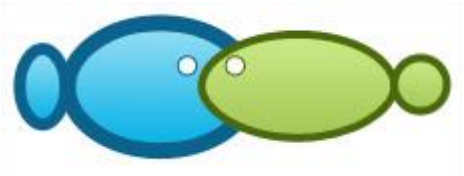

Phytochemical screening, antioxidant and anti-inflammatory activities of green macroalga *Valonia aegagropila* from coastal waters of Kora-kora, North Sulawesi, Indonesia

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Abstract. The objectives of this research were to screen the phytochemical compounds of *Valonia aegagropila* and analyze their antioxidant and anti-inflammatory activities. Samples of algae were collected from the intertidal zone of Kora-kora Beach by exploration method. Algae samples were extracted by maceration method using 96% ethanol. Phytochemical screening was carried out by qualitative and quantitative methods while bioactive substances were analyzed by gas chromatography-mass spectrometry (GC-MS). Antioxidant activity was analyzed using the DPPH method, while anti-inflammatory activity was carried out *in vivo* on the blood hematocrit of experimental animals. Research results showed ethanol crude extract of green alga *V. aegagropila* contained alkaloids, saponins, tannins, flavonoids and phenolics but steroids were not detected. The highest concentration was for phenolics, followed by flavonoids, tannins, alkaloids and saponins respectively. Based on the GC-MS analysis, there were 49 compounds identified in the ethanol extract; antioxidant, and anti-inflammatory activity is obtained from the 10 active compounds with the highest concentration. *V. aegagropila* had very strong antioxidant activity with IC_{50} of 21.31541 and moderate anti-inflammatory activity with IC_{50} of 136.41951.

Key Words: active compounds, GC-MS, pharmacologic activity, seaweed.

Introduction. Green algae are eukaryotic organisms that can photosynthesize, as they contain chlorophyll a and b, carotenoid pigments, and bioactive compounds (Pereira 2021; Mantiri et al 2021). In general, green algae have a complex thallus structure, from single cells to filaments, colonies, and various levels of tissue organization as well as branching morphology. Unicells were round to elongated, with or without flagella, scales, walls or other coverings (Lewis & McCourt 2004). *Valonia* spp. is a coenocytic green algae belonging to the Siphonocladales order. Its thallus consists of several very large cells or one giant cell (Satoh et al 2000).

According to Pereira (2021), *Valonia* spp. is distributed globally in the Northeast Atlantic, Adriatic Sea, Mediterranean, East Atlantic, and tropical and subtropical areas include the Philippines, Vietnam, and Indonesia. In the intertidal zone of Mantehage Island, North Sulawesi, Indonesia, Kepel et al (2019) identified 45 different seaweed species including Rhodophyta (Galaxauraceae and Gracilariaceae), Phaeophyta (Dictyotaceae, Scytosiphonaceae and Sargassaceae), and Chlorophyta (Caulerpaceae, Halimedaceae, and Valoniaceae such as *Valonia fastigiata* and *Valonia aegagropila*). In Minahasa Peninsula coastal waters, Tombokan et al (2020) found 19 species of macroalgae such as *Valonia aegagropila*.

The distribution of green macroalgae in diverse environments caused these organisms to contain many different active compounds (Ganesan et al 2019). Bioactive compounds such as alkaloids, flavonoids, phenolics, tannins, terpenoids and steroids have antioxidant and anti-inflammatory potential so that they can be used in pharmaceutical industries (Palaniyappan et al 2023). Rabecca et al (2020) reported green alga *Codium tomentosum* contains alkaloids, phenolics, flavonoids and tannins. Another research conducted through membrane stabilization and protease inhibition tests showed this species had anti-inflammatory activity.

Phytochemical analysis on red alga *Halymenia durvillei* showed this species contain phytochemical compounds such as alkaloid, flavonoid, tannin, saponin, triterpenoid and phenolic and has antioxidant activity of 39.25 µg/ml due to the phycoerythrin pigment which existed on the red algae (Mantiri et al 2021). Moreover, Rumengan and Mantiri (2015) reported that extract of green alga *Dictyosphaeria cavernosa* also has antioxidant activity.

Some algae were previously reported to contain a variety of compounds having phytochemical, antioxidant and anti-inflammatory activities. To the best of our knowledge, *Valonia aegagropila* has never been studied, therefore the aims of this research were to screen phytochemical compounds of this species and analyze their antioxidant and anti-inflammatory activities.

