

Preliminary Characterization of Crude Lectins Fractions of Red Macroalgae Species Collected from the Southern Coast of Gunungkidul Indonesia

Choirul Anam^{#*}, Danar Praseptiangga^{*}, Nurrahmi Dewi Fajarningsih⁺, Ekowati Chasanah⁺, Ahmad Yunus[§]

[#] Graduate School Program of Sebelas Maret University (UNS), Kentingan, 57126, Surakarta, Indonesia
E-mail: choiroelanam@staff.uns.ac.id

^{*} Department of Food Technology, Sebelas Maret University (UNS), Kentingan 57126, Surakarta, Indonesia.
E-mail: dpraseptiangga@staff.uns.ac.id

⁺ Research Center for Marine and Fisheries Product Processing and Biotechnology,
Ministry of Marine Affairs and Fisheries, Jakarta, Indonesia.
E-mail: ^{#3}ekowatichasanah@gmail.com; ^{#4}nurrahmi_dewi@yahoo.com

[§] Department of Agrotechnology, Sebelas Maret University (UNS), Kentingan 57126, Surakarta, Indonesia.
E-mail: yunus.uns7@yahoo.com

Abstract— Lectins or hemagglutinins are specific sugar-binding proteins (glycoproteins) of non-immune origins that typically agglutinate certain cells and/or precipitate glycoconjugates. This study aimed to evaluate the activity of hemagglutination, protein content, and initial characterization (the stability towards pH, temperature, divalent cation and inhibition of hemagglutination activity by sugars and/or glycoproteins) crude lectins fractions of red macroalgae from the coast of Gunungkidul Beach, Yogyakarta. Crude lectins fractions was taken from red macroalgae extracted by phosphate buffer saline (PBS) pH 7 at 4 oC for over night. Precipitation using ammonium sulfate to reach 75% saturation, precipitates that have been dissolved and then put into a dialysis bag with a cut off molecular weight of 10 kDa. The dialysis process lasts for eight hours by replacing the dialysis solution (PBS pH 7) every two hours. The result of dialysis called as crude lectins fractions. The findings showed that hemagglutination activity using trypsin treated rabbit erythrocytes of crude fractions of red algae lectins *Nitophyllum punctatum* species, *Helminthora divaricata*, and *Acanthophora spicifera* were 26, 26, and 213, respectively. The protein contents were 8,123.47 µg / ml; 9,545.65 µg / ml; and 7,368.45 µg / ml. Although the three red macroalgae species tend to maintain a relatively stable pH, their temperature tolerance was unstable, and were unaffected by divalent cations. The carbohydrate-binding specificities assay of the red macroalgae species showed the occurrence of inhibition in certain glycoproteins and not simple sugars.

Keywords— red macroalgae; lectin; crude fraction; hemagglutination; preliminary characterization.

I. INTRODUCTION

Indonesia is a mega biodiversity country which famously known for its macroalgae species richness [1]. This diverse marine life is the source of rich biological compounds [2] and has the potential to be developed into a variety of active ingredients from the sea which can then add value to Indonesia's natural resources. Red macroalgae primarily from the genus *Eucheuma*, *Gracilaria*, and/or *Kappaphycus* have been widely developed in several regions of Indonesia [3]. The biodiversity prospecting keeps continue to develop commercially-valuable products for pharmaceutical,

cosmetic and other applications which has potential economic value for Indonesia [4].

Compared to green and brown algae, red algae (*Rhodopyta*) contain more primary and secondary metabolite compounds. Red algae (*Rhodopyta*) are well known as producer of phycocolloids such as agarose, agar, carrageenan, and a great variety of secondary metabolites [3] such as flavanoid, alkaloids, terpenoids, α -tocopherol β -carotene, and β -apo-8'-carotenal, as antioxidant free-radical scavengers [1]. The rich potential of red algae in Indonesia has provided a good seaweed-based industry prospect and promises to be developed. Nutraceuticals produced from

marine derivative products, including those from macroalgae (*seaweed*), are an alternative source of ingredients which has a very important function on human health and nutrition [5], [6], [7].

Due to its composition, red algae have the capacity for use in functional foods. Moreover, a high variety of bioactive metabolites was obtained from red algae [8], such as brominated phenols, nitrogen heterocyclics, brominated oxygen heterocyclics, kainic acids, phenazine derivatives, guanidine derivatives, amino acids and amines, polysaccharides sulfate, sterols, and prostaglandin [9]. An increasing amount of studies show great of interests on lectins, one of the bioactive compounds of algae. While lectin (hemagglutinin) is widespread in nature, algae are good candidates as a new source of lectins. Lectins possess the ability of binding carbohydrates and cell agglutination. It is often used as a tool to demonstrate glycans (carbohydrates) structure differences and numerous biological activities associated with its binding and interaction with glycans. [5], [10], [11]. This suggests that lectins can be used as one of tools in glycomics and in medical fields [5]. Other functions of lectins are in phenomenon related to the binding specificity of carbohydrate, cellular communication, self-defense, fertilization, cell development, apoptosis, and anti-inflammatory [12].

The term "lectin" was derived from the Greek word "legere" which means "to select" or "to bind". Lectins are specific carbohydrate-binding proteins (glycoproteins) of non-immune origins that typically agglutinate certain cells and/or precipitate glycoconjugates, which have at least two sides of binding, agglutinate animal cells (especially erythrocytes, with or without enzyme treatment) and/or precipitate polysaccharides, glycoproteins, and glycolipids [10], [13].

Each molecule of lectin usually has two or more carbohydrate-binding sites. Lectins are usually multivalent and can bind to clusters of carbohydrates on the surface of erythrocytes and erythrocyte agglutination. In general, lectin-carbohydrates interactions are similar to antibody and antigen mechanisms. Lectins can be used to study immunological, cell biology, membrane structure, mitogenic activity, antitumor activity, and cancer [14]–[16].

Macroalgal lectins molecular mass are much lower than those derived from land plants. Moreover, they appear to have no affinity for simple sugars and strong specificity for complex oligosaccharides and glycoproteins [17], [18]. These characteristics show the potential of lectins of macroalgae to be used in future applications, such as specific probes, biomarker, and drug delivery/targeting systems. Since it has low molecular weight, it is expected to have a smaller antigenic effect [18]. Lectins from macroalgae are generally monomeric with isoelectric points from 4 to 6, and have a proportion of acidic amino acids. Furthermore, there are no divalent cations requirement for their biological activity [18].

