

Antioxidant Activity of β -Carotene Derived from *Spirulina platensis*: An *in vitro* and *in silico* Study

Lia Kusmita¹, Amira Nahdi¹, Ratih Pangestuti^{2,*}

¹Stifar Yayasan Pharmasi Semarang, Jl Letjend Sarwo Edie Wibowo KM 1 Plamongsari Pucanggading Semarang Indonesia; ²Research Centre for Food Processing and Technology (PR TPP), National Research and Innovation Agency (BRIN), Yogyakarta 55861, Indonesia

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Abstract

The search for natural antioxidants has intensified due to concerns regarding the side effects of synthetic compounds. *Spirulina (Arthrospira) platensis*, a cyanobacterium, is recognized for its rich content of bioactive compounds, particularly β -carotene, a powerful antioxidant pigment. This study aimed to isolate and identify β -carotene from *S. platensis* and evaluate its antioxidant activity using both *in vitro* and *in silico* approaches. The extraction of β -carotene was performed using a methanol–acetone solvent system, followed by fractionation with diethyl ether and isolation through preparative thin-layer chromatography (TLC). The identity of β -carotene was confirmed via TLC, UV-Vis spectrophotometry (λ_{max} at 452 and 478 nm), and High-performance liquid chromatography (HPLC) (retention time 30.65 min). The *in vitro* antioxidant activity was measured using the DPPH assay, which indicated an IC_{50} of 476 ± 0.32 mg/L for the β -carotene isolate, demonstrating greater potency than the crude fraction. *In silico* molecular docking against catalase, glutathione reductase, and superoxide dismutase (SOD) revealed that β -carotene exhibited stronger binding affinities (ΔG up to -9.34 kcal/mol) than the reference compound quercetin. Absorption–distribution–metabolism–excretion–toxicity (ADMET) predictions indicated high intestinal absorption and blood-brain barrier permeability, but poor water solubility and oral bioavailability based on Lipinski's criteria. The compound was found to be non-mutagenic, non-hepatotoxic, and exhibited no cardiotoxicity via human ether-à-go-go-related gene (hERG) I inhibition. These results affirm the antioxidant potential of β -carotene from *S. platensis* and open its potential application in pharmaceutical and nutraceutical formulations, particularly for non-oral delivery systems.

Keywords: *Spirulina platensis*; β -Carotene; Antioxidant activity; DPPH assay; Molecular docking; ADMET prediction

1. Introduction

The quest for effective antioxidants has gained momentum due to the increasing awareness of the detrimental effects of free radicals in promoting oxidative stress and their role in various chronic diseases (Alamoudi, 2019). Free radicals, distinguished by their unpaired electrons, can trigger chain reactions that harm cellular components, such as lipids, proteins, and DNA, potentially leading to pathological conditions (Girgis *et al.*, 2024). Oxidative stress occurs when the production of free radicals surpasses the body's antioxidant defences, underscoring the significance of exogenous antioxidants in maintaining cellular homeostasis (Lobo *et al.* 2010). Antioxidants counteract oxidative damage by neutralizing free radicals through electron donation or scavenging mechanisms (Sayed *et al.*, 2022). Although synthetic antioxidants such as butylated hydroxyanisole, butylated hydroxytoluene, and tert-butylhydroquinone are widely used, concerns regarding their toxicity and carcinogenic potential have fuelled the demand for safer, natural alternatives (Ayoub and Mehta, 2018; Neethu *et al.*, 2017).

Natural antioxidants, which are abundant in plant-based foods and microorganisms, are increasingly favoured because of their safety profiles and synergistic effects of multiple bioactive compounds within complex extracts (Mohan and Kakkar, 2020; Pourreza, 2013). These synergistic interactions often enhance the antioxidant activity beyond that of isolated synthetic compounds (Fajriah *et al.*, 2021). However, the effectiveness of natural antioxidants can vary based on factors such as their stability, bioavailability, and interactions with other dietary components (Batinić-Haberle *et al.*, 2010). Among natural sources, *Spirulina (Arthrospira) platensis*, a cyanobacterium rich in nutrients and pigments, has attracted significant interest because of its antioxidant potential (Kumar *et al.*, 2022).

The antioxidant activity of *S. platensis* is largely due to its wide range of bioactive components, including chlorophylls, carotenoids, phycocyanin, and phenolic acids (Gurugubelli *et al.*, 2023). Among these, β -carotene is a significant carotenoid with strong antioxidant capabilities. It effectively neutralizes free radicals and prevents lipid peroxidation, particularly by quenching singlet oxygen, a highly reactive species (Genç *et al.*, 2020). In addition to

* Corresponding author. e-mail: pangestuti.ratih@gmail.com.

β -carotene, other carotenoids, such as zeaxanthin and lutein, also enhance the antioxidant capacity of *Spirulina*. Carotenoids derived from algae offer distinct benefits over terrestrial sources, including reduced production costs, simpler extraction methods, and improved environmental sustainability (Christaki *et al.*, 2012; Galasso *et al.*, 2017).

While the general antioxidant activity of *S. platensis* is well-documented, there is a scarcity of studies specifically focusing on β -carotene or examining its molecular interactions in detail. To address this research gap, the present study aims to isolate and identify β -carotene from *S. platensis* extracts, assess its antioxidant activity using *in vitro* DPPH assays, and elucidate its mechanism of action through *in silico* molecular docking with key antioxidant-related enzymes. By integrating experimental and computational methodologies, this study seeks to provide a comprehensive understanding of the antioxidant potential of β -carotene from *S. platensis* and support its targeted development for nutraceutical or pharmaceutical applications.

2. Materials and Methods

2.1. Samples and Materials

The *S. platensis* samples were taxonomically identified by E. Siahaan at the Marine Bioindustry Centre, Indonesian Institute of Sciences (LIPI), under reference number B-167/III/DI.02/9/2021. Before analysis, the microalgae powder was stored at -20°C . All the solvent and reagent used in this study were analytical-grade obtained from Merck (Germany) and Sigma-Aldrich (USA).

2.2. Extraction

To extract the compounds, 50 g of *S. platensis* powder was immersed in 200 mL of methanol–acetone solution (7:3, v/v) at ambient temperature for 10 min, with occasional stirring to enhance solvent absorption. This extraction process was repeated thrice under identical conditions to optimize metabolite recovery, and the resulting extracts were filtered using Whatman No. 1 paper. The combined filtrates were concentrated under reduced pressure using a rotary evaporator (Heidolph, Germany) at a temperature below 30°C to prevent the degradation of heat-sensitive substances. The concentrated crude extract was partitioned with diethyl ether in a separating funnel at a 1:1 (v/v) ratio. The organic phase was collected and rinsed with distilled water to remove the polar impurities. The solvent from the diethyl ether fraction was evaporated under a gentle nitrogen stream until completely dry. The dried fractions were stored in airtight containers, shielded from light, and maintained at -20°C until further analysis (Kusmita *et al.* 2015).

