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Inhibition breast carcinogenesis via PI3K/AKT pathway using bioactive compounds of Strychnine tree (*Strychnos nux-vomica*): *in silico* study

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ABSTRACT

Breast cancer poses a significant global health challenge, with a notable prevalence in Indonesia. Given the intricate nature of breast cancer progression and classification, precise treatment strategies are imperative, particularly targeting signaling pathways like PI3K/AKT, pivotal in cell growth, proliferation, survival, and apoptosis. Bioactive compounds from the Strychnine tree demonstrate potential in enhancing apoptotic effects and inhibiting breast carcinogenesis. This potential is explored through *in silico* studies. This research aims to analyze potential targets of Strychnine tree compounds, along with binding energy and stability between ligands and receptors. Employing bioinformatics target analysis, molecular docking, and molecular dynamics simulation, the study reveals AKT1 as a potential target of Strychnine tree compounds. These compounds inhibit AKT1 at both active and allosteric sites, displaying notably low binding energy scores. For example, brucine exhibits a binding energy of -10.83 kJ/mol at the active site, surpassing the standard capivasertib. However, lupeol, with a binding energy of -11.14 kJ/mol, falls short of the MK-2206 standard at the allosteric site. Molecular dynamics simulations expose fluctuations in parameters like RMSD, RMSF, and binding energy within the initial 5 ns. In conclusion, Strychnine tree compounds, such as brucine and lupeol, showcase potential AKT1 inhibition at both active and allosteric sites, enhancing apoptotic effects. However, the stability of these compounds in binding to their receptors within the first 5 ns of the simulation warrants further investigation for prolonged interactions.

Keywords: PI3K/AKT pathway, AKT1, strychnine tree compounds, target analysis, molecular docking, molecular dynamics

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INTRODUCTION

Breast cancer is a major global health issue, with a notable proportion of cases reported from Indonesia. According to a research published by the International Agency for Research on Cancer (IARC), 16.6% of all new instances of cancer in Indonesia are breast cancer, out of a total of 396,914 cases. The report also shows the percentage of deaths due to breast cancer in Indonesia to be 9.6%. IARC also reports that breast cancer patients worldwide, including in Indonesia, are predominantly women over the age of 40 (Global Cancer Observatory 2020; Global Cancer Observatory 2021).

The intricate nature of breast cancer's progression and classification necessitates precise treatment strategies that target the signaling pathways involved in breast carcinogenesis, one of them being the PIP3K/AKT pathway. A key component of cell development, proliferation, survival, and apoptosis is the PI3K/AKT pathway. Research on the application of targeted therapy on the PI3K/AKT pathway is also substantial. The utilization of bioactive chemicals obtained from plants, such as those from the strychnine tree, has been increasingly investigated in the development of medications for breast cancer (Martorana et al., 2021; Saraswati & Agrawal, 2013; Victor et al., 2016; Yuan et al., 2023; Zhu et al., 2022).

The Strychnine tree (*Strychnos nux-vomica*) is known to contain compounds such as alkaloids, iridoid glycosides, triterpenoids, and organic acids like strychnine, brucine, adenosine, lupeol, catechol, and maltol that have the potential to inhibit breast carcinogenesis (Chen et al., 2014; Enkhtaivan et al., 2015; Joy et al., 2016; Saraswati & Agrawal, 2013; Victor et al., 2016; Zhang et al., 2012). The majority of the Strychnine tree's anti-cancer studies have been carried out both in vitro and in vivo. Additional study is required to assess the pharmacological processes, efficacy, precise receptor targets, and stability of receptor interactions of strychnine tree substances, notwithstanding the encouraging results of these studies on their anti-cancer activities. Further research is crucial to create formulations that are safer and more effective (Eldahshan & Abdel-Daim, 2015; Guo et al., 2018; Lu et al., 2020).

Numerous studies have been carried out using *in silico* technology to find and develop new medications, including those for breast cancer. Through *in silico* research, predictions can be made about the activity of a compound with receptor targets involved in cellular biological processes and the pathways related to that activity (De Vivo et al., 2016; Hermawan, et al., 2021; Hermawan, et al., 2021; Hermawan, et al., 2021; Roy et al., 2022; Roy et al., 2016). *In silico* research has proven to complement the development of new, specific, and safe drugs in the pharmaceutical field The predictive results of these *in silico* studies can serve as hypotheses for further research, especially in the field of oncology.

