (pH 7.0) were added (final soluble starch concentration in the reaction mixture was 0.035%) to each reaction mixture and all reaction mixtures were kept for additional 15 min at same conditions. Subsequently, 700 µL of DNS reagent was added to the samples and the reaction mixture was boiled for 10 min the reactions were stopped. Finally, all samples reached to thermal equilibrium were measured at 540 nm compared to blank by a spectrophotometer (Perkin Elmer, Lambda 25). After measuring enzyme activity, inhibitor concentrations were plotted against relative activities % to determine $IC_{50}$ value which is the inhibitor concentration inhibits 50% of the enzyme activity representing inhibition efficiencies of each inhibitor molecules. The percentage inhibition effect was measured using the following Formula I as the effect on the amylase activity of each organic molecule:

$$\text{Formula I: Inhibition } (\%) = \left[ {}^{(\mathrm{Ac} - \mathrm{As})}\big/_{\mathrm{Ac}} \right] \times 100$$

Ac: Absorbance of control, As: Absorbance of sample [27].

### Determination the inhibition type and the Ki value of α-amylase inhibitory activities

To reveal the mechanism of enzyme inhibition, and determination of Michaelis Menten constant (Km), Vmax and inhibition constant (Ki) values, the Linewear-Burk graphics were plotted for different substrate concentrations at fixed Compound VII concentration using GraFit enzyme kinetical program (Version 7.0 Erithacus Software). Ki of Compound VII effect on amylase enzyme activity was calculated by employed Formula II using to uncompetetive inhibition model of the program.

$$\text{Formula II: } V = {}^{[V\mathrm{max}/(1+[\mathrm{I}]/K\mathrm{i})]}\Big/_{[\,K\mathrm{m}/(1+[\mathrm{I}]/K\mathrm{i})]}$$

Where V is the reaction velocity; Vmax is the limiting velocity; Ki is defined as the rate constant for kinetically binding inhibitor to the enzyme, the inhibition constant, Km is the Michaelis constant; [I] and [S] are the concentration of inhibitor and substrate, respectively [9, 28].

### α-Amylase molecular modeling study

The crystal structure of target protein, α-amylase (PDB ID: 1BAG) was obtained from Protein Data Bank (www.rcsb.org/pdb) [29]. Then, it was pre-processed, modified and refined using AutoDock Tools 1.5.6 and AutoDock Vina software for all compounds [30]. Ligand optimizations were performed by Gaussian 03 program. Discovery Studio 4.1 Visualizer was used to show binding interaction of molecules with the target site. After the calculation of docking scores were produced, the best fitted ligand to target protein were determined [31].

### Cell culture

Mammalian pancreatic cancer (AR42J, Cat# 93100618) and mouse embryonic fibroblast cell lines (CF-1, Cat# SCRC-1040) were purchased from ECACC (UK, England) and ATCC (VA, USA), respectively. The cells were cultured in RPMI-1640 (for AR42J cells) or DMEM (for CF-1 cells) with 10% heat inactivated, 1% penicillin/streptomycin. Complete culture media were filtered by a 0.22 µm filter (Aisimo, London, England), and warmed at 37°C before use. Cells were incubated supplied at 37 °C humidified with 5% $CO_2$ [32].

### Drug preparation and treatment

Acarbose and Compound VII were dissolved in DMSO. Acarbose, a standard α-amylase inhibitor, was used as a positive control [33] and was also included in cytotoxicity experiment. Final solvent (DMSO) concentrations were adjusted to 0.1% within the medium during cell culture [34].

### Measurement of cell viability

Cell viability was evaluated with trypan blue staining supplemented with fixative [35]. This method is based on the differences in cell membrane permeability between live and dead cells. Dead cells with disrupted membrane are seen dark blue/black under the brightfield tool of microscope due to the dye intake within the cell, whereas dye cannot diffuse into live cells with intact membrane. Briefly, $5x10^3$ of AR42J and CF-1 cells were seeded into each well of the sterile 96-well cell culture plates. Media was refreshed after overnight incubation followed by treatment with various concentrations of Compound VII (0,001-5 µM). Control cells were untreated. Subsequently, 0.4% trypan blue solution was added to each well for 10 min at room temperature, were washed with phosphate buffered saline (PBS) for three times. Cells were then treated with fresh 4% paraformaldehyde (PFA) for 30 min at room temperature. The fixative was washed with 1xPBS. The cells were visualized under Axio Vert inverted microscope (Carl Zeiss, Germany) and images for each treatment were captured. Dead/Live cells were analyzed using count tool of Adobe Photoshop software. Cell counts were transformed to percentages and viability (%) of treated and untreated cells was compared.

### *In vitro* α-amylase inhibitory studies

*In vitro* α-amylase inhibitory studies on AR42J cell line was performed using slight modifications on the microplate-based starch-iodine method [36]. The degraded amount of starch was used to estimate the activity of α-amylase. AR42J cells were cultured onto T25 flasks at the density of $5x10^4$ cells overnight. Cells were treated with Compound VII (0.05-4 µM) or acarbose (0.05-500 µM) for 48h). Following incubation, 0.5% soluble starch were added to the cells for 1 h. 100 µL samples taken from the flasks were added to sterile 96-well plates and 100 µL of iodine reagent (0.5% iodine and 5% potassium iodide) was then added. Finally, all samples were measured at 540 nm by a microplate reader (Molecular Devices Versamax, California, USA). After measuring *in vitro* α-amylase activity for reduce soluble starch digestion via Compound VII and acarbose was plotted against relative activities (%) to determine $IC_{50}$ value (the concentration inhibiting 50% of the enzyme activity). The percentage of inhibition was measured the Formula III as the effect on the amylase activity of Compound VII and acarbose as standard drug was using an online calculator (https://www.aatbio.com/tools/ic50-calculator) [37].

$$\text{Formula III: Inhibition } (\%) = \left[ {}^{(\mathrm{Ac} - \mathrm{As})}/_{\mathrm{Ac}} \right] \times 100$$

Ac: absorbance of control, As: absorbance of sample.

