Antioxidant and antibacterial compounds of *Gracilaria salicornia* from the coastal waters of Nain Island, Indonesia

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Abstract. Macroalga *Gracilaria salicornia* is used as a raw material for the preparation of agar, which has antioxidant and antibacterial activities. The aims of this research were to determine the antioxidant and antibacterial activities, as well as chemical compounds that act as antioxidants and antibacterial in *G. salicornia* from the coastal waters of Nain Island, North Sulawesi, Indonesia. In this study, *G. salicornia* was extracted by maceration method using 96% ethanol. The extract was partitioned successively with hexane, ethyl acetate and water. The extracts obtained were then subjected to phytochemical screening, analyzed for total phenolic content, antioxidant activity and antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*. The chemical compound content in the ethanol extract of *G. salicornia* was analysed by gas chromatography-mass spectrometry (GC-MS). The results showed that the ethanol extract and all partitioned fractions contained alkaloids, flavonoids, tannins, saponins and phenols, while triterpenoids were only found in the ethanol extract. The hexane fraction contained the highest total phenolics (39.64 mg GAE g^{-1} extract) and antioxidant activity (IC_{50} 1.38 mg mL^{-1}). The hexane fraction also had the highest antibacterial activity against *E. coli* (8.33 mm) and especially against *S. aureus* (15.33 mm). Based on the GC-MS analysis, there were 99 compounds identified in the ethanol extract. Among the 10 compounds with the highest concentration, the compounds having antioxidant activity were heptadecane; 2-pentadecanone, 6,10,14-trimethyl-; phytol and 17-(1,5-dimethylhexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H- cyclopenta[a]phenanthren-3-ol. Antibacterial compounds were: 9-nonadecene; hexadecanoic acid, methyl ester; trans-13-octadecenoic acid, methyl ester; phytol; diisooctyl phthalate; 17-(1,5-dimethylhexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-ol and cholest-4-en-3-one.

Key Words: ethanol, GC-MS, macroalgae, partition, phytochemicals.

Introduction. Macroalgae provide many benefits for human welfare. Utilization of macroalgae as raw material for medicines in the pharmaceutical industry (marine pharmacology) continues to be developed as a new and renewable economic resource. *Gracilaria salicornia* is one of the red macroalgae species useful as a raw material for agar preparation and safe for consumption (Vuai & Mpatani 2019). In North Sulawesi, Indonesia, *G. salicornia* grows in the coastal waters of Mantehage (Kepel et al 2019), in the Minahasa Peninsula in the rainy season (Kepel et al 2019) and in the dry season (Kepel et al 2020).

G. salicornia has been traditionally used by people in Ilocos Norte, Philippines to cure stomach aches, asthma, coughs, and diarrhea (Dumilag & Javier 2022). Several studies revealed that *G. salicornia* has antioxidant activity (Ghannadi et al 2016; Widowati et al 2021; Bahrun et al 2023) and antibacterial activity (Vijayavel & Martinez 2010; Rasooli et al 2015; Ramezanpour et al 2021). Antioxidant is essential in preventing oxidative damage caused by reactive oxygen and nitrogen species. Oxidative damage is responsible in the emergence of various diseases such as atherosclerosis, Alzheimer's disease, cancer (Forman & Zhang 2021) and diabetes mellitus (Yaribeygi et al 2020).

Antibiotics are medicines that prevent and heal diseases caused by bacterial infections (MacLean & Millan 2019). The irrational use of antibiotics, such as inappropriate dosage and administration time, can cause genetic changes in bacteria resulting in the emergence of antibiotic resistant bacteria. Thus, antibiotics become ineffective in killing bacteria, thus increasing medical costs and the risk of morbidity and mortality (Lestari et al 2018). Resistance to antimicrobial agents is one of the major threats to public health and global development, and directly contributed to 1.27 million global deaths in 2019 (WHO 2023). Developing new antibiotics from antibacterial compounds is a solution to prevent the emergence of antibiotic resistance (Geta 2019).