The research by Zhou et al. (2020) indicates that *Strychnos nux-vomica* has the potential as an anticancer agent by binding to several potential targets such as AKT1, EGFR, ALB, MAPK1, EGF, VEGFA, CCND1, SRC, Jun, Casp3, HSP90AA1, ESR1, MAPK8, and FN1. However, this study did not analyze the potential binding of strychnine tree compounds to these receptor targets or the stability of compound-protein interactions. Another study using strychnine tree compounds was conducted by Reynaldi & Setiawansyah (2022), indicating that the compounds strychnine, brucine, and secoxyloganin from the plant *Strychnos lucida* R.Br have the potential to bind to the estrogen α receptor but have affinity energies no better than 4-hydroxytamoxifen. However, this study did not conduct potential target analysis beforehand. The finding by Zhou et al. (2020) and Reynaldi & Setiawansyah (2022) indicate that certain strychnine tree chemicals may block receptors connected to the PI3K/AKT pathway, thus further we is needs to be conducted.

Based on the above considerations, we are interested in conducting *in silico* research on compounds from strychnine trees that may prevent breast cancer by blocking the PI3K/AKT pathway. We will explore potential targets, binding energies, and the stability of ligand-receptor interactions for compounds such as strychnine, brucine, adenosine, lupeol, catechol, and maltol. These compounds are primarily found in the seeds of the strychnine tree and are available in databases. Through the PI3K/AKT pathway, they may be able to prevent breast cancer.

MATERIALS AND METHOD

Materials

The hardware used in this research included a Lenovo ThinkPad T460s laptop with an Intel Core i7 6th gen processor, 8 GB RAM, 256 GB SSD, and Windows 10. The software used includes applications such as Cytoscape, Autodock tools/MGL tools, MarvinSketch, YASARA Dynamics, YASARA View, and Biovia Discovery Studio Visualizer. Additionally, web servers such as http://stitch.embl.de, https://string-db.org/, https://bioinfogp.cnb.csic.es/tools/venny/, https://www.ncbi.nlm.nih.gov/, http://www.swisstargetprediction.ch/, and https://pubchem.ncbi.nlm.nih.gov/ were utilized. The compounds investigated in this study were strychnine, brucine, adenosine, lupeol, catechol, and maltol from the strychnine tree.

Methods

Potential target analysis

The search for Direct Target Proteins (DTP) of active compounds from *S. nux-vomica* was conducted using STITCH (http://stitch.embl.de). The search for Indirect Target Proteins (ITP) of active compounds from S. nux-vomica was performed using STRING (https://string-db.org/). Target proteins not found in STITCH and STRING were searched using Swiss Target Prediction (http://www.swisstargetprediction.ch/). Breast cancer gene targets were searched using the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/). Potential Target Therapeutic Genes (PTTG) were identified using Venn diagrams on the Venny 2.1 server (https://bioinfogp.cnb.csic.es/tools/venny/). A Protein-Protein Interaction (PPI) Network was constructed using STRING (https://string-db.org) with a confidence score > 0.7 and visualized using the Cytoscape software. Genes with a degree score greater than 10 were analyzed using the CytoHubba plugin (Brown et al., 2015; Chin et al., 2014; Daina et al., 2019; Hanif, et al., 2021; Kuhn et al., 2014; Liao et al., 2019; Szklarczyk et al., 2015).