2.3. Identification and Isolation of β -Carotene

The presence of β -carotene in the diethyl ether fraction was confirmed by thin-layer chromatography (TLC) using silica gel GF254 as the stationary phase. Chromatographic separation was performed using a mobile phase composed of n-hexane, acetone, and diethyl ether in a ratio of 6:3:2 (v/v/v). The resulting chromatograms were visualized under visible light, where β -carotene was identified by its characteristic yellow-orange bands. For further purification, preparative thin-layer chromatography (TLC)

was performed using the same solvent system. The distinct yellow β -carotene band was carefully scraped from the silica gel plate, eluted with an appropriate solvent, and collected for subsequent analyses (Wang *et al.*, 1995).

2.4. UV-Vis Spectrophotometry and High-performance liquid chromatography (HPLC) Analysis

The isolated β -carotene fraction was dissolved in acetone and analysed using a UV–Vis spectrophotometer (Shimadzu UV-1700, Japan) over a wavelength range of 350–800 nm. The resulting absorption spectrum was compared with that of a genuine β -carotene standard and reference values from the literature to confirm the identity of the compound. For additional verification and measurement, high-performance liquid chromatography (HPLC) was performed using a Shimadzu system with a photodiode array (PDA) detector and a column temperature controller. The separation process was performed using a Shim-pack VP-ODS C18 column (250×4.6 mm i.d.) under isocratic conditions with methanol and acetonitrile as the mobile phase (70:30, v/v). The flow rate was set to 1.0 mL/min, and the column temperature was maintained at 25°C . β -Carotene was detected at 420 nm, with chromatographic peaks identified by comparing retention times and absorption spectra to those of a β -carotene standard.

2.5. *In Vitro* Antioxidant Activity

The antioxidant properties of the fractions and isolated β -carotene were assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method, as described by (Samanta *et al.* 2016) with minor adjustments. In summary, 1 mL of the test sample solution at different concentrations was combined with 3 mL of a 0.08 mM DPPH solution in methanol. The mixtures were thoroughly mixed using a vortex mixer to ensure uniformity and then incubated in the dark at room temperature for 45 min to complete the reaction. The reduction in absorbance was recorded at 517 nm using a UV–Vis spectrophotometer (Shimadzu UV-1700, Japan). A control solution was prepared by mixing 3 mL of DPPH solution with 1 mL of methanol under identical conditions. Methanol served as a blank. Antioxidant activity was quantified as the percentage of DPPH radical inhibition, calculated using the following equation:

$$\% \text{ Inhibitory} = \frac{[DPPH]_0 - [DPPH]_s}{[DPPH]_0} \times 100\%$$

$[DPPH]_0$ = Initial DPPH concentration

$[DPPH]_s$ = Final DPPH concentration remaining

2.6. *In Silico* Antioxidant Activity

2.6.1. Molecular Docking Simulation

The interaction between β -carotene and antioxidant-associated enzymes was assessed using *in silico* molecular docking. AutoDockTools 1.5.6 was used to predict the binding affinity and interaction patterns of β -carotene within the active sites of specific enzymes. The proteins targeted in this study included catalase (PDB ID: 1DGH), glutathione reductase (PDB ID: 1XAN), and superoxide dismutase (SOD; PDB ID: 4A7G), all sourced from the RCSB Protein Data Bank (<https://www.rcsb.org/>). Protein

structures were prepared using BIOVIA Discovery Studio 2021 by eliminating water molecules and heteroatoms, incorporating hydrogen atoms, and saving the modified receptors in the *pdb format. The 3D structure of β -carotene was acquired from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>) in *sdf format, converted to *pdb format, and optimized through energy minimization with the addition of Gasteiger charges. Docking simulations were conducted to position the ligand within each enzyme's catalytic pocket, and the binding affinities were determined based on the binding energy (kcal/mol). The docking protocol was validated by redocking the native ligand to its respective receptor-binding site. The method was deemed valid when the root mean square deviation (RMSD) between the docked and crystallographic poses of the native ligand was less than 2 Å. Lower RMSD values indicate greater reliability of the docking process, ensuring that the predicted binding poses of β -carotene closely match biologically relevant interactions (Pandey *et al.*, 2019; Ijoma *et al.*, 2024).

2.6.2. Drug-Likeness and Absorption–distribution–metabolism–excretion–toxicity (ADMET) Prediction

To evaluate the drug-likeness of β -carotene, Lipinski's Rule of Five was utilized, which is a widely recognized guideline in drug discovery for estimating oral bioavailability. According to this rule, a compound may exhibit poor absorption or permeability if it satisfies any of the following criteria: a molecular weight exceeding 500 Da, more than 10 hydrogen bond donors, or a calculated log P (ClogP) greater than 5 (or or MlogP >4.15). These physicochemical properties serve as an initial screening tool to determine the suitability of molecules for drug development. Additionally, Absorption, Distribution, Metabolism, Excretion, and Toxicity (ADMET) predictions were performed to further assess the pharmacokinetic and safety profiles of β -carotene. The evaluated parameters included intestinal absorption (Caco-2 cell permeability), blood–brain barrier (BBB) penetration, metabolic stability concerning cytochrome P450 (CYP2D6) interactions, total clearance (excretion), and potential toxicity risks. Toxicological assessments also considered Ames

mutagenicity and inhibition of human ether-à-go-go (hERG) potassium channel types I and II, which are critical indicators of cardiotoxicity. Collectively, these analyses provide a comprehensive prediction of the potential of β -carotene as a bioactive candidate (Rahardhian *et al.*, 2025).

2.6.3. Software and Computational

The preparation of these proteins, which involved the elimination of water molecules, addition of hydrogen atoms, and optimization of receptor structures, was conducted using BIOVIA Discovery Studio (v21.1.0.2.20298, Dassault Systèmes). Molecular docking simulations were performed using AutoDock Tools (version 1.5.6, Sep_17_14 release), which facilitated ligand preparation, assignment of Gasteiger charges, grid box setup, and docking process. Binding affinities, docking poses, and interaction profiles were analysed on the same platform, and ligand–protein complex visualizations were created using BIOVIA Discovery Studio to depict hydrogen bonding, hydrophobic interactions, and spatial orientation within the active site (Abdulkareem *et al.*, 2025).