G. salicornia has potential to be developed as a medicinal raw material source to find new antibiotics, especially for the treatment of diseases caused by oxidative stress. This research aimed to determine the antioxidant and antibacterial activities, as well as chemical compounds that act as antioxidants and antibacterial in *G. salicornia* from the coastal waters of Nain Island, North Sulawesi, Indonesia.

Material and Method

Sample collection. This research was carried out from December 2022 to September 2023. The research location was in the coastal waters of Nain Island, Wori District, North Minahasa Regency (Figure 1). Macroalgae samples were collected from Nain Island waters, cleaned and then identified according to the thallus morphology by referring to the identification books of Calumpong & Meñez (1997), Trono (1997), Kepel & Baulu (2013). Afterwards, samples were put in plastic bags, stored in a cool-box and brought to the Laboratory of the Marine Biology, Faculty of Fisheries and Marine Science. Identification of antioxidant and antibacterial activities was carried out at the Pharmacy and Chemistry Laboratories, Faculty of Mathematics and Natural Sciences, Sam Ratulangi University.

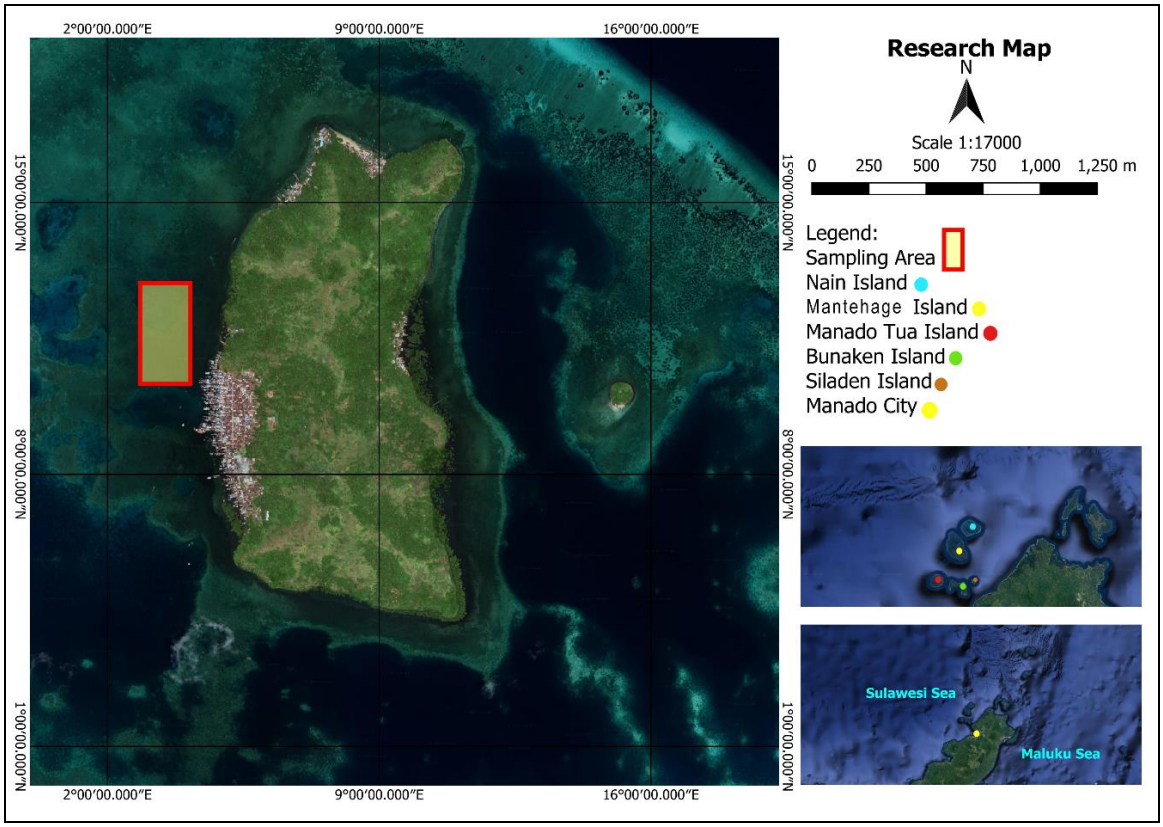

Figure 1. Map of research location

Morphological identification of macroalgae. Identification of *G. salicornia* from the coastal waters of Nain Island was carried out through observing its morphological characteristics. The thallus is cylindrical, smooth, segmented, forming dense clumps, height reaching up to 7 cm. The attachment apparatus is disc-shaped. Thallus branching is polystichous on the main thallus. The color of the thallus is yellowish green to orange (Figure 2). This alga grows in coral, rock and sandy habitats.

Figure 2. Macroalga *Gracilaria salicornia*.

Sample extraction. *G. salicornia* was cut into small pieces of about 1 cm, then air-dried for 6 days, blended and sieved with a 60-mesh sieve to obtain powder. A total of 200 g of powder was macerated with 1 L of 96% ethanol for 24 hours, then filtered with 15 µm pore size filter paper. Ethanol extract of *G. salicornia* was obtained by evaporating the solvent using a rotary evaporator. The ethanol extract obtained was then partitioned using