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1. Introduction

Among the various diseases caused by viruses, the 2019 coronavirus disease (COVID-19) caused by a new coronavirus (SARS-CoV-2) was first detected in December 2019 and has since become a global pandemic (Chan et al., 2020; Chen et al., 2020). This virus has been reported as a new member of the β-coronavirus genus. It is closely related to severe acute coronavirus respiratory syndrome (SARS-CoV) and several bat coronaviruses (Zhou et al., 2020). Compared to coronavirus types previously found (SARS-CoV and MERS-CoV), the SARS-CoV-2 virus shows faster human-to-human transmission. Thus, the World Health Organization (WHO) has established public health emergencies worldwide (Chan *et al*. 2020; Chen *et al*., 2020). Additionally, this virus's targeted therapeutic compounds and effective treatment options are still minimal. Therefore, efforts are needed to design new drugs that can be used as SARS-CoV-2 antivirus candidates with virtual drug screening methods. To date, no effective antiviral therapy has been found. However, several broad-spectrum antivirals have been recommended, such as the Nucleoside analogs and HIV-Protease Inhibitors (lopinavir, ritonavir), as an alternative to temporary therapy until specific antivirals are found (Zhou *et al*., 2020). Additionally, aside from vaccine development, repurposing approved antiviral drugs (e.g., remdesivir) is a practical clinical approach to overcome the SARS-CoV-2 global pandemic (Wu et al., 2020). Nevertheless, designing broad-spectrum antiviral agents that are effective against a wide range of SARS-CoV-2 and other emergent classes of viruses could be a sound strategy (Cho and Glenn, 2020; Hall and Ji, 2020).

The broad spectrum of antiviral drugs can be divided into two mechanisms of action: 1) by inhibiting the interactions between virus particles outside of cells and the receptors on the cell surface, thus preventing infection (Hangartner et al., 2006; Kim et al., 2012) and 2) by stopping

viral genome replication to minimize the production of new virus particles (Clercq, 2004; de Wilde et al., 2018). Previous studies revealed that the Angiotensin Converting Enzyme-2 (ACE2) receptor has a significant role in SARS-CoV-2 infection (Wan et al., 2020; Zhang et al., 2020). SARS-CoV-2 recognizes this receptor to facilitate the entry of the virus into the target cell. In addition, the binding site between the ACE2 receptor and SARS-CoV has been identified at the molecular level (Zhang et al., 2020). Therefore, inhibiting this interaction with ligands on the ACE2 receptor site can be a potential approach in the preliminary drug discovery for COVID-19 therapy (Zhang et al., 2020).

Microalgae and cyanobacteria are essential organisms at the basis of most aquatic ecosystems. They can produce various high-value bioactive compounds (Cuellar-Bermudez et al., 2015; de Morais et al., 2015; Hayes, 2012a), as high-value lipophilic and hydrophilic-like compounds (c.a. tocopherols, polyphenols, tannins, and flavonoids) and pigments (c.a. carotenoids, astaxanthin, lutein, and phycobiliprotein) (Cuellar-Bermudez et al., 2015; Gastineau et al., 2014; Hayes, 2012b; Prasetiya et al., 2020). The diversity of these bioactive compounds makes microalgae and cyanobacteria ideal candidates for extensive applications, including the pharmaceutical industry, as a source of new drugs (Olaizola, 2003; Vo et al., 2011; Yim et al., 2004).

One of the applications of bioactive compounds produced by microalgae and cyanobacteria for the pharmaceutical and health sectors concerns the development of drugs with antiviral activity. Indeed, several microalgal species have been identified as potential sources of antiviral compounds. For example, spirulan polysaccharides and cyanovirin-N derived from *Arthrospira* sp. and *Nostoc ellipsosporum*, respectively, are reported to inhibit the spread of the virus HIV-1, HIV-2, HSV, influenza (de Morais et al., 2015; El-Baz et al., 2013; Smee et al., 2008). In addition, marennine-like pigments produced by the blue diatoms from the genus *Haslea* have been shown

to display antiviral activity against the HSV-1 virus (Gastineau et al., 2014, 2012). Furthermore, several other compounds such as lutein, astaxanthin, and terpenoids produced by the genus *Chlorella* microalgae can also inhibit HPV virus, hepatitis B and C types, including viruses with severe acute respiratory syndrome SARS-CoV (de Morais et al., 2015; Silva et al., 2018).