Material and Method

Research location. This research was conducted in September 2023 on the coastal area of Kora-kora Beach, East Lembean District, Minahasa Regency at coordinates 1°09'22.2"N 125°01'32.3"E (Figure 1). This location was selected because various types of algae including green algae grow abundantly in this area. Samples of green macroalgae were gathered using the exploration method. The collected samples were put in plastic bags, stored in a cool box and then brought to the Laboratory of Molecular Biology, Faculty of Fisheries and Marine Sciences. After cleaning, samples were identified morphologically by referring to the identification manuals of Calumpong and Meñez (1997), Trono (1997) and Kepel and Baulu (2013). Analysis of antioxidant and anti-inflammatory activities were carried out at the Pharmacy Laboratory, Faculty of Mathematics and Natural Sciences, Sam Ratulangi University.

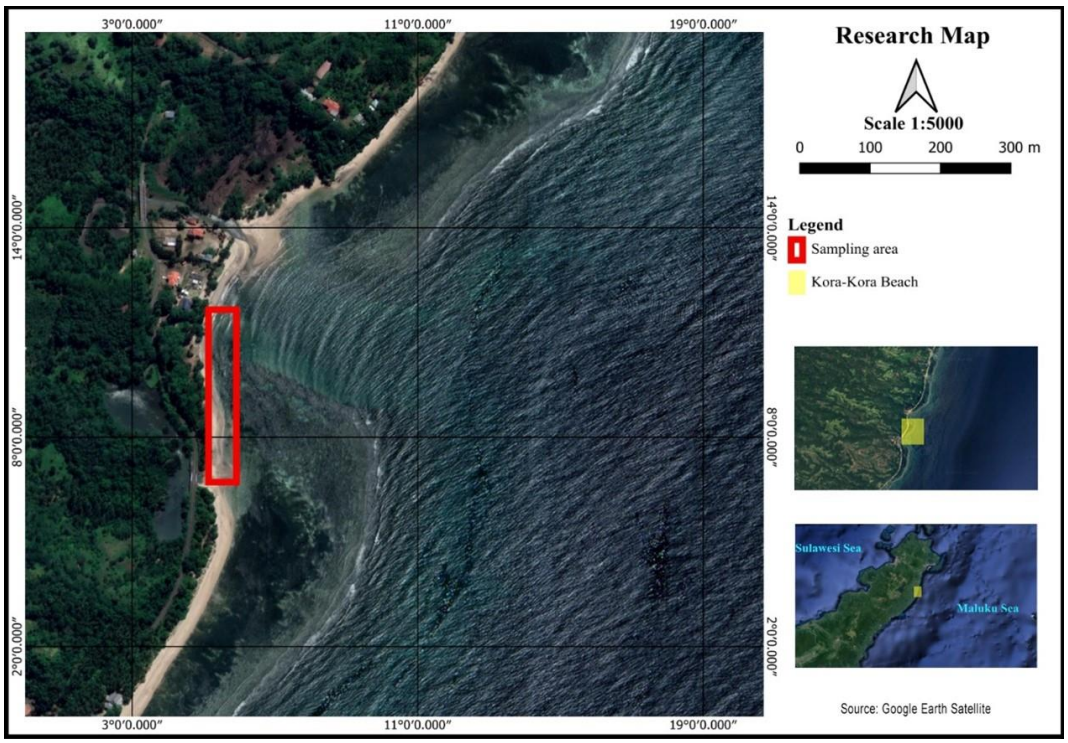

Figure 1. Research site (map generated using Google Earth).

Sample extraction. Macroalgae samples were cut into small pieces and dried in an electric oven at room temperature for 6 days. After drying, samples were ground and sieved with a 40-mesh sieve to obtain a homogenous powder. Furthermore, as much as 10 g of powder was placed into a closed vessel, then 150 mL of 96% ethanol was added until all the powder was submerged. The soaking process was carried out for 3 days with several stages. In the first 24 hours, all the filtrate was removed, put into a container and placed in the refrigerator. Then, 150 mL of 96% ethanol was added again into powder debris. After 24 hours, the filtrate was taken and combined with the first filtrate. Debris was re-added again for third time with 150 mL of 96% ethanol and soaked for 24 hours. The filtrate formed was taken and combined with the first and second filtrates. All the collected filtrates were concentrated using a rotary evaporator at a temperature of 55°C then placed in an oven at a temperature of 40°C until a thick crude extract was obtained. The crude extract obtained was then stored in a freezer at -20°C (Kanjana et al 2011). This crude extract was then used for identification of the bioactive compounds content using gas chromatography-mass spectrometry (GC-MS).

