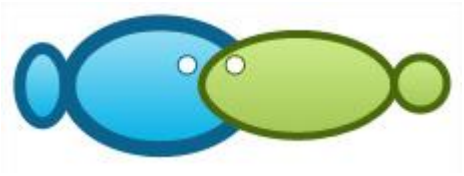

Biomineral characterization and antibacterial activity of marine algae *Tricleocarpa fragilis* from Kora-kora coastal waters of Minahasa Regency, Indonesia

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Abstract. This study aimed to determine the mineral composition and identify the bioactive compounds contained in red algae *Tricleocarpa fragilis* and to test its antibacterial activity of *T. fragilis* against *Vibrio harveyi*. The scanning electron microscope analysis and energy dispersive X-ray were used to show the morphology of the particles and the mineral composition contained in this algae. Identification of bioactive compounds used the Harborne method. Antibacterial activity test used the Minimum Inhibitory Concentration (MIC) test and Minimum Bactericidal Concentration (MBC) test. The scanning electron microscope analysis showed the particle morphology of *T. fragilis*. Enlargement of *T. fragilis* nanoparticle images was carried out on a scale of 200x, 1000x, and 10,000x. Six specimens analyzed using energy dispersive X-ray showed that the red algae *T. fragilis* contained biomineral compound elements respectively C> Ca> Pb> Pt> S> Ni> Fe> Mg> Na> Al> Mn> Cr> Se> P. Screening for bioactive compounds shows that *T. fragilis* positively contains alkaloids, triterpenoids, steroids, tannins, flavonoids and saponins. Antibacterial bioactivity test results showed that *T. fragilis* has antibacterial activity against *V. harveyi* bacteria. In n-hexane extract the concentration of 90% was determined as MIC value.
Key Words: bioactivity, bacteria, extract, minimum inhibitory concentration, phytochemical screening.

Introduction. *Tricleocarpa fragilis* has a lush thallus shape, with the total height of thallus measured from holdfast to the apex of 6-9 cm, while the thallus measured from holdfast to the first branch is 0.5-1.0 cm (Figure 1). The algae have dichotomous and multiaxial branches at part of round branch and the leaf width of 0.2 cm. The fresh algae have red color. Its habitat is in rocks at flat coral reef areas (Kepel & Baulu 2013). *T. fragilis* is one type of red algae that produces carrageenan (Trono & Ganzon-Fortes 1988), steroid and triterpenoid compounds (Lobban & Wynne 1981). Robinson (1991) stated that some steroid and triterpenoid compounds had antibacterial activity. Macroalgae produce several bioactive components that fall into the group of fats, fatty acids, polysaccharides, and pigments and secondary metabolites such as alkaloids, phenols, lectins and terpenes (Perez et al 2016). Several studies conducted previously have identified bioactive compounds in algae and tested their ability to inhibit gram-negative, gram-positive and resistant bacteria (Singkoh 2011; Zen et al 2015; Panden et al 2019; Singkoh et al 2019). The purpose of this study was to determine the mineral composition and to identify bioactive compounds contained in the red algae *T. fragilis* and to test its antibacterial activity against *Vibrio harveyi.*

Figure 1. *Tricleocarpa fragilis* (original).

Material and Method. Samples of red algae were taken from the Beach of Atep Oki Village, East Lembean District, Minahasa Regency, Province of North Sulawesi, Indonesia (Figure 2). Several species of red, green, and brown algae grew naturally in this area. Samples were packaged in plastic bags and then placed in a cool box.