Potential protease inhibitors of metabolites produced by microalgae and cyanobacteria are very promising. Hence, preliminary studies show that some active compounds such as scytovirin, sulfoglycolipid, lutein, palmitic or linoleic acid can be used as inhibitors of different viruses (De Morais et al., 2015; Gastineau et al., 2012; Santoyo et al., 2012; Silva et al., 2018; Vitale et al., 2015). Some of them have been hypothesized as potential inhibitors of viruses related to SARS (Pendyala et al., 2021; Smee et al., 2008). Recent *in silico* studies from (Al-Khafaji et al., 2020) and (Naidoo et al., 2021) demonstrated that some cyanobacterial proteins could serve as potential inhibitors against SARS-CoV-2. Nevertheless, both studies have yet to confirm their results with *in vitro* experiments. These promising results should be confirmed, and further study is needed regarding the potential of marine bioactive compounds from microalgae and cyanobacteria as SARS-CoV-2 virus inhibitors.

The present study aims to obtain structural insight into the ACE2 receptor of SARS-CoV-2 and to identify which microalgal and cyanobacterial bioactive compounds could be used as potential SARS-CoV-2 inhibitors. Therefore, a list of bioactive compounds was retrieved from the established database, targeting the ACE2 receptor to specifically develop anti-COVID-19 drugs. Furthermore, a compound selected by virtual screening using molecular docking analysis (AutoDock VINA software) was further validated by molecular dynamics simulation and tested *in vitro* using surface plasmon resonance (SPR) to identify possible molecular interaction with the

ACE2 receptor. Indeed, the previous study demonstrated that SPR is a promising method for characterizing various binding interactions (Zhu et al., 2021).

2. Materials and methods

2.1 Software and data collection

Molecular docking simulations were carried out using AutoDock VINA software, downloaded from the website of The Scripps Research Institute (http://vina.scripps.edu/). Visualization of the docking results was performed with BIOVIA Discovery Studio Visualizer 2020. The protein's crystal structure was downloaded from the Protein Data Bank website (www.rcsb.org). ChemDraw Ultra 8.0 (PerkinElmer Inc.) was used to draw 2D and 3D structures and optimize ligand geometry. Data collection strategies and structure of compounds from potential microalgae and cyanobacteria as antivirals were carried out through MarinLit database searches (http://pubs.rsc.org/marinlit/) and publications related to active compounds from marine organisms from 1970 to 2020.

2.2 Ligand preparation and target receptors for molecular docking

The 2D structure of the compound was prepared with ChemDraw Ultra 8.0 and then converted to a 3D structure using the Chem 3D program. All ligands were saved in PDB format. The pdb format of the crystal structure of ACE2 receptor in complex with their inhibitors was downloaded from the RCSB Protein Data Bank (http://www.rcsb.org). The complex for ACE2 and its inhibitor, MLN-4760, was available under PDB ID 1R4L. The MLN-4760 is a cell-permeable, bioavailable, highly potent inhibitor of ACE2 (IC_{50} = 440 pM against soluble human ACE2) that exhibits good selectivity over bovine carboxypeptidase A and porcine ACE. Afterward, the Biovia Discovery

Studio 2020 (BDS) was used to separate the protein from the inhibitor. Concomitantly, undesired molecules were also eliminated. Both protein and inhibitor molecules were then saved into two different pdb files (target receptor and control ligand). Finally, the inhibitor molecule file was put aside, whereas the protein file was loaded into Autodock Tools 1.5.6 software for further preparation steps. These steps cover the addition of polar hydrogen and Kollman charges, removing non-polar hydrogen atoms, and converting into pdbqt format following (Afriza et al., 2018; Khayrani et al., 2021). The file conversion into pdbqt format allowed the file to be loaded in Autodock Vina for molecular docking simulation (Huey et al., 2012).

