

In Vitro and In Silico Evaluation of *Cladophora* sp. as an Anti-Inflammatory Agent via COX-2 (Cyclooxygenase-2) Inhibition

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Abstract. *Cladophora* sp. is known to contain chemical constituents with pharmacological properties. The secondary metabolites present in *Cladophora* sp. include alkaloids, phenolic compounds, saponins, and terpenoids, and it has been reported to exhibit anti-inflammatory activity. To evaluate its potential as an anti-inflammatory agent, a study was conducted using in silico and in vitro approaches. The in silico analysis involved screening the physicochemical characteristics and pharmacokinetic profiles of the compounds. Meanwhile, the in vitro analysis was performed using a bovine serum albumin (BSA) protein denaturation assay. Docking studies with the receptor protein showed that compounds from the ethanol extract of *Cladophora* with lower binding affinity to COX-II (PDB ID: 5kir) compared to the control drug rofecoxib were (2R)-5-hydroxy-7-methoxy-2-phenyl-3,4-dihydro-2H-1-benzopyran-4-one (-9 kcal/mol) and pinocembrin (-8.9 kcal/mol). The compound (2R)-5-hydroxy-7-methoxy-2-phenyl-3,4-dihydro-2H-1-benzopyran-4-one showed 100% similarity in amino acid residues with the control, forming hydrogen bonds at His90 and Arg513. The in vitro anti-inflammatory assay produced a linear regression equation of $y = 352.52x - 1506.3$ with an r^2 value of 0.9179, and an IC_{50} value of 82.664 ppm, indicating strong anti-inflammatory activity. Further studies are recommended to isolate (2R)-5-hydroxy-7-methoxy-2-phenyl-3,4-dihydro-2H-1-benzopyran-4-one for subsequent in vitro and in vivo anti-inflammatory evaluations.

Keywords: Antiinflammatory, *Cladophora* sp, In Vitro, In Silico.

1 Page layout

Cladophora sp. is a green alga that is widely distributed in the waters of South Malang. This alga is known to contain various chemical compounds with pharmacological potential. Previous studies have reported that *Cladophora* sp. exhibits antioxidant and antibacterial activities [1]. The secondary metabolites identified in *Cladophora* sp. include alkaloids, phenolic compounds, saponins, and terpenoids [2]. Alkaloids, phenolics, terpenoids, and steroids have been reported to possess anti-inflammatory properties [3],[4].

Inflammation is a biological response of an organism to tissue damage resulting from cellular injury. Anti-inflammatory agents are administered to reduce inflammatory responses. These agents work by suppressing cyclooxygenase II (COX II), thereby inhibiting the synthesis of

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pro-inflammatory chemical mediators. Anti-inflammatory drugs commonly used to control and suppress inflammation include nonsteroidal anti-inflammatory drugs (NSAIDs) and steroidal anti-inflammatory drugs (SAIDs). However, NSAIDs are known to cause adverse effects, such as gastrointestinal and peptic bleeding, hypertension, and renal failure [5],[6]. Therefore, the exploration of alternative anti-inflammatory agents derived from *Cladophora* sp. is necessary.

Cladophora sp. is known to contain secondary metabolites belonging to the flavonoid group, which are recognized for their potential anti-inflammatory activity. Previous studies on *Cladophora* species have demonstrated strong antioxidant potential [13]. Nevertheless, studies specifically investigating their anti-inflammatory potential are still lacking. Therefore, the exploration of the anti-inflammatory activity of *Cladophora* sp. was conducted using both *in silico* and *in vitro* approaches.

The *in silico* approach was employed to predict the binding affinity of chemical compounds from *Cladophora* sp. to receptor proteins. COX II was selected as the target receptor protein to evaluate the anti-inflammatory potential of the compounds present in *Cladophora* sp. Chemical compounds that show good binding affinity to COX II are expected to inhibit the synthesis of pro-inflammatory mediators [7]. The binding ability of *Cladophora* sp. compounds was interpreted by comparing them with a control compound, sodium diclofenac. In addition, the pharmacokinetic profiles of *Cladophora* sp. compounds as oral drugs were predicted based on ADMET parameters (absorption, distribution, metabolism, excretion, and toxicity).

The active chemical constituents of *Cladophora* sp. collected from Tamban Beach, Malang, have not previously been investigated. In this study, the chemical composition of *Cladophora* sp. was characterized using LC-HRMS (Liquid Chromatography–High Resolution Mass Spectrophotometry). Based on the presence of chemical compounds in *Cladophora* sp. that are suspected to possess anti-inflammatory activity, exploratory research was conducted using *in silico* and *in vitro* approaches. The *in silico* analysis focused on evaluating the binding affinity of *Cladophora* sp. compounds in inhibiting COX II, as well as predicting their pharmacokinetic profiles. Meanwhile, the *in vitro* approach assessed the anti-inflammatory activity of *Cladophora* sp. using the Bovine Serum Albumin (BSA) protein denaturation method.

