Screening the Activity of Limonene and Its Derivatives in Inhibiting the Enzymes MMP-2, MMP-9, and Cyclin A2 in Triple Negative Breast Cancer through Molecular Docking

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Abstract: Breast cancer is among the diseases with the highest mortality rate in women in the world. The triple negative cancer subtype with aggressiveness and metastatic ability causes the sufferer to be difficult to treat. Targeted treatment efforts of natural ingredients such as limonene and its derivatives are more profitable due to their easy excretion process. Screening the activity of compounds through docking specifically provides convenience in the synthesis process in the laboratory. Limonene compounds and their derivatives will be interpreted against the enzymes MMP-2 (PDB ID: 3AYU), MMP-9 (PDB ID: 4H1Q) and Cyclin A2 (PDB ID: 2V22) involved in the cellular function of triple-negative breast cancer using PyRx 9.0 software. The docking results showed that the limonelyl salicylate compound provided the best binding affinity value against the enzymes MMP-2, MMP-9, and Cyclin A2 with successive values of -7.7, -8.8, and -6.7 kcal/mol.

Keywords: Limonene derivatives; Molecular docking; Triple negative breast cancer

Introduction

Cases of breast cancer patients in women around the world in 2020 reached 24.5%, with a mortality rate of 15.5%. The triple negative breast cancer subtype is a cancer type with the lowest prognosis rate, the highest level of aggressiveness and potential to metastasize, and the lowest ER-, PR-, and HER2-values (Charan et al., 2020; Kaur et al., 2012; Pitarch et al., 2021). To date, the triple negative cancer subtype remains a challenge in clinical trials in identifying treatment mechanisms to help sufferers.

The breast cancer cells 4T1 and MDA-MB 231 represent the triple-negative breast cancer subtype (Hero et al., 2019; Silva, 2016). The matrix metalloproteinases (MMPs) family of enzymes, which includes MMP-2 and MMP-9 (Bassiouni et al., 2021; Visse et al., 2003), are expressed in breast cancer cells 4T1 and MDA-MB 231 (Farahani et al., 2021; Li et al., 2017;

Lyu et al., 2019). The extracellular matrix is lowered by these enzymes, which promotes tumor invasion and metastasis (Li et al., 2014; Ning et al., 2016; Tauro et al., 2018; Yousef et al., 2014). Additionally, triple negative cancer expresses the Cyclin A2 enzyme, which is important for the cell cycle (Lu et al., 2020).

In preclinical experiments, the lipophilic monoterpene limonene from citrus oil has been shown to have both chemopreventive and chemotherapeutic effects (Miller et al., 2013). The negative effects can be reduced by using limonene as an anticancer active component in the breast. This is supported by a study by Haag et al. (1992), which showed that giving limonene to rats orally could prevent the growth of breast cancer cells without having any negative side effects. Alpha-terpineol, perillyl alcohol, perillic acid, and perillyl aldehyde are just a few of the molecules changed by limonenes that exhibit different bioactivities (Chen et al.,

