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Original Article

COMBINING LIQUID CHROMATOGRAPHY WITH TANDEM MASS SPECTROMETRY (LC-MS/MS) TECHNIQUE, SPECTROPHOTOMETERVALIDATION AND AN *IN SILICO* STUDY OF 96% ETHANOL EXTRACT OF *SPIRULINA PLATENSIS*

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ABSTRACT

Objective: This study was to analyze the component sofa 96% ethanol extract of *Spirulina platensis* by the LC-MS/MS technique, then validate them with the spectrophotometer technique using the C-phycocyanin standard and an *in silico* study approach as an antioxidant property of *S. platensis* against inflammatory.

Methods: Chromatographic resolution was attained with a Phenominex C18 (50 mm×2.6 mm, 3 µm) stationary column technique, validation using C-phycocyanin standard using the spectrophotometer technique, and an *in silico* study of c-phycocyanin using molecular docking analysis.

Results: Tentative active compounds such as flavonoid (Maltol and Morin), peptide (Cyclo Pro-Ala, Cyclo Pro-Pro, and Thymine), and phenol (m-Aminophenol, N-Methyltyramine, and Tyramine) have been identified from a 96% ethanol extract of *S. platensis* by LCMS/MS analysis. The concentration of c-phycocyanin in the 96% ethanol extract of *S. platensis* is 229, 2µg/ml. According to our *in silico* study, c-phycocyanin demonstrates potential as an anti-inflammatory agent.

Conclusion: The LC-MS/MS technique can detect flavonoid, peptide, and phenolic components in the 96% ethanol extract of *S. platensis*. A spectrophotometer can identify the validation equation of c-phycocyanin in a 96% ethanol extract of S. *platensis*. Based on our *in silico* study, c-phycocyanin demonstrate the capability to prevent inflammatory activity.

Keywords: C-phycocyanin, In silico study, LC-MS/MS, Spectrophotometer, *Spirulina platensis*

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INTRODUCTION

Spirulina platensis is a cyanobacterium filamentous microalga that contains various flavonoids, among other phytochemicals. These flavonoids enhance antioxidant activity, potentially offer protection against oxidative stress and inflammation [1, 2], and anticancer properties [3, 4]. *Spirulina platens s*can be used as a preventive supplement since it is high in protein, carbohydrates, polyunsaturated fatty acids, sterols, and other essential components [5, 6]. *Spirulina platensis* contains 60-70% dry-weight protein [7], essential amino acids, carotenoids, lipids, vitamins E, C, and selenium [8, 9]. It has been reported that the consumption of *S. platensis* could prevent or manage metabolic syndrome disorders [10-12]. *Spirulina platensis* has long been utilized for nutrition in Mexico, Africa, and Asia, especially in Indonesia. In Indonesia, *S. platensis* is cultivated using freshwater aquaculture.

Spirulina platensis and its pigments such as c-phycocyanin, carotene, xanthophyll, and chlorophyll, exhibit exogenous antioxidant properties [13]. C-phycocyanin, rich in blue-green microalgae, is known to break the radical chain to inhibit reactive oxygen species and oxidative stress, act as a hepatoprotection, and help reduce blood glucose levels. C-phycocyanin can reduce free radicals, minimize nitrite production, suppress nitric oxide synthase (iNOS) expression, and inhibit lipid peroxidation [2, 14, 15]. Because c-phycocyanin is soluble in water, cells must first be disrupted using physical or chemical methods to extract the pigment [16-18]. C-phycocyanin prevents oxidative stress and cell damage *in vitro* in the hypoxia model employing the myoblast cell line H9c2 [19, 20].

This study aimed to explore and identify the most potent *S. platensis* compounds from the Indonesian medicinal algae. Using liquid chromatography coupled with mass spectrophotometry (LC-MS/MS) for precise identification and quantification, the study developed and validated an LC-MS/MS analytical procedure. The validation included a spectrophotometric analysis using a c-phycocyanin standard. Additionally, the *in silico* research was conducted to evaluate the antioxidant properties of c-phycocyanin from *S. plarensis* against oxidative stress and inflammation.