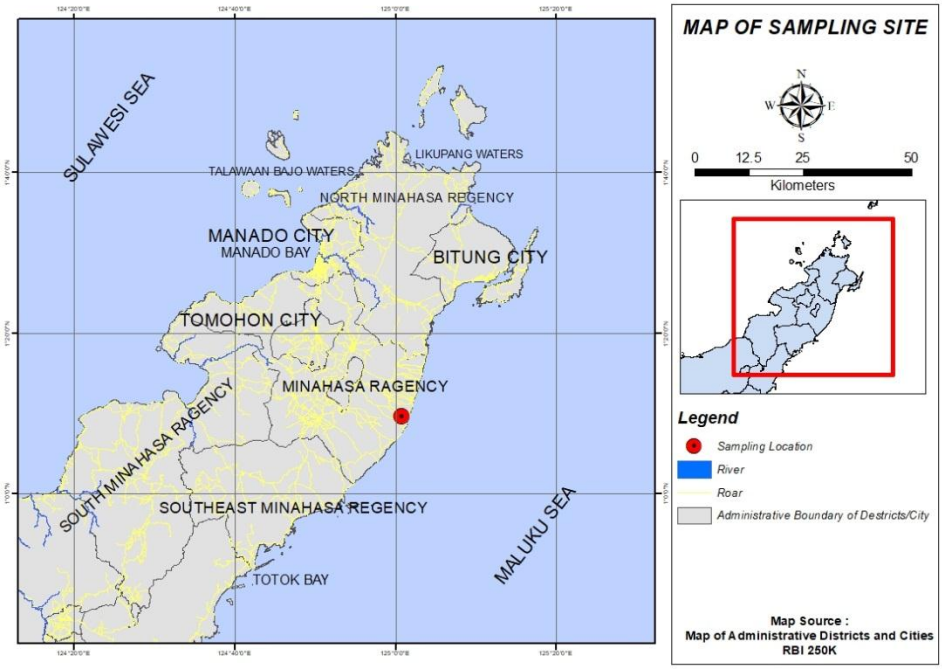


Figure 2. Map of sampling site.

Preparation of G. oblongata flour. The algae were first washed and then soaked in fresh water for one night to remove dirt, and then rinsed under running water so that the algae was really clean and then drained. Fresh cleaned algae were ground to powder using a grinder, and dried for about 18 hours to reduce the water content. After that the algae were ground again and then sieved to get the flour. Analysis of red algae flour was carried out in the Basic Science Center of Bandung Institute of Technology. Observation of nanoparticles was done through Scanning Electron Microscope (SEM) while main composition and chemical compounds of the flour were analyzed by the Energy Dispersive X-Ray Spectrometer (EDS).

Preparation of G. oblongata extract. The extraction method used in the study was maceration. The sample was cut into small pieces and then crushed. The crushed sample was then immersed in 95% ethanol solvent with a ratio of solvent and sample of 2:1 for 24 hours and then macerate was filtered using Whatman paper no. 1. This first filtrate was stored in a bottle while the sample pulp was soaked again with the same solvent and ratio. Similar procedure was performed in the third maceration for 24 hours. The filtered maceration results were then collected and evaporated using a rotary vacuum evaporator at a temperature of 45°C to form a thick extract. The result of this thick extract was an extract with a concentration of 100%. The extract was stored at a temperature of 18°C to prevent the loss of compounds contained in the extract.