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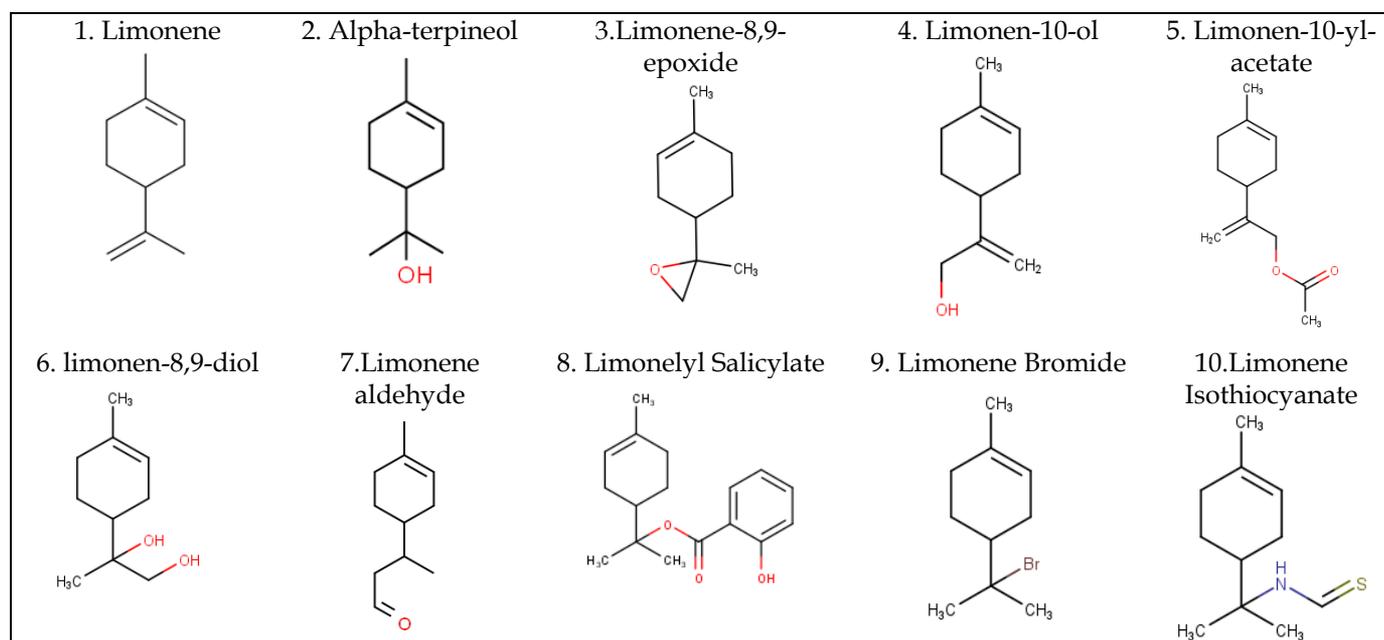2018; Gouveia et al., 2018; Lima et al., 2020; Terpou, 2014).

Figure 1. Structure of limonene and its derivatives

Modern strategies with in silico techniques are used to predict and evaluate the early stages, thus helping to increase the success and acceleration of drug discovery. This method starts with the creation of the targeted molecular structure, which is then screened through docking against the specific target receptor. The best predictions of drug molecules at the molecular level can be used as drug candidates to be synthesized and tested in-vitro. In addition to reducing costs, experiments carried out in the laboratory became more targeted and reduced trial and error (Zloh et al., 2018).

Docking is carried out specifically through active sites (binding pockets) on proteins in order to obtain accurate results. These binding pockets can be opened, closed, and adapted to regulate binding with ligands, transporting a compound towards or away from a protein (Stank et al., 2016). These methods for detecting small molecule binding sites in proteins are obtained through software (Wang et al., 2019).

The Protein Data Bank's receptor database provides access to proteins that have been identified by binding pockets. Appropriate protein screening is selected based on the presence of native ligands bound to the cavity or inside of the protein. The location of the active sites on the protein area is limited, so it is necessary to visualize the box size and amino acid residues that make up the protein binding bag through PyMOL software so that docking is only carried out on that area (Seeliger et al., 2010). The 3-dimensional structures of the enzymes MMP-2 (PDB ID: 3AYU), MMP-9 (PDB ID: 4H1Q), and Cyclin A2 (PDB ID: 2V22) were docked using PyRx 9.0.

The docking results evaluated the value of binding affinity and visualized the interaction of compounds with amino acid residues using LigPlot.

Method

Materials and Equipment

The tool used is a computer with the Samsung brand, intel core i3 windows 10. Docking is done using AutoDock Vina, which is contained in the PyRx 9.0 software. Receptor preparation using Discovery Studio Visualizer, protein-active side Grid Box visualized with PyMol (version 4.6.13560), and Ligplot+ (version 2.2.4). The 3D structures of the enzymes MMP-2, MMP-9, and Cyclin A2 were downloaded from the RSCB Protein Data Bank (https://www.rcsb.org/). The 3D structure of the limonene and its derivatives (Table 1) was drawn using MarvinSketch software (version 21.10).

Preparation of Ligands and Receptors

The ligand structure of limonene compounds and their derivatives was drawn through marvinSketch software (version 21.10). 3D receipts are saved in.sdf format. Then the 3D structures of the enzymes MMP-2 (PDB ID: 3AYU), MMP-9 (PDB ID: 4H1Q), and Cyclin A2 (PDB ID: 2V22) were downloaded from the RSCB Protein Data Bank (https://www.rcsb.org/). Receptor preparation using Discovery Studio Visualizer. The same receptor chains, water molecules, and native ligands are eliminated. The receptor is only inserted into

one of the chains and cofactors in the form of metal atoms, then stored in PDB format.

