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Novel Anti-Cancer Agents: Potential of Protease Enzymes from *Vibrio alginolyticus* as a Breast Cancer Therapy for MCF-7 Cells

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Abstract

This study explores the potential of *Vibrio alginolyticus* protease enzyme as a cytotoxic agent against MCF-7 breast cancer cells, using molecular docking and cytotoxicity assays. The docking analysis revealed that the native ligand interacts strongly with key residues at the active site of the target protein, including hydrogen bonds with residues ARG394 (2.034 Å) and GLU353 (1.920 Å), as well as hydrophobic interactions with residues PHE404, LEU391, MET421, and ILE424, with distances ranging from 4.664 Å to 5.355 Å. These interactions demonstrate a high affinity between the ligand and the target protein, indicating potential for enzyme-based cancer therapy development. Cytotoxicity testing using the resazurin assay showed that *V. alginolyticus* protease enzyme exhibited an IC_{50} value of 1.63 µg/mL, indicating significant cytotoxic effects on MCF-7 cells. At concentrations above 2.5 µg/mL, the enzyme demonstrated strong cytotoxic effects, with morphological changes in the cells, such as shrinkage and detachment. In contrast, at lower concentrations, the cytotoxic activity was limited. The observed decrease in cell viability at high concentrations suggests that the enzyme works by disrupting key proteins or pathways involved in cancer cell proliferation and survival. Both *in silico* and *in vitro* analyses support the possibility that *V. alginolyticus* protease targets essential proteins involved in apoptosis regulation and structural protein degradation in cancer cells. These findings provide new insights into the development of enzyme-based cancer therapies, warranting further research to understand the specific mechanisms behind cancer cell death induction.

Keywords: *Vibrio alginolyticus*, Protease, Cytotoxicity, MCF-7 cell, Molecular docking

Introduction

Cancer is a disease that occurs when cells in the body grow uncontrollably [1]. Normally, cells in the body have a well-regulated life cycle: they grow, divide to replace cells that are dead or damaged, and then die through a process called apoptosis (programmed cell death) [2]. This cycle is controlled by genes and complex biological mechanisms. However, in cancer, this regulatory mechanism becomes disrupted [3,4]. Mutations or genetic changes in the DNA of cells cause them to lose control over their growth and division. As a result, the cells continue to divide endlessly, even when the body does not need them. Furthermore, cancer cells fail to undergo apoptosis as they should, allowing them to survive and multiply indefinitely. [5]. There are many types of cancer, depending on the type of cell affected and where these cells are located in the body [6]. Cancer can be treated with various methods, including surgery, chemotherapy, radiation therapy, and targeted therapy, depending on the type and stage of the cancer [7-10]. One type of cancer that continues to

attract significant research attention is breast cancer. Breast cancer occurs when cells in the breast tissue start to grow and divide uncontrollably, forming a mass known as a tumor. This tumor can be benign (not spreading to other parts of the body) or malignant (cancerous, which can spread to other parts of the body) [1,11,12].

Despite significant advancements in diagnosis and treatment, various limitations must be addressed. Breast cancer treatment often causes significant side effects, such as weakness, fatigue, and other functional impairments [13,14]. The MCF-7 cell line is a breast cancer cell line derived from a female patient diagnosed with adenocarcinoma of the breast in 1970 [15,16]. This cell line is well-known for its ability to respond to estrogen, thanks to the presence of estrogen receptors on its surface, making it a highly valuable model for research on hormone-responsive breast cancer [16]. The MCF-7 cell line has been a mainstay in breast cancer research for over 40 years, providing consistent and reliable data. The success of this cell line is also supported by its ease of access and cultivation in laboratories, which facilitates researchers in developing new therapies and studying breast cancer biology. Additionally, the genetic variability in MCF-7 cells allows for in-depth exploration of the pathogenesis mechanisms of breast cancer and the development of more specific therapies. The widespread use of MCF-7 cells in research has helped scientists understand the progression of breast cancer, the response to various treatments, and the mechanisms of treatment resistance.

Despite the extensive use of MCF-7 cells in breast cancer research, there remains a significant gap in exploring novel therapeutic targets, particularly involving proteases. Proteases, enzymes that break down proteins Natsir *et al.* [17], Pratiwi *et al.* [18], play a crucial role in various physiological and pathological processes, including cancer progression and metastasis. However, studies investigating the potential of proteases in inhibiting the growth and proliferation of MCF-7 breast cancer cells are still limited and underexplored. In this research, we aim to address this gap by evaluating the potential of specific proteases as inhibitors of MCF-7 cell growth, providing a new perspective on therapeutic strategies for hormone-responsive breast cancer. This study aims to uncover unique mechanisms through which proteases can suppress tumor progression, as demonstrated through *in silico* and *in vitro* testing, while also providing a foundation for developing more targeted therapies that can complement existing treatments and minimize adverse side effects.

Although *in silico* approaches, such as molecular modeling and molecular docking, provide important preliminary insights into the potential interactions between ligands and target proteins, several limitations should be considered**.** The validity of *in silico* results heavily depends on the quality of the protein structures used, docking parameters, and the simplified representation of the biological environment**.** Moreover, computationally predicted interactions do not always fully reflect the dynamics and complexity of real biological systems, including ligand stability in plasma, bioavailability, metabolism, and potential synergistic or antagonistic effects with other biomolecules within cells**.** Therefore, the in silico findings in this study need to be further validated through *in vitro* and *in silico* assays to ensure the biological efficacy and safety of the identified candidate compounds**.**

Materials and methods

In silico methods

Protein modelling, prediction, and structural analysis

Protein modeling follows the steps on the SWISS-MODEL server [19]. The printing stages are: determination of target protein sequence, identification of protein template, model creation, and evaluation model. The template protein is identified on the server swissmodel.expasy.org/interaktif. The next data, DNA obtained from the sequencing results, then translated into amino acid sequences with the help of BioEdit [20,21]. Then, target proteins in their FASTA format are sent to the SWISSMODEL server. The template identification process will produce several protein templates with their parameters [22]. The protein template is selected according to the parameter identity. Protein model creation is done by selecting the template protein generated on the server swissmodel.expasy.org/interactive/RGPEKv/templateS/. The choice of one template protein generally produces one or more models.