Phytochemical screening. Samples of algae were cut into small pieces and then soaked for 24 hours in 96% ethanol. After immersion, the extract was filtered and evaporated with a rotary vacuum evaporator at 45°C to reduce the water content until a thick extract was obtained. Phytochemical screening included alkaloids, flavonoids, tannins, saponins, phenolics, triterpenoids and steroids analyzed through qualitative and quantitative methods following the procedure of Harborne (1987). Qualitative phytochemical analyses were conducted using 6 samples of 50 mg each for the following tests: alkaloid test, flavonoid test, tannin test, saponin test, steroid and triterpenoid test and phenolics test. Quantitative phytochemical analyses were conducted for tannin content (0.5 g sample extract), phenol content (100 ml sample extract), flavonoid content (100 mg sample extract), alkaloid content (10 mg sample extract) and saponin content (10 mg sample extract).

Gas chromatography-mass spectrometry (GC-MS) analysis. Gas chromatography-mass spectrometry (GC-MS) analysis of *Valonia aegagropila* 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. The injector temperature was 230°C, split flow 50 mL min^{-1}, split less 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}. One sample of 5 g of *V. aegagropila* sample extract was analyzed.

Antioxidant activity test. Antioxidant activity was measured using DPPH radical method using 0.1 mM (1,1-diphenyl-2-picrylhydrazyl). DPPH powder was dissolved in methanol (p.a). Blank solution, 2 mL of 0.15 mM DPPH, was put into a test tube, then added with 2 mL of methanol, stirred until homogeneous, incubated in a dark room for 30 minutes, and then absorbed using a UV-Vis spectrophotometer at a wavelength of 517 nm.

Five extract samples were analyzed. Alga extract was dissolved in methanol to get concentrations of 10, 20, 30, 40 and 50 ppm. Afterward, a total of 0.25 mL of each sample solution 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 minutes. The absorbance of the sample was read on a Shimadzu 1800 UV-Vis spectrophotometer at a wavelength of 517 nm. The same procedure was carried out for the control.

Free radical scavenging activity is expressed as IC_{50} (inhibitory concentration value). The smaller the IC_{50} value, the higher the antioxidant activity. Radical scavenging activity expressed in inhibition percentage was calculated using the following formula (Meyer et al 1982):

$$\% \text{ Inhibition} = \frac{(C-D)-(A-B)}{(A-B)} \mathbf{X}\ 100$$

where: A = sample absorbance, B = absorbance control sample, C = absorbance of negative control, D = absorbance blank.

Anti-inflammatory test. Anti-inflammatory test of *Valonia aegagropila* extract was carried out *in vivo* by taking blood sample from mice as test animals. The ethical guidelines of animal care and use were followed during the experiment and were approved by the Research Committee of our institutions (approval Description of Ethical Approval No. 015/EC/KEPK-KANDOU/I/2024). A total of 5 (five) Wistar mice were prepared and acclimatized for seven days.

The phases of the anti-inflammatory test consisted of animal preparation, blood sampling (hematocrit cells), production of hematocrit cell suspensions and production of a solution that includes a pH 7.4 (0.15 M) phosphate damp solution, an isosaline solution, a hyposaline solution, the concentration of sample extract and sodium diclofenac. A total of five Wistar mice were anesthetized first, followed by hematocrit cells taken from the retro-orbital sinus of the eye. Each 3 mL of blood was inserted into 5 EDTA tubes and centrifuged at a speed of 3000 rpm for 15 minutes at room temperature. The supernatants formed were separated, then the residues were moved into the centrifugation tube, and the isosalin solution was added and re-centrifugated. The volume of the hematocrit cells was measured and resuspended with isosaline solution until a suspension was obtained with a 10% concentration, then stored at a temperature of 40^{o}C.