hexane, ethyl acetate and water as solvents. Partitioning was carried out by dissolving 3 g of ethanol extract in 50 mL of water, which were put in a separating funnel, hexane was added, the mix was shaken and left until two layers were formed, water layer at the bottom and hexane layer at the top. The hexane layer was removed and evaporated using a rotary evaporator to obtain hexane fraction extract. Ethyl acetate was added to the water layer at the bottom, and processed again as in the previous procedures until ethyl acetate and water fractions were obtained. *G. salicornia* samples with four treatments, namely ethanol extract, hexane fraction extract, ethyl acetate fraction extract and water fraction extract were used for phytochemical screening, analysis of total phenolic content, antioxidant and antibacterial activity tests.

Phytochemical screening. Phytochemical screening of *G. salicornia* extract was carried out using the Harborne method (1998). Phytochemical analysis included the determination of alkaloids, phenolics, flavonoids, tannins, triterpenoids, saponins and steroids.

GC-MS analysis. GC-MS analysis of *G. salicornia* ethanol extract was carried out using HP-5MS UI column with 30 m length, maximum temperature of 325/350°C, and UHP helium (He) as gas carrier. Injector temperature was 230°C, split flow 50 mL min^{-1}, splitless time 1 minute front inlet flow 1.00 mL min^{-1}, MS transfer line temperature 250°C, ion source temperature 200°C, mass list range (amu) 40-500, and purge flow 3 mL min^{-1}.

Total phenolic content analysis. The total phenolic content in the extracts was analyzed following the Folin-Ciocalteu method (Jeong et al 2004), with modifications. 0.1 mL of 50% Folin-Ciocalteu reagent was added to a total of 0.1 mL of 1000 µg mL^{-1} sample extract, then vortexed for 3 min. 2 mL of 2% Na_2CO_3 solution were added to the mixture, which was then stirred and stored in a dark room for 30 min. The absorbance of the sample was read on a Shimadzu 1800 UV-Vis spectrophotometer with a wavelength of 750 nm. The total phenolic content was obtained by entering the sample absorbance value into the linear regression equation of the gallic acid calibration curve, and expressed as mg gallic acid equivalent (GAE) g^{-1} extract.

