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Original Article

Biological and computational evaluation of carbazole-based bis-thiosemicarbazones: A selective enzyme inhibition study between α-amylase and α-glucosidase

Hasan Sahin[1], Alev Arslanturk Bingul[2], Ibrahim Fazil Sengul[3], Murat Bingul[4]

[1]Dicle University, Faculty of Pharmacy, Department of Pharmacognosy, Diyarbakır, Turkiye
[2]Dicle University, Faculty of Science, Department of Chemistry, Diyarbakır, Turkiye
[3]Gebze Technical University, Faculty of Science, Department of Chemistry, Kocaeli, Turkiye
[4]Dicle University, Faculty of Pharmacy, Department of Basic Pharmaceutical Sciences, Diyarbakır, Turkiye

ORCID IDs of the authors: H.Ş. 0000-0002-8325-8116; A.A.B. 0000-0003-0744-0968; İ.F.Ş. 0000-0001-9386-4892; M.B. 0000-0002-3909-0694

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ABSTRACT

Background and Aims: Carbazole heterocyclic systems are an important class of chemicals that have been reported as valuable antidiabetic agents in the literature. Uncoincidentally, the ayurvedic antidiabetic plant *Murraya koenigii* Spreng (Curry tree) was the source of the first carbazole alkaloids. Another important class of chemicals in terms of antidiabetic activity is thiosemicarbazones. The hybridization of these fragments can create new potential inhibitors for α-amylase and α-glucosidase enzyme inhibitions, which is one approach controlling post-prandial hyperglycemia in type 2 diabetes patients.
Methods: The four carbazole-based thiosemicarbazone compounds (**4a-d**) have been selected from the group library and α-amylase and α-glucosidase inhibition potencies have been evaluated. A molecular modelling study has also been carried out to provide a complementary study on how the molecules behave in terms of the enzymes' catalytic properties. .
Results: All compounds showed higher potencies than the standard acarbose in terms of α-glucosidase inhibition and very low inhibitions toward α-amylase compared to acarbose. Having the number of hydrophobic interactions determine the potency of the compounds was crucial with compound **4a** being shown to provide the highest number of conventional H bonds and the highest percentage of inhibition values for both enzymes.
Conclusion: Carbazole-based thiosemicarbazone compounds have been found to be promising candidates in terms of both their potency and relative selectivity for developing new inhibitors that lack the usual side effects of current drugs.

Keywords: α-amylase, α-glucosidase, carbazole, molecular docking, thiosemicarbazones

INTRODUCTION

Natural compounds have been used as models to identify new active molecules in organic chemistry. 9H-Carbazole was discovered as an anthracene derivative in 1872 (Graebe & Glaser, 1872) and has been illustrated as an important heterocyclic compound model. Afterward, the interest in carbazole derivatives increased significantly through the strong antimicrobial effects of the alkaloid named murrayanine that was isolated from *Murraya koenigii* Spreng (Schmidt, Reddy, & Knölker, 2012a). Numerous studies conducted along these lines have identified carbazole derivatives as valuable therapeutic targets due to their antidiabetic, anti-

Address for Correspondence:
Murat BİNGÜL, e-mail: muratbingul1983@gmail.com

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HIV, antitumor, antibacterial, and neuroprotective potentials. After 150 years, many natural and synthetic carbazole derivatives are now available for different diseases including cancer in the pharmaceutical market (Tan, Sharma, & An, 2022).

The natural compounds and traditional medicines have been used for the discovery of new naturally active synthetic compounds.. For example, *M. koenigii*, from which the first bioactive carbazole alkaloids were obtained, is used to treat diabetes in traditional Indian medicine (i.e., Ayurveda). Many pharmacognostic studies conducted in this context have attributed the hypoglycemic effect of the plant to its carbazole derivatives (Kesari, Kesari, Singh, Gupta, & Watal, 2007; Patel et al., 2016). Non-insulin dependent type 2 diabetes patients constitute the majority of *diabetes mellitus* patients with altered blood glucose levels, and α-amylase and α-glucosidase inhibitors are used on these patients to prevent postprandial hyperglycemia and its complications. In this way, these enzymes are prevented from hydrolyzing polysaccharides in foods into monosaccharides. Currently, acarbose, miglitol, and voglibose are the active pharmaceutical compounds prescribed for this purpose. The search for new inhibitors continues due to the side effects (e.g., abdominal pain, nausea, vomiting and meteorism) from these non-selective inhibitors. Many studies are also found to have reported carbazole derivatives to be potent α-glucosidase inhibitors (Adib et al., 2019; Dhameja & Gupta, 2019; Iqbal et al., 2017).

