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Original Article

α-amylase Inhibitor Based on Oleanolic Acid: *In vitro* and *in silico* Studies

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Abstract: Alpha-amylase (α-amylase) is a critical enzyme in carbohydrate metabolism, catalyzing the hydrolysis of α-1,4-glycosidic bonds in starch. Inhibition of α-amylase activity is an effective strategy for managing metabolic disorders like diabetes and obesity by reducing postprandial glucose levels. This study evaluated the α-amylase inhibitory potential of oleanolic acid (OA), a pentacyclic triterpenoid extracted from *Panax bipinnatifidus* Seem., using a combined *in vitro* and *in silico* approach. *In vitro*, the assays demonstrated that OA inhibited α-amylase with an IC_{50} value of 198.73 µg/mL, which, while less potent than the standard inhibitor acarbose (IC_{50} ~ 22.48 µg/mL), showed promise as a natural compound with inhibitory activity. *In silico* molecular docking studies provided insights into the interaction mechanisms between OA and α-amylase. OA displayed a strong binding affinity with a binding energy of -8.33 kcal/mol, significantly lower than acarbose (-2.90 kcal/mol). Key residues, including TYR62, ASP197, GLU233, HIS101, and LEU162, were identified as contributors to hydrogen bonding and hydrophobic interactions with OA. Although acarbose formed more hydrogen bonds with residues such as GLU233, ASP300, and GLY304, OA exhibited stable binding characteristics and a distinct interaction profile. These findings suggest that while OA has lower *in vitro* potency compared to acarbose, its strong binding affinity and interaction with critical active site residues make it a promising candidate for further optimization and development as a natural α-amylase inhibitor. The study highlights the potential of OA for therapeutic applications in metabolic disorder management, emphasizing the need for structural modifications or combination strategies to enhance its efficacy.

Keywords: α-amylase, oleanolic acid, *Panax bipinnatifidus* , *in vitro, in silico.*

1. Introduction

Enzyme α-Amylase (α-1,4 glucan-4-glucanohydrolase, E.C.3.2.1.1) is an endohydrolytic enzyme that catalyzes the random cleavage of α-1,4-glycosidic bonds within starch molecules, hydrolyzing polysaccharides into products with α-anomeric configuration, such as dextrins and oligosaccharides. These products can further be broken down into glucose and maltose by other enzymes [1]. α-amylase can be derived from

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plants, animals, and microorganisms; however, enzymes from fungal and bacterial sources always hold an advantage in applications [2]. Inhibiting the activity of α-amylase, such as sapoinins in *Panax bipinnatifidus* Seem., presents a promising avenue for controlling conditions related to abnormal glucose metabolism, such as diabetes and obesity [3]. α-amylase inhibitors reduce postprandial blood glucose levels by impeding the complex carbohydrate hydrolysis process, thereby improving blood glucose control and minimizing complications associated with hyperglycemia.

Oleanolic acid (OA) (3b-hydroxyolean-12-en-28-oic acid), is a pentacyclic triterpenoid, a conjugated acid of oleanolate, widely occurring in various plant species in nature in the form of free acid or as a precursor aglycone for triterpenoid saponins, serving as a secondary metabolite [4]. OA and its derivatives exhibit diverse pharmacological properties, such as hepatoprotective, anti-inflammatory, antioxidant, or anticancer activities [5]. Recent studies have emphasized its potential as an α-amylase inhibitor, demonstrating its utility in controlling diabetes and related metabolic disorders. The inhibitory mechanism of oleanolic acid against α-amylase involves its interaction with the active site of the enzyme, impeding substrate binding and catalysis. The ability of oleanolic acid to regulate α-amylase activity underscores its therapeutic potential in controlling postprandial hyperglycemia and enhancing insulin sensitivity.

In vitro experiments provide direct assessment of α-amylase inhibition activity, yielding important insights into the therapeutic relevance of oleanolic acid sourced from *P. bipinnatifidus*. Meanwhile, *in silico* studies, particularly molecular docking and molecular dynamics simulations, provide valuable tools to elucidate the molecular interactions between oleanolic acid and α-amylase at the molecular level. Through analytical techniques, we aim to predict the binding relationships and interaction energies between oleanolic acid and α-amylase, thereby enhancing our understanding of their complex relationship.