Preparation of receptor and ligand structures

The initial step for docking simulations is identifying the receptor and ligand to be tested. The receptor used in this study is the target protein from the MCF-7 breast cancer cell line, with the PDB code 5U2B, available on the server (https://www.rcsb.org/structure/5U2B). This receptor structure is then prepared using Biovia Discovery Studio 2021. The ligand structure can be obtained by searching the available databases on the PubChem server (https://www.ncbi.nlm.nih.gov/pccompound) [23-25].

Docking simulation and visualization of results

To begin the docking simulation, first, open the PyRx application and select the Vina Wizard mode as the main interface for the docking process. The next step is to upload the target protein and ligand files by clicking the "Load Molecules" button. The protein file should be in .pdb format; the same process applies to uploading the ligand file. Once the files are uploaded, PyRx automatically converts both the protein and ligand files into PDBQT format, the standard format AutoDock Vina requires for docking simulations [26,27].

After the files are prepared, the next task is to define the grid box, which will serve as the search area around the protein's active site where the ligand will bind. The grid box can be configured through the "Grid" tab, where you can adjust the size and position of the grid box based on the active site information obtained from literature or the position of the native ligand within the PDB structure. The grid box dimensions (X, Y, Z) should be set to cover the target area to ensure optimally accurate docking results.

Once the grid box is set correctly, choose the target protein and ligand from the available molecule list, then click "Start" to initiate the docking simulation using AutoDock Vina. After the simulation, PyRx will display the binding affinity scores for each ligand pose within the protein's active site. These scores are generally reported in energy units (kcal/mol), where more negative values indicate stronger and more stable interactions between the ligand and the protein.

The final step is to save the docking results. The best pose from the simulation can be saved in PDBQT format and exported in PDB format for further analysis using other software like BIOVIA Discovery Studio. This software can visualize and understand the molecular interactions between the ligand and the target protein in more detail.

The analysis using SwissADME helps evaluate the pharmacochemical properties of a compound to assess its potential as a drug candidate. The analyzed parameters include physicochemical properties, lipophilicity, solubility, pharmacokinetics, drug-likeness, and medicinal chemistry aspects. Physicochemical properties provide information about molecular size, polarity, and possible interactions within biological systems. Lipophilicity and solubility determine how a compound is distributed in the body, where being either too hydrophobic or too hydrophilic can affect its absorption and effectiveness.

Cytotoxicity test on MCF-7 Cells (*in vitro*)

Workflow

The process begins with culturing cells in a 96-well plate, then incubating at 37 °C with 5% CO_2 until cell growth reaches at least 70% confluency. Once this condition is achieved, the cells are treated with specific samples and re-incubated for 48 h at the same temperature and CO_2 concentration. Subsequently, the working reagent of resazurin is added to each well to initiate the reaction. The final step involves measuring the absorbance using a multimode reader to analyze the reaction results.

Media/positive control/sample preparation

Preparation starts with preparing liquid culture media using Roswell Park Memorial Institute Medium (RPMI) complete, which contains 10% Fetal Bovine Serum (FBS) and 50 µL of antibiotics per 50 mL of medium. Next, a positive control (Cisplatin) is prepared for use in this assay. The sample is diluted to a specific final concentration as a stock solution, ensuring the solvent is non-toxic to the cells. The antiproliferation assay working solution is also prepared using Resazurin Sodium Salt-Powder, BioReagent.

Cell preparation

Cell preparation begins by ensuring that the cells used have reached a minimum confluency of 70%. The medium on the dish is discarded, and the cells are rinsed twice with 1 mL of PBS to remove any remaining medium. Then, 1 mL of Trypsin-EDTA solution is added to the dish, followed by incubation for 5 min to

detach the cell layer. Cell dispersion is confirmed using an inverted microscope, where the cells appear floating. The dispersed cells are then transferred into a tube containing media and centrifuged at 3000 rpm for 5 min. After centrifugation, the supernatant is discarded, and the cell pellet is re-suspended in a tube containing media.

Seeding cells into a 96-well plate

The process starts with determining the cell count and viability using the trypan blue exclusion method. The cell suspension is resuspended in media to reach a final density of 170,000 cells/mL (targeting 17,000 cells per well). For cell counting, 10 µL of trypan blue solution is prepared in a sterile microtube, mixed with 10 µL of cell suspension, and homogenized. The hemacytometer and its coverslip are cleaned with 70% ethanol and dried. Using a pipette, 10 µL of the cell-trypan blue mixture is gently introduced into one side of the hemacytometer chamber. The number of viable (healthy) cells is then counted to determine the total cell count per mL. After determining the cell count, the seeding process is performed by culturing the cells in a 96-well plate. The seeded plate is incubated for 24 h until the cells reach a minimum confluency of 70%, at 37 °C with 5% CO_2.

Cell treatment with samples/positive control/negative control

The treatment begins by preparing 8 microtubes (1.5 mL) labeled according to the target dilution concentrations. The stock sample is diluted using media to produce 8 concentration variants. Next, the 96-well plate containing cells is removed from the incubator. The plate is labeled along the left margin to mark rows receiving the standard treatment and rows receiving the sample treatment. The media in each well is then discarded. Using a micropipette, 100 µL of each sample and positive control is taken from the microtubes and added to the corresponding wells on the 96-well plate containing cells. Once all treatments are completed, the plate is re-incubated for 48 h at 37 °C with 5% CO_2.

