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5-*O*-Acetylpinostrobin as a Potential Therapeutic Agent Against Breast Cancer: Cytotoxic Effects on T47D Cell Line and Molecular Docking Predictions

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ABSTRACT

Breast cancer is the leading cause of cancer-related death among women worldwide. This disease is commonly treated with doxorubicin, a chemotherapeutic agent associated with cardiotoxic side effect. Recently, pinostrobin, a compound isolated from the rhizome of *Boesenbergia pandurata* Roxb., has also shown potential in inhibiting the growth of human breast cancer cells (T47D). To enhance the bioavailability, reduce toxicity, and increase selectivity, the compound has been modified through the *Schotten–Baumann* reaction to produce 5-*O*-Acetylpinostrobin. Therefore, this research aimed to evaluate the cytotoxic effect of 5-*O*-Acetylpinostrobin against T47D cells, selectivity toward Vero cells, and interaction with estrogen receptor alpha (ERα) (PDB ID: 5T1Z) through molecular docking. Cytotoxicity was assessed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay against T47D and Vero cells. Molecular docking was performed using AutoDockTools software (v1.5.7) and docking interaction was visualized on BIOVIA Discovery Studio. The result showed that the 50% inhibitory concentration (IC_{50}) values of 5-*O*-Acetylpinostrobin and pinostrobin against T47D were 53 µM and 156 µM, respectively. Cytotoxic concentration 50 (CC_{50}) values against Vero cells were 1155 µM and 3303 µM for 5-*O*-Acetylpinostrobin and pinostrobin, respectively, while selectivity index (SI) was 22 and 21, respectively. Furthermore, molecular docking showed that 5-*O*-Acetylpinostrobin had a favorable interaction with ERα, with a binding affinity of –7.98 kcal/mol. These results suggested that 5-*O*-Acetylpinostrobin had potential as anti-breast cancer agent and could be further developed as a co-chemotherapeutic agent.

Keywords: 5-*O*-Acetylpinostrobin, Breast cancer, Cytotoxic effect, Molecular docking, T47D cell line.

Introduction

Breast cancer is the leading cause of cancer-related death among women, with an estimated 2.3 million new cases and 9.95 million death globally in 2020.[1] This disease develops when the epithelial cells from the ductus or lobules of the breast increase uncontrollably. Furthermore, breast cancer originates from ductus hyperproliferation, which is consistently driven by several carcinogenic stimuli, causing the growth of a benign tumor or metastatic carcinoma.[2] The treatment of breast cancer includes surgery, radiation, and chemotherapy, with drug delivery contingent upon the specific form of breast cancer.[3] Doxorubicin, a chemotherapeutic drug, is an anticancer agent whose mechanism of action includes the reduction of topoisomerase II and DNA intercalation.

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This reduction leads to the inhibition of DNA (Deoxyribonucleic Acid) and RNA (Ribonucleic Acid) synthesis, alongside the formation of semiquinone radicals and reactive oxygen species.[4] Doxorubicin directly damages cancer cells DNA and strengthens the apoptosis pathway through inhibition of Bcl-2, increasing the effectiveness of cancer treatment.[5] However, doxorubicin use is limited by its toxic effect on multiple organs, including cardiotoxicity and diabetic cardiomyopathy, hepatotoxicity, nephrotoxicity, as well as diabetes-like symptoms, impaired adipose metabolism, neurotoxicity, and reproductive disease.[6] The existence of these effects prompted the search and development for drugs with specific targets and high selectivity from natural ingredients, including the plant *Boesenbergia pandurata* (Roxb.), as an alternative anticancer agents.