Grid Box Docking Setup

The grid box is determined using PyMol (version 4.6.13560) at the position of the native ligand contained in the receptor. The grid boxes for the enzymes MMP-2 (PDB ID: 3AYU), MMP-9 (PDB ID: 4H1Q), and Cyclin A2 (PDB ID: 2V22) are found in Table 1.

The Molecular Docking Method

Built-in receptors and ligands are inserted through the 'load molecule' toolbar. Then, click 'Open Babel' and select all the ligands to be interacted with the receptor. Energy minimization is performed on all ligands and converts the 'ligand.pdb' format to the pdbqt format. After that, click on the Vina Wizard, select the receptor and all ligands, and click 'Run Vina'. The determination of the grid box is carried out according to the active side of the protein that has been determined (Table 1). After that, running docking is carried out. Affinity data binding is stored in '.csv' format and interaction results are stored in '.pdbqt' format (Dallakyan et al., 2015).

Table 1. Protein Codes and Grid Box

Protein Code	Grid Box
MMP-2 (PDB ID: 3AYU)	Center: X (1.825), Y (-11.935), Z (-4.867) Dimension (Å): X (53.500), Y (51.600), Z (46.678)
MMP-9 (PDB ID: 4H1Q)	Center: X (29.606), Y (4.946), Z (19.187) Dimension (Å): X (37.148), Y (46.563), Z (24.970)
Cylin A2 (PDB ID: 2V22)	Center: X (40.109), Y (24.281), Z (-0.477) Dimension (Å): X (21.518), Y (20.544), Z (26.869)

Visualization of Docking Results

The 2-dimensional interaction between the receptor and ligand can be observed using Discovery Studio visualizer software and Ligplot+ (version 2.2.4).

Druglikeness, ADME, and Toxicity Prediction

Absorption, distribution, metabolism, excretion, and toxicity will be predicted for compounds with the best results based on docking. Predictions will be made via the SwissADMET web server (http://www.swissadme.ch/index.php#) (Daina et al., 2017).

Result and Discussion

Preparation of Ligands and Receptors

Water at the receptors must be removed because ligands can interact with water molecules, forming hydrogen bonds. This interferes with the interaction of ligands with receptors so that docking scores are obtained not only from ligand interactions with

receptors but also with water molecules (Elokely et al., 2013). In the structure of the PDB ID 3AYU (MMP-2) and 4H1Q (MMP-9) receptor structures, there are metal ions Zn^{+2} and Ca^{+2}. These metal ions do not need to be eliminated in receptor preparations because they have an important role in the binding of ligands with proteins, regulating the stability of protein structures and acting as catalytics between target receptors and ligands. The receptor is only left with one of the chain receptors and metal ions only. For MMP-2, chain A is used. MMP-9 is used for either chain A or B, while for Cyclin A2, it is used by either chain B or D.

Molecular Docking

The parameters used after docking are the accuracy of conformation and orientation ("pose") of the ligand into the active site of the target receptor determined by the RMSD (Root Mean Square Deviation) value ≤ 2Å (Bhojwani et al., 2019). Then, evaluate the value of binding-free energy (ΔG) or binding affinity between ligands and receptors, a great interaction when the value gets smaller (Yunta, 2016).

The docking results of limonene compounds and their derivatives as a whole obtained an RMSD value of 0 Å, so that the accuracy of the ligand position with the receptor when docking was valid. Then, the value of binding affinity (ΔG) against the enzymes MMP-2, MMP-9, and Cyclin A2 is shown in Table 2. Based on the docking results, compound number 8 (limonelyl salicylate) provides the best binding affinity values against the 3AYU, 4H1Q, and 2V22 receptors with successive values of -7.7, -8.8, and -6.7 kcal/mol.

Table 2. Molecular Docking Results of Limonene Compounds and Their Derivatives

Compound	ΔG (kcal/mol)		
	MMP-2 (3AYU)	MMP-9 (4H1Q)	Cyclin A2 (2V22)
Nattive ligand	0	-9.2	-7.6
1	-5.3	-6.7	-5.3
2	-5.7	-7.1	-5.3
3	-5.3	-6.9	-5
4	-6	-7	-5.2
5	-5.5	-7.4	-5.8
6	-5.9	-6.9	-5.3
7	-5.5	-7	-5.5
8	-7.7	-8.8	-6.7
9	-5.4	-6.9	-5.3
10	-5.7	-6.8	-5.4

Visualization of Ligand Interaction with Receptors

Compound 8 (limonelyl salicylate) gives the best binding affinity results. Visualization of the interaction of ligands with amino acid residues is seen in Figure 2. The interaction of hydrogen and hydrophobic from salicylic acid compounds with MMP-2, MMP-9, and Cyclin A2 receptors can be seen in Figure 3. The green

color describes the hydrogen interaction, while the red color describes the hydrophobic interaction. The interaction of limonelyl salicylate with amino acid residues from receptors is summarized in Table 3.

