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Investigation of Chemical Compounds from Phomopsis Extract as Anti-Breast Cancer Using LC-MS/MS Analysis, Molecular Docking, and Molecular Dynamic Simulations

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Abstract. Since 2014, we have successfully isolated endophytic fungi from the leaves of Indonesian Annona muricata, exhibiting potential anti-breast cancer properties. The analysis of Internal Transcribed Spacer (ITS) showed the identified fungi species as *Phomopsis* sp. The ethyl acetate extract derived from *Phomopsis* sp. inhibited MCF7 cells (IC_{50} <20 ppm) and reduced the number and volume of nodules in *Sprague-Dawley* rats with breast cancer. However, molecular mechanism underlying the action of this extract in breast cancer treatment remains unclear. Therefore, this study aimed to identify the active compounds in *Phomopsis* extract and to predict anti-breast cancer mechanism through HER2 inhibition using MD and MDS. Using LC-MS/MS, 44 compounds were successfully identified, and 16 have the potential to be anti-cancer and obey Lipinski's rule. *In silico* studies were performed on the human epidermal growth factor receptor 2 (HER2). Subsequently, molecular docking results showed that the most negative affinity energy was 3-[(4-hydroxyphenyl)methyl]-octahydropyrrolo[1,2-a]pyrazine-1,4-dione (-9.4 kcal/mol), better than trastuzumab as a comparison ligand. Molecular dynamic simulations (MDS) of protein-ligand complexes showed prominent inhibition of HER2, as shown by dynamic trajectory analysis. Based on these results, 3-[(4-hydroxyphenyl)methyl]-octahydropyrrolo[1,2-a]pyrazine-1,4-dione was identified as a promising HER2 inhibitor for breast cancer.

Keywords: Breast cancer; Docking; HER2; Molecular dynamic; Phomopsis

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1. Introduction

Soursop leaves (*Annona muricata*) have been widely studied as medicinal plants that can be used as an alternative treatment for breast cancer (Dalal and Medithi, 2022). Using these leaves poses challenges as it demands precise harvesting timing and can negatively impact soursop fruit production, which is an important national agricultural commodity. Hence, the investigation of endophytic organisms in soursop leaves, known for their anticancer potential, becomes crucial. These symbiotic microorganisms, residing within host plants, can be isolated using plant growth media (Gouda *et al.*, 2016). Endophytic organisms are closely related to their hosts, resulting in the transfer of genetic material and the production of secondary metabolites that are the same as their hosts (Kusari, Singh, and Jayabaskaran, 2014). Previous studies have shown that endophytic organisms in soursop leaves with the best anticancer potential are fungi, and the best solvent is ethyl acetate. According to ITS analysis, the type of endophytic fungi found in Indonesian *Annona muricata* leaves with anti-breast cancer potential belongs to *Phomopsis* sp. category (Minarni *et al.*, 2017).

In vitro studies indicate that the ethyl acetate extract from *Phomopsis* sp. shows a cytotoxic impact on breast cancer cells. It effectively inhibits MCF-7 cell proliferation with an IC50 below 20 ppm, while demonstrating safety for normal cells (Minarni *et al.*, 2017). *In vivo* studies have also shown that *Phomopsis* extract at a dose of 20 mg/kgBW significantly decreased the number and volume of breast tumors in DMBA-induced *Sprague-Dawley* rats compared to the negative control group (Asyura *et al.*, 2017). Molecular mechanism by which this extract can improve breast cancer is not yet known.

Cancer occurs due to the abnormal proliferation of cells in the body, resulting in uncontrolled cell growth (Maman and Witz, 2018). To prevent the development of cancer cells, therapy is directed at inhibiting the receptors that play a role in inhibiting the cell cycle and cancer cell proliferation (Jazieh *et al.*, 2020; Mutebi *et al.*, 2020). Subsequently, several proteins play a role in breast cancer, including the human epidermal growth factor 2 receptor (HER2).

