Rosmarinic Acid from *Orthosiphon aristatus* Potentially Targets Estrogen Receptor-Alpha in Breast Cancer: *In-silico* Study

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Abstract

Breast cancer is the most common cancer among women. Tamoxifen, a widely used estrogen receptor-alpha (ER-α) inhibitor, is effective but often causes side effects, necessitating the search for alternative inhibitors from natural sources. *Ortosiphon aristatus*, also known as cat's whiskers, is a medicinal plant traditionally valued for its anti-inflammatory and antioxidant properties. Recent studies suggest its bioactive compounds may exhibit anticancer activity by inducing apoptosis in cancer cell lines. This study explores the potential of *O. aristatus* metabolites as ER-α inhibitors using computational approaches. Nine metabolites were assessed for their physicochemical properties based on Lipinski's rule of five and ADMET predictions, followed by pharmacophore-based virtual screening with LigandScout and molecular docking with AutoDock. The results showed that all tested compounds complied with Lipinski's rule, and most met ADMET criteria. Among these, rosmarinic acid was identified as one of the hit compounds based on pharmacophore screening, exhibiting binding interactions comparable to 4-hydroxytamoxifen with the ER-α amino acid residues HIS524 and GLY521. It also demonstrated a binding energy of -8.02 kcal/mol and a low inhibition constant (Ki) of 1.31 μM. These findings highlight the potential of *O. aristatus* and rosmarinic acid for further evaluation as candidates against ER-α in breast cancer cells.

Keywords: *breast cancer, estrogen receptor-alpha,* Orthosiphon aristatus, in silico.

INTRODUCTION

Breast cancer is a condition where there is uncontrolled cell growth in the breast tissue, causing a lump in the breast area (Angahar, 2017). In 2022, 2.3 million women in the world were affected with breast cancer, and 670,000 women died from breast cancer, based on data from WHO. Data from The Global Cancer Observatory shows that in Indonesia, breast cancer ranks first as the cancer with the most cases, reaching 66,271 cases with more than 22 thousand cases of death.

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One of the protein biomarkers of breast cancer is estrogen receptor alpha (ER-α). Overexpression of ER-α causes abnormal proliferation of breast lobular and ductal epithelium that becomes malignant and forms tumors (Clusan, *et al*., 2023). Therefore, breast cancer therapy can be treated using an ER-α antagonist, such as tamoxifen. Besides acting as an antagonist, tamoxifen can act as an estrogen receptor agonist that can trigger proliferation in the endometrium. This may increase the risk of endometrial cancer after long-term therapy (>2 years), especially in postmenopausal women with pre-existing uterine abnormalities (Hermansyah, *et al*., 2024; Emons, *et al*., 2020). Furthermore, tamoxifen can cause dangerous side effects such as bleeding and endometrial cancer risk. Hence, alternative ER-α antagonists from other resources must be developed (Fauzi, *et al*., 2024; Dermawan, *et al*., 2019). Many studies are looking for ER-a candidates from natural products. Natural products are considered more effective with minimum side effects and are readily metabolized and absorbed in the body (Wangchuk, 2018). Some plants have anticancer activity but need further research for compounds such as cat's whisker (*Orthosiphon aristatus*).

Empirically, *O. aristatus* can be used as a traditional medicine to treat rheumatism, cough, gout, colds, kidney stones, diabetes, and as a diuretic (Rafi, *et al*., 2021). The chemical compounds contained in *O. aristatus* include flavonoids (eupatorin and sinensetin), polyphenols (rosmarinic acid), and others (Septyani & Sinta, 2021). Some of the active compounds contained in *O. aristatus* provide anticancer activity, as listed in Table 1. To obtain new candidate compounds in breast cancer treatment against estrogen receptor alpha, an *in silico* study with a molecular docking method was performed using these bioactive compounds from *O. aristatus*.

Table 1. Anticancer activities of active compounds found in *O. aristatus*.

