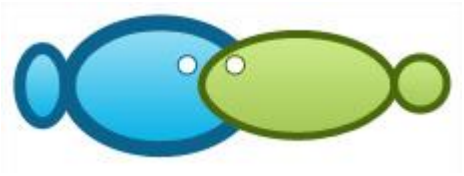

Phytochemical screening, antioxidant and antibacterial tests on red algae, *Halymenia durvillaei*, and phycoerythrin pigments

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Abstract. The red algae, *Halymenia durvillaei*, grow in the coastal waters of North Sulawesi and have been successfully cultivated. The purpose of the study was to conduct phytochemical screening, antioxidant and antibacterial tests on phycoerythrin pigments and thallus of *H. durvillaei*. The algal sampling was carried out by cruising the Totok Bay waters. The content of water-soluble phycoerythrin pigments were obtained by the freezing-liquefaction method, then analyzed spectrally with UV-Vis at wavelengths of 400-600 nm. The maceration method was performed in ethanol solvents for the extraction process and a subsequent phytochemical screening was carried out. The results of the phytochemical tests obtained on phycoerythrin pigments and algal thallus showed alkaloids in higher concentrations, followed by flavonoids, tannin, saponins, triterpenoids and phenolics. Steroids' presence was not detected. Inhibitory concentration IC_{50} values obtained in thallus and phytoerytrhin pigments were 39.255 and 30.961 µg mL^{-1}, respectively. The results of antibacterial analysis for *Staphylococcus aureus* and *Escherichia coli* showed that phycoerythrin pigments (90% concentration) had an inhibiton zone of 12.5 and 11.83 mm and *H. durvillaei* thallus (100%) had an inhibiton zone of 14.33 and 13.33 mm, respectively.
Key Words: seaweeds, extraction, toxicity test, *Staphylococcus aureus, Escherichia coli*.

Introduction. Macroalga or seaweed is one of the marine biological resources of economic value and has good benefits for humans and the surrounding environment, for example as food, raw material for cosmetic products and medicinal ingredients (Pakidi & Suwoyo 2016). According to Bold & Wynne (1985), algae are classified into classes, namely Rhodophyceae (red algae), Phaeophyceae (brown algae) and Chlorophyceae (green algae). Each class of algae has a certain color characteristic due to the presence of the type of pigment it contains. Pigments in algae not only play an important role in the process of photosynthesis, but also contain bioactive compounds with various potential applications in human health, such as antioxidants, antibacterials and antipyretics. These bioactive compounds can act as biopharmaceutical preparations (Karaki et al 2013).

Besides chlorophyll and carotenoid pigments, the red algae *Halymenia durvillaei* produce a significant amount of phycoerythrin pigments (Mantiri & Ampou 2001). This pigment is red and can easily dissolve in water. Based on test results using the DPPH method (2,2-diphenyl-1-picrylhydrazyl), phycoeritrin has an antioxidant potential (Pumas et al 2012), it can slow down and even inhibit the oxidation of a substance, and can protect cells from the impact of free radical attacks.

Antioxidant, antibacterial and anticancer activities have been studied in several species of algae (Mantiri et al 2019; Singkoh et al 2021). It is important to study the benefits of phycoerythrin pigments through each of the bioactive compounds they contain, as well as in the pigment-producing full-spectrum algae. Thus, the purpose of the study was to conduct a phytochemical screening, antioxidant and antibacterial tests on phycoerythrin pigments and thallus of *H. durvillaei*.

Material and Method

Sampling. Sampling was carried out by exploring the coastal waters of Totok Bay in September 2020 (Figure 1). The samples of algae found were taken by removing the algae from the substrate to which they were attached, regardless of the growth rate of the algae. Algae were first cleaned with sea water to remove dirt and adhering sediment, then rinsed with distilled water. The algae were then weighed and placed in a plastic container that has been marked, placed in a cool box containing ice, and taken to the Laboratory of Biomolecular and Marine Pharmacy, Faculty of Fisheries and Marine Sciences, Sam Ratulangi University. The morphological identification of the algae was carried out based on Trono (1997), to ensure that their species was *H. durvillaei*.

