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Biological evaluation and molecular docking of Indonesian *Gracilaria salicornia* as antioxidant agents

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ABSTRACT: The antioxidant activity of *Gracilaria salicornia* extract was investigated to develop natural product-based chemotherapeutic agents using more efficient and straightforward methods. The efficacy was determined through free radical scavenging activity against DPPH, phytochemical assays, GC-MS analysis, and molecular docking analysis through NADPH Oxidase (NOX) protein (PDB ID: 2CDU). The best antioxidant activity of several extracts was shown by ethyl acetate extract with an IC_{50} value about 179.81±6.38 µg/mL, classified as moderate activity. Based on the phytochemical assay, the extract contains alkaloids, steroids, phenolics, flavonoids, and saponin compounds. Further analysis of the extract by GC-MS showed the presence of secondary metabolites that have been shown to have bioactivity as antioxidant and anticancer agents, such as L-(+)-ascorbic acid 2,6-dihexadecanoate, cholest-5-en-3-ol (3.beta.), 1,2-benzenedicarboxylic acid, and phytol. The activity was also supported by molecular docking analysis. Cholest-5-en-3-ol (3.beta.), 1,2-benzenedicarboxylic acid, and phytol showed outstanding interaction with the target protein's active site (binding energy -10.90, -7.11, and -6.22 kcal/mol, respectively). The binding energy of cholest-5-en-3-ol (3.beta.) was significantly higher than the native ligand. The binding energy describes the potential of the compound to suppress ROS production by inhibiting NOX protein activity. These findings revealed that the phytochemicals of *G. salicornia* can be developed as a chemotherapeutic agent. This approach can be used as a guide in developing natural product-based chemotherapeutic agents.

KEYWORDS: *Gracilaria salicornia*; secondary metabolites; chemotherapy; antioxidant; molecular docking.

1. INTRODUCTION

Cancer is one of the diseases categorized as a leading cause of death. This is inextricably linked to the high number of deaths, particularly in countries with advanced economies. The number of deaths increases and is even predicted to become the highest mortality rate worldwide in the next few decades [1]. In 2018, the Global Cancer Observatory (GLOBOCAN) reported that out of 18.1 million cancer patients, 53.04% died [2]. The emergence of the disease is caused by the activity of Reactive Oxygen Species (ROS), which are produced as a result of metabolism and exposure of pollution from the environment [3].

Free radicals, such as ROS, play an essential role in the immune system [4] and cell survival [5]. Inhibiting ROS production in the body can treat cancer and other inflammatory diseases. This can be addressed by blocking the primary source of intracellular ROS, Nicotinamide Adenine Dinucleotide Phosphate Oxidases (NOX) [6–8]. Exogenous antioxidants that efficiently decrease ROS are also required [9]. Antioxidants are the main natural products that act as ROS inhibitors and reduce oxidative stress [10].

Researchers are currently interested in natural antioxidants, especially those obtained from macroalgae [11,12]. Furthermore, natural products such as macroalgae are easy to get, cheaper, and have lower cytotoxicity effects [13]. *Gracilaria* is a genus of macroalgae that is a potential source of antioxidant compounds to be developed as chemotherapeutic agents [14]. This potential is also supported by its nutritional contents so that residents in several countries on the Asian continent, especially in Indonesia, *Gracilaria* used as a complementary/food additive [15]. The development of chemotherapeutic agents from *Gracilaria* is also supported by isolated compounds that generally have higher activity when compared to commercial antioxidants [14]. However, the research that has been carried out takes a long time and is relatively expensive.

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Seeing the urgent requirement for chemotherapeutic agents, a more effective and efficient approach will be carried out. The molecular docking approach can certainly be easier, cheaper, and faster to determine the potential bioactivity of *G. salicornia*. The present study was the first to use a molecular docking technique to examine and analyze the potential of *G. salicornia*'s phytochemical as an antioxidant through the inhibition of NOX protein.

2. RESULTS AND DISCUSSION

2.1 Extraction and phytochemical assay

The graded maceration technique of extracting samples yielded four types of extracts with varying levels of polarity depending on the solvent used. Through this method, the compounds in the obtained extract were separated based on their polarity, making it easier to find promising compounds to develop according to the desired target. Polar extract dominated the sample with a yield of 1.95%, according to the weight of the extract produced (Table 1). Semipolar extracts, on the other hand, showed the lowest yield following nonpolar extracts (n-hexane and chloroform extracts). Previous experiments on *G. edulis* samples using the same method revealed that the ethyl acetate extract similarly had the lowest yield [16].

Table 1. Weight of *G. salicornia* extract

Extract	Weight (g)
n-Hexane	1.16
Chloroform	3.24
Ethyl acetate	0.62
Methanol	9.74

The extracts were then analyzed for phytochemicals to determine the class of secondary metabolite (Table 2). The results revealed identical phytochemical profiles in nonpolar (n-hexane and chloroform) and semipolar extracts (ethyl acetate). The only difference was in the concentration of each compound class, which was assessed based on the intensity of the color or precipitate produced. The phytochemical assay of the methanol extract did not show a positive reaction to the alkaloid and steroid test as in other extracts. Positive reactions only occurred in phenolic and flavonoid tests which were also found in semipolar and nonpolar extracts. Another result that showed a striking difference was a positive reaction with the saponin test, only found in methanol extract.