HER2 is a transmembrane receptor tyrosine kinase, part of the EGFR family, facilitating the rapid growth of breast cancer cells (Feng *et al.*, 2018). In normal cells, HER2 activation regulates processes such as proliferation, motility, and survival through cell signaling pathways. However, in breast cancer, overexpression of HER2 occurs predominantly through amplification of the HER2 gene. This overexpression is associated with a more aggressive phenotype and is also an important predictive biomarker of the response to HER2-targeted therapies, such as trastuzumab (Yu *et al.*, 2020). HER2 testing is recommended for all patients with invasive breast cancer to determine appropriate treatment options.

Before performing *in vivo* molecular analysis, it was necessary to determine the content of the ethyl acetate extract of *Phomopsis*. Considering the involvement of various proteins in breast cancer incidence, an initial in silico analysis is imperative to predict the influence of compounds in this extract on specific proteins. In this study, bioinformatics analysis was performed using molecular docking (MD) and molecular dynamic simulations (MDS). Subsequently, MD is a computational method used in molecular modeling to predict the preferred orientation of one molecule to another when a ligand and target are bound to each other to form a stable complex (Kaur *et al.*, 2019). Lipinski's Rule of Five was an important criterion in screening compounds before docking, serving as a rule of thumb to evaluate drug-likeness and assess the probability of chemical compounds becoming an oral drug in humans. Biologically active molecules must meet these five conditions for their potential use as oral drugs. These rules are related to molecular properties that are

important for drug pharmacokinetics in the human body, such as absorption, distribution, metabolism, and excretion (ADME) (Benet *et al.*, 2016).

MDS is a computer simulations method used in the theoretical study of biological molecules, such as proteins and nucleic acids, to analyze the physical movement of their constituent atoms and molecules over time. This method has been applied to thoroughly examine dynamic of biological molecules, their complexes, and their conformational changes by providing detailed information about their fluctuations and conformational changes (Badar *et al.* 2022). Furthermore, it is often used to study protein-ligand docking interactions in the search for new drug candidates. In MDS, atoms and molecules are allowed to interact for a fixed period, thereby providing a view of dynamic "evolution" of a system (Liu *et al.*, 2018). Simulations can be used to study various properties of a system, such as its thermodynamic, transport, and structural properties.

In silico studies on *Phomopsis* exist, but have not been associated with breast cancer. Several studies have investigated anticancer activity of *Phomopsis,* but they differ in observed proteins and *Phomopsis* sources, often derived from different plant endophytes with distinct active compounds. Therefore, this study aimed to identify the active compounds in *Phomopsis* ethyl acetate extract and predict molecular mechanism of action of these compounds against HER2 through MD and MDS.

2. Methods

The entire series of studies were conducted in Bogor, Indonesia. Extraction process was carried out at BRIN Cibinong. LCMS and *in silico* study was carried out at IPB University,

2.1. Phomopsis Extraction and Identification Chemical Compounds

In this study, the analytical material used was *Phomopsis* sp. isolate (endophytic fungi from Indonesian *Annona muricata* leaves) that was obtained from BRIN Indonesian Culture Collection (InaCC), labeled "Sir-G5". *Phomopsis* was first inoculated in Yeast Malt Agar (YMA) and then cultivated in Yeast Malt Broth (YMB) for 21 days. The culture was then extracted with ethyl acetate for 24 h using the maceration method and dried in a rotary evaporator to remove the solvent (Figure S1) (Minarni *et al.*, 2017). Additionally, chemical compounds were identified in *Phomopsis* extract using LC-MS/MS Thermo Scientific Vanquish Flex Ultra High-Performance Liquid Chromatography (UHPLC) tandem Q Exactive Plus Orbitrap High-Resolution Mass Spectrometer (Saravanakumar *et al.*, 2021).