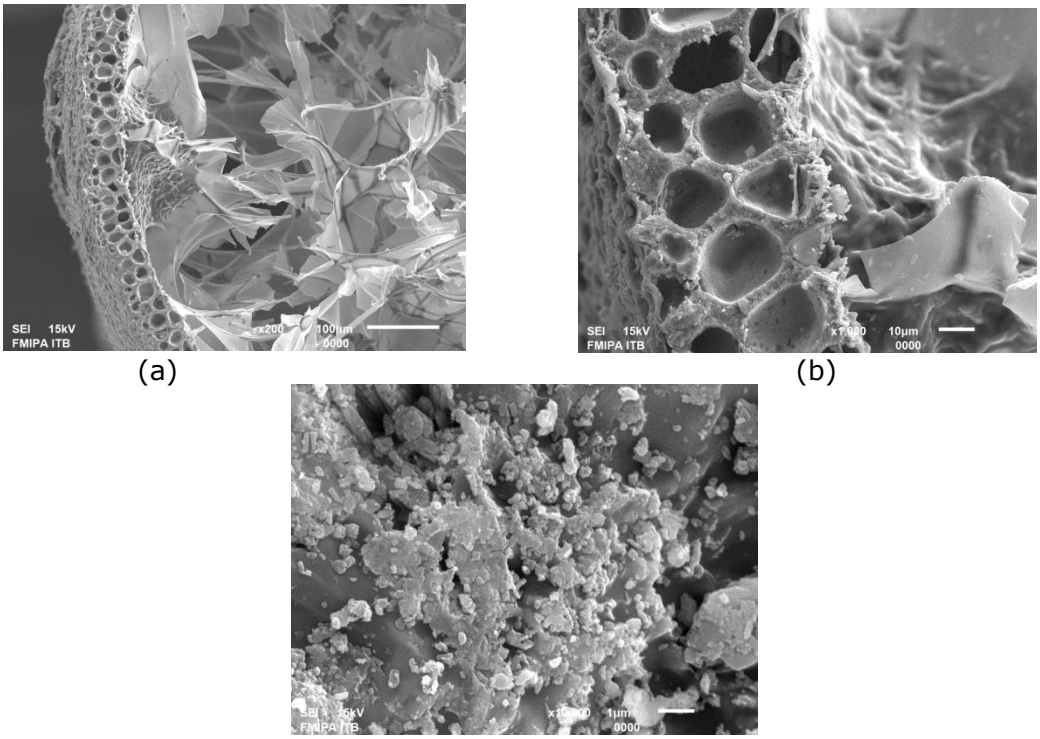
Fractionation. The crude extract obtained was placed into a separating funnel, dissolved with 96% ethanol, added an n-hexane solvent in the ratio of 1: 1 v/v, and then shaken in a separating funnel until homogeneous. It was allowed to form ethanol layer of n-hexane layer. Each layer was placed in a different container. The n-hexane layer was then evaporated using a rotary evaporator until dried, then weighed and this result was called the n-hexane fraction. Furthermore, the ethanol layer was added with 100 mL of distilled water, partitioned with ethyl acetate solvent in a ratio of 1:1 v/v and shaken in a separating funnel until homogeneous, left to allow forming two layers, namely ethanol and ethyl acetate layers. Each layer was held in a different container. The ethyl acetate layer was then evaporated using a rotary evaporator until dried and then weighed and this result was called the ethyl acetate fraction. The ethanol layer was added with 100 mL of distilled water and then partitioned with chloroform solvent in a ratio of 1:1 v/v, shaken in a separating funnel until homogeneous and left to form two layers, namely ethanol and chloroform layers. Each layer was held in a different container. The chloroform layer was then evaporated using a rotary evaporator until dry and then weighed and this result was called the chloroform fraction. The ethanol layer was evaporated using a rotary evaporator until dried and then was weighed (Harborne 1984 modification). The four fractions obtained were used in antibacterial testing.

Phytochemical screening. Phytochemical screening of *T. fragilis* extract included examination of compounds of alkaloids, flavonoids, tannins, terpenoids/steroids and saponins. The analytical method used is based on Harborne (1984).

Antibacterial activity test. The antibacterial activity test of *T. fragilis* used the turbidity measurement method using spectrophotometer analysis to determine the MIC value and the pour plate method to determine the MBC value. Minimum Inhibitory Concentration (MIC) is a test to determine the minimum concentration that can inhibit bacterial growth. MIC test was conducted referring to the research of Munfaati et al (2015). MBC test is an advanced test that aims to find out the lowest or minimum concentration that can kill bacteria in total on growing media. The MBC test used the pour plate method which refers to the research of Munfaati et al (2015). The bioactivity test begins with the sterilization of the instrument that will be used during the study then continues with the making of the media, making a series of test concentrations and both controls, bacterial suspensions, and finally the determination of MIC and MBC values.

Data analysis. Data of MBC (quantitative) were analyzed statistically through one way ANOVA using SPSS for windows at significance level of 95%.