This integrated approach, combining experimental methods and analysis *in vitro* and *in silico*, aims to comprehensively evaluate the α-amylase inhibitory activity of oleanolic acid from *P. bipinnatifidus*. cultivated in Vietnam. By elucidating its mode of action and molecular interactions, this study contributes to the exploration of natural compounds for diabetes control. Furthermore, these findings may provide insights for future drug discovery efforts targeting α-amylase inhibition and pave the way for the development of novel therapeutic agents sourced from plants.

2. Materials and Methods

2.1. Chemicals and Reagents

The Acarbose, α-amylase from porcine pancreas (Type VI-B, >= 5 units/mg solid), starch, and iodine reagents were purchased from Sigma-Aldrich (Merck Vietnam Ltd.). The OA with a purity of 99% was provided by Associate Professor Dr. Nguyen Huu Tung from the Faculty of Pharmacy, Phenikaa University, Hanoi, Vietnam [6].

2.2. α-Amylase Inhibitory Assay

The α-amylase inhibitory activity of saponins, including OA from *P. bipinnatifidus* and acarbose, was evaluated using a 96-well Microtiter™ microplate assay at various concentrations (Acarbose: 5, 10, 20, 30, and 50 µg/mL; OA: 50, 100, 150, 200, and 250 µg/mL). The assay utilized a modified starch-iodine colorimetric method to assess α-amylase inhibition.

In the assay, 50 µL of phosphate buffer at pH ~ 7.4 (Na_2PO_4/NaH_2PO_4, 0.2 M) was combined with 10 µL of α-amylase solution in phosphate buffer (0.5 unit/mL). The mixture was incubated in an IN30 incubator (Memmert GmbH + Co. KG, Schwabach, Germany) for 15 minutes at 37 °C. Subsequently, 10 µL of 1% (w/v) starch solution was added, and the

incubation continued for an additional 15 minutes at 37 °C. The reaction was stopped by adding 40 µL of 1 M HCl. The presence of starch was visualized with 100 µL of iodine reagent (5 mM I_2 and 5 mM KI). Absorbance was measured at 620 nm using a HiPo MPP-96 Microplate Photometer-Biosan (Riga, latvia).

The control reaction, representing 100% enzyme activity, did not contain any saponins from *P. bipinnatifidus*. To account for the absorbance of saponins alone, a control reaction without enzyme was performed. Inhibition of enzyme activity was calculated as follows: % inhibition = $(1 - A_{Sample}/A_{Control}) \times 100\%$. The IC_{50} values, defined as the concentration of saponins that inhibited 50% of α-amylase activity, were determined by plotting α-amylase inhibition (%) against sample concentration (µg/mL).

2.3. Preparation of Receptor

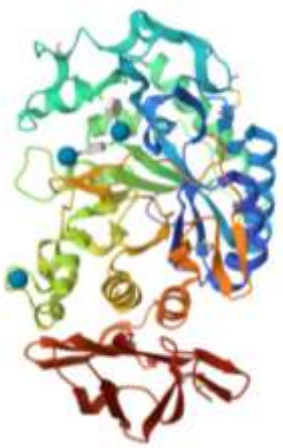

Figure 1. The 3D structure of the enzyme 1OSE was downloaded from the Protein Data Bank.

The 3D structure of the enzyme α-amylase, with PDB ID: 1OSE, was obtained from the Protein Data Bank (http://www.rcsb.org/pdb) (Figure 1). This structure was chosen based on its frequent use in ligand-protein interaction research and its well-documented relevance. The downloaded structure was processed and optimized using BIOVIA Discovery Studio Visualizer 2024. Initially, all water molecules, unwanted ligands, and non-protein atoms such as metal ions were removed to ensure that only the protein structure was used in subsequent docking studies. This step helps eliminate any potential interference during ligand-protein interaction analysis. Polar hydrogen atoms were then added to facilitate hydrogen bond formation, a critical component of ligand binding stability. Missing residues within the protein structure were repaired, and the entire structure was optimized to enhance its stability and ensure accurate docking results. The binding site of the protein was identified using AutoDock 1.5.7. The Lamarckian Genetic Algorithm (LGA) wasemployed to guide the docking process by optimizing binding parameters and ensuring accurate prediction of ligand-receptor interactions [7].