MATERIALS AND METHODS

Extraction of *spirulina platensis*

S. platensis powder from PT Algae park, Klaten-Indonesia (1000 g) was macerated using 96% ethanol and left to stand for 48 h at room temperature. The solution was then filtered to obtain precipitate, which was further macerated using 96% ethanol. A rotary evaporator was used to evaporate the solvent, which was then freeze-dried.

Materials

The tools used for the in silico study are a laptop (HP ENVY x360 13-AG0023AU). AMD Ryzen 7 2700 8GB 1TB Win 13 (Logical Processors 8) 8GB of memory RAM, a 64-bit operating system, an x64-based processor, and an Autodock 1.5.7 System. Micropipette 1000 µl**, 1.5 ml microtubes, tips, 96-well plate Biologyx, glassware, analytical balance (Sartorius). The chemicals used were Ethanol (BrataChem), Water proinjection (Sigma Aldrich), and a spectrophotometer (Varisocan, Thermo).

LCMS/MS analysis of a 96% ethanol extract of *Spirulina platensis*

The liquid chromatography-tandem mass spectrophotometry (LC-MS/MS) was conducted in PT Saraswanti INDO GENETECH, Bogor-Indonesia, under certificate number: SIG. LHP. VII.2023.211434171. the methodology employed was based on protocol 18-16/MU/SMM/-

SIG (LCMS/MS), using a Quadrupole Time-of-Flight (QTOF) system. [21, 22]. Mass spectra were acquired using the AB Sciex 3200 Q-Trap LCMS/MS with the Perkin Elmer FX 15 UHPLC system (MA, USA). The negative ion mass spectra were obtained from the LC Q-Trap MS/MS detector in full ion scan mode (100 to 1200 m/z for the full scan and 50–1200 m/z for the MS/MS scan) at a scanrate of 0.5 Hz. The hyphenated system was supported with mass spectrometry software and a spectral library provided by ACD Labs (TO, CA). Analyte separation was carried out on a pre-packed C18 (4 × 250 mm, 5 µm, Phenomenex) column with a gradient mobile phase comprising water (solvent A) and ethanol with 1% acetonitrile (solvent B), each containing 0.1% formic acid and 5 mmol ammoniumformate. The gradient program commenced with 80% to 90% solvent B from 0.01 to 11.00 min with a flow rate of 1.0 ml/min. The injection volume was set to 20 µl**. All chromatographic procedures were performed at ambient temperature, and the corresponding peaks from the TOF Analyser of the 96% ethanol extract of *S. platensis* were identified by comparison with the literature data/ACD labs mass spectral library i. e., Flavonoid, Peptide, and Phenols.

Preparation of quality and calibration standard solutions

A 100 µg/ml solution of S. platensis extract in 96% ethanol was prepared using the mobile phase. Quality and calibration controls were processed with plasma blank samples containing flavonoid, peptide, and phenol standards. Eight calibration levels (1, 5, 35, 150, 350, 600, 900, and 1200 ng/ml) were created using the spike method on plasma blanks. Lower-QC (3 ng/ml), Medium-QC (6 ng/ml), and Higher-QC (9 ng/ml) solutions were also prepared. All processed solutions were stored at −20 °C until analysis.

Protocol for sample preparation

Each spiked plasma sample of 50µl was mixed with 250µl of methyl alcohol having0.1%HCOOHtoprecipitatetheproteins present in the mixture. The resultant mixture was subjected to vortex mixing for 10 min. Then these sample solutions were centrifuged for20 min at 4.0 °C. After that, 150µl of supernatant liquid was relocated to polypropylene tubes, from which an aliquot of 5µl of samples was infused into the LC-MS/MS system.