Molecular docking simulation

The RCSB PDB (https://www.rcsb.org/) provided the target proteins. Before conducting molecular docking simulations, validation was performed using native ligands obtained from PDB (PDB ID: 3MVH for active site and PDB ID: 3O96 for allosteric site). Ligands obtained from https://pubchem.ncbi.nlm.nih.gov/ in sdf format were converted to pdb format using https://cactus.nci.nih.gov/translate/. Ligand files were opened in the MarvinSketch application and their conformers were calculated using the MMFF9 force field Modified (Hanif et al., 2020; Surya & Praveen, 2021). With Biovia Discovery Studio Visualizer, the receptor from RCSB PDB was prepared. Next, using the Autodock Tools program, the native ligand was docked to the target receptor in order to look for 3D conformations. The studied result is shown as RMSD, and a value of less than 2 Å is accepted as indicative of a reliable molecular docking outcome. Using the Autodock Tools tool, the test compounds were docked to the target protein taking into account the binding site grid box size in Angstrom as well as the center of mass coordinates of the structure. Each compound's binding energy value was determined using the LGA parameter. Using the DS Biovia tool, the result with the lowest possible binding energy was chosen for additional amino acid residue visualization and analysis. Modified from Tapiory et al. (2020), Al-Khodairy et al. (2013), Rizvi et al. (2013) and Zardecki et al. (2016).

Molecular dynamics simulation

YASARA Dynamics software was used for molecular dynamics simulations, employing AMBER14 as the force field. Na^+ and Cl^- ions were added for neutralization at physiological pH 7.4. The TIP3P water model was used for the solvation step. Preparation steps included minimization and heating to 310K. For 5 ns, simulations with a time step of 2.5 fs were run in an NPT ensemble at constant temperature and pressure, followed by a production step for 100 ps. The results of RMSD, RMSF, and binding energy were analyzed using Ms. Excel (Ouassaf et al., 2021).

Data Analysis

The data analysis employed is descriptive analysis to illustrate and elucidate data from bioinformatics target analysis, molecular docking, and molecular dynamics simulations of the strychnine tree in inhibiting AKT1 in breast carcinogenesis.

RESULT AND DISCUSSION

In silico studies on bioactive compounds from the strychnine tree can demonstrate how these bioactive compounds may be able to stop breast cancer from developing by blocking the PI3K/AKT pathway. This *in silico* study encompasses the search for potential targets, molecular docking simulations, and molecular dynamics simulations.

Potential target analysis

After obtaining target proteins from STITCH, STRING, and Swiss Target Prediction, an analysis was conducted to obtain PTTG (Potential Therapeutic Target Gene). The results indicated that out of 654 target proteins and 5326 breast cancer genes, 369 PTTGs were obtained (Figure 1). These results led to the construction of a proein-protein interaction network using STRING for additional analysis. Cytoscape was used to find the top 10 hub genes with CytoHubba plugin. According to the analysis, the top 10 hub genes are those with the highest level of interaction. Bioinformatics target analysis shows that bioactive compounds from the strychnine tree have the potential to interact with target proteins, including TP53, AKT1, GAPDH, IL6, CTNNB1, EGFR, ESR1, INS, SRC, and PTEN (Figure 2).

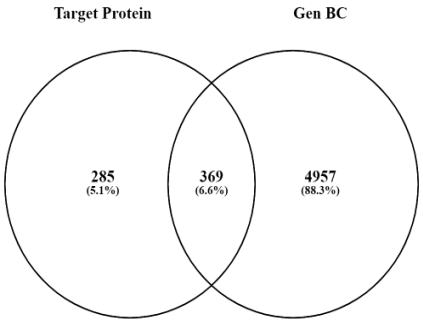


Figure 1. Results of PTTG (Potential Therapeutic Target Gene) of bioactive compounds from the strychnine tree

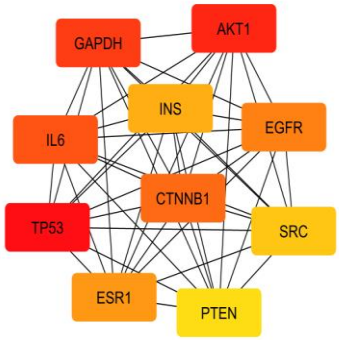


Figure 2. Top 10 genes that interact most intensely from Cytoscape analysis.