Analysis of anti-inflammatory activity against stabilization of erythrocyte membranes of Wistar rats had three stages, namely: 1) treatment groups, divided into 5 groups based on the concentration of extracts used, i.e. extract 50, 100, 150, 200 and 250 ppm. To each group 0.5 ml of hematocrit cells were added, 1 mL of phosphate buffer, 1 mL of extract solution, and 2 mL of hyposaline solution; 2) a positive control solution was made by mixing 0.5 mL hematocrit cell suspension, 1mL pH phosphatic acid pH 7.4 (0.15 M), 1 mL of Na diclofenac solution and 2 mL of hyposaline solution; 3) the negative control solution contained 0.5 mL hematocrit cell suspensions, 1 mL pH 7.4 (0.15.M) phosphatic acid, 1 mL isosaline solution, 2 mL hyposaline solution. The solution was incubated at 37°C for 30 minutes and centrifuged at a speed of 5000 rpm for 10 minutes. The superfluid obtained was measured for its absorption with UV-Vis spectroscopes at a wavelength of 577 nm. The value of IC_{50} *V. aegagropila* extract was calculated by making a linear regression equation between concentration (X) and % inhibition (Y).

Results and Discussion

Identification of macroalgae. Macroalga used in this study has a transparent thallus with yellow green to old green and are tightly arranged. Thalli consists of large vesicular clavicular segments of 3-13 mm in length and 2-4 mm in diameter. The alga also has adhesive devices with irregular bonding and vesicles that stick to each other. The vesicles are slightly narrowed in the base and are structured with two to five small vesicles that appear from the top of the stem vesicle. This macroalga is found in shallow waters, sticking to a hard substrate between the mounds, forming clusters or spreading plates. Morphological identification revealed that this species is *Valonia aegagropila* (Figure 2).

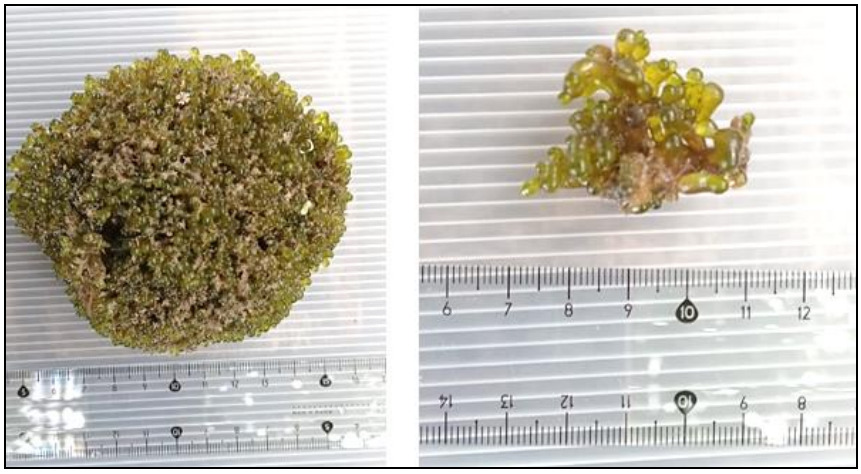

Figure 2. *Valonia aegagropila* (original image).

Valonia aegagropila is a green macroalga that contains bioactive compounds useful for the biopharmaceutical industry. The ability of this species to produce bioactive compounds is influenced by environmental factors, especially good air quality.

Phytochemical screening. *Valonia aegagropila* extracted with ethanol contains alkaloid secondary metabolite compounds, flavonoids, tannins, saponins and phenolics. Steroid and triterpenoid compounds were not found in ethanol extracts (Table 1). Likewise, research conducted by Mantiri et al (2021) also found no steroid compounds in the ethanol extract of red algae *Halymenia durvillei*.

Table 1

Phytochemical compounds of *Valonia aegagropila*

Compound types	*Reagent*	*Qualitative*	*Quantitative (mg/g)*
Alkaloid	Dragendorf	+	0.8914
	Wagner	+	
	Meyer	+	
Flavonoid	Mg, HCl	+	1.1341
Tannin	$FeCl_3$	+	1.1154
Saponin	HCl	+	0.1400
Steroid	CH3COOH, HCl	-	-
Triterpenoid	CH3COOH, HCl	-	-
Phenolic	$FeCl_3$	+	2.9698

Qualitative and quantitative phytochemical screening showed that *V. aegagropila* contains alkaloids as much as 0.8914 mg/g, flavonoids 1.1341 mg/g, tannins 1.1154 mg/g, saponins 0.1400 mg/g and phenolics 2.9698 mg/g. Phenolic compound is the most abundant phytochemical on *V. aegagropila,* followed by flavonoid, tannin, alkaloid and saponin (Table 1).