3. Results

3.1. Isolation and Identification of β -carotene

The pigment composition of the diethyl ether fraction of *S. platensis* was examined using thin-layer chromatography (TLC). The separation profile is depicted in Figure 1A, and the corresponding pigment identification is detailed in Table 1. To obtain purified β -carotene, preparative TLC was performed using silica gel GF254 as the stationary phase and a solvent mixture of hexane, ether, and acetone (6:3:2, v/v/v) as the mobile phase (Figure 1B). The distinct yellow band corresponding to β -carotene was carefully scraped, eluted, and collected for further analysis. The identity of the isolated compound was confirmed using analytical TLC against an authentic β -carotene standard. Both the isolate and the standard exhibited identical R_f values (0.98), providing compelling evidence that the isolated pigment was β -carotene (Figure 1C).

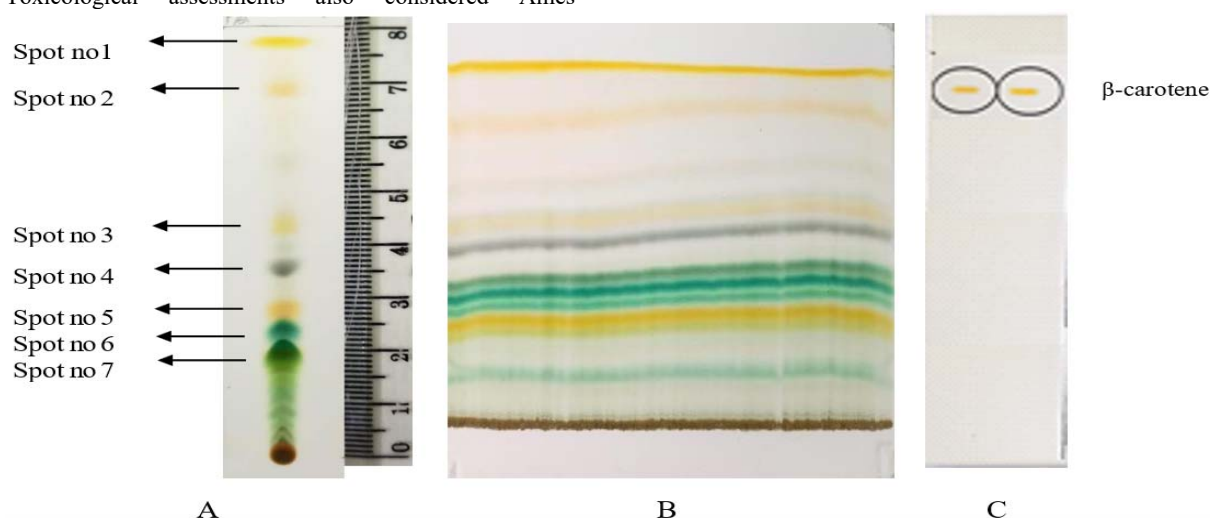
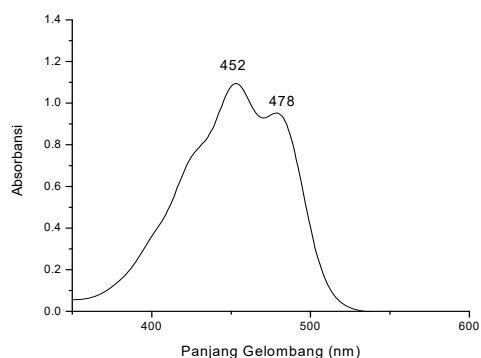


Figure 1. Separation Pattern of Diethyl Ether Fraction Stain of *S. platensis* extract (A), Isolation of β -carotene using preparative-TLC, with a stationary phase of silica gel GF 254 and a mobile phase composed of hexane, ether, and acetone in a 6:3:2 ratio (B), TLC identification of β -carotene isolate (left) and β -carotene standard (right) (C)

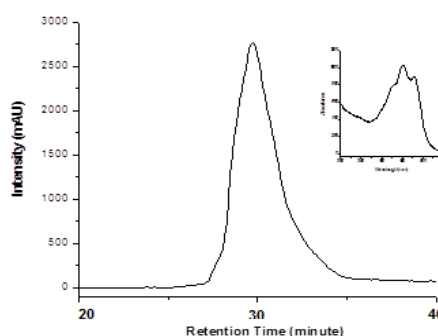
Table 1. Results of Pigment Identification in Diethyl Ether Fraction of *S. platensis*

No	Colour	Rf	Identification Ref
1	Yellow	0.98	β -carotene (Britton, 1985)
2	Orange	0.86	Carotene (Britton, 1985)
3	Yellow	0.55	Carotene (Britton, 1985)
4	Gray	0.45	Pheophytin <i>a</i> (Kusmita, 2015)
5	Orange	0.35	Xanthophyll (Kusmita, 2015)
6	Bluish green	0.30	Chlorophyll <i>a</i> (Kusmita, 2015)
7	Dark green	0.24	Chlorophyll <i>b</i> (Kusmita, 2015)

*Rf: the retention factor



A

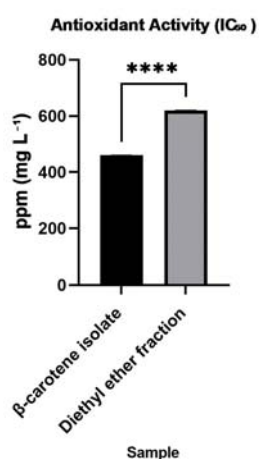


B

Figure 2. Spectrum of β -carotene isolate from the *S. platensis* (A) and HPLC profile β -carotene isolate. The detection was conducted at a wavelength of 420 nm, with a flow rate of 1 mL per min⁻¹ (B).

3.2. *In vitro* antioxidant activity

The antioxidant capacity of the β -carotene isolate was assessed using the DPPH radical-scavenging assay, and the results are shown in Figure 3. This assay is extensively utilized to evaluate the free radical scavenging potential of natural compounds because of its simplicity, sensitivity, and reproducibility.

**Figure 3.** Antioxidant activity diethyl ether fraction and β -carotene isolate from *S. platensis* using DPPH scavenging assay (****= significant, p value <0.0001, n=5)

The structure of the isolated pigment was confirmed using UV-Vis spectrophotometry (Figure 2A). To further substantiate these findings, the β -carotene isolate was subjected to high-performance liquid chromatography (HPLC) analysis, employing detection at 420 nm with a flow rate of 1 mL min⁻¹ (Figure 2B).

