Anti-inflammatory potential of λ-carrageenan by inhibition of IL-6 receptor: *in silico* study

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Abstract. In some cases, the immune system in COVID-19 patients leads to the release of excess cytokine production (cytokine storm), which will potentially develop into pneumonia. Interleukin 6 (IL-6) plays the role of pro-inflammatory cytokine, it is a receptor mediated signalling system. Macroalgae is well known as a source of valuable bioactive substances with potential biological activities. Among them is the sulphated polysaccharide lambda-carrageenan (λ-CGN) which has been reported as an anti-inflammatory agent. However, its mechanism of action against IL-6 production is currently unknown. This study aims to predict potential molecular mechanisms of λ-CGN chemical compound against IL-6 expression through *in silico* study. Chemical compound of λ-CGN and target protein in this study were obtained from the pubchem and protein data bank (PDB). The molecular docking prediction was conducted with PyRx software, the result is λ-CGN compound showing strong binding energy to bind target protein IL-6 receptor with the value of -5.9 kcal/mol. Based on the results of *in silico* study, the sulphated polysaccharide λ-CGN potentially inhibits IL-6R expression by binding ligand pocket with six conventional hydrogen bonds (amino acid residus: His256, His 257, Trp 219, Arg 231, and Asp 221) and two carbon hydrogen bonds (amino acid residus: THR 218 and GLN 220). Binding with these amino acid residues potentially contributes to IL-6 receptor structural change which could result in functional change. Hence, further studies related to *in vitro* and *in vivo* investigations would be interesting to further understand the inhibitory mechanism of λ-CGN against IL-6.
1. Introduction

Coronavirus disease (COVID-19) was discovered for the first time in Wuhan, China, in December 2019. COVID-19 is caused by the SARS-CoV-2 virus (severe acute respiratory syndrome coronavirus 2). COVID-19 spread rapidly worldwide and became a global pandemic infecting around the world. The infected patients showed symptoms such as flu, dry cough, fever, fatigue, or muscle soreness. It has been well known that cytokine has an important role in the human immune system against viral infection [1]. In some cases, the immune system COVID-19 patients led to the release of excess cytokine excessively (cytokine storm), which will be develop into pneumonia [2]. Cytokine storm also plays important role in causing ARDS (Acute Respiratory Distress Syndrome) and multi-organ functional disorders due to exaggerated immune response [3]. Thus, suppressing the cytokine storm in COVID-19 patient becomes solution to prevent COVID-19 progression.

Interleukin 6 (IL-6) role as pro-inflammatory cytokine mediates the cytokine signalling system. The function of IL-6 as a mediator for signal inducement occurs due to some emerging in the immune system. IL-6 is secreted in an infectious area and would further trigger a warning signal to the entire body. However, excessive amount of IL-6 production could potentially cause tissue damage. Hence, in most cases, over-expression of IL-6 plays a critical role in the prognosis and mortality of COVID-19 patients [4].

The IL-6 blockers and inhibitory drugs which are recommended by the WHO are tocilizumab and sarilumab. Whereas there are also some IL-6 blockers from derived from herbal medicines such as flavonoids, alkaloids, terpenoids, and glycosides that have biological effects in vitro and in vivo [5].

