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Screening of Bioactive Compounds and Antioxidant Activity of Ale-ale Shellfish (Meretrix meretrix) Crude Extracts from West Kalimantan, Indonesia

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Abstract
The ale-ale shellfish (Meretrix meretrix) is a member of the Bivalvia class of the phylum Mollusca. It is a marine commodity that is widely used as a functional food ingredient and is hereditarily believed to have properties that may cure several types of diseases by the local community in Ketapang Regency, West Kalimantan. The purpose of this study was to determine the chemical components and antioxidant activity of Meretrix meretrix shellfish crude extract. Phytochemical screening of the M. meretrix was carried out qualitatively using phytochemical reagents, while the determination of antioxidant activity was carried out using the free radical scavenging of the DPPH method by measuring using a UV-Visible spectrophotometer. The results of the phytochemical screening of the shellfish extract showed that the components it contained were alkaloids, flavonoids, saponins, and steroids. In contrast, the results of the antioxidant test showed that the methanol extract of M. meretrix shellfish had a strong antioxidant activity with the lowest IC50 value of 84.46 ppm.

Keywords: Meretrix meretrix, Screening, Phytochemical, Scavenging, DPPH

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INTRODUCTION
Bivalves and gastropods are members of the Mollusc phylum. It is one of the marine animal phyla, which is very potential as a candidate source for bioactive compounds both in pharmaceutical dosage forms and as functional food ingredients. Ale-ale shellfish (*Meretrix meretrix*) is a member of Bivalvia that is relatively abundant in tropical waters as a source of animal protein, easy to obtain, and relatively cheap. The peptides, depsipeptides, sesquiterpenes, squalene, terpenes, alkaloids, polypropionates, nitrogen compounds, macrolides, prostaglandins, and some fatty acid derivatives, are found in various species of mollusk. These compounds have the potential for biological activity, which is very important in the world of health (Balcázar et al., 2006; Blunt et al., 2006). Several active compounds that have been isolated from bivalve animals have been used as antioxidants, anticancer, cytotoxic, antiviral, antibacterial, antifungal, and enzyme inhibitors related to the body's metabolic function (Defer et al., 2009; Tadesse et al., 2008; Zhou et al., 2012). Several marine and freshwater organisms produce secondary metabolites that have pharmacological activities (Pringgenies, 2010). Several studies have been carried out on bivalves and gastropods as nutraceuticals and as pharmaceuticals. Those include research on goldfish (*Atactodea striata*) (Mutaqin, 2009); *Cerastoderma edule* (Cardiidae), *Ruditapes philippinarum* (Veneridae), *Ostrea edulis* (Ostreidae), *Crepidula fornicata* (Calyptraeidae), *Buccinum undatum* (Buccinidae) (Defer et al., 2009); ipong-ipong snail
(*Fasciolaria salmo*) (Nurjanah et al., 2011); and Scallops (*Amusium pleuronectes*) (Suptijah et al., 2013) and sand clams (*Semele cordiformis*) (Kalsum et al., 2020). Antioxidant activity is one of the topics of biological activity testing that has become the center of attention of researchers both for land plants or animals as well as those that live in marine waters. Antioxidants are active compounds produced by living things naturally or synthetically, which play a role in preventing the free radical oxidation process (Amarowicz, 2009; Darmawati et al., 2016). Free radical exposure is often associated with various types of degenerative diseases such as inflammation of the joints, cancer, diabetes, premature aging. The main triggers for the formation and exposure of free radicals include internal influences such as continuous normal cell metabolism, unhealthy lifestyles such as adequate eating and rest, and external influences such as environmental pollution, excessive UV exposure, and cigarette smoke (Bhaigyabati et al., 2011). The formation of more free radicals in the environment makes it increasingly necessary to search for antioxidants that are widely available in nature (Hardiningtyas et al., 2014). In this research, the *M.meretrix* shellfish extraction will be carried out with a polarity gradient solvent, and then each crude extract will be tested for chemical components and antioxidant testing with the DPPH free radical scavenging method.

MATERIALS AND METHODS
Sampling and Identification of Samples
Samples of *M. meretrix* shellfish were taken on the coast of Ketapang Island and identified at the Marine Science Laboratory of the Faculty of Math and Natural Sciences, Tanjungpura University. Sample processing includes extraction, phytochemical analysis, and testing of antioxidant

activity carried out at the Research Laboratory of the Department of Chemistry, Faculty of Math and Natural Science, Tanjungpura University, Pontianak Indonesia.