Explanation: TP53 (Tumor Protein 53), AKT1 (Serine/Threonine Kinase 1), GAPDH (Gyceraldehyde 3-Phosphate Dehydrogenase), IL-6 (Interleukin-6), CTNNB1 (Catenin (Cadherin-Associated Protein) Beta 1), EGFR

(Epidermal Growth Factor Receptor), ESR1 (Estrogen Receptor 1), INS (Insulin), SRC (Protein Kinase Src), PTEN (Phosphatase and Tension homolog deleted on chromosome ten)

The analysis results with Cytoscape indicate that the more intense the red color formed, the stronger the potential of that protein as a target for bioactive compounds. This is based on the degree of interaction between proteins and compounds obtained from databases like STITCH, STRING, and Swiss Target Prediction, which predict protein-compound, protein-protein and compound-compound interactions based on supporting information and evidence from text mining, experiments, databases, co-expression, neighborhood, gene fusion, and co-occurrence (Chin et al., 2014; Kuhn et al., 2014; Szklarczyk et al., 2015).

Targeting TP53 as a direct target protein (DTP) indicates a strong association with bioactive compounds derived from the strychnine tree. TP53 is a target protein obtained from STITCH, which analyzes the potential interactions between compounds and proteins. The results obtained indicate that TP53 has a strong potential interaction with compounds from the strychnine according to the supporting information that is currently accessible (Hermawan et al., 2020; Kuhn et al., 2014). However, TP53 as a tumor suppressor gene (TSG) has a high mutation rate, making it less suitable as a drug target (Bull & Doig, 2015; Giacomelli et al., 2018; Gonzalez-Garcia & Mlachila, 2017). Therefore, the next potential target for inhibiting breast cancer development is *AKT1*. This finding suggests that this putative target is connected to the PI3K/AKT pathway (Martorana et al., 2021).

Interfering with AKT can increase apoptosis and negatively impact cell growth, proliferation, and survival by disrupting the PI3K/AKT/mTOR signaling pathway. Cell growth and survival are decreased by AKT inhibition, which controls a number of protein molecules and transcription factors including mTOR, cyclin D, and CDK4/6. AKT also regulates mTOR, 4E-BP1, eIf4E, and P70S6K1, which can reduce cell proliferation. Moreover, AKT regulates Bad, Bcl-2, and Bcl-xL to enhance apoptosis (Dong et al., 2021; Fusco et al., 2021; Hein et al., 2014; Liu et al., 2020; Tapia et al., 2014). Protein AKT1 was then used as the receptor target in molecular docking simulations.

Molecular docking simulation

To comprehend how the ligand and receptor interact, as well as to anticipate the binding energy or affinity, molecular docking simulation was used (Pantsar & Poso, 2018). Since AKT1 is a crucial component of multiple signaling pathways involved in cell growth, proliferation, survival, and metabolism, targeting it is one tactic to slow the advancement of breast cancer (Hua et al., 2021; Martorana et al., 2021; Zhu et al., 2022). On the active and allosteric locations, molecular docking simulations were run. The PDB file 3MVH was utilized by the active site with the standard drug capivasertib, while the allosteric site used the PDB file 3O96 with the standard drug MK-2206. Both active and allosteric sides showed validation results with RMSD below 2 Å. represents the binding energy of the native ligand at -11.22 kJ/mol when it binds to AKT1 at the active site. The binding energy of the standard, capivasertib, is -10.56 kJ/mol. With a binding energy of -10.83 kJ/mol, brucine is the strychnine tree compound with a higher binding energy than the standard compound. At the allosteric site, the natural ligand having a binding energy of -12.82 kJ/mol with AKT1. The binding energy of the used standard, MK-2206, is -11.39 kJ/mol. Lupeol, with a binding energy of -11.14 kJ/mol, is the strychnine tree compound with the lowest value binding energy, while still not much better than the standard. Different types of interactions between ligands and receptors affect the magnitude of affinity energy (Arthur et al., 2021).