The presence of alkaloid compounds was dominant in the chloroform and ethyl acetate extracts. Similar results have been reported by Sakthivel et al., (2016) [16] that the ethyl acetate extract of *G. edulis* has a high alkaloid content. Meanwhile, the presence of flavonoid and saponin compounds in methanol extract has been reported by Dayuti, (2018) [17] on the species *G. verrucosa.*

Several studies to compare the phytochemical profiles of the *Gracilaria* were summarized in Table 2. The phytochemical profiles of the *Gracilaria* are diverse, as seen by the previous research. Environmental conditions, species, and solvents employed in the extraction process significantly influence the findings. According to Aroyehun et al., (2019) [18], seasonal fluctuations also contributed considerably to the diversity of the phytochemical composition of the *Gracilaria*.

Table 2. Phytochemical profile of the *Gracilaria*

Location	Species	Solvent	Phytochemical					
			Alkaloid	Steroid	Terpenoid	Phenolic	Flavonoid	Saponin
Palk Bay, India [19]	*G. corticata*	MeOH 70%	NA	NA	NA	4.00 ± 0.35 (mg GAE/g)	3.33 ± 0.12 (mg GAE/g)	NA
	G. edulis	MeOH 70%	NA	NA	NA	3.4 ± 0.21 (mg GAE/g)	2.5 ± 0.08 (mg GAE/g)	NA
Pulo Aceh, Indonesia [20]	*G. verrucosa*	MeOH 96%	-	√	√	√	-	-

(continued on next page)

Location	Species	Solvent	Phytochemical					
			Alkaloid	Steroid	Terpenoid	Phenolic	Flavonoid	Saponin
Tamil Nadu, India [21]	*G. corticata*	Ethanol	NA	NA	NA	6.81 ± 0.18 (mg GAE/g)	26.49 ± 0.05 (mg RE/g)	NA
	G. edulis	Ethanol	NA	NA	NA	6.75 ± 0.17 (mg GAE/g)	28.18 ± 1.01 (mg RE/g)	NA
	G. crassa	Ethanol	NA	NA	NA	7.77 ± 0.15 (mg GAE/g)	29.45 ± 1.25 (mg RE/g)	NA
	G. salicornia	Ethanol	NA	NA	NA	8.52 ± 0.43 (mg GAE/g)	31.45 ± 0.35 (mg RE/g)	NA
	G. verrucosa	Ethanol	NA	NA	NA	7.89 ± 0.05 (mg GAE/g)	27.39 ± 0.12 (mg RE/g)	NA
Gulf of Mannar [22]	*G. dura*	MeOH	NA	-	√	√	√	-
Kalpitiya, Sri Lanka [23]	*G. edulis*	MeOH	2875.54 ± 22.29 (µg PE/g)	NA	NA	1007.81 ± 54.21 (µg GAE/g)	541.02 ± 51.84 (µg QE/g)	NA
		Hexane Fr.	2875.54 ± 22.29 (µg PE/g)	NA	NA	760.85 ± 37.75 (µg GAE/g)	688.60 ± 9.55 (µg QE/g)	NA
		Chloroform Fr.	2875.54 ± 22.29 (µg PE/g)	NA	NA	560.85 ± 55.08 (µg GAE/g)	289.39 ± 9.55 (µg QE/g)	NA
		EtOAc Fr.	1073.75 ± 45.88 (µg PE/g)	NA	NA	2414.51 ± 50.34 (µg GAE/g)	1461.49 ± 75.22 (µg QE/g)	NA
		Aqueous Fr.	522.34 ± 67.13 (µg PE/g)	NA	NA	1704.69 ± 43.16 (µg GAE/g)	786.95 ± 62.04 (µg QE/g)	NA
Talango Island, Indonesia [17]	*G. verrucosa*	Ethanol 75%	√	NA	-	NA	-	√
		MeOH 75%	√	NA	-	NA	√	√
Naozhou Island, South China [24]	*G. lemaneiformis*	Ethanol 70%	NA	√	√	√	NA	NA
Red sea, Saudi Arabia [25]	*G. dendroides*	Ethanol	NA	NA	NA	NA	√	NA
Makassar Strait, Indonesia [26]	*G. verrucosa*	Acetone	√	NA	√	-	√	NA
		Ethanol	√	NA	√	-	√	NA
		MeOH	√	NA	√	√	√	NA
Gulf of Mannar [27]	*G. verrucosa*	MeOH	√	-	√	√	√	√
Semarang, Indonesia [28]	*G. verrucosa*	EtOAc	-	√	-	√	√	-
		MeOH	√	√		√	√	√
Selayar Island, Indonesia (Present Study)	*G. salicornia*	n-Hexane	+	+	-	+	+	-
		Chloroform	++	++	-	+	+	-
		EtOAc	++	+	-	++	+	-
		MeOH	-	-	-	+	++	++

Note: (-): No reaction; (+): Weak-intensity reaction; (++): Moderate-intensity reaction; (√): Confirmed; (NA): Not Analyze

2.2 Antioxidant activity

The antioxidant activity of *G. salicornia* was analyzed based on free radical scavenging activity against DPPH (2,2-Diphenyl-1-picrylhydrazyl). This method has been widely applied to determine the antioxidant activity. The results showed a linear correlation between the concentration of the extract and scavenging activity (%) (Figure 1). The highest antioxidant activity was shown in the ethyl acetate extract, with a scavenging activity of 42.82±1.39% at a concentration of 160 µg/mL. Meanwhile, the chloroform extract had the lowest activity at the same concentration, with a value of 30.06±0.60%. The different activity was caused by the different compounds contained in each extract. This finding was in line with the research conducted by Gunathilaka et al., (2019) [23].