Another study related to green algae revealed this green alga was rich in phytochemical compounds. Research by Windyaswari et al (2020) showed *Ulva lactuca* has phytochemical compound including alkaloids, flavonoids and mono-sesquiterpenoids. Tarigan et al (2023) reported *Ulva reticulata* has alkaloids and steroids/terpenoids, flavonoids, saponins, tannins and phenolics. Palaniyappan et al (2023) reported green alga *Caulerpa racemosa* contains phenolics with antioxidant activity, tannins with anti-inflammatory potential and flavonoids with both antioxidant and anti-inflammatory activities. *V. aegagropila* was also expected to have antioxidant and anti-inflammatory activities due to the presences of phytochemical compounds. Aroyehun et al (2020) confirmed *V. aegagropila* contains flavonoids and phenolic acids which are natural antioxidants that function as reducing agents or inhibitors of peroxide radicals such as reactive oxygen species dangerous for human cells. The effectiveness of these natural

antioxidants in biological systems is measured by the inhibition of oxidation through lipid peroxidation or their ability to bind free radicals.

Gas chromatography-mass spectrometry (GC-MS) analysis. GC-MS chromatogram from ethanol extract of *V. aegagropila* showed 49 peaks (Figure 3) with 10 active compounds having the highest concentration. The composition of phytochemical compounds on *V. aegagropila* determined through GC-MS analysis was shown in Figure 3 while compounds having the highest concentration based on the relative area percentage were presented in Table 2.

The selection of 10 active compounds is based on the percentage of the relative area and retention time. Retention time is a qualitative identification tool of a targeted active compound. This parameter is used to determine the type of active compound based on existing references (Nugraha & Nandiyanto 2021). Based on Nichols (2024), the relative percentage of area indicated the magnitude of the concentration of active compounds in the analyzed sample. The active compounds analysis using GC-MS in this study showed the content of 3,7,11,15-Tetramethyl-2-hexadecen-1-ol and phytol. The highest acetate with a relative area of 21.69% found at peak 31, followed by compounds at peak 23, namely Hexadecanoic acid, ethyl ester, Pentadecanoic acid, ethyl ester and Hexadecanoic acid, 2-methyl-, methyl ester.

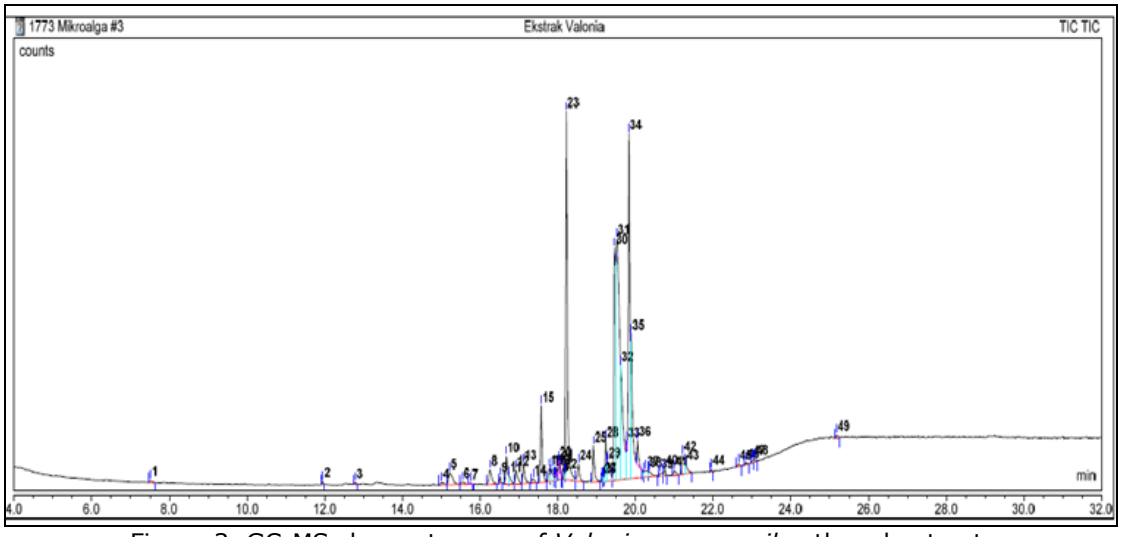

Figure 3. GC-MS chromatogram of *Valonia aegagropila* ethanol extract.