Results and Discussion. The results of SEM analysis showed the particle morphology of *T. fragilis*. Enlargement of *T. fragilis* nanoparticle images was carried out on a scale of 200x, 1000x, and 10,000x. Figure 3 shows the shape and size of nanoparticles obtained through observations using SEM. Based on SEM data, it can be seen that the nanoparticles of *T. fragilis* were not uniform, porous at the edges and sizes tended to vary.



(a) (b)

(c)

Figure 3. Morphological analysis of *Tricleocarpa fragilis* using SEM (a). 200x magnification, (b) 1000x and (c) 10,000x.

T. fragilis contains elements of biomineral compounds dominated by C (carbon) of more than 40%. From the 6 specimens analyzed using EDS, the average elements of biomineral compounds present in *T. fragilis* are shown in Figure 3. In addition to the carbon element, *T. fragilis* is also composed of O (oxygen) 39.86%, Ca (calcium) 14.5%, Pb (lead) 3.53%, Pt (platinum) 3.13%, S (sulfur) 1.5%, Ni (nickel) 0.34%, K (potassium) 0.19%, Fe (iron) 0.19%, Co (cobalt) 0.19%, Zn (zinc) 0.16%, Mg (magnesium) 0.15%, Na (sodium) 0.14%, Al (aluminum) 0.12%, Mn (manganese) 0.09%, Cr (chromium) 0.05%, Se (selenium) 0.04 %, and P (phosphorus) 0.02%.

Trono & Ganzon-Fortez (1988) stated that several types of algae (*Caulerpa lentillifera, Dictyota* spp., *Kappaphycus alvarezii*, *Gracilaria coronopifolia*, *Gracilariopsis longissima*, *Hypnea cervicornis*, *Chondrophycus tronoi*, *Sargassum* spp., *Turbinaria conoides, Ulva lactuca*) have quite high mineral contents of Ca, K, Mg, Na, Cu, Fe, and Zn. Elements of Fe and Mn are also found in the red algae *Gracilaria* sp. and *Hypnea* sp. (Awalina 1993), where these two elements are interrelated in the process of photosynthesis. Awalina (1993) reported that Fe and Mn played an important role in physiological processes, especially in photosynthesis, which affected the growth and proliferation of algae. Fe and Mn are important parts of many enzymes, cytochromes and porphyrins. Wiessner (1962) stated that the deficiency of Fe and Mn elements was closely related to metabolic functions involving metal ions, for example a lack of Fe can lead to chlorosis and inhibition of growth as a result of reduced photosynthetic units. Algae growth was also influenced by the element of Fe which played a role in helping nitrogen metabolism and carbon assimilation (Lobban & Wynne 1981). Liu et al (2000) reported that Fe was required to maintain the growth of algae cells. Kakita & Kamishima (2006) stated that there was a relationship between Fe regulation and the metabolism of nitrogen in macroalgae.

Elements of Cu (0.0204 ppm) and Zn (0.0849) were also found in *Dictyosphaeria versluysii* (Radiena 2018). Zn was one of the essential micro minerals that functions as an antioxidant because Zn is a cofactor of 240 enzymes in the system of metabolism, cofactors of essential enzymes such as lactate dehydrogenase, alkali, phosphatase and carbon anhydrase (Lagana et al 2007; Naz et al 2016). Hidayat et al (2014) reported that Zn nanoparticles have an antibacterial and antioxidant function. Some researchers

Rajendra et al (2010), Liu et al (2000), Padmavathy & Vijayaraghavan (2008) reported the ability of nano-ZnO as an antibacterial against *Escherichia coli*, of which growth inhibition is directly proportional to the concentration of nano-ZnO. Nano-ZnO is known to distort and damage bacterial cell membranes, which causes leakage of intracellular contents and ultimately results in bacterial cell death. Jin et al (2009) reported that nano-ZnO has strong antimicrobial activity against three pathogenic bacteria (*Listeria monocytogenes*, *Salmonella enteritidis* and *E. coli* O157: H7).