Antioxidant activity test. Antioxidant activity was analyzed by the DPPH method (Brand-Williams et al 1995). Each sample of *G. salicornia* extract (ethanol extract, hexane fraction extract, ethyl acetate fraction and water fraction) was dissolved in absolute ethanol to get various concentration of 0, 100, 200, 300, 400 and 500 µg mL^{-1}. Next, 0.25 mL of sample was added to 2 mL of 1,1-diphenyl-2-picrylhydrazyl (DPPH) 91.3 µM solution in absolute ethanol, vortexed and incubated at 37°C for 30 min. The absorbance of the sample was read on a Shimadzu 1800 UV-Vis spectrophotometer with a wavelength of 517 nm. The same procedure was carried out for the control (absolute ethanol). The change in solution color from purple to yellow indicated the efficiency of the radical antidote. Radical scavenging activity (RSA) was calculated as the percentage reduction in DPPH concentration, with the equation (Chen et al 2013):

$$\%\text{RSA} = \left(\frac{\text{control absorbance} - \text{sample absorbance}}{\text{control absorbance}}\right) x\ 100$$

Antioxidant activity was expressed as the concentration of antioxidant required to reduce 50% of the initial DPPH concentration or control (inhibition concentration 50%, IC_{50}). The IC_{50} value was calculated from the linear regression equation between sample concentration (x-axis) and %RSA (y-axis), with a value of y=50%.

Antibacterial activity test. Antibacterial activity of *G. salicornia* extract against gram-positive *S. aureus* and gram-negative *E. coli* bacteria was determined using the well diffusion method (Marfuah et al 2018). Distilled water was used as a control. The diameter of the clear zone formed around the well was measured using a caliper.

Results and Discussion

Phytochemical screening. *G. salicornia* contained secondary metabolite compounds of alkaloids, flavonoids, tannins, saponins and phenolics in the ethanol extract as well as in the hexane, ethyl acetate and water fractions. Triterpenoid compounds were only found in the ethanol extract (Table 1). Research conducted by Mantiri et al (2021) did also not find steroid compounds in the red algae *Halymenia durvillei*.

Table 1

Secondary metabolites of *Gracilaria salicornia* extract

Secondary metabolites	*G. salicornia*			
	Ethanol	*Hexane*	*Ethyl acetate*	*Water*
Alkaloid				
Dragendorff's reagent	+	+	+	+
Wagner's reagent	+	+	+	+
Meyer's reagent	+	+	+	+
Phenolics	+	+	+	+
Flavonoids	+	+	+	+
Tannins	+	+	+	+
Triterpenoids	+	-	-	-
Saponins	+	+	+	+
Steroids	-	-	-	-

Note: +: detected; -: undetected.

Several research reports showed that the profile of secondary metabolite content in *G. salicornia* extract is diverse. Julyasih (2022) reported that ethanol extract of *G. salicornia* from Sanur Beach, Bali, Indonesia contained phenolics, flavonoids and terpenoids, and did not contain steroids, alkaloids or saponins. Methanol extract of *G. salicornia* from the Persian Gulf, Iran contained alkaloids, flavonoids, tannins, triterpenoids, saponins and steroids (Ghannadi et al 2016), while from Tidung Island Coastal Region, Indonesia, the extract contained flavonoids, saponins and steroids (Widowati et al 2021).

Furthermore, hexane and ethyl acetate extracts of *G. salicornia* from Selayar Island, Indonesia, contained alkaloids, phenolics, flavonoids and steroids (Bahrun et al 2023). Flavonoids were present in all *G. salicornia* extracts previously reported. Differences in secondary metabolite content of *G. salicornia* extracts are caused by the environmental conditions and the type of solvent used in the extraction process. In addition, changes in temperature and radiation influence the phytochemical content of *G. salicornia* (Narrain et al 2023). The biochemical composition of *Gracilaria* is also influenced by seasonal changes (Aroyehun et al 2019).

GC-MS analysis. There were 99 chemical compounds identified in *G. Salicornia* ethanol extract (Figure 3). Based on the percent relative area of each peak, the 10 compounds having the highest cncentration in the ethanol extract of *G. salicornia* are presented in Table 2.

