Bioactivities of red seaweed extracts from Banten, Indonesia

T S Khatulistiani*, D Noviendri, I Munifah and S Melanie

Research and Development Center for Marine and Fisheries Product Processing and Biotechnology, Ministry of Marine Affairs and Fisheries, Indonesia

*E-mail: tiaraemerys@gmail.com

Abstract. Red seaweed has many advantages and can be utilized for cosmetics and pharmaceutical products for its rich bioactive content such as R-Phycoerythrin and phenols. This study was aimed to determine the R-phycoerythrin and phenolic contents, also the bioactivities of red seaweed extracts from Indonesia. The samples of Eucheuma sp., Gelidium sp., Eucheuma spinosum, Halymenia sp., and Rhodopeltis sp. were extracted by maceration method with distilled water (ratio 1:2, w/v) for 24 hours at cold temperature. The R-phycoerythrin content was determined by UV/VIS spectrophotometry at λ 455, 565, and 592 nm. Total Phenolic Content (TPC) was determined using Folin-Ciocalteu method. The antioxidant and antityrosinase were determined using Ferric Reducing/Antioxidant Power (FRAP) and mushroom tyrosinase assays.

The highest yield of extract (2.73%, w/w) and R-PE (0.1056 mg/mL) were obtained from Halymenia sp., with highest phenolic content 8.79±1.21 GAE/mg, as well as in Eucheuma sp. (7.05±0.87 GAE/mg, p > 0.05). Halymenia sp. and Eucheuma sp. extracts showed antioxidant content of 19.40±0.37 and 19.08±0.66 AA mg/g, with tyrosinase enzymes of 40.09±2.01% and 41.36±3.8% (1,000 ppm). Thus, Eucheuma sp. and Halymenia sp. extracts have high potential activities, while Halymenia sp. was potentialy used as natural colorant for cosmeticals and nutraceutical.

1. Introduction

Macroalgae (seaweeds) have been consumed by human being for centuries. It is nutritious because of high protein, vitamin, and mineral contents. Macroalgae can be divided into three groups, i.e. green seaweed (Chlorophyta), brown seaweed (Phaeophyta) and red seaweed (Rhodophyta) (Rajasuloc et al 2009). Red seaweed is one of the macroalgae with high economical value. Eucheuma/Kappaphycus, Porphyra and Gracilaria were massively exploited for agar or carrageenan industry (Jimenez and Robaina 2015). Red seaweeds have a wide range of colour, from greenish, yellow, to brownish in shallow clear water and become blackish-purple in deep water. Phycoerythrin pigment in red seaweeds was accountable to construct the blackish-purple colour (Venugopal 2011).

Red seaweeds have many advantages to fulfil human needs. Not only for the food and carrageenan industry, but the pigment can also be used as a natural colourant. The natural colourant can be applied to food colouring, cosmetics colouring, and pharmacist industry. Red seaweeds have a protein's pigment complex named phycobiliproteins and classified into four types: i.e. phycoerythrin (PE)/ purple colour;
phycoerythrocyanin (PEC)/ orange colour; phycocyanins (PC)/blue colour; and allophycocyanins (AP)/bluish-green colour (Sekar and Chandramohan 2007). Phycoerythrin is the most dominant pigment in the red algae, which gave the algae blackish-purple colour. Phycoerythrin also classified into three types based on their absorption spectra, such as B-phycoerythrin (B-PE), R-phycoerythrin (R-PE) and C-phycoerythrin (C-PE) (Kawser et al 2011).

R-PE was the major phycobiliprotein in red seaweed. R-PE can be used as a natural food colourant or natural dye. Moreover, it also has many capabilities to support pharmaceutical industries and cosmeceutical industries. The proteins on R-PE are highly water-soluble and stable (Dumay et al 2013). The definition of the cosmeceutical industry is the combination of topical cosmeceutical industries. The proteins on R-PE has the potential capability as the protective role of antioxidants (Moon and Shibamoto 2009).