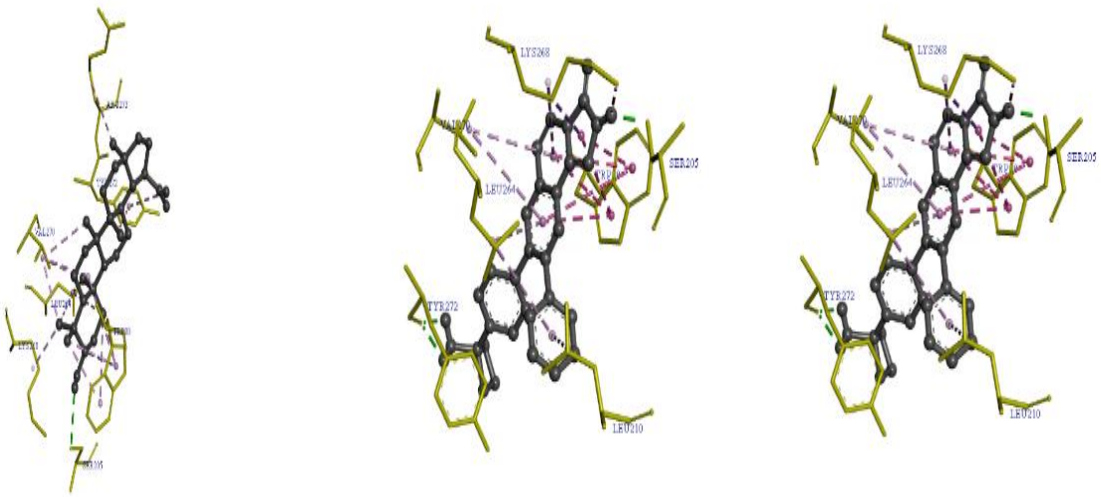
Ionic, hydrogen bonding, hydrophobic, and Van Der Waals interactions are a few examples of the chemical interactions that have a substantial impact on binding energy. Phi (π) interactions can stabilize ligand conformations and facilitate the insertion of ligand structures into the binding pocket on the receptor protein, whereas carbon hydrogen bonds are weak interactions. High hydrogen bonding is known to contribute significantly to the low binding energy produced (Arthur et al., 2021; Pantsar & Poso, 2018). Brucine and capivasertib compounds have one hydrogen bonding interaction each, at residues Lys158 and Glu228, respectively (Figure 3). While lupeol has one hydrogen bonding

interaction at residue Ser205, MK-2206 has two hydrogen bonding interactions at residues Tyr272 and Ser205 (Figure 4).

Table 1. Results of molecular docking simulation for the active and allosteric sites of AKT1 in the strychnine tree compound

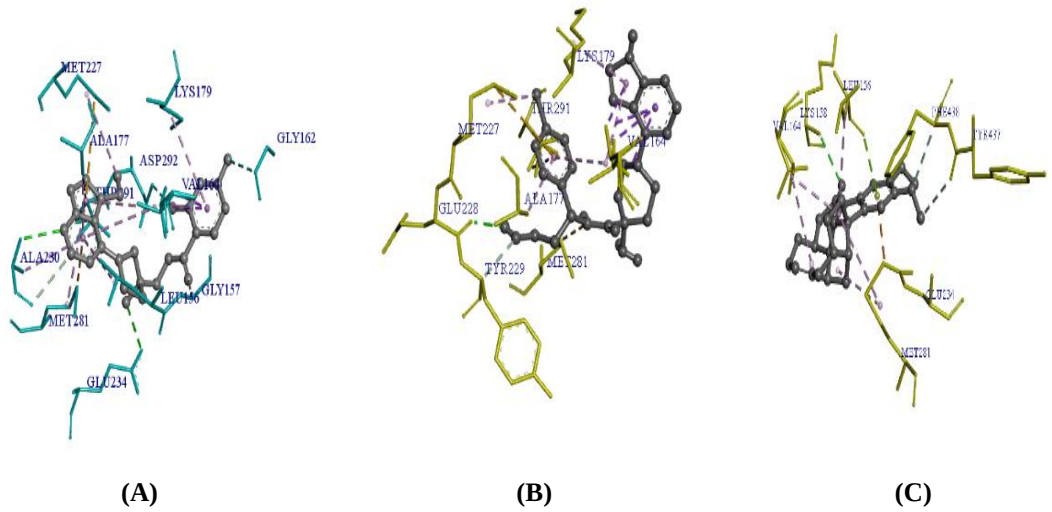
Compound	Binding Energy (kJ/mol)	
	Active Site	Allosteric Site
Native	-11.22	-12.82
Capivasertib	-10.56	-
MK-2206	-	-11.39
Strychnine	-10.19	-9.34
Brucine	-10,83	-10.04
Adenosine	-6.90	-5.76
Lupeol	-9.71	-11.14
Catechol	-4.89	-4.43
Maltol	-4.81	-4.46

