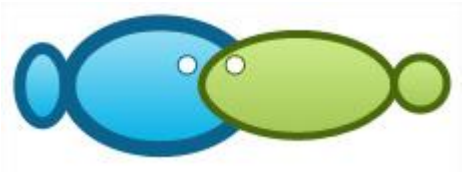

# Crude extracts of *Kappaphycus alvarezii* algae cultivated in several seaweed production centers in North Sulawesi, Indonesia as immunostimulant

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**Abstract**. The use of natural ingredients as immunostimulants to improve fish immunity continues to develop. This study is to determine the quality of *Kappaphycus alvarezii* extract from 3 cultivation locations in North Sulawesi and to compare the amount of carrageenan between non-ATC and ATC products in the form of SRC (Semi Refined Carrageenan). As immunostimulant, antioxidant activity was tested to measure $IC_{50}$ of antioxidant activity. Results showed that there were differences in the quality of *K. alvarezii* extracts among 3 research locations. The highest carrageenan content was found Likupang NSMEC, 63.80% for non-ATC seaweed and 64.43% for the SRC, respectively. The percent of carrageenan content from Arakan Village was the lowest, 44.43% for non-ATC and 52.55% for SRC, respectively, while Talengen Bay had 46.14% for non-ATC and 57.16% for SRC. Alkaline treatment (SRC) can increase the percent of carrageenan. *K. alvarezii* extract is known to have bioactive ingredients as immunostimulants. The best antioxidant activity was recorded in the extract from Arakan Village with $IC_{50}$ value of 22.04 μg $mL^{-1}$, followed by Talengen Bay, 27.6 μg $mL^{-1}$, and NSMEC, 28.45 μg $mL^{-1}$, respectively. The $IC_{50}$ value is very strong because it is <50μg $mL^{-1}$.
**Key Words**: fish immunity, semi refined carrageenan, carrageenan, $IC_{50}$.

**Introduction**. Aquaculture business that has become an industrial activity has encouraged producing good quality aquaculture product. The current intensive cultivation system can trigger the emergence of serious problems related to environmental degradation which result in attacks of both infectious and non-infectious diseases in cultured fish. Preventive measures and optimal disease control can be done by increasing the fish body resistance through immune-simulants. These are substances that can activate cells of non-specifically defensive mechanisms against infections. Immunostimulants can be obtained from nature, one of which is seaweed. *Kappaphycus alvarezii* is a type of seaweed that has been widely cultivated in Indonesia because it is highly needed in various food and non-food industries including the pharmaceutical industry.

*K. alvarezii* is carrageenan-producing tropical seaweed, which is only found in the Philippines, Indonesia, Malaysia, Vietnam and several other tropical countries. The Philippines produces about 50% of all carrageenophytes in the world, and other 25% are produced by Indonesia. Seaweed is a multicellular alga containing an immunologically active substance. The use of seaweed so far is still limited to carrageenan and agar products. Marine algae are rich in sulfate polysaccharides (SPs), such as carrageenan found in red algae, and have many beneficial bioactive compounds as anti-coagulant, antiviral, antioxidant, anticancer and immune modulation activators (Wijesekara et al 2011). According to Ridlo & Paramesti (2009), polysaccharides are known to be essential components for all organisms and have various biological vital functions, such as antitumor, anti-inflammatory, anticoagulant, anticomplementary, immunostimulant and antiviral. The potential of seaweed in the field of disease control is still not much explored and exploited. Several studies have shown that seaweed has prospects that are still open to its development in relation with disease control.

Several types of polysaccharides have been known to be very important in the formation of immune cells (Hou & Chen 2005). Immunostimulants are an alternative use of vaccines and antibiotics as a protection against fish diseases. Polysaccharides from red algae (carrageenan) can increase phagocytic macrophage activity and are able to fight against bacterial infections in fish (Castro et al 2004). The administration of seaweed extract has increased the number of hemocytes and phagocytic activity of shrimp *Penaeus vannamei* (Ridlo & Paramesti 2009).