2.3 Preparation of grid and molecular docking parameters

The grid parameter files were prepared with the help of AutoDockTools 1.5.6. As for its input and output, Vina uses a similar pdbqt molecular structure file format used by AutoDock. The pdbqt files were generated interactively and viewed using MGLTools. The lowest binding energy and Root Mean Square Deviation (RMSD) were further analyzed.

2.4 Molecular docking simulations

Positive control docking was performed using the Autodock Vina program to find the coordinates and parameters that are most appropriate on the active sites. The observed docking parameters were amino acid residues and hydrogen bonds, and the RMSD value should not be more than 2 Å. As for target receptors that are not complex with ligands, blind docking was performed to determine the binding site of the ligand with the receptor.

2.5 Virtual screening of potential compounds and molecular interaction analysis

Virtual screening was performed using PaDEL-ADV software (http://www.yapcwsoft.com/dd/padeladv/) following the protocol from (Hardianto et al., 2018). This program reads each ligand file in pdbqt format, previously prepared using AutodockTools (see sections 2.2 and 2.3). Autodock Vina was then used to dock the ligand with the receptor. Individual binding modes were extracted from the output pdbqt file using vina_split. Results for each binding mode were extracted from the log file and saved into the csv file. The log file and all the related pdb and pdbqt files were then compressed into a zip file. The docking results were sorted by their free energy binding of compounds and by the most histogram groups. Afterward, the compound with the lowest binding energy or maximum multitarget receptors was chosen for further analysis of the interaction between the ligand compound and its receptor.

Autodock tools integrated with the Autodock Vina generated grid maps for each ligand atom. The grid boxes were made to include one site at a time and perform docking. Each coordinate used in the grid box is described in Table 1, with 1 Å spacing on the grid box. Autodock Vina produces energy binding for each molecule and ten models with different conformations and energy. The lowest binding energy was considered as the ligand with the maximum binding affinity. Additionally, Visualization from docking analysis was performed using Biovia Discovery Studio by examining the number and type of interactions between the ligand and the target protein and its native ligand that was used as the control.

2.6 Molecular dynamic simulations

The molecular dynamic simulation was carried out on the ACE2 protein system with potential inhibitors from screening results on the ACE2 binding site. This simulation used the GPU with the program pmemd.cuda on AMBER20 (Case et al., 2021). Initial minimization was performed using 1000 steps of steepest descent. Afterward, 2000 steps of conjugate gradient minimization were

carried out by applying 5 kcal mol^{-1} harmonic force resistance. The force resistance was gradually released from all heavy atoms (including side chains) to only atoms in the mainframe with the same minimization protocol. Additionally, 5000 steps of unimpeded conjugate gradient minimization were performed to eliminate spatial collisions.

Afterward, the system was set to 310 K in increments (0-100 K; 100-200 K; 200-310 K for 20 ps each) for 60 ps. Moreover, the system density and pressure were equilibrated for 1000 ps. The resistance force was released gradually during the equilibrium phase. Then the production stage was carried out for 200 ns. The time-step in the production phase was set to 2 fs. The MD simulation trajectories were then analyzed using the cpptraj module on AmberTools 20.

2.7 CPC isolation from Arthrospira platensis

Dry powder of *A. platensis* was obtained from the Jepara Aquaculture Research Facility (BBPBAP Jepara), Indonesia. A total of 20 mg of *A. platensis* dry biomass with 5 mL of sodium phosphate buffer (0.1 M, pH 7) used and subjected to repeated maceration extraction (ME) and then vortexed (Vortex V1 plus, BOECO, Germany). The cell debris was removed by centrifugation (Tomy MX-307, Tomy Seiko Co.ltd., Japan) at 3140× g for 5 min. The supernatant was then pooled and marked as crude extract and the extraction solvent was colorless. The CPC crude extract was then purified following protocol from (Liao et al., 2011) and the purity of CPC was calculated. The C-PC was then freeze dried (BIOBASE BK-FD10PT, China) for *in vitro* experiments.

