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Composition of Pigments and Antioxidant Activity in Edible Red Seaweed *Halimena durvilae* Obtained from North Sulawesi

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Abstract : This study was carried out to identify the pigment and antioxidant activity extracted from *Halimena durvilae*. Hexane, acetone and ethanol were used as extraction solvent. The pigments were measured consisting of chlorophyll a, chlorophyll b, total chlorophyll, chlorophyll c_1+c_2 , fucoxanthin, carotenoids, phycocyanin and phycoerythrin, while 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging and ferric reducing antioxidant power (FRAP) were used to study their antioxidant activity. Total Phenolic content (TPC) were also investigated. The result showed hexane extract respectively containing highest level of all pigments. The lowest value of pigment was phycocyanin, recorded for hexane, acetone and ethanol extract were 0.7875 ± 0.08 ; 0.1475 ± 0.08 and $0.1565 \pm 0.02 \mu\text{g g}^{-1}$ dried weight. Ethanol extract exhibited the lowest TPC ($7.605 \pm 0.383 \mu\text{g GAE(Gallic acid equivalent)g}^{-1}$). Good value of radical scavenging of DPPH was acetone extract (scavenging activity of IC_{50} $1.211 \pm 0.03 \text{ mg ml}^{-1}$). The highest Reducing power was acetone, $0.17 \pm 0.01 \text{ uM Fe}^{2+}/\text{mg}$ extract, respectively. Thus *H. durvilae* could be used as natural pigment and antioxidant source which is potential to be applied in food product as functional food.

1. Introduction

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Seaweeds or marine macro algae are the prospective renewable resource in marine environment. About 6,000 species of seaweeds have been identified and are classified into green algae (Chlorophyceae), brown algae (Phaeophyceae) and red (Rhodophyceae) (Devi *et al.*, 2011, Chandini *et al.*, 2008). The color in case of green seaweeds is due to the attendance of chlorophyll a and b; beta-carotene (a yellow pigment) and varioustypical xanthophylls (Yellowish or brownish pigments). Fucoxanthin is the prominent of the xanthophylls pigment which is responsible for the color of brown seaweeds. This compounds masks the other pigments such as Chlorophyll a and c and other xanthophylls. Phycoerythrin and phycocyanin accountable for the color of red seaweeds and mask the pigments such as Chlorophyll a and beta-carotene (Gupta and Abugannam, 2011). Anthocyanins belonging to the flavonoid group are another group of pigments which are responsible for the red, purple, and blue colour. Anthocyanins exhibited a high possible as colorants because of their low toxicity (Özkan and Bilek, 2014).

Macroalgae have been reported to have more than 2400 natural products of profitable significance in pharmaceutical, biomedical and nutraceutical industries. They have been utilized as ingredients in human and animal food preparations owing to their outstanding source of bioactive compounds which consist of sulfated polysaccharides, polyphenols, diterpenes, protein, essential fatty acids, fatty fiber vitamins and minerals (Chinnadurai *et al.*, 2013, Özkan and Bilek, 2014, Chandini *et al.*, 2007). Among functional ingredients identified from marine algae, natural pigments have obtained specific attention as they have been found to

show many advantageous biological activities such as antioxidant, anticancer, anti-inflammatory, anti-obesity, anti-angiogenic and neuroprotective activities. (Pangestuti and Kim, 2011).

Humans are impacted by many free radicals especially reactive oxygen species (ROS). ROS constitutes superoxide (O_2^-), hydroxyl (HO^\bullet), hydrogen peroxide (H_2O_2) and nitric oxide (NO). These molecules are unsteady and highly reactive, and can harm cells by chain reactions, such as lipid peroxidation or configuration of DNA adducts. Extreme amounts of ROS may be injurious because they can damage essential biomolecules: proteins, DNA and lipids and other cells which consequences various diseases disorders such as cancer, diabetes, stroke, cataract, myocardial infarction, atherosclerotic and Parkinsons diseases (Wu *et al.*, 1988; Chew *et al.*, 2008). In order to diminish or avoid this damage of the human body by ROS antioxidants are believed to be protective, all cells perpetually contain antioxidants (Wu *et al.*, 1998, Halliwell *et al.*, 1995, Tapiero *et al.* 2002).