Macroalgae is rich in various bioactive compounds which show adverse biological activity inhibited proinflammatory TNF-α and IL-6 expression IL-10 in vivo study [6]. Macroalgae Sargassum cristaefolium have potential activity as antioxidant with phytochemical composition total phenolic content (44.95 ± 2.62 mg GAE/g extract) and total flavonoid content (70.27 ± 3.59 mg QE/g extract) [7]. Based on the in vitro analysis in the HeLa cell model, Sargassum cristaefolium could protect DNA from UVA irradiation and also in vivo study protect from UVA exposure [8]. Carrageenan is a sulphated polysaccharide found in Rhodophyceae red macroalgae. Based on the commercial point of view, carrageenans can be categorized into kappa carrageenan (k-CGN), iota carrageenan (i-CGN), and lambda carrageenan (λ-CGN), which differ in the solubility and also the content and the position of sulphate groups on the galactose. Carrageenan has been previously shown to exhibit biological activities such as antitumor, antiviral, and anti-inflammatory. Both k-CGN and λ-CGN also demonstrate selective cytotoxicity against tumor cells [9]. Carrageenan also showed anti-inflammatory activity by reducing inflammatory cytokines levels and symptoms, which has the potential to reduce viral load and viral clearance. Among the different types of carrageenan, λ-CGN is the most hydrosoluble, compared with k-CGN, and i-CGN [10]. λ-CGN has biological properties such as anticoagulant and antiviral. Based on the cell culture assays, revealed that the λ-CGN efficiently inhibited influenza virus with EC50 values ranging from 0.3 to 1.4 μg/mL and also inhibited SARS-CoV-2 with an EC50 value of 0.9 ± 1.1 μg/mL. No toxicity effect to the host cells at concentrations up to 300 μg/mL. Polyanionic compound in λ-CGN have antiviral activity by targeting viral attachment to cell surface receptors and preventing virus entry [11].

However, previous studies have not yet shown the potential molecular mechanism of the chemical compound of λ-CGN to act as an anti-inflammatory inhibition against IL-6. Inhibition of IL-6 over-expression is critical for suppression of the cytokine storm which could potentially lead to severe outcomes in COVID-19 patients. This study aims to predict potential molecular mechanisms of λ-CGN chemical compound as an anti-inflammatory inhibition of IL-6 through in silico study. In silico study is able to predict the mechanism of candidate compound of biological pathways, binding energy, types of molecular interactions and dynamics, based on the specific objectives of a study [12].

2. Materials and methods

2.1. Materials

In this in silico study was conducted with hardware, a hp desktop PC with specifications of processor Intel® Core™ i5, Windows 10 Home; 8 GB DDR4-3200 MHzRAM. The software used in this study included PyRx for molecular docking simulation, PyMol for sterilization and visualization ligand
interaction with target protein, and Discovery Studio 2016 Client for analysis of the docking simulation [13].

2.2. Target protein and ligand preparation
Target protein sample IL-6 receptor (IL6R) was collected from protein data bank (PDB: https://www.rcsb.org/), then sterilized using PyMOL software. While, the chemical compound λ-CGN as a ligand was obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov/) with PubChem CID: 101231953.

2.3. Initial screening study
Lipinski rules (http://www.scfbio-iitd.res.in/software/drugdesign/lipinski.jsp) and PASS online analysis (http://www.way2drug.com/passonline/) were conducted for initial screening study. Lipinski rules, that is molecular weight less than 500 Da, number of acceptor hydrogen bonds less than 10, number of donor hydrogen bonds less than 5, and high lipophilicity less than 5, two rules are minimum requirements [14]. This study is important to predict the compound in penetrating the semi-permeable membrane. PASS online analysis to predict the potential activation (pa) and potential inhibition (pi) the compound. The principal is the value of pa must be higher from pi.

2.4. Molecular docking study
After the compound has been approved by Lipinski rules and PASS online analysis, then the molecular docking was conducted by PyRx software to estimate the value of binding energy (kcal/mol) between the ligand and ILR6. In this study blind docking was conducted for prediction ligand binding IL6R.

2.5. Protein ligand interaction
This analysis to predict the position of interaction and chemical bond type formed when the λ-CGN compound binds to IL6R. This analysis was carried out the Discovery Studio 2016.

2.6. Molecular visualization
PyMol software is used for molecular visualization, which consists of representative coloring and structural selection study.