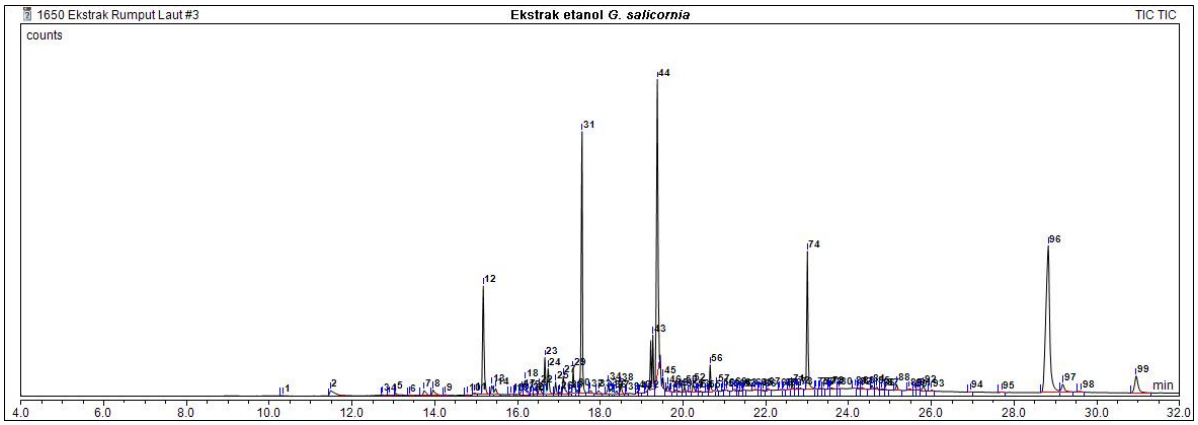

Figure 3. GC-MS chromatogram of *Gracilaria salicornia* ethanol extract.

The concentration of the more common compound in the ethanol extract of *G. salicornia* was 17-(1,5-Dimethylhexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15, 16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-ol or cholest-5-en-3-ol with a relative area of 23.12%, followed by phytol compounds (18.08%) and hexadecanoic acid, methyl ester (13.13 %) (Table 2). The presence of cholest-5-en-3-ol and phytol compounds in the ethanol extract of *G. salicornia* from Nain Island, North Sulawesi in this study supported the research results of Bahrun et al (2021, 2023). Chloroform extract of *G. salicornia* from the Selayar Islands, South Sulawesi, Indonesia contained cholest-5-en-3-ol (14.18%) and phytol (2.41%) (Bahrun et al 2021), while the ethyl acetate extract contained cholest-5-en- 3-ol (11.85%) and phytol (3.45%) (Bahrun et al 2023). The composition of cholest-5-en-3-ol and phytol compounds in this study was higher than that in the two previous studies. The different composition of cholest-5-en-3-ol and phytol compounds in *G. salicornia* extracts between this study and that of Bahrun et al (2021, 2023) was caused by differences in polarity level of the solvents used, namely ethanol, ethyl acetate and chloroform. Ethanol had the highest level of polarity, followed by ethyl acetate and chloroform. Different environmental conditions where *G. salicornia* grows, namely the waters of North Sulawesi and South Sulawesi, influence the chemical composition of this alga.

Table 2

Major compounds in *Gracilaria salicornia* ethanol extract identified through GC-MS