C-phycocyanin

C-phycocyanin, sourced from PT Algaepark in Klaten, Indonesia, was used as the primary phytochemical standard at a concentration of 1 mg/ml. Water purification systems were used for all research involving water. The spectrophotometric quantification of c-phycocyanin was performed using a series of standard dilutions. A 1 mg/ml C-phycocyanin solution was serially diluted to 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml, 0.0625 mg/ml, and 0 mg/ml (blank) in 1.5 ml microtubes.

Ligand and protein preparation for an *in silico* study

C-phycocyanin as a ligand from phytoconstituents of *S. platensis* was downloaded from the PubChem database (http://pubchem.ncbi.nlm.nih.gov) in SDF structure format. The structure of c-phycocyanin (Structure2D_CID_6438349) was converted from the SDF to the PDB structure format using Marven Sketch software. The prepared PDB protein is used in the virtual screening docking method of PyRx tools.

The protein used for in silico study is the TNF-α protein (PDB ID 7JRA). TNF-protein preparations were obtained from the UniProt website (https://www.uniprot.org/uniprotkb/P01375/entry#structure). This protein classification is a cytokine in *Homo sapiens*, and an expression system in *Escherichia coli* BL21 (DE3), with no mutation. TNF-α has 3 chains: A, B, and C chains, with sequence lengths of 160, and has 2 native ligands, VGY and GOL [23].

The 3D protein crystal structures were retrieved from the RCSB Protein Data Bank with protein preparation. Using AutoDockTools version 1.5.7 software and protein optimization, the water was removed, hydrogen polar only was added, hydrogen non-polar was merged, and Gasteiger charge was added. The docking was performed using AutoDock4. The run Genetic Algorithm (GA) was set to 100 times. The docking analysis was performed using PyMOL version 2.4.1 and Discovery Studio Visualizer for 3D visualization.

The grid parameter file was prepared and optimized in several grid points with the lowest RMSD (<2Å). The compounds of the screening results were re-docked with selected grid boxes. Based on molecular docking validation with a re-docking method between 7JRA and its native ligand (VGY), the optimum grid box is 30x30x30 with a binding energy value of −1.78 Å, an RMSD value of 2.72 Å, and an inhibition constant value of 213.97 uM. The optimum grid center is x =-14.265, y =-2.177, and z =-26.652 with 0.375 Å spacing for the default setting. The native ligand VGY has bindings with Gly127, Tyr195, Leu196, Tyr227, Tyr135, Leu233, Gly198, and Ile123.

The docking analysis results displayed binding affinity values as several approximations were used to model protein-ligand interactions. RMSD with a zero value was used to predict the highest predicted total binding energy to a ligand and was then selected for analysis of 3D structures and 2D visualizations of ligand–residue interactions at their proper docking positions. The ligand-protein interactions were visualized using the software Discovery Studio Visualizer, and we observed the formation of hydrogen and hydrophobic bonds with the active site.

RESULTS AND DISCUSSION

LCMS/MS analysis of a 96% ethanol extract of *Spirulina platensis*

LCMS/MS analysis of a 96% ethanol extract of *Spirulina platensis*is shown in fig. 1 and table 1. A few compounds have been identified and enlisted based on the literature data and the Advanced Chemistry Development (ACD) Labs based mass spectral library i. e., flavonoid, peptide, and phenol.

In this present study, possible active compounds such as flavonoid (Maltol and Morin), peptide (Cyclo Pro-Ala, Cyclo Pro-Pro, and Thymine), and phenol (m-Aminophenol, N-Methyltyramine, and Tyramine) have been identified from a 96% ethanol extract of *S. platensis* through LCMS/MS analysis. Until recently, the most common chemicals identified from *S. platensis* extract have been flavonoids, peptides, phenolic compounds, carotenoids, phycobiliproteins, chlorophyll, polyunsaturated fatty acids, sulphated polysaccharides, and sterols [24].