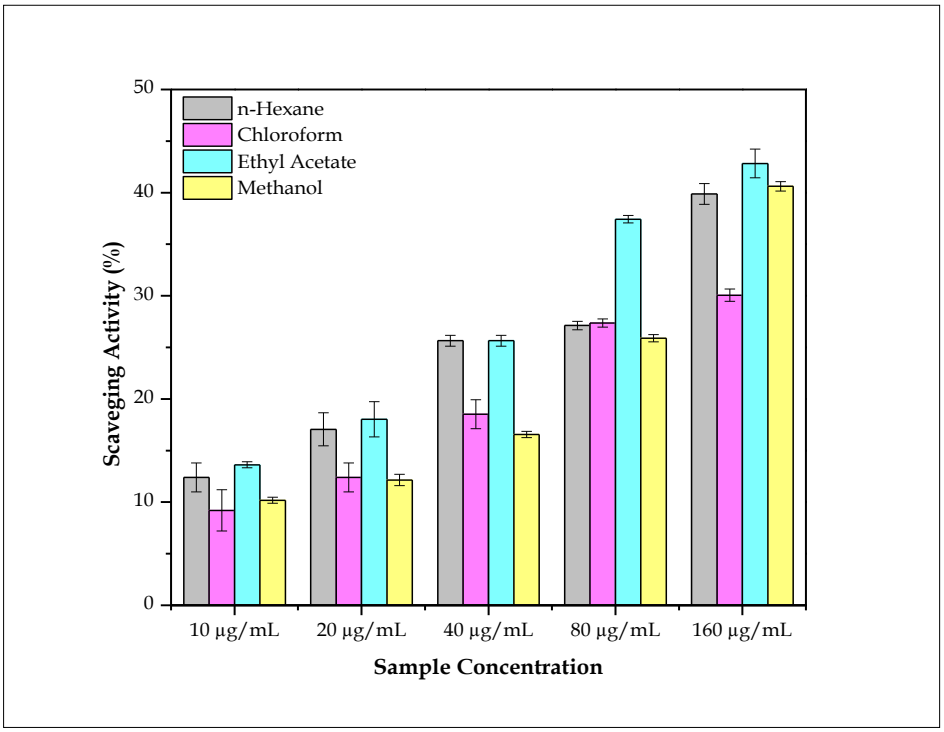


Figure 1. Antioxidant activity of *G. salicornia* extracts

Based on the IC_{50} value, it is known that the whole extract of *G. salicornia* can inhibit the DPPH radical. Ethyl acetate extract with IC_{50} 179.81±6.38 µg/mL showed the best inhibition, the activity was classified as moderate activity. Similar results were shown in the *G. edulis* species but with the lower activity (IC_{50} 3170 µg/mL) [23]. Antioxidant analysis of *Gracilaria* in other studies revealed that *G. salicornia* and *G. corticata* from the Persian Gulf had IC_{50} of 730 and 540 µg/mL, respectively [29], while *Gracilaria sp* from the Brazilian coast and Johor Bahru waters had IC_{50} of >1000 µg/mL [30] and 5600 µg/mL [31]. The antioxidant activity of the previous study is still lower than our finding.

The presence of phenolic compounds is one of the explanations for the strong antioxidant activity of ethyl acetate extract compared to other extracts. According to Mahomoodally et al., (2020) [9] and Mateos et al., (2020) [32], phenolic compounds significantly affect antioxidant activity. This fact is in line with the results obtained. Namely, the ethyl acetate extract has higher phenolic compounds than other extracts (Table 2). The presence of phenolic in *Gracilaria* has been reported in *G. corticata* [30] and *G. salicornia* [29]. Several bromophenol-based structures were also isolated from *G. edulis* and *G. secundata* with antioxidants activity [33]. The antioxidant ability of phenolic compounds is related to the ability of electron or proton donors of these compounds to stabilize DPPH radicals [34]. However, the presence of alkaloids and flavonoid compounds, of course, also contributes to this activity. The flavonoid compounds have been reported to provide a protective effect from radical compounds on cell membranes [27,35].

2.3 Gas Chromatography-Mass Spectrometry (GC-MS) analysis

GC-MS is a technique for analyzing thermally stable compounds, volatile compounds with a boiling point of < 300°C, and lipophilic or nonpolar organic compounds such as steroids, lipids, and aromatic

hydrocarbons. This method is used because it can detect low concentrations of compounds [36,37]. GC-MS spectra in Figure 2 shows that there were 55 peaks of the compound identified. Based on the area of each peak, six major compounds amounted totally to 58.27% dominated the components in the extract (shown in Table 3). These compounds have a high similarity with the data in the libraries, as indicated by the similarity index between 91-98%.