3.3. *In silico* antioxidant activity

The interaction between β -carotene and the target proteins catalase, glutathione reductase (GR), and superoxide dismutase (SOD) plays a crucial role in strengthening the body's antioxidant defense system. β -Carotene, as a lipophilic antioxidant, can bind to the active site or surface of proteins through hydrophobic interactions and non-covalent bonds, thereby helping stabilize the enzyme structure and increasing its efficiency in neutralizing free radicals. The antioxidant activity of β -carotene was further evaluated *in silico* using the molecular docking method, as illustrated in Figure 4.

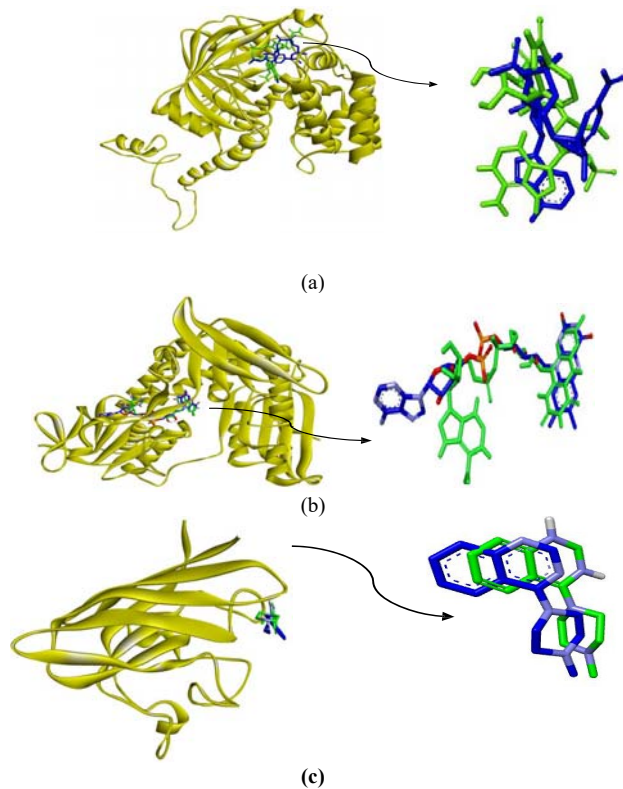
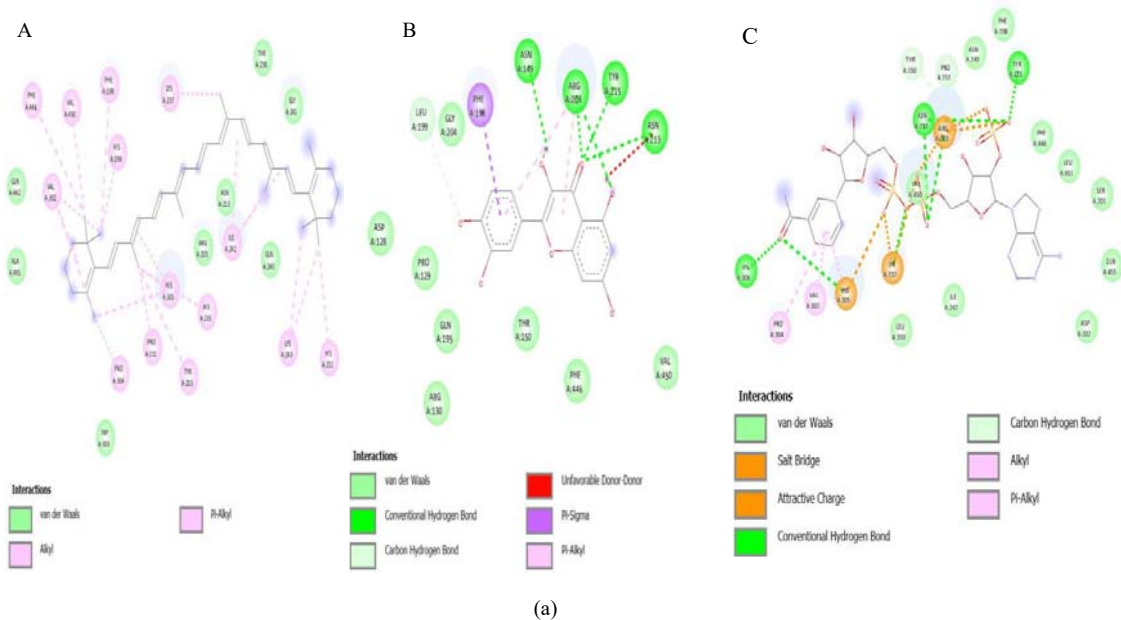


Figure 4. Overlay visualization of the IDGH (a); IXAN (b); and 4A7G (c) complex with natural ligands, depicted in blue before and in green after the redocking process.

The validation process yielded RMSD (Root Mean Square Deviation) values, which are instrumental in assessing conformational differences or comparing ligand positions post-redocking with their original positions on the protein. A smaller RMSD value indicates a reduced likelihood of ligand displacement from its original position. An acceptable RMSD value for this redocking process is 3 Å (Syahputra *et al.*, 2022). The RMSD values obtained for redocking with catalase, glutathione reductase, and superoxide dismutase (SOD) proteins were 3.25 Å, 3.16 Å, and 1.41 Å, respectively. The RMSD value that met the criteria was observed in the redocking with superoxide dismutase (SOD). Following the completion of the validation process, molecular docking of the β -carotene compound with the three proteins was conducted to ascertain the optimal docking result or antioxidant activity among them. In this analysis, quercetin served as a reference compound. The binding results were visualized in 2D using the Biovia Discovery Studio application, as depicted in Figures 5a, b, and c.