2.8 Surface Plasmon Resonance-Based Competition Assay

2.8.1 ACE2 immobilization

ACE2 was immobilized onto the BA1080 standard SPR chip with a refractive index of 1.61, purchased from NanoSPR LLC, US. First, the chip must be cleaned by soaking it in piranha solution (v/v 3:1 (NH_4OH : 30 wt.% H_2O_2) at room temperature) for 10 s then streamed with tap water, soapy water, acetone, and distilled water, respectively. Afterward, the SPR chip was dried using an air blower. After the SPR chip was clean, the 150 µl 3-MPA 10 mM was dropped onto the SPR chip surface and let for 30 min. Then, it was rinsed with PBS solution. Afterward, the EDC-NHS solution was dropped and incubated for 30 min. Afterward, it was rinsed with PBS solution and dried using an air blower. Furthermore, the chip was installed in the NanoSPR 8 chip holder (NanoSPR LLC, US) for immobilizing the ACE2. Moreover, the ACE2 was flowed onto the SPR chip with a flow rate of 40 µL min^{-1} for 5 min, allowed to stand for 30 min and then flowed with PBS for 10 min. Afterward, 1% BSA was flowed onto the SPR chip to block the residual active sites with the same parameters as ACE2 immobilization.

2.8.2 SPR screening measurements

In this study, SPR screening was performed using Nano SPR 8 (NanoSPR LLC, US), and its dynamic responses were observed by SPR intensity (RU) versus time parameters. The ACE2 - CPC interactions were performed with 5 min baseline, 40 min association process, and 30 min dissociation process using PBS as the media with 40 µL min-1 flow rate. Afterward, the prepared CPC solution used had a 5 – 125 µM in the PBS solution.

2.8.3 Kinetic binding analysis

The kinetic binding analysis was performed to obtain binding kinetic and affinity parameters of ACE2 – CPC. In this study, the association and dissociation dynamic responses of CPC by ACE2

were followed using the Pseudo-second-order and Avrami kinetic adsorption models. These models were represented by equations 1 and 2, respectively, with slight modifications (Ahmad et al., 2015; Vargas et al., 2011). From this model, the observed rate (k_{obs}) could be used to obtain the value of the association rate (k_{on}) and dissociation rate (k_{off}) through linear regression of the C vs. k_{obs} with the relationship as shown in equation 3 (Schasfoort, 2017; Swinney et al., 2014).

$$(\Delta RU/_{Ro})_t = \frac{(\Delta RU/_{Ro})_m^{\,2} k_2 t}{1+k_2(\Delta RU/_{Ro})_m t};\ h_o = k_2(\Delta RU/_{Ro})_m^{\,2} \quad (1)$$

$$(\Delta RU/_{Ro})_t = (\Delta RU/_{Ro})_m \{1 - \exp[-(k_{AV}t)]^{n_{AV}}\} \quad (2)$$

$$k_{obs} = k_{on}[C] + k_{off} \quad (3)$$

Where $(\Delta RU/_{Ro})_t$ is the dynamic response at a specific time, t is the time, h_o is the initial adsorption rate, k_2 is the adsorption rate of the Pseudo-second-order, n_{AV} is the Avrami's constant, and k_{AV} is the adsorption rate on the Avrami model (Ahmad et al., 2015; Vargas et al., 2011). Parameters k_2 and k_{AV} are the k_{obs} values.