Table 2

Major compound in *Valonia aegagropila* ethanol extract identified through GC-MS with pharmacologic activity

No	*Compound name*	*Group of bioactive compounds*	*Peak*	*Ret. time (min)*	*Ret. area (%)*	*Chemical formula*	*PUB CHEM CID*	*Pharmacology activity*
1	Phytol	Diterpenoid	31	19.53	21.69	$C_{20}H_{40}O$	5280435	Antioxidant, antinociceptive (Santos et al 2013)
2	3,7,11,15-Tetramethyl-2-hexadecen-1-ol		31	19.53	21.69	$C_{20}H_{40}O$	5366244	Anti-inflammatory, anti-microbial, anti-diabetic (Taj et al 2021)
3	Hexadecanoic acid, ethyl ester	Fatty acid ester	23	18.22	14.76	$C_{18}H_{36}O_2$	12366	Anti-inflammatory, anti-microbial (Pucot et al 2021)
4	Pentadecanoic acid, ethyl ester		23	18.22	14.76	$C_{17}H_{34}O_2$	38762	Immunomodulation antioxidant (Watson & Butterworth 2022)

5	(E)-9-Octadecenoic acid, ethyl ester	34	19.83	13.78	$C_{20}H_{38}O_2$	5364430	Antioxidant & anti-microbial (Adeyemo et al 2021)
6	Ethyl oleate	34	19.83	13.78	$C_{20}H_{38}O_2$	5363269	Antifungal (Abubacker & Devi 2014)
7	cis-9-Octadecenoic acid, propyl ester	35	19.88	9.74	$C_{21}H_{40}O_2$	5356106	Antioxidant, anticholinesterase (Nazir et al 2021)
8	Methyl stearate	30	19.46	8.84	$C_{19}H_{38}O_2$	8201	Anti-inflammatory (Baeshen et al 2023)
9	Heptadecanoic acid, 16-methyl-, methyl ester	30	19.46	8.84	$C_{19}H_{38}O_2$	110444	Antioxidant (Mazumder et al 2020)
10	Pentadecanoic acid, methyl ester	15	17.57	3.27	$C_{16}H_{32}O_2$	23518	Antioxidant (Sulieman & Ibrahim 2022)

Based on Table 2, the active compounds from the ethanol extract of the *V. aegagropila* analyzed by GC-MS are classified into 2 groups of secondary metabolites, namely diterpenoids and fatty esters. Active compounds in the diterpenoid group such as phytol and 3,7,11,15-Tetramethyl-2-hexadecen-1-ol, phytol is assumed to have pharmacological activities such as antioxidant, anti-inflammatory, antimicrobial, antinociceptive and anti-diabetic. According to Taj et al (2021) and Santos et al (2013), the identification of active compounds with GC-MS shows a high phytol content in this green alga. Phytol is a diterpenoid compound with a branched chain of unsaturated alcohol that can function as an antioxidant by producing enzymes to suppress oxidative stress.

Meanwhile, the other 8 compounds are Hexadecanoic acid, ethyl ester; Pentadecanoic acid, ethyl ester; (E)-9-Octadecenoic acid ethyl ester; ethyl oleate; cis-9-Octadecenoic acid, propyl ester; methyl stearate; Heptadecanoic acid, 16-methyl-, methyl ester and Pentadecanoic acid, methyl ester are classified as fatty acid esters. These compounds have pharmacological activities such as antioxidants, anti-inflammatory, immunomodulation, anticholinesterase, antimicrobial and anti-fungal (Table 2).

The pharmacological activity of diterpenoid fatty acids and esters has been studied in several species of green macroalgae. According to Wirawan et al (2022), the green macroalgae *Caulerpa macrophysa* and *Caulerpa cylindracea* collected from Bali Sea waters contain hexadecanoic acid and phytol which have antioxidant, anti-inflammatory, anti-microbial and anti-bacterial activities. Furthermore, research by Tarigan et al (2023) showed that the active compound of *U. reticulata* also contains phytol and 3,7,11,15-Tetramethyl-2-hexadecen-1-ol which are classified as terpenoids with antioxidant and immunostimulant activities. In addition, it contains hexadecanoic acid and palmitic acid which are classified as fatty acids that have antioxidant and anti-inflammatory activities.