Prediction of Druglikeness, ADME, and Toxicity

The prediction of "drug similarity (druglikeness)" follows Lipinski's rule. These predictions were made to determine the physicochemical properties of the docking interactions obtained and match those properties with the listed drug molecules (Daina et al., 2017). The rules of Lipinski include, compounds have hydrogen bond donors ≤ 5, have hydrogen bond acceptors ≤ 10, molecular weights ≤ 500 Da, Clog P ≤ 5, (Mlog P ≤4.5). Lipinski's rule relates to the good solubility and permeability of the drug. Based on the data in Table 3, the limonelyl salicylate complies with the Lipinski rule.

The results of Adsorption, Distribution, Metabolism, and Excretion (ADME) show that limonelyl salicylate can be well absorbed by the gastrointestinal tract. However, in the attribution process, the compound is able to pass through the blood-brain barrier (BBB). This Blood-Brain Barrier prevents the entry of most drugs into the central nervous system from the blood (Pardridge, 2012). Furthermore, in the metabolime process, limonelyl salicylate can inhibit the enzymes CYP1A2, CYP2C19, and CYP2C9. Drugs that inhibit the enzymatic pathway CYP can cause an increase in the concentration of other drugs (in the blood plasma) metabolized by the same pathway, resulting in toxicity or side effects of the drug (McDonnell et al., 2013). Then, in the excretion process, the compound cannot be transported by the P-glycoprotein (P-Gp) substrate, which means that chemical compounds are difficult to excrete from cells (Li et al., 2014).
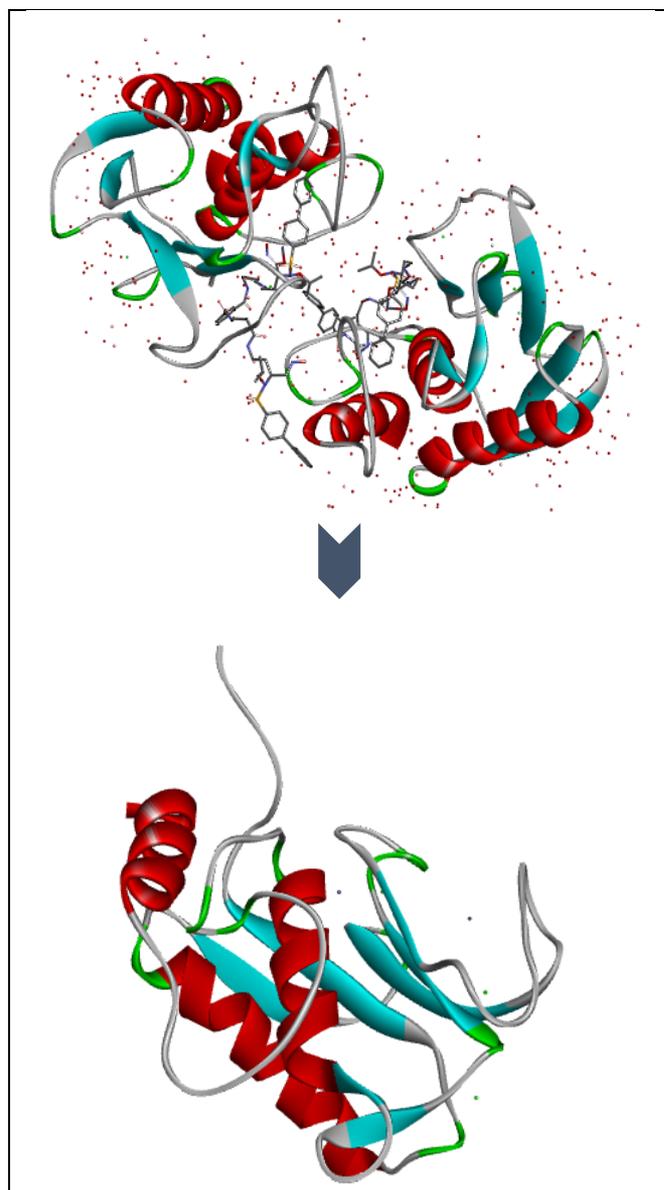
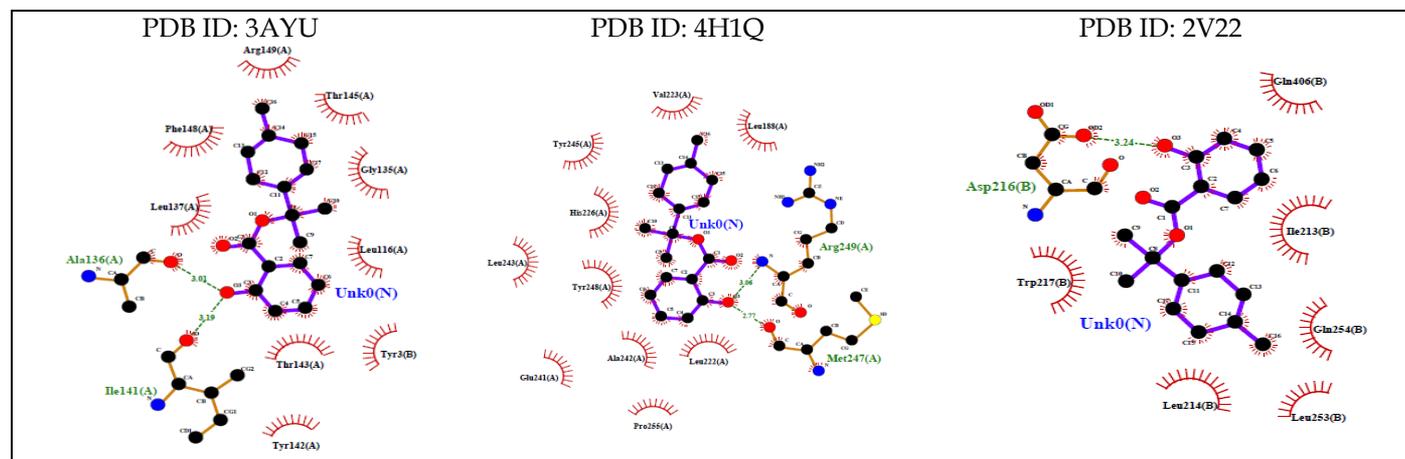