2.8.4 Isotherm analysis

In this study, Isotherm analysis was performed to predict the binding interaction behavior between CPC and ACE2. This was obtained by making a curve *C vs* $\Delta RU/_{Ro}$ fitted using a non-linear regression according to adsorption isotherm models. Additionally, the curve follows the Brouers-Sotolongo (BS) adsorption model with the modified equation shown below (Karoui et al., 2020; Vargas et al., 2011):

$$\Delta RU/_{Ro} = (\Delta RU/_{Ro})_m \times \left(1 - e^{-K_{BS}C^{\alpha}}\right) \quad (4)$$

Where $\Delta^{RU}/_{Ro}$ is the dynamic response of SPR (%), $(\Delta^{RU}/_{Ro})_m$ is the maximum adsorption capacity, C is the concentration of CPC, K_{BS} is the Brouers–Sotolongo constant or isotherm constant, and α is the surface heterogeneity constant.

3. Results and discussion

3.1 Molecular docking analysis for ACE2 receptor

In this study, docking analysis between the control ligand and protein ACE2 showed five hydrogen bonds with several amino acids, such as Arg^{273}, Thr^{371}, Tyr^{515}, Pro^{346}, and Thr^{371} (Table 2). Apart from the main hydrogen bonds, other interactions were also found, two types of electrostatic bonds with Arg^{514} and Arg^{518}, as well as hydrophobic interaction between ligand and amino acids Tyr^{510} and His^{345}. From the docking analysis on several bioactive compounds from microalgae and the various interactions that occur between these compounds and the ACE2 receptor, a total of three potential candidate compounds were selected based on the number of interactions that resemble the native ligand one, as well as the number of overall interactions involved. Phycocyanobilin, phycocyanin, and 7-dehydroporiferasterol peroxide have 5, 4, and 3 hydrogen bonds, respectively, with several hydrophobic interactions. Although not many interactions were formed compared to the native ligand, some of these compounds have approximately similar potential as the native ligand, especially for the phycocyanobilin compound with five hydrogen bonds and three hydrophobic interactions. Based on theoretical and experimental correlations between H-bond pairings and their effects on ligand binding affinity, H-bonds could enhance receptor-ligand interactions when both the donor and acceptor have either significantly stronger or significantly weaker H-bonding capabilities than the hydrogen and oxygen atoms in water (Chen et al., 2016). Thus, our virtual screening revealed that the binding affinity of the screened compounds was similar to or higher than that of the control ligand of ACE2 (MLN-4760).

The docking prediction using Autodock VINA software revealed that among the different molecules tested, phycocyanobilin could have various interactions with the ACE2 receptor. In this receptor, phycocyanin has a docking score of -9.7 kcal mol^{-1}, which is lower than the ACE2' native ligand with four hydrogen bonds formed in their amino acids' residues (Asn^{131}, Arg^{255}, Glu^{388}, and Asp^{251}; Fig. 1a). Additionally, a total of seven hydrophobic bonds were also observed in the amino acid residues (Arg^{255}, Phe^{256}, Ala^{135}, Phe^{486}, His^{487}, Tyr^{492}, and Tyr^{497}; Fig. 1a). On the other hand, phycocyanobilin has a slightly higher docking score (-9.5 kcal mol^{-1}) compared to that of phycocyanin by forming six hydrogen bonds with the residues of amino acids Lys^{363}, Thr^{371}, Arg^{518}, His^{345}, Glu^{406}, and Glu^{145} (Fig. 1b). Apart from the hydrogen bonds, phycocyanobilin also forms electrostatic interactions with the ACE2 receptor at the residue Arg^{273} as well as hydrophobic interactions phi – sigma (Tyr^{510} and Phe^{274}) and phi – alkyl (His^{378} and Pro^{346}). From docking prediction, it can thus be assumed that the binding affinity of phycocyanin and phycocyanobilin is lower than those of the native ligand. This result suggests that these compounds can potentially be used as ACE2 inhibitors.