Thiosemicarbazones are derived from the condensation reaction of aldehyde functionality and thiosemicarbazides and additionally have attracted great attention due to the presence of R^1R^2C=N-NH-(C=S)-NR^3R^4 moiety. The conjugated *N-N-S* system provides an important therapeutic potential to thiosemicarbazone-based systems, with interesting interactions occurring with the biomolecules (Antholine, Knight, Whelan, & Petering, 1977; Richardson, 2002; Shahabadi, Kashanian, & Darabi, 2010). Specifically, thiosemicarbazone fragments have been reported as important targets for antidiabetic studies. A new series of benzoxazinone-thiosemicarbazones have been reported as potent inhibitors of aldo-keto reductases, which are important enzymes for the polyol pathway (Shehzad et al., 2019). Tok et al. (Tok, Küçükal, Baltaş, Tatar Yılmaz, & Koçyiğit-Kaymakçıoğlu, 2022) reported on a range of thiosemicarbazones derived from substituted sulfonyl acetophenone and evaluated for α-amylase and glucosidase inhibition potency. They also carried out kinetic studies of the designated compounds and revealed their competitive mode of binding. A series of indole-based thiosemicarbazones have also been designed and tested for identifying aldose reductase (ALR2) and aldehyde reductase (ALR1) potency (Shehzad et al., 2021), which also concluded the reported compounds to display selective potential toward complications associated with *diabetes mellitus*.

As a result, this study aims to explore the antidiabetic potentials of hybrid molecules derived from carbazole and substituted thiosemicarbazides. The main consideration is to create multiple binding patterns between the designated compounds and different binding pockets on the α-amylase and α-glucosidase enzymes. The individual antidiabetic potentials of each fragment can help increase the inhibitory activity and provide better compounds as more promising inhibitors. Four compounds (**4a-d)** have been chosen from our groups` chemical library containing promising fragments (carbazole and thiosemicarbazones) and tested them against the responsible enzymes for an antidiabetic study. The computational study has also been carried out to understand the binding behaviors of the compounds in terms of the enzymes' catalytic sides. The practical results obtained from the bioassays have been compared with the theoretical data from the molecular docking study. This study is a complementary report that provides an important comparison of practical and theoretical data for antidiabetic study.

MATERIALS AND METHODS

Chemicals and physical measurements

The general synthetic procedure for the known compounds has been reported in the appropriate reference (Bingul et al., 2019). All commercially available reagents and standards, as well as the α-amylase type VI-B (E.C. 3.2.1.1 from porcine pancreas, lyophilized powder), 2-chloro-4-nitrophenyl-α-D-maltotrioside (CNP-G3), sodium chloride, sodium azide, acarbose, Dimethyl sulfoxide (DMSO), p-nitrophenol, α-D-glycopyranoside (PNPG), and α-glucosidase type I (E.C. 3.2.1.20, from *Saccharomyces cerevisiae*, lyophilized powder) as used for the biological assays were purchased from Sigma Aldrich (St. Louis, MO) and carried out without further purification. All other chemicals were of analytical grade.

The α-Glucosidase inhibition assay

The α-glucosidase inhibitory activity was performed using the method described by Schmidt et al. (Schmidt, Lauridsen, Dragsted, Nielsen, & Staerk, 2012b). In brief, 90 µL of 0.1 M phosphate buffer (pH 7.5, 0.02% NaN_3), a 10 µL test sample dissolved in DMSO, and 80 µL of enzyme solution (well concentration of 0.05 U/mL) were added to each well. The mixture was incubated at 28 °C for 10 min before adding PNPG to a final volume of 200 µL (final well concentration of 1.0 mM). A blank was used consisting of the enzyme, substrate, and test solvent instead of the sample. Absorbance was measured at 405 nm every 40 s for 35 min. BioTek Power Wave XS microplate photometer with built-in incubator and controlled by the program GEN5 9ver. 2.05.20050 was used for the incubation and absorbance measurements. The α-glucosidase inhibitory activity was expressed as percentage inhibition and calculated using the following formula:

$$\text{Inhibition \%} = (\text{Slope}_{\text{blank}} - \text{Slope}_{\text{sample}}) / \text{Slope}_{\text{blank}} * 100 \quad (1)$$

Acarbose was used as the positive control, and all measurements were performed in triplicate (Student's t-test with $p < 0.05$ showing significance).