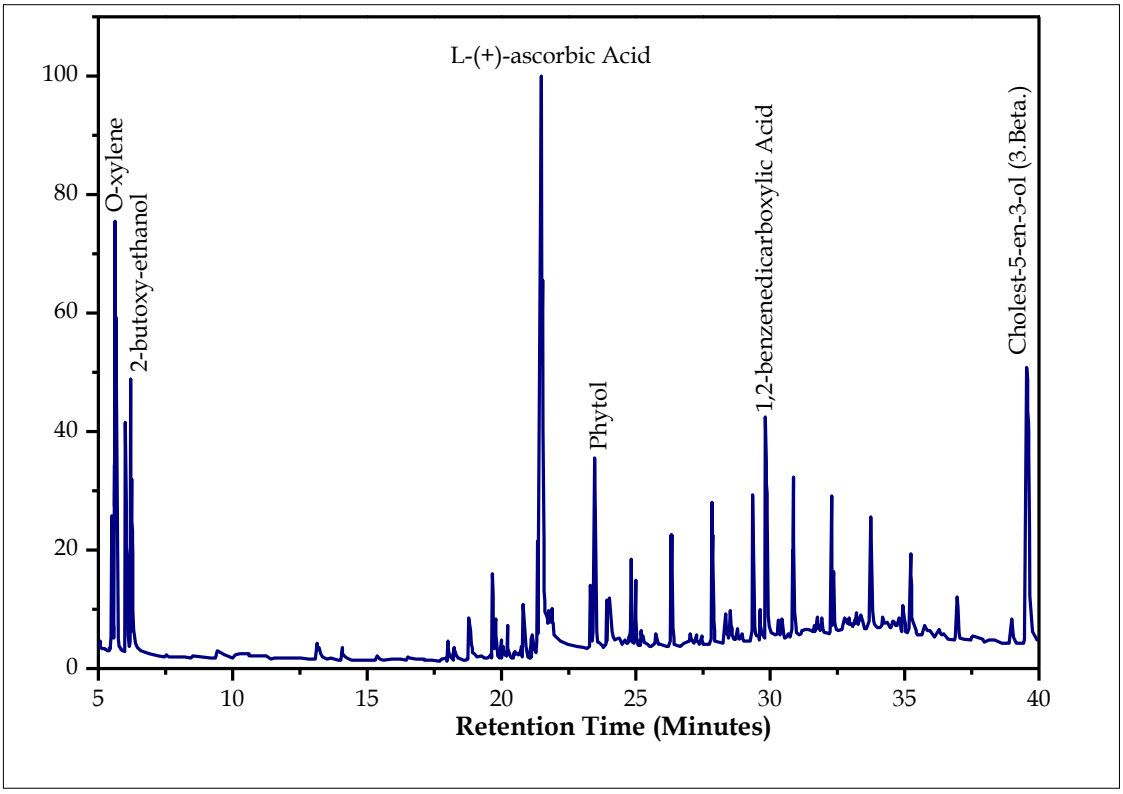


Figure 2. GC-MS spectra of ethyl acetate extract

Refers to the relative area of each peak, L-(+)-ascorbic acid 2,6-dihexadecanoate had the highest composition (27.79%). The compound had not been reported in other *Gracilaria* species. This compound has the same basic structure as ascorbic acid, which has been proved to be strong antioxidant. The presence of a hydrophobic chain in these structures has increased the activity of compounds as enzyme inhibitors [38]. L-(+)-ascorbic acid 2,6-dihexadecanoate has also been reported to contribute to the antioxidant activity of ethyl acetate extract of *Sargassum wightii* [39]. Another report showed that L-(+)-ascorbic acid 2,6-dihexadecanoate effectively inhibited ROS production, DNA damage, and induced apoptosis [40].

Table 3. Major compound in ethyl acetate extract

Retention Time (Minutes)	Area (%)	SI (%)	Name	PubChem ID	Class of Compound
21.53	27.79	91	L-(+)-ascorbic acid 2,6-dihexadecanoate	54722209	Ester
39.61	11.08	95	Cholest-5-en-3-ol (3.beta.)	304	Steroid
5.64	7.73	98	O-xylene	7237	Aromatic hydrocarbon
29.88	4.45	95	1,2-Benzenedicarboxylic acid	109429	Carboxylic acid
6.23	3.77	97	2-Butoxy ethanol	8133	Glycol ethers
23.50	3.45	97	Phytol	5280435	Diterpene

Other compounds belonging to the sterol group, namely cholest-5-en-3-ol (3.beta.), were also found in this study. This compound is commonly reported as a component of *Gracilaria* such as *G. birdiae, G. caudate* [41], and other red algae, namely *Halymenia durvillei* [42]. Cholest-5-en-3-ol (3.beta.) has been reported to have

anticancer activity [43]. The presence of sterol compounds has also been reported in the species *G. salicornia* that lives in the Persian Gulf (near Bandare-Abbas city) [44].

1,2-Benzenedicarboxylic acid and phytol compounds were also found in this research. The presence of these compounds has been reported in other *Gracilaria* species. Sheeja et al., (2016) [45] have isolated phytol compounds as components of the methanol extract of *G. edulis*. Meanwhile, the 1,2-benzenedicarboxylic acid compound was found in methanol extracts *G. corticata* [46]. 1,2-Benzenedicarboxylic acid and phytol compounds have also been identified as components of red algae (*Jania rubens, Corallina mediterranea,* and *Pterocladia capillacea*) [47]. These compounds have been reported to have antioxidant activity [46,47] and anticancer [16,43].