Natural antioxidants are chosen by consumer due to worry on the toxic and carcinogenic effect of synthetic antioxidant (Mantanjun *et al.*, 2007, Ahnet *et al.*, 2003). Natural antioxidant considered safe for use as ingredients in medicine, dietary supplements, nutraceuticals and cosmetics with the intent of improving consumer health, reducing the belongings of damaging diseases and other broader aspects of immune system function (Shahidi, 2009, Pangestuti and Kim 2011, Yip *et al.*, 2014). Additional, there are facts obtainable in the literature to show the potential defensive properties of seaweeds against oxidative stress in target tissues and lipid oxidation in foods. Consequently, consumption of antioxidant and addition of antioxidant in food materials protect the body as well as against oxidative stress. Although seaweeds possess extensive applications in food and pharmaceutical industries, the elements and antioxidant activities of many types of seaweeds in Indonesian area are still unexplored. Hence, the present study was proposed to explore the pigments and antioxidant properties of *H. durvillae* which grows plentifully in North Sulawesi.

2. Materials and methods

Sample preparation and extraction

Sufficient amount of *H. durvillae* was collected from Arakan Village, Manado North Sulawesi. The sample was completely washed with seawater and fresh water to eliminate epiphytes, dirt particles and shells. It was then brought to the laboratory followed with shading-drying for two days and oven-dried at 50 °C for 3 days and ground using mixer without producing heat and transformed to powder.

The dried sample was extracted using hexane, acetone and ethanol (1 : 10 w/v). The samples were incubated for overnight at room temperature in a dark place. Extraction was repeated three times till the sample became colorless. The procedure was carried out in triplicates. The extracts were filtered and concentrated in vacuum rotary evaporator at 40 °C. The extracts were stored in dark glass bottle for future analysis.

Chemicals and reagents

1,1-diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma Aldrich, Follin-Ciocalteu's phenol, butylated hydroxytoluene (BHT), sodium carbonate (Na_2CO_3), potassium dihydrogen phosphate (KH_2PO_4), iron (III) chloride hydrate ($FeCl_3$), Trichloroacetic acid (TCA) and Potassium ferricyanide $K_3Fe(CN)_6$ purchased from Merk. All other solvent and chemicals were of analytical grade.

Pigment.

The pigments extracted using different solvents were quantified using UV-Visible spectrophotometer by reading the absorbance at their respective wavelengths and using the formulae given below:

- Chlorophyll a ($mg\ g^{-1}$) = $[12.7 (A_{663}) - 2.69 (A_{645}) V] / (1000 \times W)$ (Arnon, 1949).
- Chlorophyll Chl b ($mg\ g^{-1}$) = $[24.0 \times 0.645 - 4.68 - A_{663} V] / (1000 \times W)$ (Arnon, 1949).
- Total Chlorophyll ($mg\ g^{-1}$) = $[20.2 (A_{645}) + 8.02 (A_{663}) V] / (1000 \times W)$ (Jeffrey *et al.*, 1961).
- Chlorophyll C1+C2 ($mg\ g^{-1}$) = $[24.36 \times A_{630} - 3.73 \times A_{664}]$ (Jensen and Jensen, 1959 & Duxbury and Yentsh, 1956).
- Carotenoids ($\mu g\ g^{-1}$) = $[7.6 (A_{480}) - 1.49 (A_{510}) V] / (1000 \times W)$ (Seely *et al.*, 1972).
- Fucoxanthin ($mg\ g^{-1}$) = $A_{470} - 1.239 (A_{631} + A_{581} - 0.3 \times A_{664}) - 0.0275 \times A_{664} / 141$ (Sudhakaret *et al.*, 2013).

-Phycoerythrin ($\mu\text{g g}^{-1}$) = $[(A_{564} - A_{592}) - (A_{455} - A_{592}) 0.20] 0.12$ (Beer and Eshel, 1985)
 -Phycocyanin ($\mu\text{g g}^{-1}$) = $[A_{618} - A_{645}) - (A_{592} - A_{645}) 0.15] 0.15$ (Beer and Eshel, 1985);
 here, A = Absorbance at particular wavelength;
 V = Total volume of the pigment extract;
 W = Weight of the sample used for extraction.