### Statistical analysis

All experiments were performed as at least three independent repeats and the results were given as arithmetic mean ± standard deviations. Statistical analyzes were carried out using SPSS (Statistics Program for Social and Science) software (Version13.0.1). The suitability of the data to normal distribution was evaluated with the Kolmogorov-Smirnov test. One-Way ANOVA test was used for parametric data and then Post-hoc Tukey test for multiple comparisons between the groups. *p* value less than 0.05 was considered as statistically significant.

## RESULTS AND DISCUSSION

T2DM is one of the rapidly increasing diseases of our age. It is predicted that the measures taken and must be followed due to the COVID 19 pandemic will cause adverse effects especially on the young population and the rate of spread will come in the form of a Tsunami [6]. An effective treatment approach for treatment T2DM is to reduce food-related hyperglycemia by reducing the digestion of carbohydrates taken through the inhibition of carbohydrate-breaking enzymes [3]. The widely used component of many drugs are five-membered heterocyclic rings such as 1,2,4-triazole, 1,2,3-triazole derivatives. They have a wide spectrum of biological activities such as anti-cancer [18], anti-viral [11], anti-depressant [38], anti-bacterial [39], analgesic [40], anti-HIV [16], diuretic [12], anti-inflammatory [17], anti-tuberculosis [41] and COVID-19 associated anti-fungal [19]. Recent studies have shown that the easy synthesis of Schiff and Mannich base derivatives of 1,2,4-triazoles utilized their use in various applications, in particular biology and chemistry [42, 43].

### α-Amylase inhibition activity

In this study, α-amylase inhibition potentials of some 1,2,4-triazole derivatives [44] (Table 1) were examined. Because of the different behavioral profile of hydrophobic groups in their structures of each organic molecule used in the study, the concentration ranges of inhibitor molecules were also different in the inhibition studies [45] (Table 2). Soluble starch (0.035%) was used as a substrate, and inhibition studies were performed at optimum pH (7.0) and temperature (40 °C) values for α-amylase activity. Relative activities were plotted against inhibitor concentrations and the inhibitor concentration at which 50% decrease in the activity was determined as the $IC_{50}$ value (Table 2). Compound VII among the studied molecules had the lowest $IC_{50}$ value as 150 μM (Table 2). In the presence of other inhibitor molecules except of Compound VII, inhibition of α-amylase was observed in the range of 25-62% even at the highest inhibitor concentrations tested (Table 2). $IC_{50}$ value of acarbose for α-amylase inhibition was determined as 235 μM.

**Table 1.** 1,2,4-triazole derivatives were evaluated for *Bacillus subtilis* α-amylase inhibition[15]

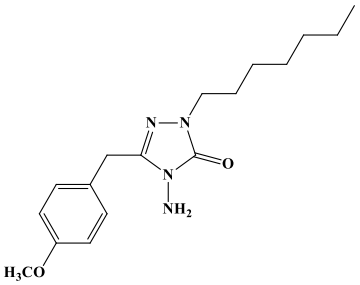
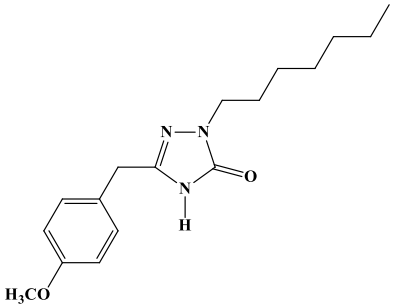
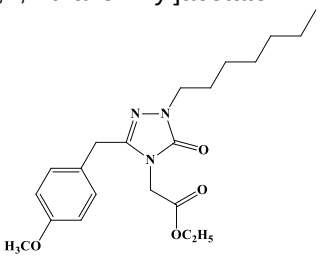
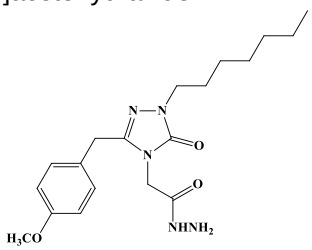
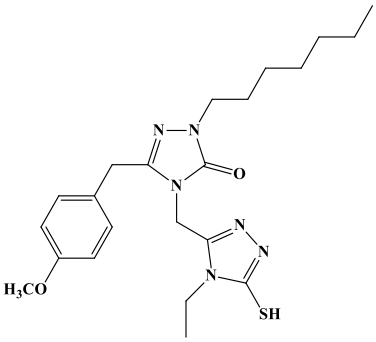
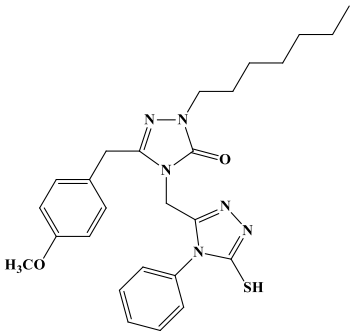
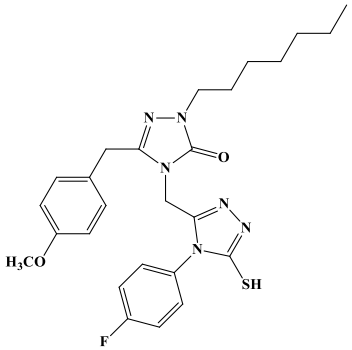
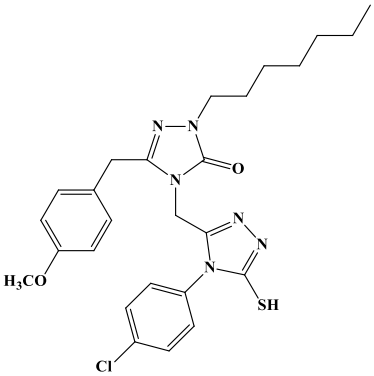
| Compound code/Nomenculature/ Formulas |
|---|
| **I.** 4-amino-2-heptyl-5-(4-methoxybenzyl)-2,4-dihydro-3*H*-1,2,4-triazol-3-one  |
| **II.** 2-heptyl-5-(4-methoxybenzyl)-2,4-dihydro-3*H*-1,2,4-triazol-3-one  |
| **III.** Ethyl [1-heptyl-3-(4-methoxybenzyl)-5-oxo-1,5-dihydro-4*H*-1,2,4-triazol-4-yl]acetate  |
| **IV.** 2-[1-heptyl-3-(4-methoxybenzyl)-5-oxo-1,5-dihydro-4*H*-1,2,4-triazol-4-yl]acetohydrazide  |
| **V.** 4-[(4-ethyl-5-mercapto-4*H*-1,2,4-triazol-3-yl)methyl]-2-heptyl-5-(4-methoxybenzyl)-2,4-dihydro-3*H*-1,2,4-triazol-3-one  |
| **VI.** 2-heptyl-4-[(5-mercapto-4-phenyl-4*H*-1,2,4-triazol-3-yl)methyl]-5-(4-methoxybenzyl)-2,4-dihydro-3*H*-1,2,4-triazol-3-one  |
| **VII.** 4-{[4-(4-fluorophenyl)-5-mercapto-4*H*-1,2,4-triazol-3-yl]methyl}c-2-heptyl-5-(4-methoxybenzyl)-2,4-dihydro-3*H*-1,2,4-triazol-3-one  |
| **VIII.** 4-{[4-(4-chlorophenyl)-5-mercapto-4*H*-1,2,4-triazol-3-yl]methyl-2-heptyl-5-(4-methoxybenzyl)-2,4-dihydro-3*H*-1,2,4-triazol-3-one  |