Adding resazurin reagent and measuring absorbance

The first step involves discarding the media from each well on the 96-well plate. A working solution is then prepared by mixing 9 mL of media with 1 mL of Resazurin Sodium Salt-Powder, BioReagent (using a ratio of 10 µL reagent to 90 µL media). 100 µL of this solution is added to each well in the microplate. The plate is then incubated for 1 – 2 h until a color change is observed. This process leverages the properties of the Resazurin reagent, which is reduced inside live cells from the blue resazurin compound (with no intrinsic fluorescence) to the red resorufin compound, which is highly fluorescent. Therefore, this conversion is proportional to the number of metabolically active cells and can be quantitatively measured. The final step involves measuring the absorbance at 570 nm (with a reference wavelength of 600 nm as in the **Figure 1**) using a multimode reader to determine cell metabolic activity.

Results and discussion

In the study conducted by Natsir *et al.* [17], the bacterial species in the MBS-L3 isolate were discovered and identified as capable of expressing protease, with a species similarity identification percentage of 98.93% to *Vibrio alginolyticus* strain NBRC 15630, as visualized in **Figure 2**.

In this context, molecular docking is employed to evaluate potential interactions between the protease enzyme and molecular targets in MCF-7 cancer cells (breast cancer), the results of the *in-silico* analysis can be seen in **Figure 3**, and the results of the study can be seen in **Table 1**. Docking results of a target protein of Human Breast Cancer MCF-7 Cells, aiming to explore the protease's potential as a cancer therapy candidate, either through direct proteolytic mechanisms or as a model for inhibitor development. Thus, this research opens new opportunities for developing enzyme-based therapeutic agents or innovative anticancer drug designs.

The determination of the 3D structure of protein molecules from *V. alginolyticus* bacteria isolated from Losari Beach sediment (MBS-L3) holds significant relevance in understanding the bacteria's ability to produce protease enzymes and its potential in molecular docking studies against MCF-7 cancer cells. Identification supporting the bacteria's capability to express protease. Protease enzymes play a significant role in protein degradation and have broad applications, including anticancer therapy [17].

Determining the hypothetical 3D protein structure enables visualization of active sites and key residues involved in proteolytic activity, forming the basis for molecular interaction studies.

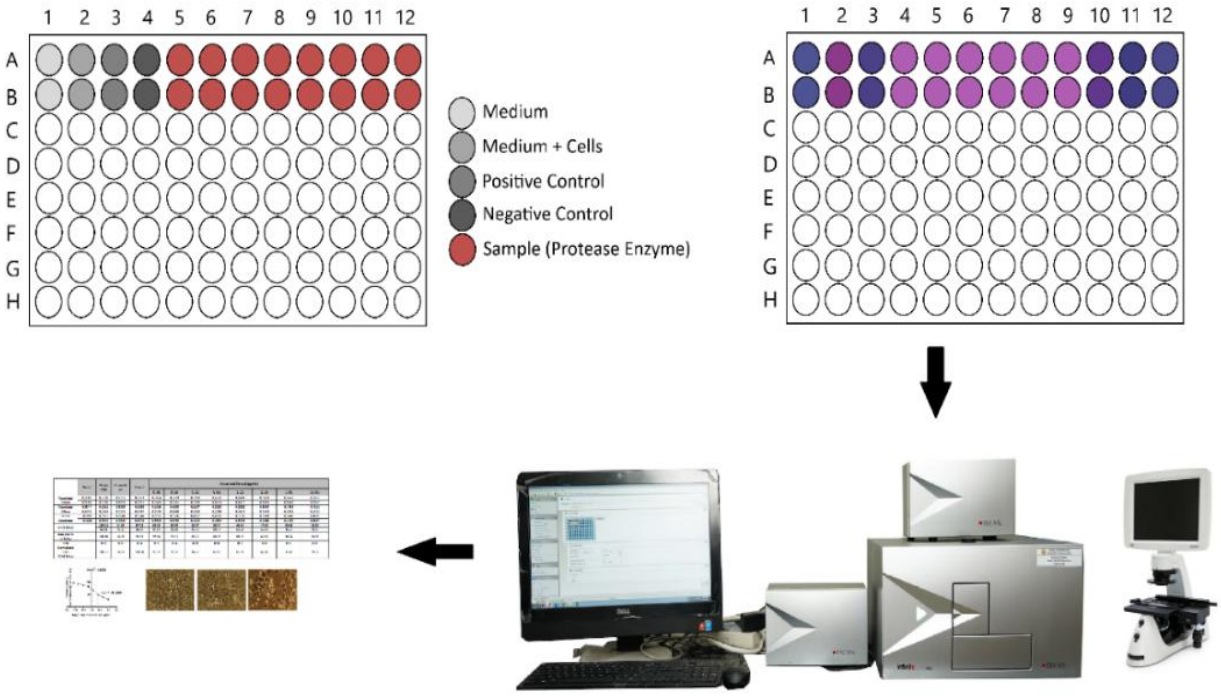

Figure 1 Plate illustration using a 96-Well Plate with Resazurin reagent measuring the absorbance at 570 nm (with a reference wavelength of 600 nm) using a multimode reader to determine cell metabolic activity.