The rhizome of *Boesenbergia pandurata* (Roxb.) has several potential pharmacological effect, including antiviral, antioxidant, anticancer, and anti-inflammatory properties.[7,8] Among several bioactive constituents, pinostrobin is characterized by its anticancer properties.[9] The efficacy of this compound as a DNA topoisomerase inhibitor is related to its capacity to fragment DNA in T47D cells.[10] Therefore, obstructing the activity of DNA topoisomerase with inhibitor compounds leads to the formation of Protein-Linked DNA Breaks (PDBs), including DNA damage and cessation of cancer cells replication.[11] Compared to breast anticancer drug available on the market, pinostrobin activity is still lower.[12] To increase its activity, structural modifications were conducted

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through the *Schotten-Baumann* reaction to produce 5-*O*-Acetylpinostrobin.[13]

Based on the above, this research aimed to determine the cytotoxic effect of pinostrobin and 5-*O*-Acetylpinostrobin on T47D cells using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The compound selectivity test was also conducted on Vero cells using the same method. Meanwhile, the anticancer potential of 5-*O*-Acetylpinostrobin and pinostrobin against the estrogen receptor (ER) was predicted *in silico* via molecular docking using the estrogen receptor alpha (ERα) with PDB ID: 5T1Z.

Materials and Methods

Compounds

Samples of pinostrobin and 5-*O*-acetylpinostrobin were obtained from the Research Center, *In Vitro-1* Laboratory, Faculty of Pharmacy, Universitas Airlangga, Indonesia. These samples were synthesized in a previous study.[13] The chemical structures and purities of the compounds were confirmed by Thin Layer Chromatography (TLC) and melting point determination prior to use. Further confirmation of the synthesized compounds was carried out by UV spectrophotometry, FT-IR spectrophotometry, Nuclear Magnetic Resonance (^{1}H-NMR and ^{13}C-NMR) spectroscopy, and mass spectrometry (MS).

In vitro anticancer activity screening

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test was used to assess the compounds anticancer efficacy. A 96-well plate was seeded with T47D and Vero cells at a density of 5000 cells per well. Pinostrobin and 5-*O*-Acetylpinostrobin dissolved in dimethyl sulfoxide (DMSO) at varying doses (10, 50, 150, 250, 500, 750, and 1000 µM) were applied to the cells, and they were incubated for 24 hours at 37 °C. Following incubation, each well received 100 µL of MTT solution (0.5 mg/mL), which was then incubated at 37°C for three to four hours to allow the MTT to react with living cells and create purple formazan crystals. 100 µL of 10% sodium dodecyl sulfate (SDS) in 0.01 N HCl was added to each to halt the process. Following incubation, each well received 100 µL of MTT solution (0.5 mg/mL), which was then incubated at 37°C for three to four hours to allow the MTT to react with living cells and create purple formazan crystals. 100 µL of 10% sodium dodecyl sulfate (SDS) in 0.01 N HCl was added to each well to halt the reaction. A microplate reader (EZ Read 2000 model, Biochrom, UK) was used to measure the absorbance of the resultant solution at 570 nm. It was established what concentration was required to stop 50% of T47D cell proliferation (IC_{50}).[14]

Selectivity evaluation

To evaluate chemical selectivity in Vero cells, including whether pinostrobin and 5-*O*-Acetylpinostrobin were lethal to cancer cells exclusively, a selectivity test was conducted using the MTT assay. Cytotoxic concentration 50 (CC_{50}), which was found to reduce cell viability by 50% in comparison to the untreated control, was employed as a selectivity criterion. The selectivity index (SI), which compared each compound IC_{50} values in both normal.[15]

In silico study

Protein preparation

The receptor used was estrogen receptor alpha (ERα) with PDB ID: 5T1Z obtained from RSCB Protein Data Bank website (http://rcsb.org). It was downloaded in .pdb format, with the unused parts (water, solvent, unused chains) removed. Meanwhile, polar hydrogen, Kollman charge, and the position of the grid box were set using AutoDockTools software (v1.5.7).[16,17]