Antioxidant, based on its function was classified into two kinds: biological antioxidant and synthetic antioxidant. Every plant extract, mixture or single compound from any organism which can prevent the oxidation was called natural or biological antioxidant (Kusumawati and Gunawan 2013). Many unhealthy conditions have been prevented by antioxidants. Nowadays, research to explore natural antioxidants in many fields were expanded, consist of phenolic compound, flavonoids, and vitamins that have the potential capability as the protective role of antioxidants (Moon and Shibamoto 2009).

People are more interested to consume natural products or natural ingredients that have less harmful effects for their healthiness. This is one of the reasons of the increasing application of natural antioxidant in cosmeceutical and pharmaceutical industries (Kusumawati and Gunawan 2013). The evaluation of potential antioxidants from seaweed has been studied by many researchers. The evaluation mostly focused on ethanol and methanol extracts which is will affect high production costs for large scale production. Possibly, extraction using water as a solvent will be more convenient and inexpensive for large scale production. The advantage of red seaweed water extract has not been attained yet by the industry in Indonesia, to expand R-PE beneficial for healthiness, the aims of this study are focused to determine R-Phycoerythrin and total phenolic content and its bioactivities (antioxidant and antityrosinase properties) from Binuangeun, in the south coast of West Java Province.

2. Materials and methods

2.1. Plant materials
Five species of red algae (Eucheuma sp., Gelidium sp., Eucheuma spinosum, Halymenia sp., and Rhodopelitis sp.) were sampled from Binuangeun, a place at the southern coast of West Java (6°50′4″S - 105°52′49″E) at about 4 m depth. Samples were rinsed with seawater before packed into plastic bag and storage at -20°C until the extraction process.

2.2. Chemicals
Ultra-filtrated water as solvent was taken from Adrona Crystal Water Purifier in Instrument Laboratory, Research and Development Center for Marine and Fisheries Product Processing and Biotechnology, Ministry of Marine Affairs and Fisheries, Indonesia. K₃HPO₄ and KH₂PO₄ were used as chemical ingredients to make buffer phosphate solution. Folin Ciocalteu (MERCK ) and sodium carbonate (MERCK ) were used as total phenolic content (TPC) reagent, and ascorbic acid (MERCK ) as antioxidant standard. Meanwhile, 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ- Sigma-Aldrich) as FRAP reagent and gallic acid (Sigma-Aldrich ) were used as TPC standard.

Red algae extract was separated from the seaweed using K3 Series, Centrifuge Scientific. Seaweed extracts were dried using Freeze Dryer (Coolsave, Scanfac, Chemo Science. R-Phycoerythrin (P-PE)
were analyzed using Perkin Elmer Pricency, Spectrophotometer. Antityrosinase, TPC test, and antioxidant assay were analyzed using Multiskan Go, Thermo Scientific.

2.3. Extraction
The ratio of water and sample was 1:2 (w/v), where 100 g of red algae were extracted by 200 mL buffer solution pH 7 and incubated for 24 hours. The supernatants were separated using Whatman filter paper and dried using freeze dryer. The dry extracts were stored at 4°C and used as a sample for antitirosynase and antioxidant assay.

2.4. Phycoerythrin analysis
Phycoerythrin analysis was using 5 g of red algae extracted by 5 mL of buffer phosphate solution pH 7 (ratio m: v; 1: 1) and incubated for 24 hours. The supernatants were separated using Whatman filter paper and centrifuged at 2000 rpm for 20 minutes to separate crude extract from red algae. Pigment concentrations were evaluated using Perkin Elmer Pricency, Lambda 25 Uv per Vis spectrophotometer with various wavelength of phycoerythrin absorption spectra (455, 565, and 592 nm, according to Wenno 2014). The R-PE concentrations were estimated by the equation below (Beer and Eshel 1985):

\[
[R-PE] \text{ (mg mL}^{-1}\text{)} = \frac{[(A565 - A592) - (A455 - A592) \times 0.20]}{0.12} \times \text{V} \times \text{C} 
\]