The first study of the occurrence of algal lectins was conducted by Boyd *et al.* [19], which utilized human erythrocytes for screening. Then [20], [21], show the existence of algae lectins using animal and human erythrocytes (A, B, O, and AB). Hori *et al.* [22] reported that hemagglutination activity is more easily detected when

erythrocytes are enriched enzymes. It has been widely reported that algal lectins are distributed in different regions, such as in Japan [11], [13], [17], [23], [24] American [25], [20], [21], Brazil [14], [26]–[29], China [30], Antarctica [31], [32], Algeria [33], Vietnam [34], Belgium [35], India [36], [37], and more than 250 algal species reported to contain lectins [38].

Marine algae, especially the red macroalgae are promising sources of novel lectin molecules [37]. This study examines the activity of hemagglutination and initial characterization (the stability towards pH, temperature, divalent cation and inhibition of hemagglutination activity by sugars and glycoproteins), crude fractions of red macroalgae lectins from the coast of Gunungkidul Beach, Yogyakarta. To the best of our knowledge, there had not been any research that studied the lectins from red macroalgae in coastal waters of the south coast of Gunungkidul, Daerah Istimewa Yogyakarta, Indonesia. The present study aimed to initiate the study of lectins from red macroalgae obtained from the southern coast of Gunungkidul, Yogyakarta. It is also expected that the study could provide information on the usage of several species of red macroalgae as a source of active compound from marine products, mainly lectins.

II. MATERIALS AND METHODS

A. Materials

Red macroalgae samples were collected from the coastal coast of Gunungkidul, Yogyakarta, Indonesia. The algae were cleaned, washed, transferred to freezer in the laboratory, and then protected at -20°C for advanced use. Rabbit blood erythrocytes was obtained from Balai Breeding and Cultivation of Non-Ruminant Cattle of Central Java Province in Solo, Indonesia. Sodium dihydrogen phosphate (NaH_2PO_4), di-Sodium Hydrogen Phosphate (Na_2HPO_4), Ammonium sulfate ($(\text{NH}_4)_2\text{SO}_4$), Sodium chloride (NaCl) from Merck, Germany.

The BCA Kit Assay reagent from Thermo Fisher ScientificTM, USA was used. Trypsin enzyme, D-glucose, D-mannose, D-galactose, L-fucose, L-rhamnose, D-xylose, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, and Lactose used in this study were from Sigma-Aldrich, USA. While the glycoproteins used were Transferrin, Asialo-Transferrin, Yeast mannan, Bovine Thyroglobulin (BTG), Asialo-BTG, Bovine Submaxillary Mucin (BSM), Porcine Thyroglobulin (PTG), Asialo-PTG, Asialo-BSM, Fetuin, Asialo-Fetuin (Sigma-Aldrich, USA). Acetate, Tris-HCl, Sodium carbonate, Sodium bicarbonate, EDTA, Magnesium chloride (MgCl_2), Calcium chloride (CaCl_2), were from Sigma-Aldrich, USA. Wholly the chemicals used in each stage of this study have the highest purity levels.

B. Methods

The extraction process in this study modified the method employed by Praseptiangga *et al.* [11]. 100 g of algae was defrosted and then cut into pieces. The size was further reduced by using blender and with the addition of liquid nitrogen. The macroalgae powder was then added a phosphate buffer of 0.02 M pH 7 containing 0.85% NaCl (phosphate buffer saline / PBS) at a ratio of 1: 2 (w / v).

Afterwards, the solution was homogenized by using the stirrer at 4°C.

Centrifugation was then performed at 8000 rpm for 30 min at 4°C. The result of centrifugation process was taken its supernatant to be precipitated to produce 75% saturation with the addition of ammonium sulfate. The supernatant was kept overnight at 4°C and re-centrifuged (8000 rpm) for 30 min at 4°C. The recovered precipitate was reconstituted by adding limited amount of buffers. It was then dialysed using PBS for overnight with PBS replacement treatment 4 times every 2 hours. Then, the inner fraction was centrifuged (10,000 rpm) for 30 min at 4°C. The supernatant obtained from this centrifugation will be used as an analysis material called a salting-out fraction and stored at -20°C before being tested.

1) *Hemagglutination assay*: The hemagglutination activity of the crude fraction of lectins was tested with rabbit erythrocytes, each of which was not treated with the enzyme (non-treated) and treated with trypsin-treated enzymes, as performed by Hori et al. [39]. To perform the analysis of hemagglutination activity, 96-well microtiter V-plate with two-fold dilution was used. Native (non-treated) and trypsin-treated rabbit erythrocytes were added into each well. Afterwards, it was incubated for 1 hour at room temperature. Hemagglutination was observed macroscopically and judged as positive in the case that more than 50% of erythrocytes in the well were agglutinated. Hemagglutination activity was expressed as a titer, the reciprocal of the highest two-fold dilution exhibiting positive hemagglutination [34].

2) *Protein contents*: The protein content was determined by the BCA Protein Assay Kit method using 96-well microplate flat bottom [40]. This protein content test used 9 standard proteins of 25 µl each inserted into 18 wells (two replicates). While for the other wells, 25 µl sample were inserted, each repeated twice. All wells were then given 200 µl working reagent (reagent BCA) (Thermo Fisher Scientific TM, USA) and incubated for 30 minutes at 37 ° C. The reaction after contacting with the BCA will proceed at room temperature, but if incubated at higher temperature acceleration in the colour development is seen [40]. There is no reaction to stop the colour development, it will continue as long as there are reagents present. Therefore, each test should always include a standard curve that is created in parallel with the test sample on each analysis to minimize errors and precise accuracy [40]. Klarbring states that the reaction will take place at room temperature. However, if incubation is carried out at higher temperatures, the intensity of the color enhancement also remains visible. The flexibility has put advantage of using BCA analysis. The protein content was obtained by use of a UV-vis spectrophotometer at a wavelength of 562 nm.

3) *Initial Characterization of the Lectins Crude Fractions*: Initial characterization was conducted by using Praseptianga et al.'s method [11]. The stability towards pH was determined by conducting dialysis 500 µl of test sample for one night with 100 ml 50 mM buffer with pH numbers at room temperature of 4°C ranging from 3-10. Then, we performed the dialysis with PBS for 8 hours at 4°C with buffer replacement every 2 hours. The samples'

hemagglutination activity were tested by involving positive, negative, and serial dilution controls. The temperature stability was determined by incubating 500 µl of the test solution for 30 minutes at temperature ranging between 30°C to 100 °C. Subsequently, the cooling process and hemagglutination assay were performed. Divalent cation's stability was established by performing dialysis to 500 µl of the test solution at 4 °C for 8 hours towards EDTA in PBS. The examination of hemagglutination activity on internal fraction obtained utilized three treatments, namely without adding divalent cation (Ca²⁺ dan Mg²⁺), by adding CaCl₂, and by adding MgCl₂. Positive, negative and dilution controls were included on the analysis.

