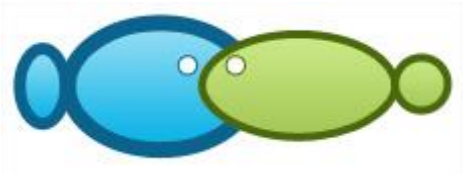

Antioxidant activities of culture supernatant *Haslea ostrearia* adapted in Indonesia

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Abstract. Among marine diatoms, *Haslea ostrearia* has a peculiarity in releasing a water soluble blue-green pigment called marennine. This pigment is responsible of the oyster greening phenomenon in the Atlantic coast of France. The purified form of this pigment has biological activities, such as antibacterial and antioxidant activities, that could be advantageous in aquaculture. However, the purification of this pigment requires a long process and it is unfeasible for its direct application. The present study aims to conduct phytochemical tests to determine the type of bioactive compounds present in the culture supernatant of *Haslea ostrearia* (Blue Water, BW) adapted in Indonesia and to test the antioxidant activities of this BW usingthe DPPH assay. The phytochemical screening showed that the BW contained alkaloid compounds. However, no positive results were observed for flavonoid and phenolic compounds. Additionally, the antioxidant activity of BW *H. ostrearia* was considered very strong, with an IC_{50} value up to 9.09 mg L^{-1}, which is higher than that of vitamin C (2.65 mg L^{-1}), yet lower than that of chlorophyll (28.56 mg L^{-1}). The culture supernatant of *H. ostrearia* containing marennine could potentially be used as a natural antioxidant, more feasible than its purified form, for further applications in aquaculture.
Key Words: Blue Water, marennine, marine diatom, natural antioxidant, pigment.

Introduction. To date, the use of compounds with antioxidant properties is increasingly desired by the public. Antioxidants are compounds that can neutralize or reduce free radicals, and inhibit oxidation in living cells (Rahal et al 2014). When the number of free radicals exceeds the capacity of the organism's cells to neutralize them, oxidative stress will occur, possibly leading to cell, tissue and organ damage (Ames et al 1993; Rahal et al 2014). Therefore, external sources of antioxidants are required to achieve the balance between antioxidants and free radicals in the living cells. External antioxidant compounds consist of synthetic and natural antioxidants. The former are less favored due to their adverse effects such as cell defects, inflammation and carcinogenic effect in long-term usage. Therefore, natural antioxidants are preferable to use since they do not have adverse effects in living organisms. These compounds can be derived from plants, animals or microorganisms, for instance, tocopherol, vitamin C, vitamin A, vitamin E, β-carotene, flavonoid, carotenoid and phenolic compounds (Rahal et al 2014).

Recently, microalgae have attracted attention as a source of high value compounds, including natural antioxidants (Maadane et al 2015; Mourelle et al 2017). These compounds could be pigments including carotenoids, phenolic compounds, sulfated polysaccharides and long-chain polyunsaturated fatty acids (Pouvreau et al 2008; Prasetiya et al 2016; Tan et al 2016).

Among microalgae, *Haslea ostrearia* has a particular ability in producing and releasing a water-soluble blue pigment named marennine, which is responsible for the oyster greening phenomenon on the French Atlantic coast (Gastineau et al 2014, 2018; Prasetiya et al 2016). Although the exact molecular structure of this pigment remains unclear, it has biological activities such as allelopathy (Pouvreau et al 2007; Prasetiya et al 2016), and antibacterial activities (Falaise et al 2016), including antioxidant activity (Pouvreau et al 2008). Recent findings revealed that other *Haslea* species from different regions (not only in the Northern, but also in the Southern Hemisphere) can also produce marennine-like pigment, displaying similar biological activities to those of marennine from *H. ostrearia* (Gastineau et al 2016, 2012; Prasetiya et al 2020) that could be potentially applied in aquaculture by inhibiting the growth of pathogens and increasing the survival rate of cultured organisms (Turcotte et al 2016; Falaise et al 2016). However, the purification of this pigment requires a long and relatively expensive process, thus hampering its feasibility for application in aquaculture. Therefore, the efficiency of utilization of the pigment in form of culture supernatant (hereafter named as blue water, BW) of *H. ostrearia* needs to be assessed.

The purpose of the present study was to characterize the BW from *H. ostrearia* using the phytochemistry method and to determine the antioxidative capacities of BW as a baseline information for its viability in the field of aquaculture.