2.2. Constructing Database of Phomopsis Extract Chemical Compounds

The resulting compounds from LC-MS/MS analysis were then searched for their 2D structures. Databases constructing the 2D structures were collected from PubChem (https://pubchem.ncbi.nlm.nih.gov) and ChemSpider (http://www.chemspider.com) in the SDF format (Kim *et al.*, 2019). A 2D structure is required for the druggability analysis of each chemical compounds. Drugability was predicted using the SwissADME database (http://www.swissadme.ch) and bioavailability prediction by Lipinski's "rules of five" (Daina, Michielin, and Zoete, 2017). Ligands showed high bioavailability potential when they adhere to Lipinski's rules, which include molecular weight <500 Da, log P <5, H-bond donors <5, H-bond acceptors <10, and molar refraction 40-130) (Chagas, Moss, and Alisaraie, 2018). These rules serve as a parameter for assessing drug bioavailability, specifically related to the ADME properties of a drug.

2.3. Ligand and Protein Preparation for MD

The 2D structure was converted to 3D and saved in PDB format for MD analysis. The 3D structures of *Phomopsis* compounds acted as ligands (file type. pdb). Ligand optimization was performed using *AutoDock Tools 1.5.6,* by adjusting the torsion ligands

and were saved in the PDBQT format (Sahlan *et al.*, 2023). The protein used in this *in silico* study was human HER2 (PDB: 3PP0). The data were downloaded from the PDB database (Protein Data Bank) (http://www.rscb.org/pdb). Additionally, MD was performed using *AutoDock Vina* version 1.2.3. All data were processed using Intel Pentium Core i7 hardware (16 GB RAM, Windows 10, 64-bit). The HER2 receptor in PDB format was prepared using the Discovery Studio Visualizer by removing water molecules and other ligands attached to their structure. Hydrogen atoms were added using AutoDock Tools 1.5.6, and the files were saved in PDBQT format (Fitrilia *et al.*, 2020).

Grid-box validation was performed with a target root-mean-square deviation (RMSD) value of less than 2 Å. The selected ligands were subjected to MD using the *AutoDock Vina* application by being attached to the receptor target. Docking results were scored and the best affinity energy was determined based on the most negative ΔG value. The ligand's binding area to the target receptor was identified, and the selected ligand underwent MDS using YASARA software.

2.4. MDS

Phomopsis chemical compounds exhibited strong affinity during docking on HER2, with the most robust interaction observed for a specific ligand, showed by the most negative ΔG value. The selected ligand was then analyzed for its interaction stability with HER2 through MDS using YASARA Structure version 19.9.17 with the AMBER14 force field (Prasasty and Istyastono, 2020; Bhadra and Siu, 2019). The cell extension on each side around the solute was measured at 10 Å from the cube box wall with periodic boundary conditions. MD simulations were performed for 30 ns. The stability of the ligand-protein complex interaction was observed based on the RMSD of the ligand and RMS fluctuations (RMSF).

3. Results and Discussion

3.1. Chemical Compounds of Phomopsis Extract

Analysis of *Phomopsis* sp. extract using LC-MS/MS successfully identified 44 chemical compounds (Table S1). A review of the literature on the 44 compounds identified in *Phomopsis* extract shows their potential applications as antibacterial, anticancer, antioxidant, anti-inflammatory, and antimicrobial agents, and as raw materials for industrial purposes (Figure S3). All the compounds had molecular weight of less than 500 Da. Based on a literature review, there were 16 compounds with anticancer activity, and all complied with more than three of Lipinski's rules (Table 1). These 16 compounds were subjected to molecular docking (MD) analysis.

Table 1 ADME properties of selected compounds conform to Lipinski's rules

	Compounds	Molecular Weight (Da)	H-bond donor	H-bond acceptors	log p	Molar refractivity
1.	7-Hydroxycoumarine	162.140	1	3	1.32	42.776
2.	Sorbic acid	112.130	1	2	0.48	27.377
3.	Cyclo(phenylalanyl-prolyl)	244.120	1	4	0.72	66.811
4.	3-[(4-hydroxyphenyl)methyl]-octa hydropyrrolo[1,2-a]pyrazine-1,4-dione	260.116	0	4	-1.4	60.945
5.	1,3,7-Trihydroxy-6-methoxy-4,5-diisoprenylxanthone	410.173	3	6	5.16	114.206
6.	4-Methoxychalcone	238.099	0	2	3.6	72.800
7.	Dibenzoylmethane	224.084	0	2	3.14	66.162
8.	4-(hydroxymethyl)benzoic acid	152.047	2	3	0.88	39.324
9.	Citral	152.120	0	1	2.32	50.465
10.	9-Oxo-10(E),12(E)-octadecadienoic acid	294.400	1	3	5.06	87.384
11.	3-[(1-Carboxyvinyl)oxy]benzoic acid	208.037	2	5	1.36	50.805