In fig. 2, the intensity of maltol is 3.83, cyclo (Pro-Pro) is 4.25 and morin is 14.00. These three main components have a high intensity of more than 20,000. This result is shown in table 2**.**

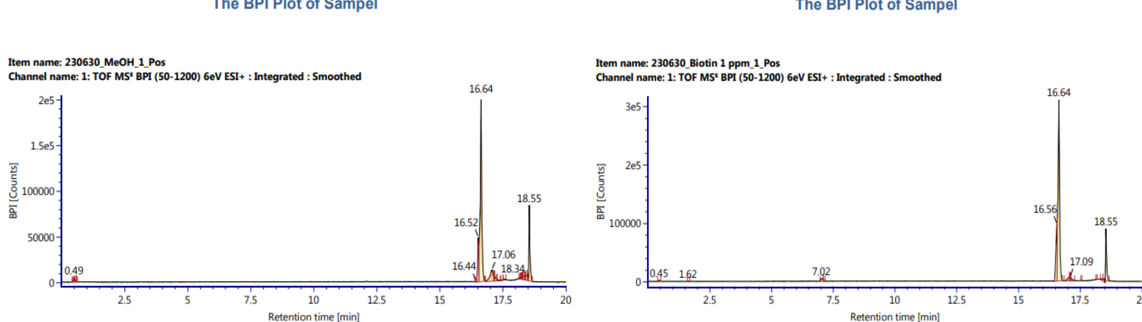


Fig. 1: The BPI Plot of 96% ethanol extract of *Spirulina platensis*

Table 1: Confirmed component summary of 96% ethanol extract of *Spirulina platensis*

Component name	Formula	Identification status	Observed RT (min)	Mass error (ppm)	Total fragments found	Isotope match Mz RMS PPM	Isotope match intensity RMS percent	Response	Adducts
Biotin	C10H16N2O3S	Identified	7.03	0.2	12	0.86	2.75	18169	+H
Biotin	C10H16N2O3S	Identified	7.10	-0.6	14	0.93	2.83	18194	+H
Cyclo(Pro-Ala)	C8H12N2O2	Identified	3.16	-0.9	2	2.22	8.55	8789	+H
Cyclo(Pro-Ala)	C8H12N2O2	Identified	3.17	-0.8	4	1.24	8.44	7722	+H
Cyclo(Pro-Pro)	C10H14N2O2	Identified	4.24	-0.8	5	1.07	9.70	20112	+H
Cyclo(Pro-Pro)	C10H14N2O2	Identified	4.25	-0.8	10	2.12	9.75	19671	+H
Maltol	C6H6O3	Identified	3.82	-0.4	1	1.21	5.54	30077	+H
Maltol	C6H6O3	Identified	3.83	-0.8	1	0.98	4.48	30691	+H
m-Aminophenol	C6H7NO	Identified	0.95	0.4	3	1.98	5.18	3041	+H
m-Aminophenol	C6H7NO	Identified	0.94	-1.6	4	3.56	9.50	2588	+H
Morin	C15H10O7	Identified	14.05	0.7	8	0.76	4.07	63525	+H
Morin	C15H10O7	Identified	14.00	0.3	5	0.64	3.63	46487	+H
N-Methyltyramine	C9H13NO	Identified	8.04	-0.3	9	0.56	5.54	6198	+H
N-Methyltyramine	C9H13NO	Identified	8.05	-3.4	7	3.64	6.17	6281	+H
Thymine	C5H6N2O2	Identified	2.03	4.3	3	4.55	8.53	368	+H
Thymine	C5H6N2O2	Identified	2.03	1.0	1	1.83	9.29	403	+H
Tyramine	C8H11NO	Identified	4.62	-1.7	2	4.09	6.67	4509	+H
Tyramine	C8H11NO	Identified	4.66	-1.5	2	1.52	9.61	4567	+H