**Table 2.** α-Amylase inhibition potentials of evaluated organic compounds

| Inhibitor Compounds | $IC_{50}$, µM | Inhibition Level | | Binding Affinity ($\Delta G$, kcal.mol$^{-1}$) |
|---|---|---|---|---|
| | | % | [I] µM | |
| I | ›540 | 33.1±0.2 | 540 | -7.4 |
| II | ›9625 | 45.0±0.1 | 9625 | -7.3 |
| III | ›1300 | 29.0±0.1 | 1300 | -6.7 |
| IV | ›415 | 25.2±0.1 | 415 | -8.0 |
| V | ›1000 | 45.3±3.6 | 1000 | -7.5 |
| VI | ›700 | 39.2±0.1 | 700 | -7.9 |
| VII | 150 | 84.2±1.7 | 670 | -8.2 |
| VIII | 163 | 62.1±0.2 | 650 | -8.3 |
| Acarbose | 235 | 53.7±1.1 | 240 | - |

It is suggested that the Compounds VII and VIII are more potent than acarbose as α-amylase inhibitors. Gonzaga and coauthors observed α-glucosidase inhibition in the presence of 3 of 24 synthesized 1-phenyl-1*H*- and 2-phenyl-2*H*-1,2,3-triazole derivatives and $IC_{50}$ values of these three molecules was found as 145 µM, 201 µM and 281 µM [45]. Nawaz and coauthors observed α-amylase inhibition for 5-amino-nicotinic acid derivatives with $IC_{50}$ values ranged 12.17±0.14 to 37.33±0.02 µg.mL$^{-1}$ [8]. In studies conducted with wheat flour, Tu and coauthors defined a molecule with 37.58 µg.mL$^{-1}$ $IC_{50}$ value for α-amylase activity [46]. On the other hand, $IC_{50}$ value for inhibition by acarbose was found between 16.7 µM and 774 µM [8, 21, 47].

Besides the synthetic organic compounds, some natural extracts *i.e.* from plants were frequently used for potential of amylase inhibition [21, 48, 49]. For instance, extracts from different species of *Cinnamomum zeylanicum* were shown to have $IC_{50}$ value 57 µg.mL$^{-1}$ [48]. Although 1,2,4 triazole compounds have high potential as drug candidate, there is a limited understanding on their anti-diabetic potential. This study here presents a significant potential of the Compound VII to be used in controlling diabetes.

## Kinetic study of α-amylase inhibition

To understand inhibition types, the mechanism of enzyme inhibition, and determination of Km, Vmax, and Ki values, the Linewear-Burk graphics were plotted for different substrate concentrations (50 µM and 150 µM) at fixed Compound VII concentration using GraFit enzyme kinetical program (Version 7.0 Erithacus Software) (Figure 1, Table 3). Previous studies have shown that molecules derived from similar starting molecules have similar inhibitory effects and mechanisms [50]. Activity measurements showed that Km and Vmax values were 0.68% and 0.66 µmol min$^{-1}$, respectively when Compound VII was not included in the reaction mixture. However, in the presence of 50 µM and 150 µM Compound VII, the Km and Vmax values decreased to 0.07% and 0.12 µmol.min$^{-1}$, and 0.03% and 0.04 µmol.min$^{-1}$, respectively (Table 3). This indicates uncompetitive inhibition in which both Km and Vmax values decrease with increasing inhibitor concentration (Figure 1). According to this, it can be said that the Compound VII caused α-amylase inhibition by binding to a region rather than the active site of the enzyme, and the enzyme-substrate complex reversibly formed with some weak interactions. Ki value in the presence of Compound VII was also calculated as 6.43±0.12 µM.

