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In Silico Studies of Carotenoid Antioxidants in Sand Lobster (*Panulirus homarus*) as Candidate Anti-Breast Cancer Compounds

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ABSTRACT

Breast cancer is the most diagnosed and deadliest type of cancer among women worldwide. In Indonesia, breast cancer ranks as the most prevalent and deadly cancers, with 58,256 cases and 22,692 deaths. This research aims to analyze the potential of carotenoid antioxidants found in the sand lobster (*Panulirus homarus*) as breast cancer treatment candidates through an in silico approach. In this study, six carotenoid compounds were evaluated: dinoxanthin, diadinoxanthin, zeaxanthin, lutein, astaxanthin, and violaxanthin, using 4-hydroxytamoxifen, genistein, and exemestane as standard ligands, and tamoxifen, metformin, and letrozole as positive controls. The 3D crystal protein structures used for molecular docking were obtained from the Protein Data Bank (PDB), including estrogen receptor alpha (PDB ID: 3ERT), estrogen receptor beta (PDB ID: 1X7J), and aromatase (PDB ID: 3S7S) as target receptors. Subsequently, bioactivity and bioavailability analyses of the test compounds were performed. Proteins and test compounds were prepared, followed by molecular docking against the estrogen receptor alpha, estrogen receptor beta, and aromatase, with visualization performed using Discovery Studio. Molecular dynamics simulations were performed to evaluate the stability of binding interactions. Toxicity prediction was carried out using the Protox Web Server (https://tox-new.charite.de/protox_II/). The in silico results indicate that two of the six tested compounds, lutein and violaxanthin, showed favorable predicted binding interactions with estrogen receptor alpha, estrogen receptor beta, and aromatase. Additionally, in silico toxicity predictions indicated favorable toxicity profiles for these compounds, suggesting their potential as candidates for further investigation against breast cancer.

Keywords: Bioactivity, Bioavailability, Lutein, Toxicity, Violaxanthin

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1. Introduction

Breast cancer ranks first among cancer cases in Indonesia [1]. The breast is an organ composed of epithelial tissue, adipose tissue, and fibrous connective tissue. Histologically, epithelial tissue consists of lobules and ducts, which function in milk production and transportation of milk [2]. Lobules are small functional units responsible for milk production, while ducts are channels connecting lobules to the nipple. Adipose tissue provides shape and cushioning, while fibrous connective tissue supports the breast structure [3]. Alterations in these epithelial cells can lead to breast cancer, which often begins in the milk ducts and is referred to as ductal carcinoma. Breast cancer can spread to the surrounding adipose or fibrous tissues. A key factor influencing the development of breast cancer is the hormone estrogen [4].

Estrogen is a steroid hormone crucial in developing and regulating the female reproductive system and secondary sexual characteristics [5]. Additionally, estrogen contributes to maintaining bone health, controlling cholesterol levels, and preserving skin elasticity and vascular tissue integrity. In normal cells, estrogen supports the growth and differentiation of healthy cells, particularly in breast tissue and reproductive organs [6]. However, under conditions of excessive estrogen activity, estrogen can also contribute to breast cancer development by stimulating the growth of estrogen-dependent cancer cells. Estrogen functions in breast cancer by binding to estrogen receptors (ER) in breast cells, subsequently activating signaling pathways that promote cell proliferation and resistance to apoptosis. Estrogen receptor activation stimulates epithelial cell proliferation in breast cancer through increased estrogen receptor expression and the induction of estrogen receptor-mediated gene transcription. Estrogen receptors mediate the effects of estrogen and anti-estrogen agents on cells and are predominantly found on the nuclear membrane of target cells [7]. Estrogen receptors are utilized to determine the sensitivity of breast cancer lesions to anti-estrogen therapy and to assess the efficacy of preventive chemotherapy in women at high risk for breast cancer [8]. In breast cancer, estrogen receptors bind estrogen, which then activate genes that support cancer cell growth. Mutations in estrogen receptors can result in abnormal expression in target cells, predisposing them to malignancy and triggering epithelial cell proliferation in breast cancer [9].

There are two central estrogen receptors involved in breast cancer: estrogen receptor alpha (ER-α) and estrogen receptor beta (ER-β) [10]. Both receptors are found in breast tissue and often indicate a potentially more aggressive cancer [11]. Activation of ER-α by estrogen triggers gene transcription that supports cancer cell proliferation and survival, making ER-α a primary target in hormonal therapy for breast cancer [12]. On the other hand, ER-β can act as a tumor suppressor by inhibiting cell proliferation and promoting cell differentiation [13].

Additionally, the conversion of hormone precursors into estrogen within breast tissue, facilitated by aromatase, is another critical target in breast cancer treatment. Aromatase is an enzyme that plays a significant role in breast cancer by converting androgens into estrogen in breast and adipose tissues [14]. Estrogen production can influence the growth of estrogen-dependent cancers, particularly estrogen-responsive breast cancer. Elevated levels of aromatase can increase estrogen levels in the body, thereby triggering the onset of breast cancer [15]. The activity of ER-α, ER-β, and aromatase can be inhibited by carotenoids [16].

Carotenoids are natural pigment compounds that impart yellow, orange, and red colors. They belong to the fat-soluble carotene group and are chemically composed of long chains of carbon and hydrogen atoms [17]. Carotenoids exhibit antioxidant properties and contribute to aromatase inhibition. Additionally, carotenoids can suppress the elevation of ER-α and ER-β levels, which may

influence the proliferation of epithelial cells in breast cancer [16]. Sand lobster (*P. homarus*) is a natural source of carotenoid antioxidants that are needed for candidate anti-breast cancer compounds.

Carotenoids identified in sand lobster (*P. homarus*) include dinoxanthin, diadinoxanthin, zeaxanthin, lutein, astaxanthin, and violaxanthin. Among these, three compounds; lutein, zeaxanthin, and astaxanthin, are known for inhibiting free radicals [18]. However, molecular-level information regarding the activity, interactions, and safety of the carotenoid compounds found in *P. homarus* remains limited. Therefore, this study aimed to evaluate the bioactivity, bioavailability, molecular docking, molecular dynamics, and toxicity profiles of carotenoid antioxidants from *P. homarus* against ER-α, ER-β, and aromatase using an in silico approach.