2 Experimental Section

2.1 Materials

The material used in this research are *Cladophora* of simplisia; ethanol p.a; NaCl; distilled water; glacial acetic acid; bovine serum albumin (BSA); Tris Buffer Saline (TBS).

2.2 Materials

2.2.1 Extraction of *Cladophora* ethanolic extract

Extraction was carried out using the maceration method with ethanol as the solvent. The type of maceration applied was kinetic maceration for 24 hours. The obtained extract was then evaporated using an evaporator. The concentrated extract was subsequently screened for bioactive compounds using LC-HRMS.

2.2.2 Formatting author names and author affiliations

A total of 150 mg of tris base and 1.10 g of NaCl were dissolved in distilled water up to a volume of 100 mL. The pH was adjusted to 6.2–6.5 (pathological pH) using glacial acetic acid, after which distilled water was added to reach a final volume of 125 mL in a beaker. A 0.2% BSA solution was prepared by dissolving 100 mg of Bovine Serum Albumin (BSA) in Tris Buffer Saline (TBS) up to a volume of 50 mL in a measuring flask.

The concentration of *Cladophora* sp. ethanol extract was varied at 25 ppm, 50 ppm, 100 ppm, and 200 ppm. A volume of 0.5 mL of each sample was taken, and 0.2% BSA was added to a final volume of 5 mL. The mixtures were incubated at room temperature (25 °C) for 30 minutes, followed by heating at 85 °C for 3 minutes. The solutions were then allowed to stand at room temperature for another 30 minutes. The absorbance of the solution was measured using a UV–Vis spectrophotometer at 660 nm. A positive control (sodium diclofenac) was prepared and measured using the same procedure as the test solutions [8].

The percentage of inhibition was calculated using the following equation:

$$\% \text{ inhibition} = \frac{\text{abs negative control} - \text{abs test solution}}{\text{abs negative control}} \times 100\% \quad (1)$$

2.2.3 ADME Prediction

Bioactive compounds from the ethanol extract of *Cladophora* identified by LC-HRMS were selected using PASS Online with $P_a > 0.5$. The ADME profiles of the selected bioactive compounds were then predicted using admetlab 3.0.

2.2.4 Molecular Docking

Selected bioactive compounds was downloaded from the Pub Chem server at <https://pubchem.ncbi.nlm.nih.gov>. The CID of each active compound was recorded. The 3D structure of each was downloaded in sdf format. The COX II receptor proteins were downloaded from the server (<https://www.rcsb.org/>). The obtained proteins were then downloaded and saved in pdb format. The docking process was carried out using the Pyrx program (autodock vina). The docking results were then visualized using the pymol program. To see the interaction between the receptor and the ligand using the Biovia Discovery Studio program.

3 Results and Discussion

3.1 Metabolite profiling ethanolic extract of *Cladophora* sp.

Metabolite profiling is a qualitative and quantitative analysis of various metabolite compounds present in a biological sample. The result of metabolite profiling ethanolic extract of *Cladophora* sp. from LC-HRMS has fifty-one active compounds. The active compound content of *Cladophora* sp. with an abundance of more than 1% is shown in Table 1 below. Based on the results of the metabolite profiling, the active compounds with the highest abundance is (2R)-5-hydroxy-7-methoxy-2-phenyl-3,4-dihydro-2H-1-benzopyran-4-one (14.088%). This compound belongs to the flavonoid group, more specifically to the flavanone subclass, which is one of the subgroups of polyphenolic secondary metabolites. Flavonoid compounds known can directly inhibit the lipoxygenase pathway in inflammation, inhibiting eicosanoid biosynthesis and inactivating free radicals that can attract inflammatory

mediators. Besides that, phenolic can inhibit inflammatory mediators [8]. The content of other compounds in *Cladophora* sp. that are greater than 10% are Bis (2-ethylhexyl) phthalate (11.724%) and Diisobutylphthalate (11.688%). This study is consistent with previous research, which also found dibutyl phthalate (DBT) in the green alga *Ulva* sp., with levels ranging from 50% to 80% [9].