4) *Hemagglutination-Inhibition assay*: The test of hemagglutination-inhibition involved both qualitative and quantitative methods. On the qualitative tests, 25 µl of saline was inserted in well, by adding 25 µl of the crude fraction of lectins and 25 µl of sugars or glycoproteins which were incubated for one hour at room temperature, plus 25 µl TRBC, and a macroscopic observation was performed after incubation at room temperature for one hour. The result of inhibitory activity is used to express the lowest concentration of sugar or glycoprotein capable of inhibiting erythrocyte haemagglutination perfectly. [34]. Positive results are shown by the formation of the dot, while the negative results with the formation of the carpet. The positive result of this qualitative test initiated a follow-up quantitative test regarding its inhibitory activity.

For the quantitative test of the hemagglutination-inhibition, 25 µl of saline were added into the well. Subsequently, 25 µl of glycoprotein were added and diluted. Afterwards, 25 µl lectins was added which then incubated at room temperature for 1 hour. Thereafter, 25 µl TRBC was added, then incubated at room temperature for 1 hour, and observed macroscopically. The sugars used in the test was D-glucose, D-mannose, D-galactose, L-fucose, L-rhamnose, D-xylose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, and Lactose. While the glycoproteins used was Tranferin, Asialo-Transferrin, Yeast mannan, Bovine Thyroglobulin (BTG), Asialo-BTG, Porcine Thyroglobulin (PTG), Asialo-PTG, Bovine Submaxillary Mucin (BSM), Asialo-BSM, Fetuin and Asialo-Fetuin.

III. RESULTS AND DISCUSSION

A. Hemagglutination Assay

Through the screening process of red algae collected from the coastal coast of Gunungkidul Regency, Yogyakarta, Indonesia, 9 types of red macroalgae were identified. The identification of red macroalgae was conducted by Department of Biology Education, Faculty of Teacher Training and Education, Sebelas Maret University Surakarta Indonesia, and Indonesian Institute of Sciences, Center for Plant Conservation Botanic Gardens, Bogor Indonesia. To get the crude fractions of lectins, extraction was performed and hemagglutination assay was conducted as a routine assay to detect the presence of hemagglutinins (lectins) by their hemagglutination activities. Rabbit erythrocytes are more well-suited than erythrocytes from other animals in terms of agglutinins screening [40]. The highest

hemagglutination activity by with a titer of 2^{13} was shown by the crude fraction of *Acanthophora spicifera* lectin with trypsin enzyme treatment in rabbit erythrocytes used in hemagglutination assay.

In general, treatment with trypsin enzyme showed an increase in hemagglutination activity of crude fractions of red algae lectins (Table 1). The change in the normal structure of cell surface came as a result of the treatment of trypsin enzymes in erythrocytes, which then affected the change of the binding-site lectins in red blood cells.

As a result, there was a possibility of significant decrease on the lectin's ability to recognize binding sites and bind glycoproteins on the surface of red blood cells treated with protease enzymes [10]. Fernandes states that erythrocyte treatment with protease enzymes is often used to remove glycoprotein fragments from RBC membranes, thus enhancing the proximity of RBC and improving access between antibodies to antigens in the blood [41]. Sometimes lectin is not detected due to steric hindrance in the lectin-carbohydrate interaction. As a result, the pretreatment with enzymes in erythrocytes is required for hemagglutination testing [42].

TABLE I
HEMAGGLUTINATION ACTIVITY OF CRUDE LECTINS FRACTIONS OF RED MACROALGAE

No	Name of Species	Native erythrocytes	Trypsin-Treated erythrocytes
1	<i>Acanthopora muscoides</i>	2^4	2^2
2	<i>Nitophyllum punctatum</i>	2^3	2^6
3	<i>Helminthora divaricata</i>	2^5	2^6
4	<i>Gelidiella acerosa</i>	0	0
5	<i>Jania longifurca</i>	2^4	2^4
6	<i>Acanthophora spicifera</i>	2^7	2^{13}
7	<i>Hypnea pannosa</i>	2^2	2^3
8	<i>Gelidium robustum</i>	0	0
9	<i>Digenea simplex</i>	0	0

Lectins agglutinated because of the characteristic property of polyvalent proteins along with their affinity towards specific carbohydrates on the cell surface [16]. The examination of hemagglutination assay in each alga can reveal the hemagglutination activity shown by different titer. The rationale behind this phenomenon is the differences in algae species and climatic factors such as sampling time as well as the intensity of sunlight and different ocean currents [43]. Moreover, the sampling method contributed to the difference. When the harvesting was done through the hard way, it can prematurely damage the walls and cell membranes thus affecting the lectins obtained.

It is advisable that liquid nitrogen was used for the plant tissue extraction process, liquid nitrogen plays an important role in obtaining good extraction quality and hence, it has been used extensively for extraction from fresh leaf and other tissues. The most appropriate extraction comes from 100 μ m powder [21]. In this study, several red macroalgae from species which were tested for their hemagglutination activity show the following outcomes: *Eucheuma denticulatum* 2^9 ; *Grateloupia filicina* 2^7 ; *Halymenia*

maculata 2^6 ; *Hypnea boergesenii* and *H. Nidulans* 2^5 ; *H. Valentiae* 2^4 ; [34] *Hydropuntia eucheumatoides* 2^8 ; *H. Edulis* 2^4 ; *H. spinella* 2^9 [38]; *H. japonica*, *H. Cervicornis*; *H. musciformis*, *H. Boergesenii*; *H. Nidulans*; and *H. valentiae* have titers 2^8 , 2^3 , 2^7 , 2^5 , 2^5 , and 2^4 [15], [34], [43]. Different species of the same genus can be distinguished by the presence or lack of agglutinin activity through different animal erythrocytes [21].

B. Protein Contents

In the last fifty years, comprehensive analysis and results of nutrition research have shown that proteins from algae are comparable to conventional vegetable proteins and have high quality. So mass production has been made in certain high-protein content to close predictions called "protein gaps" [44]. Protein content have been significantly demonstrated by some macroalgae species. This proves that high protein from macroalgae, can be made for ideal initial steps and promising macroalgae as the representative source of marine protein-derived for the formation of bioactive peptides. [45]. Macroalgae species have varying protein content (3-47%(w/w) dry weight. In general, red macroalgae species have higher protein content than green and brown algae species. Algal peptide is shown to have properties such as antibacterial, opioid, immuno-modulatory, antihypertensive and antithrombotic activity [46]. Galland-Irmouli *et. al* stated that red seaweed contains high protein content [43]. Since most of red and green seaweeds have protein contents higher than 20%, they possess high commercial values. However, the large number of polysaccharides present in the cell walls hampered the dissolution during the extraction process [47].