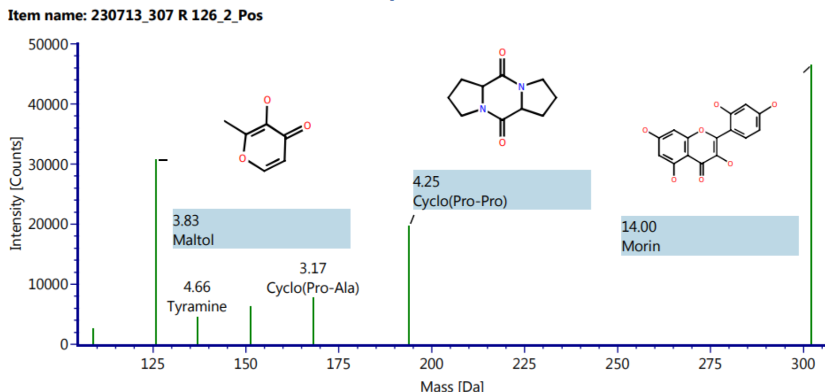


Fig. 2: Component plot of maltol, cyclo (Pro-Pro) and morin

Table 2: ESI mode with positive value

ESI mode	Compound name	Result
(+)	Flavonoid (Maltol)	Positive
(+)	Flavonoid (Morin)	Positive
(+)	Peptide (Cyclo Pro-Ala)	Positive
(+)	Peptide (Cyclo Pro-Pro)	Positive
(+)	Peptide (Thymine)	Positive
(+)	Phenol (m-Aminophenol)	Positive
(+)	Phenol (N-Methyltyramine)	Positive
(+)	Phenol (Tyramine)	Positive

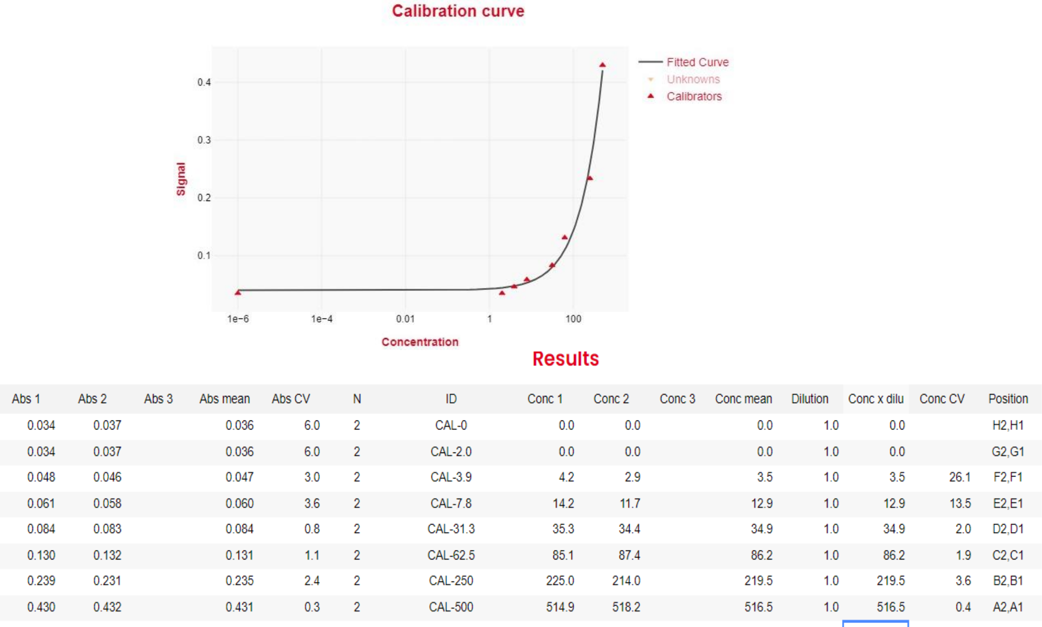
In this present study, LC-MS/MS analysis of a 96% ethanol extract of *S. platensis* revealed flavonoid, peptide, and phenolic compounds. The most general compounds that have been reported in the 96% ethanol extract of *S. platensis* are flavonoids, peptide and phenolic compounds, carotenoids, phycobiliproteins, chlorophyll, polyunsaturated fatty acids, sulfated polysaccharides, and sterols. These reports strongly suggest that bioactive compounds such as the flavonoid present in the 96% ethanol extract of *S. platensis* are Maltol and Morin, and then peptide and phenol.