Compounds	Anticancer Activity	Cancer Cell Line Applied To	IC_{50}	References
Eupatorin	Breast cancer	MCF-7 and MDA-MB-231 cells	5 μg/mL	Razak, *et al*., 2019
Ladanein	Leukemia	K562 cells	10.4±2.0 μM	Alkhatib, *et al.*, 2009
Sinensetin	Breast cancer	T47D cells	159.049±12.9 μg/mL	Arifianti, *et al*., 2020
Salvigenin	Colorectal cancer	HCT-116 cells	1.5 and 1.8 μM	Castano, *et al.*, 2024
Tetramethyl scutellarein	Breast cancer	MCF-7 cells	0.53 μM	Manthey and Guthrie, 2002
5-Hydroxy-6,7,3',4'-tetramethoxyflavone	Glioblastom	U87MG cells T98G cells	78 μM 30.5 μM	Papapetrou, *et al*., 2024
Rosmarinic Acid	Breast cancer	MDA-MB-231 cells MDA-MB-468 cells	321.75±9.75 μM 340.45±7.57 μM	Messeha, *et al.*, 2020
Curcumin	Breast cancer	MCF-7 cells	26.30 μg/mL	Halimatushadyah, *et al*., 2023
7,3',4'-Tri-O-methylluteolin	Breast cancer	MCF-7 cells	8 and 12 μg/mL	Sudh,a *et al.*, 2018

METHODS

Lipinski Rule of Five Prediction

Lipinski Rules of Five prediction was performed to analyze the drug-likeness of the test compound and determine its ability as an oral preparation. The canonical smiles of the test compounds were copied from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). Physicochemical properties, including molecular weight, permeability, and number of hydrogen donors and acceptors, were analyzed using a free online website, Mcule Property Calculator (https://mcule.com/apps/property-calculator/).

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ADMET Prediction

ADMET prediction was carried out to predict the pharmacokinetic profile (absorption, distribution, metabolism, excretion) and toxicity of the test compound. Human Intestinal Absorption (HIA %), CaCo-2, Plasma Protein Binding (PPB %), Blood Brain-Barrier (BBB), mutagenic and carcinogenic potential were performed on PreADMET page https://preadmet.webservice.bmdrc.org/.

Pharmacophore Screening

Pharmacophore screening was done to identify hit test compounds that have similarities with the pharmacophore and provide activity. The target receptor (ER-α) with PDB ID 1SJ0 and the active and decoy databases were downloaded from https://dude.docking.org/targets and prepared by converting the file format of nine test compounds into .ldb. Then, pharmacophore modeling was carried out with LigandScout 4.4.5 using the active database, which had been categorized into clusters, to obtain ten pharmacophore models (Fa'aizah, *et al*., 2024).

Next, pharmacophore validation was carried out using 10 pharmacophore models, the active database marked in green and the decoy database marked in red. After that, the modeling with the best ROC Curve was selected and used with the test compound database to obtain the hit compound.

Preparation of the Ligand and Receptor

The ligands we used were three-dimensional structures of ten test compounds downloaded on https://pubchem.ncbi.nlm.nih.gov/ and optimized through the MM2 function using Chem3D Pro 12. All test compounds were protonated by adding hydrogen atoms. Then, a Gasteiger charge was added using AutoDockTools-1.5.6 and saved in pdbqt format. We also use 4-hydroxytamoxifen, an active metabolite from tamoxifen, as a comparative drug.

The receptor used was ER-α (PDB ID: 1SJ0), downloaded from the Protein Data Bank (https://www.rcsb.org/structure/1SJ0) in .pdb format. This receptor was chosen since this PDB ID was also selected for the pharmacophore screening, ensuring consistency between the docking and screening processes. Using the same PDB ID (1SJ0) allowed us to maintain structural alignment and relevance in evaluating ligand interactions. Furthermore, it was the only available structure in the DUD.E database suitable for studying ER-α inhibitors, making it a reliable and practical choice for our further analysis.

Later, the water molecules on the protein were removed, and the natural ligand was separated from the receptor using the BIOVIA Discovery Studio 2020. Then, using AutoDock 4.2.6, ER-α was protonated by adding hydrogen atoms (polar only) and given a Kollman charge. The results of the receptor preparation were saved in pdbqt format.

Docking Validation

Molecular docking was validated through a redocking procedure of the native ligand to the receptor using the Lamarckian genetic algorithm with 100 runs in AutoDock. The parameter observed from this process is the root mean square deviation (RMSD) value. The RMSD value of the ligand position after the redocking procedure should be lower than 2.0 Å.