TABLE II
PROTEIN CONTENTS OF CRUDE LECTINS FRACTIONS

No.	Name of Species	Protein Content (μ g/ml)*
1.	<i>Acanthopora muscoides</i>	2,228.05
2	<i>Nitophyllum punctatum</i>	8,123.47
3.	<i>Helminthora divaricata</i>	9,545.65
4.	<i>Gelidiella acerosa</i>	1,612.70
5.	<i>Jania longifurca</i>	926.15
6.	<i>Acanthophora spicifera</i>	7,368.45
7.	<i>Hypnea pannosa</i>	1,768.55
8.	<i>Gelidium robustum</i>	1,543.95
9.	<i>Digenea simplex</i>	2,348.47

Note *) Result of dilution 10x

The highest protein content (Table 2) was produced in *Helminthora divaricata* 9,545.65 μ g / ml. Anam *et al.* [48] reported that the red alga, *Acrocystis nana* had a protein content of 513.28 μ g / ml. This suggests that the species of red algae used greatly influence the levels of protein obtained. The successful extraction of algae proteins is also positively influenced by the availability of protein molecules, which are substantially inhibited by high viscosity and anionic cell wall polysaccharides, such as carrageen in red seaweed or alginate in brown seaweed [49]. Cell interference methods and the selection of appropriate chemical reagents

were utilized to improve the efficiency of algae protein extraction [50].

Khairunnisa reported that some red algae from Manado, Indonesia for *Galaxaura rugosa* 488.25 $\mu\text{g} / \text{ml}$, *Hydropuntia fragillissima* 457.45 $\mu\text{g} / \text{ml}$ and *Amphiroa edulis* 525,30 $\mu\text{g} / \text{ml}$ [15]. Red algae *G. foliifera*, *G. Acerosa*, *L. Tronoi* and *G. lichenoides* collected from Binuangeun Beach, Banten Indonesia only accounted for less than 2000 $\mu\text{g} / \text{ml}$ [10]. The protein contents of green algae *spirogyra sp* from Brazil was about 153 $\mu\text{g} / \text{ml}$ [2]. The variation of macroalgae protein does not only exist among the species, but also depends on the season. Joubert *et al.* [51] support this argument by stating that season, temperature and location where seaweed is harvested may affect the protein content.

For example, based on the annual monitoring of *palmata* harvested on the Atlantic coast of France, the protein levels in winter and spring vary between 9-25%, and reach its peak in May [52]. The *U. pinnatifida* that originates from New Zealand also significantly influences protein content and amino acid composition [53]. Similarly, *Gracilaria cervicornis* and *S. vulgare's* protein contents are influenced by seasons. The protein contents negatively correlated with temperature and salinity [54]. The average protein content of red algae is above 1 mg / ml depending on saturation of ammonium sulfate [55].

Different protein concentrations indicate the difference in protein solubility in water. On the other hand, differences in protein levels might be caused by the differences in characteristics possessed by each protein [56]. Studies on three *Chlorella* strains protein levels may be affected by temperature and availability of N, Protein decreases and lipids increase over time with N-deficiency [57]. According to Lutfiyanti, the divergence in proteins concentration indicates the differences in the natural conditions of the compound, the extraction methods used, the sample particle size, the condition and storage time, the extraction time, and the ratio of the number of solvents to the number of samples [58].

C. Stability to pH, Temperature, and Divalent Cations

The objective of this analysis is to determine the stability of the crude fraction of lectins against changes in pH, temperature and divalent cations. With the pH range from 5-10, the crude fraction of *Helminthora divaricata* lectins was proven to not under the influence of pH. In comparison, *Acanthophora spicifera* had a wider range of pH (3-10). Hemagglutination activity of *Nitophyllum punctatum* is stable against neutral pH 6-8 (Figure 1). The activity of hemagglutination of red macroalga *Acrocystis nana* [48] collected from the same area showed stable pH at 6-7, although it slightly decreased at pH 3-5 and pH 8-10.

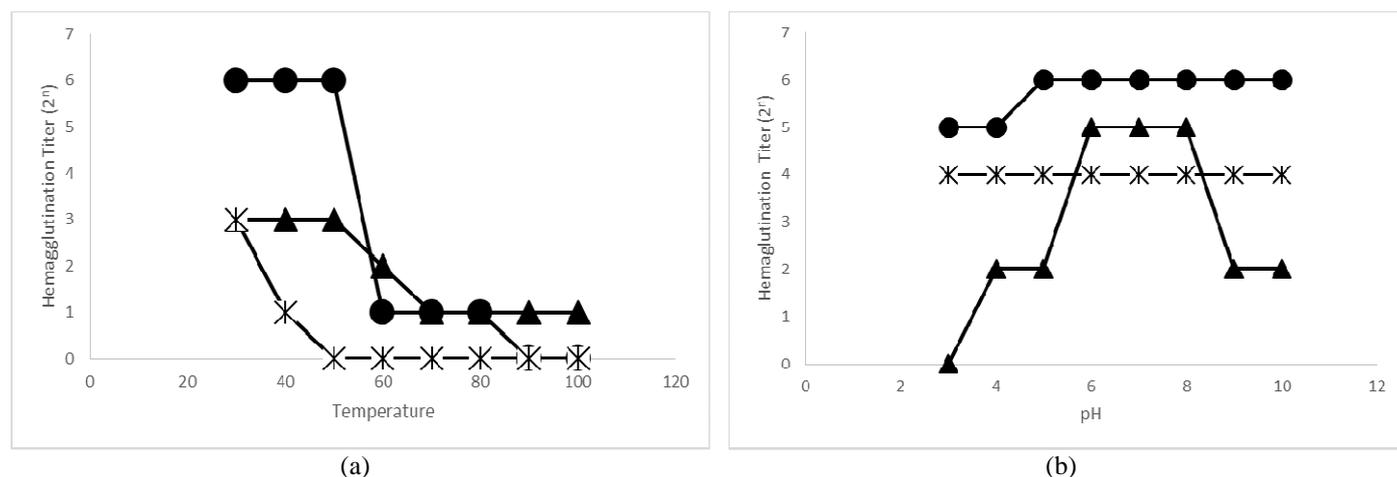


Fig. 1 The effect of pH (a) and Temperature (b) on hemagglutination activity of crude lectins fractions from red algae *Nitophyllum punctatum* (\blacktriangle), *Helminthora divaricata* (\bullet), and *Acanthophora spicifera* (\times)

The majority of algal lectins are able to retain their activity on a wide pH range (5-9) but will experience a slight decrease under acidic and alkaline conditions [34]. However, the Algerian *Pterocliadiella capillacea* alga remains active in the pH range of 2 - 12 [33]. Some unaffected red algae were *Kappaphycus alvarezii*, *Euचेuma denticulatum*, and *Gracilaria euchematoides* [34]. Different characteristics of proteins possessed may contribute to these results [56]. Moreover, extreme pH conditions may cause the occurrence of protein denaturation [59].