Compounds	Molecular Weight (Da)	H-bond donor	H-bond acceptors	log p	Molar refractivity
12. (+)-ar-Turmerone	216.320	0	1	3.44	71.076
13. 3-Allyl-2-hydroxybenzoic acid	178.062	2	3	1.81	48.967
14. Ferulic acid	194.058	2	4	1.50	51.329
15. Chalcone	208.089	0	1	3.58	66.248
16. Hydroxycinnamic acid	150.067	1	2	1,70	42.399

Extraction process was carried out using ethyl acetate, a rarely used solvent. The process generally uses harmless solvents such as distilled water (Sulistiawati *et al.*, 2023). However, according to this study, most endophytic fungi were extracted using this solvent, as well as in previous investigations (Minarni *et al.*, 2017). The final extraction process ensured that extract was free of ethyl acetate (Table S2).

3.2. MD and Energy Affinity

The 16 selected chemical compounds were subjected to MD to the HER2 receptor (PDB 3PP0). Table 2 shows docking results, showing affinity energy values, inhibition constants, and binding site similarity (BSS) for the assessed compounds. The compounds exhibiting the most favorable docking results possessed the lowest affinity energy, approaching the values of the native and comparative ligands. Affinity energy is widely used as a determinant of "docking scores " (Sahlan *et al.*, 2020).

Table 2 Energy affinity and BSS from MD with HER2

Compounds	Energy affinity (kcal/mol)	Inhibition constants (µm)	BSS (%)	Hydrogen bond	Hydrophobic interaction
• ***Native Ligand:*** 2-{2-[4-({5-chloro-6-[3-(trifluoromethyl)phenoxy]pyridin-3-yl}amino)-5H-pyrrolo[3,2-d]pyrimidin-5-yl]ethoxy}ethanol	-11.1	0.007		1	19
• ***Compare Ligand:*** Trastuzumab	-7.5	3.136	40	4	7
• 3-[(4-hydroxyphenyl)methyl]-octahydropyrrolo[1,2-a]pyrazine-1,4-dione	-9.4	0.126	55	1	11
• Chalcone	-9.3	0.15	70		15
• 4-methoxy chalcone	-9.3	0.15	55		12
• 1,3,7-Trihydroxy-6-methoxy-4,5-diisoprenylxanthone	-9.3	0.15	55	2	13
• Dibenzoylmethane	-8.9	0.294	55		11
• Cyclo(phenylalanyl-prolyl)	-8.7	0.413	45		9
• *(+)-ar-Turmerone*	-8.1	1.138	60		13
• *9-Oxo-10(E),12(E)-octadecadienoic acid*	-7.4	3.713	70		17
• 3-[(1-Carboxyvinyl)oxy]benzoic acid	-7.4	3.713	55	3	9
• 3-Allyl-2-hydroxybenzoic acid	-7.4	3.713	45	2	10
• Ferulic acid	-6.9	8.643	45	2	8
• 7-hydroxycoumarin	-6.8	10.234	30	1	6
• Hydrocinnamic acid	-6.4	20.117	50	2	9
• 4-(hydroxymethyl)benzoic acid	-6.2	28.205	40	3	6
• *Citral*	-6.1	33.397	45		10
• *Sorbic acid*	-5.6	77.735	35	2	8

The compounds 3-[(4-hydroxyphenyl)methyl]-octahydropyrrolo[1,2-a]pyrazine-1,4-dione shows the most negative affinity energy (-9.4 kkal/mol) for the HER2 receptor among *Phomopsis* extract compounds, showing a robust affinity. It hydrophobically interacts with

four key amino acids in the active site: Thr862, Asp863, Phe864, and Lys753 (Figure 1). These four active sites also interact hydrophobically with the native ligand. This compounds exhibit high lipophilicity and pharmacokinetic properties, as showed by its negative LogP value (Table 1). The compound's high lipophilicity suggests excellent interaction with hydrophobic environments, enhancing its bioavailability. Molecules with increased lipophilicity typically show improved permeability through the enterocyte phospholipid bilayer, emphasizing the compound's potential efficacy.