Table 1. The active compounds ethanolic extract of *Cladophora* sp

No	Name	Calc. MW	RT [min]	Area (Max.)	% abundance
1	(2R)-5-hydroxy-7-methoxy-2-phenyl-3,4-dihydro-2H-1-benzopyran-4-one	270.08895	12.197	803287442	14.088%
2	Bis(2-ethylhexyl) phthalate	390.27612	17.48	668477133.6	11.724%
3	Diisobutylphthalate	278.1516	13.588	665284568.2	11.668%
4	Adenine	135.05438	0.835	471454202.4	8.268%
5	Hexadecanamide	255.25609	14.434	413891998.2	7.259%
6	Perillartine	165.11527	5.063	292782420.9	5.135%
7	2,6-N-tert-Butylpyridine	191.16725	7.406	249423512.3	4.374%
8	α -Eleostearic acid	278.22426	12.521	152338755.7	2.672%
9	Diisobutylphthalate	278.15162	13.746	151453448.3	2.656%
10	Uracil	112.02744	0.991	151446345.2	2.656%
11	Isophorone	138.1044	5.416	150547700.1	2.640%
12	Arachidonic acid	304.23989	15.121	124611599.4	2.185%
13	Palmitoleic acid	254.22437	12.399	124481239.5	2.183%
14	4-methyl-5-oxo-2-pentyl-2,5-dihydrofuran-3-carboxylic acid	212.10458	4.645	115579552.6	2.027%
15	Monobutyl phthalate	222.08873	13.592	95675164.04	1.678%
16	Triphenylphosphine oxide	278.0862	10.151	90031375.53	1.579%
17	α -Pyrrolidinopropiophenone	203.13089	4.382	79771603.27	1.399%
18	6-Methyl-2-pyridinemethanol	123.06845	0.833	70219290.63	1.231%
19	8-Hydroxyquinoline	145.05283	3.295	64675608.64	1.134%
20	3-Hydroxy-2-methylpyridine	109.05298	0.969	60539716.36	1.062%

3.2 Anti-inflammatory activity ethanolic extract of *Cladophora* sp.

Anti-inflammatory activity was tested using the protein denaturation method. Protein denaturation is one of the causes of inflammation, resulting in damage to the secondary, tertiary, and quaternary structures of proteins. This damage is caused by heat and denaturing agents, which reduce the biological function of proteins. The results of Anti-inflammatory activity of ethanolic extract of *Cladophora* sp. are shown in Table 2.

Table 2. Antiinflammatory activity ethanolic extract of *Cladophora* sp.

Sample	IC ₅₀ (ppm)			IC ₅₀ - SD
	I	II	III	
Ethanolic extract of <i>Cladophora</i> sp.	82,014	85,720	82,664	83,466 – 1,97
Natrium diclofenac	38,33	37,802	40,760	38,964– 1,577

Based on the data, IC₅₀ value ethanolic extract of *Cladophora* sp into strong activity category, while natrium diclofenac into very strong activity category [10]. The ethanolic extract of *Cladophora* sp. exhibits strong anti-inflammatory activity, which is presumed to

be due to the presence the most abundance active compounds of a flavonoid compound, namely(2R)-5-hydroxy-7-methoxy-2-phenyl-3,4-dihydro-2H-1-benzopyran-4-one. Flavonoids can interact with proteins through hydrogen bonding and hydrophobic interactions, which help maintain the stability of the protein’s tertiary structure [11]. Thus, flavonoids prevent protein denaturation caused by external factors such as heat or oxidative stress.

3.3 Screening pharmacokinetic of ethanolic extract *Cladophora* sp.

The ethanol extract of *Cladophora* sp. contains fifty-one active compounds. These active compounds were screened using PASS Online with Pa value > 0.5. A Pa value greater than 0.5 but less than 0.7 (0.5 < Pa < 0.7) indicates that the compound has high biological activity at the laboratory scale and has potential for the development of new drug candidates with the corresponding bioactivity [14]. The screening results revealed fourteen compounds with potential antihypertensive activity. The physicochemical and pharmacokinetic profile screening results of the ethanol extract of *Cladophora* sp. are presented in Table 3 below.

Table 3. Screening pharmacokinetic and physicochemical of ethanolic extract *Cladophora* sp.