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>GAD69877.1 hypothetical protein VAL01S_01_03020, partial [Vibrio alginolyticus NBRC 15630
= ATCC 17749]
TTITSLVATADPSVWTGDVVVPSTSELNVGLLVRDYQDLSGNTGSQNTAYSMPITPTIHLDVINDVTGVE
SVTVSGSSERFEDGEFIDIKVVDADGTEATGMATVLSDSWTTDLDLSGLKEGVVTIYVNGTNKLSASAEE
AQATFNYDRFASTASLGAVYNRYFSDNKTLKNAA
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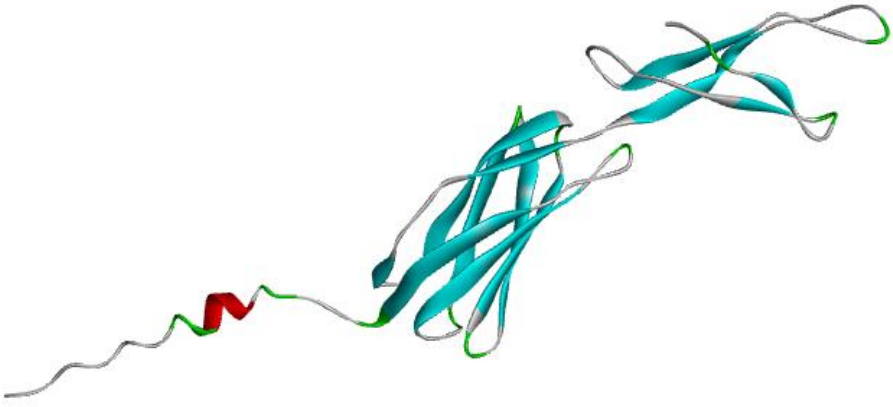

Figure 2 3D structure visualization of *Vibrio alginolyticu* s isolate MBS-L3 from Losari beach sediment.

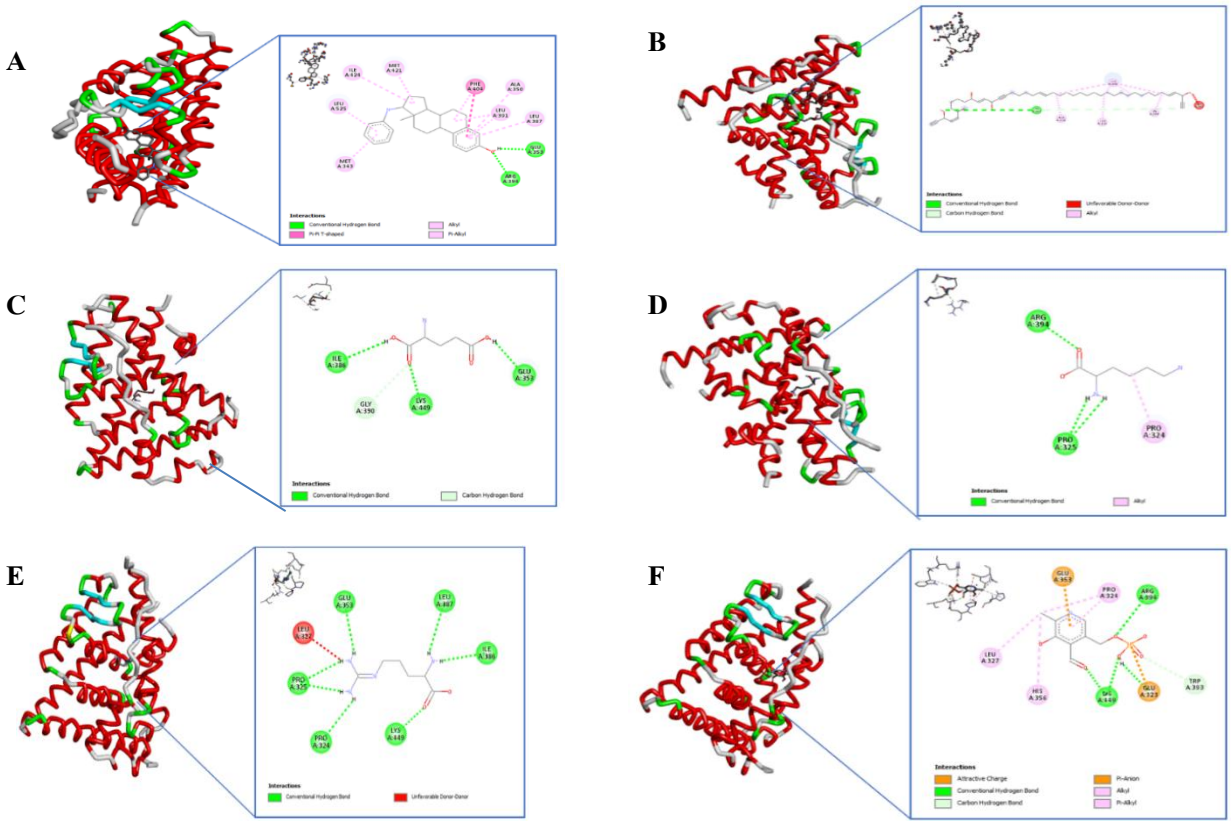

Figure 3 Docking Visualization of Breast Cancer MCF-7 Cell : A). (8~{r},9~{s},13~{s},14~{s},17~{s})-13-Methyl-17-Phenylazanyl-6,7,8,9,11,12,14,15,16,17-Decahydrocyclopenta[a]phenanthren-3-Ol (control +); compound resulting from enzyme hydrolysis with skim milk/casein as a substrate; B). Casein phosphopeptide/Tetratetraconta-4,17,21,27,40-pentaen-1,12,15,43-tetrayne-3,6,11,14,42-pentol; C). glutamic acid/(2S)-2-Aminopentanedioic acid; D). Lysine/(2S)-2,6-diaminohexanoic acid; E). Arginine/(S)-2-Amino-5-guanidinopentanoic acid; F). β-laktoglobulin/pyridoxal 5-phosphate.

Table 1 Docking results of target protein of Human Breast Cancer MCF-7 Cells with native ligand and suspected ligand from enzyme hydrolysis with casein and skim milk substrates.