Extraction M. meretrix samples

This extraction was following the methods reported by (Elkhamlichi et al., 2017). The *M. meretrix* shellfish sample were separated from their shells, washed thoroughly and chopped, then extracted by maceration using n-hexane solvent for 3 x 24 hours and separating the pollen solution every 1x24 hours. Then the extracted waste is extracted again with a solvent whose polarity is sequentially ethyl acetate and methanol. The resulting extracts were concentrated using a vacuum rotary evaporator at a temperature of 30-40°C. The resulting extract yield is calculated by the equation:

$$Total\ extract\ yield = \frac{extract\ weight\ obtained}{Initial\ weight} \times 100\%$$

Screening of Bioactive Compounds

Screening for bioactive compounds of methanol, ethyl acetate, and n-hexane extracts was done using the methods reported by (Warsidah et al., 2020).

Steroids

The extract solution was added with chloroform and Libermann-Burchard reagent, the formation of green color indicates the presence of steroid compounds, and red color indicates the presence of triterpenoids.

Alkaloids

Identification of alkaloid compounds using the Mayer test and Wagner test, in the Mayer test, the extract solution is added with Mayer's reagent, and the formation of a white precipitate indicates the presence of alkaloids, while in the Wagner test, the extract solution is added with two drops of Wagner's reagent and the formation of a brown precipitate indicates an alkaloid compound.

Saponins

Aquades were added to the extract solution and shaken vigorously. The formation of foam 1-10 cm, which is stable and not less than 10 minutes, indicates the presence of saponins.

Flavonoids

The extract solution was added with magnesium powder and 2N HCL. The appearance of red, yellow, or orange indicates the presence of flavonoid compounds.

DPPH method antioxidant activity test

The extract of *M. meretrix* shellfish was dissolved using methanol (polar) as a solvent made with concentrations of 40, 80, 120, 160, and 200 ppm. Ascorbic acid was used as a comparative antioxidant and positive control made by dissolving it in methanol with a concentration of 3, 6, 9, 12, and 15 ppm. The DPPH solution to be used is prepared by dissolving DPPH crystals in methanol with a concentration of 0.2 mM. The process of making DPPH 0.2 mM solution is carried out in low-temperature conditions and protected from sunlight (LeeWei and Ismail, 2012; Purwaningsih, 2012).

The extract solution and the comparative antioxidant solution that had been made were taken 2 ml each and reacted with 2 ml of the DPPH 0.2 mM solution in different and labeled test tubes. The mixture was then incubated for 30 minutes, and its absorbance was measured using a UV-VIS spectrophotometer at the maximum wavelength. The maximum wavelength is determined by scanning the blank solution at a wavelength of 400-800 nm. The absorbance of the blank solution was also measured to calculate the percent inhibition. The blank solution was prepared by reacting 2 ml of methanol solvent with 2 ml of 0.2 mM DPPH solution in a test tube. Next, the antioxidant activity of each sample and the comparison antioxidant is expressed as percent inhibition, which is calculated by the following equation:

$$\%\ inhibition = \frac{Control\ absorbance - sample\ absorbance}{Control\ absorbance} \times 100\%$$

The sample concentrations and the percentage of inhibition are plotted on the x and y axes, respectively, in the linear regression equation. This equation is then used to determine the IC50 (Inhibition Concentration 50) value of each sample expressed by the value of y = 50 and x, which will be obtained as IC50 then entered in the equation y = a + bx.

RESULTS AND DISCUSSION

Sample Preparation

The sample used in this research was *M. meretrix* shellfish from the coast of Ketapang Regency, West Kalimantan Province. The *M. meretrix* sample was chopped to expand the surface of the sample to facilitate the entry of organic solvents into the cell and extract secondary metabolites of *M. meretrix* more effectively.

Extraction

The extraction method used in this research is maceration with a multilevel extraction method, which is based on the like dissolve like principle, which means that polar compounds will dissolve easily in polar solvents and non-polar compounds will readily dissolve in non-polar solvents. The maceration process lasts for 3x24 hours with a ratio between the sample and the solvent (1: 1); every 1x24 hours is filtered; and the change of the solvent-based on its polarity is non-polar, semi-polar, and polar. Stirring during the extraction process aims to increase the collision of particles between the sample and the solvent, thereby increasing the process of releasing bioactive components from the tissue and dissolving them in the solvent. The use of solvents based on polarity in the maceration process alternately is n-hexane (non-polar), ethyl acetate (semi-polar), and methanol (polar). Initial extraction using non-polar solvents aims to separate lipids from the sample so as not to block the release of bioactive components in the extraction process with subsequent solvents (Pebrian, 2010). The final step to obtaining a crude extract from the sample is to do evaporation using a vacuum rotary evaporator with a moderate temperature of about 30-40°C to prevent damage to the bioactive components in the evaporation process.