Ligand preparation

The 2D structures of the test ligands were prepared using the ChemBio Draw 2D program and saved in .cdxml format. The 2D structures were further copied into the ChemBioDraw 3D program and minimized with MMFF94, which were saved in .mol2 and .sdf formats.[18] AutoDockTools software (v1.5.7) was used to prepare ligand by adding nonpolar hydrogens and Gasteiger charges. Subsequently, a torsion tree was added, and ligand was saved in the .pdbqt format for docking.[16]

Docking method validation

Validation was performed to show that the selected docking parameters could reduce the androgen receptor ligand. The process included recoupling natural ligand into the active site of the receptor or protein. However, redocking was carried out on a grid box of size 40 x 40 x 40, with a center at x: 24.752, y: -1.814, and z: 7.460. The docking validation was performed by saving the cocrystallized ligand in .pdbqt format and calculating the Root Mean Square Deviation (RMSD), which was found to be less than 2 Å, indicating a successful validation.[19,20]

Molecular docking

Docking of the test ligand was performed using the same grid box sizes and positions as in the validation process for each receptor. Based on the parameters evaluated, amino acid residue as well as the lowest ΔG and Ki values were used to determine which ligand was the most effective as a breast cancer drug.[21] Protein-ligand interactions were visualized in both two and three dimensions using Biovia Discovery Studio 2021 Client.

Statistical analysis

Statistical analysis was performed using SPSS 25 software (IBM SPSS Statistics, Chicago, IL, USA). Data were analyzed using one-way analysis of variance (ANOVA) and Tukey's multiple comparisons test. $P < 0.05$ was accepted as statistically significant.[22]

Results and Discussion

In vitro anticancer activity

The cytotoxic effect of the test compounds (pinostrobin and 5-*O*-Acetylpinostrobin) against T47D cells were evaluated in terms of their IC_{50} values, which is defined as the concentration needed to inhibit 50% of cell growth. Lower IC_{50} values indicate a more potent cytotoxic effect. That means a stronger cytotoxic effect correlate with lower IC_{50} value.[23] As presented in Figure 1, pinostrobin, 5-*O*-Acetylpinostrobin, and 5-fluorouracil showed cytotoxic effect against T47D cells, with IC_{50} values of 156 µM, 53 µM, and 915 µM, respectively. IC_{50} value of 5-*O*-Acetylpinostrobin was approximately 3 and 17 times lower than that of pinostrobin and 5-fluorouracil, respectively. Based on the classification criteria established by the United States National Cancer Institute, 5-*O*-Acetylpinostrobin fell in the category of moderately active compounds, showing potential as a promising anticancer agent.[23]

Selectivity of the test compounds

Evaluation of the selectivity index (SI) of the test compounds showed that 50% cell death was not achieved by the highest concentration tested against Vero cells. The CC_{50} value in Figure 1 was determined using cell viability curve, and estimation was carried out through probit analysis. The results showed CC_{50} values of 3303 µM and 1155 µM for pinostrobin and 5-*O*-Acetylpinostrobin, respectively. These high values suggested that the two compounds had a weak cytotoxic effect against normal cells.[24] CC_{50} also served as a reference for determining the selectivity of the test compounds, where higher values signified less toxicity to Vero cells. Selectivity is assessed using selectivity index (SI), which is calculated as the ratio of the cytotoxic concentration (CC_{50}) to the bioactive concentration (IC_{50}).[24]

Generally, chemicals with low bioactive concentrations are often more desirable. In the drug development process, SI serve as an important parameter used to determine whether further investigations should be conducted.[15] Based on the classification criteria carried out in previous research,[25] a compound is considered selective when SI value is ≥ 3. A lower SI of ≥ 2 but < 3 indicate a bioactive but less selective compound, and those with a value of < 1 are often considered as toxic. The IC_{50} and CC_{50} values were used to calculate the SI, as shown in Figure 1. According to these criteria, 5-*O*-Acetylpinostrobin showed potential as anticancer agent against T47D cells and selectivity over Vero cells.