Figure 2. Receptor preparations of MMP-9 (PDB ID: 4H1Q)

Figure 3. Two-dimensional visualization of the interaction of limonelyl salicylate with target receptors using LigPlot

Table 3. Interaction of Limonelyl Salicylate Compounds with Amino Acids at Target Receptors

Compound	PDB's ID	ΔG (kkal/mol)	Bond Interaction	Amino Acid
Limonelyl Salicylate (8)	3AYU	-7.7	Hydrogend	Ile141, Ala136
			Hydrophobic	Leu137, Phe148, Arg149, Thr145, Gly135, Leu116, Tyr3, Thr143, Tyr142
	4H1Q	-8.8	Hydrogend	Met247, Arg249
			Hydrophobic	Leu188, Val223, Tyr245, His226, Tyr248, Leu243, Glu241, Ala242, Leu222, Pro255
	2V22	-6.7	Hydrogend	Asp216
			Hydrophobic	Gln406, Ile213, Leu253, Gln254, Leu214, Trp217

Table 4. Druglikeness, ADME, and Toxicity Prediction

Parameters	Result
Drug-Likeness (Lipinski's Rules)	Yes
Gastrointestinal absorption	High
BBB permeation	Yes
P-gp substrate	No
CYP1A2 inhibitor	Yes
CYP2C19 inhibitor	Yes
CYP2C9 inhibitor	Yes
CYP2D6 inhibitor	No
CYP3A4 inhibitor	No

Conclusion

Computational interactions of limonene compounds and their derivatives based on binding affinity obtained the best results in limonelyl salicylate compounds. The value of binding affinity to docking on the active side of the enzymes MMP-2, MMP-9, and Cyclin A2 was, respectively, -7.7; -8,8; -6.7 kcal/mol. Based on SwissADMET predictions, salicylic acid compounds meet the Lipinski rule. However, limonelyl salicylate still has limitations on its metabolic processes in the body.

Acknowledgements

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