2.4. Ligand Preparation

The 3D structure of oleanolic acid, a known α-amylase inhibitor, was retrieved from the PubChem database (http://pubchem.ncbi.nlm.nih.gov/) in .sdf format. This compound was chosen for its potential to bind to the enzyme's active site, thereby inhibiting its activity. The structure underwent energy minimization and torsional angle adjustment to optimize its conformation for molecular docking. Subsequently, the optimized structure was converted to .pdbqt format using AutoDock Tools, which included critical information about the ligand's torsional degrees of freedom and atomic charges. This format is essential for accurate docking simulations and ensures the ligand remains in a biologically relevant conformation during the process. Acarbose, a standard α-amylase inhibitor with a well-established mechanism of competitive inhibition, was used as a control ligand in the study. This allowed for a comparative evaluation of the inhibitory efficacy of oleanolic acid against a benchmark compound. By thoroughly preparing the ligands, the study aimed to maximize the reliability of molecular docking results and provide a solid foundation for evaluating ligand-enzyme interactions.

2.5. Identification of Active Sites

The active site of the α-amylase enzyme is the primary region where ligand binding occurs, influencing the enzyme's activity. To accurately identify these sites, PrankWeb, an advanced machine learning-based tool, was employed. PrankWeb predicts potential active sites by analyzing the protein's surface geometry and physicochemical properties, such as depth, size, and the nature of surrounding amino acid residues. The tool's predictions are informed by a large database of known protein structures and binding sites. Using the 3D structure of α-amylase (PDB ID: 1OSE), PrankWeb identified potential binding pockets, which were further validated through blind docking. In blind docking, a grid box is extended to cover the entire protein surface, allowing the ligand to explore all possible binding sites without predefined constraints. This method complements PrankWeb's predictions and ensures no significant binding pockets are overlooked. The identified active sites were analyzed for their structural features and amino acid composition, providing insights into potential interaction mechanisms with ligands.

2.6. Molecular Docking

Molecular docking simulations were performed to study the interaction of oleanolic acid and acarbose with the α-amylase enzyme (1OSE). The docking studies employed AutoDock software, with the Lamarckian Genetic Algorithm (LGA) utilized for optimizing binding parameters. This method combines genetic algorithms with heuristic rules to identify the most stable binding conformations of ligands on the protein surface. Binding energy, measured in kcal/mol, was a key parameter for evaluating the stability of the ligand-receptor complexes [8]. Lower binding energy values indicate more stable complexes and stronger interactions. Additionally, the number and quality of hydrogen bonds formed between the ligands and the amino acid residues in the active site were analyzed. These bonds play a crucial role in stabilizing the ligand-protein complex and contribute to the inhibitory potential of the ligands. By comparing the binding energy and hydrogen bond interactions of oleanolic acid and acarbose, the study assessed the relative efficacy of oleanolic acid as an inhibitor of α-amylase.

2.7. Analysis Method for Docking Results

After completing the docking simulations, the results were meticulously analyzed to identify the best interaction models. The primary criteria for selecting optimal models were the binding energy and the number of hydrogen bonds formed. Models with the lowest binding energy values were prioritized, as these indicate stronger and more stable ligand-receptor interactions. The interactions were visualized using BIOVIA Discovery Studio Visualizer 2024, which provided detailed 3D and 2D diagrams of the binding interactions. These visualizations highlighted key interactions such as hydrogen bonds, electrostatic forces, and van der Waals interactions. The generated diagrams facilitated a clear understanding of the interaction mechanisms and were used for both analysis and scientific reporting. By benchmarking the results of oleanolic acid against those of acarbose, the study evaluated the inhibitory potential and bioactivity of oleanolic acid in a comparative context.

3. Results and Discussion

3.1. In vitro α-Amylase Inhibitory Activity

As shown in Table 2, the α-amylase inhibitory activity of the OA was showed and compared to the standard acarbose. While the inhibitory activity at 50 µg/mL of OA represented 18.04% with IC_{50} = 198.73 µg/mL, the inhibitory activity at 50 µg/mL of Acarbose showed 61.94% at with IC_{50} = 22.48 µg/mL.

Acarbose is an inhibitor of the enzyme α-amylase has been widely used in the treatment of type 2 diabetes. In this study, we

used acarbose as a positive control for its α-amylase inhibitory activity, with an IC_{50} value of 22.48 µg/mL. This result is consistent with previous studies. In prior research, the enzyme inhibition effect of acarbose was reported to be 15.47 µg/mL [9], 83.33 µg/mL [10], and 14.1 µg/mL [11].

The results of OA extracted from *P. bipinnatifidus* showed approximately 8.84 times lower inhibitory activity compared to acarbose. However, with an IC_{50} value of 198.73 µg/mL, it still represents a promising result and holds significant potential for application.