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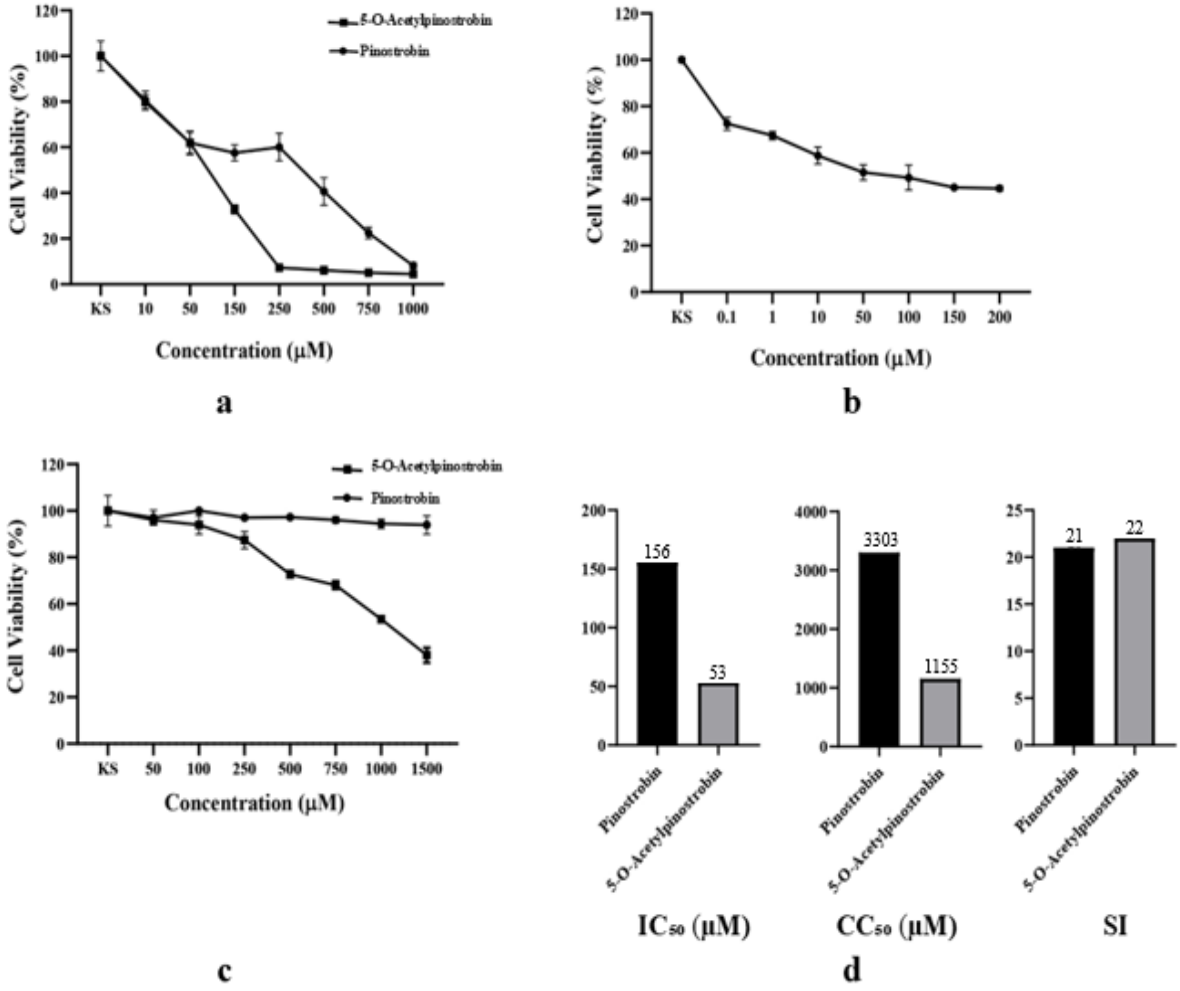