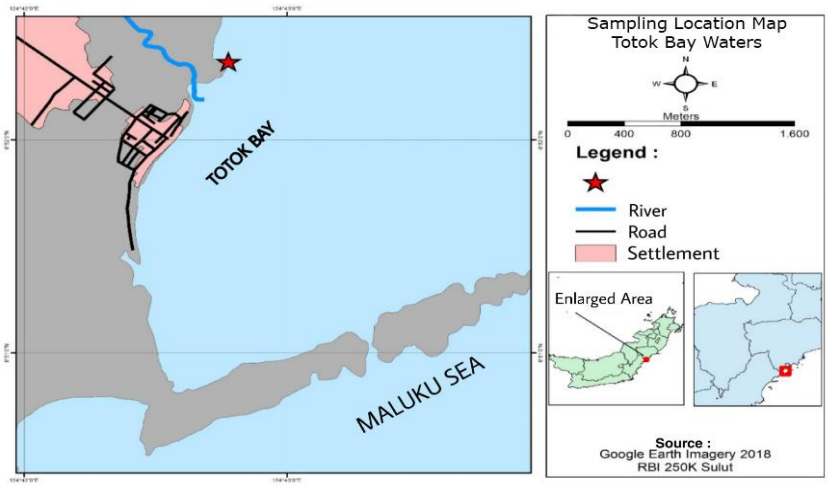

Figure 1. Sampling site.

Pigment extraction. Extraction of phycoerythrin pigment from red algae *H. durvillaei* was carried out at the Laboratory of Biomolecular and Marine Pharmacy, Faculty of Fisheries and Marine Sciences, Sam Ratulangi University, through soaking, freezing and thawing procedures, for seven days, performed on 500 g of algae. Subsequently, the phycoerythrin pigment solution was filtered using gauze, sterile cotton and Whattman No. 1 filter paper. Pigment absorption was carried out with a UV-Vis Spectrophotometer at a wavelength of 400-600 nm (Figure 2). The pigment in algae has been tested for toxicity using the Brine Shrimp Lethality Test method on *Artemia salina* and a probit analysis.

Figure 2. Red algae *Halymenia durvillaei* and phycoerythrin pigment (original photo).

Phytochemical screening of phycoerythrin and algal pigments. Phytochemical screening was carried out on the algal thallus and phycoerythrin pigment. The algae were

cut into small pieces and then soaked for 24 hours in 95% ethanol solvent, while the phycoerythrin pigment in the form of a solution was mixed with 95% ethanol solvent. Both have a ratio of 1:2 each. After immersion, the extract in ethanol was filtered and evaporated with a Rotary Vacuum Evaporator at 45ºC to reduce the water content and obtain a thick extract. Phytochemical screening stages include testing for alkaloids, flavonoids, tannins, saponins, phenolics, triterpenoids and steroids following Harborne (1984).

Antioxydant activity test. Antioxidant activity test on *H. durvillaei* was carried out with 0.1 mM DPPH radical (1,1-diphenyl-2-picrylhydrazil). DPPH powder was dissolved in methanol pro-analysis (p.a). The maximum wavelength of DPPH was 517 nm. The blank solution, 2 mL of 0.15 mM of DPPH, was put into a test tube, added 2 mL of methanol, stirred until homogeneous, incubated in a dark room for 30 minutes, then the absorption was measured with a UV-Vis Spectrophotometer at a wavelength of 517 nm. Algae extract solution was made on 50 mg of algae sample dissolved in 50 mL of ethanol p.a, then macerated for 24 hours. Likewise, the phycoerythrin pigment extracted from the algal thallus was taken 50 mL and then added with ethanol p.a. 100 mL, left for 24 hours. The active solution of the algal thallus and the phycoerythrin pigment was evaporated. Each of these extract concentrations was made in methanol 20, 30, 40, 50 and 60 ppm. Free radical scavenging activity can be expressed by the IC_{50} (inhibitory concentration) value, which is the concentration of the test compound that resulted in 50% loss of the free radical activity. The smaller the IC_{50} value, the higher the antioxidant activity. The radical scavenging activity is expressed as the percentage of inhibition which can be calculated using the following formula (Meyer at al 1982):

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

Where:
A - sample absorbance;
B - absorbance control sample;
C - absorbance of negative control;
D - absorbance blank.