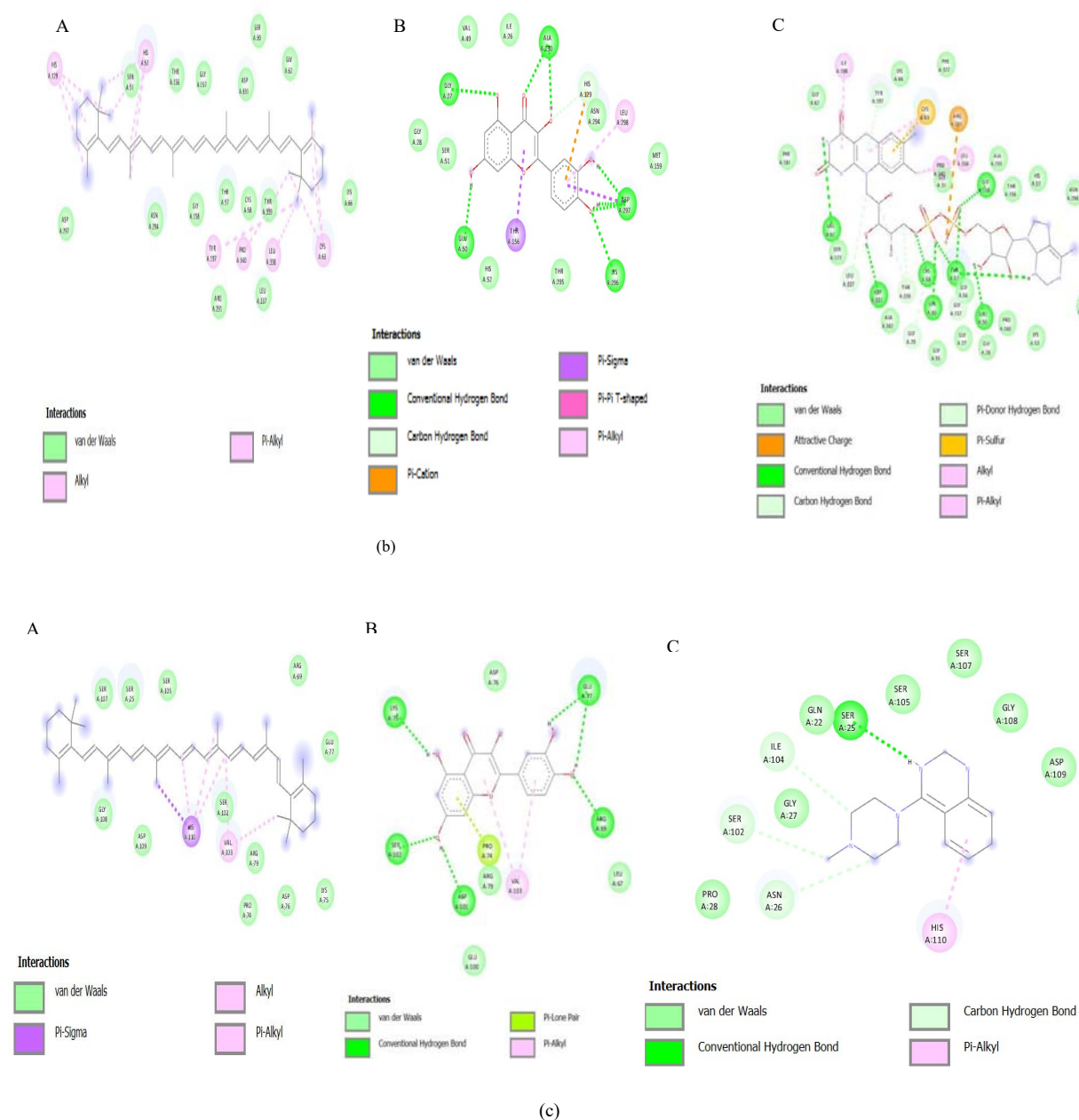


Figure 5. 2D Visualization of molecular docking results β -carotene [A], Quercetin [B], dan Natural ligand [C], PDB ID: 1DGH (a); PDB ID: 1XAN(b); and PDB ID: 1XAN (c).

Table 2. Results of Molecular Docking Analysis of β -carotene and Other Compounds

No.	Coumponds	ΔG (Kkal/mol)	Ki (μM)	Amino acid residue interactions
1DGH (Catalase Enzyme)				
1.	β -carotene	-8.30	0.83	Ala 445, Gln 442, Phe 446, Val 302, Val 450, Phe 198, His 194, Trp 303, Pro 304, Pro 151, Tyr 215, His 305, His 235, Lys 237, Arg 203, Asn 213, Ile 242, Gln 240, Lys 243, His 211, Thr 238, Gly 241
	Quercetin	-7.94	1.50	Asp 128, Pro 129, Gln 195, Arg 130, Thr 150, Phe 446, Val 450, Leu 199, Gly 204, Phe 198, Asn 149, Arg 203, Tyr 215, Asn 213
3.	NADPH dihydro nicotinamide adenine dinucleotide phosphate (Natural ligand)	-7.84	1.79	Lys 306, Pro 304, Val 302, His 305, Lys 237, Val 450, Leu 310, Ile 242, Asn 213, Arg 203, Thr 150, Pro 151, Asn 149, Phe 198, Tyr 215, Phe 446, Leu 451, Ser 201, Gln 455, Asp 202
XAN (Glutathione Reductase Enzyme)				
1.	β -carotene	-9.34	0.14	His 129, Asp 297, Ser 51, His 52, Asn 294, Thr 156, Gly 157, Gly 158, Tyr 197, Pro 340, Leu 338, Arg 291, Leu 337, Thr 57, Cys 58, Thr 339, Asp 331, Ser 30, Gly 62, Cys 63, Lys 66
2.	Quercetin	-8.32	0.79	Gly 28, Ser 51, Glu 50, His 52, Thr 156, Thr 295, Lys 296, Asp 297, Gly 27, Val 49, Ile 26, Ala 130, His 129, Asn 294, Leu 294, Met 159
3.	Flavin adenine dinucleotide (Natural ligand)	-9.65	0.084	Phe 181, Gly 62, Ile 198, Tyr 197, Lys 66, Cys 63, Phe 372, Arg 291, Pro 340, Gly 31, Leu 338, Gly 158, Ala 155, Thr 156, His 52, Asn 294, Val 61, Ser 177, Leu 337, Asp 331, Ala 342, Gly 29, Thr 339, Cys 58, Ser 30, Gly 55, Thr 57, Gly 56, Gly 157, Gly 27, Glu 50, Gly 28, Pro 160, Lys 53, Met 159
Protein 4A7G (Superoxide Dismutase (SOD) Enzyme)				
1.	β -carotene	-9.06	0.23	Ser 107, Ser 25, Ser 105, Gly 108, Asp 109, His 110, Ser 102, Val 103, Arg 79, Pro 74, Asp 76, Lys 75, Glu 77, Arg 69
2.	Quercetin	-8.08	1.20	Lys 75, Ser 102, Asp 101, Pro 74, Arg 79, Val 103, Leu 67, Arg 69, Glu 77, Asp 76, Glu 100
3.	4-(4-methylpiperazin-1-yl) quinazoline (Natural ligand)	-5.21	151.90	Pro 28, Asn 26, Ser 102, Gly 27, Ile 104, Gln 22, Ser 25, Ser 105, His 110, Ser 107, Gly 108, Asp 109

In the development or discovery of new drugs, there are guidelines used to evaluate and select the characteristics of compounds that will be used for drugs so that it can be determined whether the compound can be used for oral drugs or not. These guidelines are known as Lipinski's Rule of Five. This guideline states that if a compound has good oral bioavailability, the following five parameters must be met: the compound must have a molecular weight of no more than 500 Dalton, lipophilicity (Log P) or the tendency of the compound to dissolve in fat <5, have fewer than 5 hydrogen donors, have no more than 10 hydrogen acceptors in the compound, and have a molar refractivity index between 40 and 130 (Lipinski *et al.*,

2001). The Lipinski prediction results for β -carotene isolates are presented in Table 3.