Figure 1: Cytotoxic effect of pinostrobin, 5-*O*-Acetylpinostrobin, and 5-fluorouracil using MTT assay. (a) Effect of pinostrobin and 5-*O*-Acetylpinostrobin on T47D cell viability (b) Effect of 5-fluorouracil on T47D cell viability (c) Effect of pinostrobin, 5-*O*-Acetylpinostrobin on vero cell viability, (d) Comparison between IC_{50}, CC_{50}, and SI values of 5-*O*-Acetylpinostrobin and pinostrobin

In silico study results

Docking method validation

The analytical method validation was carried out by redocking native ligand 4,4'-[(1Z)-1-(4-ethoxyphenyl)but-1-ene-1,2-diyl]diphenol. This process aimed to compare the position of the native ligand on the target protein to that of the test ligands. Redocking results of native ligand against ERα with PDB ID: 5T1Z gave RMSD values of 0.652 Å, 0.669 Å, and 0.694 Å, respectively. This indicated that the conformation of the test ligand obtained through redocking was similar to the native ligand. When the RMSD value is less than 2 Å, it means that the test ligand conformation successfully passed the validation.[26-29]

Molecular docking

Molecular docking was conducted using 5-*O*-Acetylpinostrobin and pinostrobin tested on ERα with PDB ID: 5T1Z (Table 1). In this context, T47D cells, characterized by overexpression of ER and progesterone receptor (PR), served as molecular docking model. ERα is a type of steroid receptor located in nucleus of cells and is responsible for regulating gene expression in cancer development through transcription factors and signal transduction pathways.[17,30]

The binding affinity values in Table 2 showed that both test compounds were capable of inhibiting ERα in silico. However, 5-*O*-Acetylpinostrobin showed better activity compared to pinostrobin, as proven by the binding affinity values of -7.98 kcal/mol and -7.11 kcal/mol, respectively. The high conformational stability of ligand complex with the target receptor was shown by a lower binding affinity value.[18,31-33] Furthermore, the inhibition constant values obtained were 1.41 µM and 6.18 µM for 5-*O*-Acetylpinostrobin and pinostrobin, respectively. Smaller values indicate that the chemical bind more strongly to the receptor.[18,34]

The interactions of both compounds were evaluated with identical amino acid residues as the native ligand. 5-*O*-Acetylpinostrobin interacted with Leu384, Met343, Thr357, Glu353, Leu540, and Leu525, while pinostrobin interacted with Leu384, Met343, Thr347, Met528, Leu536, Leu540, Phe425, and Ile424. The highest bond energy between pinostrobin pentanoate and receptor contact was also provided by the hydrophobic bond, Van der Waals forces. The results showed that interactions with hydrophobic amino acids dominated the active site of the receptor.[27] These bonds minimized the exposure of nonpolar residues to water, enhancing hydrophobic effect. Hydrophobic interactions contributed to enhancing the stability of the binding between the ligand and receptor.[33] Molecular docking results suggested that 5-*O*-Acetylpinostrobin and pinostrobin had optimum interactions with ERα (PDB ID: 5T1Z), as shown in Table 3.

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Table 1: 2D and 3D structures of test ligands