2.4 Molecular docking and ADME-TOX properties analysis

Molecular docking analysis is a method for predicting the compound's activity against a certain protein based on its binding energy. The application of this method is one of the most effective and efficient solutions in drug discovery [48,49]. Molecular docking also accelerates the development of natural products-based medicine [52]. Another significant contribution is identifying compounds with promising therapeutic activity [53].

This study was the first to evaluate the potential of secondary metabolites of Indonesian *G. salicornia* to inhibit the NOX protein. There were six compounds which were the main components in the highest antioxidant activity extract, were used as ligands. The molecular docking results of these compounds were compared to native ligand, in this case, FAD compound that acts as NOX cofactor. The native ligand superimposes before and after redocking had a Root-Mean Square Deviation (RMSD) < 2 Å (1.46 Å), indicating that the method was valid. The results of molecular docking expressed by the value of binding energy summarized in Table 4 provide an overview of the effectiveness of the interactions between the ligand and protein.

Table 4. Molecular docking results

Ligand	Binding Energy (kcal/mol)	Conventional H bond (Bonding Distance (Å))
L-(+)-ascorbic acid 2,6 dihexadecanoate	-4.06	ALA45 (2.29) ILE44 (2.94; 3.00) GLY43 (1.81)
Cholest-5-en-3-ol (3.beta.)	-10.90	THR112 (1.98)
o-Xylene	-4.37	-
1,2-Benzenedicarboxylic acid	-7.11	ASP282 (1.97; 2.89) SER115 (2.07) LYS134 (1.76)
2-Butoxy ethanol	-4.33	THR112 (1.98) THR9 (1.99) HIS10 (2.51)
Phytol	-6.22	THR112 (1.83) THR112 (2.96)
Native	-8.65	HIS10 (2.95; 2.48; 2.74) ALA11 (1.73) LYS134 (2.01) THR301 (2.14)

Based on the binding energy measured through the interaction between the ligand and the protein, it was known that cholest-5-en-3-ol (3.beta.) was bind strongly to the protein's active site, due to the lowest binding energy about -10.90 kcal/mol. This energy was even lower when compared to the binding energy of the native ligand, which only has an energy of -8.65 kcal/mol. This indicates the stability of the interaction between the two molecules. The results of this study were supported by the fact that the smaller the energy resulting from the interaction, the compound indicates suitability for the protein's active site [54].

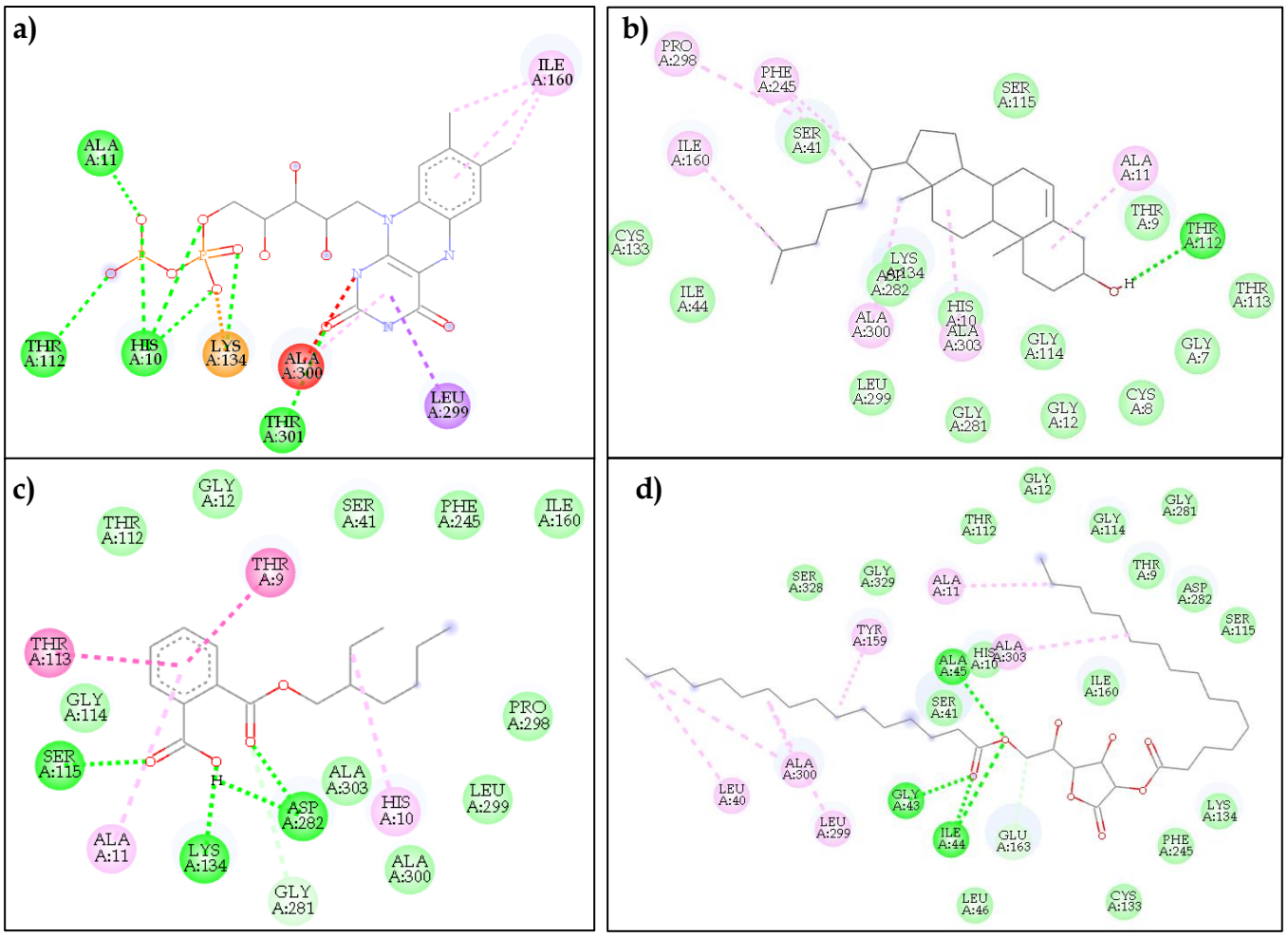
In addition, the interaction of 1,2-benzenedicarboxylic acid and phytol can also be categorized as having a good interaction. The two compounds produced binding energies of -7.11 and -6.22 kcal/mol, respectively. However, the energy higher than native ligands, the binding energy less than-5.0 kcal/mol could be