No.	*Retention time (minute)*	*Relative Area (%)*	*Compound name*	*Chemical formula*	*Pharmacology*	*PubChem CID*
12	15.17	6.49	Heptadecane	$C_{17}H_{36}$	Anti-inflammatory, antioxidant (Kim et al 2013)	12398
18	16.18	1.76	9-Nonadecene	$C_{19}H_{38}$	Anti-microbial, anti-fungi (Yu et al 2010)	5364436
23	16.66	1.85	Neophytadiene	$C_{20}H_{38}$	Anti-inflammatory (Bhardwaj et al 2020),	10446
24	16.74	1.71	2-Pentadecanone, 6,10,14-trimethyl-	$C_{18}H_{36}O$	Antioxidant, larvicidal against *Anopheles* larva (Morah & Uduagwu 2017)	10408
31	17.56	13.13	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	Antibacterial (Shaaban et al 2021)	8181
43	19.27	4.00	trans-13-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	Anti-inflammatory (Khan et al 2022) Anti-microbial (Adepoju et al 2021)	5364506
44	19.38	18.08	Phytol	$C_{20}H_{40}O$	Antioxidant, anti-microbial, anxiolytic, autophagy and apoptosis inducer, anti-inflammatory, immune modulator (Islam et al 2018)	5366244
74	23.00	7.25	Diisooctyl phthalate	$C_{24}H_{38}O_4$	Anti-microbial, insecticidal (Huang et al 2021)	33934
96	28.82	23.12	17-(1,5-Dimethylhexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-ol	$C_{27}H_{46}O$	Antioxidant, anticancer, anti-inflammatory (AlAmery & AlGaraawi 2020) Antibacterial (Bahrun et al 2021)	304
99	30.95	2.45	Cholest-4-en-3-one	$C_{27}H_{44}O$	Antibacterial, antiobesity, anti-diabetic, anti-cancer (Vasanthakumar & Kuppusamy 2022)	91477

Total phenolic content. Hexane fraction of *G. salicornia* extract (39.64 mg GAE g^{-1} extract) quantitatively contained the highest total phenols, followed by ethyl acetate fraction (25.15 mg GAE g^{-1} extract), ethanol extract (24.90 mg GAE g^{-1} extract) and water fraction (21.44 mg GAE g^{-1} extract) (Figure 4). Total phenolic content was not significantly different between ethyl acetate fraction and ethanol extract.

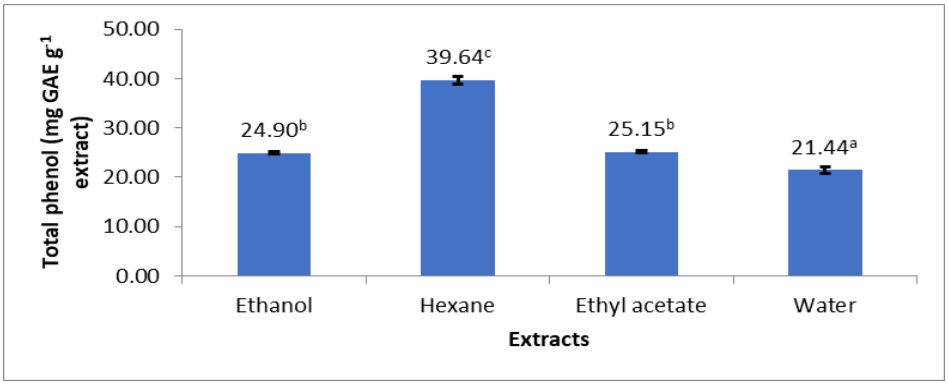

Figure 4. Total phenolic content of *Gracilaria salicornia* extract; values with different superscripts are significantly different ($p<0.05$).

The total phenolic content of *G. salicornia* in various types of solvents had been reported by several previous studies. In *G. salicornia* methanol extract, the total phenolic content was 0.012 mg GAE g^{-1} dry extract in the study of Ghannadi et al (2016), 0.015 mg GAE g^{-1} dry extract in the study of Sanger et al (2019) and 0.051 mg GAE g^{-1} dry extract was obtained by Ramezanpour et al (2021). Total phenolic content in ethanol extract was 0.14 mg GAE g^{-1} dry extract (Vijayavel & Martinez 2010) and 0.044 mg GAE g^{-1} dry extract (Sanger et al 2018). Total phenolic content in acetone extract was 0.072 mg GAE g^{-1} dry extract (Sanger et al 2018). Total phenolic content in water extract was 0.073 mg GAE g^{-1} dry extract (Ramezanpour et al 2021). In this study, the total phenolic content of *G. salicornia* was greater than previously reported in other studies.