Fitton (2005) reported that mineral content of algae was higher than plants originating from land. The content and composition of each chemical compound depended on the type, age of harvest, and the state of the environment in which the algae grew. This causes the content and composition of each algae differed from each other. This is in accordance with the Venugopal (2018) report, which states that the type of mineral depends on the habitat of each seaweed. There are variations in the amount of minerals, organic components in the bottom of the waters, function of depth, distance from the land and other environmental factors.

Phytochemical screening. Phytochemical screening tests were carried out to identify bioactive compounds contained in the extract of *T. fragilis* which have activity in treatment as antibacterial according to Harborne (1984). The screening results can be seen in the Table 1.

Table 1

Phytochemical screening results of *Tricleocarpa fragilis* ethanol extract from Kora-kora Minahasa coastal waters

Phytochemical screening	*Results*
Alkaloid Dragendorf	+
Alkaloid Wagner	+
Alkaloid Meyer	+
Triterpenoid	+
Steroid	+
Tannin	+
Flavonoid	+
Saponin	+

Antibacterial activity. The extraction results from each fractionation were divided to make three series of concentrations, namely 30%, 60%, and 90%. This difference in concentration series aimed to find out which concentration series showed the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values for *V. harveyi*. Bioactivity tests conducted were to determine the presence of antibacterial biological activity in ethanol, ethyl acetate, n-hexane and *G. oblongata* chloroform extracts tested on *V. harveyi* bacteria. Testing the antibacterial activity of *T. fragilis* extract against Vibrio bacteria was carried out by the liquid dilution method by determining the value of Drinking Inhibitory Concentration or Minimum Bactericidal Concentration using the parameter of absorbance value of each extract concentration series. MIC testing was determined by measuring the difference in the value of OD pre and post-incubation with the formula OD (ΔOD) = post-incubation - pre-incubation. If it shows a negative value it means that there is a decrease in absorbance value, which means a decrease in the number of microbial cells that have been incubated for 18 hours, whereas if the value of ΔOD is positive means there is no reduction in the number of cells, or growth in the number of cells after incubation (Munfaati et al 2015).

As can be seen in Table 2, result showed that the value of ΔOD was positive for ethanol extract at a concentration of 30% with 0.495; likewise in the negative control treatment (aquades), the value was 0.415. Negative control did not showed a decrease in the value of ΔOD because aquades did not have compounds to inhibit bacterial growth. The positive value indicated that there was still an increase in the growth of *V. harveyi* bacteria. The negative value was found in ethanol extract with a concentration of 60%,

the value of ΔOD decreased by -0.034 and 90% concentration by -0.358. Likewise, n-Hexane extract was started at a concentration of 30% ΔOD value of -0.168; 60% of -0.199; concentration of 90% -1.817 and in ethyl acetate extract the value of ΔOD -0.495 and concentration of 90% -0.527. A negative ΔOD value was also shown on the positive control with a ΔOD value of -0.304. Based on the above results, the *T. fragilis* extract with n-Hexane solvent at a concentration of 90% was determined as the Minimum Inhibitory Concentration (MIC) in inhibiting the growth of *V. harveyi* bacteria.

Table 2

OD Value in MIC Test of *Tricleocarpa fragilis* extract on *Vibrio harveyi* bacteria

Treatment		*Value OD*		*ΔOD*
		Preincubation	*Postcubation*	
Ethanol	30%	0.404	0.900	0.495
	60%	1.906	1.872	-0.034
	90%	2.344	1.985	-0.358
Chloroform	30%	0.623	1.160	0.537
	60%	0.124	0.413	0.289
	90%	0.348	0.495	0.147
N-Hexane	30%	1.911	1.742	-0.168
	60%	1.518	1.318	-0.199
	90%	2.616	0.799	-1.817
Ethyl acetate	30%	0.836	1.340	0.504
	60%	2.968	2.472	-0.495
	90%	2.279	1.752	-0.527
Positive control		0.819	0.515	-0.304
Negative control		0.057	0.472	0.415

The result showed that the highest average number of colonies growing on NA media was in negative control (Table 3) while the least was in positive control (cotrimoksazole) and in the N-hexane fraction at concentration of 90%.