The stability of the crude fraction of algal lectins *Nitophyllum punctatum* and *Helminthora divaricata* were unstable when the temperatures were above 50°C, whereas *A. spicifera* was unstable against temperature changes from 30°C to 100°C (Fig. 1). Some algal lectins possess the thermostable characteristic. However, it does not help at

temperature of 100°C for 30 minutes [17]. In general, algal lectins are reported to be fairly stable, yet their hemagglutination activity begins to drop at a warming temperature of 40°C to 50°C. Species of *Hypnea valentiae* was stable to heat [34], while the thermolabile species were *Pterocliadiella capillacea* [60] and *Amansia rhodantha* [38]. On the other hand, it should be noted that hemagglutination activities of *Anadyomene plicata*, *Avainvillea erecta*, and *Hypnea valentiae* agglutinins were resistant to temperature changes, owing to the fact that they did not change even while at 100°C for 30 minutes. This unusual thermostability is also reported for lectins isolated from red algae *Hypnea japonica* [39] and *Hypnea musiformis* [27].

Algal hemagglutination activity is generally independent of the presence of divalent cations [34]. Similarly, the findings of the present study showed that there was no

influence on hemagglutination activity. Aside from using Ca^{2+} dan Mg^{2+} ions in the form of CaCl_2 and MgCl_2 salts as divalent cations, Mn^{2+} or Zn^{2+} ions in the form of MnCl_2 or ZnCl_2 salts were used in the testing of activity's stability against divalent cations [61]. Several species of red algae which were not affected by divalent cations include *Hypnea japonica*, *Solieria robusta*, *Plumaria elegans*, and *Palmaria palmata* [18]. While the affected species were *Gelidiopsis scoparia*, *Ptilota serrata*, *P. filicina*, *Enantiocladia duperreyi* and *Pterocladia capillacea* [38], [60].

D. Hemagglutination-Inhibition Assay

The hemagglutination-inhibition assay was performed by involving both qualitative and quantitative methods. The qualitative test was conducted to find out which sugars/glycoproteins are able to inhibit the activity of crude fractions of lectins, while the quantitative test was used to observe the minimal concentration of sugars/glycoproteins needed to inhibit the activity of crude lectins fractions. If a lectin is capable of being inhibited by a sugar/glycoprotein, it has the binding specificity to the sugar/glycoprotein. The sugar and glycoprotein concentrations used were 100 mM and 2,000 $\mu\text{g} / \text{ml}$, respectively. The crude fractions of *N. punctatum*, *H. divaricata* and *A. spicifera* lectins showed negative results on various types of sugars (monosaccharides and disaccharides), but some showed positive results in glycoproteins. This is consistent with Hori *et al's* findings, which reported that most algae lectins have no affinity for monosaccharides. Therefore, the hemagglutination activity is not inhibited by monosaccharides or disaccharides [17].

On the qualitative tests, 25 μl of saline was inserted in well, by adding 25 μl of the crude fraction of lectins and 25 μl of sugars / glycoproteins which were incubated at room temperature for 1 hour, and 25 μl of TRBC which were incubated for 1 hour at room temperature and then being observed macroscopically. Positive results are shown by the formation of the dot, while the negative results with the formation of the carpet. The positive result of this qualitative test initiated a follow-up quantitative test regarding its inhibitory activity.

For the quantitative test of the hemagglutination-inhibition, 25 μl of saline were added into the well. Subsequently, 25 μl of sugars/glycoproteins were added and diluted. Afterwards, 25 μl lectins was added which then incubated at room temperature for 1 hour. Thereafter, 25 μl TRBC was added, then incubated at room temperature for 1 hour, and observed macroscopically.

On the glycoproteins which were being analyzed, *A. spicifera* showed a weak inhibition profile (Table 3). Based on the results of this preliminary study, Asialo-BSM has the strongest inhibition on the crude fraction of *N. punctatum* and *H. divaricata* lectins with inhibitory concentrations of 250 $\mu\text{g} / \text{ml}$. Many species of red algae have been reported to have a lectin which has the specificity of binding to carbohydrates in the form of complex glycoproteins or high mannose N-glycans. This lectin-glycan interaction further triggers many biochemical responses which in the end lead to their extensive use as one of valuable tools in glycomics and biomedical studies [16].

Purified lectins from *Palmaria palmata* showed specificity of GluNAc and GalNAc, purified lectins from

Ptilota serrata has specificity against D-fucose and D-galactose, and purified lectin from *P. plumosa* against α -D-galactose [18]. Although the majority of algal lectins have no affinity for monosaccharides and oligosaccharides [17], they have more specific glycoproteins binding capabilities [25], [62].

TABLE III
HEMAGGLUTINATION-INIBITION ASSAY OF THE SALTING-OUT FRACTIONS FROM *N. PUNCTATUM*, *H. DIVARICATA*, AND *A. SPICIFERA* WITH SUGARS AND GLYCOPROTEINS

No	Sugars or Glycoprotein	Minimum Inhibition concentration ($\mu\text{g}/\text{ml}$)		
		<i>N. punctatum</i>	<i>H. divaricata</i>	<i>A. spicifera</i>
1	Monosaccharides and disaccharide (mM)	-	-	-
2	Glycoproteins ($\mu\text{g}/\text{mL}$)			
	<i>N-Glycans</i>			
	High-mannose type			
	Yeast mannan	>2000	>2000	>2000
	Complex type			
	Asialo-Transferrin	>2000	>2000	>2000
	Transferrin	>2000	>2000	>2000
	Complex type and high-mannose type			
	Bovine Thyroglobulin (BTG)	>2000	>2000	1000
	Asialo-BTG	>2000	1000	>2000
	Porcine Thyroglobulin (PTG)	>2000	>2000	>2000
	Asialo-PTG	>2000	>1000	>2000
	<i>O-Glycans</i>			
	Bovine submaxillary mucin (BSM)	>2000	>2000	>2000
	Asialo-BSM	250	250	>2000
	<i>N/O-Glycans</i>			
	Fetuin	>2000	>2000	>2000
	Asialo-Fetuin	>2000	>2000	>2000