2. Materials and Methods

This research involved six carotenoid pigments: dinoxanthin, diadinoxanthin, zeaxanthin, lutein, astaxanthin, and violaxanthin, with standard ligands: tamoxifen, genistein, and exemestane, as well as drug ligands for comparison: tamoxifen, metformin, and letrozole. Carotenoid pigments were sourced from PubChem(https://pubchem.ncbi.nlm.-nih.gov/). The 3D crystal structure data of the proteins used for molecular docking were obtained from the Protein Data Bank (PDB) (https://www.rcsb.-org/), including ER-α (PDB Code: 3ERT), ER-β (PDB Code: 1X7J), and aromatase (PDB Code: 3S7S) as anticancer receptors.

2.1 Analysis of carotenoid bioactivity

The biological activities of carotenoid compounds were predicted using PASS Online (Prediction of Activity Spectra for Substances), a web-based computational tool for predicting the biological activity spectrum of chemical compounds based on their chemical structures [19]. Six carotenoid compounds, namely dinoxanthin, diadinoxanthin, zeaxanthin, lutein, astaxanthin, and violaxanthin, together with three reference compounds (tamoxifen, metformin, and letrozole), were analyzed. The canonical SMILES of all compounds were retrieved from PubChem (https://pubchem.ncbi.nlm-.nih.gov) and submitted to the PASS Online server (https://www.way2drug.com/passon-line/) to predict their biological activities. The predicted Pa values obtained from PASS Online were used for subsequent analyses.

2.2 Analysis of carotenoid bioavailability

Bioavailability prediction was performed to evaluate the drug-likeness and physicochemical properties of the test compounds using the Swiss ADME web server (http://www.swissadme.ch) [20]. The prediction was based on Lipinski's Rule of Five, which assesses molecular weight (≤500 Da), lipophilicity (LogP ≤5), hydrogen bond donors (≤5), hydrogen bond acceptors (≤10), and molar refractivity (40-130) to estimate the oral bioavailability of small molecules [21].

2.3 Geometry optimization of test compounds

Ligands used were six test compounds from *P. homarus* obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov/): dinoxanthin (CID: 6443021), zeaxanthin (CID: 5280899), astaxanthin (CID: 5281224), diadinoxanthin (CID: 6449888), lutein (CID: 5281243), and violaxanthin (CID: 448438). The SDF file format of the ligands was converted to PDB format using OpenBabel version 2.4.1. Next, the geometry of the test compounds was optimized using Avogadro version 2.0 with a Density Functional Theory (DFT) approach and a 6-31G(d) basis set to achieve a stable conformation of the compounds [22].

2.4 Validation of molecular docking methods

After obtaining and optimizing the test compounds in PDB format, the test compounds were prepared using Autodock

Tools version 1.5.7. The prepared ligands were saved in pdbqt format, ensuring that they were ready for docking simulations. The preparation receptors used in this research were obtained from the Protein Data Bank: ER-α (3ERT), ER-β (1X7J), and aromatase (3S7S). The crystal structure of the macromolecules was prepared using Discovery Studio version 24.1.0.23298 and Autodock Tools version 1.5.7 [24]. Validation was performed through redocking, using standard ligands present in the receptor. Molecular docking was conducted by redocking the standard ligands into their respective receptors using Autodock Tools version 1.5.7 and Discovery Studio version 24.1.0.23298. The validity was assessed using the Root Mean Square Deviation (RMSD) value, employing a genetic algorithm (GA) method with 100 GA runs [25].

2.5 Molecular docking and visualization of carotenoid antioxidant

To determine the binding affinity of carotenoid pigments, molecular docking was performed against ER-α (3ERT), ER-β (1X7J), and aromatase (3S7S) using AutoDock Tools software. The grid box was centered on the active binding site of each receptor. For ER-α (3ERT), the grid box center was set at x = 29.597, y = 36.671, and z = 39.192 with a grid size of 48 × 48 × 48. For ER-β (1X7J), the grid box center was set at x = 30.128, y = −1.997, and z = 24.206 with a grid size of 48 × 48 × 48. For aromatase (3S7S), the grid box center was set at x = 86.441, y = 54.407, and z = 46.382 with a grid size of 50 × 50 × 50. The docking region encompassed the active-site amino acid residues surrounding the co-crystallized ligand. The best docking pose was selected based on the lowest (most negative) predicted binding affinity score generated by AutoDock [26]. Molecular docking results were visualized using Discovery Studio version 24.1.0.23298 for docking complexes and 2D interactions.

2.6 Molecular dynamic simulation

Molecular dynamics simulations were analyzed using YASARA Dynamics version 23.5.19.W.64 for 100 nanoseconds with 401 snapshots and the AMBER14 force field [27]. First, the script was checked in YASARA Dynamics version 23.5.19.W.64, then parameters were set, such as temperature to '300K' and the simulation duration to 100 nanoseconds.

2.7 Toxicity prediction

Toxicity prediction was analyzed using the ProTox Web Server (https://tox-new.charite.de/protox_II/index.php?site=compound_input) with input provided in the form of the compound's SMILES structure. The toxicity parameters analyzed include LD50, toxicity class, mutagenicity, carcinogenicity, hepatotoxicity, and immunotoxicity. The prediction was performed using the QSAR model implemented by ProTox-II [28].

3. Results and Discussion

3.1 Analysis of carotenoid bioactivity

The results of the carotenoid bioactivity screening analysis via PASS online are shown in Table 1.

Table 1. Results of PASS online screening.