The α-Amylase inhibition assay

The α-amylase inhibitory activity was measured using the method described by Okutan et al. (Okutan, Kongstad, Jäger, & Staerk, 2014) with the minor changes. In brief, 80 µL of 0.1 M phosphate buffer (pH 6.0, 0.02% NaN_3), a 20 µL test sample dissolved in DMSO, and 80 µL of enzyme solution (well concentration of 0.05 U/mL) were added to each well. After incubation at 37°C for 10 min, the reaction was started by adding CNP-G3 to a final volume of 200 µL (final well concentration of 1.0 mM). A blank was used consisting of the enzyme, sub-

strate, and test solvent instead of the sample. Absorbance was measured at 405 nm at for 30m. A background sample consisting of enzyme, sample, and buffer in place of the substrate for each compound was employed to eliminate the effect of the absorbance for analytes that absorbs at 405 nm. The BioTek Power Wave XS microplate photometer with built-in incubator controlled by the program GEN5 (ver. 2.05.2005) was used for the incubation and absorbance measurements. The α-amylase inhibitory activity was expressed as percentage inhibition ± SD and calculated using the following formula:

$$\text{inhibition } \% = (A_{blank} - A_{sample}) / A_{blank} * 100 \quad (2)$$

Acarbose was used as the positive control, and all measurements were performed in triplicate (Student's t-test with $p < 0.05$ showing significance).

Molecular modeling

Receptor preparation

The crystallographic structures of α-amylase and α-glucosidase were respectively obtained from the Protein Data Bank (Berman, et al., 2000) as 1B2Y (crystallographic structures of α-amylase, 3.20 Å) (Nahoum et al., 2000) and 3W37 (crystallographic structures of α-glucosidase1.70 Å) (Tagami et al., 2013). The crystal structures were cleaned from all ingredients contained in the PDB file apart from the amino acid residues using BIOVA Ds Visualizer. The program MGLTools was used to add missing residues, hydrogen atoms, and charges and to remove non-polar hydrogen atoms.

Ligand preparation

Three-dimensional structures of the synthesized ligands **4a-d** were drawn using the Biovia DS Visualizer (Biovia, 2019) and displayed using the Chimera (UCSF) software package (Pettersen et al., 2004). All molecules were firstly optimized using the semi-empirical AM1 method, with the optimization taking place using Gaussview 5.0.9 (Frisch et al., 2009). AM1-Bcc (the Austin model with bond and charge correction) atomic partial charges for the ligands were determined using the antechamber module of the package program AMBER v.11. (Jakalian, Jack, & Bayly, 2002).

Docking study

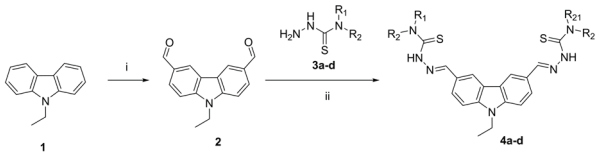
This research performed the docking studies using Dock 6.5. Grid generations were computed by centering a box to the binding sites of the receptors. Grid boxes were centered to the binding sites of the enzymes with the x, y, and z coordinates of 66.521, 51.261, and 12.259 for the α-amylase and 39.434, 33.431, 6.003 for the α-glucosidase, with the dimensions being defined as 40 40 40 Å for the enzymes. The Lamarckian genetic algorithm was used with a population of 300 individuals and docking settings of 2,500,000 maximum energy evaluations, and 54,000 maximum generations to give 250 runs.

RESULTS AND DISCUSSION

Chemistry

Synthesis of the Carbazole-Based Bis-Thiosemicarbazones 4a-d

Syntheses of the targeted carbazole-based bis-thiosemicarbazones **4a-d** were achieved using two reactions, starting with the Vilsmeier Hack formylation of *N*-ethly carbazole **1** in the presence of $POCl_3$ and DMF followed by the Schiff base condensation reaction of the corresponding carbazole 3,6-dicarbaldehyde **2** with the thiosemicarbazides **3a-d** under acidic conditions for good yields (Figure 1). The syntheses and characterization analyses (Fourier Transform Infrared) FT-IR, (Nuclear Magnetic Resonance) NMR, and (High Resolution Mass Spectroscopy) HRMS) of the final compounds **4a-d** have already been discussed in a previous study (Bingul et al., 2019).