Further research verified the antioxidant capacity of Spirulina, establishing a basis for its development as a functional food. Spirulina has antioxidant peptides [25]. Our study found that a 96% ethanol extract of *S. platensis* contains peptides such as Cyclo Pro-Ala, Cyclo Pro-Pro, and Thymine, which may have antioxidant properties.

C-phycocyanin standard of 96% ethanol extract of *Spirulina platensis*

To determine whether or notc-phycocyanin is bound in the 96% ethanol extract of *Spirulina platensis*, a calibration curve was constructed by comparing the c-phycocyanin standard curve. As we know, c-phycocyanin is cultivated in Indonesia. Standard values for calibration using 1 mg/ml of c-phycocyanin range from serial dilution to 1 mg/ml of c-phycocyanin. Calibration curves were usedin Hycult Biotech 4-parameter logistic (4PL) across the operating range, and calibration was employed for quantitative analysis using 4PL regression. The concentration of c-phycocyanin in the 96% ethanol extract of *Spirulina platensis* is 229.2µg/mlas shown in fig. 3. This study shows that a 96% ethanol extract of *S. platensis* containsc-phycocyanin, a phycobiliprotein-like peptide that acts as an antioxidant and anti-inflammatory[15,19,26,27]. A study

of s-phycocyanin in an animal model of Acute Myocardial Infarct showed that it prevents oxidative stress and inflammation because c-phycocyanin down-regulates iNOS, COX2, and phospho-NFκB p65, reducing the mRNA synthesis of IL1b and TNF-α [15, 19].



ID	Abs 1	Abs 2	Abs 3	Abs mean	Abs CV	N	ID	Conc 1	Conc 2	Conc 3	Conc mean	Dilution	Conc x dilu	Conc CV	Position
CAL-0	0.034	0.037		0.036	6.0	2	CAL-0	0.0	0.0		0.0	1.0	0.0		H2,H1
CAL-2.0	0.034	0.037		0.036	6.0	2	CAL-2.0	0.0	0.0		0.0	1.0	0.0		G2,G1
CAL-3.9	0.048	0.046		0.047	3.0	2	CAL-3.9	4.2	2.9		3.5	1.0	3.5	26.1	F2,F1
CAL-7.8	0.061	0.058		0.060	3.6	2	CAL-7.8	14.2	11.7		12.9	1.0	12.9	13.5	E2,E1
CAL-31.3	0.084	0.083		0.084	0.8	2	CAL-31.3	35.3	34.4		34.9	1.0	34.9	2.0	D2,D1
CAL-62.5	0.130	0.132		0.131	1.1	2	CAL-62.5	85.1	87.4		86.2	1.0	86.2	1.9	C2,C1
CAL-250	0.239	0.231		0.235	2.4	2	CAL-250	225.0	214.0		219.5	1.0	219.5	3.6	B2,B1
CAL-500	0.430	0.432		0.431	0.3	2	CAL-500	514.9	518.2		516.5	1.0	516.5	0.4	A2,A1
Sample 1	0.241	0.243		0.242	0.6	2	Sample 1	227.8	230.6		229.2	1.0	229.2	0.9	A3,A4

Fig. 3: Calibration curve of C-phycocyanin

In silico results of molecular docking analysis

C-Phycocyanin has a binding affinity of-4.46 kcal/mol and an inhibition constant of 536.16 uM, with 1 atom in H-bond TYR195, compared to the native ligand, in fig. 4. The result of amino acid mapping in the c-phycocyanin complex areTyr195, Tyr227, Tyr135, Ile131, Leu133, Gly192, Ile134, Gly193, Leu133, and Leu233.