The values as shown above represented the lowest concentration of sugar (mM) and glycoprotein ($\mu\text{g} / \text{ml}$) of which actually inhibit the activity of hemagglutination titer of 4. The inhibitory profile of hemagglutination activity varies considerably, depending on the origin of algae species [34]. Lectins of several species of red algae whose hemagglutinin activity is inhibited by glycoproteins, such as *Amansia rhodantha* (asialo-transferin at 62.5 $\mu\text{g} / \text{ml}$, PTG at 31.2 $\mu\text{g} / \text{ml}$; asialo-PTG at 31.2 $\mu\text{g} / \text{ml}$; fetuin at 250 $\mu\text{g} / \text{ml}$, asialo-fetuin at 62.5 $\mu\text{g} / \text{ml}$), *Rhodymenia anastomosans* (yeast mannan at 125 $\mu\text{g} / \text{ml}$, PTG at 250 $\mu\text{g} / \text{ml}$; asialo-PTG at 125 $\mu\text{g} / \text{ml}$; fetuin at 1,000 $\mu\text{g} / \text{ml}$; asialo-fetuin at 1,000 $\mu\text{g} / \text{ml}$), *Gelidiopsis scoparia* (asialo-transferin at 1,000 $\mu\text{g} / \text{ml}$; asialo-PTG 125 $\mu\text{g} / \text{ml}$; asialo-fetuin at 125 $\mu\text{g} / \text{ml}$); and *Laurencia perforate* (asialo-transferrin at 1,000 $\mu\text{g} / \text{ml}$; asialo-PTG 500 $\mu\text{g} / \text{ml}$, asialo-fetuin 1,000 $\mu\text{g} / \text{ml}$) [36]. As for green algae *C. barbatum* was inhibited by Porcine thyroglobulin (PTG) 500 $\mu\text{g} / \text{ml}$, Asialo-PTG 250 $\mu\text{g} / \text{ml}$, Bovine submaxillary mucin 500 $\mu\text{g} / \text{ml}$ and Asialo-BSM 500 $\mu\text{g} / \text{ml}$ [11].

IV. CONCLUSION

The crude lectins fractions of *Nitophyllum punctatum*, *Helminthora divaricata*, and *Acanthophora spicifera* from

the coast of Gunungkidul possess incredible potential to become the source of lectin with hemagglutination activity of 2^6 , 2^6 , 2^{13} and protein contents of 8.123,47 $\mu\text{g} / \text{ml}$; 9.545,65 $\mu\text{g}/\text{ml}$; 7.368,45 $\mu\text{g}/\text{ml}$, respectively. The results show that *Nitophyllum punctatum* and *Helminthora divaricata* can bear 50°C of temperature, while *Acanthophora spicifera* is not resistant to heat. The crude fraction of the lectin was shown as stable against all range of pH, independent from divalent cations and did not inhibit by simple sugars. *Nitophyllum punctatum* and *Helminthora divaricata* have inhibitory specificity in Asialo-BSM glycoprotein with inhibitory concentrations of 250 $\mu\text{g} / \text{ml}$. Thus, the coastal red algae of Gunung Kidul Yogyakarta Indonesia have the potential to be purified as a source of lectins. It is expected that this study can provide useful bioprospecting of lectins as the active compound of red macroalgae. The purification process and its characterization are currently being performed.

ACKNOWLEDGEMENTS

We are sincerely grateful to Sebelas Maret University (UNS) that has supported this research through the research grant of PNBPN UNS. Research facilities and equipments supported by Research Center for Marine and Fisheries Product Processing and Biotechnology, Ministry of Marine Affairs and Fisheries, Republic of Indonesia is gratefully acknowledged. We also would like to express our gratitude to Miss Tri Nurhayati, Miss Martha Arum Nugraheni, Miss Astri Suryani, Mr. Bhagaz Putra Perdhana and Mr. Banget Simanjuntak for their assistances and technical supports.