Molecular Docking

Molecular docking simulations of test compounds as ligands and ER-α as macromolecules were performed in AutoDock using the Lamarckian Genetic Algorithm with 100 runs. The coordinates used are the best grid coordinates obtained from the previous validation, which were Grid Box Size (x = 30.885; y = -1.067; Z = 23.464 Å), Grid Coordinate (x = 40; y = 40; z = 40 Å) with a distance of 0.375 Å. The parameters observed were binding energy and inhibition constant. Then, visualization was carried out using the BIOVIA Discovery Studio to identify the bond between the ligand and the receptor and the amino acids involved in the interaction.

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RESULTS

Lipinski Rule of Five Prediction

First, the physicochemical properties of all bioactive compounds listed in Table 1 were evaluated using Lipinski's rule of five. A compound is considered to comply with the Lipinski rule if it meets at least three out of four criteria: molecular weight <500 Da, log octanol/water partition coefficient (Log *P*) <5, hydrogen bond donors ≤5, and hydrogen bond acceptors ≤10 (Lipinski, 2004). The analysis revealed that all tested compounds satisfied all Lipinski criteria (Table 2), indicating their potential for further development as candidates for oral administration.

Table 2. Lipinski's rule of five screening results for bioactive compounds in *O. aristatus*.

Compounds	Molecular Weight (<500 Da)	Log *P* (<5)	Hydrogen Bonds		Result
			Donor (<5)	Acceptor (<10)	
Eupatorin	344.31	2.90	2	7	Meet the criteria
Ladanein	314.29	2.89	2	6	Meet the criteria
Sinensetin	372.37	3.50	0	7	Meet the criteria
Salvigenin	328.32	3.19	1	6	Meet the criteria
Tetramethyl Scutellarein	342.34	3.49	0	6	Meet the criteria
5-Hydroxy-6,7,3',4'-tetramethoxyflavone	358.34	3.20	1	7	Meet the criteria
Rosmarinic Acid	360.31	1.76	5	8	Meet the criteria
Curcumin	368.38	3.37	2	6	Meet the criteria
7,3',4'-Tri-O-methylluteolin	328.32	3.19	1	6	Meet the criteria

ADMET Prediction

The pharmacokinetic predictive analysis of bioactive compounds in *O. aristatus* revealed that all compounds, except rosmarinic acid (moderate absorption), satisfied the criteria for human intestinal absorption (%HIA). Sinensetin exhibited the highest %HIA at 98.89. The compounds' permeability in the gut was evaluated using CaCo-2 values, which serve as an in vitro model of human colon carcinoma. A compound is considered to have good permeability with a CaCo-2 value ≥70 nm/sec, while values between 4 and 70 nm/sec indicate moderate permeability (Ghannay, *et al*., 2020; Abdurrahman, *et al*., 2021). All tested compounds demonstrated moderate permeability, with tetramethyl scutellarein showing the highest CaCo-2 score of 53.769.

The parameters used to assess distribution included plasma protein binding (%PPB) and blood-brain barrier (BBB) permeability. The %PPB describes how well a compound binds to plasma proteins for distribution in the bloodstream. For optimal distribution and pharmacological effects, compounds should not bind too strongly to plasma proteins, as this can lead to safety concerns and side effects (Suherman and Maulidya, 2023). All test compounds had %PPB values below 90%, indicating weak binding to plasma proteins. Regarding BBB permeability, compounds were categorized as well absorbed (BBB >2), moderately well absorbed (BBB 0.1–2), or poorly absorbed (BBB <0.1) (Deli, 2011). Rosmarinic acid was the only compound moderately well absorbed at the BBB, while the remaining compounds were poorly absorbed. Toxicity profiling assessed the mutagenicity and carcinogenicity of the test compounds. Mutagenicity was predicted using the Ames test, which evaluates the growth of several strains of

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Salmonella typhimurium requiring histidine for survival (Hengstler, 2001). All test compounds, except curcumin, were predicted to be mutagenic. Based on ADME-Tox predictions, ladanein was identified as carcinogenic in mice, while all compounds were predicted to be carcinogenic in rats (Table 3). Therefore, the bioactive compounds in *O. aristatus* exhibited favorable pharmacokinetic profiles, but further *in vivo* validation is necessary to address those pharmacokinetic profiles.