Table 3. Prediction Results of 5 Lipinski Parameters of β -carotene Isolates

No.	Molecular Weight	Log P	Donor Hydrogen Bond	Acceptor Hydrogen Bond	Surface Area
1.	536.888	12.6058	0	0	247.375

After predicting parameter values using Lipinski's rule, ADMET testing using pk-CSM was then performed to predict the pharmacokinetic activity and toxicity of β -carotene (Table 4).

Table 4. ADMET Prediction of β -carotene Compounds as Antioxidants

PharmacokineticModel Name Parameters	Prediction	Unit
Absorption	Water solubility	-7.252
	CaCO ₂	1.239
	Intestinal absorption (human)	90.393
	Skin permeability	-2.739
	P-glycoprotein substrate	No
	P-glycoprotein I inhibitor	No
	P-glycoprotein II inhibitor	Yes
Distribution	VDss (human)	0.202
	Fraction unbound (human)	0
	BBB Permeability	0.92
	CNS permeability	-1.074
Metabolism	CYP2D6 substrate	No
	CYP3A4 substrate	Yes
	CYP1A2 inhibitor	No
	CYP2C19 inhibitor	No
	CYP2C9 inhibitor	No
	CYP2D6 inhibitor	No
	CYP3A4 inhibitor	No
Excretion	Total Clearance	1.061
	Renal OCT2 substrate	No
Toxicity	AMES toxicity	No
	Max. tolerated dose-0.51 (human)	
	hERG I inhibitor	No
	hERG II inhibitor	Yes
	Oral Rat Acute Toxicity (LD50)	2.134
	Oral Rat Chronic Toxicity (LOAEL)	0.624
	Hepatotoxicity	No
	Skin Sensitisation	No
	T.Pyriformis toxicity	0.319
	Minnow toxicity	-3818

4. Discussion

Among the separated pigments, β -carotene was identified as a yellow spot with an R_f value of 0.98 (Spot 1), which aligns with previously reported data (Britton, 1985). Owing to its highly nonpolar nature, β -carotene migrates further in normal-phase chromatography systems, resulting in a high R_f value (Butnariu, 2016). Other identified pigments included additional carotene derivatives, xanthophylls, pheophytin *a*, and chlorophylls

a and *b*, reflecting the broad spectrum of photosynthetic and accessory pigments in *S. platensis*. *S. platensis* contains chlorophyll *a* pigment and its derivatives, which have antioxidant activity (Alotaiby *et al.*, 2024). Maharani *et al.*, (2024), also showed that the pigments in *S. platensis* are carotenoids and chlorophyll and its derivatives.

The successful isolation and confirmation of β -carotene from *S. platensis* highlights its potential as a significant bioactive carotenoid. Given its antioxidant properties and role as a precursor of vitamin A, β -carotene enhances the functional and nutraceutical value of *S. platensis*. Furthermore, the co-occurrence of other pigments, such as chlorophylls and xanthophylls, suggests a synergistic contribution to the antioxidant capacity of the extract, which is consistent with the DPPH assay results presented later in this study. Chlorophyll and its derivatives, namely pheophytin and pheophorbide, have high antioxidant activity. Testing was carried out using the FRAP, TEAC, and DPPH methods (Alotaiby *et al.*, 2024).

The spectrum of the isolate exhibited two distinct absorption maxima within the wavelength range of 400–600 nm, with peak values at 452 and 478 nm. This absorption pattern, characterized by the presence of two well-defined maxima, is consistent with the spectral features of carotenoid. Notably, the recorded absorption maxima closely aligned with the reference values for β -carotene, as reported by Britton (1995) and Rodriguez-Amaya *et al.* (2004), further substantiating the successful isolation of this compound. The appearance of dual absorption peaks can be attributed to the extensive conjugated double-bond system of β -carotene. This conjugation lowers the energy gap between the ground and excited states, allowing the molecule to absorb visible light at longer wavelengths. The fine structure observed in the spectrum arises from electronic transitions within the polyene chain, which are a distinctive feature of carotenoid molecules (Telegina, *et al.*, 2023).

The chromatographic profile from HPLC revealed a predominant peak with a retention time of 30.65 min, which aligned with the elution characteristics of β -carotene documented in the literature under comparable conditions. Additionally, the spectral profile associated with this chromatographic peak exhibited maximum absorbance at 452 and 478 nm, corroborating the identification of the isolate as β -carotene.

The integration of evidence from TLC, UV-Vis spectroscopy, and HPLC strongly supports the identification of the isolated pigment as β -carotene. The concordance between the chromatographic retention time and distinctive spectral characteristics reinforces the validity of this identification. This multi-method verification is particularly crucial, given the structural similarity of carotenoid derivatives, which frequently co-occur in microalgal extracts. The use of complementary analytical approaches, therefore, minimizes the risk of misidentification and increases confidence in distinguishing closely related compounds. By integrating chromatographic behavior, spectral characteristics, and quantitative patterns, the analysis becomes more robust, especially when dealing with complex matrices rich in overlapping metabolites (de Rosso *et al.*, 2007).

The β -carotene isolate demonstrated an IC_{50} value of $460 \pm 0.33 \text{ mg L}^{-1}$, whereas the diethyl ether fraction exhibited a higher IC_{50} of $620 \pm 0.25 \text{ mg L}^{-1}$. Kusmita *et*

al. (2023) research tested the antioxidant activity of β -carotene in *S. polycystum*. The results obtained showed that β -carotene in spirulina had the same activity as β -carotene in *S. polycystum*. Given that lower IC₅₀ values are indicative of stronger antioxidant capacity, the markedly reduced IC₅₀ observed in the purified β -carotene clearly suggests that the isolated pigment possesses substantially greater radical-scavenging activity compared to the crude fraction. This enhanced bioactivity can be explained by the intrinsic structural properties of β -carotene, which is characterized by an extensive conjugated polyene chain composed of alternating double and single bonds. Such a configuration allows the π -electrons to become highly delocalized, enabling efficient stabilization of unpaired electrons through resonance. As a result, β -carotene is able to neutralize a broad range of reactive species, particularly singlet oxygen and peroxy radicals, by acting as an effective electron donor or energy quencher (Young *et al.*, 2018).