considered the optimum interaction [55]. This interaction indicates a synergy with the activity of these compounds, which have been reported to have antioxidant and anticancer activities.

Interactions of additional ligands such as L-(+)-ascorbic acid 2,6-dihexadecanoate, o-xylene, and 2-butoxy ethanol were regarded as ineffective because of the higher energy than -5.0 kcal/mol. The binding energies of these ligands were -4.06, -4.37, and -4.33 kcal/mol, respectively. However, it has been reported that L-(+)-ascorbic acid 2,6-dihexadecanoate has excellent binding energy with PI3K protein (a protein involved in most cancers) [39].

The binding energy is strongly influenced by the type and number of bonds formed between the ligand and the protein [56]. Visualization of the docking results with Discovery Studio (Figure 3) shows that there has been an interaction between the ligand and the active site of the target protein. The interaction between cholest-5-en-3-ol (3.beta.) and the protein was dominated by hydrophobic interactions (Van der Walls and Pi-Alkyl interaction). Conventional hydrogen bond accompany the hydrophobic interactions through amino acids THR112 1.98 Å. These interactions contribute to the stability of binding of cholest-5-en-3-ol (3.beta.) with the protein. The binding of the cholest-5-en-3-ol (3.beta.) molecule has also been reported as an effective ligand against COVID-19 virus infection by inhibiting the 3CL-Mpro protein [42].

Despite having fewer hydrogen bonds than native ligands, the cholest-5-en-3-ol (3.beta.)-NOX protein complex has higher binding energy. This was because the complex had less intermolecular energy than the native-NOX protein complex. These interactions had intermolecular energies of -12.69 and -11.33 kcal/mol, respectively. The higher intermolecular energy in the native-NOx complex was caused by the formation of an unfavorable interaction. This was clearly seen in the complex's 2D interaction (Figure 3a). Previous studies have reported that this interaction may contribute to the decreased stability of ligand-protein interactions [57]. This contribution will be observed with a decrease in the effectiveness of the ligand-protein interaction, which was characterized by a higher binding energy.



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e) **f)**

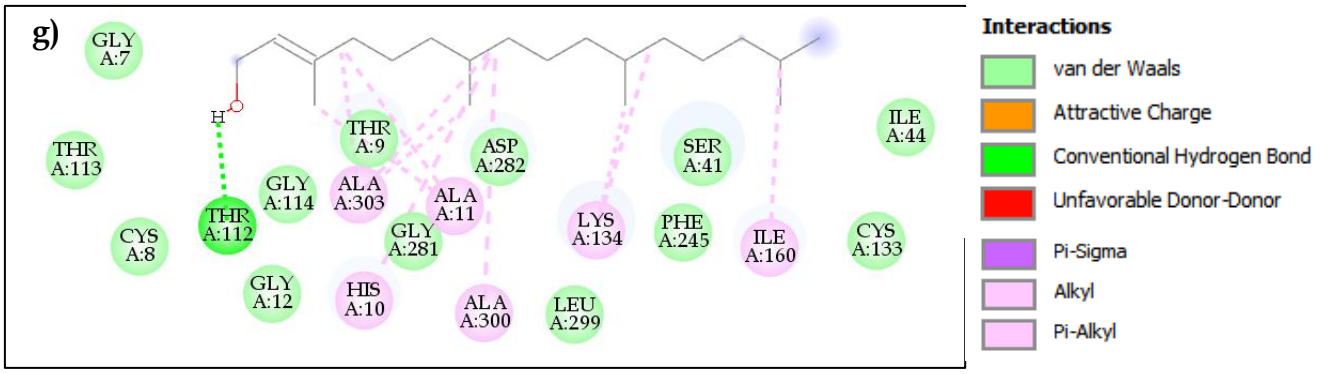

Figure 3. Ligands Interaction againts NOX Protein a) Native ligand; b) Cholest-5-en-3-ol (3.beta.); c) 1,2-Benzenedicarboxylic acid; d) L-(+)-ascorbic acid 2,6 dihexadecanoate; e) o-Xylene; f) 2-Butoxy ethanol; g) Phytol

In contrast to the observation of binding energy in the 1,2-benzenedicarboxylic acid-NOX complex, the stability of the interactions formed was a major contribution from the hydrogen bonds formed. In addition, the compound also undergoes internal structural stabilization through intramolecular hydrogen bonds with a distance of 1.64 Å. These interactions resulted in the lowest internal energy among the analyzed ligands, which was -1.61 kcal/mol.