Affinity energy is an important parameter in determining the quality of MD results, signifying the strength of the interaction between the ligand and the active site of the receptor (Du *et al.*, 2016). The affinity energy, or change in *Gibbs* free energy (ΔG), represents the driving force of all chemical reactions in nature to measure the capacity of a system to perform maximum work at constant temperature and pressure (Popovic and Minceva, 2020). Protein binding to a ligand occurs when the change in affinity energy is negative. A more negative value signifies a spontaneous reaction, showing a favorable and energetically favorable interaction between the protein and ligand. The affinity energy is directly proportional to the inhibition constant (Ki). The Ki value can predict the ability of a compounds to inhibit its target protein (Muttaqin, 2019). A lower Ki value shows a better inhibition ability.

Although the BSS value of this compounds was not as high as that of chalcone, (+)-ar-Turmerone and 9-Oxo-10(E),12(E)-octadecadienoic acid, it contained many hydrogen bonds and hydrophobic interactions. Hydrogen bonds play an important role in protein folding, protein-ligand interactions, and catalysis (Chen *et al.*, 2016). The quantity and arrangement of hydrogen bonds significantly influence the binding affinity between a ligand and a receptor. Hydrophobic interactions, characterized by nonpolar molecules in water, are essential for protein folding and also contribute to stabilizing ligand binding to the receptor. Subsequently, these interactions play crucial roles in determining the strength and stability of the overall ligand-receptor complex (Bogunia and Makowski, 2020).

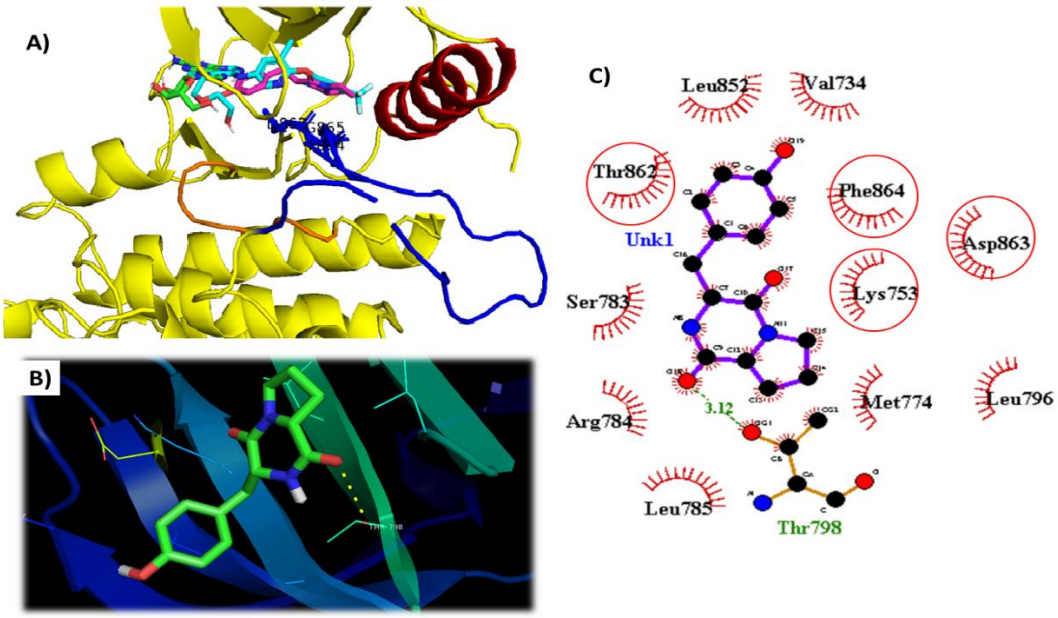

Figure 1 Visualization of the tested ligand (3-[(4-hydroxyphenyl)methyl]-octahydropyrrolo[1,2-a]pyrazine-1,4-dione) and HER2 interactions. (A) Superimpose the tested ligand with the native and comparative ligand on HER2 (yellow = kinase domain; red = alpha-helix C; orange = catalytic loop; green = activating loop; magenta stick = compound;