Furthermore, the purification process likely removes interfering compounds—such as proteins, carbohydrates, or other pigments—that may compete with or hinder the antioxidant potential of β -carotene in the crude extract. By concentrating the active carotenoid and eliminating non-functional matrix components, the isolate exhibits improved accessibility and reactivity toward free radicals, thereby enhancing its measured antioxidant potency (Stahl, *et al.*, 2003). This relationship between purification and increased bioactivity has also been highlighted in previous carotenoid research, emphasizing that matrix effects can significantly influence antioxidant test outcomes. These structural characteristics enable β -carotene to act as an efficient quencher of free radicals. The findings of this study are consistent with the report by Christiana *et al.* (2008), who demonstrated that β -carotene in *Spirulina platensis* powder exhibits antioxidant activity approximately 5–6 times stronger than that of chlorophyll *a*. Their results reinforce the concept that β -carotene is one of the major contributors to the overall antioxidant profile of *S. platensis* and plays a central role in its protective biochemical functions. The agreement between the present data and earlier literature strengthens the validity of β -carotene as a potent natural antioxidant with high physiological relevance.

Overall, the superior activity of the purified β -carotene relative to the crude pigment fraction underscores the importance of purification strategies for maximizing functional and bioactive properties of carotenoid-rich materials. Enhancing pigment purity not only improves analytical accuracy in antioxidant assays but also holds practical implications for the development of high-value bioproducts, including nutraceuticals, functional foods, and natural antioxidant formulations.

The analysis of β -carotene binding with three proteins revealed that this compound exhibits a more negative binding energy (ΔG) value compared to quercetin, which served as a positive antioxidant control. This value, along with the inhibition constant (K_i), is utilized to assess the strength of a ligand's binding to a specific site on a protein or receptor. In its interaction with the catalase protein (1DGH), β -carotene and quercetin demonstrated binding energy values of -8.30 kcal/mol and -7.94 kcal/mol, respectively (Table 2). When bound to glutathione reductase, β -carotene and quercetin exhibited binding

strengths of -9.34 kcal/mol and -8.32 kcal/mol, respectively. Furthermore, in interaction with the superoxide dismutase (SOD) protein (4A7G), the binding energies of β -carotene and quercetin were -9.06 kcal/mol and -8.08 kcal/mol, respectively (Table 2). The more negative binding energy of β -carotene suggests a stronger binding affinity to the proteins or target receptors, resulting in enhanced bond stability and improved antioxidant activity (Krinsky *et al.*, 2005). The more negative binding energy of β -carotene suggests a stronger and more stable interaction with the target antioxidant enzymes, which in turn indicates its potential to enhance the overall antioxidant mechanism. In this study, β -carotene exhibited varying affinities toward catalase, glutathione reductase, and SOD. Among these proteins, the β -carotene–SOD complex demonstrated the most robust binding, as reflected by the most negative binding energy compared to the other two protein complexes. Interestingly, this strong affinity was achieved despite the involvement of fewer interacting amino acid residues, suggesting that the quality of interaction plays a more critical role than the quantity of residues involved (Meng, *et al.*, 2011).

The interaction of β -carotene with the three antioxidant proteins did not involve hydrogen bonding. Instead, the docking results showed that the binding was predominantly driven by hydrophobic interactions, including π -alkyl, alkyl, and van der Waals forces. Although hydrophobic interactions are typically weaker than hydrogen bonds, they collectively contribute to the stabilization of the ligand–protein complex and support the maintenance of protein conformational integrity (Lohita Sari *et al.*, 2022). The dominance of these interactions is consistent with the physicochemical nature of β -carotene, which is a highly hydrophobic compound characterized by a long-conjugated hydrocarbon chain. This structural feature limits the formation of polar or electrostatic interactions, particularly hydrogen bonds, due to the absence of electronegative functional groups capable of interacting with donor or acceptor atoms on the protein surface.

Overall, the strong hydrophobic-driven binding of β -carotene to SOD, catalase, and glutathione reductase supports its role as a potent antioxidant molecule. The stability of these interactions, especially with SOD, may contribute to the enhanced scavenging of reactive oxygen species, reinforcing the biological relevance of β -carotene in modulating oxidative stress responses.

In binding with the three proteins, β -carotene and quercetin do not form bonds with specific amino acid residues, according to existing literature. The amino acid residues involved in bonding with the catalase protein include Arg112, which forms a salt bridge with the heme carboxylate radical; Tyr358, which regulates reactivity by donating electrons to the catalase, particularly within the iron-heme group; and Arg354, which contributes to oxidative stability by generating an electrostatic field through protonation. Meanwhile, the amino acid residues involved in bonding with the glutathione reductase protein include Tyr407, which plays a protective role in the human protein complex; and His82 along with Cys90, or alternatively His75, Met79, Phe78, and Asp81, which are located at the enzyme's disulfide bond active site (Hossen *et al.*, 2022).

Although quercetin does not bind to specific key amino acid residues, it forms conventional hydrogen bonds during its interaction with the proteins, an important type of bond commonly found in human biological systems. The interaction between quercetin and the catalase protein results in four hydrogen bonds, involving Asn149, Arg203, Tyr215, and Asn213. Its interaction with the glutathione reductase protein forms five hydrogen bonds with Gly27, Ala130, Glu50, Lys296, and Asp297. Meanwhile, its binding with the SOD protein involves five hydrogen bonds formed with Lys75, Ser102, Asp101, Glu77, and Arg69.

The interaction between β -carotene and the three proteins produced several amino acid residues that were the same as those found in the binding between quercetin and proteins with conventional hydrogen bonds. The amino acid residues that were the same in the interaction with catalase protein were Arg 203, Tyr 215, Asn 21, and those in the interaction with glutathione reductase protein were Asp 297. In the binding of β -carotene with SOD protein, the amino acid residues obtained are Lys 75, Ser 102, Glu 77, and Arg 69, which have π -alkyl and alkyl bonds (hydrophobic interactions), but in the binding of quercetin with SOD, these bonds are hydrogen bonds.