3. Results and Discussions
The target protein IL-6 receptor (IL6R) was obtained from a data bank (PDB: https://www.rcsb.org/) with PDB entry 1n26 [15]. Then sterilization using PyMOL software to remove a water molecule, another ligand, and another protein which sticks around the target protein. While the chemical compound 3D structure of λ-CGN was obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) with PubChem CID: 101231953 (Figure 1).

![Figure 1: 3D structure of λ-CGN.](image-url)
λ-CGN as a drug candidate for IL6R inhibitor was firstly screened using Lipinski rules (http://www.scfbio-iitd.res.in/software/drugdesign/lipinski.jsp). The rules explain that a chemical compounds with high probability as drug like molecules must be approved at least by two rules from five rules such as molecular weight ≤500 Da, high lipophilicity ≤5, hydrogen bond donors ≤5, hydrogen bond acceptors ≤10, and molar refractivity between 40-130 [14]. The result of Lipinski rules showing that λ-CGN is approved by that rules (Table 1).

| Compound                  | MW (Dalton) | HBD | HBA | LOGP     | MR (g/mol) |
|---------------------------|-------------|-----|-----|----------|------------|
| Lambda-Carrageenan (λ-CGN)| 594.000     | 3   | 19  | 1.297    | 109.809    |

Table 1 shows the result of Lipinski rules, in general describes the solubility of a certain compound to penetrate the cell membrane by passive diffusion and also determine the physicochemical properties of compound as like determine the hydrophobic/hydrophilic characteristics of a compound to pass cell membrane. The molecular weight of λ-CGN is 594.000 dalton, it represents based on the Lipinski rules, λ-CGN can’t enter membrane cells because of molecular weight more than 500.000 dalton. It needs advanced research to make molecular weight smaller (≤500.000 Da). While, the number of hydrogen donors and acceptors describes the high hydrogen capacity, then increase in the amount of energy required for the absorption process to happen [14]. The hydrogen bond donors of λ-CGN is 3, it represents λ-CGN donates 3 electrons to another atom, based on the Lipinski rules it is approved. The hydrogen bond acceptors of the λ-CGN is 19, it represents λ-CGN needs 19 electrons from another atom. The log P value represents the solubility coefficient in fat/water which has a range of -0.4 - 5. The log P value of λ-CGN is 1.297, it is approved by the Lipinski rules. The larger the log P value, the more hydrophobic the molecule would be. The molecules which are too hydrophobic tend to have a high level of toxicity, because it will be disconnected longer in the lipid bilayer membrane and more widely distributed in the body, so the selective effect of the compound to target protein is reduced. The more negative the log P value, would result in non-permeability of the molecule [16,17]. It can be concluded that λ-CGN was approved by Lipinski rules, then the potential of anti-inflammatory activity from λ-CGN is predicted by PASS online analysis web server (http://www.way2drug.com/passonline/) with Pa>0.3. The results showed that λ-CGN as anti-inflammatory activity with the probability of activation (Pa) is 0.471 and probability of inhibition (Pi) is 0.066. This indicates that when the λ-CGN enters the human body, it would potentially interact with the target protein IL6R because the Pa value is higher than the Pi value.

The compounds that were approved by the Lipinski rules and PASS online analysis, were then analysed for their binding energy to the molecular complex. In this case, it is between λ-CGN and target protein IL-6 receptor (IL6R). This was performed on PyRx software with a grid docking center X: 22.0579 Y: 48.1673 Z: 102.5437 and dimensions (Å) X: 62.0645 Y: 55.6789 Z:102.5437. This study conducted the blind docking method because the functional domain of the target protein is unknown [18].
Table 2: The result of molecular docking simulation

| Compound                  | PubChem CID | Binding Energy (kcal/mol) |
|---------------------------|-------------|--------------------------|
| Lambda-Carrageenan (λ-CGN) | 101231953   | -5.9                     |
| Ibuprofen (control)       | 3672        | -5.9                     |