REFERENCES

- [1] A. Tanduwinata, H. A. Istiqomah, J. Jamilah, N. L. K. Caesaria, R. R. Saputra, and A. Aulanni'am, "Potensi Bioaktif Ekstrak Alga Merah (Gracillaria Verrucosa) Terhadap Kadar Malondialdehid (Mda) Dan Gambaran Histologi Paru Tikus Putih (Rattus novergicus) Pasca Induksi Formalin," *Molekul*, vol. 10, no. 2, p. 82, 2015.
- [2] D. B. de Alencar *et al.*, "Chemical composition of volatile compounds in two red seaweeds, *Pterocladia capillacea* and *Osmundaria obtusiloba*, using static headspace gas chromatography mass spectrometry," *J. Appl. Phycol.*, vol. 29, no. 3, pp. 1571–1576, 2017.
- [3] "Andi Parenrengi dan Silaeman_ 2007 Mengenal Rumput Laut_ Media Akuakultur Vol 2 No .1 2007.pdf."
- [4] "Andi Parenrengi-2012- Budidaya Rumpit Laut- 03_Edisi-I-2008-budidaya-rumput-laut-penghasil-karaginanFILEminimizer hal 3-4.pdf."
- [5] D. Praseptianga, "Development of Seaweed-based Biopolymers for Edible Films and Lectins," in *IOP Conference Series: Materials Science and Engineering*, 2017, vol. 193, no. 1.
- [6] P. V. S. Rao, V. A. Mantri, and K. Ganesan, "Mineral composition of edible seaweed *Porphyra vietnamensis*," *Food Chem.*, vol. 102, no. 1, pp. 215–218, 2007.
- [7] D. Praseptianga, "Algal Lectins and Their Potential Uses," *Squalen Bull. Mar. Fish. Postharvest Biotechnol.*, vol. 10, no. 2, pp. 89–98, 2016.
- [8] R. E. Cian, S. R. Drago, F. S. De Medina, and O. Martínez-Augustin, "Proteins and carbohydrates from red seaweeds: Evidence for beneficial effects on gut function and microbiota," *Mar. Drugs*, vol. 13, no. 8, pp. 5358–5383, 2015.
- [9] J. C. Lee *et al.*, "Marine algal natural products with anti-oxidative, anti-inflammatory, and anti-cancer properties," *Cancer Cell International*, vol. 13, no. 1, 2013.
- [10] N. D. Fajarningsih *et al.*, "Penapisan Senyawa Hemagglutinin dari Makroalga Asal Pantai Binuangeun , Banten , Indonesia Screening of Hemagglutinins of Macroalgae Collected from Binuangeun Coast , Banten , Indonesia," pp. 19–26, 2015.
- [11] D. Praseptianga, M. Hirayama, and K. HORI, "Purification, Characterization, and cDNA Cloning of a Novel Lectin from the Green Alga, *Codium barbatum*," *Biosci. Biotechnol. Biochem.*, vol. 76, no. 4, pp. 805–811, 2012.
- [12] N. Sharon and H. Lis, "History of lectins: From hemagglutinins to biological recognition molecules," *Glycobiology*, vol. 14, no. 11, pp. 53–62, 2004.
- [13] I. J. Goldstein, R. C. Hughes, M. Monsigny, T. Osawa, and N. Sharon, "What should be called a lectin? [2]," *Nature*, vol. 285, no. 5760, p. 66, 1980.
- [14] I. L. Ainouz and A. H. Sampaio, "Screening of Brazilian Marine Algae for Hemagglutinins," *Bot. Mar.*, vol. 34, no. 3, pp. 211–214, 1991.
- [15] U. I. N. Syarif, H. Jakarta, F. Kedokteran, D. A. N. Ilmu, and P. S. Farmasi, "Penapisan lektin pada alga yang diambil dari perairan pantai manado," 2015.
- [16] R. S. Singh and A. K. Walia, "Lectins from red algae and their biomedical potential," *J. Appl. Phycol.*, no. July, 2017.
- [17] K. Hori, K. Miyazawa, and K. Ito, "Some common properties of lectins from marine algae," *Hydrobiologia*, vol. 204–205, no. 1, pp. 561–566, 1990.
- [18] D. J. Rogers and K. Hori, "Marine algal lectins: new developments," *Hydrobiologia*, vol. 260–261, no. 1, pp. 589–593, 1993.
- [19] C.-C. Tsai *et al.*, "Cyanovirin-N Inhibits AIDS Virus Infections in Vaginal Transmission Models," *AIDS Res. Hum. Retroviruses*, vol. 20, no. 1, pp. 11–18, 2004.
- [20] J. Fabregas, J. Liovo, and A. Munoz, "Hemagglutinins in Red Seaweeds," *Bot. Mar.*, vol. 28, no. 12, pp. 517–520, 1985.
- [21] J. Fabregas, A. Muñoz, and J. Llovo, "Hemagglutinins in brown seaweeds," *J. Exp. Mar. Bio. Ecol.*, vol. 97, no. 2, pp. 213–219, 1986.
- [22] K. Hori, C. Oiwa, K. Miyazawa, and K. Ito, "Evidence for Wide Distribution of Agglutinins in Marine Algae," *Bot. Mar.*, vol. 31, no. 2, pp. 133–138, 1988.
- [23] R. Okamoto, K. Hori, K. Miyazawa, and K. Ito, "Isolation and characterization of a new hemagglutinin from the red alga *Gracilaria bursa-pastoris*," *Experientia*, vol. 46, no. 9, pp. 975–977, 1990.
- [24] T. Mori *et al.*, "Isolation and characterization of Griffithsin, a novel HIV-inactivating protein, from the red alga *Griffithsia* sp.," *J. Biol. Chem.*, vol. 280, no. 10, pp. 9345–9353, 2005.
- [25] K. T. Bird, T. C. Chiles, R. E. Longley, A. F. Kendrick, and M. D. Kinkema, "Agglutinins from marine macroalgae of the southeastern United States," *J. Appl. Phycol.*, vol. 5, no. 2, pp. 213–218, 1993.
- [26] I. L. Ainouz, A. H. Sampaio, A. N. A. Lucia, P. Freitas, N. Maria, and B. Benevides, "COMPARATIVE STUDY ON HEMAGGLUTININS FROM THE RED ALGAE *Bryothamnion seaforthii* AND *Bryothamnion triquetrum*," vol. 7, no. 1, pp. 15–19, 1995.
- [27] C. S. Nagano *et al.*, "Purification and characterization of a new lectin from the red marine alga *Hypnea musciformis*," *Protein Pept. Lett.*, vol. 9, no. 2, pp. 159–166, 2002.
- [28] L. G. Do Nascimento-Neto *et al.*, "Characterization of isoforms of the lectin isolated from the red algae *Bryothamnion seaforthii* and its pro-healing effect," *Mar. Drugs*, vol. 10, no. 9, pp. 1936–1954, 2012.
- [29] A. S. de Oliveira *et al.*, "Detection, purification and characterization of a lectin from freshwater green algae *Spirogyra* spp," *An. Acad. Bras. Cienc.*, vol. 89, no. 3, pp. 2113–2117, 2017.
- [30] Z. Yi and L. Hai-sheng, "Screening of agglutinins in marine algae from Fujian coast of China," *Chinese J. Oceanol. Limnol.*, vol. 20, no. 3, pp. 256–260, 2002.
- [31] B. de Souza and D. Teixeira, "A survey of antarctic algae for agglutinins," *Oecologia Bras.*, vol. 11, no. 1, pp. 122–130, 2009.
- [32] B. W. S. Souza, F. K. Andrade, D. I. A. Teixeira, A. Mansilla, and A. L. P. Freitas, "Haemagglutinin of the antarctic seaweed *Georgiella confluens* (Reinsch) Kylin: Isolation and partial characterization," *Polar Biol.*, vol. 33, no. 10, pp. 1311–1318, 2010.
- [33] Y. Necib, A. Bahi, S. Zerizer, C. Abdennour, and M. Salah Boulakoud, "Immunomodulatory activity of argan oil (*Argania spinosa* L)," *Int. J. Pharm. Sci. Rev. Res.*, vol. 23, no. 1, pp. 57–59, 2013.
- [34] H. Le Dinh, K. Hori, and N. H. Quang, "Screening and preliminary characterization of hemagglutinins in Vietnamese marine algae," *J. Appl. Phycol.*, vol. 21, no. 1, pp. 89–97, 2009.
- [35] D. Huskens and D. Schols, "Algal lectins as potential HIV microbicide candidates," *Mar. Drugs*, vol. 10, no. 7, pp. 1476–1497, 2012.
- [36] R. Mangaiyarkarasi, L. Kannan, M. Girija, and S. Gnanamurthy, "Screening of Indian marine macro algae (seaweeds) for haemagglutinin activity," *Int. J. Adv. Research*, vol. 2, no. 4, pp. 43–