Total phenolic content (TPC)

The TPC of the extracts was measured using Follin Ciocalteu method as described by Ganesan *et al.*, (2008) with modification. 50% Follin Ciocalteu's phenol reagent (1 ml) was added to 0.1 ml extract and vortexed. Furthermore, added with 7% Na_2CO_3 (1 ml), and the reaction mixture was then incubated at room temperature for 30 min. The absorbance measured at 750 nm. TPC was expressed in terms of $\mu\text{g gallic equivalents (}\mu\text{GAE)/g}$ dried samples.

1,1-diphenyl-2-picrylhydrazyl (DPPH) assay

DPPH-scavenging potential of different concentrations of extracts was measured based on to test the scavenging ability of stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals by the seaweeds antioxidant. DPPH assay was measured using the method describe by Chew *et al.*, (2008) with modification. Briefly, 2 ml of 93 μM DPPH (solution in methanol) was added to 0.5 ml extract at various dilutions. The mixture was then vortexed vigorously and left for 20 minutes at room temperature in dark condition. The absorbance was measured at 517 nm and its activity was expressed as percentage of DPPH scavenging activity relative to the control, using the following equation:

$$\% \text{ Inhibition} = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100\%.$$

Ferric Reducing Antioxidant Power (FRAP).

Reducing power of seaweed extract was determined by the method prescribed by Chew *et al.*, (2008). The antioxidant activity of the standards was estimated by using the increasing in absorbance caused by the generated ferrous ion. Briefly, 1.0 ml of extracts at various dilutions were mixed with 1 ml of 0.2 M phosphate buffer, pH 6.6 and 50 μl potassium ferricyanide (1%). Reaction mixture was incubated at 50°C for 20 minutes. After incubation, 1 ml of trichloroacetic acid (10%) was added and centrifuged at 3000 rpm for 10 minutes. From the upper layer, 1 ml solution was mixed with 1 ml sterile water and 0.5 ml FeCl_3 (0.1%). Absorbance of all solution was measured at 700 nm. The FRAP value was expressed in terms of $\mu\text{M Fe}^{2+}/\text{mg}$ extract.

Statistical analysis

All experiments were conducted in triplicate. The means of parameters pigment composition, total phenol content and antioxidant activity presented as mean \pm standard deviation.

3.Result and Discussion

Pigment

Regarding photosynthetic pigment a significant variation in content was recorded among the hexane, acetone and ethanol solvent studied (Table 1). The results show that *H. durvillae* contain highest amount of chlorophyll C1+C2 ($2.26864 \pm 0.04 \text{ mg g}^{-1}$ dried weight) whereas, the concentration of phycoerythrin and phycocyanin were found lowest, in hexane were 2.33 ± 0.253 and $0.7875 \pm 0.08 (\mu\text{g g}^{-1})$. Naziret *et al.*, (2013), the concentration of carotenoids, phycoerythrin and phycocyanin in red seaweeds were found higher than green seaweeds. Pereira *et al.*, 2012 reported that red seaweeds *Gracilaria dumingensis* possesses more phycocyanin than green strain. Red seaweeds observed are insignificant variation in chlorophyll and phycocyanin (Plastino *et al.*, 2004). Pereira *et al.*, 2012, seaweeds contain three main photosynthetic pigments i.e. chlorophylls, carotenoids and phycobilins. These pigments provide protection against high light intensity and also support in light absorption and energy transfer to the reaction centre.

Table 1. Quantification of photosynthetic pigments of *H.durvilae* in different solvents

Parameter	Hexane	acetone	ethanol
Chlorofil a (mg g^{-1})	0.181382 ± 0.01	0.01713 ± 0.01	0.02181 ± 0.05
Chlorofil b (mg g^{-1})	0.038453 ± 0.001	0.00315 ± 0.001	0.01918 ± 0.006
Total Chlorofil (mg/g^{-1})	0.219787 ± 0.05	0.02038 ± 0.001	0.04097 ± 0.002
Total chlorofil c1+ c2 (mg g^{-1})	2.26864 ± 0.04	0.08763 ± 0.001	0.90726 ± 0.22
Carotenoid ($\mu\text{g g}^{-1}$)	28.654 ± 0.212	2.642 ± 0.035	5.96 ± 0.3241
Fucoxantin (mg g^{-1})	-0.37143 ± 0.03	0.023399 ± 0.005	-0.0198 ± 0.04
Pycoerytrin ($\mu\text{g g}^{-1}$)	2.33 ± 0.253	0.48 ± 0.018	1.13 ± 0.081
Phycocyanin ($\mu\text{g g}^{-1}$)	0.7875 ± 0.08	0.1475 ± 0.002	0.1565 ± 0.08