Compound	Pa	Activity
Dinoxanthin	0,856	Antioxidant
Diadinoxanthin	0,641	Antioxidant
Zeaxanthin	0,766	Antioxidant
Lutein	0,609	Antioxidant
Astaxanthin	0,758	Antioxidant
Violaxanthin	0,749	Antioxidant

Screening results through PASS Online showed that all carotenoid compounds had Pa values >0.3. Compounds with Pa values exceeding 0.3 are predicted to exhibit the corresponding biological activity [29]. Among the carotenoids analyzed, dinoxanthin exhibited the highest predicted antioxidant activity (Pa = 0.856), followed by zeaxanthin (0.766), astaxanthin (0.758), violaxanthin (0.749), diadinoxanthin (0.641), and lutein (0.609). These findings indicate that all

carotenoid compounds exhibit predicted antioxidant activity, with differences in Pa values reflecting variations in the probability of exhibiting antioxidant activity. Antioxidant activity is directly relevant to breast cancer because oxidative stress and excessive reactive oxygen species (ROS) contribute to DNA damage, cellular proliferation, tumor progression, and resistance to apoptosis in breast cancer. Therefore, the predicted antioxidant activity of these carotenoids provides a biological basis for their investigation as potential anti-breast cancer compounds [30, 31].

3.2 Analysis of Carotenoid Bioavailability

Results of the bioavailability analysis of carotenoids based on Lipinski's Rule of Five are presented in Table 2. Lipinski's Rule aims to evaluate the potential of these compounds to act as effective drug candidates that the body can absorb. Lipinski's Rule of Five involves several key criteria: molecular weight (MW) ≤500 daltons, log *P* ≤5, hydrogen bond donors ≤5, hydrogen bond acceptors ≤10, and molar refractivity between 40-130 [21].

Table 2. Bioavailability analysis based on Lipinski's Rule of Five.

No.	Compound	Criteria Based on Lipinski's Rule of Five				
		MW (Dalton)	Log *P* (≤5)	Hydrogen donor (≤5)	Hydrogen acceptor (≤10)	Molar refractivity (40-130)
1.	Dinoxanthin	642.91	8.68	2	5	196.39
2.	Diadinoxanthin	582.85	9.03	2	3	184.91
3.	Zeaxanthin	568.87	9.83	2	2	186.76
4.	Lutein	568.87	9.72	2	2	186.76
5.	Astaxanthin	596.84	8.65	2	4	187.16
6.	Violaxanthin	600.87	8.68	2	4	185.80

Based on the SwissADME analysis, none of the six carotenoid compounds fully satisfied Lipinski's Rule of Five. All compounds exhibited molecular weights greater than 500 Da and consensus LogP values exceeding 5, indicating high molecular size and lipophilicity. In addition, the molar refractivity values of all compounds exceeded the recommended range of 40-130. However, all compounds fulfilled the hydrogen bond donor (≤5) and hydrogen bond acceptor (≤10) criteria. These findings suggest that the investigated carotenoids may exhibit limited oral bioavailability because of their relatively high molecular weight and lipophilicity.

Nevertheless, Lipinski's Rule of Five serves as a guideline for evaluating drug-likeness rather than an absolute requirement, and many bioactive natural products may violate one or more of these criteria while still exhibiting pharmacological activity [32].

3.3 Geometry optimization of test compounds

Results of the geometry optimization of the test compounds are presented in Table 3, which compares the initial and final energy of the optimized structures.

Table 3. Initial and final energy values of optimized compounds.

Compound	Initial Energy (kJ/mol)	Final Energy (kJ/mol)
Dinoxanthin	432,288	381,696
Diadinoxanthin	468,41	438,287
Zeaxanthin	564,437	506,927
Lutein	471,251	451,259
Astaxanthin	696,545	613,435
Violaxanthin	525,965	480,910

Geometry optimization of the test compounds performed using the DFT method with the 6-31G(d) basis set proved to yield accurate results. Optimization results showed a significant decrease in energy between the initial and final states of the

carotenoid compounds. The geometry optimization method effectively predicts the geometric structure of compounds [23].

Reduction in potential energy indicates that the final geometric structure is more stable than the initial structure and the optimized structure will serve as the basis for further analyses, including calculations of the test compound's electronic properties and vibrational spectra [33]. Optimized potential energy is not lost but rather utilized to achieve an even distribution of atoms within the test compound's structure. Even the distribution of atoms suggests that the geometry optimization successfully identified a lower-energy conformation, which is the primary goal of the optimization process [34].

3.4 Validation of molecular docking methods (Redocking)

The results of the redocking are shown in Table 4 and Fig. 1

Table 4. Redocking validation results of the standard ligands.

Ligand	Binding Affinity	RMSD
4-hydroxytamoxifen	-11.91	1.23
Genistein	-9.10	1.88
Exemestane	-9.47	0.70

Based on the redocking results (Table 4 and Fig. 1), all target receptors used in this in silico study have an RMSD value < 2.0 Å. These redocking results also show that the AutoDockTools software is capable of repositioning the ligand to its original position with a shift of < 2.0 Å between the natural ligand and the redocked ligand, thus meeting the validity criteria. Finally, redocking is considered valid if the RMSD value of the redocking results is ≤ 2.0 Å [35].

Redocking results were followed by the docking of several receptors with carotenoid compounds.

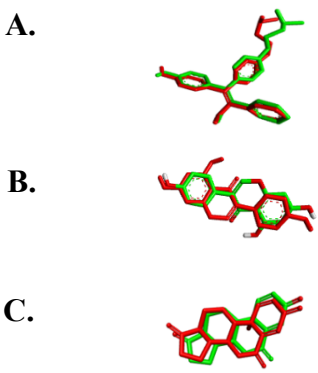


Fig. 1. Visualization of the molecular docking validation results: (A) Standard ligand 4-hydroxytamoxifen (green) and redocked ligand (red). (B) Standard ligand genistein (green) and redocked ligand (red). (C) Standard ligand exemestane (green) and redocked ligand (red).