Table 3. ADME and toxicity prediction results for bioactive compounds in *O. aristatus*.

Compounds	Absorption		Distribution		Toxicity		
	HIA (%)	CaCo-2 (nm/sec)	PPB (%)	BBB	Mutagen	Carcinogen	
						Mouse	Rat
Eupatorin	93.49	7.14	85.46	0.03	Mutagen	-	+
Ladanein	93.37	9.77	87.68	0.08	Mutagen	+	+
Sinensetin	98.89	51.22	86.24	0.02	Mutagen	-	+
Salvigenin	96.49	33.06	87.41	0.02	Mutagen	-	+
Tetramethyl Scutellarein	98.44	53.77	88.12	0.06	Mutagen	-	+
5-Hydroxy-6,7,3',4'-tetramethoxyflavone	96.81	30.48	85.83	0.01	Mutagen	-	+
Rosmarinic Acid	62.49	20.25	86.24	0.10	Mutagen	-	+
Curcumin	94.40	20.07	88.03	0.09	Non- mutagen	-	+
7,3',4'-Tri-O-methyl luteolin	96.49	31.47	87.12	0.02	Mutagen	-	+

Pharmacophore Screening

Among the ten pharmacophore models generated, models 1 and 2 demonstrated the best performance, yielding 184 hit compounds and the highest AUC value of 96% compared to the other models. Out of the nine test compounds, five were identified with functional groups that closely matched the interaction patterns of the target receptor (Table 4). The highest fit score was observed for ladanein (46.02), while the lowest was recorded for rosmarinic acid (44.31). These findings suggest that the chemical properties of these ligands align well with the features of the native ligand structure-based pharmacophore model.

Table 4. Pharmacophore screening results for bioactive compounds in *O. aristatus*.

Compounds	Pharmacophore Fit Score
Ladenin	46.02
Eupatorin	44.55
Salvigenin	44.52
5-Hydroxy-6,7,3',4'-tetramethoxyflavone	44.37
Rosmarinic Acid	44.31

Molecular Docking

The molecular docking validation results showed an RMSD value of 0.84˚Å, indicating the method is valid. All test compounds exhibited negative binding energy values in the molecular docking study involving the bioactive constituents in *O. aristatus*, suggesting spontaneous activity to interact against ER-α. However, the binding energy values of the test compounds were all higher than the bond energy value of 4-hydroxytamoxifen, which was -11.31 kcal/mol. Furthermore, none of the test compounds had an inhibition constant value lower than 5.12 nM. Among the nine test compounds, 7,3',4'-Tri-O-methylluteolin had the lowest inhibition constant at 0.38282 μM, followed by rosmarinic acid at 1.31 μM (Table 5). The compound that interacts with amino acids similar to the 4-hydroxytamoxifen was rosmarinic acid, which has hydrogen bonds GLY521 and HIS524 (Figure 1).

Table 5. Molecular docking results of bioactive compounds in *O. aristatus* toward ER-α