Table 5. ADME-TOX properties

Ligand	Lipinski Rules				Toxicology		
	MW	HBA	HBD	M Log P	Cramer rules	Genotox/ Non-genotox	Mutagenicity
L-(+)-ascorbic acid 2,6 dihexadecanoate	**652.94**	8	2	**4.64**	**High**	No/No	No
Cholest-5-en-3-ol (3.beta.)	386.65	1	1	**6.34**	Intermediate	No/No	No
o-Xylene	106.17	0	0	3.85	Low	No/No	No
1,2-Benzenedicarboxylic acid	264.32	4	1	3.18	Low	No/**Yes**	No
2-Butoxy ethanol	118.17	2	1	0.61	Low	No/No	No
Phytol	296.53	1	1	**5.25**	Low	No/No	No

Note: MW: Molecular weight (<500 g/mol); HBA: H-bond acceptors (<10); HBD: H-bond donors (<5); M Log P: Moriguchi Lipophilicity (≤4.15)

The ADME-TOX analysis is another critical parameter in the in silico study of chemical compounds. This analysis will provide an overview of the compound's pharmacological and toxicological properties. Pharmacological properties of compounds based on Lipinski rules of five include molecular weight (<500 g/mol), H-bond acceptors (<10), H-bond donors (<5), and Moriguchi lipophilicity (≤4.15). A compound with less than two violations is said to have good pharmacologic properties or drug-likeness [58]. Most of the compounds examined complied with these requirements, except for L-(+)-ascorbic acid 2,6-dihexadecanoate. This compound showed two violations, namely molecular weight >500 g/mol (652.94 g/mol) and Moriguchi lipophilicity >4.15 (4.64), indicating the compound has poor pharmacological properties. In line with these data, the toxicological analysis of L-(+)-ascorbic acid 2,6-dihexadecanoate showed a high category of toxicity based on the Cramer rules. As a result, L-(+)-ascorbic acid 2,6-dihexadecanoate did not meet the ADME-TOX criteria. Meanwhile, other compounds met the ADME-TOX criteria. However, high doses of cholest-5-en-3-ol (3.beta.) compounds should be avoided. This was indicated by the toxicity analysis with the Cramer rules, which was included in the intermediate category. The compound also needs to be considered because it was non-genotox. The non-genotoxic carcinogenic compounds, in general, do not cause damage to DNA directly

and generally do not have mutagenicity [59] as the results obtained, making them safer to use than genotoxic carcinogenic compounds. The summary of the ADME-TOX analysis can be seen in Table 5.

3. CONCLUSION

G. salicornia extract contains various phytochemicals depending on the polarity of the extraction solvent used. Each of these extracts had moderate antioxidant activity, with the best activity shown by ethyl acetate extract (IC_{50} 179.81±6.38 µg/mL). The following compounds dominated the extract were: L-(+)-ascorbic acid 2,6-dihexadecanoate, cholest-5-en-3-ol (3.beta.), o-xylene, 1,2-benzenedicarboxylic acid, 2-butoxy ethanol, and phytol. The presence of L-(+)-ascorbic acid 2,6-dihexadecanoate itself has never been reported in *Gracilaria* species. Meanwhile, molecular docking between the bioactive components of the extract against the NADPH Oxidase (NOX) protein showed an outstanding interaction. The compounds cholest-5-en-3-ol (3.beta.), 1,2-benzenedicarboxylic acid, and phytol had binding energies of -10.90, -7.11, and -6.22 kcal/mol, respectively. This analysis indicates that the presence of cholest-5-en-3-ol (3.beta.), 1,2-benzenedicarboxylic acid, and phytol compounds can be a candidate to be developed as chemotherapeutic agents. This result also shows the prospect of developing antioxidant compounds of non-phenolic to suppress ROS production by inhibiting NADPH oxidase (NOX) protein. Based on this research, *G. salicornia* extract can be developed as a source of chemotherapeutic agents.

4. MATERIALS AND METHODS

4.1 Chemicals and reagents

The solvents used in the maceration process were n-hexane, ethyl acetate, and methanol with technical grade and chloroform with chemical grade purity. Technical grade solvents were purified first through a distillation process before use. The antioxidant assay used methanol p.a and 2,2-diphenyl-1-picrylhydrazyl (DPPH) (obtained from Sigma Aldrich). The phytochemical assay used $FeCl_3$, $Pb(CH_3COO)_2$, Wagner, Meyer, Dragendorf and Lieberman Burchard reagent.

4.2 Collection of *G. salicornia* algae

The sample was taken from the Selayar Islands, South Sulawesi, Indonesia (5°54'59.45 "S, 120°26'43.98 "E). The sample was identified as *G. salicornia* based on National Research and Innovation Agency (Oceanography Laboratories) and morphological analysis based on Guiry & Guiry, 2020 [60].