3.5 Molecular docking and visualization of carotenoid antioxidant docking

3.5.1 Molecular docking of ER-α (3ERT)

The molecular docking results between ER-α and the six test compounds, and tamoxifen as the positive control, are presented in Table 5. Here, lutein exhibited the lowest binding affinity value (-9.9 kcal/mol). In molecular docking, superior docking results are indicated by a more negative binding affinity value than that of the reference compound. A more negative binding affinity reflects a stronger and more stable ligand-receptor interaction [36-37]. A negative binding affinity indicates stronger and more stable ligand-receptor interactions, suggesting greater inhibitory potential, whereas a positive binding affinity reflects weak and unstable interactions with reduced inhibitory potential [38, 39].

Table 5. Results of molecular docking of *P. homarus* carotenoid antioxidant compounds on ER-*α* (3ERT).

Compound	Binding Affinity (kcal/mol)	Residue Interaction	Type of Bond
Lutein	-9,9	VAL533, LEU346, LEU525, ALA350, LYS529, TRP383	Hydrophobic interactions
Tamoxifen (control)	-9,7	MET421, LEU525, ALA350, LEU346, LEU387, LEU391, MET388	Hydrophobic interactions
Astaxanthin	-9,3	TYR526, MET522, LEU525, TRP383, LEU536	Hydrophobic interactions
Zeaxanthin	-9,1	MET522, LEU525, TYR526, LEU536, LEU539	Hydrophobic interactions
Diadinoxanthin	-9,0	LYS529, LEU525, ALA350, TRP383, LEU346, MET343, LEU387	Hydrogen bond and hydrophobic interactions
Violaxanthin	-8,8	VAL533, LYS529, LEU525, TYR526	Hydrophobic interactions
Dinoxanthin	-8,2	LEU536, TRP383, LEU525, TYR526, MET522	Hydrogen bond and hydrophobic interactions

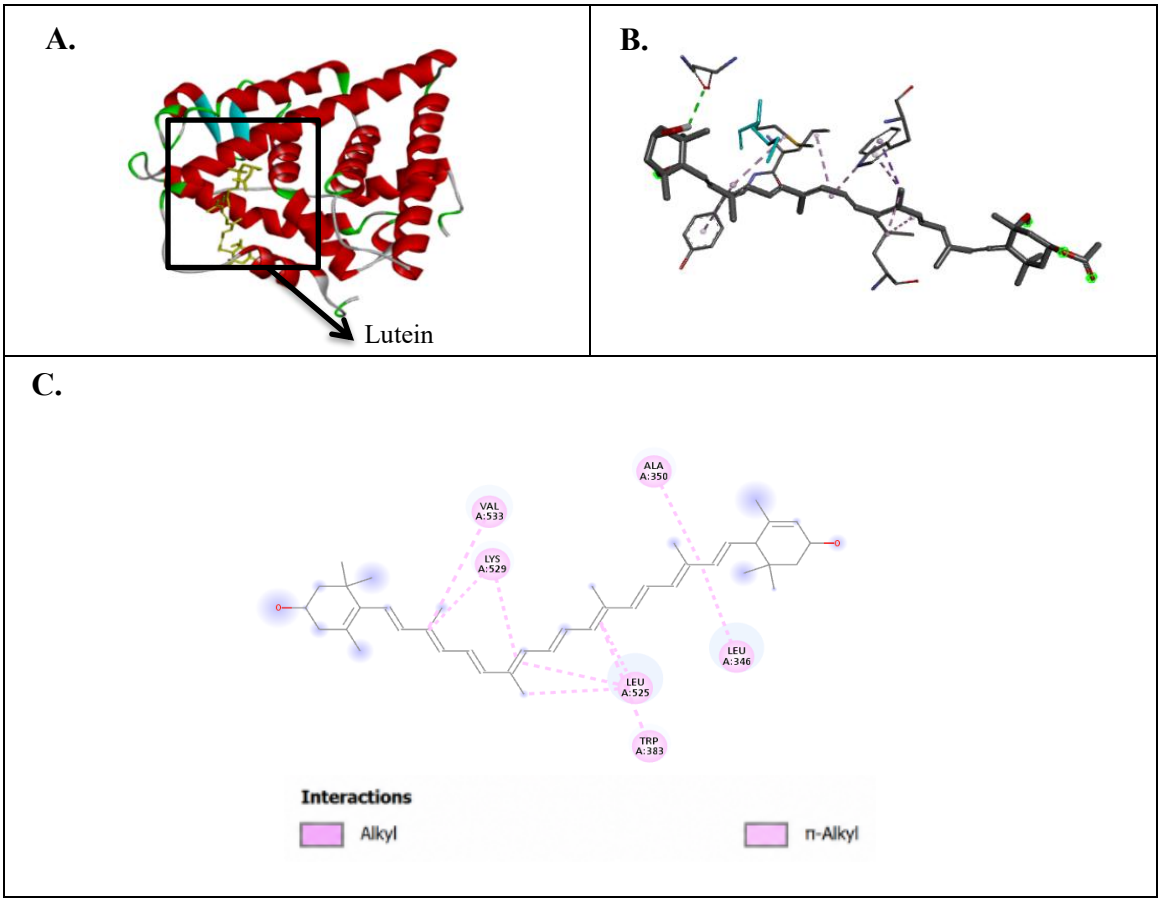


Fig. 2. Visualization of molecular docking results: A. Docked complex (lutein with ER-α); B. 3D Interaction; C. 2D Interaction (interaction of amino acid residues with lutein).

Fig. 2 visualizes the molecular docking results between the receptors and the selected test compounds. Visualization of molecular docking provides a direct view of the ligand-binding site and the interactions between the ligand and receptor. It also facilitates the identification of key amino acid residues involved in ligand binding and receptor recognition [41].

Lutein, as visualized in Fig. 2, exhibited the most negative predicted binding affinity among the tested compounds and control. Interaction between lutein and the ER-α is reinforced by hydrophobic interactions. Hydrophobic interactions contribute to ligand-receptor binding by promoting favorable contacts between nonpolar regions of the ligand and receptor. These interactions enhance binding stability by reducing unfavorable contacts with surrounding water molecules and strengthening the protein-ligand complex [42].