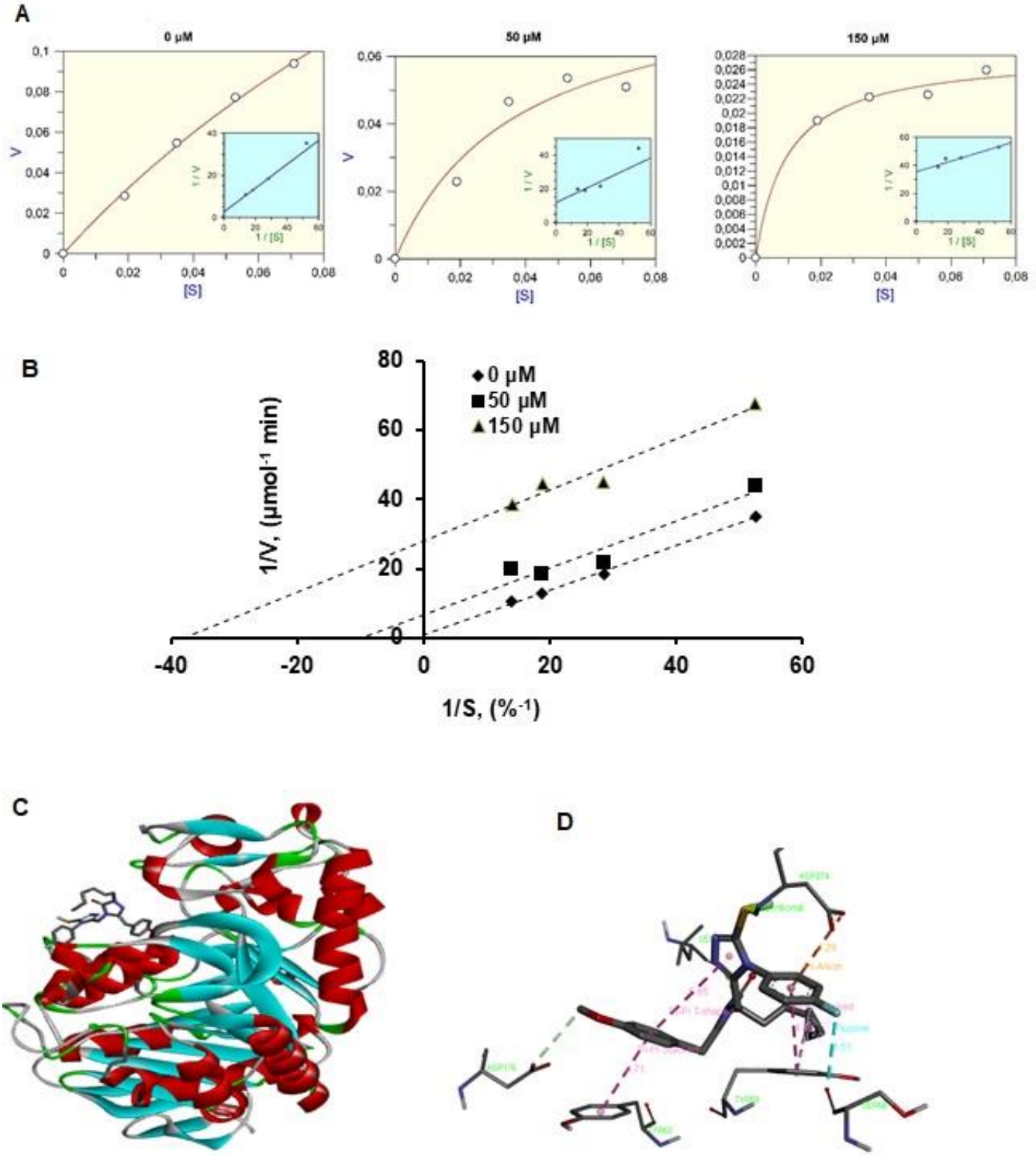



**Figure 1.** A) Lineweaver–Burk plots obtained with variable starch concentrations at fixed Compound VII concentration. B) Determination of α-amylase inhibition type in the presence of Compound VII. C) Predicted conformation of the Compound VII inside the binding pocket of *Bacillus subtilis* α-amylase. D) Micro environment which shows various types of interactions of the compounds atoms with the amino acid residues.

**Table 3**. The type of α-amylase inhibition in the presence of the Compound VII and the calculated kinetic parameters -

| [Compound VII] (μM) | $K_m$ (%) | $V_{max}$ (μmol.min$^{-1}$) | Type of inhibition | $K_i$ (μM) |
|---|---|---|---|---|
| 0 | 0.68 | 0.66 | | |
| 50 | 0.07 | 0.12 | Uncompetetive | 6.43±0.12 |
| 150 | 0.03 | 0.04 | | |

Dandekar and coauthors reported that they observed uncompetitive inhibition and Ki values of range of 2.49-47.60 μM for an α-amylase by dehydrodieugenol B isolated from *Ocimum tenuiflorum* [3]. Shahzad and coauthors observed competitive inhibition for an α-amylase in the presence of novel C-2 symmetric molecules with Ki value as 3.865 μM [1]. Ki value and inhibition type for α-amylase inhibition by wheat bran was reported to be as 17.24 μg.mL$^{-1}$ and noncompetitive, respectively [46]. Balbaa and coauthors observed noncompetitive inhibition for an α-amylase in the presence of 4-amino-3-(D-glucopentyl-1-yl)-5-mercapto-1,2,4-triazoles and their 3-methyl analogues with Ki value 0.363 mM [51].

## Molecular docking calculation

The most rational and authentic approaches in the drug design and discovery are the investigation of the binding efficiencies and molecular interactions of the ligands with target proteins by molecular docking [1, 8, 43]. The docking analysis was performed with active site of amylase (PDB ID: 1BAG) and the results exhibited good binding interaction with receptor. Docking scores ranging from -8.3 to -6.7 can be seen in Table 2, and various interactions between target protein and ligands such as hydrogen bond, π-π stacking interaction and π-cation interaction were also mainly observed. The binding models (2D and 3D) of Compound VII with protein receptor are illustrated in Figure 1. The title compounds showed hydrogen bond interaction with six amino acids residues including SER 56, TYR 59, TYR 62, ASP 176, ASP 274, and LEU 142. Along with π-π stacking interaction with TYR 59 and TYR 62, it also displayed π-cation interaction with ASP 176. Thus, the docking studies show that multimerization enhances the binding affinity and occupies the highest docking score.

## Cytotoxicity activity

Cytotoxic concentration of Compound VII on AR42J cells was first determined by the trypan blue method supplemented with fixative [35], and amylase inhibition studies were performed using non-cytotoxic conditions not to obtain false negative results (Figure 2). Thus, Figure 2 shows the amylase inhibition induced by the non-cytotoxic dose of Compound VII. Experiments were also performed for CF-1 cells, control cells which are not amylase overexpressing. Again, non-cytotoxic doses were used as well, and there was no significant difference in cell viability after treatment with Compound VII compared to untreated counterparts ($p$<0.05) (Figure 2).