Material and Method

Culture conditions and BW production. Marine diatom *H. ostrearia* was used in this study. This species was obtained from the laboratory of Mer Molécules Santé (MMS) Le Mans Université, France. *H. ostrearia* was cultured in the Laboratory of Microbiology and Molecular Biotechnology, Faculty of Fisheries and Marine Sciences, Padjadjaran University, Indonesia. This culture was grown under non-axenic conditions in sterilized 500 mL Erlenmeyer flasks, containing 250 mL of artificial seawater medium (Mouget et al 2009; Prasetiya et al 2016) at 20±1°C. Cultures were maintained at an irradiance of 50 µmol photons m^{-2} s^{-1} provided by Philips TLD 36 W/965 fluorescent tubes. Irradiance was measured with a lux meter application (IX LightMeter) that was previously calibrated with the Li-Corr LI-189 quantum meter, in a 24:0 h light/dark cycle.

BW was obtained by harvesting the culture of *H. ostrearia* at the 12^{th} day of culture by filtering the culture supernatant of *H. ostrearia* on a Millipore filter (0.22 µm) prior to the measurement. Afterwards, different concentrations of BW were set up (2, 2.5, 3, and 3.5 ppm) prior to the antioxidant activity tests.

Estimation of marennine concentration. Marennine concentration in the culture supernatant of *H. ostrearia* was estimated following the method of Prasetiya et al (2019a, 2019b, 2016). The concentration (C) of marennine (g L^{-1}) was calculated according to the following formula:

$$C = A\lambda max / (\varepsilon\lambda max \times l)$$

Where: $A\lambda max$ is the absorbance at the peak wavelength in the red region (674 nm); $\varepsilon\lambda max$ is the specific extinction coefficient at the peak wavelength; l is the cuvette path length.

Phytochemical qualitative analysis of BW. The BW solutions from *H. ostrearia* adapted in Indonesia were previously concentrated using a rotary evaporator (Heidolph Co., standard model), until the concentrate was formed. These concentrates were assessed for phytochemical compounds by using the following standard methods described by Gul et al (2017).

Tests for alkaloids. A few drops of chloroform were added to 0.05 g of BW sample and stirred. Afterward, a few drops of NH_4OH were added to the mixture and homogenized. The solution was filtered and the filtrate was dissolved in 10 drops of 2N H_2SO_4, where

the 2 layers are formed. The upper layer (chloroform layer) was collected and tested with Meyer and Wagner reagents (Houghton & Raman 1998). The sample with positive alkaloids was indicated by the formation of white-yellowish and brown precipitates for Meyer and Wagner reagents, respectively.

Tests for flavonoids. The presence of flavonoids was assessed using the Shinoda and alkaline tests. Briefly, in Shinoda tests, pieces of magnesium ribbon and concentrated HCl were mixed with aqueous BW, and the appearance of pink color showed the presence of flavonoids. In the alkaline reagent test, a total of 2 mL of 2.0% NaOH mixture was mixed with aqueous BW. The sample with flavonoids was indicated by the yellow color produced, which became colorless when 2 drops of diluted acid were added to the mixture.

Tests for phenols. 0.05 g of concentrated BW was dissolved in 1 mL of 70% ethanol in a reaction tube. 0.5 mL of the mixture was placed in a secondary reaction tube, then 2 drops of 5% $FeCl_3$ solution were added. Sample with phenol content was indicated by the formation of green or green-blueish color.

DPPH free radical scavenging assay of BW. To determine antioxidant activity, 2,2-diphenyl-1-picryl-hydrezyl (DPPH) was used as free radical as described in Jun et al (2003). Briefly, 4 different concentrations of BW and also chlorophyll, as positive control, were prepared, in concentrations of 2, 2.5, 3 and 3.5 ppm. Afterwards, DPPH was added to the samples with a 1:2 ratio (v/v) that consists of 1 mL of BW or chlorophyll and 2 mL of DPPH 0.1 mM. The mixture was incubated at 37°C for 30 min. The absorbance of the sample was measured at 517 nm by using a UV-Vis spectrophotometer. Ascorbic acid was also used as a standard antioxidant. All readings were conducted in triplicate. The GraphPad Prism 5.0 software for Mac OS was used to calculate IC_{50}. The IC_{50} was calculated from the percentage of inhibition in each sample to evaluate the antioxidant activity. This IC_{50} value shows the concentration of sample solution needed to inhibit 50% of DPPH free radicals. A lower IC_{50} value obtained in each sample means that the samples have a better ability in inhibiting free radicals. Decrease in absorbance indicated increased radical scavenging activity, which was determined by the following formula (Pouvreau et al 2008):

Inhibition (%) = (A – A1/A) x 100

Where: A is the absorbance of the control/blank; A1 is the absorbance of the test samples. The antioxidant activity was derived from the IC_{50} value, which is negatively correlated. The IC_{50} value of each samples was categorized according to Jun et al (2003), as described in Table 1.