4.3 Preparation of *G. salicornia* extract

Sample that has been taken was immediately washed using seawater several times to remove impurities then rinsed with distilled water and dried. After the drying process, the sample was then cut into pieces and crushed using a grinding machine. The crushed sample was then extracted by the graded maceration technique using n-hexane, chloroform, ethyl acetate, and methanol as solvents (ratio 1:8). The filtrates were collected and evaporated using a rotary evaporator to obtain concentrated extracts.

4.4 Phytochemical assay

The class of compounds that make up each extract was analyzed with phytochemical assay according to Harborne, (1998) [61]. The extracts were analyzed using phenolics, flavonoids, alkaloids, saponins, terpenoids, and steroids test.

4.4.1 Phenolic test

A sample of 1 mL was added with 1% $FeCl_3$ solution, then shaken and observed for color changes. The presence of phenolic compounds is characterized by the formation of green, red, purple, blue, or black colored solutions.

4.4.2 Flavonoid test

A sample of 1 mL was added with a few drops of $Pb(CH_3COO)_2$ solution and then observed the precipitate. The formation of a yellow precipitate indicates the presence of flavonoids compounds.

4.4.3 Alkaloid test

A sample of 1 mL was added with a few drops of Wagner, Meyer, or Dragendorf reagents, and then observed the precipitate formation. The presence of alkaloid compounds is characterized by the formation of brown precipitate with Wagner reagent, yellowish-white precipitate with Meyer reagent, and orange to brownish red precipitate with Dragendorf reagent.

4.4.4 Terpenoid and steroid test

A sample of 1 mL was added with a few drops of Lieberman Burchard reagent, and the color change was observed. A positive reaction indicates the presence of terpenoid if a red or purple solution is formed, while a green or blue solution indicates the presence of steroids.

4.4.5 Saponin test

A sample of 1 mL was added with hot distilled water and shaken vigorously. The presence of saponins is indicated by the formation of a stable foam with a height of 1-3 cm.

4.5 Antioxidant activity assay

The antioxidant activity of *G. salicornia* extracts were analyzed based on Chakraborty et al., (2019) [12] with minor modifications. The sample stock solution (500 µg/mL) was pipetted as much as 0.1, 0.2, 0.4, 0.8, and 1.6 mL, respectively, to make a series of concentrations. Then, 1 mL of 0.4 mM DPPH solution was added, and the volume was made up to 5 mL with methanol to make a mixture with a series of concentrations of 10, 20, 40, 80, and 160 µg/mL. The mixtures were incubated for 30 minutes at room temperature and protected from light exposure. The absorbance of the mixtures and control solution was measured using a UV-Vis spectrophotometer at the maximum wavelength of the DPPH solution. Antioxidant activity was expressed in IC_{50} obtained by linear regression equation from sample concentration against scavenging activity (%). The value of scavenging activity (%) was obtained through the following equation:

$$\text{Scavenging activity (\%)} = \frac{(A_0 - A_i)}{A_0} \times 100\%$$

Note, A_0 = Control absorbance
A_i = Sample absorbance

4.6 GC-MS analysis

The *G. salicornia's* extract with the best antioxidant activity was then analyzed the phytochemical using the GC-MS instrument (Shimadzu GC-MS 2010 brand Gas Chromatography-Mass Spectrometry plus). The column used is SH-Rxi-5Sil MS type (30 m x 0.25 mm) with FID detector (operated in EI mode at 70 eV). The ion source and interface temperatures are 200 °C and 280 °C, and solvent cut time is 3 minutes, 400-700 m/z. The sample was injected at an injector temperature of 250 °C with splitless mode, pressure 76.9 kPa, and flow rate 14 mL/min. The analysis was carried out with the column initial temperature of 70 °C, holding time 2 minutes, and the temperature was raised to 200 °C at a rate of 10 °C/min. The final temperature of the column is 280 °C, holding time is 9 minutes at a rate of 5 °C/min. The abundance of each compound was expressed in the relative area (%), and identification was made by comparing the retention time and mass spectra from the library (NIST and WILEY 9).

4.7 Molecular docking and ADME-TOX properties analysis

The chemicals identified in the GC-MS extract were used as ligands in the molecular docking analysis. The ligands structures were created and optimized by the MMFF94 method using Chem Draw Ultra Professional 15.0. The structures were saved in 3D with PDB format. The crystal structure of the NADPH oxidase (NOX) protein (PDB ID: 2CDU) as the target protein was downloaded from the Protein Data Bank (http://www.rcsb.org/-pdb) and removed from native ligands and water molecules using AutoDock Tools 1.5.6. Next, the docking parameters were set according to Abuelizz et al., (2017) [62] and set the grid box size at 40 x 40 x 40 Å, central grid point at 4.627, -0,555, 3.985 and spacing at 0.375 Å. This stage was carried out using AutoDock4 with the help of AutoDock Tools 1.5.6 [63]. The docking results were visualized using Discovery Studio Visualizer. However, the docking method was validated first. Validation was performed by comparing the native ligand's pose before and after redocking to obtain the RMSD value. Pharmacological and toxicological (ADME-TOX) properties analysis was also carried out. The SwissADME server

(http://www.swissadme.ch/) was used to analyze ADME properties to determine whether the chemicals had the potential to be drugs [64]. Toxicological properties were analyzed using Toxtree Version 3.1.0 [65–67].

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