Table 1. The α-amylase inhibitory activities of Olenolic acid from *P. bipinnatifidus* Seem

Sample No.	Acarbose		OA	
	Conc. (µg/µL)	Inhibition (%)	Conc. (µg/µL)	Inhibition (%)
1	5	39.85 ± 0.19	50	18.04 ± 0.52
2	10	44.94 ± 0.48	100	29.36 ± 0.76
3	20	49.81 ± 0.92	150	38.06 ± 0.98
4	30	54.68 ± 0.84	200	52.76 ± 0.67
5	50	61.94 ± 0.63	250	59.74 ± 0.59
Linear regression equation		y = 0.472x + 39.387 R^2 = 0.9739	y = 0.2136x + 7.552 R^2 = 0.9911	
IC_{50}		22.48 ± 0.51	198.73 ± 2.35	

3.2. Identification of the Active Site of α-amylase

The best model with the lowest binding energy (kcal/mol^{-1}) and the highest number of hydrogen bonds was selected for further visualization. The binding interactions between the ligand and receptor were displayed using BIOVIA Discovery Studio Visualizer, providing advanced tools for analyzing molecular interactions, generating 2D and 3D plots, and creating high-quality images for publication purposes (Figures 3, 4).

The docking simulations for both oleanolic acid and acarbose were conducted using identical grid parameters, ensuring a consistent and reliable comparison between the two compounds. The grid parameters for α-amylase enzyme inhibition were set as follows: grid spacing at 0.375 Å, a grid box dimension of 90 × 90 × 90, and the grid center positioned at coordinates X = 37.575, Y = 33.065, and Z = 5.408. It is important to note that the grid box parameters were chosen to be identical for both ligands to maintain consistency in the docking process.

Key residues within the active site, such as TYR62, HIS101, and GLU233, were identified as particularly significant due to their high probability scores and established roles in the enzyme's catalytic mechanisms. TYR62 is likely involved in substrate binding and stabilization. HIS101 plays a crucial role in proton transfer, which is essential for the enzyme's catalytic activity, while GLU233 is important for stabilizing the transition state and participating in acid-base catalysis. Additionally, ASP197 contributes to transition state stabilization and proton transfer, whereas LEU162 is important for maintaining the structural integrity of the active site.

Identifying these key amino acid residues provides crucial insights for further comparative analyses, enabling a deeper understanding of the binding interactions and structural relationships between the modeled α-amylase and reference templates. These insights also facilitate the evaluation of potential inhibitors, such as oleanolic acid and acarbose,

in the context of their interaction with the enzyme's active site.

3.3. Molecular Docking

The study of α-amylase complexed with oleanolic acid (OA) was conducted to investigate binding interactions and potential inhibition mechanisms of OA. According to the data presented in Table 2, OA demonstrates strong binding affinity with α-amylase, exhibiting a notable binding energy of -8.33 kcal/mol^{-1}. The binding interactions are illustrated in the 2D and 3D interaction diagrams (Figure 3). The results demonstrate a range of non-covalent interactions contributing to the stability of oleanolic acid binding in the active site of α-amylase.

Table 2. Interaction and binding energies of ligand with α-amylase

Pubchem ID	Compound Name	Binding Energy kcal/mol^{-1}	Interaction	Amino Acid Residues
10494	Oleanolic acid	-8.33	Conventional Hydrogen Bond	VAL 163, ASP 197
			van Der Waal	TRP 58, HIS 101, LEU 165, ARG 195, GLU 233, ASP 356
			Carbon Hydrogen Bond	ASP 300
			Pi-Sigma	TRP 59
			Alkyl, Pi - Alkyl	TYR 62, LEU 162, ALA 198, HIS 299, HIS 305
41774	Acarbose	-2.90	Conventional Hydrogen Bond	GLU 233, ASP 300, GLY 304, HIS 305, GLY 306
			van der Waals	TRP 58, TYR 62, HIS 101, LEU 162, VAL 163, ALA 198, ILE 235, HIS 299, GLY 308, GLY 309
			Carbon Hydrogen Bond	ASP 197

Oleanolic acid forms conventional hydrogen bonds with ASP 197 and HIS 305, crucial for anchoring the ligand in the binding site and enhancing its binding affinity. Additionally, a hydrophobic interaction with VAL 163, albeit weaker, further stabilizes the ligand in the enzyme's active site. Hydrophobic interactions play a critical role in oleanolic acid binding, with alkyl interactions involving TYR 62, ALA 198, HIS 299, and LEU 165 contributing to maintaining a hydrophobic environment around the ligand, crucial for stable binding. Moreover, pi-alkyl interactions with TYR 62 and TRP 59 facilitate stacking and stabilizing aromatic systems within the binding pocket, essential for proper orientation and binding of the ligand. Pi interactions, specifically pi-sigma interactions, are observed with LEU 162 and TRP 58, influencing the ligand's electronic properties and further enhancing its binding affinity to α-amylase. Several residues, including ARG 195, ASP 197, ASP 300, ASP 356, GLU 233, and HIS 101, participate in van der Waals interactions with OA. These non-covalent interactions contribute to overall stability by providing an additional surface area for ligand binding in the enzyme's active site.