Note: Native compound = compound obtained from PDB binding with protein. Standard drug for active site= capivasertib. Standard drug for allosteric site= MK-2206



(A) **(B)** **(C)**

Figure 3. Molecular docking simulation results with AKT1 protein at the active site. (A) Native compound (B) Standard capivasertib. (C) Compound brucine



(A) **(B)** **(C)**

Figure 4. Molecular docking simulation results with AKT1 protein at the allosteric site. (A) native compound. (B) standard MK-2206. (C) compound lupeol

By competing via ATP to bind to AKT at the ATP binding site, inhibitors can inhibit AKT through the active site. Inhibition of AKT by the inhibitor can also occur through the allosteric site by targeting the PH domain and then maintaining AKT in an inactive form by preventing the protein from migrating to the plasma membrane where activation by the upstream kinase occurs (Andrikopoulou et al., 2022). Active site inhibitors have high potential but may have side effects, requiring dose restrictions. Some active site inhibitors of AKT that have been studied are capivasrtib (AZD5363) and ipatasertib (GDC0068), which have undergone clinical trials phases I and II. The efficacy of allosteric inhibitors in clinical studies is still modest despite their greater selectivity and lack of competition. MK-2206 and miransertib (ARQ092) are two examples of AKT allosteric inhibitors. Research suggests that these inhibitors can reduce side effects and toxicity (Landel et al., 2020; Mundi et al., 2016). The interaction between bioactive compounds from the strychnine tree and AKT1 will then be further analyzed for stability through molecular dynamics simulations.

Molecular dynamics simulation

Next, employing YASARA Dynamics software, the outcomes of molecular docking were used to analyze molecular dynamics simulations. In order to gather data regarding the temporal dynamics of interactions between ligands and proteins as well as binding sites, molecular dynamics simulations were run. Furthermore, these simulations were run in a setting that replicated human physiological parameters in order to ascertain the stability of the relationships between these two molecules (Chairunisa et al., 2023; Ivanova et al., 2018). Trajectory analysis employed Root Mean Square Deviation (RMSD), Root Mean Square Fluctuation (RMSF), and binding energy.

The use of molecular dynamics simulation time varies depending on the type of research. In drug development studies, some molecular dynamics research uses a time period of 20 ns to depict the stability of interactions between ligands and receptors. Other studies also utilize longer times such as 50 ns, 70 ns, 100 ns, up to 200 ns to illustrate the stability of ligand-receptor interactions (Al-Karmalawy et al., 2021; Castro et al., 2008; Fakih et al., 2021; Mohimani et al., 2017; Sakkiah et al., 2013; Wang et al., 2022). However, according to Ouassaf et al. (2021), simulations are conducted for a period of 5 ns to depict the potential of a compound as a drug candidate.

The RMSD evaluations show how a protein alters from its starting structural shape to its final position (Aier et al., 2016). Within the range of 0.514-2.127 Å, the average RMSD of the native (1.577 Å) and capivasertib (1.710 Å) at the active site of AKT1 is lower than the normal RMSD (1.758 Å) and brucine

(1.772 Å). The average RMSD under normal conditions falls within the ligand RMSD range, i.e., 1,577-1,772 Å. The average normal RMSD on the allosteric site (2.070 Å) is lower than the RMSD of lupeol (2.231 Å) within the range of 0.718-2.527 Å. Meanwhile, the average normal RMSD on the allosteric side falls within the ligand RMSD range, i.e., 1.882-2.231 Å (Figure 5A & 5B). Generally, normal RMSD values are higher than when the protein binds to the ligand, indicating that the chemical interactions between the ligand and protein stabilize the interaction of these two molecules. The mean normal RMSD within the ligand's range However, RMSD indicates that the interaction between the ligand and the protein it bonds to has a negligible impact on the stability of protein structure (Kalasariya et al., 2022; Weng et al., 2021).