Thiosemicarbazides	R_1	R_2	Carbazole-based bis-Thiosemicarbazones
3a	H	H	4a
3b	Me	H	4b
3c	Me	Me	4c
3d	Et	H	4d

Figure 1. Reagents, conditions and compounds: i) $POCl_3$, DMF, reflux, 24 h, ii) Thiosemicarbazides (3a-d), EtOH, CH_3COOH, r.t.

Biological studies

α-Amylase and α-Glucosidase inhibitory activity

Table 1 shows the α-glucosidase and α-amylase inhibition potencies of the four compounds **4a-d** at the 800 μM concentration, with acarbose being used as the standard. The inhibition concentrations revealed the compounds to be more potent than the standard acarbose in the case of α-glucosidase. The % inhibition value of acarbose against α-glucosidase was determined as 70.95% at 1,250 μM, which is in accordance with the previously reported value (Schmidt et al., 2012b An IC_{50} value of 900 μM was found for acarbose for the same method). All four compounds (**4a-d**) provided more than 50% inhibition of the enzyme at 800 μM concentrations. The compound **4c** was found to be the least active compound with 57.84% inhibition. The compounds **4b** and **4d** provided similar inhibitory patterns, with respective values of 79.73% and 73.21%. The compound **4a** was determined to be the most promising candidate for the α-glucosidase inhibition, with 96.72% inhibition.

In the case of the α-amylase enzyme, the standard acarbose provided 93.23% inhibition at 1 μM concentration. The inhibition percentages of the compounds **4a-d** ranged between 80.58%-93.05% at 800 μM, which reveals the compounds to have very low potency toward α-amylase compared to acarbose. The compounds **4c** and **4d** provided similar inhibitory patterns with respective values of 82.24% and 80.58%, while the compounds **4a** and **4b** were determined as the most potent compounds, with respective inhibition values of 93.05% and 88.29%.

Non-selective inhibition of the above-mentioned enzymes is thought to be associated with the usual side effects (e.g., abdominal pain, nausea, vomiting, meteorism, diarrhea) of the drugs in the market. α-Amylase is responsible for hydrolyzing polysaccharides such as amylose and amylopectin to oligosac-

charides such as maltose and maltotriose. It is located in saliva and pancreatic secretions. Cleaving the bonds in oligosaccharides to form monosaccharides such as glucose is the duty of α-glucosidase, which is in the small intestine. Relative greater inhibition of α-amylase compared to α-glucosidase may result in incompletely digested polysaccharides (carbohydrates) reaching the intestine and causing abnormal fermentation (Apostolidis & Lee, 2010; Beidokhti et al., 2020). All tested compounds (**4a-d**) have better inhibitory profiles than acarbose, which is more than 1,250 times more sensitive to amylase according to the current results. Compound **4a** has particularly promising results, with higher potency and selectivity compared to the standard.

The structural evaluations of the compounds revealed the presence of any substitution on the N-edge of the thiosemicarbazones to decrease the inhibition potency for both enzymes. The unsubstituted compound **4a** provided the best inhibition for α-amylase and α-glucosidase enzymes, and its inhibition potency behavior toward the designated enzymes was found to be identical to the methyl-substituted compound **4b**. Although, the ethyl-substituted counterpart **4d** showed similar inhibition values for the enzymes, a dramatic difference was obtained for the dimethyl-substituted thiosemicarbazone **4c**. The compound was a promising candidate for the α-amylase enzyme; however, its inhibition potency had decreased to 57.84% for the α-glucosidase enzyme.

Molecular docking

The computational study has been carried out to understand the behavior of the compounds on the catalytic side of the enzyme pockets. The study has evaluated interactions between the amino acid residues and the chemical fragments on the compounds and attempted to make a logical explanation of how the molecules demonstrate their inhibition potencies.

Figures 2-5 show the 2D interactions of the compounds with the α-amylase enzyme. The conventional hydrogen bonds have been identified as stronger interactions, with the number of interactions determining the compounds' inhibition potencies. Compound **4a** should importantly be noted to have also been determined as the most active candidate for the computational study, as it has the highest number of conventional hydrogen bonds. His201, Ile235, Glu233, Asp197, and Asp300 are the residues that form hydrogen bonds with the thiosemicarbazone fragments of the molecules. Compound **4b** resulted in two different hydrogen bonds through His201 and Tyr151; however, compounds **4c** and **4d** demonstrated only hydrophilic interactions (i.e., carbon-hydrogen, pi alkyl). Pi-donor and pi-sulfur interactions are the common bindings for all compounds through the His or Asp and Tyr or Trp residues.