cyan stick = native ligand; green stick = comparative ligand); (B) visualization in 3D; and (C) visualization in 2D (red circles = HER2 active sites; dot-dot lines = hydrogen bond).

3.3. MDS

MDS was performed to identify the stability of the protein-ligand complexes through dynamic trajectories. This process can be performed using various tools, such as YASARA, AMBER, and graphical processing units (GPUs) (Prasasty and Istyastono, 2020; Suhartanto *et al.*, 2018). Some data can be obtained from MDS, including RMSD, RMSF, solvent access for surface area (SASA), and radius of gyration (Rg).

The RMSD of liganded HER2 was calculated for the initial model over the 30 ns MDS period as shown in Figure 2. The stability of the protein-ligand complex was first assessed based on RMSD calculations, considering both ligand movement and conformation. The selected test ligand for MDS on HER2 was 3-[(4-hydroxyphenyl)methyl]-octahydropyrrolo[1,2-a]pyrazine-1,4-dione. Based on the RMSD graph of ligand movement on HER2 (Figure 2A), the structure showed a short increase in the first 4 ns and a sharp increase from 5 to 10 ns, with an RMSD value of >3.0 Å. The ligand movement reached equilibrium over 30 ns with an RMSD value of approximately 2.0 Å. In contrast, the RMSD graph of ligand conformation showed conformational stability for 30 ns with an RMSD value of around 1.2-1.4 Å (Figure 2B). The binding of the selected ligand to HER2 did not significantly affect the conformational stability of HER2 during simulations time, as shown in the RMSD graph of HER2, which tended to be stable with an RMSD value of approximately 3.0 Å.

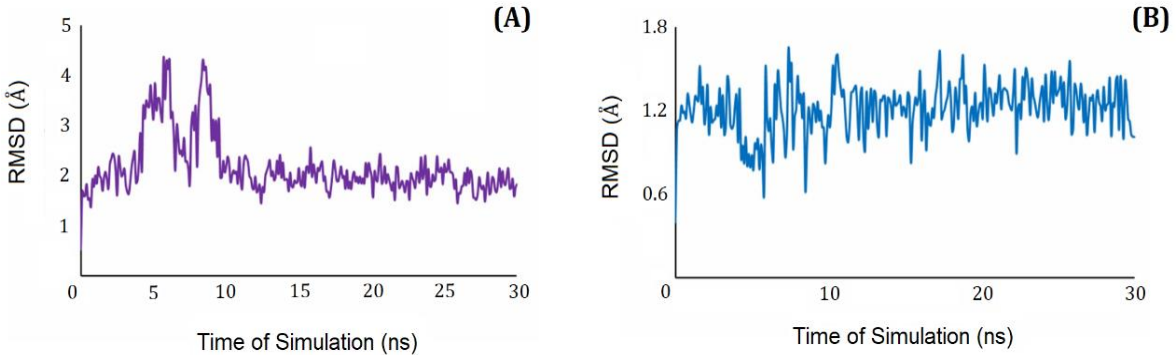

Figure 2 RMSD of 3-[(4-hydroxyphenyl)methyl]-octahydropyrrolo[1,2-a]pyrazine-1,4-dione on HER2: ligand movement (A), ligand conformation (B)

The RMSF profile comparative analysis of the HER2 protein bound to the ligand molecule showed no significant fluctuations in the HER2 catalytic site (Figure 3A). Although some regions within the protein-ligand complex showed a moderately high degree of mobility with fluctuations ranging between 1 and 4 Å, these were not considered significant for this study because the main focus was on the catalytic dynamic behavior.