Carrageenan is obtained through the extraction process of algae *K. alvarezii* (Duduku et al 2008; Campo et al 2009). The chemical structure of the carrageenan is distinguished by its type, Kappa-carrageenan, iota-carrageenan, and lambda-carrageenan. Kappa carrageenan is the most abundant species in nature produced by *K. alvarezii* (Reen 1986). *K. alvarezii* contains carrageenan in the kappa-carrageenan group with a relatively high content, about 50% of the dry weight (Winarno 1996).

Carrageenan is the main constituent of the polysaccharide of *K. alvarezii* cell wall. The amount varies because it is influenced by ecological factors, such as light, nutrient, surface waves and temperature. According to Pamungkas (1987), the content of chemical compounds in marine algae is influenced by season, habitat and plant age. Good seaweed growth will produce good quality carrageenan, and the quality is influenced by water quality, while water pH and temperature affect the yield. The viscosity is influenced by salinity, the gel strength is influenced by nitrate concentration, and the ash content is affected by phosphate (Kreckhoff et al 2015).

North Sulawesi is a producer of *K. alvarezii,* but it has not been optimally utilized yet. For this reason, the quality of *K. alvarezii* extract cultivated in 3 locations was studied in order to use as immune-stimulant. The extracts were compared in the form of ATC (Akali Treatment Carrageenan) or semi refined carrageenan (SRC) and non-ATC.

## Material and Method

***Research sites***. The location of the study was determined by simple random sampling (Clark & Hosking 1986 in Radiarta et al 2013). The determination of the culture location is done by taking a certain distance from the coastline towards the sea estimated to be representative and taking into account of tidal factors, so that the seaweed stocked was submerged in the water and not directly exposed to sunlight when receding occurs. The protection factor was also considered because the waters with too high waves are not suitable, because they can damage the cultivation infrastructure. The existence of seaweed-eating pests was also considered to ensure the achievement of the research objectives.

The cultivation was carried out for 6 weeks at 3 centers of seaweed production in North Sulawesi, i.e. Talengen Bay, Sangihe Islands Regency, Arakan Village, South Minahasa Regency and North Sulawesi Marine Education Center (NSMEC) area, Likupang, North Minahasa Regency. Harvesting age was determined based on previous research to get the best seaweed harvest, after 40 planting days (Wenno et al 2012) or 45 days of planting (Marseno et al 2010).

The profile of the research location used as a cultivation site for *K. alvarezii* is shown in Figure 1.