The interaction between lutein and ER-α involved several amino acid residues, including VAL533, LEU346, LEU525, ALA350, and LYS529. Hydrophobic residues such as VAL533, LEU346, and LEU525 contribute to stabilizing the ligand within the binding pocket, whereas the polar residue LYS529 may reinforce complex stability. In addition, ALA350 helps optimize ligand positioning within the active site [43, 44].

A combination of hydrophobic interactions between VAL533, LEU346, LEU525, ALA350, and LYS529 results in lutein having the most negative binding affinity compared to the other test compounds and controls. The lower (more negative) binding affinity indicates that lutein has strong inhibitory potential against ER-α. Thus, lutein can potentially suppress breast cancer proliferation by inhibiting the receptor's activity. These findings are consistent with previous experimental evidence. In one study, lutein was shown to selectively inhibit the proliferation of breast cancer cells through ROS-mediated mechanisms while exhibiting minimal toxicity toward normal mammary epithelial cells [45]. Lutein also induced cell-cycle arrest and promoted apoptosis, highlighting its selective anticancer activity against breast cancer cells. Therefore, the present molecular docking results are consistent with previous in vitro evidence and further support the potential of lutein as a promising anti-breast cancer compound.

3.5.2 Molecular docking of ER-β (1X7J)

The molecular docking results between ER-β and the six test compounds, with genistein and metformin included as control compounds, are presented in Table 6. Metformin was included as a control compound based on its reported antiproliferative and anticancer effects in breast cancer [47].

Table 6. Results of molecular docking of *P. homarus* carotenoid antioxidant compounds on ER-*β* (1X7J).

Compound	Binding Affinity (kcal/mol)	Residue Interaction	Type of Bond
Violaxanthin	-7,3	ARG346, VAL361, PRO358, ILE355.	Hydrogen bond and hydrophobic interactions
Dinoxanthin	-7,2	HIS394, GLU366, VAL361, ILE355, PRO358, HIS279	Hydrogen bond and hydrophobic interactions
Astaxanthin	-7,1	ARG386, ALA382, HIS464, MET379, LEU374	Hydrogen bond and hydrophobic interactions
Zeaxanthin	-7,1	LEU374, MET379, ALA382, ARG386	Hydrogen bond and hydrophobic interactions
Diadinoxanthin	-6,9	HIS464, MET379, LEU374, LYS368, ARG386	Van der waals, hydrogen bond, and hydrophobic interactions
Lutein	-6,9	HIS464, ALA382, MET379, LEU374, LYS368	Hydrophobic interactions
Genistein (Control)	-5,2	LEU476, ALA302, LEU339, PHE356, LEU343, HIS475, GLU305	Hydrogen bond and unfavorable donor
Metformin (Control)	-5,0	GLU305, ARG346, LEU339	Hydrogen bond and electrostatic interactions

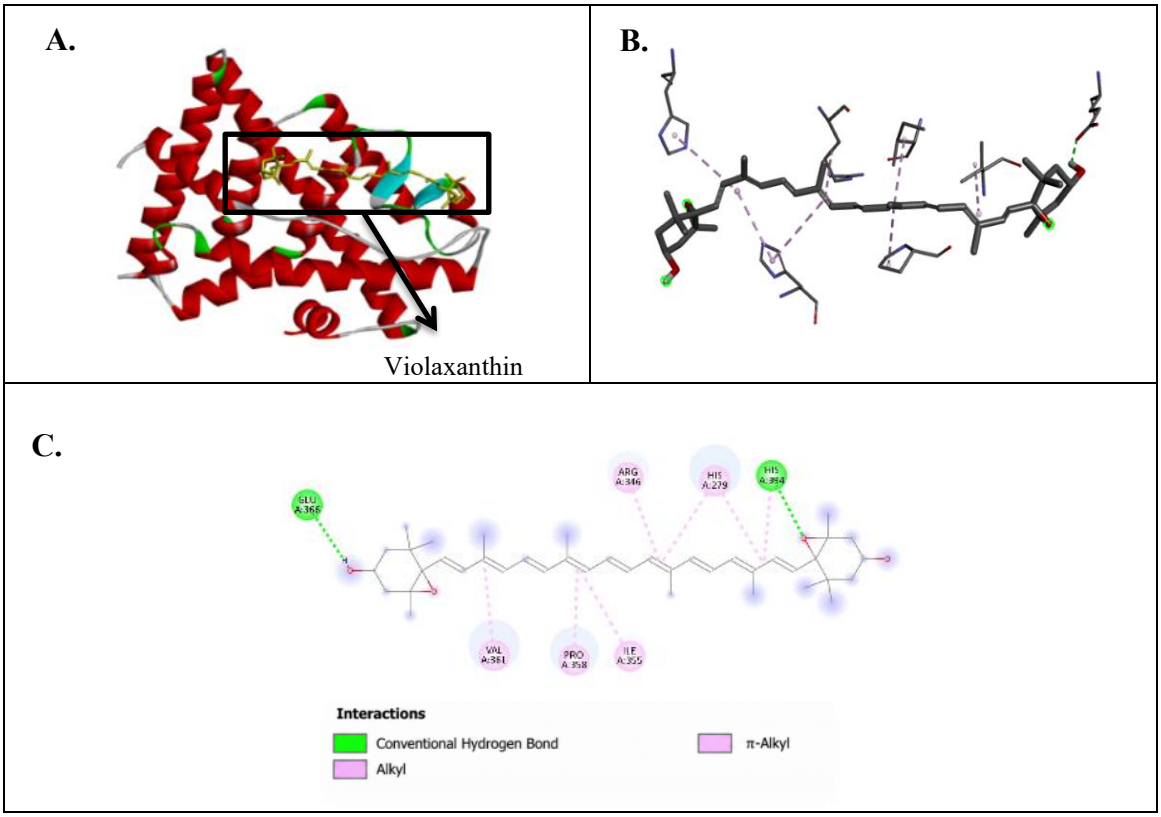


Fig. 3. Visualization of molecular docking results: A. Docked complex (violaxanthin with ER-β); B. 3D interaction; C. 2D interaction (interaction of amino acid residues involved in hydrogen bond and hydrophobic interactions with violaxanthin.