Table 1

Different categories of antioxidant activity with DPPH assay

Intensity	*IC_{50} (ppm)*
Very strong	< 50
Strong	50-100
Moderate	101-250
Weak	250-500

Statistical analysis. All data regarding phytochemical screening were analyzed descriptively. All data concerning inhibition were analyzed using one-way ANOVA with a 5% significance level. The Tukey HSD post-test was conducted when significance between samples was observed. All statistical analyses were performed by using GraphPad Prism 5.0 for Mac OS.

Results and Discussion

Estimation of marennine concentration. In this study, the BW concentration from the culture supernatant of *H. ostrearia* adapted in Indonesia was 3.74 mg L^{-1}. This result is lower than the concentration of BW found in the culture of *H. ostrearia* in France, which ranged from 5 to 7 mg mg L^{-1} (Gastineau et al 2014; Prasetiya et al 2016). The low marennine concentration in culture supernatant of *H. ostrearia* adapted in Indonesia is probably due to the biological response of this species to environmental factors, like light. The species adapted in Indonesia was maintained at 50 µmol photons m^{-2} s^{-1} with 24 h of light photoperiod. This condition is different to that of *H. ostrearia* culture in France, where 50 µmol photons m^{-2} s^{-1} of irradiance were used and 14 h of light photoperiod. Indeed, previous findings from Mouget et al (1999) and Prasetiya et al (2016) demonstrated that the strong light irradiance affects the cell ultrastructures of *H. ostrearia*, shrinking chloroplasts that can eventually affect pigment production.

Phytochemical analysis. The phytochemical screening for BW from *H. ostrearia* was carried out to determine qualitatively the presence or absence of a class of bioactive compounds that are potential antioxidants counteracting free radicals. The results of the phytochemical screening tests are qualitatively presented in Table 2.

Table 2

Phytochemical analysis of BW from *Haslea ostrearia* culture adapted in Indonesia

Phytochemical analysis	*Blue water*	*Visual observation*
Alkaloids		
Meyer	+	Formation of white precipitate
Wagner	+	Formation of brown precipitate
Flavonoids		
Concentrated HCl	-	No color change
NaOH 10%	-	No color change
Phenols	-	No color change

Note: (-) - negative; (+) - positive results.

Based on the phytochemical analysis, BW of *H. ostrearia* samples showed positive results for alkaloids. However, negative results were found in the samples for flavonoids and phenols (Table 2). Alkaloid compounds in phytochemical screening tests showed positive results, which are marked by the formation of white and brown precipitates. Alkaloids are semi-polar compounds. The alkaloid test shows positive results because it is characterized by the formation of precipitate and discoloration to brown after reacting with Mayer's reagents (Gan et al 2017). These reagents contain Hg and KI metals, which will form brownish precipitates with alkaloid compounds (Gan et al 2017; Gul et al 2017). Alkaloids are a group of organic compounds found in nature. They generally include alkaline compounds with one or more hydrogen atoms, usually in combination as part of a cyclic system (Morales & Jimenez-Perez 2001; Wang et al 2016). Alkaloids are the largest group of secondary metabolites and are considered a heterogeneous group. To date, approximately 5500 different structures of alkaloids have been discovered (Wang et al 2016).

The present study revealed that phenolic compounds were undetected in the BW samples. This result is in contrast with that of Pouvreau et al (2006a), who found that the culture supernatant of *H. ostrearia* contains phenols. This contradictory result could be due to the different conditions of culture, which may affect the pigment production and variation of pigment content.

Phytochemical screening for flavonoids in BW of *H. ostrearia* showed negative results, indicated by the absence of formation of red, yellow and brown in the amyl alcohol layer. The absence of flavonoid compounds in BW could probably be due to their low quantity in the sample. Flavonoids are one of the secondary metabolites that are

important to living organisms. They are involved in various processes, such as protection from ultraviolet rays and pigmentation (Wang et al 2016).

DPPH free-radical scavenging activity. The highest percentage of inhibition obtained was at 60.31, 22.35, and 11.44% for ascorbic acid, BW and chlorophyll, respectively (Figure 1). This result shows that BW of *H. ostrearia* samples is able to reduce the DPPH radical. Additionally, the ability of BW in hampering the DPPH radical was significantly higher than that of the chlorophyll pigment ($p<0.05$). The percentage of inhibition from marennine in form of BW in this study is comparable to marennine in its purified form (Pouvreau et al 2008).