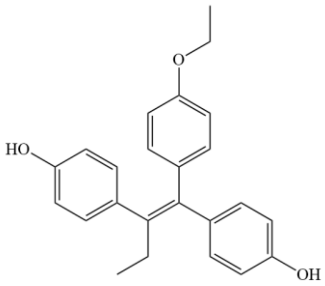
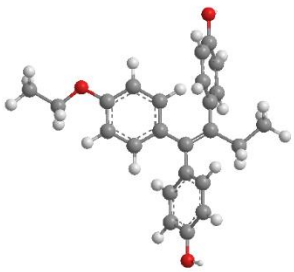
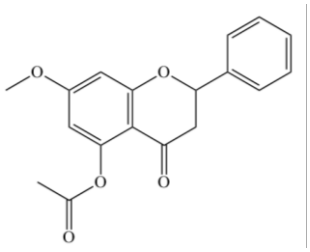
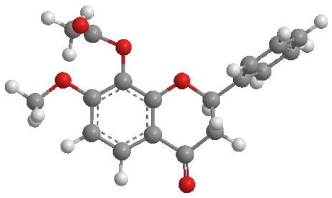
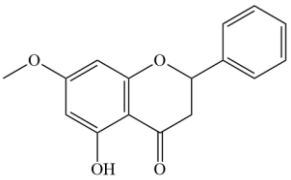
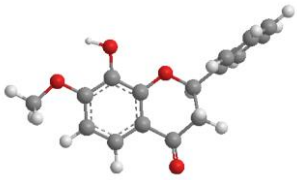
Ligand	2D Structure	3D Structure
Native ligand 5T1Z		
5-*O*-Acetylpinostrobin		
Pinostrobin		

Table 2: Molecular docking results of 5-*O*-Acetylpinostrobin and pinostrobin with estrogen receptor (ERα) with PDB ID: 5T1Z

Ligand	Binding Affinity (kcal/mol)	Inhibition Constant (μM)	Amino Acid Residue
Native ligand 5T1Z	-11.95	0.00174	**Leu384[a], Met343[a], Thr347[a], Met528[a], Leu536[a], Glu353[b], Leu540[g], Leu525[g], Phe425[g], Ile424[g], Leu346[a], Ile424[a], Phe425[a], Leu387[b], Arg394[b], Met421[c], Phe404[d], Met528[g], Met343[g], Leu536[g]**
5-*O*-Acetylpinostrobin	-7.98	1.41	**Leu384[a], Met343[a], Thr347[a], Leu540[g], Glu353[b], Leu525[g], Leu428[a], Trp383[a], Phe404[a], Leu387[e], Ala350[g], Leu 391[g], Met388[g]**
Pinostrobin	-7.11	6.18	**Leu384[a], Met343[a], Thr347[a], Leu540[g], Phe425[g], Ile424[g], Leu42[a], Trp383[a], Phe404[a], Arg394[a], Leu349[a], His524[b], Leu346[f], Leu525[f], Ala350[g], Met421[g]**

Description: Van der waals[a], Conventional Hydrogen Bond[b], Pi-sulfur[c], Pi-Pi T-shape[d], Amino-Pi stacked[e], Pi-sigma[f], Alkyl/ Pi-Alkyl[g]. Bold indicates similarity of interaction with the same amino acid as the native ligand.