Violaxanthin, as visualized in Fig. 3, exhibited the most negative predicted binding affinity (-7.3 kcal/mol) among the tested compounds and control. The interaction was stabilized by hydrogen bonds and hydrophobic interactions, which are essential for forming stable and specific ligand-receptor complexes. Hydrogen bonds facilitate interactions between polar groups of the ligand and amino acid residues, whereas hydrophobic interactions occur between nonpolar regions of the ligand and hydrophobic amino acid residues, thereby contributing to ligand orientation and binding stability within the receptor binding pocket [49].

The interaction between violaxanthin and ER-*β* involved several amino acid residues, including ARG346, VAL361, PRO358, and ILE355. ARG346, a polar residue, may contribute to hydrogen bond formation and stabilization of the protein-ligand complex. In contrast, VAL361 and ILE355, which are hydrophobic residues, enhance hydrophobic interactions within the binding pocket, whereas PRO358 helps maintain the structural conformation of the binding site and facilitates ligand accommodation [50-52].

The present molecular docking results demonstrated that violaxanthin exhibited the strongest binding affinity toward ER-*β* among all tested compounds. However, molecular docking predicts only the binding affinity between a ligand and its target and cannot determine whether a compound acts as an agonist or antagonist. Previous in vitro studies demonstrated that violaxanthin isolated from *Dunaliella tertiolecta* inhibited the proliferation of

MCF-7 breast cancer cells and induced apoptosis, supporting its anticancer potential [53]. Therefore, the present findings suggest that ER-β may represent one of the potential molecular targets of violaxanthin, although further in vitro and in vivo studies are required to clarify its mechanism of action and validate its therapeutic potential.

3.5.3 Molecular Docking of Aromatase (3S7S)

The molecular docking results between aromatase and the six test compounds and positive control are presented in Table 7. Here, letrozole was used as the positive control as it is a standard aromatase inhibitor widely used in the treatment of hormone receptor-positive breast cancer [31].

Table 7. Results of molecular docking of *P. homarus* carotenoid antioxidant compounds on aromatase (3S7S).

Compound	Binding Affinity (kcal/mol)	Residue Interaction	Type of Bond
Violaxanthin	-8,3	LYS448, TYR441, MET149	Hydrophobic interactions and conventional hydrogen bond (donor)
Diadinoxanthin	-8,2	TYR441, LYS150, PRO146, MET149, LYS448, ARG288	Hydrogen bond and hydrophobic interactions
Letrozole (control)	-8,2	SER478, VAL370, ARG115, VAL373, CYS437, ILE133	Hydrogen bond and hydrophobic interactions
Zeaxanthin	-8,2	TYR441, MET149, LYS150	Hydrophobic interactions
Astaxanthin	-8,0	LYS448, GLU357, TYR361, TYR441, MET149, PHE147	Hydrogen bond, hydrophobic interactions, and unfavorable interactions
Lutein	-7,9	MET149, TYR441, TYR361	Hydrogen bond and hydrophobic interactions
Dinoxanthin	-7,7	GLN428 and LYS 440	Hydrogen bond

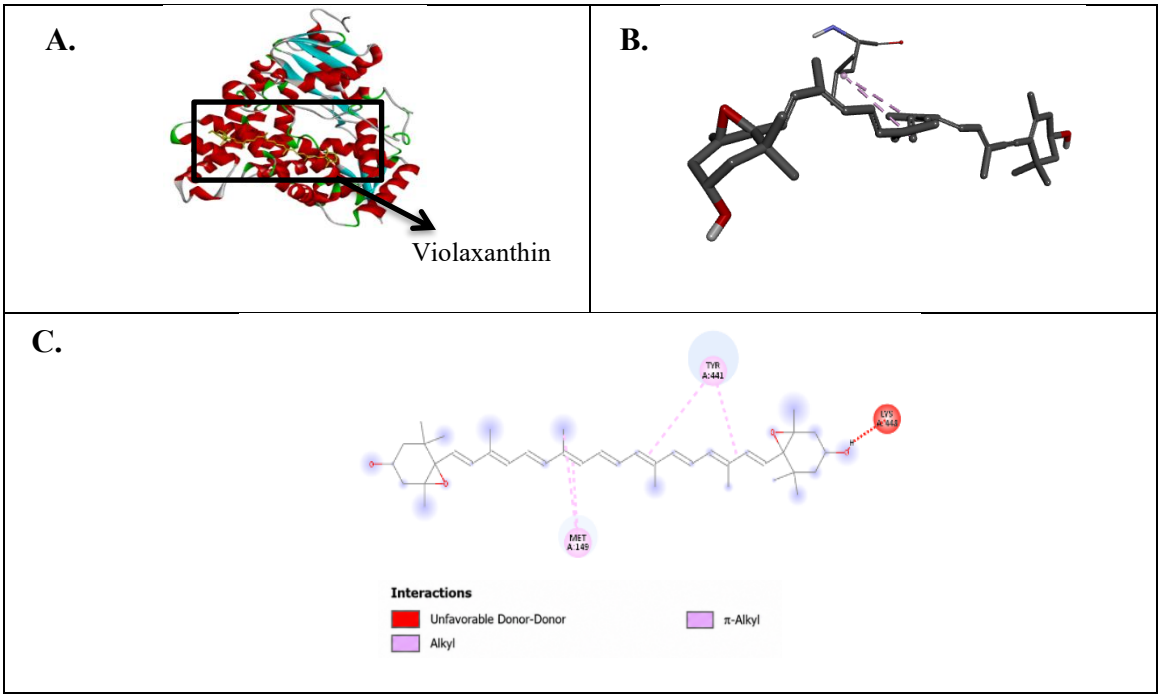


Fig. 4. Visualization of molecular docking results: A. Docked complex (violaxanthin with aromatase); B. 3D interaction; C. 2D interaction (interaction of amino acid residues and hydrophobic, and donor bonds with violaxanthin).