Extraction using three types of solvents with different levels of polarity will produce different amounts of extract yield

(Nurjanah et al., 2011). The yield is the ratio between the weight of the extract produced and the initial weight of the sample used and expressed in percent (%). The yield value of the extract from each solvent can be seen in the bar chart in Figure 1.

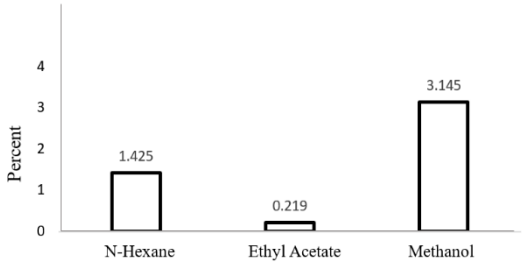


Figure 1. The percentage of crude extracts of *M. meretrix*

The use of different types of solvents, especially with varying levels of polarity in the maceration process, resulted in different yield percentages of the extract. The highest percentage yield value of methanol extract was 3.1%, while the lowest yield percentage value of ethyl acetate extract was 0.2%. Some factors can cause a high percent yield value in methanol solvent. One of the factors is methanol as a solvent, which has a low molecular weight, then makes it easy to form hydrogen and water bonds in the sample tissue. Also, methanol is one of the solvents capable of dissolving almost all organic compounds in good samples, especially those that are polar (Jos et al., 2011). Fats are a subgroup of lipids called triglycerides and are non-polar components. The lack of lipid components in non-polar extracts is thought to make it easier for the next solvent to extract polar and semi-polar components. So that the percent yield in methanol solvent is higher than n-hexane extract. The high or low level of the extract obtained can be influenced by the level of compatibility between the solvent used and the sample to be extracted or the polarity of the solvent and the compounds contained in the sample (Senja et al., 2015; Yanuarizki, 2013). The yield of the extract resulting from the maceration process can be influenced by several factors, including extraction time, type of solvent, comparison of the number of samples and solvents, extraction temperature, and sample particle size (Dewi et al., 2020; Shalaby and Shanab, 2013).

Bioactive Compounds of M. meretrix Extracts

The bioactive compounds test was carried out to determine whether there was a bioactive component contained in the crude extract of *M. meretrix,* which had potential as an antioxidant compound. The bioactive compounds contained in the crude extract can be identified by qualitative phytochemical testing techniques. The tests carried out consisted of four bioactive components, namely alkaloids, flavonoids, steroids, and saponins, based on the fact that many antioxidant compounds are contained in alkaloids, steroids, flavonoids, and saponins (Kannan et al., 2009; Warsidah et al., 2020). The bioactive components of the phytochemical test results in the complete *M. meretrix* shellfish extracts are shown in Table 1. The test results in Table 1 show that the methanol extract contains the most types of bioactive components, namely alkaloids, flavonoids, and saponins, while the n-hexane and ethyl acetate extracts have the same two types of bioactive components, namely alkaloids and steroids.

Table 1. Bioactive compounds of *M. meretrix* extracts

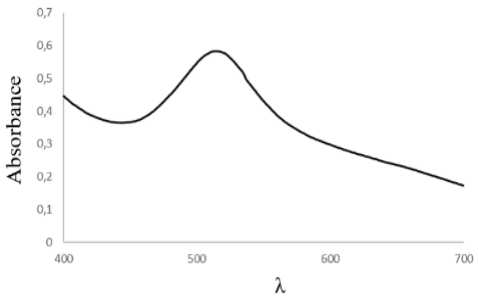
Sample	Phytochemical test				
	Alkaloid		Flavonoid	Steroid	Saponin
	Mayer	Wagner			
Methanol extract	+	-	+	-	+
Ethyl acetate extract	+	+	-	+	-
n-hexane extract	+	+	-	+	-

This shows the distribution of bioactive components based on the polarity of the solvent used. In polar solvents (methanol), there are three types of bioactive components, namely alkaloids, flavonoids, and saponins. In contrast, in semi-polar (ethyl acetate) and non-polar (n-hexane) solvents, two types of bioactive components are found, namely alkaloids and steroids. According to (Harborne, 1998), polar solvents can extract alkaloid compounds of phenolic components, carotenoids, and tannins. Semi-polar solvents are capable of extracting terpenoid and alkaloid compounds. Steroids detected in n-hexane extract (non-polar) and ethyl acetate extract (semi-polar). Those due to the precursor of steroid formation is cholesterol, which is non-polar and normally produced by reproductive organs, so steroids are more easily dissolved in non-polar solvents (Nurjanah et al., 2011).