All the values are mean \pm SD of triplicates

Carotenoids have highest content in hexane extract, $28.654 \pm 0.212 \mu\text{g g}^{-1}$. Carotenoids scavenge oxygen radicals and reduce oxidative stress. Thus, they denote antioxidant activity. The carotenoids are used in food, nutraceutical, and pharmaceutical preparations by their applications as colorants and their provitamin A activity. One of the most consumed carotenoid groups of pigments are responsible for the yellow, orange, and red colour of many foods, maintain to be intensely explored mainly because of their health-promoting properties. Carotenoids are unstable when they're exposed to light or oxygen because of their properties as highly conjugated and powerfully colored isoprenoid plant compounds (Özkan and Bilek, 2014).

Variation in the pigment concentration is a reply to environmental variations that allows an organism to adjust under a specific habitat. Increased temperature, heavy metal accumulation and ill-inclined light due to extreme exploitation of natural resources and unrestrained anthropogenic activities, induces too much production of ROS which causes injury to biological membranes and unfavorably affect a number of physiological processes (Nasir et al., 2015). Pigment shows several capability to maintain immune system, to help prevent cancer and is being utilized in cancer therapy, to aid to invigorate and energize the body detoxification of the liver, to normalize blood pressure and to struggle bad odors, bad breath as well as body odor by reason of the magnesium salts that it contains (Ferruzzi and Blakeslee, 2007).

Total Phenol Content

Various studies have concentrated on the biological activities or phenolic compounds, which are potential antioxidants and free radical-scavenger. Early research reported that marine seaweeds extracts, particularly their polyphenols, have antioxidant activity. The most important active compounds in different seaweeds extracts have been revealed to be the phlorotannins. The total polyphenol content (expressed as gallic acid equivalent) of *H.durvilae* extracts is shown in Fig.1. It was observed that hexane, acetone and ethanol extracts had TPC 23.235 ± 1.011 ; 22.151 ± 1.967 ; $7.605 \pm 0.383 \mu\text{g GAE g}^{-1}$. Duan et al., (2006) observed TPC ($73.7 \text{ mg GAE g}^{-1}$) in ethyl acetate soluble fraction of red algae *Purcellotia*. The TPC in brown algae, *Papenfussiella kurono* was $0.18 \text{ mg catechin equivalent/g}$ in ethanolic extract (Kuda et al., 2005).

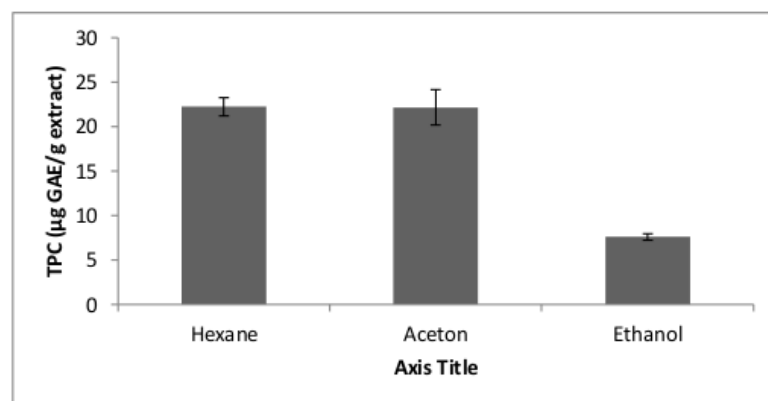


Fig. 1. Total Phenolic Content of *H. Durvilae* extract. Data are expressed as mean \pm SD (n=3).