Violaxanthin, as visualized in Fig. 4, exhibited the most negative predicted binding affinity (-8.3 kcal/mol) among the tested compounds and control. The interaction was stabilized by hydrogen bonds and hydrophobic interactions, both of which play essential roles in forming stable and specific ligand-receptor complexes. Hydrophobic interactions facilitate ligand accommodation within the receptor binding pocket and contribute to binding stability by reducing unfavorable interactions with surrounding water molecules, whereas hydrogen bonds enhance binding specificity through interactions between polar groups of the ligand and amino acid residues of the receptor [56, 57].

The interaction between violaxanthin and aromatase involved several amino acid residues, including LYS448, TYR441, MET149, and MET143. LYS448, a polar residue, may contribute to hydrogen bond formation and stabilization of the protein-ligand complex. TYR441 possesses both aromatic and polar characteristics, enabling hydrophobic interactions as well as hydrogen bond formation. In contrast, MET149 and MET143 are hydrophobic residues that strengthen ligand binding through hydrophobic interactions within the receptor binding pocket [58-60].

A combination of hydrophobic and hydrogen bond interactions involving LYS448, TYR441, MET149, and MET143 contributed to violaxanthin exhibiting the lowest (most negative) binding affinity among all test compounds and the positive control. Aromatase inhibition may reduce estrogen biosynthesis and consequently suppress the proliferation of estrogen-dependent breast cancer cells. These findings are consistent with previous experimental evidence demonstrating the anticancer potential of violaxanthin. Previous in vitro studies showed that violaxanthin isolated from *D. tertiolecta* inhibited the proliferation of MCF-7 breast cancer cells and induced apoptosis, supporting its anticancer activity [53]. Although the inhibitory effect of violaxanthin on aromatase has not yet been experimentally confirmed, the present molecular docking results suggest that aromatase may represent one of the potential molecular targets underlying its antiproliferative activity. Therefore, further in vitro and in vivo studies are required to validate this predicted molecular interaction.

3.6 Molecular dynamics simulation

The molecular docking in this study had several limitations that should be considered when interpreting the predicted binding interactions. The carotenoid compounds investigated are relatively large and structurally flexible molecules, which may result in multiple possible conformations during ligand binding. In addition, the protein structures used for docking were obtained from X-ray crystallographic structures, which represent experimentally determined conformations of the receptors at a specific structural state. The use of a relatively rigid receptor structure may therefore not fully capture the conformational flexibility and induced-fit effects that can occur during ligand binding. Consequently, the predicted binding affinities and interactions should be interpreted as computational estimates rather than definitive evidence of biological activity. Molecular dynamics simulations and further experimental validation are therefore required to provide additional evidence for the stability and biological relevance of the predicted interactions [38, 55].

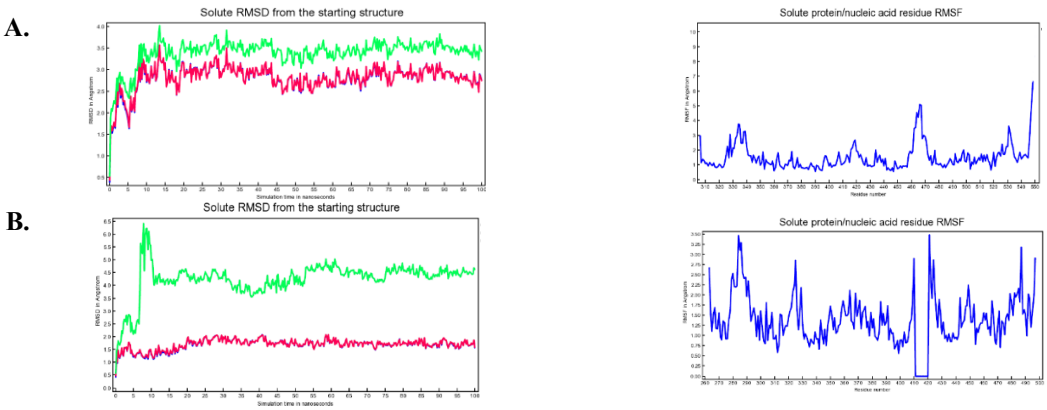
Molecular dynamics simulations between ER-α, ER-β, and aromatase with the top three test compounds from docking were carried out using Yasara Dynamics software version 23.5.19.W.64 for a duration of 100.00 nanoseconds with 401 snapshots, utilizing the AMBER14 force field, resulting in 400 simulations. Molecular dynamics simulations evaluate the stability of the protein-ligand interactions under conditions

that closely mimic the human physiological environment over a specified period. RMSD and Root Mean Square Fluctuation (RMSF) are key metrics in assessing protein stability [61]. RMSD is commonly used in 3D molecular geometry to compare the changes or movements of molecular conformations [62] and helps assess the stability of protein conformations, which play a crucial role in various biological processes, such as enzymes involved in metabolism or receptor protein functioning in cell signaling. Significant changes in RMSD may indicate structural alterations that could affect protein function or its interaction with ligands, potentially influencing health or disease outcomes [63].

RMSF measures the deviation between the position of particles and several reference positions. Unlike RMSD, which assesses the overall structural change, RMSF is calculated for each protein residue, evaluating how much each residue fluctuates during the simulation. RMSF values generally represent the conformational shifts of each amino acid residue, providing insight into the flexibility of the protein. RMSF is determined from the time point when the potential energy shows minimal fluctuation. RMSF serves to evaluate the fluctuations of the amino acid residues that compose the protein during the simulation [62]. RMSF is used to assess the flexibility and dynamics of proteins, which are crucial in biological processes such as ligand binding, protein-protein complex formation, and signal transduction. For instance, regions with high fluctuation may indicate flexible sites that are important for protein interactions or movement in biological processes. In drug development, understanding these fluctuations can help in designing molecules that bind more effectively or modify the function of target proteins [64].

Table 8. Quantitative analysis of mean RMSD and RMSF values of ligand–receptor complexes during 100-ns molecular dynamics simulations.