Conversely, acarbose, a commonly used control substance, exhibited lower binding

energy, achieving only -2.90 kcal/mol^{-1} (Table 2) when interacting with α-amylase (Figure 4). The conventional hydrogen bonds between acarbose and amino acid residues such as GLU 233, ASP 300, GLY 304, HIS 305, and GLY 306 contribute to the stability of the ligand within the active site. Simultaneously, van der Waals interactions with several amino acid residues, including TRP 58, TYR 62, HIS 101, LEU 162, VAL 163, ALA 198, ILE 235, HIS 299, GLY 308, and GLY 309, play a critical role in stabilizing the ligand-enzyme complex.

When comparing the results from this study to those of Okechukwu et al., [12], similar binding interactions were noted for acarbose with α-amylase. In their analysis, acarbose exhibited a binding energy of -8.8 kcal/mol with significant contributions from hydrogen bonds to residues such as TRP 59, GLN 63, and ASP 300. These findings reinforce the importance of specific hydrogen bond interactions and van der Waals forces in ligand stabilization. The slight variation in binding energy may arise from methodological differences, including docking algorithms and structural optimizations employed.

This comparison underscores the reliability of our molecular docking results and highlights the critical role of van der Waals and hydrogen bonding interactions in stabilizing the acarbose-α-amylase complex. The findings also align with prior studies, affirming the validity of the docking model and its potential for predicting enzyme-inhibitor interactions.

In comparison, oleanolic acid (OA) exhibited a superior binding affinity with α-amylase, driven by a diverse array of non-covalent interactions, including hydrogen bonds, hydrophobic interactions, and pi interactions. This suggests the potential of OA as a potent inhibitor against the α-amylase enzyme, with the capability to effectively compete with acarbose in inhibiting this enzyme's activity.

However, a comparison between the *in vitro* and *in silico* results for α-amylase inhibition by both acarbose and OA reveals a contradiction between these two outcomes. In the in vitro assay, the α-amylase inhibitory activity of OA at a concentration of 50 µg/mL was only 18.04%, with an IC_{50} of 198.73 µg/mL, whereas acarbose achieved a 61.94% inhibitory activity with an IC_{50} of merely 22.48 µg/mL. This indicates that despite OA's strong binding affinity observed in the *in silico* study, its actual inhibitory efficacy is lower compared to acarbose.

This discrepancy can be explained by several factors. *In silico* simulations only model molecular interactions at a theoretical level, based on the 3D structure of the enzyme and ligand, but cannot fully account for kinetic factors, biological conditions, and the real-world environment within a living organism. In vitro experiments, on the other hand, are conducted under more realistic conditions, where factors such as solubility, chemical stability, and interactions with other components in the test sample can significantly impact the enzyme inhibition efficacy.

Additionally, the molecular structure and size of OA may affect its ability to access and bind to the active site of the enzyme under in vitro conditions, leading to reduced efficacy compared to the *in silico* predictions. Factors such as hydrophobicity, solubility, and penetration into the aqueous environment may limit OA's ability to fully express its enzyme inhibition potential as predicted *in silico*.

Finally, differences in the accuracy of *in vitro* and *in silico* measurement methods may also contribute to the divergent results. *In silico* focuses on theoretical interactions at the molecular level, while in vitro assesses the overall inhibitory capacity of a compound, including factors related to the actual biological environment. This underscores the importance of utilizing both methodologies to obtain a more comprehensive understanding of the efficacy of bioactive compounds.