No	Molecule	HIA	MDCK	BBB	VDss	CYP2D6-sub	Lipinski
1	(2R)-5-hydroxy-7-methoxy-2-phenyl-3,4dihydro-2H-1benzopyran-4-one	00.01	1.75E-05	00.27	0,369444444	0,059722222	Accepted
2	Bis(2-ethylhexyl) phthalate	0.001	1.62E-05	0.015	1.427	00.12	Accepted
3	Arachidonic acid	0.033	7.84E-05	0.002	0,510416667	0,652083333	Accepted
4	Palmitoleic acid	00.03	3.80E-05	0.043	0,377083333	0,203472222	Accepted
5	Ethyl palmitoleate	0.005	2.95E-05	0,175694444	2.417	0,176388889	Accepted
6	2-Pyridylacetic acid	0.009	5.47E-05	0,1375	0,228472222	0,155555556	Accepted
7	Ethyl myristate	0.001	1.89E-05	0,161111111	1.629	0.056	Accepted
8	4-methoxy-6-[(E)-2phenylethenyl]-2Hpyran-2-one	0.014	1.38E-05	0,155555556	0,426388889	0,061111111	Accepted
9	Ethyl oleate	0.005	2.69E-05	0,0875	2.898	0,121527778	Accepted
10	2,3dihydroxypropyl 12methyltridecanoate	0.005	3.49E-05	0,253472222	0,540277778	0.048	Accepted
11	Eicosapentaenoic acid ethyl ester	0.031	0.000160727	0.009	2.653	0,665972222	Accepted
12	Pinocembrin	0.006	1.62E-05	0.085	0,429861111	0,497222222	Accepted
13	Monoolein	0.070138889	3.95E-05	0,090972222	0,517361111	0,093055556	Accepted
14	Phthalic acid	0.071	1.02E-05	0,277083333	0,141666667	0.055	Accepted

Description: HIA is human intestinal absorption; MDCK is Madin Darby Canine Kidney; BBB is Blood Brain Barrier;VDss is volume distribution is staeady state

The physicochemical profiles of the fourteen active compounds comply with Lipinski’s Rule of Five. Drug compounds extract of ethanol *Cladophora* sp. comply with Lipinski’s Rule of Five are considered to have good potential as oral drug candidates. This study is consistent with previous research on the green alga *Valoniopsis pachynema*, in which the contained chemical compounds comply with Lipinski’s rule of five and showed potential as candidates for orally administered drugs [15].

The pharmacokinetic profile of the ethanol extract of *Cladophora* sp. includes Human Intestinal Absorption (HIA), Madin-Darby Canine Kidney (MDCK), Blood-Brain Barrier (BBB), Volume of Distribution at Steady State (VDss), and Cytochrome P450 2D6 substrate (CYP2D6-sub). The fourteen compounds identified in the ethanol extract of *Cladophora* sp. are predicted to have excellent absorption potential and good potential to penetrate the bloodbrain barrier. The ethanol extract of *Cladophora* sp. shows VDss values within the

excellent category. CYP2D6-sub refers to compounds that can act as substrates metabolized by the CYP2D6 enzyme, converting them into more easily excreted metabolites. A compound with a score of 0 is categorized as a non-substrate, whereas a score of 1 indicates a substrate or inhibitor. Based on the obtained data, the compounds from the ethanol extract of *Cladophora* sp. are predicted to act as substrates or inhibitors of the CYP2D6 enzyme [12]. This study is in line with previous research on the Active Compounds Fucoidan and Alginate Derived from *Sargassum* Sp which also pharmacokinetic propertie identified was antiinflammation candidate [4].

3.4 Molecular docking antiinflammation of ethanolic extract *Cladophora* sp.

Molecular docking is an in silico method used to determine the potential of active compounds from the ethanol extract of *Cladophora* as anti-inflammatory agents by binding them to the COX II receptor protein. Before performing the docking, it is necessary to validate the docking method by redocking the COX II protein with its native ligand (rofecoxib). The validation results are shown in Figure 1.



Fig. 1. Validation of COX II protein redocking with the native ligand (rofecoxib).

From the validation results, an RMSD value of 1.611 Å was obtained. The validation is considered valid because the RMSD value is less than 2 Å. This indicates that the docking method used can predict the ligand position with an error of less than 0.2 nanometers, which is still within the natural fluctuation range of the protein structure. The docking results of the ethanol extract with the COX II receptor protein are presented in Table 4 below.

Table 4. Molecular Docking Results of *Cladophora* sp. Ethanol Extract with COX II

No	Name	CID	Binding Af finity (Kca l/Mol)	Ikatan Hidr ogen	Ikatan Van der Walls
1	(2R)-5-hydroxy-7methoxy-2-phenyl-3,4dihydro-2H-1benzopyran-4-one	4101463	-9	HIS 90; AR G 513	GLN 192; ALA 516; ILE 517; SER 353; TYR 3 55; LEU 531; TRP 387; GLY 526; MET 522
2	Bis(2-ethylhexyl) phthalate	8343	-5.2	HIS 90; AR G 513	GLN 192; ALA 516; ILE 517; PHE 518; ARG 120; VAL 116; TYR 355
3	Arachidonic acid	444899	-7.6	TYR 387; HIS 214	HIS 486; TYR 385; ALA 202; THR 206; PHE 2 10; ASN 382; THR 212; HIS 386
4	Palmitoleic acid	445638	-4.5	Gln 203	HIS207; PHE 210; THR 206; ALA 202; TYR 3 85 ; TRP 387; LEU 390; LEU 294; VAL 447; V AL 295; VAL 444