- 52, 2014.
- [37] R. S. Singh, S. R. Thakur, and P. Bansal, "Algal lectins as promising biomolecules for biomedical research," *Crit. Rev. Microbiol.*, vol. 41, no. 1, pp. 77–88, 2015.
- [38] D. Le Hung, B. M. Ly, V. T. D. Trang, N. T. D. Ngoc, T. Le Hoa, and P. T. H. Trinh, "A new screening for hemagglutinins from Vietnamese marine macroalgae," *J. Appl. Phycol.*, vol. 24, no. 2, pp. 227–235, 2012.
- [39] "Hori 1986 - Preliminary Characterization of Agglutinins from Seven Marine Algal Species.pdf" .
- [40] E. Klarbring, "Studies of the BCA assay for determination of total protein in allergen extracts," 2011.
- [41] H. P. Fernandes, C. L. Cesar, and M. de L. Barjas-Castro, "Electrical properties of the red blood cell membrane and immunohematological investigation," *Rev. Bras. Hematol. Hemoter.*, vol. 33, no. 4, pp. 297–301, 2011.
- [42] P. Paiva, F. Gomes, and T. Napoleão, "Antimicrobial activity of secondary metabolites and lectins from plants," ... *Educ. Top.*, pp. 396–406, 2010.
- [43] "Hori K, K Miyazawa, K. Ito, 1981, Hemagglutinins in marine algae, Bull of The Japan Soc of Scienc Fish, Vol 47, 793-798."
- [44] E. W. Becker, "Micro-Algae as source of protein," *Biotechnol. Adv.*, vol. 25, pp. 207–210, 2006.
- [45] P. A. Harnedy and R. J. Fitzgerald, "Bioactive proteins, peptides, and amino acids from macroalgae," *J. Phycol.*, vol. 47, no. 2, pp. 218–232, 2011.
- [46] B. a Murray and R. J. FitzGerald, "Angiotensin converting enzyme inhibitory peptides derived from food proteins: biochemistry, bioactivity and production," *Curr. Pharm. Des.*, vol. 13, pp. 773–791, 2007.
- [47] J. Fleurence, "Seaweed proteins: Biochemical, nutritional aspects and potential uses," *Trends Food Sci. Technol.*, vol. 10, no. 1, pp. 25–28, 1999.
- [48] C. Anam *et al.*, "Preliminary Characterization of Crude Lectin Fraction of the Red Alga, *Acrocystis nana* from Wediombo Beach of the Southern Coast of Java Island, Gunung Kidul, Yogyakarta, Indonesia," in *IOP Conference Series: Materials Science and Engineering*, 2017, vol. 193, no. 1.
- [49] J. Fleurence, "The enzymatic degradation of algal cell walls: A useful approach for improving protein accessibility?," *J. Appl. Phycol.*, vol. 11, no. 3, pp. 313–314, 1999.
- [50] S. Bleakley and M. Hayes, "Algal Proteins: Extraction, Application, and Challenges Concerning Production," *Foods*, vol. 6, no. 5, p. 33, 2017.
- [51] Y. Joubert and J. Fleurence, "Simultaneous extraction of proteins and DNA by an enzymatic treatment of the cell wall of *Palmaria palmata* (Rhodophyta)," *J. Appl. Phycol.*, vol. 20, no. 1, pp. 55–61, 2008.
- [52] A. V. Galland-Irmouli *et al.*, "Nutritional value of proteins from edible seaweed *Palmaria palmata* (Dulse)," *J. Nutr. Biochem.*, vol. 10, no. 6, pp. 353–359, 1999.
- [53] A. Y. Zhou, J. Robertson, N. Hamid, Q. Ma, and J. Lu, "Changes in total nitrogen and amino acid composition of New Zealand *Undaria pinnatifida* with growth, location and plant parts," *Food Chem.*, vol. 186, pp. 319–325, 2015.
- [54] E. Marinho-Soriano, P. C. Fonseca, M. A. A. Carneiro, and W. S. C. Moreira, "Seasonal variation in the chemical composition of two tropical seaweeds," *Bioresour. Technol.*, vol. 97, no. 18, pp. 2402–2406, 2006.
- [55] S. Dali *et al.*, "Bioaktivitas Antibakteri Fraksi Protein Alga Merah *Gelidium Amansii* dari Perairan Cikoang Kabupaten Takalar , Sulawesi Selatan," No. 6.
- [56] A. Kusumaningrum, G., A. Alamsjah, M., and D. Masithah, E., "Uji Kadar Albumin dan Pertumbuhan Ikan Gabus (*Channa striata*) dengan Kadar Protein Pakan Komersial yang Berbeda," *J. Ilm. Perikan. dan Kelaut.*, vol. 6, no. 1, pp. 25–29, 2014.
- [57] V. Ördög *et al.*, "Effect of temperature and nitrogen concentration on lipid productivity and fatty acid composition in three *Chlorella* strains," *Algal Res.*, vol. 16, pp. 141–149, 2016.
- [58] R. Lutfiyanti, W. F. Ma'ruf, and E. N. Dewi, "Aktivitas Antijamur Senyawa Bioaktif Ekstrak *Gelidium latifolium* terhadap *Candida Albicans*," *J. Pengolah. dan Bioteknol. Has. Perikan.*, vol. 1, no. 1, pp. 26–33, 2012.
- [59] V. K. Roy, N. S. Kumar, and G. Gurusubramanian, "Proteins – structure , properties and their separation by SDS- polyacrylamide gel electrophoresis," *Sci. Vis.*, vol. 12, no. 4, pp. 170–181, 2012.
- [60] S. R. M. Oliveira, A. E. Nascimento, M. E. P. Lima, Y. F. M. M. Leite, and N. M. B. Benevides, "Purification and characterisation of a lectin from the red marine alga *Pterocladia capillacea* (S.G. Gmel.) Santel. & Hommers.," *Rev. Bras. Botânica*, vol. 25, no. 4, pp. 397–403, 2002.
- [61] S. Wang, F. Zhong, Y. Zhang, Z. Wu, Q. Lin, and L. Xie, "Molecular Characterization of a New Lectin from the Marine Alga *Ulva pertusa*," vol. 36, no. 2, pp. 111–117, 2004.
- [62] T. C. Chiles and K. T. Bird, "A comparative study of animal erythrocyte agglutinins from marine algae," *Comp. Biochem. Physiol. -- Part B Biochem.*, vol. 94, no. 1, pp. 107–111, 1989.