Antioxidant activity. Antioxidant analysis showed that *V. aegagropila* has antioxidant activity with an IC_{50} of less than 50 ppm. The average IC_{50} value of antioxidant activity was found to be 21.31541 ppm (Table 3). Low IC_{50} value indicates very strong antioxidant activity. Based on Molyneux (2004), a compound has very strong antioxidant activity if the IC_{50} value is less than 50, strong (50-100), medium (100-150), and weak (151-200). Research conducted by Sianipar et al (2022) found antioxidant activity of green macroalga *Ulva lactuca* has 118 ppm of IC_{50} which is classified as medium activity. *Caulerpa racemosa* has moderate antioxidant activity with IC_{50} 104.46 ppm (Palaniyappan 2023) but *Ulva reticulata* has a high potential antioxidant activity with IC_{50} 53.00 ppm (Tarigan 2023). The diversity of IC_{50} can be caused by extrinsic and intrinsic factors. Extrinsic factors could be differences in water conditions such as the availability of nutrients, climate and weather where the algae grew (Mirghani et al 2018).

Table 3

Antioxidant activity of *Valonia aegagropila*

Concentration (ppm)	*Mean absorbance*	*Mean inhibition (%)*	*Mean IC_{50}*
10	0.489	41.12	
20	0.406	51.12	
30	0.370	55.38	21.31541
40	0.314	62.21	
50	0.246	70.36	
Control DPPH	0.830	-	

Anti-inflammatory activity. Analysis of anti-inflammatory activity contained in *Valonia aegagropila* was conducted by observing the stability of the red blood cell membrane in experimental animals. Table 4 displayed *V. aegagropila* had an inhibition value of more than 20% at 100 ppm with an IC_{50} value of 136.419 which was classified as moderate anti-inflammatory activity. The anti-inflammatory activity contained in this green macroalga at 100 ppm can inhibit the hemolysis process of red blood cells. This is almost similar to the control sample using diclofenac sodium at the same concentration. The inflammatory activity in samples would directly be proportional to the increase in concentration with anti-inflammatory ability being slightly different from the control. Research conducted by Kurnia et al (2019) showed anti-inflammatory activity of *Chlorella vulgaris* at a concentration of 100 ppm was of 34.952% with an IC_{50} of 83.852 while control had inhibition value of 71.268% with an IC_{50} of 54.149 at the same concentration. Inhibition value of more than 20% has anti-inflammatory properties and can be used as a reference value for drug development (Kurnia et al 2019; Williams et al 2008). This report confirmed that *Valonia aegagropila* has weak anti-inflammatory activity as compared to that of *Chlorella vulgaris*.

Table 4

Anti-inflammatory activity of *Valonia aegagropila*

Concentration (ppm)	*Control*		*Valonia aegagropila*	
	Average inhibition (%)	*IC_{50}*	*Average inhibition (%)*	*IC_{50}*
50	43.17		16.29	
100	54.37		49.93	
150	66.37	79.67793	58.52	136.41951
200	72.45		65.92	
250	84.59		75.41	

Inflammation is a reaction of the immune system due to tissue injury. This inflammatory process will increase the release of inflammatory mediators and increase levels of phagocytes and free radicals that can cause cell and tissue damage (Sanniyasi et al 2023). Phytochemical compounds such as alkaloids, tannins, and phenolics contained in green macroalgae have anti-inflammatory activity effects. Alkaloids can inhibit the expression of several pro-inflammatory factors, such as cytokines, lipid mediators, histamine, and enzymes involved in the inflammatory response (Souza et al 2020). Tannin is anti-inflammatory by inhibiting chemical mediators and pro-inflammatory cytokines (Rocha et al 2022). Phenolics have anti-inflammatory effects by inhibiting enzymes in forming nitric oxide (NO) and directly scavenging NO (Cotas et al 2020). Fatty acids and terpenoids contained in *V. aegagropila* have anti-inflammatory effects by controlling the inflammatory process through the biosynthesis of lipid mediators derived from phospholipid membranes (Lopes et al 2020).

Conclusions. *Valonia aegagropila* is a green macroalga found in the coastal waters of Kora-Kora, Minahasa Regency. Based on phytochemical analysis, secondary metabolites

contained in this green macroalga include alkaloids, saponins, tannins, flavonoids and phenolics. GC-MS analysis revealed that 49 active compounds were present in the ethanol extract of algae. Among these, 10 active compounds, which belong to the classes of diterpenoids and fatty acid esters, demonstrated antioxidant and anti-inflammatory properties, as well as other activities such as anti-microbial, anti-fungal, anticholinesterase, anti-diabetic and immunomodulation.

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

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