Antioxidant activity. The antioxidant activity of *G. salicornia* extract was expressed in IC_{50} value (Figure 5). The IC_{50} value is negatively correlated with antioxidant activity. A smaller IC_{50} value means a greater antioxidant activity. The hexane fraction of *G. salicornia* extract tended to have greater antioxidant activity than that of ethanol extract. The IC_{50} values of ethyl acetate and water fraction were relatively small. The lowest antioxidant activity was observed in the water fraction.

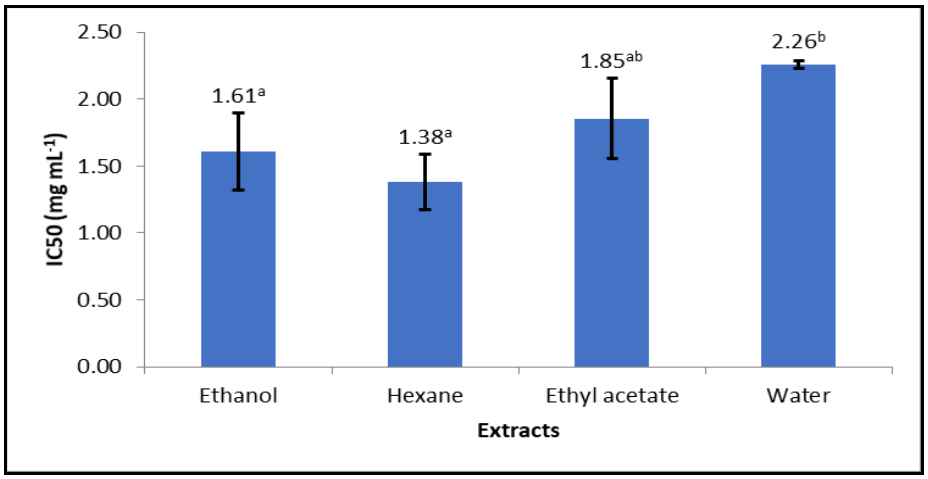

Figure 5. Antioxidant activity of *Gracilaria salicornia* extract; values with different superscript are significantly different ($p<0.05$).

The antioxidant activity of *G. salicornia* extract was positively correlated with its total phenolic content (Figures 4 and 5). The hexane fraction had an antioxidant activity relatively higher than other extracts. This hexane fraction also contained the highest total phenols. Phenolic compounds have antioxidant activity due to their ability to react with free radicals, either through hydrogen atom transfer, single electron transfer, or transition metal chelation (Zeb 2020). Several studies regarding the antioxidant activity of *G. salicornia* with various solvents using the DPPH method are presented in Table 3. Each extract has a different IC50 value.

Table 3

Antioxidant activities of *Gracilaria salicornia* extract using various solvents

Solvent	*IC_{50} (mg mL^{-1})*	*Location*
Acetone	1.24	*G. salicornia* Arakan, North Sulawesi, Indonesia (Sanger et al 2018)
Ethanol	8.01	*G. salicornia* Arakan, North Sulawesi, Indonesia (Sanger et al 2018)
	2.88	*G. salicornia* coast of North Sulawesi, Indonesia (Sanger et al 2021)
Ethyl acetate	0.18	*G. salicornia* Selayar Islands, South Sulawesi, Indonesia (Bahrun et al 2023)
	0.73	*G. salicornia* Persian Gulf, Iran (Ghannadi et al 2016)
Methanol	5.6	*Gracilaria* sp., Johor Bahru, Malaysia (Assaw et al 2018)
	12.81	*G. salicornia* Nain Island, North Sulawesi, Indonesia (Sanger et al 2019)
	119.7	*G. salicornia* Persian Gulf, Iran (Ramezanpour et al 2021)

Based on GC-MS analysis, the presence of antioxidant activity in *G. salicornia* extract was caused by its antioxidant compounds (Table 2). Phytochemical compounds in *G. salicornia* extract identified as having antioxidant activity included heptadecane (Kim et al 2013), 2-pentadecanone, 6,10,14-trimethyl- (Morah & Uduagwu 2017), phytol (Islam et al 2018), and 17 -(1,5-dimethylhexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-ol (AlAmery & AlGaraawi 2020).