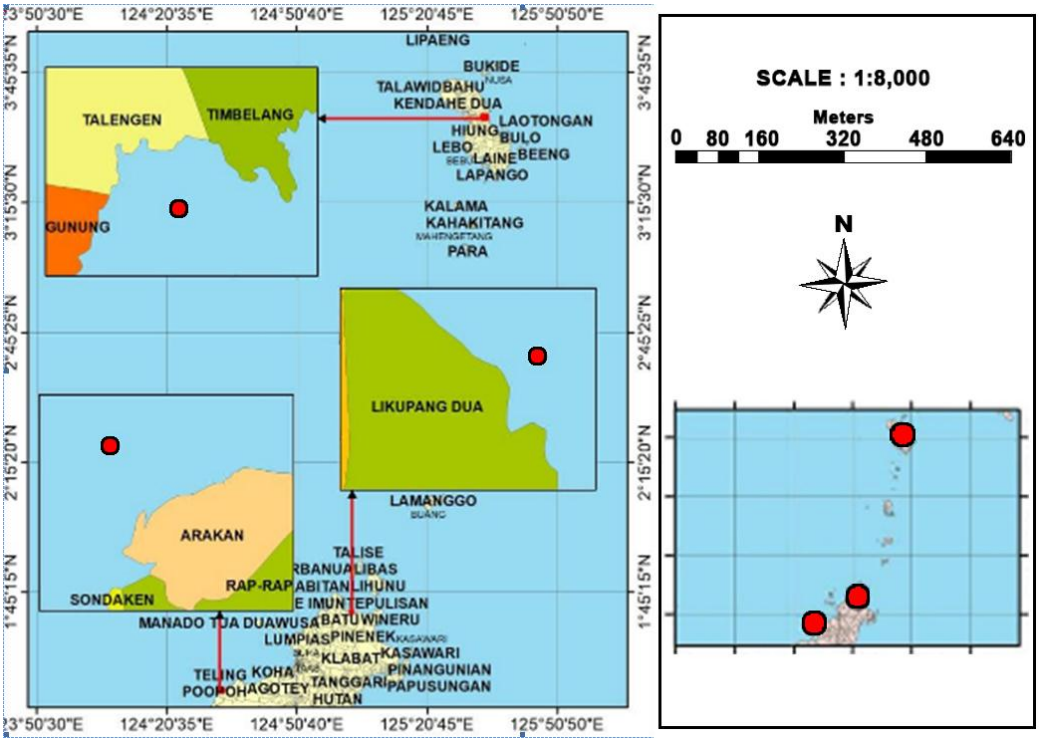

Figure 1. Map of research locations. Red dots are culture sites where the samples were taken.

***Production of semi refined carrageenan (SRC)***. The harvested seaweed was sun-dried for 3 days. Some were treated with alkali (Alkali Treatment Carrageenan, ATC) to produce semi carrageenan (Semi Refined Carrageenan, SRC). The dry seaweed was soaked for 30 min., washed and cooked in potassium hydroxide solution (8% KOH) for 2 hours at 80°C. It was then cooled at room temperature and washed with distilled water to remove KOH and dry it again to characterize carrageenan analysis.

***Yield***. Five grams of seaweed were added with dilute NaOH solution until all the seaweed was submerged ±100 mL and the sample pH was around 8.5-9. The sample was heated at 70-90°C for 3 hours. The gel solution was filtered hot with gauze using Filtering Flash and a Vacuum pump containing ±25 mL of Isopropyl alcohol. The filtrate was stored in petri dish heated in an oven at 60°C for 24 hours. After the cool, the petri dish is weighed The carrageenan was then estimated as:

$$\text{Carrageenan \%} = \frac{(\text{Empty cup + sample after heating}) - \text{The weight of empty cup}}{\text{Sample weight}} \times 100$$

***Antioxidant extraction***. Extraction of antioxidant components was carried out according to Gulcin et al (2004). One gram of sample was mixed with 400 mL of ethanol and heated to 70°C for 2 hours while stirring. The mixture was then filtered through a vacuum filter using whatman filter paper No. 1. Ethanol was evaporated in vacuum evaporator at 50°C until the concentrated extract was produced. The extract was trasferred in a dark bottle and stored in freezer.

***Antioxidant activity test***. Testing was carried out using DPPH method according to Gulcin et al (2004). Antioxidant activity was determined by measuring the DPPH free radical concentration as follows: 1 mL of sample extract was added with 1 mL of DPPH trace (0.16 g $L^{-1}$) in methanol then shaken. The absorbance was measured after 30 min. at room temperature at a wavelength of 517 nm. As blank, methanol was used in the same way. For $IC_{50}$, a standard of 0.125, 0.500, 0.750 mL was made from the sample.

$$\text{DPPH inhibitor activity (\%)} = \frac{A0 - A1}{A0} \times 100\ (\%)$$

Where: A0 = absorbent control and A1 = absorbing sample.

## Results and Discussion

***Water quality***. The representative research location was randomly determined by considering the physical, chemical, and biological factors. These are very important for the seaweed growth and the carrageenan quality. The measurements of water quality, such as temperature, brightness, current velocity, depth, pH, salinity, nitrate ($NO_3$), and phosphate in 3 research locations indicated that all parameters met the requirements for the location of *K. alvarezii* cultivation.

Temperature is an important physical factor, and its change tends to affect many chemical processes that occur simultaneously in plant and animal tissues and could affect the biota as a whole. Water temperature can affect several physiological functions of seaweed such as photosynthesis, respiration, metabolism, growth and reproduction (Ain et al 2014). High temperature can increase the photosynthetic rate since the photosynthesis-regulating of the plant will work optimally. Seawater temperature is influenced by sunlight, depth, currents and tides (Poncomulyo et al 2006). Water temperature range for the growth of *K. alvarezii* is 27–30$^0$C (Setiyanto et al 2008; Parenrengi et al 2006), with a maximum daily fluctuation of 4$^0$C and can increase carrageenan content (Lundsor 2002). This is supported by Yunque et al (2011) that the growth triggering substances contained in the culture media will work more optimally if is supported by the right pH and temperature conditions.