Amino acid residue fluctuations on the protein during simulation are demonstrated by the RMSF analysis (Kalasariya et al., 2022). The RMSF values at the active site of AKT1 range from 0.72-1.09 Å for Val164 residues and 0.54-0.78 Å for Met281 residues. For Val164 residues, the RMSF of brucine is higher than the normal RMSF. The allosteric site RMSF ranges from 0.740-1.860 Å for Lys268 residues, 0.520-1.590 Å for Trp80 residues, 0.710-1.140 Å for Leu264 residues, 0.740-1.1370 Å for Val270 residues, and 0.750-1.930 Å for Ser205 residues. In general, the allosteric site RMSF under normal conditions is higher than the ligand RMSF, except for Ser205, Leu264, and Val270 residues where the RMSF of lupeol is higher. MK-2206 RMSF at Val270 residues also has a higher value than its normal RMSF (Figure 5C & 5D). The results of RMSF analysis at the active and allosteric sites show varied outcomes. Higher RMSF values indicate that amino acid residues do not have strong interactions at the amino acid binding site with their ligands, indicating suboptimal binding between them. Nonetheless, the low RMSF values obtained are less than 2 Å, suggesting that the ligand and its protein still have a strong level of binding (De Vita et al., 2021; Umar et al., 2023).

Binding energy provides information on the binding affinity formed during the simulation period. The free binding energy (ΔG) that is derived from the combined potential energy of the protein, ligand, and ligand, along with the solvation energy of all three, is known as binding energy (Chen et al., 2015; Riandono & Istyastono, 2023). The results of binding energy analysis at the active site of AKT1 are less favorable within the 5 ns timeframe. The binding energy values within the 5 ns timeframe are negative, with average binding energy values successively -157.001 kJ/mol, -199.920 kJ/mol, and -194.716 kJ/mol for native, capivasertib, and brucine. Similarly, the analysis of binding energy at the allosteric site of AKT1 is less favorable within the 5 ns timeframe, where the binding energy values within the 5 ns timeframe are negative, with average binding energy values successively -47.485 kJ/mol, -69.797 kJ/mol, and -23.752 kJ/mol for native, MK-2206, and lupeol **(**Figure 5E & 5F**).** The results of molecular dynamics analysis indicate low stability between the bioactive compounds from the strychnine tree and the AKT1 protein. Factors influencing this include instability in the initial binding mode, mismatched force field parameters, and the limited simulation time, suggesting that new stability may occur after 5 ns (Kalasariya et al., 2022; Liu et al., 2017).

Simulations of molecular dynamics was out with distinct proteins for the allosteric site (PDB ID: 3O96) and the active site (PDB ID: 3MVH). Overall, molecular dynamics simulation results show that the stability of protein conformations during the simulation period is largely unaffected by the ligand-protein interaction, both at the active and allosteric sites (Kalasariya et al., 2022; Weng et al., 2021). The ligand and its protein bind relatively strongly, as evidenced by the RMSF values at the active and allosteric sites (De Vita et al., 2021; Umar et al., 2023). At the conclusion of the simulation, if the values are still changing, there may be a chance that conformational changes have occurred, indicating less stable connections between the ligand and the receptor. This implies that longer simulation times would be required to get better results because the anticipated stability might not materialize for up to 5 ns (Aier et al., 2016; Kalasariya et al., 2022). Less stable interactions with the AKT1 protein are shown by the examination of the energies of all ligands. Positive binding energy values yield positive results in binding energy analysis utilizing YASARA software (Chen et al., 2015).