Compounds	Binding Energy (kcal/mol)	Inhibition Constant (μM)	Interaction with Amino Acid		
			Hydrogen Bonds	Van Der Waals Bonds	Others
4-Hydroxytamoxifen (Comparative Drug)	-11.31	0.00512	GLY A: 521 HIS A: 524	-	LEU A: 346; LEU A: 384: MET A: 388; LEU A = 349; ALA A:350; LEU A = 525
Eupatorin	- 6.9	8.69	CYS A: 530	MET A: 421 GLY A: 521 THR A: 347	ASP A: 351; ALA A: 350; LEU A: 525; LEU A: 346; LEU A: 428; ILE A: 424; PHE A: 425; PHE A: 404; MET A: 343; HIS A: 524
Ladanein	-8.74	391.16 A	SP A: 351 CYS A: 530	-	ALA A: 350; THR A: 347; LEU A: 384; LEU A: 354; MET A: 388; TRP A: 383
Sinensetin	-7.51	3.1	-	-	MET A: 343; MET A: 421; MET A: 388; LEU A: 391; PHE A: 404; LEU A: 428; PHE A: 425; ILE A: 424; HIS A: 524; TRP A: 383; LEU A: 525; CYS A: 530; ALA A: 350
Salvigenin	- 7.57	2.84	-	MET A: 421 GLY A: 521 TRP A: 383	ASP A: 351; TRP A: 383; PHE A: 425; PHE A: 404; ILE A: 424; LEU A: 428; LEU A: 346; LEU A: 525; HIS A: 524; MET A: 343; ALA A: 350 CYS A: 530; THR A: 347
Tetramethyl Scutellarein	-7.19	5.36	-	-	ASP A: 351; THR A: 347; CYS A: 530; MET A: 343; TRP A: 383; ALA A: 350; LEU A: 346; LEU A: 350; LEU A: 387; LEU A: 391; LEU A: 525; MET A: 388; MET A: 421; PHE A: 404
5-Hydroxy-6,7,3',4'-tetramethoxyflavone	-7.64	2.5	CYS A: 530	PHE A: 425 LEU A: 525 THR A: 347 MET A: 343 PHE A: 404 LEU A: 349 LEU A: 536	LEU A: 346; ALA A: 350; LEU A: 354; LEU A: 387; TRP A: 383; ASP A: 351
Rosmarinic Acid	-8.02	1.31	ASP A: 351 HIS A: 524 GLY A: 521	-	THR A: 347; CYS A: 530; ALA A: 350; ILE A: 424; LEU A: 525
Curcumin	-8.56 5	33.29	GLU A: 353	-	LEU A: 387; TRP A: 383; ALA A : 350; LEU A: 346; PHE A: 404
7,3',4'-Tri-O-methyl luteolin	-8.75	0.38	ARG A: 394 LEU A: 387	-	HIS A: 524; ILE A: 424; LEU A: 346; LEU A: 349; LEU A: 391; LEU A: 525; MET A: 421; PHE A: 404