Marine algae contain phloroglucinol phenol (phlorotannins) which are probably excellent antioxidant, since plant phenolics can perform as ROS scavengers metal chelators and enzyme modulator and inhibit lipid peroxidation (Rodrigo and Bosco 2006). Phenol, 2-[(1-phenylethyl)thio] characterize a diverse group of pigment, extensively distributed in nature. They serve as accessory pigments to harvest light photosynthesis. Moreover, these type of pigments can give rise to rich in polyphenol compounds. Norisoprenoids resulting from the oxidative cleavage of carotenoid are signal in development, provide as antifungal and antibacterial agents and donate to their flavor and aroma. The norisoprenoid derivatives detected were present in the Rhodophyta, namely α -ionone, geranyl acetone, β -ionone, 2,3-epoxy- β -ionone, dihydroactinidiolide and 2,3-Epoxy- β -ionone (Valentão et al., 2010).

DPPH radical scavenging activity

DPPH is a compound that possesses a nitrogen free radical and is readily destroyed by a free radical scavenger. It has been used extensively as a free radical to assess reducing substances and is a valuable reagent for investigating the free radical scavenging activities of compounds. DPPH radical scavenging of *H. durvillae* are presented on Fig. 2. and expressed as presentation reduction of the initial DPPH• absorption by the tested compound.

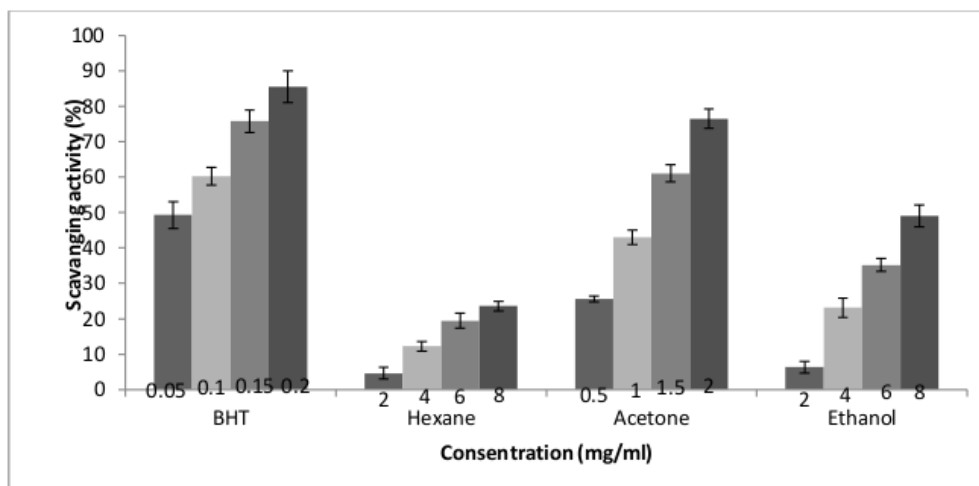


Fig 2. DPPH radical scavenging activity of *H. durvillae* extract. Data are expressed as mean \pm SD (n=3).

The best radical scavenging obtained in the acetone extract. The radical scavenging of hexane, acetone and ethanol extracts were IC_{50} 15.940 ± 1.63 ; 1.1676 ± 1.99 and 8.017 ± 2.31 mg ml⁻¹. The beneficial effect is could beside the high content phenol, it may also be related to the high content of mineral, dietary fiber and pigment. The red seaweeds are a diverse eukaryotic lineage, characterized by accessory photosynthetic pigments phycoerythrin, phycocyanin and allophycocyanins arranged in phycobilisomes. The extracts of *H. durvillae* showed better radical scavenging activity than ethanol extract of *Kappaphycus alvarezii* (Doty) (IC_{50} 3.03 mg ml⁻¹) (Kumar et al., 2008) and the extract of *Palmaria palmata* (dulse) (IC_{50} 12.5 mg ml⁻¹) (Yuan, Carrington & Walsh, 2005). Seaweeds are low in fat but have vitamins and bioactive compounds such as terpenoids, sulfated polysaccharides and polyphenol compounds, the later being a potential natural antioxidant not found in land plants (Chew et al., 2008). Algae polysaccharides participate essential function as free radical-scavengers in-vitro and antioxidant for the avoidance of oxidative damage in living organism. Their activity depends on numerous structural parameter, such as the amount of sulfation, the molecular weight, sulfation position, type of sugar and glycosidic branching. Furthermore, several reports expose that the sulfate and phosphate group in the polysaccharides cause the differences in their biological activities (Juan et al. 2005a). Dietary natural antioxidant are reported to help in preventing aging and other diseases. There are various evidences that seaweeds contain compounds with a moderately high antioxidant and antiproliferative activity (Yuan, Carrington & Walsh, 2005).