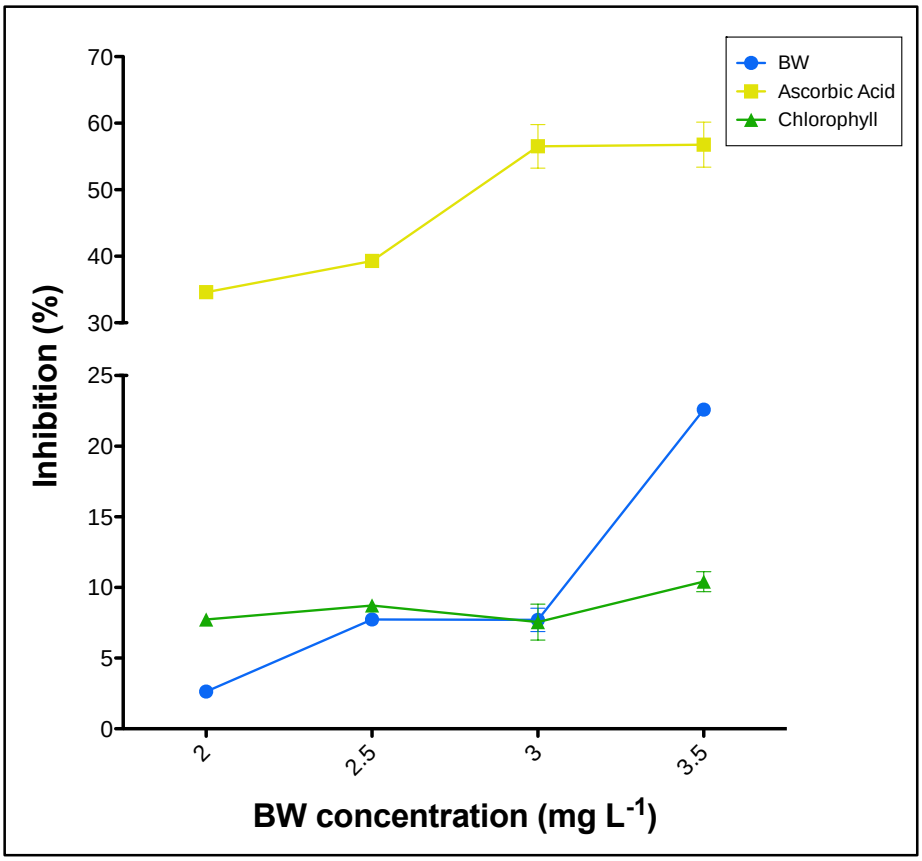

Figure 1. Inhibition of DPPH by ascorbic acid, blue water (BW) from *Haslea ostrearia* and chlorophyll pigment (n=3).

The results showed that the IC_{50} of BW from *H. ostrearia* is significantly lower (9.05; $p<0.05$) than that of chlorophyll (28.56). However, the IC_{50} of BW was significantly higher than that of ascorbic acid (Figure 2).

In the present study, the results showed that the 3 types of sample have a very strong antioxidant activity, based on the categories from Jun et al (2003). The ability of BW in inhibiting free radicals with lower IC_{50} than chlorophyll may imply that marennine in the form of BW could be potentially be used as a natural antioxidant. Nevertheless, the IC_{50} of marennine in the form of BW solution in this study was higher than its purified form, suggesting that the purified form have a stronger antioxidant activity than that of BW (Pouvreau et al 2008). Even though the antioxidant activity of marennine in purified form is better than that of BW, the utilization of the purified pigment could be impractical, especially in the field of aquaculture, due to the long-step purification process and high-cost to produce purified marennine (Prasetiya et al 2019a; Turcotte et al 2016). Therefore, the utilization of BW for aquaculture application could be considered as a better option.