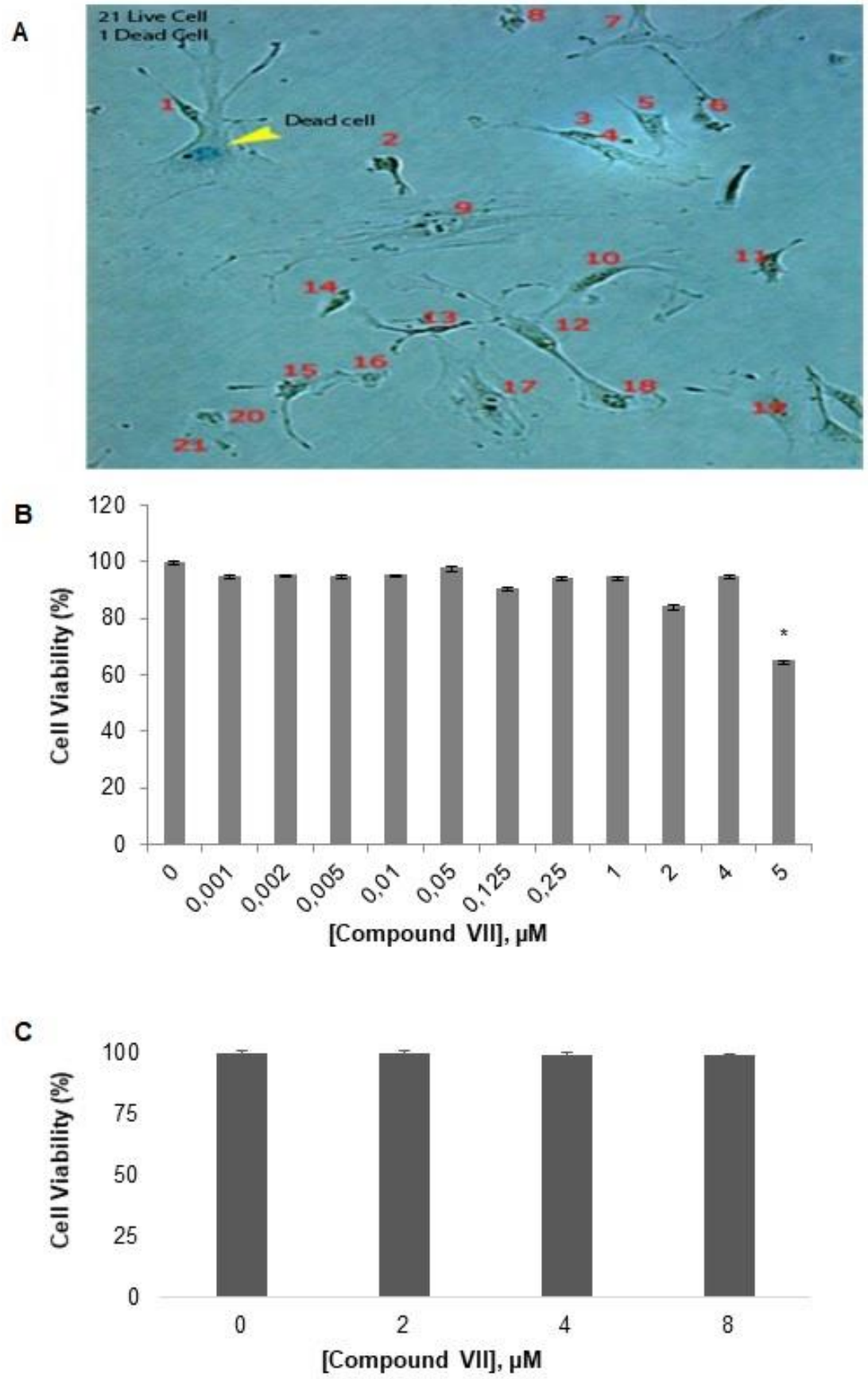



**Figure 2.** A) The concentrations of Compound VII using the Trypan blue staining supplemented with fixative. B) Cell viability (%) detected after treatment of Compound VII on AR42J cells. C) Cell viability (%) detected after treatment of Compound VII on CF-1 cells. *Statistically significant difference compared to untreated control cells ($p$<0.05).

## α-Amylase inhibitory activity *in vitro*

One of the group of chemicals in drug design for T2DM therapy is triazole-derived compounds so that a large scale of drug development studies currently focus on the design and synthesize these compounds due to their clinical importance [12, 14, 52]. This study reports that a heterocyclic molecule containing a triazole derivative the cytotoxic effect of Compound VII on AR42J cells above 5 µM, and amylase inhibition by Compound VII in a dose-dependent manner on AR42J cells. AR42J cell line is one of the best model for studies on amylase inhibition as it has been shown to overexpress amylase [53]. The Compound VII was also found to be more effective on α-amylase inhibition compared to acarbose as a positive control (Figure 3). Compound VII and acarbose were dissolved in DMSO before experiments and was used in a final concentration not exceeding 0.1% [34]. The prepared stock iodine solution was used diluted 1:100 (v/v) so that the final concentration of the starch solution was 0.125%. The $IC_{50}$ value of Compound VII was found to be 4 µM but could not be calculated for acarbose even at 500 µM which was the highest concentration used for the Compound VII. There are a range of drugs developed chemically or using biotechnological tools. Ngoh and coauthors performed the α-amylase inhibition experiments on AR42J cells of the peptides they obtained by purifying from speckled beans on $2x10^4$ cells and the $IC_{50}$ value was calculated as 8 mM [54]. In addition, in amylase inhibition studies performed on AR42J cell line with limonoid derivatives obtained by purification, it has been reported that only 41.8% inhibition of Azadiradione can be observed at 3.5 µM [55]. It can be said that the studies conducted are meaningful compared to the other examples in the literature.