Table 3

MBC extract of *Tricleocarpa fragilis* on *Vibrio harveyi* bacteria

Treatment		*Total colony*			*Total*	*Average±SD*
		U.I	*U.II*	*U.III*		
Ethanol	30%	464	480	510	1,454	485±23.352
	60%	220	208	232	660	220±12.000
	90%	109	128	135	372	124±13.453
N-Hexane	30%	114	120	110	344	115±5.033
	60%	74	86	84	244	81±6.429
	90%	22	20	18	60	20±2.000
Ethyl acetate	30%	428	394	380	1,202	401±24.684
	60%	156	164	168	488	163±6.110
	90%	116	104	124	344	115±10.066
Chloroform	30%	680	668	704	2,052	684±18.330
	60%	620	580	642	1,842	614±31.432
	90%	582	530	514	1,626	542±35.552
Positive control		0	0	0	0	0±0
Negative control		771	740	708	2,219	740±31.501

The MBC test showed there was a significant difference in total colonies growing on NA media between different extract concentrations.

The secondary metabolites content in plants has antibacterial activity that synergistically works with a variety of mechanisms of action. *T. fragilis* extract works as

an antibacterial due to the content of chemical and biomineral compounds present in these algae. Alkaloids work as an antibacterial by disrupting the constituent components of peptidoglycan in the cells of the bacteria causing the cell wall layer will not be formed properly so that the cell dies. Another mechanism is as a DNA accelerator that inhibits the topoisomerase enzyme bacteria. The terpenoid compounds are known to be active against bacteria by involving membrane breakdown by lipophilic components (Cowan 1999). As an antibacterial, steroid interacts with cell phospholipid membranes that are permeable to lipophilic compounds, causing membrane integrity to decrease and morphology changes so that cells become brittle and lysed. The function of flavonoids as an antibacterial is by binding to proteins in bacteria that inhibit the activity of enzymes and result in a disturbed process of bacterial metabolism. The lip physical properties of flavonoids can also damage bacterial cell membranes because cell membranes contain lipids which can function as a conduit through the membranes (Chusnie & Lamb 2005). Flavonoids can also inhibit energy metabolism by inhibiting the use of oxygen by bacteria. Tannins inhibit the reverse transcriptase enzyme, DNA topoisomerase, and cell wall polypeptides so that the formation of cell walls imperfectly causes bacterial cells to become lysed and eventually die due to osmotic and physical pressure (Nuriah et al 2009). Saponin compounds work as an antibacterial by diffusing through the outer membrane and vulnerable cell walls and then binding to the cytoplasmic membrane and interfering with and reducing the stability causing the cytoplasm to leak and exit the cell so that the cell dies. This presumably occurs because the steroid molecules have nonpolar and polar groups that can dissolve the phospholipid of plasma membrane (Wiyanto 2010). This shows that the more content of chemical compounds in plants, the greater the potential as an antimicrobial.

Conclusions. *T. fragilis* from the waters of Kora-kora Beach in Minahasa Regency is characterized by mineral elements content composed of C, Na, Mg, Al, P, S, K, Ca, Cr, Mn, Fe, Co, Ni, Se, Pt, PB, and Zn. These elements influence the growth and ability of *T. fragilis* to produce secondary metabolites. Screening for bioactive compounds shows that *T. fragilis* positively contains alkaloids, triterpenoids, steroids, tannins, flavonoids and saponins. Antibacterial activity test showed that *T. fragilis* has antibacterial activity against *V. harveyi* bacteria. In n-hexane extract the concentration of 90% was determined as MIC value.

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Received: 20 September 2019. Accepted: 22 October 2019. Published online: 29 October 2019.
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How to cite this article:
Singkoh M. F. O., Mantiri D. M. H., Lumenta C., Manoppo H., 2019 Biomineral characterization and antibacterial activity of marine algae *Tricleocarpa fragilis* from Kora-kora coastal waters of Minahasa Regency, Indonesia. AACL Bioflux 12(5):1814-1822.