In the case of the α-glucosidase enzyme, the interactions between the substituents and amino acid residues on the catalytic side were found to be compatible with the results obtained from the bioactivity assay (Figures 6-9). The strongest interaction was determined to have occurred for compound **4a** with Leu428 and Pro426 due to the presence of the carbazole

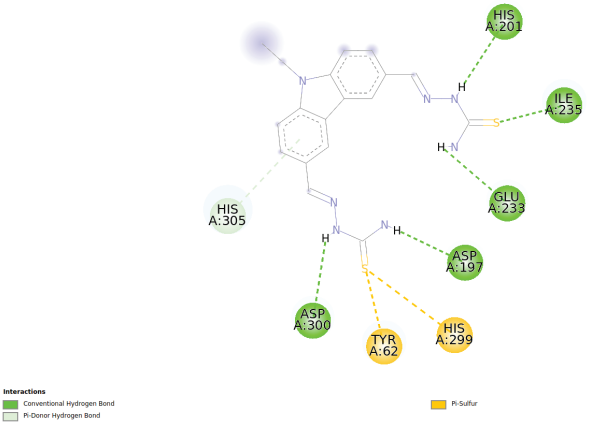


Figure 2. 2D representations of 4a to α-amylase enzyme binding site residue interactions.

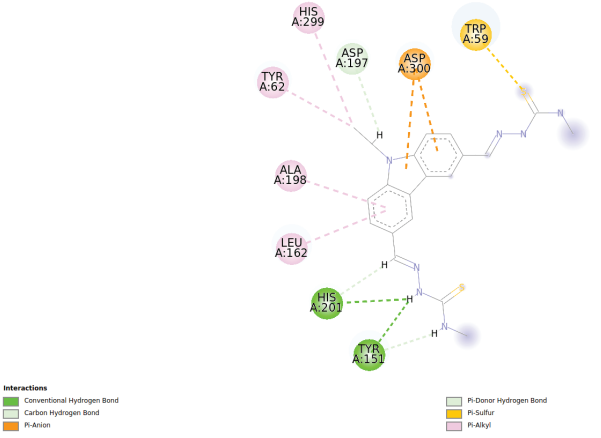


Figure 3. 2D representations of 4b to α-amylase enzyme binding site residue interactions.

Table 1: Enzyme inhibitory activity of the compounds and the standard.

Compounds (800 µM)	α-Amylase (inhibition% ± SD)	α-Glucosidase (inhibition% ± SD)
4a	93.05 ± 2.56	96.72 ± 2.66
4b	88.29 ± 0.53	79.73 ± 1.00
4c	82.24 ± 0.32	57.84 ± 1.33
4d	80.58 ± 0.96	73.21 ± 3.93
Acarbose	93.23 ± 1.26*	70.95 ± 0.46**

*C = 1 µM; **c = 1250 µM

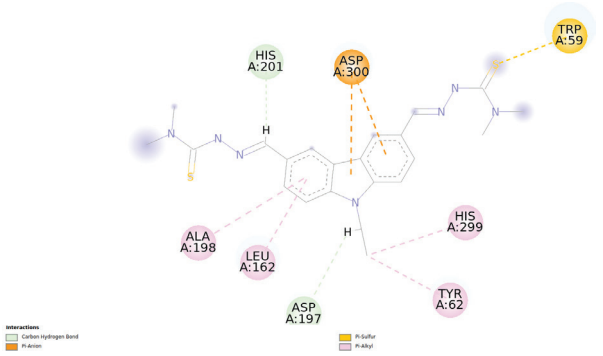


Figure 4. 2D representations of 4c to α-amylase enzyme binding site residue interactions.

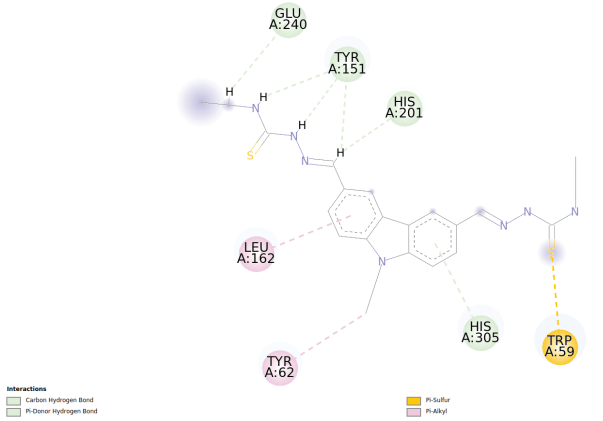


Figure 5. 2D representations of 4d to α-amylase enzyme binding site residue interactions.