Water brightness is a physical factor measuring the sunlight penetration into the waters. Light penetration could be influenced by number of suspended particle contents due to tides and depth level. Ideal water brightness is more than 1 m. Water turbidity (usually containing mud) can block the penetration of sunlight into the water and disrupt the photosynthesis. Optimal depth for seaweed growth is 3.2-7.8 m (DGEC 2009).

Currents can carry nutrients which are food for thallus seaweed. The greater the movement of water is, the higher the diffusion will be, and it could accelerate the plant growth. Currents play a role in the acquisition of food for marine algae, because they can carry the nutrients they need (Munoz et al 2012). Adequate currents have a positive effect on seaweed thallus growth (Akmal et al 2007). If the current coming to each part of thallus is the same, the chance to grow will be the same for the thallus on the edge or in the middle part of the seaweed. The greater the movement of water is, the higher the diffusion will be, and it will accelerate the metabolic processes resulting in faster plant growth. In addition, the current functions to homogenize the water mass so that fluctuations in salinity, temperature, pH, and dissolved substances could be avoided.

Water pH is needed in biochemical processes in the waters. The appropriate pH for cultivation of *K alvarezii* ranges from 8 to 8.3 and pH less than 8 will negatively affect the quality of carrageenan. Farid (2008) found that the range of pH for *K. alvarezii* growth was 7.3-8.2. The better the growth is, the higher carrageenan content will be (Munoz et al 2012). According to Effendie (2003), pH plays a role in the nitrification process to provide nitrogen for seaweed growth, and it will not occur in low pH.

Salinity affects the distribution of macroalgae in the ocean. *K. alvarezii* is stenohaline, which is susceptible to large salinity fluctuations. Most macroalgae or seaweeds have a low tolerance to salinity changes (Yuliana et al 2015). Optimum salinity could make the seaweed grow optimally, because the functional balance of the cell membrane could be maintained, especially in the osmotic regulation in the seaweed and its environmental fluids. This balance will facilitate the nutrient absorption of nutrients to support photosynthesis, so that the seaweed growth could be optimal (Sutresno & Prihastanti 2003). The salinity measurements at the study sites showed very small fluctuations, NSMEC 0.3 ppt in NSMEC, 0.2 ppt in Arakan, and 0.03 ppt Talengen, respectively. Decline in salinity could occur due to high rainfalls or due to the entry of

freshwater from the river. For this reason, the cultivation location should be far from the freshwater sources to avoid drastic salinity decline. Extreme changes in salinity could cause ice-ice. Different concentrations between fluids inside and outside the cell could encourage Golgi to keep trying to balance up to being isotonic with consequence of greater energy utilization that slow down the seaweed growth. According to Choi et al (2010) in Yuliana et al (2015), water quality parameters playing a major role in growth, thallus, color, and morphogenetic development, are salinity, because they are directly related to osmoregulation in the cells.

Nitrate is needed for seaweed growth, production and formation of food reserves in the form of carbohydrates, proteins, fats and other elements (Hayashi et al 2008). The salinity in 3 cultivation locations ranged from 0.59 to 3.12 mg $L^{-1}$, and thus, it met the location criteria, 0.9-3.5 mg $L^{-1}$ (Sulistijo 2002). Phosphate deficiency will be more critical for aquatic plants including algae plants than lack of nitrates in the waters. Phosphate range of 0.01-0.08 mg $L^{-1}$ in 3 cultivation sites gives good growth. Based on orthophosphate levels, waters are classified as oligotrophic waters with orthophosphate levels from 0.003 to 0.01 mg $L^{-1}$, mesotrophic waters with orthophosphate levels of 0.011-0.03 mg $L^{-1}$ and eutrophic waters with orthophosphate levels of 0.031-0.1 mg $L^{-1}$ (Hakanson & Bryann, 1994 in Sanusi 2006). The measurements of water quality parameters are presented in Table 1.