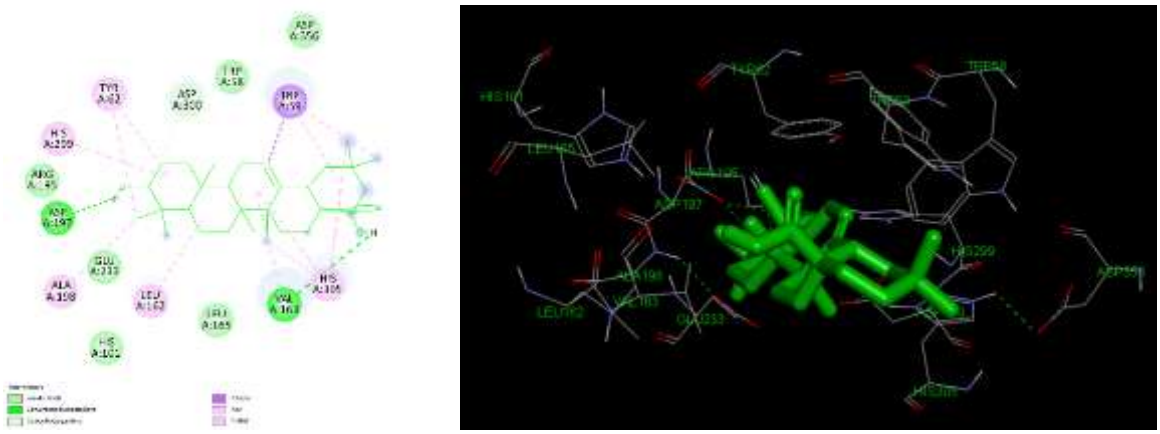

Figure 3. 2D and 3D interaction between OA and α-amylase at equilibrium state.

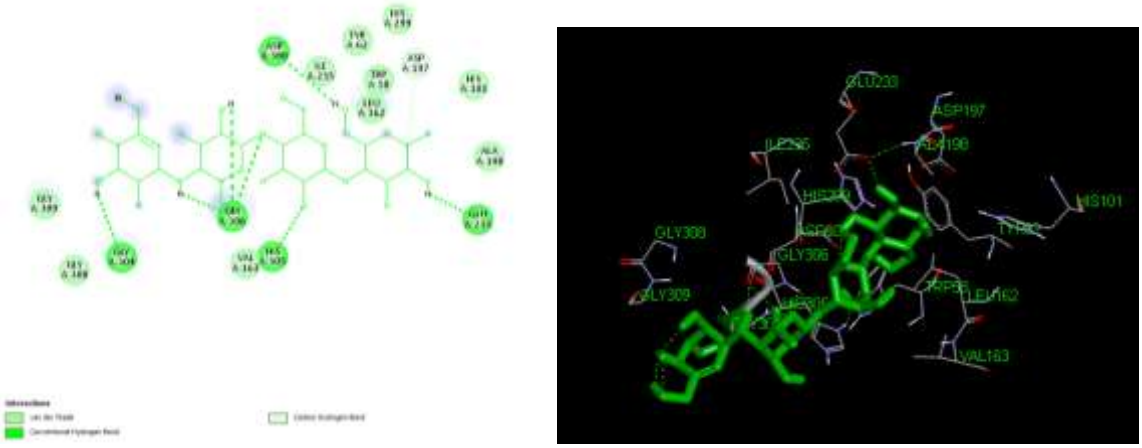

Figure 4. 2D and 3D interaction between Acarbose and α – amylase at equilibrium state.

4. Conclusion

This study provides a detailed examination of the α-amylase inhibitory effects of oleanolic acid (OA) derived from *Panax bipinnatifidus* Seem., utilizing both *in vitro* and *in silico* approaches to elucidate its interaction with the enzyme. Molecular docking results indicate that OA forms stable complexes with α-amylase, with significant binding affinity demonstrated by a binding energy of -8.33 kcal/mol^{-1}. In vitro assays further confirmed the inhibitory activity of OA, although the IC_{50} value of 198.73 µg/mL suggests that it is less effective than acarbose, which has an IC_{50} of 22.48 µg/mL. Despite this, the natural origin of OA and its diverse pharmacological properties make it a promising candidate for further research as a complementary treatment for diabetes and other metabolic disorders.

To enhance the inhibitory activity of OA in the future, specific functional group modifications could be considered. Adding polar groups, such as hydroxyl or carboxyl, may strengthen hydrogen bonding with the active sites of α-amylase, thereby increasing binding affinity. Additionally, modifying or extending alkyl side chains could improve hydrophobic interactions within the binding pocket, stabilizing OA's binding. Replacing

certain methyl groups with methoxy or other electron-donating groups may further optimize electronic interactions, potentially boosting OA's inhibitory effectiveness.

The integration of OA into therapeutic strategies could offer a natural alternative for managing postprandial hyperglycemia, contributing to the broader effort of developing plant-based therapies originating from Vietnam for metabolic diseases.

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