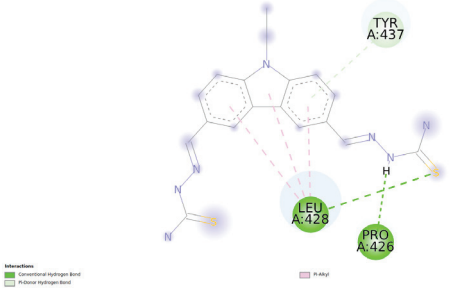


Figure 6. 2D representations of 4a to α-glucosidase enzyme binding site residue interactions.

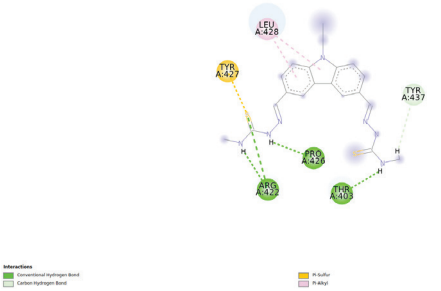


Figure 7. 2D representations of 4b to α-glucosidase enzyme binding site residue interactions.

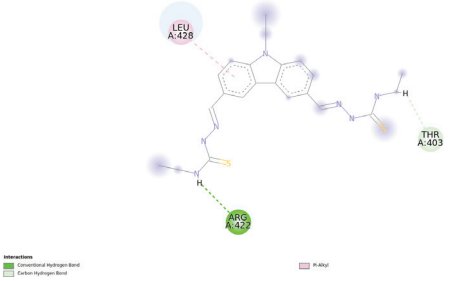


Figure 8. 2D representations of 4c to α-glucosidase enzyme binding site residue interactions.

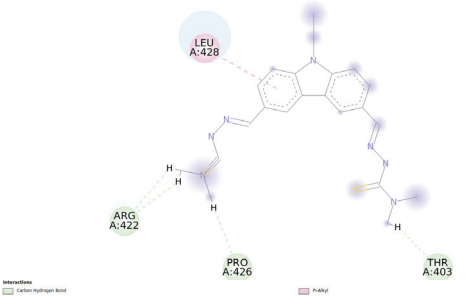


Figure 9. 2D representations of 4c to α-glucosidase enzyme binding site residue interactions.

background, sulfur atom, and amino residue on the thiosemicarbazone moiety. Similar interactions were also obtained for compound **4b** with the amino acid residues Pro426, Arg422, and Thr403. The weakest binding pattern was observed with compound **4c** due to the non-hydrogen bonding and hydrophobic interactions (i.e., C-H and pi-alkyl bonds provided the lowest potency for the α-glucosidase enzyme. As a result, compound **4d** was determined to be the third best compound and the only one with a conventional H bond to Arg422.

CONCLUSION

This study has chosen hybrid molecules derived from carbazole and thiosemicarbazones for the individually potency regarding antidiabetic study and evaluated them for their α-amylase and α-glucosidase inhibitory activity. Four compounds with different substituents on the thiosemicarbazone moiety were selected in terms of their different bonding patterns with regard to the catalytic properties of the designated enzymes. The molecular docking study was carried out to create logical explanations for the practical results obtained from the bioassays. The theoretical data obtained from the computational study is importantly noted to be compatible with the practical results. All tested compounds revealed higher potencies than acarbose in terms of α-glucosidase inhibition. Furthermore, all compounds showed very low inhibitions toward α-amylase compared to acarbose, which has been suggested for eliminating the usual side effects of acarbose. Compound **4a** was found to be the most promising candidate for both its potency and relative selectivity. The presence of an unsubstituted NH_2 functional group on the N edge of the thiosemicarbazone created more conventional H bonds with the amino acid residues and better binding ability on the catalytic side. The increased

binding ability may possibly result in better inhibition percentages for the α-amylase and α-glucosidase enzymes. The addition of any substituents on the designated area of the molecule decreased the potency due to the weaker interactions on the enzyme pockets. Overall, the carbazole-based thiosemicarbazones were found to be more potent and selective candidates for α-glucosidase enzyme inhibition.

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