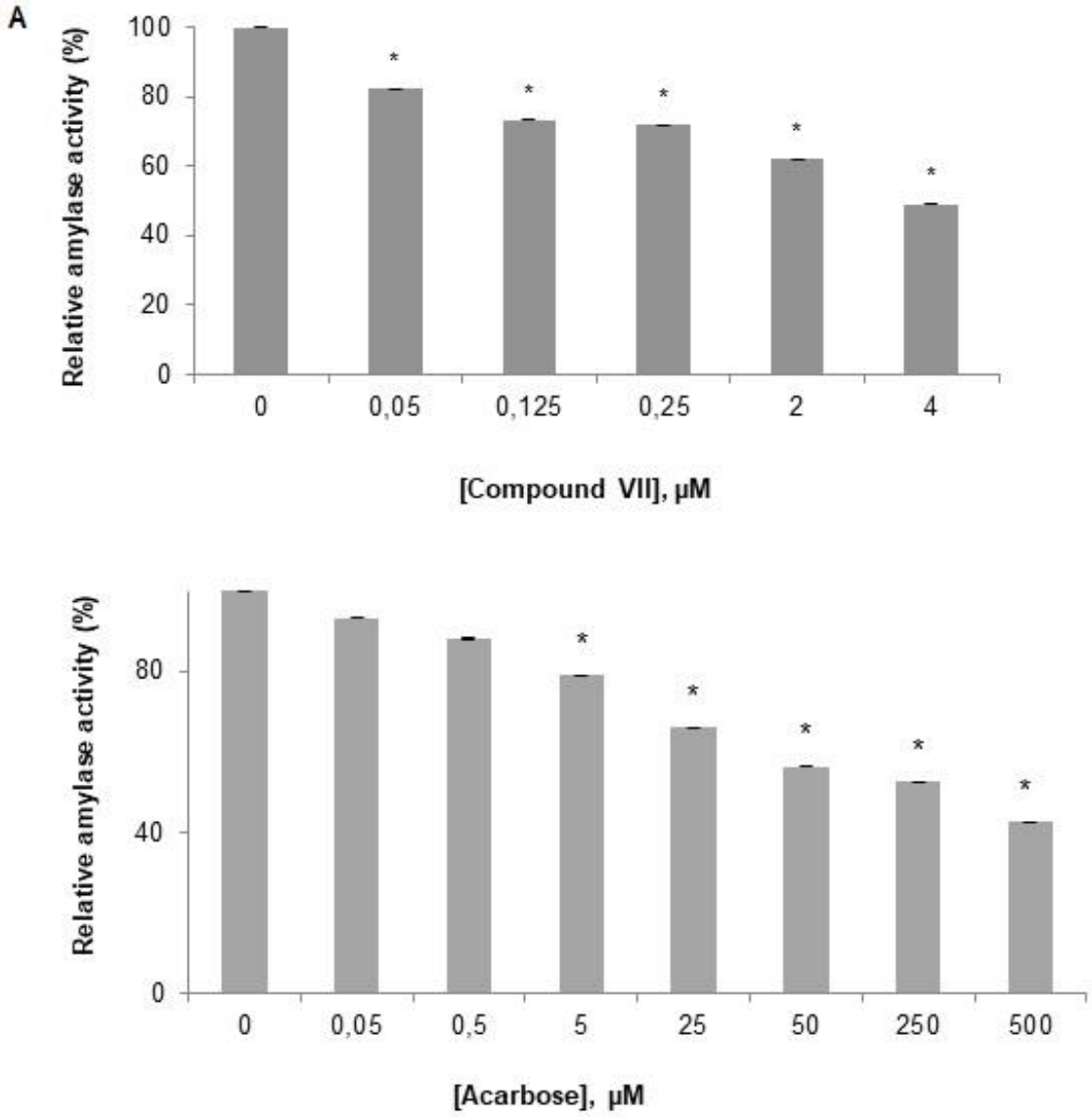



**Figure 3**. A) Amylase inhibition with compound VII on AR42J cells. B) Amylase inhibition with acarbose on AR42J cells. *Statistically significant difference compared to untreated control cells ($p$<0.05).

## CONCLUSION

Many triazole compounds are used as complementary supplies to current drug therapies, and some have been used for their inhibition of amylase activities in particular for the treatment of T2DM. AR42J cells are one of the precursor cells for T2DM, which is still used preclinically in the literature, and trials with 1,2,4 triazoles are not sufficient. In this study, eight triazole derivative compounds were examined for the first time to reveal their α-amylase inhibition potentials. Among the compounds, Compound VII was found to be the most potential (at micromolar level) for α-amylase inhibition *in silico* and *in vitro* studies. This study suggests a new drug candidate (Compound VII) for amylase inhibition, which is 2-Heptyl-4 - {[4- (4-fluorophenyl)-4,5-dihydro-5-thion-1*H*-1,2,4-triazol-3-yl] methyl}c-5-(4-methoxybenzyl)-2,4-dihydro-3*H* 1,2,4-triazol-3-one. But further biochemical and pharmacological tests should be performed for α-amylase inhibition under in vivo conditions for Compound VII.