Phycocyanobilin (PCB, $C_{33}H_{38}N_4O_6$) and phycocyanin (CPC, $C_{165}H_{185}N_{20}O_{30}$) could thus be good candidates for developing anti-COVID drugs, and more tests were conducted on these compounds. PCB is a blue phycobilin commonly found in cyanobacteria and the chloroplasts of red algae, glaucophytes, and some cryptomonads (Hayes, 2012b; Santoyo et al., 2012). This molecule has a molecular weight of 586.68 g mol^{-1}. It is present only in the phycobiliproteins, allophycocyanin (APC), and phycocyanin (CPC), of which it is the terminal acceptor of energy (de Morais et al., 2015). A previous study by (Radibratovic et al., 2016) revealed that PCB could bind to human serum albumin (HSA), a protein found mainly in the blood of humans, increasing its ability to prevent the proteolytic activity of other proteins.

3.2 Molecular dynamic simulation of PCB and CPC on ACE2 receptor

The molecular dynamic simulations of the ACE2 receptor that binds to PCB, CPC, and native ligands showed a significant change in distance (Å) from the initial conformation when compared to the structure of ACE2 Apo (Fig. 2). The dynamics of the receptor that binds to the ligand was more stable than the Apo structure. This result is indicated by the root mean square deviation (RMSD) value, which increased to 3.5 Å after 150 ns of simulation (Fig. 2). In the receptor bound to the control, the RMSD value ranged from 2 to 2.5 Å during the simulation. On the other hand, the receptor bound to the phycocyanin and phycocyanobilin compounds, the RMSD profile showed a very stable value of 1.5 Å. This stable conformation indicates favorable interaction between the ligand and the receptor, thereby changing the flexible nature of the ACE2 structure to become more rigid.

The RMSD value of the control ligand experienced a relatively high progression compared to the phycocyanin and phycocyanobilin compounds, where the shift occurred up to 15 Å at 150 ns of the simulation (Fig. 3a-c). After this period, the native ligand again dropped its RMSD value to the similar level as the phycocyanin ligand (Fig. 3). This result indicates the bond instability in the native ligand, which causes the RMSD value to increase to 15 Å.

The ACE2 protein has 597 amino acids. From the root mean square fluctuation (RMSF) profile, the ACE 2 receptor that is not bound to the ligand has a higher fluctuation than the system bound to the ligand. In contrast, as for phycocyanin and phycocyanobilin ligands, RMSF values produced a more stable profile than the native system, suggesting that these two compounds have a potential interest as inhibitors for ACE2 protein (Fig. 4).

The molecular mechanics/generalized Born surface area (MM/GBSA) method evidenced that during the MD simulation, the phycocyanin had the lowest total binding free energy (-32.8158 kJ mol^{-1}) compared to other ligands, such as phycocyanobilin (-17.162 kJ mol^{-1}) and the native ligands (-5.0989 kJ mol^{-1}, Table 3). Further hydrogen bond analysis results are also consistent with these findings, where the average number of hydrogen bonds of phycocyanin was the highest among all, followed by phycocyanobilin and the control, respectively (Fig. 5). It is also indicating that the hydrogen bond contributes to most of the interactions between ligand and ACE2 receptor. Therefore, this finding suggests that the interaction of phycocyanin and phycocyanobilin compounds with the ACE2 receptor is relatively strong, and they have stable binding free energy. To our knowledge, little is known about the potential of phycocyanin and phycocyanobilin as potential SARS-CoV-2 inhibitors by targeting the ACE2 receptor. Recent studies from Al-Khafaji et al. (2020) demonstrated that the molecular docking calculations and molecular dynamic analysis support the fact that the phycocyanin could be a potential candidate inhibitor for the ACE2 receptor towards SARS-CoV-2. On the other hand, the study by (Pendyala et al., 2021) demonstrated that phycocyanobilin potentially inhibits the binding of other SARS-CoV-2 receptors, such as the main protease (M_{pro}) and papain-like protease (PL_{pro}). The *in silico* study by similar authors revealed that the binding affinity score of phycocyanobilin with M_{pro} and PL_{pro} were -8.6 and -9.8 kcal mol^{-1}, respectively.