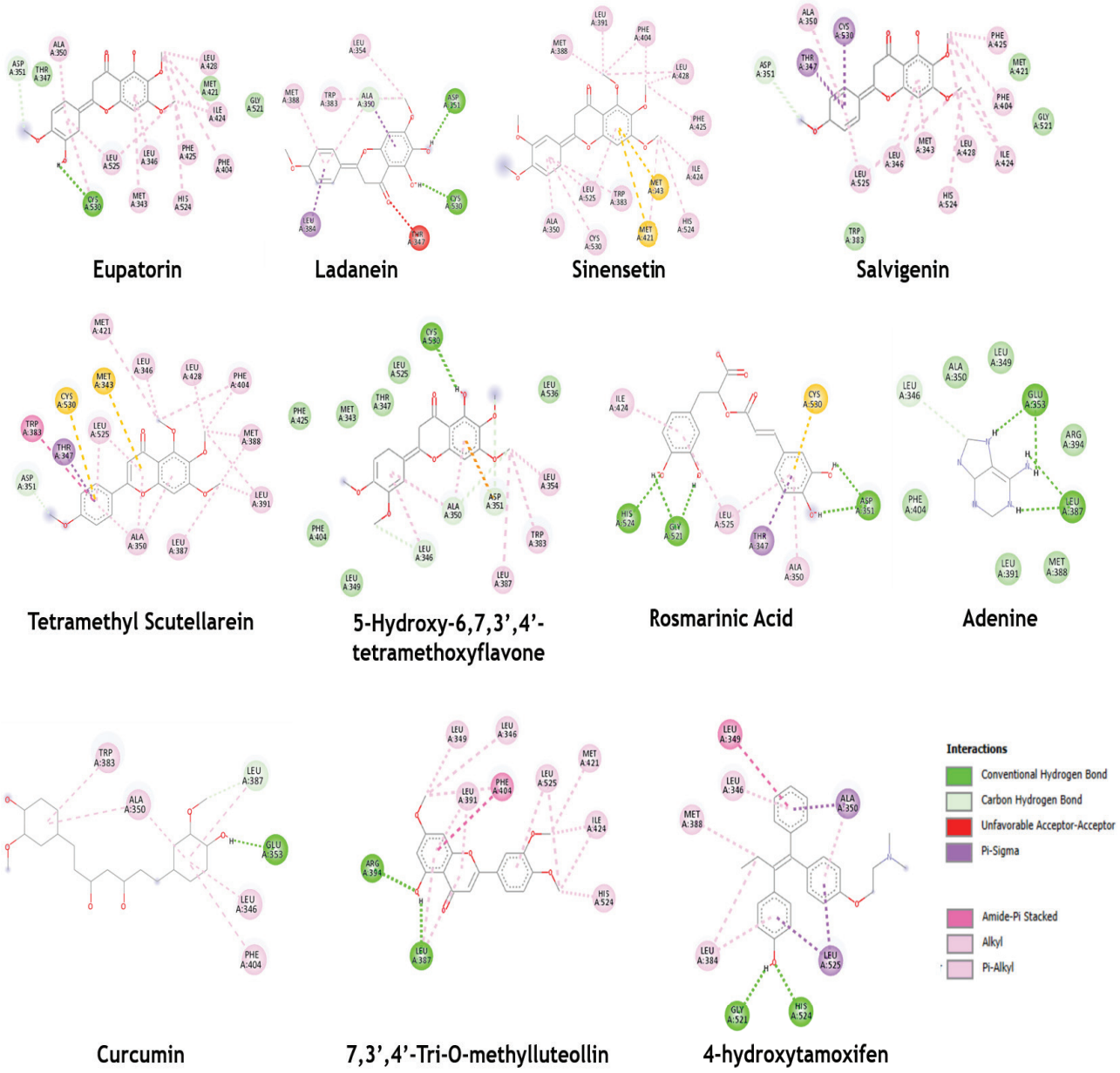

Figure 1. The 2-dimentional docking visualization from molecular docking between bioactive compounds in *Orthosiphon aristatus* toward ER-*a*.

DISCUSSION

The current study aims to evaluate whether the bioactive constituents of *O. aristatus* could potentially target ER-*α*, which is overexpressed in breast and ovarian cancers. Initially, the physicochemical properties of nine compounds were assessed based on Lipinski's Rule of Five (RO5), a guideline used to predict a compound's solubility and permeability across biological membranes and its suitability for oral administration. The analysis revealed that all tested compounds satisfied the RO5 criteria, suggesting their potential as oral drug candidates. However, it is important to note that Lipinski's rule of five is not the sole parameter for determining a compound's suitability for new drug development. Many drugs do not adhere to the Lipinski rule of five yet still exhibit pharmacological activity (Roskoski, 2023). Additionally, natural products are often considered exceptions to Lipinski's rules. Despite having higher molecular weights and numerous rotatable bonds, these compounds maintain low hydrophobicity and strong intermolecular hydrogen-bonding potential due to natural adaptations. Nevertheless, natural product leads within the Lipinski framework have achieved a comparable success rate (50%) in oral drug development (Ganesan, 2008). Furthermore, the pharmacokinetic analysis highlights the potential of *O. aristatus* bioactive compounds as oral drug candidates. The favorable absorption and distribution profiles provide a strong foundation for further development, particularly on targeted therapies. However, the toxicity concerns necessitate comprehensive validation in animal models and potential structural optimization to mitigate risks. Several strategies could also be applied to minimize risks, such as structural modifications to enhance the pharmacokinetic profile and reduce toxicity.

Virtual screening was conducted to identify test compounds with potential activity against the target protein, ER-α. Ligand-based virtual screening was performed using pharmacophore modeling to identify compounds with structural similarities, which are believed to exhibit similar activity. The pharmacophore model was validated using a database of active and decoy compounds to assess its accuracy in distinguishing between the two. Effective pharmacophore modeling is characterized by the ability to identify a high number of active compounds while minimizing decoys. Validation results, including total compound hits and ROC curves with AUC and EF values, are used to evaluate the model's performance. A pharmacophore model is considered valid if the AUC value exceeds 0.50 (Widyasari, *et al*., 2022). Following the pharmacophore screening, five of the nine tested compounds were identified as active against ER-α, as indicated by their pharmacophore fit scores. The pharmacophore fit score reflects how well geometric features in a compound align with the features of the pharmacophore model. A higher fit score indicates a greater likelihood that the compound matches the pharmacophore model and exhibits higher activity as an ER-α inhibitor (Muchtaridi, *et al*., 2017; Hariyanti, *et al*., 2021).