2.5. Total phenolic compound (TPC)
Sample preparation was done by diluting 1 mg of red algae water extract into 1 mL distilled water (10,000 ppm). Samples were homogenized using vortex. About 10 µL of diluted sample was mixed with 160 µl distilled water in microplate wells. Afterwards, 10 µL of 10% Folin-ciocalteu and 20 µL of 10% Na₂CO₃ were added into the sample in a microplate well and incubated for 60 minutes. After incubation, samples absorbance were read using microplate reader in λ 765 nm. The products of the reaction have a blue colour (Nambar et al 2013). The total phenolic compound was calculated by interpolating gallic acid standard curve (0; 6.25; 12.5; 25; 50; 100 ppm).

\[
\text{TPC (ug GAE mg}^{-1}\text{ extract) = C} \times (\text{Vm}) 
\]

C: standard curve of TPC (mg mL⁻¹)
V: extract volume (mL)
m: mass extract (g)

2.6. FRAP assay
A 300 mM acetate buffer in pH 3.6 was prepared by diluting 3.1 g sodium acetate trihydrate to 16 ml glacial acetic acid per L distilled water; 40 mM HCl was prepared by diluting 3.4 mL HCl per L distilled water; 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) was prepared by diluting 3.123 g of TPTZ per L 40 mM HCl; and 20 mM FeCl₃ 6H₂O was prepared by diluting 5.406 g per L distilled water. FRAP Reagent was made by mixing the TPTZ solution, FeCl₃ 6H₂O solution and buffer acetate solution. Ascorbic acid was used as standard curve (0, 5, 10, 20, 40, 80, 100 µM, Benzie and Strain 1999).

Sample solutions with the amount of 10 µL were added in the well, then were added with 150 µL FRAP reagent into it. Samples were incubated for 30 minutes then were read using Multiskan Go, Thermoscientific microplate reader, λ 594 nm.

2.7. Inhibitor tyrosinase
Sample stocks were prepared by diluting 1 mg red seaweed extract into 1 ml (10,000 ppm) buffer phosphate solution (pH 7) and diluted into 1,000 ppm. 70µL extract was added to the microplate well, followed by adding 30 µL of 333 unit per mL tyrosinase enzyme and then incubated for 5 minutes. After the incubation, 110 µL of 12 mM L-DOPA was added into the well as a substrate, followed by incubation
for 30 minutes. The absorbances were read using Multiskan Go, Thermo Scientific in $\lambda$ 475 nm. Kojic Acid used as a standard (62.5, 125, 250, 500 ppm). Inhibition percentage was calculated by the formula below:

$$
\text{Inhibition} \, (\%) = \frac{\text{Abs Control} - (\text{Abs Sample} - \text{Abs Control Sample})}{\text{Abs Control}} \times 100\% \quad (3)
$$

2.8. Heavy metal analysis
Heavy metal detection was conducted to the fresh red algae that had the best potential activity in this study. The heavy metal analysis was carried out in Jakarta Regional Health Laboratories, Indonesia. The analysis method using Atomic Absorption Spectrometry (AAS).

3. Results and discussion

3.1. Yields and R-Phycoerythrin analysis
Yields of water-extract from Eucheuma sp., Gelidium sp., E. spinosum, Halymenia sp. and Rhodopeltis sp. were 1.51%, 2.52%, 1.17%, 2.06% and 2.73%, respectively. The colour of Halymenia sp. extract was more pinkish-purple than the other samples. Eucheuma sp., Gelidium sp., Eucheuma spinosum, and Rhodopeltis sp. were brown in colour. The colour of the dry water-extract of red algae showed that Halymenia sp. had the highest percentage of R-PE content than other red algae.

| Sample       | Powder Appearance |
|--------------|-------------------|
| Eucheuma sp. | Brown             |
| Eucheuma spinosum | Yellow       |
| Gelidium sp. | Orange            |
| Halymenia sp. | Pinkish Purple   |
| Rhodopeltis sp. | Brown        |
Based on the colour, yield percentage, and R-PE content (as seen in table 1), *Halymenia* sp. was the best candidate as natural colourant of pinkish-purple for food, cosmetic and pharmaceutical industry. It can be used for lip tint, eyeshadow or blush-on to support natural colourant ingredients in the cosmetic industry or it can be used as natural capsule colourant in the pharmaceutical industry. Phycoerythrin as one of the major pigments from phycobiliproteins have an important contribution in natural product industries, such natural food, and cosmetic dye. However, it also need many biochemical techniques to explore its fluorescence properties to support the pharmaceutical industry as a fluorescent label (Kambel *et al* 2018).