3.3 Surface Plasmon Resonance-Based Competition Assay

The SPR method is a powerful tool for discovering protein-protein interaction (PPI) inhibitors. Moreover, this method can quantitively explain highly potent PPIs and weak fragment or low molecular weight (LMW) ligand-protein interactions. In this study, the function of phycocyanin (CPC) as an inhibitor of SARS-CoV-2 was evaluated to further validate the molecular docking

and molecular dynamic studies using the SPR technique. With this method, first, we confirmed that after the dissociation of ACE2 and BSA 1%, the dynamic response did not return to the baseline (Fig. 6). This result indicates that ACE2 and BSA 1% well bonded on the surface of the SPR chip so that the measurement of CPC binding is ready to be carried out. This SPR technique can provide information on the affinity and kinetics of CPC binding by ACE2. The ACE2 − CPC binding SPR response data at different concentrations (5-125 μM) are shown in Figure 7a-b.

To determine the efficacy of the developed inhibitor, it is necessary to evaluate the residence time (τ), where the longer the time, the more potent the drug developed (Zhu et al., 2021). The τ was obtained through the value of the dissociation rate (k_{off}) (Copeland et al., 2006). Figure 7b shows the SPR curve of the association of CPC with ACE2, which was then analyzed using an adsorption kinetic model. It was demonstrated that the association curve of SPR concentration of 5-125 μM follows the pattern of different adsorption kinetic models. Concentrations of 5 and 10 μM followed the Pseudo second-order model. In contrast, 25 – 125 μM concentrations follow Avrami's kinetic model with relative coefficient values (R^2) shown in Table S1. The obtained linear regression parameters are summarized in Table S1.

The linear regression between C and k_{obs} revealed the linearity relationship $k_{obs} = 0.00035145[C] - 0.000701142$ with $R^2 = 0.99214$ (Fig. 7c-d). From the linear equation, the computed values k_{on} and k_{off} were 0.00035145 s^{-1} μM^{-1} and 0.000701142 s^{-1}, respectively. On the other hand, the affinity constant (K_A) and dissociation constant (K_D) for ACE2 – CPC interactions were obtained using $K_D = \frac{k_{off}}{k_{on}}$ and $K_A = {}^{1}/_{K_D}$, thus the value of K_D and K_A were 1.995 μM and 0.501 μM^{-1}, respectively (Swinney et al., 2014). Additionally, the value of $\tau = 1/k_{off}$ obtained was 0.396 h, while the half-life value $(t_{1/2} = 0.693/k_{off})$ was 0.275 h (Copeland et al., 2006). It appeared that the obtained residence time in this study was relatively narrow. This result is probably caused by the

lack of strength of ACE2 in binding CPC. Nevertheless, this argument needs to be validated. Therefore, it is necessary to calculate the binding energy ($\Delta G^0 = R.T.\ Ln\ K_D$) involved during the adsorption process (Karoui et al., 2020). As a hypothesis, if the gas constant (R) is 1.987 cal K^{-1} mol^{-1} and the temperature (T) is 298 K, the value of ΔG^0 obtained is −7.77 kcal mol^{-1}.

To predict the binding mechanism of CPC by ACE2, the data were evaluated using a non-linear regression isotherm adsorption model. The curve pattern follows the Brouers-Sotolongo (BS) model with a relative coefficient value (R^2) of 0.9996. The parameters obtained from the C vs $\Delta {}^{RU}/_{Ro}$ non−linear regression are $(\Delta {}^{RU}/_{Ro})_m$= 76.087, K_{BS} of 0.00337, and α of 1.548. In the present study, the value of α was greater than 1, suggesting that the CPC binding process by ACE2 takes place through slow kinetics biosorption, and each ACE2 binds to CPC molecules with unequal energy (Karoui et al., 2020).