Subsequently, structure-based virtual screening through molecular docking was conducted to predict a complex structure interaction with a molecule, predict the binding of ligands that have activity, find new ligands, and indicate the binding affinity of compounds (Ekawasti, *et al*., 2021). From the redocking process of the native ligand, an RMSD value of 0.84 Å was obtained. The RMSD value of ≤2Å illustrates that the error in redocking is small, so it is concluded that the process is accurate and can be used as a reference in molecular docking simulation of test compounds (Amrullah, *et al*., 2023).

The molecular docking process utilized 4-hydroxytamoxifen as a reference drug, the active metabolite of the tamoxifen prodrug, which is metabolized in the liver (Effendi, *et al*., 2023). The parameters evaluated during the docking process included binding energy and inhibition constant values. Binding energy represents the energy required for the receptor to interact with the ligand

(Yunta, 2016), while the inhibition constant reflects the ligand's ability to inhibit receptor activity. A lower inhibition constant indicates a stronger ligand-receptor bond and higher inhibitory potential (Puspita, *et al*., 2022). During the ligand-receptor interaction, energy is released into the environment. If the binding energy is negligible or negative, the resulting bond affinity is considered more stable and potent (Saâd, 2016). However, none of the test compounds demonstrated binding energies or inhibition constant values lower than those of the comparator drug, 4-hydroxytamoxifen, which recorded values of -11.41 kcal/mol and 0.0051 μM, respectively. These findings suggest that the test compounds exhibit weaker binding affinities to ER-α compared to 4-hydroxytamoxifen.

The molecular docking results were visualized in 2D to compare the amino acid residues and the number of hydrogen bonds formed during interactions between each test compound and the reference drug. A ligand-test compound bond is considered more stable when more hydrogen bonds and amino acid residues are involved (Muflihunna and Sukmawati, 2023). For the reference compound, 4-hydroxytamoxifen, eight amino acid residues were identified in the interaction, including two hydrogen bonds. Among the test compounds, only rosmarinic acid demonstrated similar hydrogen bond interactions with 4-hydroxytamoxifen, specifically with the amino acid residues HIS524 and GLY521. Previous studies have reported that estradiol also binds to ER-α through hydrogen bonding with HIS524 and GLY521, while these residues interact with 4-hydroxytamoxifen through van der Waals interactions (Ervina, *et al*., 2021; Rocha-Roa, *et al*., 2023). Similarly, an *in silico* study by Ummah and Zummah (2024) showed that chlorogenic acid, a compound structurally related to rosmarinic acid, formed hydrogen bonds with HIS524 and GLY521 in ER-α. These findings highlight the significance of HIS524 and GLY521 as essential residues for receptor activity and ligand interaction.

The potential breast anticancer activity of rosmarinic acid is further supported by its reported ability to induce mitotic arrest and apoptosis in MDA-MB-468 breast cancer cells (Messeha, *et al*., 2020). This highlights rosmarinic acid in *O. aristatus* as a promising candidate for breast cancer treatment. While this study primarily utilized computational approaches, future research should explore other bioactive compounds in *O. aristatus* that may also possess anticancer properties. Furthermore, the biological activities of rosmarinic acid as a breast anticancer agent should be validated through experimental studies, particularly in ER-*α*-positive breast cancer, to confirm the findings of this *in-silico* analysis.

CONCLUSION

In summary, the bioactive constituents of *O. aristatus* satisfied all criteria of Lipinski's rule and demonstrated good to moderate results in pharmacokinetic prediction analysis. Pharmacophore modeling identified five compounds as hit compounds against ER-*α*. Among them, rosmarinic acid exhibited hydrogen bonding interactions similar to 4-hydroxytamoxifen at HIS524 and GLY521, with a binding energy of -8.02 kcal/mol. These findings suggest that *O. aristatus* and rosmarinic acid hold potential as an anticancer agent targeting ER-*α* and warrants further investigation.

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