Figure 1. Spectrophotometry scanning of a. *Eucheuma* sp., b. *Gelidium* sp., c. *Eucheuma spinosum* d. *Halymenia* sp., e. *Rhodopeltis* sp.

Figure 2. Concentration of R-Phycoerythrin in red seaweed extracts from Biniangeun Coastal Zone, Banten Indonesia.
3.2. Total phenolic content

Total phenolic content in the extracts was performed by the Folin Ciocalteu, as common method. The phenolic content was determined by equivalence to the gallic acid (EAG) concentration in the same conditions ($R^2 = 0.9994$). Total Phenolic Compound (TPC) is the basic testing method to give an estimation of antioxidant activity in some samples. It was principally because polyphenol, such as phenolic compounds, known could prevent oxidation reaction. The phenolic compound was the major reason of antioxidant activity in many plant extract (Djapiala et al 2013). The results of this TPC test are shown in table 2.

TPC results of five red seaweed samples were significantly different (P<0.05). Halymenia sp. showed the highest TPC amongst all samples. Halymenia sp. and Eucheuma sp. were significantly different with Gelidium sp., E. spinosum, and Rhodopeltis sp. Meanwhile, E. spinosum was significantly different compared to Gelidium sp. and Rhodopeltis sp. The lowest TPC result was shown by Rhodopeltis sp. Polyphenol quantity in the natural source dependents on genetic, environment, and technology factors. Hence, it will be needed to develop limitation of polyphenol losses during the process (Manach et al 2004).

Table 2. Yields percentage, TPC and FRAP analysis result of red seaweeds from Binuangeun Coastal Zone, Banten Indonesia.

| Sample          | % Yields | Total Phenolic Compound (GAE/mg) | FRAP Analysis (AA mg/g) | Antityrosinase Analysis (%) |
|-----------------|---------|----------------------------------|-------------------------|-----------------------------|
| Eucheuma sp.    | 1.51    | 7.05±0.87<sup>c</sup>            | 19.08±0.66<sup>a</sup>  | 41.36±3.80<sup>a</sup>      |
| Gelidium sp.    | 2.52    | 4.48±0.12<sup>a</sup>            | 16.84±0.48<sup>a</sup>  | 38.54±2.56<sup>a</sup>      |
| Eucheuma spinosum | 1.17    | 4.83±0.95<sup>b</sup>            | 17.33±0.73<sup>a</sup>  | 36.71±3.81<sup>a</sup>      |
| Halymenia sp.   | 2.07    | 8.79±1.21<sup>c</sup>            | 19.40±0.37<sup>a</sup>  | 40.09±2.01<sup>a</sup>      |
| Rhodopeltis sp. | 2.74    | 3.81±0.27<sup>a</sup>            | 15.81±0.57<sup>a</sup>  | 38.96±1.34<sup>a</sup>      |

<sup>a,b,c</sup> showed the results were significantly different (p<5)

3.3. Antioxidant analysis

Antioxidant activity was tested by Ferric Reducing/Antioxidant Power (FRAP) assay. FRAP assay was chosen because samples were not soluble in methanol but highly soluble in water. FRAP assay can be observed in any kind of natural fluids and aqueous or ethanolic extracts of drugs, foods, and plants (Benzie and Strain 1999). FRAP assay gives fast, reproducible results. However, the only drawback of this method is that the testing systems must be aqueous (Moon and Shibamoto 2009). FRAP is one of the antioxidant analysis methods that measure the Fe-reducing capability of antioxidants from Fe<sup>3+</sup>-TPTZ complex into Fe<sup>2+</sup>-TPTZ by electron transferred (Huang et al 2005).