Ligand	Amino acid residue	Bonding distance (Å)	Bonding type	Types of bond interactions
Native ligand (8~{R},9~{S},13~{S},14~{S},17~{S})-13-methyl-17-phenylazanyl-6,7,8,9,11,12,14,15,16,17-decahydrocyclopenta[a]phenanthren-3-ol)	**ARG394**	**2.034**	**Hydrogen Bond**	**Conventional Hydrogen Bond**
	GLU353	**1.920**	**Hydrogen Bond**	**Conventional Hydrogen Bond**
	PHE404	5.065	Hydrophobic	Pi-Pi T-shaped
	LEU391	5.355	Hydrophobic	Alkyl
	MET421	4.949	Hydrophobic	Alkyl
	ILE424	5.192	Hydrophobic	Alkyl
	PHE404	5.128	Hydrophobic	Pi-Alkyl
	ALA350	5.178	Hydrophobic	Pi-Alkyl
	LEU387	4.763	Hydrophobic	Pi-Alkyl
	LEU391	4.934	Hydrophobic	Pi-Alkyl
	MET343	4.664	Hydrophobic	Pi-Alkyl
	LEU525	5.139	Hydrophobic	Pi-Alkyl
	LYS362	2.256	Hydrogen Bond	Conventional Hydrogen Bond
	LYS362	3.105	Hydrogen Bond	Carbon Hydrogen Bond

Ligand	Amino acid residue	Bonding distance (Å)	Bonding type	Types of bond interactions
Casein phosphopeptide/Tetratetraconta-4,17,21,27,40-pentaen-1,12,15,43-tetrayne-3,6,11,14,42-pentol	LYS299	3.770	Hydrophobic	Alkyl
	LEU306	3.993	Hydrophobic	Alkyl
	LEU306	4.460	Hydrophobic	Alkyl
	LEU306	4.621	Hydrophobic	Alkyl
	LEU306	5.303	Hydrophobic	Alkyl
	LEU310	5.063	Hydrophobic	Alkyl
	ALA318	5.268	Hydrophobic	Alkyl
Glutamic acid/(2S)-2-Aminopentanedioic acid	LYS449	2.023	Hydrogen Bond	Conventional Hydrogen Bond
	GLU353	**2.064**	**Hydrogen Bond**	**Conventional Hydrogen Bond**
	ILE386	2.763	Hydrogen Bond	Conventional Hydrogen Bond
	GLY390	3.407	Hydrogen Bond	Carbon Hydrogen Bond
Lysine/(2S)-2,6-diaminohexanoic acid	**ARG394**	**2.261**	**Hydrogen Bond**	**Conventional Hydrogen Bond**
	PRO325	2.939	Hydrogen Bond	Conventional Hydrogen Bond
	PRO325	2.535	Hydrogen Bond	Conventional Hydrogen Bond
	PRO324	4.088	Hydrophobic	Alkyl
Arginine/(S)-2-Amino-5-guanidinopentanoic acid	LYS449	1.897	Hydrogen Bond	Conventional Hydrogen Bond
	LEU387	2.995	Hydrogen Bond	Conventional Hydrogen Bond
	ILE386	2.643	Hydrogen Bond	Conventional Hydrogen Bond
	PRO324	3.000	Hydrogen Bond	Conventional Hydrogen Bond
	PRO325	2.334	Hydrogen Bond	Conventional Hydrogen Bond
	PRO325	2.347	Hydrogen Bond	Conventional Hydrogen Bond
	GLU353	**2.657**	**Hydrogen Bond**	**Conventional Hydrogen Bond**
β-laktoglobulin/pyridoxal 5-phosphate	GLU323	3.436	Electrostatic	Attractive Charge
	ARG394	**2.241**	**Hydrogen Bond**	**Conventional Hydrogen Bond**
	LYS449	1.860	Hydrogen Bond	Conventional Hydrogen Bond
	LYS449	2.138	Hydrogen Bond	Conventional Hydrogen Bond
	GLU323	1.897	Hydrogen Bond	Conventional Hydrogen Bond
	TRP393	3.506	Hydrogen Bond	Carbon Hydrogen Bond
	GLU353	3.671	Electrostatic	Pi-Anion
	PRO324	4.809	Hydrophobic	Alkyl
	LEU327	4.042	Hydrophobic	Alkyl
	HIS356	4.649	Hydrophobic	Pi-Alkyl
	PRO324	4.047	Hydrophobic	Pi-Alkyl

The molecular docking analysis above reveals a close relationship between the native ligand and other ligands in terms of interactions with amino acid residues at the active site of the protein target. The native ligand, exhibits strong interactions with key residues at the active site through hydrogen and hydrophobic bonds. The hydrogen bonds occur at residues ARG394 and GLU353 with very short distances (2.034 Å and 1.920 Å), providing primary stability to the protein-ligand complex. Hydrophobic interactions involving residues like PHE404, LEU391, MET421, and ILE424 (within distances of 4.664 Å to 5.355 Å) enhance ligand binding. These hydrophobic interactions include Pi-Pi T-shaped, Alkyl, and Pi-Alkyl types, all contributing to the high affinity of the native ligand for the protein target.

On the other hand, ligands such as casein phosphopeptide, glutamic acid, lysine, arginine, and β-lactoglobulin can mimic or support the interactions demonstrated by the native ligand in various ways. Casein phosphopeptide forms hydrogen bonds with residue LYS362 at distances of 2.256 Å and 3.105 Å, accompanied by hydrophobic interactions at residues like LEU306, LEU310, and ALA318 (within distances of 3.770 Å to 5.303 Å), indicating its potential to mimic the stability of the native ligand through a combination of hydrogen and hydrophobic interactions. Glutamic acid exhibits interactions similar to the native ligand at residue GLU353 (hydrogen bond at 2.064 Å), as well as additional interactions at residues LYS449, ILE386, and GLY390, highlighting its role in creating complex stability differently while still supporting ligand binding at the active site. Lysine interacts with residue ARG394 through a hydrogen bond at 2.261 Å, also a crucial residue in the native ligand interaction.

Additionally, interactions with residues PRO324 and PRO325 (both hydrogen and hydrophobic) indicate lysine's potential to mimic the affinity of the native ligand. Arginine interacts extensively with key residues such as GLU353, LYS449, and LEU387, although its hydrophobic contribution is minor. Arginine has extensive hydrogen bonds, making it capable of creating a stable complex similar to the native ligand. β-lactoglobulin's interaction with residues like ARG394 and GLU353 directly correlates with the native ligand, especially in hydrogen bonding. In contrast, hydrophobic interactions at residues PRO324 and LEU327 provide additional stability that aligns with the native ligand mechanism.