No	Name	CID	Binding Af finity (Kca l/Mol)	Ikatan Hidr ogen	Ikatan Van der Walls
5	Ethyl palmitoleate	6436624	-5.7	GLN 203; HIS 214; A SN 382	THR 212; HIS 207; PHE 210; TYR 385; HIS 38 8; LEU 294; VAL 444
6	2-Pyridylacetic acid	85318	-6.2	-	HIS 214; LEU 390; GLN 203; TRP 387; ALA 2 02; TYR 385; THR 206; HIS 207; VAL 447
7	Ethyl myristate	31283	-5.5	-	GLN 327; SER 548; THR 549
8	4-methoxy-6-[(E)- 2phenylethenyl]- 2Hpyran-2-one	5273621	-7.8	TRP 387; H IS 214	GLN 203; LEU 294; HIS 388; TYR 385; HIS 3 86 ; HIS 207; ALA 202; THR 206; PHE 210; AS N 382; THR 212
9	Ethyl oleate	5363269	-5.8	HIS 90; AR G 513	ALA 516; GLN 192; TYR 355; SER 353; VAL 349; ALA 527; TYR 348; SER 530; GLY 526; LEU 384; PHE 381; PHE 518
10	2,3dihydroxypr opyl 12methyltrideca noate	10828227	-5.4	ARG 333; GLY 235; GLU 236	ASN 231; THR 237; GLY 225; LEU 238; GLN 241
11	Eicosapentaenoic acid e thyl ester	9831415	-6.3	GLN 241; ARG 333	LEU 238; THR 237; GLN 374; ASN 375; HIS 2 26; GLY 225; ASN 231'; ASP 229; GLU 236
12	Pinocembrin	68071	-8.9	GLU 465; GLN 461; T YR 130	PRO 154; LEU 152; GLY 135; HIS 39; ASN 34 ; CYS 47; VAL 46; ARG 44; GLY 45
13	Monoolein	5283468	-5.6	-	GLU 326; GLN 327
14	Phthalic acid	1017	-6.7	-	SER 143; ASN 144; GLU 140; TRP 139
15	Kontrol (rofecoxibe)		-8,9	HIS 90; AR G 513	GLN 192; ALA 516; ILE 517; SER 353; TYR 3 55; LEU 531; TRP 387; GLY 526; MET 522

Based on the molecular docking results of the *Cladophora* sp. ethanol extract with the COX II receptor protein, it was found that (2R)-5-hydroxy-7-methoxy-2-phenyl-3,4-dihydro-2H-1-benzopyran-4-one exhibited a binding affinity of -9 kcal/mol, which is higher than that of rofecoxib (-8.9 kcal/mol). This compound shares the same amino acid residues as rofecoxib, forming hydrogen bonds with the amino acid residues His90 and Arg513. Based on the docking results, the carbonyl group has an important role in forming hydrogen bonds with the COX II receptor protein. (2R)-5-hydroxy-7-methoxy-2-phenyl-3,4-dihydro-2H-1benzopyran-4-one is a flavonoid belonging to the flavanone subclass, with the highest relative abundance of 14% in the ethanol extract of *Cladophora* sp. This compound has potential as an anti-inflammatory agent by inhibiting the COX II receptor protein. Inhibition of the COX II receptor protein is expected to block the synthesis of inflammatory mediators [9]. This study is in line with previous research on the seaweed *Sargassum*, which also has potential as an anti-inflammatory agent by inhibiting the COX-1 and COX-2 proteins [4].

4 Conclusion

Cladophora sp. contains 51 compounds, of which 14 compounds are predicted to have anti-inflammatory activity. The ethanol extract of *Cladophora sp.* shows strong anti-inflammatory activity in an in vitro study. It has a lower binding affinity to COX II (ID PDB: 5kir) compared to the control (rofecoxib) was (2R)-5-hydroxy-7-methoxy-2-phenyl-3,4-dihydro-2H-1-benzopyran-4-one (-9 kcal / mol) those that are underway. Further research can be conducted by isolating (2R)5-hydroxy-7-methoxy-2-phenyl-3,4-dihydro-2H-1-benzopyran-4-one for in vitro and in vivo anti-inflammatory testing.

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