Antioxidants standard in this test was carried out by using ascorbic acid which is water soluble. The concentration of the sample was 1,000 ppm. Result showed that Halymenia sp. has the highest antioxidant, 19.40±0.37 AA mg/g, whilst Rhodopeltis sp. has the lowest antioxidant, 15.81±0.57 AA mg/g. The result showed linearly proportional value between the TPC test and antioxidant activity assay. It showed that Halymenia sp. had the highest TPC and also had the highest antioxidant activity. Kelman et al (2013) described that TPC is reflection of antioxidant activity. Halymenia sp., based on this study, appeared as the most potential red algae from Binuangeun to be developed as food, cosmetic or pharmaceutical raw material.
3.4. Inhibitor Tyrosinase
Tyrosinase commonly known as polyphenols oxidase, one of the ingredients that has responsibility in skin colouring. Tyrosine contains monooxygenase which is believed as a key enzyme in melanin synthesis. Tyrosine is believed to be the pigmentation process booster. The higher of tyrosine level, will darken the skin colour. Therefore, inhibition of tyrosin production is the most common mechanism used in cosmetic products in order to prevents excessive pigmentation and triggers skin whitening (Rahmadi et al 2014).

Based on the inhibitor tyrosinase assay, showed that inhibition of Eucheuma sp. was not significantly different with other samples, even though it has the highest inhibitor tyrosinase activity (41.36±3.80%). Halymenia sp. has the most potential antioxidant activity with 40.09±2.01% inhibitor tyrosinase activity, which is insignificantly different with Eucheuma sp. activity. It can be concluded that Halymenia sp. is the most potential seaweed compared with other samples in this study. Seaweeds have attracted great attention of researchers to find tyrosin inhibitor agent from natural sources. Rahmadi et al (2014) suggested that whitening agent from seaweeds have many advantages such as low production cost, more safety, and fulfill the consumen needs to use natural product for their cosmetic or skin care.

3.5. Heavy metal analysis
The heavy metal analysis was examined only on fresh Halymenia sp, the most potential red algae in this study. This analysis was conducted to confirm that Halymenia sp. from Binuangeun was less harmful from heavy metal toxicity. Metals (Arsenic/As, Lead/Sn, Cadmium/Cd, Mercury/Hg and others) can be detected in certain foods. FDA does not usually test individual food ingredient, yet examining the fresh raw material such as fresh red seaweed. Red seaweed could grow in many environments (e.g., soil or water) where some contaminants may occur naturally and, thereby, be absorbed (FDA 2018).

Table 3. Heavy Metal concentration in fresh Halymenia sp from Binuangeun Coastal Zone, Banten Indonesia.

| Heavy Metal | As  | Pb  | Cd  | Hg  | Sn  |
|-------------|-----|-----|-----|-----|-----|
| Concentration (ppb) | 12.5±0.65 | - | 1.51±0.08 | 0.03±0.01 | - |

The levels of the three metals were below the limits established by the Food Chemicals Codex (FCC) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA). Specifically, Arsenic (As) levels ranged from 97 to 2,011 ppb (FCC and JECFA limits are 3,000 ppb); Cadmium (Cd) levels ranged from 35 to 1,292 ppb (FCC and JECFA limits are 2,000 ppb); and Lead (Sn) levels ranged from 38 to 1,065 ppb (FCC and JECFA[1] limits are 5,000 ppb). Therefore, it is concluded that heavy metal content in fresh Halymenia sp. from Binuangeun was below the maximum limit.

4. Conclusion
Water as extraction solvent will be less expensive for cosmetics or pharmaceutical industry and safer for consumers. Halymenia sp. showed the best results based on the colour, R-PE content, total phenolic content and antioxidant activity. It can be concluded that Halymenia sp. was the most potential red seaweed from Binuangeun can be used as natural colourant or natural ingridient, and potential antioxidant for food, cosmetic or pharmaceutical industry.

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