This study has several similarities and differences compared to the research conducted by Abd-El-Aziz *et al.* [28], both studies demonstrate that hydrogen and hydrophobic interactions play a crucial role in the stability of the ligand-protein complex. In this study, the key residues involved in hydrogen bonding are ARG394 and GLU353, whereas in the previous study, the dominant residues are ASP657, GLN708, and CYS606. Additionally, hydrophobic interactions also contribute to enhancing the stability of the complex. In this study, Pi-Pi T-shaped, Alkyl, and Pi-Alkyl interactions with residues such as PHE404, LEU391, MET421, and ILE424 were found to contribute to ligand affinity, while in Noha's study, π interactions with PRO605 played a similar role. Regarding interaction distances, this study shows highly stable hydrogen bonding within a range of 1.920–2.261 Å, whereas Noha's study indicates that the optimal distance ranges from 2.8 – 4.3 Å, with values below 3.5 Å considered highly favorable for binding stability. Although there are similarities in the types of interactions, fundamental differences exist in the target protein and the type of ligands used. In this study, the investigated ligand interactions include casein phosphopeptide, glutamic acid, lysine, arginine, and β-lactoglobulin with a specific protein target. In contrast, Noha's study focuses more on inhibiting the Hexokinase 2 (HK-2) enzyme, which has implications for cancer therapy [28]. Furthermore, the therapeutic application of Vibrio alginolyticus protease must also consider the enzyme's structural and functional variability. As a naturally occurring marine bacterium, *V. alginolyticus* may exhibit strain-specific differences in protease expression, catalytic efficiency, and substrate specificity, which could affect reproducibility and consistency in clinical contexts. Variability due to environmental factors and horizontal gene transfer also poses a challenge for its standardization as a therapeutic enzyme. In addition, the translation of this protease to clinical or pharmaceutical applications requires addressing several practical challenges, including efficient isolation, purification, and stability of the enzyme. Large-scale production of protease may face obstacles such as low yield, cost-effectiveness, maintenance of enzymatic activity, and potential immunogenicity in humans. These challenges highlight the need for further optimization through genetic engineering, recombinant expression systems, or enzyme immobilization strategies to improve yield, scalability, and safety for therapeutic use.

Breast cancer cell line (MCF-7) cytotoxicity of protease enzymes from *V. alginolyticus*

To evaluate the cytotoxic potential of protease enzyme *V. algyniliticus*, an antiproliferative assay was conducted on MCF-7 breast cancer cells. The MCF-7 cell line was chosen as the model because it is one of the most commonly used cancer cell lines in research to assess the effectiveness of compounds or enzymes in inhibiting cancer cell growth. The assay used the resazurin method, allowing cell viability analysis based on color changes resulting from cellular metabolic

activity. The assay results provide insight into the relationship between enzyme concentration and the degree of cell viability inhibition, thereby determining the cytotoxic efficacy of the tested enzyme.

Protease Enzyme *V. algyniliticus* demonstrated cytotoxic activity against MCF-7 cells with an IC_{50} value of 1.63 µg/mL, indicating its strong potential to inhibit cell viability. The IC_{50} value represents the enzyme concentration required to reduce cell viability by 50%, with lower values reflecting more potent cytotoxic activity. At concentrations above 2.5 µg/mL, the enzyme exhibited significant cytotoxic effects, as evidenced by the predominance of blue color in the healthy plate, indicating metabolically inactive or dead cells. This suggests that high concentrations of protease enzyme *V. algyniliticus* cause substantial disruptions in cellular metabolic processes, likely through proteolytic effects on essential proteins in the membrane and within the cell. Conversely, at concentrations below 2.5 µg/mL, the enzyme's cytotoxic activity remained limited, as reflected by the predominance of pink in the well plate (**Figure 4**), indicating metabolically active cells. At these lower concentrations, the cells could maintain viability and metabolic function. These findings highlight a characteristic dose-response relationship, where increasing enzyme concentrations correlate with decreased cell viability, underscoring the potential of Protease Enzyme *V. algyniliticus* as an effective cytotoxic agent against MCF-7 cancer cells.

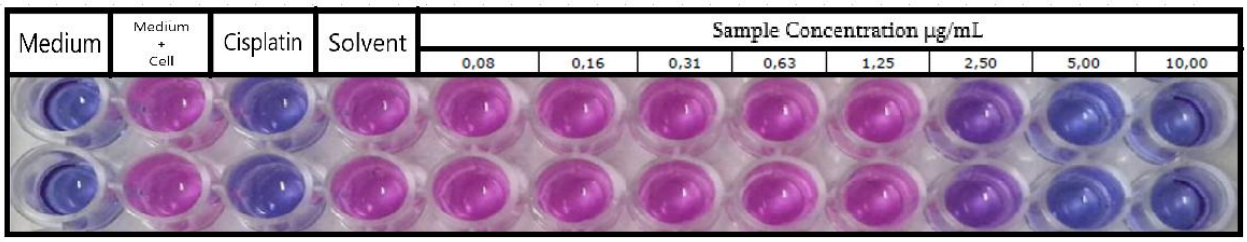

Figure 4 Well plate documentation of protease *V. algynoliticus* result on MCF-7 Cell after addiction reagent of resazurin.

Based on the morphological documentation of MCF-7 cells after treatment with protease from *V. algynoliticus*, it was observed that the cytotoxic effect exhibited a dose-dependent pattern. In the control group (medium + cells & 2% DMSO), MCF-7 breast cancer cells continued to grow well without any indication of apoptosis or significant cell death (**Figure 5**), indicating that the medium and solvent did not affect cell viability. Meanwhile, in the positive control (Doxorubicin), many cells underwent morphological changes such as shrinkage and detachment from the surface, indicating the strong cytotoxic effect of doxorubicin as a chemotherapeutic agent.