Antibacterial activity test. The sample was mashed using a blender, the resulting powder was put into a closed glass container (Gunawan & Mulyani 2004). A total of 25 g of algal powder was soaked with 96% of 25 mL ethanol solution, left for 5 days and it was occasionally stirred. After 5 days, the soaked sample was filtered using filter paper to produce a filtrate, then evaporated using a Rotary Vacuum Evaporator, so that a thick extract was obtained. The resulting viscous extract was evaporated throughout the ethanol solvent, in an oven. The extracts were weighed and stored in a closed glass container before being used for testing (Department of Health of Republic of Indonesia 1986).

The tools and materials used in this antibacterial activity study were sterilized first in an autoclave (Lay & Hastowo 1992). The test bacteria were taken with a sterile ose needle, then implanted on the agar tilted media by scraping. It was then incubated in an incubator at 37°C for 24 hours. The same treatment was carried out on each type of test bacteria. A positive control test was obtained using 500 mg of dissolved Ciprofloxacin. Distilled water served as a negative control.

The antibacterial activity test was carried out in vitro with Nutrient Agar (NA) media for bacterial inoculation (Lay & Hastowo 1992). The concentrations of *H. durvillaei* samples were 5, 10, 30, 60 and 90 mg. 50 mL of ethanol extract of the test solution, 50 mL of negative control sterile distilled water and 50 mL of Ciprofloxacin solution as a positive control were dropped different wells. Then, the Petri dishes were incubated in an incubator at 37°C for 24 hours.

Observations were carried out after 24 hours of incubation. The clear area was an indication of the sensitivity of bacteria to the antibiotics used as test material, which is expressed by the width of the inhibition zone measured in mm using a scaled ruler, as

the overall diameter minus the diameter of the well (7 mm), according to Vandepitte et al (2003). Then, the diameter of the inhibition zone indicator was mapped to a category of antibacterial power, based on the classification of Davis & Stout (1971).

Results and Discussion

Phycoerythrin pigment. *H. durvillaei*, multicellular and macroscopic red algae, has a length between 10 cm to 1 m and a form of a sheet. *H. durvillaei* taken from the sea were placed in a container with water. Phycoerythrin pigment extract from *H. durvillaei* looks bright red in aqueous solvent (Figure 2). A toxicity test of phycoerythrin pigment extract was also carried out using the Brine Shrimp Lethality test method. Observations for 24 hours showed that at a pigment concentration of 7.5 ppm the average mortality of test animals (*Artemia salina*) was 3%. Probit analysis showed that the lethal concentration (LC_{50}) was 40.10 ppm and was only able to kill 3% of the test animals. Thus, the phycoerythrin pigment extract from *H. durvillaei* was declared non-toxic (Figure 3).

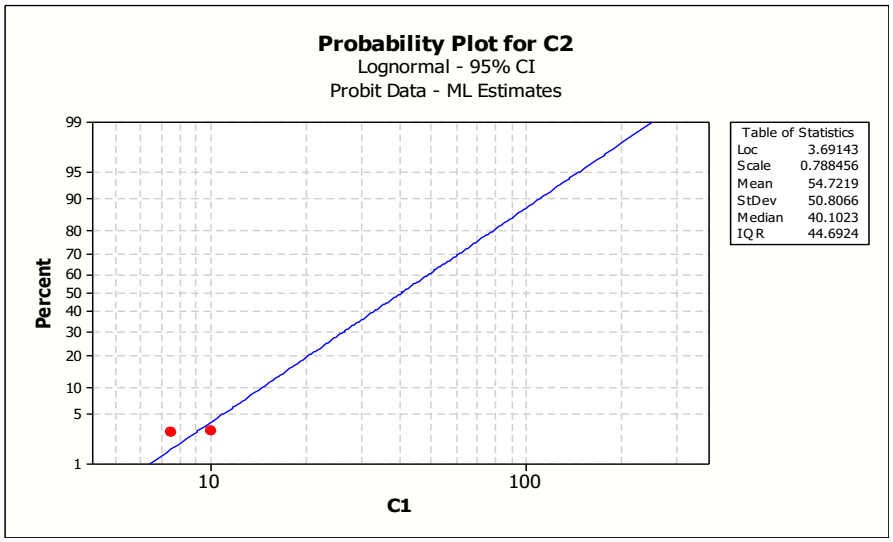

Figure 3. Probit analysis result graph.