The in-silico prediction parameters indicate that β -carotene possesses physicochemical properties that strongly influence its absorption, distribution, and overall pharmacokinetic behavior. Its molecular weight of 536.888 Da exceeds the commonly cited ≤ 500 Da threshold in Lipinski's Rule of Five (Table 3). According to Lipinski, compounds with molecular weights above this limit tend to exhibit reduced membrane permeability through passive diffusion, as larger molecules encounter steric hindrance and require higher energy to traverse phospholipid bilayers (Lipinski *et al.*, 2001). This is consistent with Benet *et al.*, (2016), who emphasized that molecular size plays a critical role in passive transport, where compounds above 500 Da typically demonstrate poor oral absorption.

The log P value of β -carotene is extremely high (log P = 12.6058), far exceeding the recommended limit of ≤ 5 for optimal drug-likeness. Log P represents the balance between hydrophilic and lipophilic characteristics; compounds with very high log P values are excessively lipophilic and tend to partition strongly into lipid membranes. β -Carotene's long conjugated hydrocarbon chain accounts for this high hydrophobicity. While lipophilicity supports membrane association, excessive hydrophobicity is associated with poor aqueous solubility, limited bioavailability, increased retention in lipid bilayers, and broader, non-specific tissue distribution (Hughes *et al.*, 2008). Hakiki *et al.* (2024) further noted that compounds with extremely high lipophilicity are more prone to off-target interactions, potentially increasing toxicity and diminishing target-binding selectivity.

Hydrogen bond donors (HBD) and hydrogen bond acceptors (HBA) are also critical parameters influencing drug absorption and permeability. β -Carotene contains zero HBD and HBA, which technically complies with Lipinski's criteria (HBD ≤ 5 , HBA ≤ 10). However, compounds with extremely low polarity and no hydrogen-bonding capability often exhibit very poor aqueous solubility, limiting their ability to dissolve in gastrointestinal fluids and reducing systemic absorption (Zhang *et al.*, 2012). Shofi (2021) emphasizes that

deviations—whether excessive or insufficient—from optimal hydrogen-bonding capacity can hinder absorption because energy is required to desolvate molecules before membrane passage.

Taken together, these properties suggest that although β -carotene can interact strongly with lipid membranes due to its high lipophilicity, its poor solubility, large molecular size, and extreme hydrophobicity reduce its suitability as a drug-like molecule under typical pharmacokinetic criteria. Its biological activity is more likely attributed to membrane-associated antioxidant roles rather than behavior expected of an orally bioavailable small-molecule drug.

In the absorption process, there is a parameter called water solubility. β -carotene compounds have low water solubility because their value is -7.252, which is less than -6 (Table 4). The CaCO_2 parameter is used to determine drug transport in the absorption process through the intestinal epithelium originating from carcinoma cells. A high CaCO_2 value indicates that the compound also has high permeability. The compound β -carotene has a CaCO_2 value of 1.239, indicating sufficient permeability since it exceeds 0.90. The parameter intestinal absorption refers to the absorption of drugs administered orally in the human intestinal tract. The absorption of compounds in the intestine is poor if this parameter value is $< 30\%$, so that β -carotene can be absorbed well by the intestine because the value of 90.393% has a high degree of absorption (Inayah *et al.*, 2024). The Log Kp parameter is an important parameter in identifying or developing drugs administered via the transdermal route. β -carotene has a Log Kp value of -2.739, indicating that this compound has sufficiently high skin permeability, making it suitable for formulation with a transdermal delivery system since its value is < 2.5 (Hakiki *et al.*, 2024).

Parameters in the distribution process are used to determine the distribution of drugs in the body on their way to their target. VDss (Steady state of volume distribution) is used to determine the distribution of drugs in the body in a steady state or equilibrium. β -carotene has a low distribution volume because its value is less than 0.71, namely 0.202 L/kg. Other parameters used to determine the permeability of compounds through the blood-brain barrier (BBB) include the Blood Brain Barrier (BBB). Prediction results indicate that β -carotene has a log BB value of 0.92, meaning that this compound can easily pass through the blood-brain barrier because the log BB value is greater than 0.3. This is also due to β -carotene being a hydrophobic molecule, enabling it to easily penetrate the BBB through the lipid bilayer (Hakiki *et al.*, 2024; Inayah *et al.*, 2024).

Prediction results indicate that β -carotene is metabolized by CYP3A4 because it acts as a substrate for CYP3A4. CYP3A4 belongs to the CYP450 enzyme family. This enzyme plays a role in biotransformation, accounting for 30.20%, and is most abundant in the liver, where it is responsible for eliminating substances that are not needed by the body or detoxification (Hakiki *et al.*, 2024). The metabolism of drug compounds occurs through reduction, oxidation, and other reactions, which increases the polarity of drug compounds, allowing them to be excreted from the body more easily (Dari *et al.*, 2022).

The prediction results at the excretion stage showed a total clearance value of 1.061 ml/min/kg. This value is a

combination of hepatic clearance and renal clearance used to determine the dose required to achieve a steady-state concentration and to determine the rate of drug clearance in the body. Another parameter is OCT2, a transporter in the kidneys that plays a role in the transport and clearance of various substances or drugs. The prediction results indicate that β -carotene does not affect the OCT2 substrate, meaning this compound is not an OCT2 substrate. Therefore, the risk of harmful interactions with OCT2 inhibitors can be minimized (Herdini and Setyawati, 2023).

The AMES toxicity test results indicate that β -carotene does not cause mutations. The hERG (human Ether-à-go-go-Related Gene) inhibition test was performed to assess whether β -carotene has the potential to induce arrhythmia. A positive result in this test suggests that a compound may cause arrhythmia and pose a risk of sudden death, as the hERG channel plays a critical role in regulating heart rhythm. The prediction results show that β -carotene does not inhibit hERG I, indicating it is unlikely to cause cardiac arrhythmia (Hakiki *et al.*, 2024). However, β -carotene shows positive inhibition of hERG II, which is involved in other tissues such as the brain. The LD50 prediction indicates a value of 2.134 mol/kg, equivalent to 1,147,950 mg/kg, placing β -carotene in toxicity class VI (>5,000 mg/kg), which is considered non-toxic. This very high LD50 value means that only at such a high dose would β -carotene cause 50% mortality in mice or other test animals. Furthermore, β -carotene is predicted not to induce liver toxicity (hepatotoxicity) or skin sensitivity.

5. Conclusion

The present work demonstrates the antioxidant relevance of β -carotene derived from *Spirulina platensis*, supported by complementary in vitro assays and molecular docking analysis. These findings underscore its potential as a natural bioactive compound suitable for further development in health-related applications. To advance its practical utilization, future studies should include in vivo validation, assessment of bioavailability, and exploration of optimized delivery systems. Such efforts will help clarify its functional role and broaden its applicability in nutraceutical and therapeutic contexts.

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