In the treatment with protease *V. algynoliticus*, cytotoxic effects were clearly observed at high concentrations (10 µg/mL and 5 µg/mL), where the number of surviving cells decreased significantly, with many cells undergoing detachment and more pronounced morphological changes. At moderate concentrations (2.50 and 1.25 µg/mL), signs of cytotoxicity began to appear; however, some cells remained viable with less noticeable morphological alterations. At low concentrations (≤ 0.63 µg/mL), the cells maintained their normal morphology, with an IC50 value of 1.63 µg/mL, classifying the cytotoxic effect of the protease enzyme as very strong against cancer cells

These results align with the *in silico* analysis, where molecular modeling showed that this protease enzyme disrupts key proteins or pathways in cancer cells that play a role in cell survival and proliferation. The interactions observed in the *in silico* study support the possibility that *V. algynoliticus* targets specific proteins involved in apoptosis regulation or structural protein degradation in cancer cells. The increasing cytotoxic effect with higher doses also confirms the predictions from the *in silico* analysis, which demonstrated the enzyme's affinity for specific target proteins crucial for maintaining cell viability. Therefore, the *in vitro* morphological findings are validated. The *in silico* results support the potential of *V. algynoliticus* protease as a therapeutic agent, requiring further research to understand its specific mechanism in inducing cancer cell death.

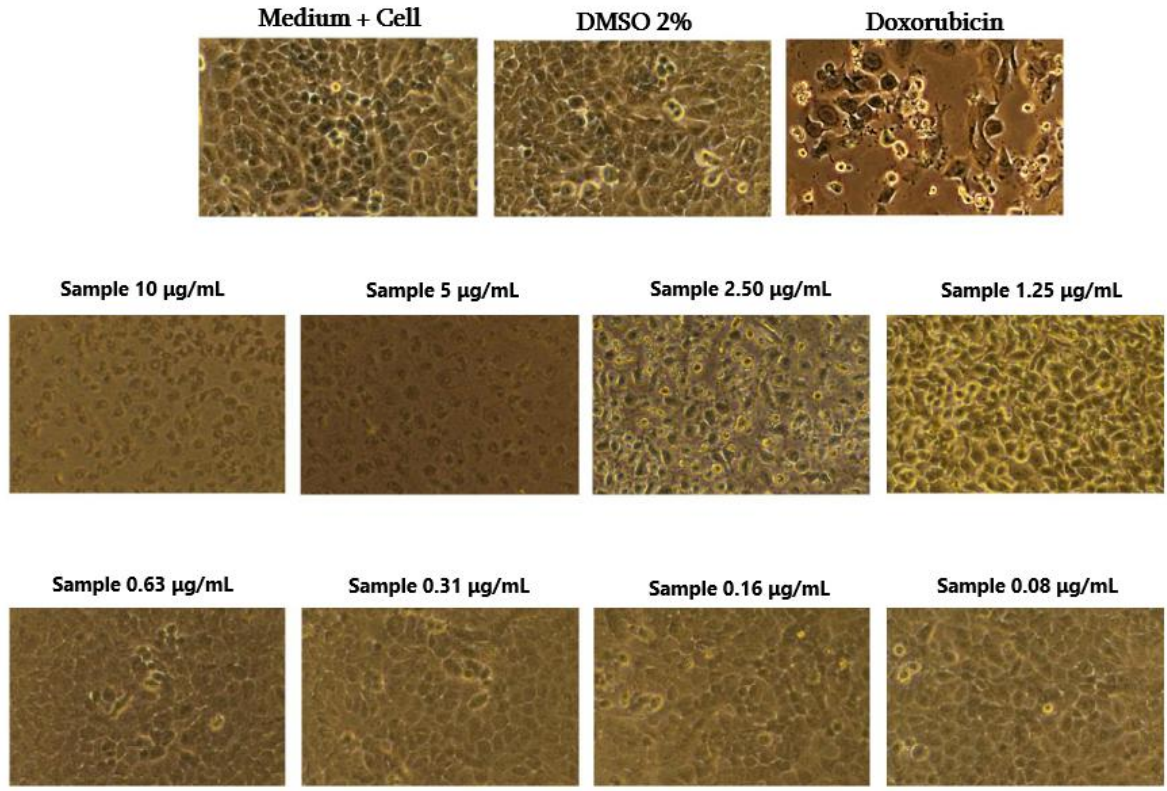

Figure 5 MCF-7 cells are damaged (apoptosis) after the addition of protease from *V. algynolyticus*.

Table 2 Results of SWISS-ADME analysis covering several parameters related to Physicochemical Properties, Lipophilicity, Water Solubility, Pharmacokinetics, Druglikeness and Medicinal Chemistry.

Parameter	Value	Native Ligand	Casein phosphopeptide	Glutamic acid	Lysine	Arginine	β-laktoglobulin
Physicochemical Properties	Formula	C24H29NO	C44H64O5	C5H9NO4	C6H14N2O2	C6H14N4O2	C8H10NO6P
	MW	347.49	672.98	147.13	146.19	174.2	247.14
	Heavy atoms	26	49	10	10	12	16
	Aromatic heavy atoms	12	0	0	0	0	6
	Fraction Csp3	0.5	0.59	0.6	0.83	0.67	0.25
	Rotatable bonds	2	28	4	5	5	4
	H-bond acceptors	1	5	5	4	4	7
	H-bond donors	2	5	3	3	4	3
	MR	108.69	209.71	32.4	38.14	44.54	53.65
	TPSA	32.26	101.15	100.62	89.34	127.72	126.76
Lipophilicity	iLOGP	3.5	8.96	0.4	0.97	0.27	−0.14
	XLOGP3	6.37	9.87	−3.69	−3.05	−4.19	−1.07
	WLOGP	5.54	8.36	−0.74	−0.47	−1.55	0.37
	MLOGP	4.87	5.61	−3.18	−2.67	−3.21	−1.91
	Silicos-IT Log P	4.52	12.98	−1.19	−0.72	−1.5	0.21
	Consensus Log P	4.96	9.15	−1.68	−1.19	−2.04	−0.51