3.4 Potential microalgae candidates for developing SARS-CoV-2 inhibitor

In the present study, our docking and molecular dynamic analysis to search for potential SARS-CoV-2 inhibitor from natural products demonstrated that phycocyanobilin, with CPC in particular, could be used as a potential inhibitor, as this compound has an excellent binding affinity and significant number type of interaction, both characteristics that resemble those of its natural ligands. Moreover, the results of these *in silico* experiments were also confirmed *in vitro* with the SPR experiment. CPC can be obtained from *Arthrospira platensis* (previously known as *Spirulina platensis*), a cyanobacteria species that is extensively cultured (Olaizola, 2003; Singh et al., 2011), mainly as a source of food, feed supplement, and natural blue pigment (Belay et al., 1996; McClane et al., 2006; Singh et al., 2011). It is worth noting that CPC from several *Arthrospira* species has also therapeutic potential but without any adverse effect to the living organisms (Chamorro et al., 1997, 1996), compared to other natural blue pigments and bioactive compounds such as marennine-like

pigments from the marine diatoms *Haslea ostrearia* and *H. nusantara* (Prasetiya et al., 2017, 2019; Prasetiya et al., 2020a; Prasetiya et al., 2020b).

In this study, CPC demonstrated potential activity as a candidate for the SARS-CoV-2 inhibitor. A previous study from McCarty (McCarty, 2007) demonstrated that CPC constitutes up to 14% of the total dry weight of *A. platensis*, in which phycocyanobilin represents 4.7% of the mass of CPC. Regarding the production of phycocyanin from *A. platensis* biomass, several studies revealed that extraction and purification is relatively simple as it can be completed with four major steps that cover; crude extract preparation, ammonium sulfate precipitation, dialyses, and anion exchange chromatography (Kumar et al., 2014) or through a hexane extraction process combined with high pressure (Seo et al., 2013).

To complete this search of compounds that could be potential candidates of SARS-CoV-2 inhibitors, our approach highlighted that the genus Chlorella also presents a significant interest. According to our docking analysis, several bioactive compounds from the database such as oxocholesterol, ergosterol, dehydroporiferasterol, and triterpenoid are produced by *Chlorella vulgaris* and they display inhibitory activity against SARS-CoV-2. To our knowledge, most *Chlorella* species mainly inhabit freshwater and are commonly found in very nutrient-rich waters. However, it is possible to find these microalgae in marine environment since a few marine species are also known (Levine and Fleurence, 2018). Chlorella strains or species are the most cultivated eukaryotic algae since they are widely used as nutraceutical and feed supplements, as well as in the pharmaceutical and cosmetics industry (Borowitzka, 1997; Li et al., 2014; Silva et al., 2018; Yasuhara-Bell and Lu, 2010). Additionally, previous studies demonstrated that several species of *Chlorella* were found to contain ergosterol as their major sterol (de Morais et al., 2015; Rahal et al.,

2014; Yasuhara-Bell and Lu, 2010). This molecule also has been reported to have the inhibitory activity to zoonotic influenza viruses, such as H5N1, H7N9, and H9N2 (Silva et al., 2018).

5. Conclusions

Compounds from natural products extracted from microalgae are potentially used for the treatment of viral diseases. In this study, we screened potential microalgal bioactive compounds and the selected hits that may inhibit SARS-CoV-2 on ACE2 receptor for COVID-19 preliminary drugs discovery. Through the combination of *in silico* and *in vitro* studies, we demonstrated that phycocyanin (CPC), which can be produced from the cyanobacteria genus *Arthrospira* (previously known as *Spirulina*), can be proposed as potential inhibitor. The molecular docking and simulation of molecular dynamics revealed that this compound potentially inhibits the binding of the ACE2 receptor and SARS-CoV-2, with the docking scores of -9.7 kcal mol^{-1}. Additionally, SPR technique also supports the fact that CPC at certain concentration strongly binds with ACE2 receptor. Therefore, the genus *Arthrospira* and possibly other phycocyanin-producer genera could be important candidates for bioprospection regarding antiviral research. However, further validation through *in-vivo* assessment is required to validate the results and to transform this potential inhibitor into clinical drugs. Additionally, our study provides valuable insights for exploring and developing novel natural anti-COVID-19 therapeutic agents from microalgae species.

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