Table 1

Water quality parameters at the research locations

| *No.* | *Parameters* | *Location* | | | *Feasibility according references* |
|---|---|---|---|---|---|
| | | *Likupang* | *Arakan* | *Talengen* | |
| 1 | Temperature (°C) | 30.1–31.4 | 31–31.2 | 29.1-29.77 | 20–33[a] |
| 2 | currents (m/sec) | 20–40 | 20–40 | 20–40 | 0.2–0.4[b] |
| 3 | Brightness (m) | 5–6.44 | 2–4.5 | 4.7–7.5 | 1–5[a] |
| 4 | Depth (m) | 5.0–9.0 | 2–9.5 | 4.7–13.5 | 2–15[b] |
| 5 | pH | 8.03-8.19 | 7.7–8.1 | 7.52–8.0 | 6–9[c] |
| 6 | Salinity (ppt) | 29.1–31.9 | 32.5–33 | 30.37–30.9 | 28–33[b] |
| 7 | Nirat (mg $L^{-1}$) | 2.16–2.54 | 3.02–3.12 | 0.58–0.60 | 1.0–3.2[a] |
| 8 | Phosphate (mg $L^{-1}$) | 0.01–0.02 | 0.08–0.08 | 0.01-0.05 | 0.021–0.1000[a] |

Note: a. BSN 2011, b. DGEC (2009), c. Atmadja et al (1996).

***Carrageenan quality***. The quality of *K. alvarezii* seaweed is based on the content of carrageenan extract. The carrageenan analysis on the seaweed cultivation in the 3 study locations found 44.43-63.80%, and even 52.55-64.43% in SRC form. These results meet the carrageenan standard set by the Ministry of Trade in 1989, 25% (Karyani 2013), and the higher standard set by FAO (2007), 40%. Previous study was also carried out on carrageenan analysis of *K. avarezii* cultivated in Minahasa Peninsula and found the yield range of 30.94-55.31% (Kreckhoff et al 2015). Thus, the overall carrageenan quality of *K. alvarezii* in North Sulawesi meets the standards of the trade department and even the carrageenan quality in these 3 research locations has met the FAO (2007) standard and is very potential to be utilized as food, including medicines (Figure 2). This quality extract is supported by the suitability of water quality in the cultivation centers. The chemical composition of the seaweed varies with individual, species, habitat, maturity and environmental conditions (FDA 1994). The quality of kappa-carrageenan is influenced by processing method, production process, climate and geography including sunlight, currents, pressure, water quality and salt content, and the rearing location (Ferdiansyah et al 2017).

Based on Figure 2, the highest quality of *K. alvarezii* extract was obtained from the cultivation area of NSMEC Likupang, 63%, for non-ATC extract and 64.43% for ATC or SRC.

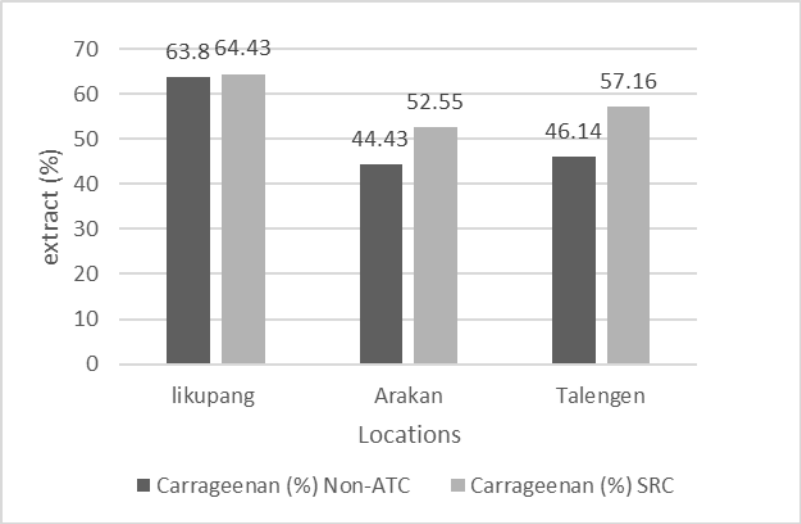


Figure 2. Percent carrageenan content of *Kappaphycus alvarezii*.