This pigment plays an important role as an accessory pigment in the photosynthesis process of red seaweed by helping chlorophyll-a in absorbing light. Phycoerythrin absorbs green light, which can mask the green color of chlorophyll, and blue from phycocyanin (Bryant 1982). The absorption spectrum of phytoerythrin pigment with UV-Vis spectrophotometer has peak wavelengths of 495 and 560 nm.

Phytochemical in phycoerythrin and algal thallus. *H. durvillaei* is a red alga that contains bioactive compounds and is very beneficial for industry. The ability of this species to produce bioactive compounds is partly due to environmental factors. The following are the results of quantitative analysis and phytochemical screening of the phycoerythrin from *H. durvillaei* (Table 1 and Table 2).

Table 1

Results of phytochemical quantitative analysis of phycoerythrin from *Halymenia durvillaei*

Name of sample	*Phycoerythrin pigment (mg mL^{-1})*	*Algal thallus (mg mL^{-1})*
Flavonoid	10.1733	11.6733
Tannin	1.5612	3.7588
Phenolic	6.8425	7.6432

Table 2

Phytochemical screening results

Group of compounds	*Reagent*	*Phycoerythrin pigment*	*Halymenia durvilllaei thallus*	*Color identification*
Alkaloid	Dragendorf	+	+	Orange
	Wagner	+	+	Brown
	Meyer	+	+	White precipitate
Flavonoid	Mg, HCl	+	+	Red
Tannin	$FeCl_3$	+	+	Green
Saponin	HCl	+	+	Bubbles/foam
Steroid	CH_3COOH, HCl	-	-	No color change
Triterpenoid	CH_3COOH, HCl	+	-	Positive (+) and negative (-)
Phenolic	$FeCl_3$	+	+	Red color change Orange brown

The average values of the phytochemical concentrations showed that flavonoid compounds were dominant in the phycoerythrin pigment and algal thallus, with 10.1733 and 11.6733 mg mL^{-1}, respectively, followed by the phenolic compounds, with 6.8425 and 7.6432 mg mL^{-1}, respectively, while the tannin compounds had the lowest concentrations, of 1.5612 and 3.7588 mg mL^{-1}, respectively. High flavonoid compounds were found in the algal thallus and phycoerythrin pigments. However, the quantitative analysis showed that the phytochemical compounds in the thallus were higher than the phytochemical content in the phycoerythrin pigments. Singkoh et al (2019) obtained flavonoid compounds concentrations which are generally the same as in this study, except for triterpenoids. Triterpenoids are nonpolar compounds that are insoluble in water, so they are not found in phytoerythrin pigments. Flavonoids are commonly found in plants, including marine algae, bound to sugars as glycosides (Sovia 2006). The benefits of flavonoids include protecting cell structure, increasing the effectiveness of vitamin C, anti-inflammatory, preventing bone loss and their antibiotic effect. Tannin compounds can function as biological antioxidants (Hagerman 2002). Phenolic compounds are known to have various biological effects as antioxidants, cell structures protectors, with anti-inflammatory and antiseptic effects (Primadini 2010).

Antioxidant test. Antioxidant test in pigment and thallus of *H. durvillaei* was carried out with concentrations of 20, 30, 40, 50 and 60 ppm with three repetitions. The results obtained are in Table 3.