**Conflicts of Interest:** The authors declare no conflicts of interest.

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## REFERENCES

1. Shahzad D, Saeed A, Larik FA, Channar PA, Abbas 2 Q, Alajmi MF, et al. Novel C-2 symmetric molecules as α-glucosidase and α-amylase inhibitors: Design, synthesis, kinetic evaluation, molecular docking and pharmacokinetics. Molecules. 2019;24:1511-27.
2. Lin X, Xu Y, Pan X, Xu J, Ding Y, Sun X, et al. Global, regional, and national burden and trend of diabetes in 195 countries and territories: an analysis from 1990 to 2025. Sci Rep. 2020;10(1):14790.
3. Dandekar PD, Kotmale AS, Chavan SR, Kadlag PP, Sawant SV, Dhavale DD, et al. Insights into the inhibition mechanism of human pancreatic alpha-amylase, a type 2 diabetes target, by dehydrodieugenol b isolated from *Ocimum tenuiflorum*. ACS Omega. 2021;6(3):1780-6.
4. Admassu H, Gasmalla MAA, Yang RJ, Zhao W. Evaluation of the *in vitro* alpha-amylase enzyme inhibition potential of commercial dried laver (*Porphyra Species*) seaweed protein hydrolysate. Turk J Fish Aquat Sc. 2018;18(4):547-56.
5. Torjesen I. Covid-19: Mental health services must be boosted to deal with "tsunami" of cases after lockdown. Bmj-Brit Med J. 2020;369.
6. Chowdhury S, Goswami S. COVID-19 and type 1 diabetes: dealing with the difficult duo. Int J Diabetes Dev C. 2020;40(3):315-20.
7. Samira N, Khedidja B, Manel L, Israa S, Mohamed Y. New inhibition detection method to evaluate the human salivary alpha-amylase activity of some drugs, molecular docking, and SAR studies. Anti-inflamm Anti-allergy Agents Med Chem. 2021;20(1):10-9.
8. Nawaz M, Taha M, Qureshi F, Ullah N, Selvaraj M, Shahzad S, et al. Structural elucidation, molecular docking, alpha-amylase and alpha-glucosidase inhibition studies of 5-amino-nicotinic acid derivatives. BMC Chem. 2020;14(1):43.
9. Kandra L, Zajacz A, Remenyik J, Gyemant G. Kinetic investigation of a new inhibitor for human salivary alpha-amylase. Biochem Biophys Res Commun. 2005;334(3):824-8.
10. Bischoff H. Pharmacology of alpha-glucosidase inhibition. Eur J Clin Invest. 1994;24 Suppl 3:3-10.
11. Wujec M, Plech T, Siwek A, Rajtar B, Polz-Dacewicz M. Synthesis and *in vitro* study of antiviral and virucidal activity of novel 2-[(4-Methyl-4*H*-1,2,4-triazol-3-yl)sulfanyl]acetamide derivatives. Z Naturforsch C. 2011;66(7-8):333-9.
12. Kumari M, Tahlan S, Narasimhan B, Ramasamy K, Lim SM, Shah SAA, et al. Synthesis and biological evaluation of heterocyclic 1,2,4-triazole scafolds as promising pharmacological agents. BMC Chemistry 2021;15(5).
13. Robertson JG. Mechanistic basis of enzyme-targeted drugs. Biochemistry-Us. 2005;44(15):5561-71.
14. Namratha B, Gaonkar SL. 1,2,4-triazoles: Synthetic strategies and pharmacological. J Pharm Pharm. 2014;6(8):73-80.
15. Wang CS, Huang Q, Wang X, Zhang YT, Ma DS, Yu YH, et al. Three new coordination polymers based on bis(4-(4*H*-1,2,4-triazol-4-yl)phenyl)methane: syntheses, structures, multiresponsive luminescent sensitive detection for antibiotics and pesticides, and antitumor activities. Rsc Adv. 2019;9(72):42272-83.
16. Huang B, Kang D, Tian Y, Daelemans D, Clercq E, Pannecouque C, et al. Design, synthesis, and biological evaluation of piperidinyl-substituted [1,2,4]triazolo[1,5-a]pyrimidine derivatives as potential anti-HIV-1 agents with reduced cytotoxicity. Chem Biol Drug Des. 2021;97:67-76.
17. Tratrat C, Haroun M, Paparisva A, Kamoutsis C, Petrou A, Gavalas A, et al. New substituted 5-benzylideno-2-adamantylthiazol[3,2-b][1,2,4]triazol-6(5*H*)ones as possible anti-inflammatory agents. Molecules. 2021;26:659.

18. Al Sheikh Ali A, Khan D, Naqvi A, Al-Blewi FF, Rezki N, Aouad MR, et al. Design, synthesis, molecular modeling, anticancer studies, and density functional theory calculations of 4-(1,2,4-Triazol-3-ylsulfanylmethyl)-1,2,3-triazole derivatives. ACS Omega. 2021;6(1):301-16.
19. Meijer EFJ, Dofferhoff ASM, Hoiting O, Buil JB, Meis JF. Azole-Resistant COVID-19-Associated Pulmonary Aspergillosis in an Immunocompetent Host: A Case Report. J Fungi. 2020;6(2):79.
20. Mohammadi-Khanaposhtani M, Rezaei S, Khalifeh R, Imanparast S, Faramarzi MA, Bahadorikhalili S, et al. Design, synthesis, docking study, alpha-glucosidase inhibition, and cytotoxic activities of acridine linked to thioacetamides as novel agents in treatment of type 2 diabetes. Bioorg Chem. 2018;80:288-95.
21. Anigboro AA, Avwioroko OJ, Ohwokevwo OA, Pessu B, Tonukari NJ. Phytochemical profile, antioxidant, alpha-amylase inhibition, binding interaction and docking studies of *Justicia carnea* bioactive compounds with alpha-amylase. Biophys Chem. 2021;269:106529.
22. Miller GL. Use of Dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem. 1959;31(3):426-8.
23. Uzun U, Akatin MY. Immobilization and some application of alpha-amylase purified from *Rhizoctonia solani* AG-4 strain ZB-34. Turk J Biochem. 2019;44(3):397-407.
24. Colak A, Col M, Canakci S, Belduz AO, Omarov I. Investigation of extracellular highly thermostable starch hydrolyzing activity from a novel thermophilic bacterium *Anoxybacillus gonensis* A4. Asian J Chem. 2008;20(2):1577-87.
25. Lineweaver H, Burk D. The Determination of enzyme dissociation-constants. J Am Chem Soc. 1934;56(3):658-66.
26. Mermer A, Demirbas N, Colak A, Demir EA, Kulabas N, Demirbas A. One-pot, four-component green synthesis, carbonic anhydrase II inhibition and docking studies of 5-Arylidenerhodanines. Chemistryselect. 2018;3(43):12234-42.
27. Chelladurai GRM, Chinnachamy C. Alpha amylase and alpha glucosidase inhibitory effects of aqueous stem extract of *Salacia oblonga* and its GC-MS analysis. Braz J Pharm Sci. 2018;54(1).
28. Srinivasan B. Words of advice: teaching enzyme kinetics. Febs J. 2021;288:2068-83.
29. Fujimoto Z, Takase K, Doui N, Momma M, Matsumoto T, Mizuno H. Crystal structure of a catalytic-site mutant alpha-amylase from *Bacillus subtilis* complexed with maltopentaose. J Mol Biol. 1998;277(2):393-407.
30. Trott O, Olson AJ. Software News and update AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. J Comput Chem. 2010;31(2):455-61.
31. Copeland RA. Evaluation of enzyme inhibitors in drug discovery: A Guide for medicinal chemists and pharmacologists, 2nd Edition. Evaluation of Enzyme Inhibitors in Drug Discovery: A Guide for Medicinal Chemists and Pharmacologists, 2nd Edition. 2013:1-538.
32. Koc E, Celik-Uzuner S, Uzuner U, Cakmak R. The detailed comparison of cell death detected by Annexin V-PI counterstain using fluorescence microscope, flow cytometry and automated cell counter in mammalian and microalgae cells. J Fluoresc. 2018;28(6):1393-404.
33. Hwang CE, Kim SC, Kim DH, Lee HY, Suh HK, Cho KM, et al. Enhancement of isoflavone aglycone, amino acid, and CLA contents in fermented soybean yogurts using different strains: Screening of antioxidant and digestive enzyme inhibition properties. Food Chem. 2021;340:128199.
34. Demir EA, Demir S, Aliyazicioglu Y. *In vitro* Cytotoxic Effect of ethanol and dimethyl sulfoxide on various human cell lines. KSU J Agric Nat. 2020;23(5):1119-24.
35. Celik Uzuner S. Development of a direct trypan blue exclusion method to detect cell viability of adherent cells into ELISA plates. Celal Bayar Uni J Sci. 2018;14(1):99-104.
36. Xiao ZZ, Storms R, Tsang A. A quantitative starch-iodine method for measuring alpha-amylase and glucoamylase activities. Anal Biochem. 2006;351(1):146-8.
37. Yusefi M, Shameli K, Ali RR, Pang SW, Teow SY. Evaluating anticancer activity of plant-mediated synthesized iron oxide nanoparticles using punica granatum fruit peel extract. J Mol Struct. 2020;1204:127539.
38. Chelamalla R, Akena V, Manda S. Synthesis of N'-arylidene-2-(5-aryl-1*H*-1, 2, 4-triazol-3-ylthio) acetohydrazides as antidepressants. Med Chem Res. 2017;26(7):1359-66.
39. Mioc M, Soica C, Bercean V, Avram S, Balan-Porcarasu M, Coricovac D, et al. Design, synthesis and pharmaco-toxicological assessment of 5-mercapto-1,2,4-triazole derivatives with antibacterial and antiproliferative activity. Int J Oncol. 2017;50(4):1175-83.
40. Hunashal RD, Ronad PM, Maddi VS, Satyanarayana D, Kamadod MA. Synthesis, anti-inflammatory and analgesic activity of 2-[4-(substituted benzylideneamino)-5-(substituted phenoxymethyl)-4*H*-1,2,4-triazol-3-yl thio] acetic acid derivatives. Arab J Chem. 2014;7(6):1070-8.
41. Pogaku V, Krishna VS, Sriram D, Rangan K, Basavoju S. Ultrasonication-ionic liquid synergy for the synthesis of new potent anti-tuberculosis 1,2,4-triazol-1-yl-pyrazole based spirooxindolopyrrolizidines. Bioorg Med Chem Lett. 2019;29(13):1682-7.
42. Murthy YLN, Govindh B, Diwakar BS, Nagalakshmi K, Rao KVR. Synthesis and bioevaluation of schiff and mannich bases of isatin derivatives with 4-amino-5-benzyl-2,4-dihydro-3*H*-1,2,4-triazole-3-thione. Med Chem Res. 2012;21(10):3104-10.
43. Joshi R, Kumari A, Singh K, Mishra H., Pokharia S. Triorganotin(IV) complexes of Schiff base derived from 1,2,4-triazolemoiety: Synthesis, spectroscopic investigation, DFT studies, antifungalactivity and molecular docking studies. J Mol Struc. 2020;1206:127639.