Antibacterial activity. The extract of *G. salicornia* had antibacterial activity against *E. coli* and *S. aureus* (Table 4). Antibacterial activity was indicated by the presence of a clear zone around the bacterial colony. Table 4 showed that the hexane fraction extract had the highest inhibitory zone diameter compared to ethanol extract, ethyl acetate fraction and water, both against *S. aureus* and *E. coli*. The hexane fraction had better sensitivity against *S. aureus* than *E. coli*.

Table 4

Antibacterial activity of *Gracilaria salicornia* extract against *S. aureus* and *E. coli*

Treatments	*Diameter of inhibition zone (mm)*	
	S. aureus	*E. coli*
control	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
Ethanol	8.00 ± 0.00^{b}	7.67 ± 0.29^{cb}
Hexane	15.33 ± 0.29^{d}	8.33 ± 0.29^{d}
Ethyl acetate	8.50 ± 0.00^{c}	7.50 ± 0.00^{b}
Water	8.17 ± 0.29^{bc}	8.00 ± 0.00^{cd}

Note: different superscripts indicate significant differences ($p<0.05$).

G. salicornia extract is able to inhibit the growth of *S. aureus* and *E. coli*. The highest antibacterial activity was observed in the hexane fraction. Hexane is a non-polar solvent that can dissolve non-polar compounds present in *G. salicornia* extract. This finding was similar with that of Saeidnia et al (2009), where the antibacterial activity of *G. salicornia* extract was greater in ethyl acetate extract than in methanol extract. *G. salicornia* ethyl acetate extract was more sensitive to *S. aureus* than *E. coli*. Ethyl acetate is more non-polar than methanol. This research finding indicates that the hexane fraction extract has the potential to be developed as an antibiotic.

The ability of *G. salicornia* extract to inhibit the growth of bacteria is caused by the antibacterial compounds contained in the extract (Table 4). Phytochemical compounds in *G. salicornia* extract identified as having antibacterial activity included 9-nonadecene (Yu et al 2010), hexadecanoic acid, methyl ester (Shaaban et al 2021), trans-13-octadecenoic acid, methyl ester (Adepoju et al 2021), phytol (Islam et al 2018), diisooctyl phthalate (Huang et al 2021), 17-(1,5-dimethylhexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11, 12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-ol (Bahrun et al 2021), and cholest-4-en-3-one (Vasanthakumar & Kuppusamy 2022).

Conclusions. *G. salicornia* extract contained secondary metabolites namely alkaloids, flavonoids, tannins, saponins and phenols in ethanol and in hexane extracts, ethyl acetate and water fractions as well. Triterpenoids was only found in the ethanol extract. Each extract had antioxidant activity. The hexane fraction presented the highest total phenolics (39.64 mg GAE g^{-1} extract) and antioxidant activity (IC_{50} 1.38 mg mL^{-1}). Hexane fraction also had the highest antibacterial activity against *E. coli* (8.33 mm) and especially against *S. aureus* (15.33 mm). Based on the GC-MS analysis, there were 99 compounds identified in the ethanol extract. Among the 10 compounds with the highest concentration, there were 4 compounds having antioxidant activity, namely heptadecane; 2-pentadecanone, 6,10,14-trimethyl-; phytol and 17-(1,5-dimethylhexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-ol, and 7 antibacterial compounds, namely 9-nonadecene; hexadecanoic acid, methyl ester; trans-13-octadecenoic acid, methyl ester; phytol; diisooctyl phthalate; 17-(1,5-dimethylhexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-ol and cholest-4-en-3-one.

Conflict of Interest. The authors declare that there is no conflict of interest.

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