Based on the molecular docking result, λ-CGN showed strong binding energy to bind target protein IL-6R receptor with the value of -5.9 kcal/mol. Ligand with lower binding energy value have potential of biological activity to bind IL6R, it represents that λ-CGN is predicted to be able to inhibit IL-6R activity. The lower binding energy allows the formation of molecular complexes in constant temperature and pressure [19]. In this study, we also used common anti-inflammatory drug ibuprofen as control to compare the binding energy to target protein IL-6R with λ-CGN [20]. The binding energy value between of ibuprofen and IL-6R was the same as λ-CGN. The distance between the hydrogen bond between λ-CGN and the target protein IL-6 receptor is shown in table 3.

![Molecular visualization of λ-CGN bind to IL-6R](image)

Figure 2: Molecular visualization of λ-CGN bind to IL-6R. The IL-6R protein displayed on transparent surface and blue cartoon structure.

After the docking simulation is complete, then visualization of the molecular docking complex was carried out through PyMol software by coloring and structural selection (Figure 2). λ-CGN as ligand have the ability to bind target protein IL-6R.

Table 3: The result of hydrogen bond distance

| Hydrogen Bond Distance (Å) | Categorize Hydrogen Bonds |
|---------------------------|--------------------------|
| 2.0                       | Strong                   |
| 2.8                       | Moderate                 |
| 3.0                       | Moderate                 |
| 3.1                       | Moderate                 |
| 3.2                       | Moderate                 |
| 3.2                       | Moderate                 |

Table 3 shows the value of hydrogen bond distance (Å) between ligand (λ-CGN) and IL-6R. There are three categories of hydrogen bonds based on the strength a ligand binding target protein, 2.2-2.5 Å (strong, mostly covalent), 2.5-3.2 Å (moderate, mostly electrostatic), and 3.2-4.0 Å (weak, electrostatic) [21]. Ligand (λ-CGN) binds the target protein (IL-6R) with two categories of hydrogen bonds, strong and moderate. Hydrogen bond distance is important to know because to decide the hydrogen bond strength is strongly dependent on the distance between acceptor and donor hydrogen [21].
**Figure 3**: Chemical interaction between λ-CGN compound with IL-6R (3D visualization).

**Interactions:**
- Conventional Hydrogen Bond
- Carbon Hydrogen Bond
- Unfavorable Donor-Donor
- Pi-Sulfur
- Pi-Alkyl

**Figure 4**: Chemical interaction between λ-CGN with IL-6R.

Figure 3 showing the 3D visualization hydrogen bonds of the complex ligand (λ-CGN) and target protein (IL-6R) was carried out through DS Visualization software. While, figure 2 showing the 2D diagram of the complex ligand-protein was conducted by DS Visualization software. When the ligand binding target protein, there are five forms of the bonds, conventional hydrogen bond, carbon hydrogen bond, unfavorable donor-donor, pi-sulfur, and pi-alkyl. Based on that bonds type, conventional hydrogen bond and carbon hydrogen bond who plays a role binding ligand pocket. Conventional hydrogen bond bind the amino acid residues such as His 256, His 257, Trp 219, Arg 231, and Asp 221. While the carbon hydrogen bond bind the amino acid residues such as THR 218 and GLN 220. The more hydrogen bonds between protein-ligand, the more stable the protein-ligand interactions [22]. Binding with these amino acid residues potentially contributes to IL-6 receptor structural change which could result in functional change. Hence, further studies related to *in vitro* and
in vivo investigations would be interesting to further understand the inhibitory mechanism of λ-CGN against IL-6.

4. Conclusion
The potential of λ-CGN as anti-inflammatory is predicted by the mechanism of inhibition IL-6R. Based on the result of Lipinski rules and PASS online analysis of λ-CGN was approved, then the molecular docking simulation was conducted with the value of -5.9 kcal/mol. Hence, further studies related to in vitro and in vivo investigations would be interesting to confirm the inhibitory activity of λ-CGN against IL-6.

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