Target Receptor	Ligand	Simulation time (ns)	Mean RMSD (Å)	Mean RMSF (Å)
ER-α (3ERT)	Lutein	100	3.39	1.59
ER-β (1X7J)	Violaxanthin	100	4.25	1.44
Aromatase (3S7S)	Violaxanthin	100	3.39	1.34



C.

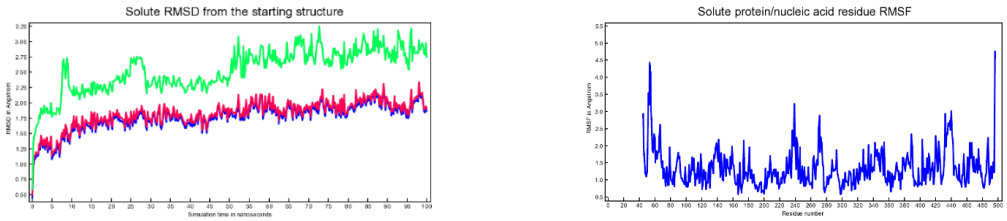


Fig. 5. The RMSD and RMSF graphs of molecular dynamics simulation results shows A**.** ER-α in complex with lutein, B. ER-β in complex with violaxanthin, and C. aromatase in complex with violaxanthin.

Quantitative analysis of the molecular dynamics simulations showed mean RMSD values of 3.39, 4.25, and 3.39 Å and mean RMSF values of 1.59, 1.44, and 1.34 Å for the ER-α–lutein, Er-β-violaxanthin, and aromatase-violaxanthin complexes, respectively (Table 8). These quantitative results are consistent with the RMSD and RMSF profiles presented in Fig. 5. In the RMSD graph, there are three colors: red (RMSD Bb), blue (RMSD Ca), and green (RMSD All). RMSD Ca refers to the RMSD calculated using only the alpha carbon (Cα) atoms from the amino acids in the protein. RMSD Bb (Backbone) is calculated using atoms in the protein backbone, typically including nitrogen (N), Cα, and carbonyl carbon (C) atoms from each amino acid residue. RMSD All is calculated using all atoms within the protein structure [65]. Based on the RMSD analysis of the receptor-ligand complexes, all systems exhibited an initial increase in RMSD during the early stage of the simulation, followed by fluctuations around relatively stable average values. The initial increase most likely reflects structural relaxation and conformational adaptation of the protein-ligand complexes after ligand binding. Subsequently, no continuous upward trend was observed, indicating that the complexes reached dynamic equilibrium during the simulation. Although some complexes exhibited relatively higher RMSD values, these fluctuations may be attributed to conformational adjustments in flexible regions of the protein rather than progressive structural destabilization [62]. Therefore, the overall RMSD profiles indicate that the protein-ligand complexes remained structurally stable throughout the 100 ns simulation. However, the RMSD graph for the ER-β-violaxanthin complex shows that the protein-ligand complex undergoes significant conformational changes at the beginning of the simulation (5-10 ns), likely due to the initial relaxation of the structure that was energy-optimized. Such rapid conformational changes early in the simulation often indicate that the protein is adjusting from its minimum energy structure to a more stable state in the simulation environment [66].

From the RMSF graph, it is observed that the flexibility of amino acid residues in the protein indicates stable interactions in the active site region that binds with the test compound. Positions of the atoms composing the amino acid residues show minimal changes during the molecular dynamic simulation, with RMSF values of <7 and findings that align with the statements of Saraswat et al., which suggest that complexes with lower RMSF values exhibit stable properties and can potentially trigger inhibition activity against the target [67]. Overall, the residues involved in the ligand-protein interaction, both from the molecular docking results and molecular dynamics simulations, demonstrate a relatively consistent presence, further supporting the stability of the interaction and its potential to exert inhibitory effects.

3.7 Toxicity prediction

The ProTox Web Server is a virtual laboratory that functions to predict the toxicity of small molecules [68]. Toxicity prediction of compounds is a crucial part of the drug development design process. Computational toxicity estimation is faster than determining toxic doses in animal testing and helps reduce the number of animal trials required. In virtual toxicity testing, the ProTox web server predicts various toxicity endpoints such as mutagenicity, carcinogenicity, hepatotoxicity, and immunotoxicity.

Table 9. Results of toxicity prediction.

Compounds	Predicted LD50 (mg/kg)	Toxicity Class	Mutagenicity	Carcinogenicity	Hepatotoxicity	Immunotoxicity
Dinoxanthin	50	2	Yes	No	No	No
Diadinoxanthin	9	2	No	No	No	No
Zeaxanthin	10	2	No	No	No	No
Lutein	10	2	No	No	No	No
Astaxanthin	4600	5	No	No	No	No
Violaxanthin	55	3	No	No	No	No

Based on the in silico toxicity prediction presented in Table 9, the tested carotenoid compounds showed different predicted toxicity profiles across the evaluated endpoints. Dinoxanthin was predicted to exhibit mutagenicity, whereas no mutagenic, carcinogenic, hepatotoxic, or immunotoxic effects were predicted for the other tested compounds. However, these predictions should be interpreted with caution because computational toxicity models provide probabilistic estimates rather than definitive biological evidence. Furthermore, structurally related xanthophyll carotenoids, including lutein, zeaxanthin, and astaxanthin, have generally demonstrated favorable safety profiles in experimental and toxicological studies [69, 70]. Therefore, additional in vitro and in vivo toxicological investigations are required to verify the predicted mutagenic potential of dinoxanthin.

4. Conclusion

This in silico study predicted that lutein and violaxanthin showed favorable binding interactions with ER-α, ER-β, and aromatase among the carotenoid compounds identified from *P. homarus*. Molecular dynamics simulations further supported the stability of the predicted ligand-receptor complexes, while in silico toxicity prediction indicated favorable safety profiles, with no significant virtual toxicity observed for both compounds. These computational findings suggest that lutein and violaxanthin may be promising candidates for further investigation as potential anti-breast cancer compounds. Nevertheless, additional experimental studies, including in vitro and in vivo investigations, are required to validate their biological activities, safety, and therapeutic efficacy before consideration for clinical application.

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