Table 3

Results of phycoerythrin pigment antioxidant test

Samples	*Concentration (µg mL^{-1})*	*Barrier activity (%)*	*IC_{50} (µg mL^{-1})*
Phycoerythrin	20	27.7074	39.25493
	30	41.2095	
	40	44.7257	
	50	62.7285	
	60	78.1997	
	DPPH control	-	
Thallus	20	33.89	30.96060
	30	55.27	
	40	59.07	
	50	71.03	
	60	75.25	
	DPPH control	-	

The antioxidant activity with methanol solvent on phycoerythrin pigment was IC_{50} 39.25493 mg mL^{-1}, while in algal thallus IC_{50} 30.96060 mg mL^{-1}. These indicate that there is a better antioxidant activity in the algal thallus compared to the phycoerythrin pigment. Antioxidant activity test on green algae *Ulva prolifera* and *Halimeda opuntia* from Totok Bay returned IC_{50} values of 448.02 and 1,433.85 mg mL^{-1} (Mantiri et al 2019). In terms of antioxidant activity, the phycoerythrin pigment is relatively similar to the algal thallus. The antioxidant activity of the extract is related to its content of secondary metabolites. One of the compounds that act as antioxidants are flavonoids, which are the largest phenolic group widely found in plants. The detected flavonoids in *H. durvillaei* quantitatively exceeded the tannins and phenolic compounds content.

Antibacterial activity. The results of the antibacterial activity test showed that the phycoerythrin sample from *H. durvillaei* had an antibacterial activity against the three bacterial test solutions. This is indicated by the presence of a clearing zone around the well. The area where bacteria are absent, called inhibition area, depends on the absorption of the antibacterial agent into the agar and on the sensitivity of the bacteria to the antibacterial agent (Table 4).

Table 4

Results of antibacterial test on phycoerythrin of *Halymenia durvillaei*

Concentration (*mg mL^{-1}*)	*Inhibition area (mm)*	
	Staphylococcus aureus	*Escherichia coli*
Phycoerythrin pigment		
5	9.83	7.83
10	11	9.33
30	11.67	10.33
60	12	11
90	12.5	11.83
Positive control (+)	18.5	14.83
Negative control (-)	0	0
Algal thallus 100%	14.33	13.33
Positive control (+)	18.5	14.83
Negative control (-)	0	0

Bacterial inhibition was categorized as weak for an inhibition area's diameter of less than 5 mm, moderate from 5 to 10 mm, strong from 10 to 20 mm and very strong for more than 20 mm (Davis & Stout 1971). The results of antibacterial test on samples of phycoerythrin extracted from *H. durvillaei*, in terms of inhibition of *Staphylococcus aureus* and *Escherichia coli* bacteria, were categorized as strong, with inhibition areas of 12.5 mm and 11.83 mm, at a concentration of 90 mg mL^{-1}. The antibacterial test on samples of *H. durvillaei* (100%) showed an inhibition against *S. aureus* and *E. coli* bacteria categorized as strong, with inhibition areas of 14.33 and 13.33 mm, compared with the research of Singkoh et al (2021), where *Padina australis* from Atep Oki beach had an inhibition area of 11.3–11.6 for *S. aureus*, *S. mutant* and *E. coli* bacteria. So, the antibacterial activity of this algal thallus is still stronger against *E. coli* and *S. aureus* bacteria. Likewise, the results of tests conducted by El-Fatimy & Said (2011) on the algae *Cystoseira* sp. from the coast of Tukra showed that the diameter of the inhibition zone in methanol solvent for *S. aureus* bacteria was 11.3 mm.

Conclusions. The results of this study show that the red algae *H. durvillaei* from Totok Bay and the phycoerythrin pigment extracted from *H. durvillaei* had bioactive compounds such as flavonoids, tannins and phenols. The results of phytochemical screening on this species' plant and on its phytoerythrin pigments show that the flavonoid compounds were dominant and the antioxidant activity of the thallus is higher than for the phycoerythrin pigment. Likewise, the antibacterial activity of both the thallus and the phytoerythrin pigment is strong e to inhibit the growth of *S. aureus* and *E. coli* bacteria.

Acknowledgements. This research was supported by Sam Ratulangi University, The Ministry of Education, Culture, Research and Technology, The Republic of Indonesia.

Conflict of interest. The authors declare no conflict of interest.

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Received: 29 September 2021. Accepted: 17 November 2021. Published online: 29 November 2021.
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How to cite this article:
Mantiri D. M. H., Kepel R. C., Boneka F. B., Sumilat D. A., 2021 Phytochemical screening, antioxidant and antibacterial tests on red algae, *Halymenia durvillaei*, and phycoerythrin pigments. AACL Bioflux 14(6):3358-3365.