***DPPH free anti-radical activities***. Carrageenan extract as an illustration material has been tested for antioxidant activity by measuring $IC_{50}$ values (Figure 3). The present study found that the $IC_{50}$ value was very strong with $IC_{50}$ <50. It is in agreement with Badarinath et al (2010) that strong antioxidant is indicated with $IC_{50}$ range of 50-100, moderate in the range of 100-150, and weak in the range of 151-200, respectively. The smaller the $IC_{50}$ value is, the higher the antioxidant activity will be. This test used DPPH solution to measure the absorption level of active ingredients. The uptake value of DPPH solution of the carrageenan extract was calculated as percent of absorption. The anti-free radical testing found the best value in carrageenan extract from Arakan with the lowest absorption rate of 22.04%, followed by Talengen, 27.6% and Likupang 28.45%. This result is in contrast to the best value of the yield from Likupang NSMEC while the $IC_{50}$ value shows a lower ability than the other 2 samples. This proves that the anti-free radical properties are not related with the yield concentration but the entire samples of *K. alvarezii* extract in the 3 cultivation centers are very strong in counteracting the free radicals.

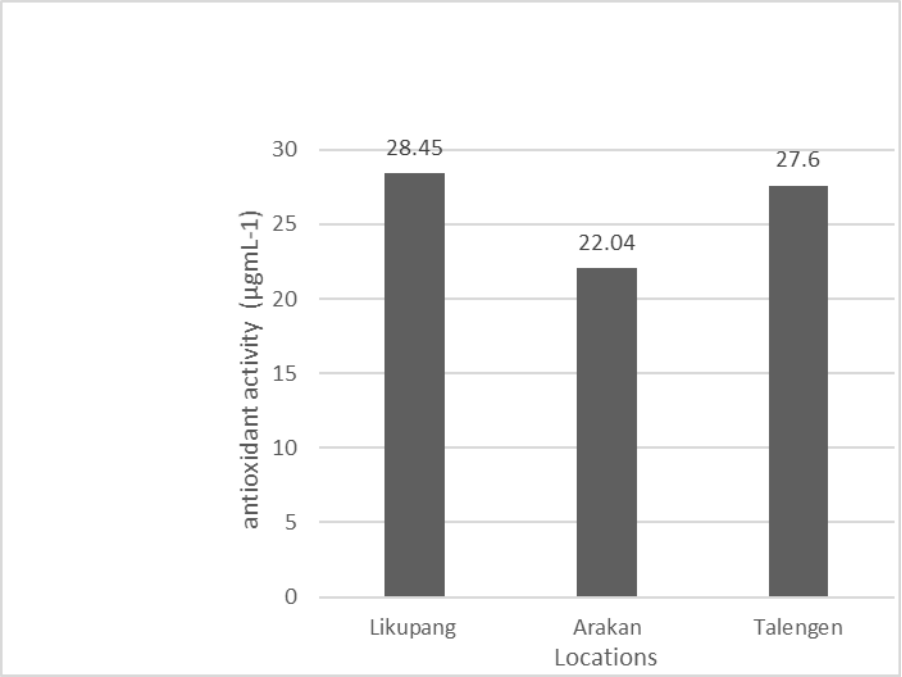


Figure 3. $IC_{50}$ antioxidant activity test.

Antioxidant activity describes the ability of the active ingredients contained in the organism's body to capture the free radicals. Phenolic or polyphenolic compounds, such as flavonoids, have the ability to reduce free radicals and also work as anti-free radicals (Giorgio 2000 in Zuhra et al 2008). The antioxidants can naturally occur in the body as normal body defense mechanisms and be derived from the intake outside the body. One of the antioxidant compound sources is plants with high polyphenol compounds. Thus, *K. alvarezii* extract could be used as an immunostimulant intake to increase the immune system, including fish.

**Conclusions**. Water quality at the study sites was eligible in *K. alvarezii* cultivation. The carrageenan concentration of *K. alvarezii* from Likupang NSMEC was the highest, 63.80%, while that in Talengen Bay was 46.14% and in Arakan Village 44.43%. With alkaline treatment, the percent of carrageenan became higher. Also, carrageenan extract could be used as an immunostimulant because the antioxidant activity was very strong with $IC_{50}$ <50µg $mL^{-1}$, 22.04 µg $mL^{-1}$ for Arakan, 27.64 µg $mL^{-1}$ for Talengen Bay, and 28.45 µg $mL^{-1}$ for NSMEC Likupang, respectively.

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