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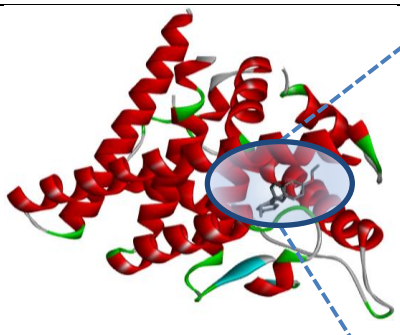
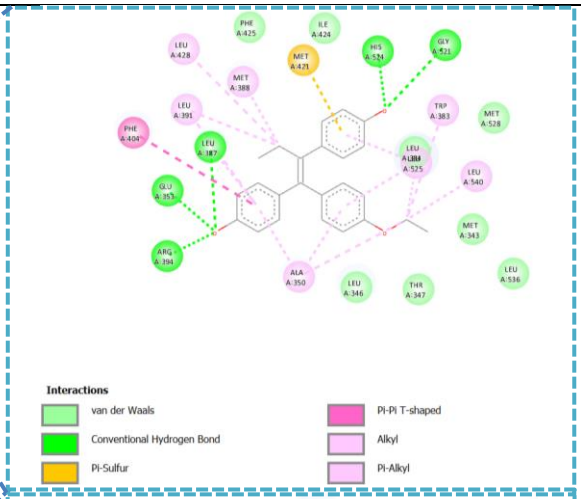
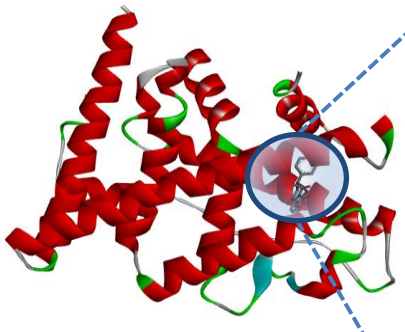
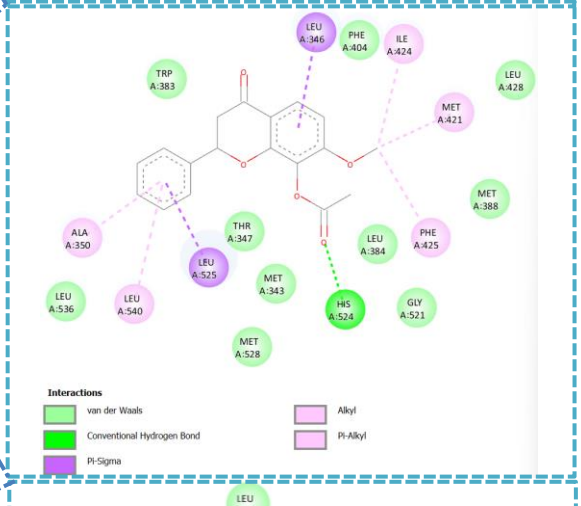
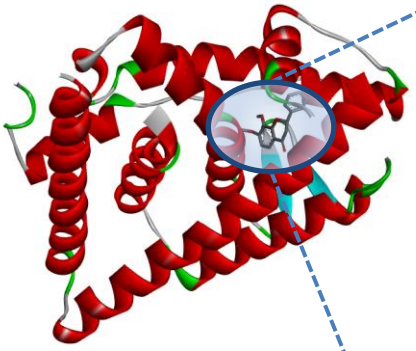
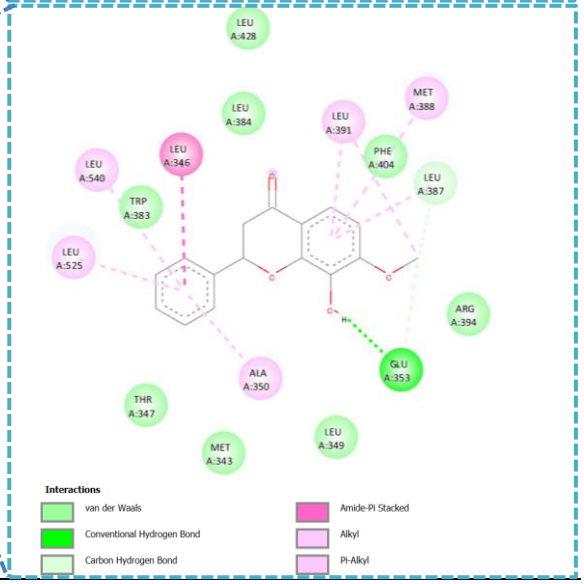
Table 3: The 3D and 2D Visualization of molecular docking interactions between ligands and estrogen receptor (ERα) with PDB ID: 5T1Z

Ligand	3D Visualization	2D Visualization
Native ligand 5T1Z		
5-*O*-Acetylpinostrobin		
Pinostrobin		

Conclusion

The results of the study showed that 5-*O*-Acetylpinostrobin has a promising cytotoxic effect against T47D cells and maintains low toxicity toward normal Vero cells. This suggests that 5-*O*-Acetylpinostrobin has the potential to be developed as a selective chemotherapeutic agent for the treatment of breast cancer. However, further research, including *in vitro*, *in vivo*, and analysis of molecular mechanism of action should be carried out to explore the benefits and safety of the compounds.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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