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Ferric reducing antioxidant power (FRAP).

The antioxidant activity in FRAP was measured based on the capability of the antioxidant compounds in the sample to reduce ferric (III) to ferrous (II) in a redox-linked colourimetric reaction that involve single electron (Chew *et al.*, 2008). FRAP assay was electron donor and it finished the oxidation chain reaction by reducing the oxidized intermediates into the stable form (Tachakittirungrod *et al.* 2007)

Fig. 3.shows that the highest reducing power of *H. durvilae* was acetone of $0.17 \pm 0.01 \mu\text{M Fe}^{2+} \text{mg}^{-1}$ extra. The reducing ability of acetone extract considerably as same as TPC. There was strong correlation ($R^2=0.96$) between the reducing power and the TPC of the seaweeds methanolic extracts expressed as phologenicol equivalents. (Matanjan *et al.*, 2008).

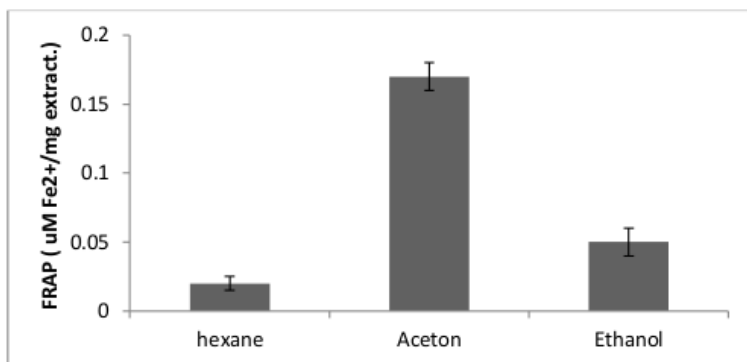


Fig.3. Ferric Reducing Antioxidant Power (FRAP) of *H.durvilae* extracts. Value are the mean \pm SD (n=3)

The reducing power of red algae *Palmaria palmate* in 1-butanol extract was $4.48 \mu\text{g}$ ascorbic acid equivalent (AAC) g^{-1} (Yuan & walsh, 2006). Polyphenols are reducing agent, and together with other dietary reducing agent such as vitamin C, E and carotenoid, referred to as antioxidant, protect the body's tissues against oxidative stress and connected pathologies such as cancer, coronary heart disease and inflammation (Tapiero *et al.*, 2002). The ethanolic extract of *K. alvarezii* showed higher inhibitory effect than did the positive control, BHT. This might be due to the presence of ascorbic acid and Vitamin A (β -carotene) content in the extract of *K.alvarezii* (Fayaz *et al.*, 2005). The reducing power property indicates that the antioxidant compounds are electron donor and can decrease the oxidized intermediates of the lipid peroxidation process, so that they can perform as primary and secondary antioxidants (Yen and Chen 1995).

In vitro antioxidant activity of κ -carrageenan oligosaccharides and their oversulfated, acetylated and phosphorylated derivatives was investigated by Juan *et al.* 2005. They are also reported that phosphorylated and sulfated glucans exhibited better antioxidant capacity than did glucans or other neutral polysaccharides, which indicated that polyelectrolytes, such as glucans sulfate or phosphate, might have enhanced scavenging activity. Moreover the sulfate content from *Porphyra yezoensis* was reported to contribute to the antioxidant activity. The cell walls of *K.alvarezii* known to be constituted of carrageenan, a sulfate polysaccharides, which may contribute to its antioxidant potential in addition to the presence of ascorbic acid, vitamin A and various phenolics (Kumar *et al.*, 2008).

Conclusions.

In the present investigation the various solvent extracts of *H.durvilae* exhibited content of chlorophyll carotenes, phycoerythrin and phycocyanin. The highest content of pigments are in hexane solvent. The antioxidant activity by DPPH assay and reducing power showed the acetone extract is highest. Thus *H.durvilae* could be used as natural pigment and source of antioxidant which is potential to be applied in food product as functional food. Future study is required for identification of the active compound in acetone extract which is responsible for highest antioxidant activity.

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