Parameter	Value	Native Ligand	Casein phosphopeptide	Glutamic acid	Lysine	Arginine	β-laktoglobulin
Water Solubility	ESOL Log S	−6.22	−8.38	1.84	1.51	2.05	−0.71
	ESOL Solubility (mg/mL)	2.11E − 04	2.79E − 06	1.01E + 04	4.68E + 03	1.95E + 04	4.80E + 01
	ESOL Solubility (mol/L)	6.07E − 07	4.14E − 09	6.86E + 01	3.20E + 01	1.12E + 02	1.94E − 01
	ESOL Class	Poorly soluble	Poorly soluble	Highly soluble	Highly soluble	Highly soluble	Very soluble
	Ali Log S	−6.84	−11.92	2.16	1.74	2.11	−1.1
	Ali Solubility (mg/mL)	5.04E − 05	8.14E − 10	2.15E + 04	7.99E + 03	2.27E + 04	1.95E + 01
	Ali Solubility (mol/L)	1.45E − 07	1.21E − 12	1.46E + 02	5.47E + 01	1.30E + 02	7.89E − 02
	Ali Class	Poorly soluble	Insoluble	Highly soluble	Highly soluble	Highly soluble	Very soluble
	Silicos-IT LogSw	−6.86	−6.12	0.89	−0.18	0.47	−0.81
	Silicos-IT Solubility (mg/mL)	4.85E − 05	5.05 − E04	1.15E + 03	9.61E + 01	5.14E + 02	3.87E + 01
	Silicos-IT Solubility (mol/L)	1.40E − 07	7.50E − 07	7.83E + 00	6.58E − 01	2.95E + 00	1.57E − 01
	Silicos-IT class	Poorly soluble	Poorly soluble	Soluble	Soluble	Soluble	Soluble
Pharmacokinetics	GI absorption	High	Low	High	High	Low	High
	BBB permeant	Yes	No	No	No	No	No
	Pgp substrate	Yes	Yes	No	No	No	No
	CYP1A2 inhibitor	No	No	No	No	No	No
	CYP2C19 inhibitor	No	No	No	No	No	No
	CYP2C9 inhibitor	No	No	No	No	No	No
	CYP2D6 inhibitor	Yes	No	No	No	No	No
	CYP3A4 inhibitor	No	No	No	No	No	No
	log Kp (cm/s)	−3.9	−3.4	−9.82	−9.36	−10.34	−8.57
Druglikeness	Lipinski #violations	1	2	0	0	0	0
	Ghose #violations	0	4	4	3	1	0
	Veber #violations	0	1	0	0	0	0
	Egan #violations	0	1	0	0	0	0
	Muegge #violations	1	3	2	2	2	0
	Bioavailability Score	0.55	0.17	0.56	0.55	0.55	0.56
Medicinal Chemistry	PAINS #alerts	0	0	0	0	0	0
	Brenk #alerts	0	2	0	0	2	2
	Leadlikeness #violations	1	3	1	1	1	1
	Synthetic Accessibility	3.72	8.39	1.81	1.75	2.47	2.44

Conclusions

This study demonstrates the potential of *Vibrio alginolyticus* protease as a cytotoxic agent against MCF-7 breast cancer cells, supported by both *in silico* and *in vitro* analyses. Molecular docking results reveal strong interactions between the native ligand and key residues at the active site of the target protein, particularly hydrogen bonding with ARG394 (2.034 Å) and GLU353 (1.920 Å), as well as hydrophobic interactions with PHE404, LEU391, MET421, and ILE424 (4.664–5.355 Å). These findings suggest a high affinity of the protease for its molecular targets, highlighting its potential role in cancer therapy. Furthermore, cytotoxicity assays using the resazurin method confirm the enzyme's strong anticancer activity, with an IC50 value of 1.63 µg/mL. At concentrations above 2.5 µg/mL, significant morphological changes such as cell shrinkage and detachment indicate substantial cytotoxic effects. In contrast, lower concentrations exhibit minimal impact on cell viability. These results suggest that the enzyme exerts its cytotoxic effects by disrupting key proteins or pathways involved in cancer cell proliferation and survival. Overall, the combination of *in silico* and *in vitro* analyses supports the potential of *V. alginolyticus* protease as a promising candidate for enzyme-based cancer therapy. Further studies are required to elucidate its precise mechanisms in inducing cancer cell death and to explore its therapeutic applications.

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Declaration of Generative AI in Scientific Writing

The author hereby declares that ChatGPT (version 4) has been used during the writing of this article to assist in the correction of grammar. The grammar starting from Abstract, background, methods, discussion and conclusion has been compiled with the help of AI. Still, all content has been critically reviewed, verified for correctness, and revised by the author. The author acknowledges the limitations of current AI technology and has verified all data and information presented. The author asserts full responsibility for the content and views expressed in this work.

Credit Author Statement

Hasnah Natsir: Conceptualization; Methodology; Supervision.

Nur Hasni Hasan: Data analysis; SWISS-ADME interpretation including physicochemical properties, lipophilicity, water solubility, pharmacokinetics, druglikeness, and medicinal chemistry.

Nur Umriani Permatasari: Structural modeling interpretation of bacterial proteins.

Wahyudin Rauf: Molecular docking study; Docking results interpretation; ADME analysis; Method design; Visualization.

Anita: Bacterial isolation and rejuvenation; Preparation for MCF-7 cancer cell testing.

Paulina Taba: Initial data processing; Data visualization.

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