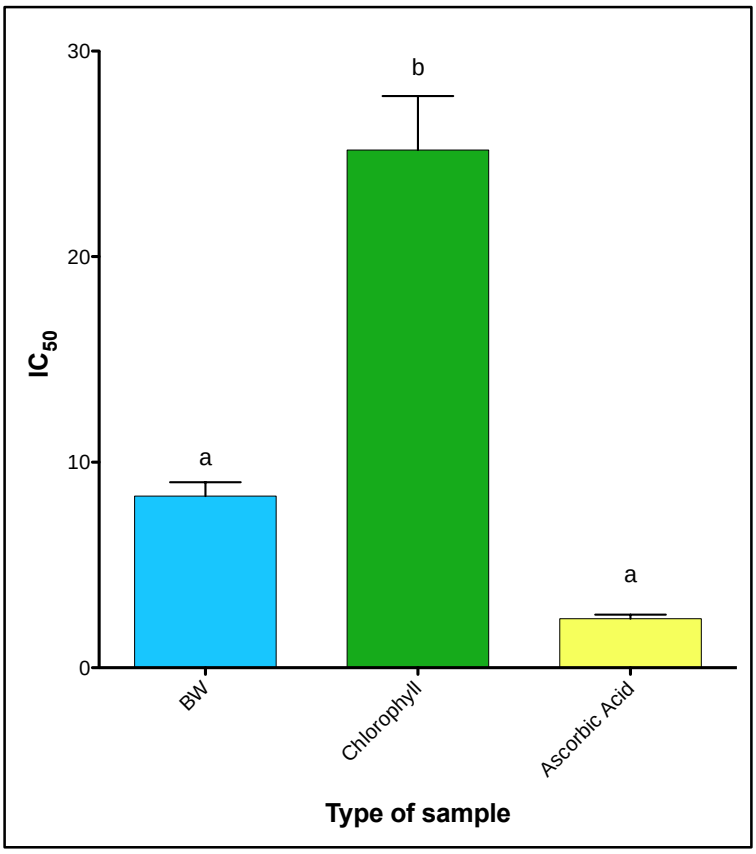

Figure 2. IC_{50} values of Blue Water (BW) of *Haslea ostrearia* in antioxidant activity assays compared with chlorophyll and ascorbic acid (n=3). Error bars with different lower case letters are significantly different ($p<0.05$).

The presence of alkaloid compounds in the BW of *H. ostrearia* may have a positive effect on its antioxidant activity. This is probably because alkaloids generally consist of basic compounds with one or more hydrogen atoms, usually in combination, as part of their cyclic system (Gan et al 2017; Gul et al 2017). Alkaloids are the largest group of secondary metabolites found in natural products and often have toxic properties, being widely used in medicine (Gan et al 2017).

The mechanism of antioxidants by alkaloids is still unclear. Yet, in general, antioxidants have similar core structure, for instance, the unsaturated benzene ring accompanied by hydroxyl groups (-OH), amino acids (-NH_2), or hydrogen (-H). These groups could bind to free radicals. Thus, they produce molecule structures that are no longer reactive (Morales & Jimenez-Perez 2001; Maadane et al 2015; Liu et al 2016). Alkaloid compounds, especially indols, can stop free radical chain reactions efficiently. Other alkaloid compounds which are antioxidants are quinolone and caffeine, which can act as a hydroxyl radical reducer, and melatonin, which plays an important role in protecting cells from the effects of radiation and drug toxicity (Morales & Jimenez-Perez 2001). However, in this study, we could not identify what types of alkaloids play a significant role in BW antioxidant activity.

Our results partially support the potential use of BW as a food additive in aquaculture application, based on two relevant properties: its natural blue coloration and its antioxidant capacity. Additionally, further considerations should be met, such as: the absence of toxicity, bioavailability, and the possibility of production on an industrial scale. The fact that there is a lack of production of natural blue colorants commercially available constitutes a great advantage for marennine. Furthermore, the pigment is highly water-soluble and non-hydrolyzable. Its blue-green dye is heat and light resistant, and it does not exhibit any significant modification in a pH ranging from 6 to 8 (Gastineau et al 2014, 2018). The apparent antioxidant properties of marennine might be beneficial to the

antioxidant system of the organisms and raise the possibility of it being used as a protective agent against the oxidative damage of food products (Pouvreau et al 2008; Gastineau et al 2014). Therefore, further metabolic and toxicological investigations are required to demonstrate that marennine could be used as a food grade supplement, but no toxicity related to the pigment has ever been reported during all the time marennine-greened oysters have been eaten by humans in the Atlantic coast of France (Piveteau 1999; Prasetiya et al 2019a, 2019b). The industrial production of marennine would require an optimization of the cultivation of *H. ostrearia* and the purification of the pigment. In fact, the method proposed by Pouvreau et al (2006b) includes semipreparative technical procedures, like ultrafiltration through membranes, but appeared to be inefficient and costly. Therefore, the utilization of BW such in the present study could offer better advantages.

Conclusions*.* The present study demonstrated that the culture supernatant (BW) from *H. ostrearia* adapted in Indonesia may be considered as a natural source of antioxidants. The pytochemical analysis revealed that marennine in the form of BW solutions contains alkaloids with better antioxidant activity compared to that of chlorophyll pigment. These results reveal that BW could be used as a natural antioxidant in aquaculture. However, further studies are needed, especially on the chronic exposure to low concentrations of BW solutions.

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