44. Bekircan O, Mentese E, Ulkerc S. Synthesis and pharmacological activities of some new 2-[1-Heptyl-3-(4-methoxybenzyl)-5-oxo-1,5-dihydro-4*H*-1,2,4-triazol-4-yl]acetohydrazide derivatives. Z Naturforsch B. 2014;69(9-10):969-81.
45. Gonzaga D, Senger MR, da Silva FD, Ferreira VF, Silva FP. 1-Phenyl-1*H*- and 2-phenyl-2*H*-1,2,3-triazol derivatives: Design, synthesis and inhibitory effect on alpha-glycosidases. Eur. J Med Chem. 2014;74:461-76.
46. Tu J, Chen J, Zhu S, Zhang C, Chen H, Liu Y. Inhibition of wheat bran and it's active compoments on alpha-glucosidase *in vitro*. Pharmacogn Mag. 2013;9(36):309-14.
47. Oudjeriouat N, Moreau Y, Santimone M, Svensson B, Marchis-Mouren G, Desseaux V. On the mechanism of alpha-amylase-Acarbose and cyclodextrin inhibition of barley amylase isozymes. Eur J Biochem. 2003;270(19):3871-9.
48. Madushika Wariyapperuma WAN, Kannangara S, Wijayasinghe YS, Subramanium S, Jayawardena B. Fungal pretreatment to enhance the yield of phytochemicals and evaluation of alpha-amylase and alpha-glucosidase inhibition using *Cinnamomum zeylanicum* (L.) quills pressurized water extracts. Lett Appl Microbiol. 2021;72(2):196-205.
49. Wang Z, Peng S, Peng M, Wang C. Isolation of polyphenol compounds from olive waste and inhibition of their derivatives for alpha-glucosidase and alpha-amylase. Nat Prod Res. 2020;34(16):2398-402.
50. Holdgate GA, Meek TD, Grimley RL. Mechanistic enzymology in drug discovery: a fresh perspective. Nat Rev Drug Discov. 2018;17(2):115-32.
51. Balbaa M, Mansour H, El-Sawy H, El-Ashry ESH. Inhibition of some hepatic glycosidases by the diseco nucleoside, 4-amino-3-(D-glucopentitol-1-yl)-5-mercapto-1,2,4-triazole and its 3-methyl analog. Nucleos Nucleot Nucl. 2002;21(10):695-708.
52. Hameed S, Kanwal, Seraj F, Rafique R, Chigurupati S, Wadood A, et al. Synthesis of benzotriazoles derivatives and their dual potential as alpha-amylase and alpha-glucosidase inhibitors *in vitro*: Structure-activity relationship, molecular docking, and kinetic studies. Eur J Med Chem. 2019;183:111677.
53. Siow HL, Tye GJ, Gan CY. Pre-clinical evidence for the efficacy and safety of alpha-amylase inhibitory peptides from cumin (*Cuminum cyminum*) seed. J Funct Foods. 2017;35:216-23.
54. Ngoh YY, Tye GJ, Gan CY. The investigation of alpha-amylase inhibitory activity of selected Pinto bean peptides via preclinical study using AR42J cell. J Funct Foods. 2017;35:641-7.
55. Ponnusamy S, Haldar S, Mulani F, Zinjarde S, Thulasiram H, RaviKumar A. Gedunin and azadiradione: Human pancreatic alpha-amylase inhibiting limonoids from Neem (*Azadirachta indica*) as anti-diabetic agents. Plos One. 2015;10(10).