SASA serves as a geometric measure of protein-surface interactions in an external solvent environment. SASA value (nm^2 or $Å^2$) was directly proportional to the proportion of amino acids in the protein exposed to the solvent environment (Figure 3B). The disruption of SASA alters the amino acids exposed to the solvent, consequently affecting the overall conformation of the protein (Chen and Panagiotopoulos, 2019). SASA analysis of the HER2 protein-ligand complex showed that the values tended to be stable, with an average area of 14000 $Å^2$. This showed that the ligand in the HER2 catalytic pocket did not cause an increase in solvent exposure to the protein surface. In contrast, this ligand does not disrupt the conformation of HER2 protein folding.

The radius of gyration (Rg) serves as an indicator of conformational equilibrium, reflecting the compaction of the protein structure through folding and unfolding processes

(Liu *et al.*, 2017). Based on the calculation of the Rg value of the HER2 protein-ligand complex (Figure 3C), the Rg value did not show significant deviations. The Rg values tended to stabilize at approximately 19.8 - 20.0 Å. This shows that the presence of the ligand in the catalytic pocket of HER2 did not induce a substantial change in the conformational equilibrium, particularly in protein folding.

Molecular dynamic of the HER2 and the ligand complex over 30 ns are shown in Figure 4. Based on dynamic trajectory analysis, the tested ligand showed good stability in binding to the HER2 catalytic pocket. A greater stability of the ligand when docked to the target protein implies a stronger binding affinity. This suggests that the ligand is more effective in inhibiting or interfering with the catalytic activity of the target protein.

The MD and MDS results showed that 3-[(4-hydroxyphenyl)methyl]-octahydropyrrolo[1,2-a]pyrazine-1,4-dione has the potential to inhibit HER2, a protein that plays a role in the incidence of breast cancer. Given the low relative abundance of 3-[(4-hydroxyphenyl)methyl]-octahydropyrrolo[1,2-a]pyrazine-1,4-dione in *Phomopsis* extract, additional steps are essential to increase its quantity. One method is isolating the target compounds, facilitating a more concentrated and effective use for potential applications. One of the advantages of natural extract is the synergy between their constituent compounds. In this study, the compounds that could inhibit HER2 was 3-[(4-hydroxyphenyl)methyl]-octahydropyrrolo[1,2-a]pyrazine-1,4-dione, but another compounds could interact with other proteins, such as Thymidine Kinase, p53, or cyclin-dependent kinase. Therefore, it is necessary to analyze other proteins involved in the incidence of breast cancer.