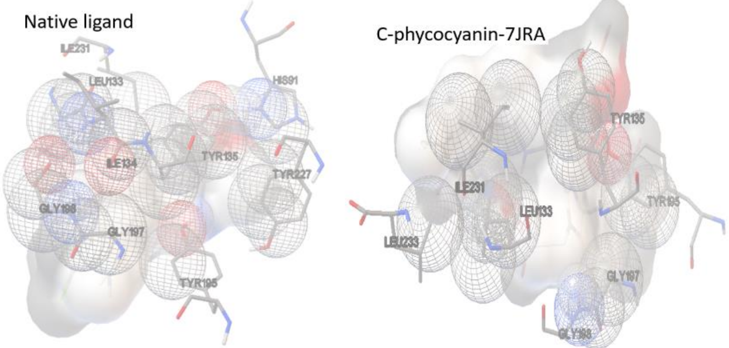


Fig. 4: Results of the complex interaction c-phycocyanin-7JRA

The molecular docking pose for the native ligand with the TNF-α receptor binding site showed several important amino acid residues, such as His91, Tyr135, Tyr227, Ile231, Leu133, Tyr195, Gly198, Ile134, and Gly193. Through comparison with the c-phycocyanin conformation in the binding sites of TNF,Tyr195, Tyr227, Tyr135, Ile131, Leu133, Gly192, Ile134, Gly193, Leu133, and Leu233 with 1H-bond play an important role in enhancing ligand's affinity. This complex shows an H-bond interaction with Tyr195 that can interact with the TNF-α protein for inflammation.

C-phycocyanin, an antidiabetic inhibitor, inhibits α-amylase and α-glucosidase by binding to the active site and disrupting substrate-enzyme interaction [28]. C-phycocyanin has a binding energy of-4.46 kcal/mol to TNF, and it is the most common phytoconstituent that interacts with TNF-α. Our molecular docking investigation revealed that c-phycocyanin had the best amino acid bond interaction with the 7JRA protein. The findings suggest a significant connection between c-phycocyanin and 7JRA. It could be hypothesized that c-phycocyanin has the potential to prevent the activity of inflammatory agents. C-phycocyanin, known as one of the bioactive phytoconstituents with important biological functions in natural plant compounds, is available on *Spirulina platensis* [29, 30].

C-phycocyanin can reduce inflammation and enhance the antioxidant capacity of the liver and kidney via the gut microbiota and their metabolites [28]. Its antioxidant effect on the liver may be mediated by GSH and related enzymes, while in renal tissue, it works by activating the NRF2 pathway [31]. *In silico* studies with molecular docking showed the important residues involved in Hydrogen bonds and hydrophobic interactions [12, 32, 33]. In this study, we found that the C-phycocyanin-7JRA complex has a Hydrogen bond, in line with several studies in silico. Thus, C-phycocyanin was predicted to

be ananti-inflammatory candidate. The analysis of the correlation between the Hydrogen bond and some hydrophobic interactions predicted they had good activity as anti-inflammatory activators.

CONCLUSION

AnLC-MS/MS technique was developed to measurea 96% ethanol extract of *S. platensis*. In this present study, LC-MS/MS analysis of a 96% ethanol extract of *S. platensis* revealed flavonoid, peptide, and phenolic compounds. The validation equation for c-phycocyanin in a 96% ethanol extract of *S. platensis* was found to be 229.2µg/ml. C-Phycocyanin has a binding affinity of-4.46 kcal/mol and an inhibition constant of 536.16 uM, with 1 atom in H-bond TYR195. C-phycocyanin shows potential in inhibiting the activity of an inflammatory agent in molecular docking analysis.

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AUTHORS CONTRIBUTIONS

TS gathers, analyzes data, and finalizes the manuscript. FF, ARP, and NSH conceptualized the study, supervised data collection and analysis, and contributed to manuscript preparation and review. All authors contributed to completing the manuscript.

CONFLICT OF INTERESTS

The authors report no financial or other conflicts of interest in this work.

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