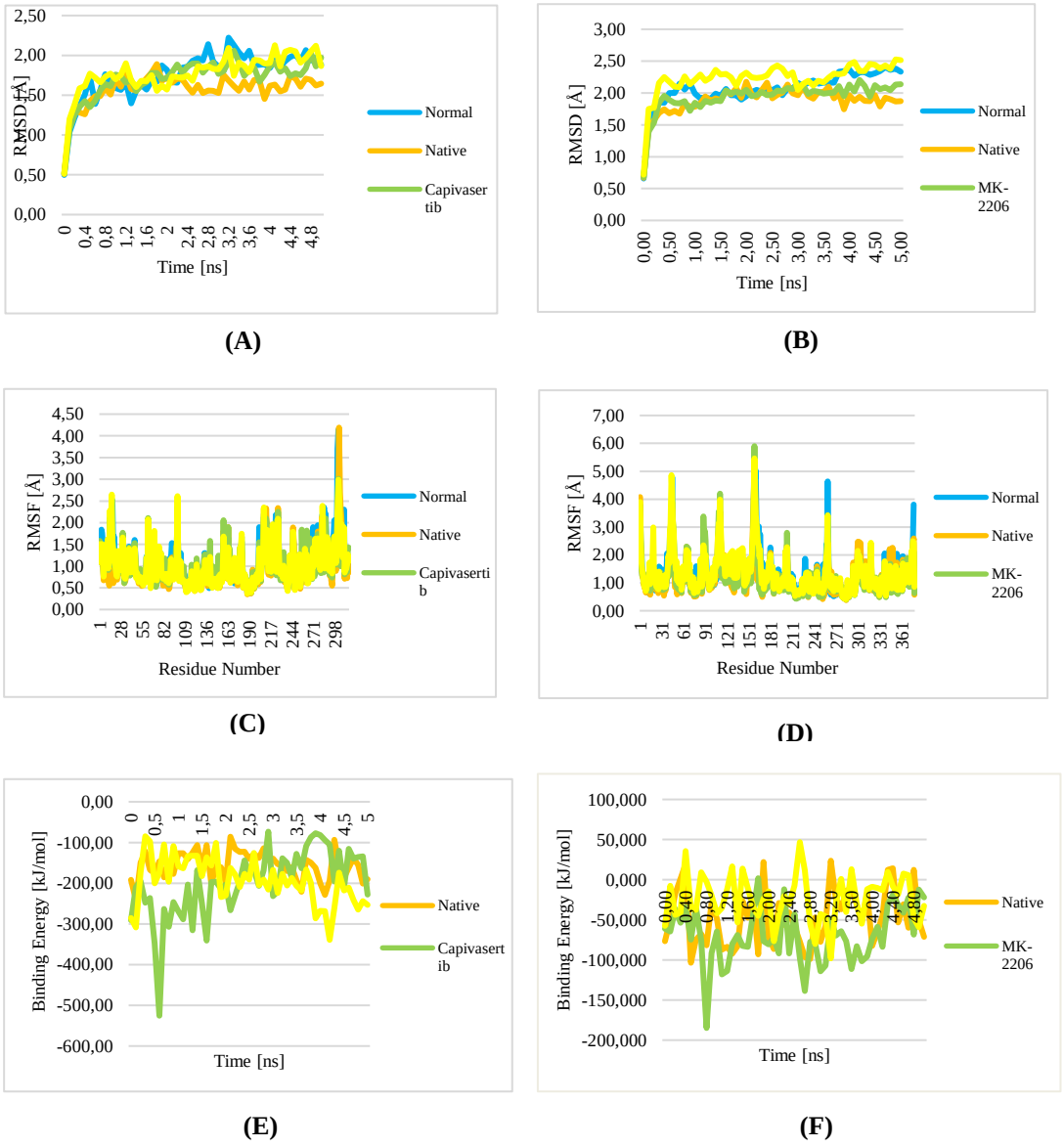


Figure 5. Results of molecular dynamics simulation with AKT1 protein and strychnine tree compounds. (A) analysis result of AKT1 RMSD on the active site. (B) analysis result of AKT1 RMSD on the allosteric site. (C) analysis result of AKT1 RMSF on the active site. (D) analysis result of AKT1 RMSF on the allosteric site. (E) analysis result of AKT1 binding energy on the active site. (F) analysis result of AKT1 binding eEnergy on the allosteric site

Explanation: Normal: when the protein is not bound to any ligand

CONCLUSION

The potential of bioactive compounds from the strychnine tree (*Strychnos nux-vomica*) can be demonstrated by *in silico* and molecular dynamics research to inhibit breast cancer development through the PI3K/AKT pathway by targeting AKT1. This has the potential to reduce cell proliferation, migration, angiogenesis, and metastasis, as well as enhance apoptotic effects. AKT1 protein inhibition is feasible at both the active and allosteric sites. However, the stability of the interaction between bioactive

compounds from the strychnine tree and AKT1 still shows less favorable results due to the limitations of the tools used, resulting in a simulation time of only 5 ns. Further research is needed using a 20 ns or more time frame for molecular dynamics simulation. To further corroborate the results of this investigation, in vitro and in vivo experiments can be carried out.

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