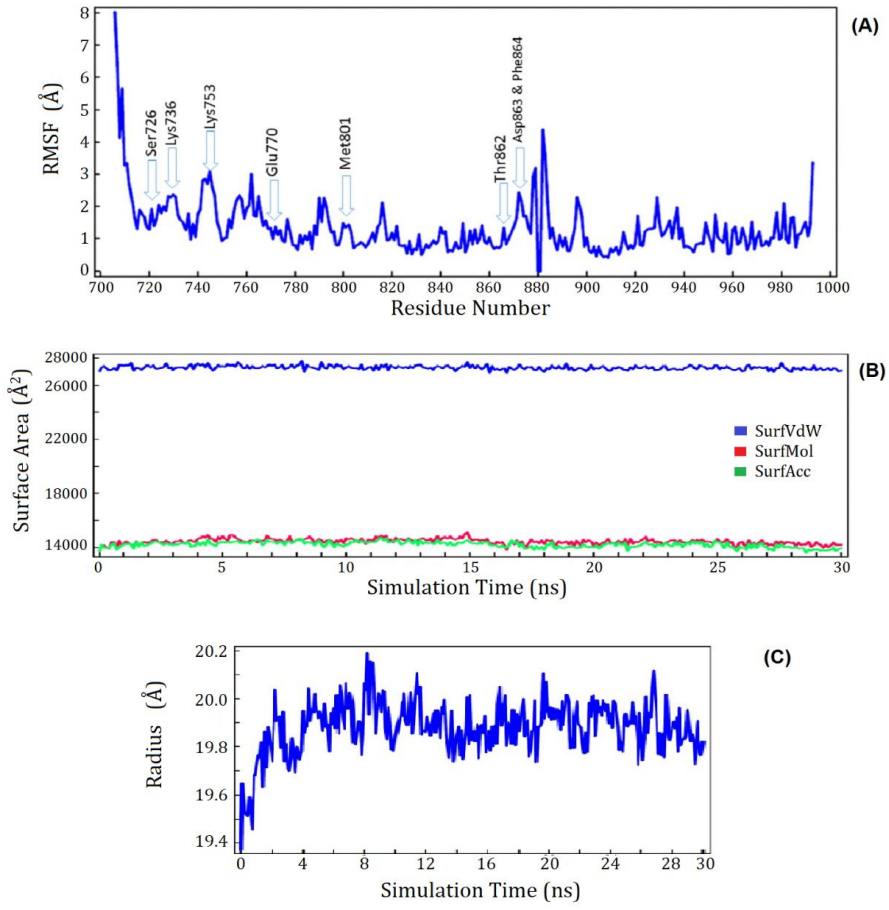

Figure 3 Cα RMSF values (A), SASA (B), and Rg (C) of HER2 that complexed with ligands in 30 ns

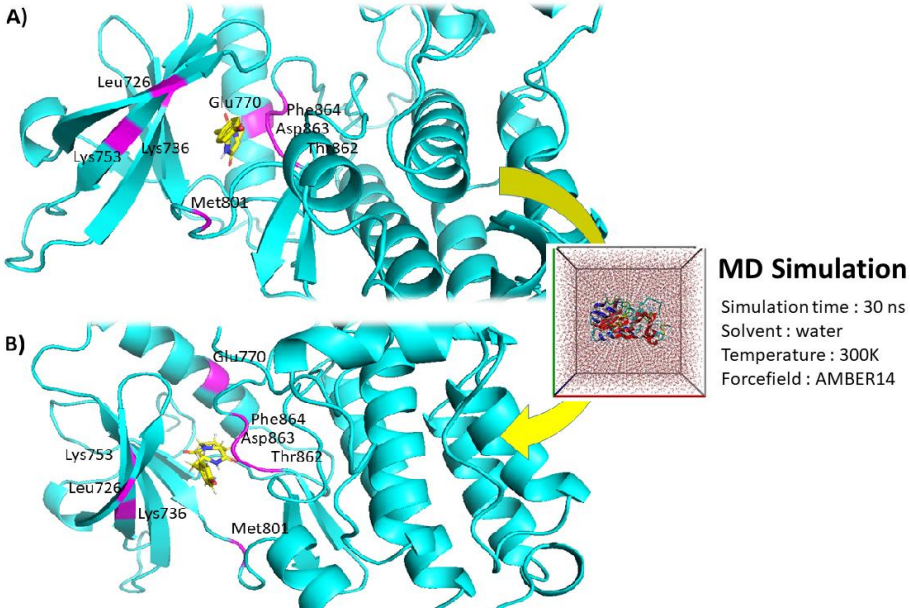

Figure 4 3D visualization of HER2-ligand complex, before (A) and after (B) MD simulations

4. Conclusions

LCMS analysis successfully identified 44 chemical compounds in extract of *Phomopsis* sp. 16 of which have the potential to be anticancer and meet Lipinski's rules. Additionally, MD results showed that the most negative energy affinity for HER2 receptors was 3-[(4-hydroxyphenyl)methyl]-octahydropyrrolo[1,2-a]pyrazine-1,4-dione (-9.4 kcal/mol). MDS of the protein-ligand complex showed prominent HER2 inhibition as shown by dynamic trajectory analysis. The compounds 3-[(4-hydroxyphenyl)methyl]-octahydropyrrolo[1,2-a]pyrazine-1,4-dione was identified as HER2 inhibitor, which can be developed for breast cancer therapy.

Acknowledgments

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