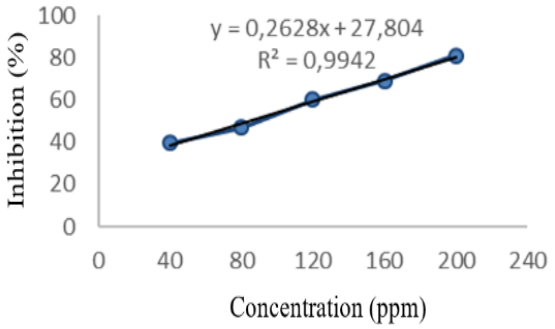
Antioxidant Activity of M meretrix crude extracts

Measurement of antioxidant activity using the DPPH method is a quantitative test method using UV-Vis spectrophotometry at a wavelength of 514 nm, which is the maximum wavelength for direct measurement of DPPH, to obtain the absorbance value of the test sample (Figure 2).

Figure 2. Graph of the maximum wavelength's DPPH



The absorbance value in this study is the value of the results of reducing DPPH compounds as free radicals by the sample of the test solution, which is thought to have antioxidant activity. Antioxidant testing will produce a relationship between sample concentration and percent inhibition. This



relationship is used to obtain a linear equation to determine the value of inhibitory concentration (IC50) (Figure 3, Figure 4, and Figure 5).

Figure 3. Graph of the linear equation of the *M. meretrix* methanol extract

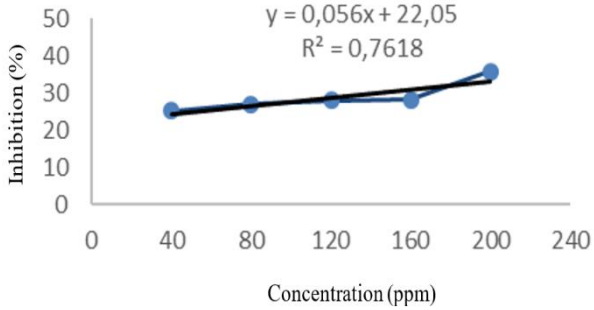


Figure 4. Graph of the linear equation of the *M. meretrix* ethyl acetate extract

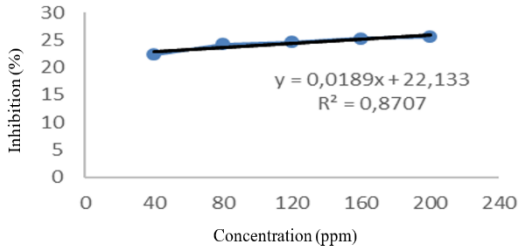


Figure 5. Graph of the linear equation of the *M. meretrix* n-hexane extract

The IC50 value is defined as the concentration of a compound in reducing free radicals at 50%. The smaller the IC50 amount, the higher the free radical scavenging activity (Molyneux, 2004). The results of the antioxidant activity test are displayed in Table 2. The IC50 value is inversely related to the percentage value of inhibition or the ability of the compound to inhibit free radical activity, which is related to the concentration of an extract. Based on Table 2, it can be seen that the higher the sample concentration in the test solution, the higher the percentage of inhibition in inhibiting free radicals. It is inversely proportional to the IC50 value. The lower the value obtained indicates, the greater free radical scavenging activity. Based on the IC50 value, the antioxidant activity can be classified into five groups (Molyneux, 2004).

Based on the classification in Table 3, the methanol extract is classified as strong, while the ethyl acetate and n-hexane extracts are classified as very weak because they have an IC50 value of more than 200 ppm.

Table 2. Antioxidant Activity Test Results of *M. meretrix* crude extracts

Sample	% Inhibition					IC_{50} (ppm)
	40 ppm	80 ppm	120 ppm	180 ppm	200 ppm	
Methanol extract	39.55	46.99	60.02	69.07	81.07	84.46
Ethyl acetate extract	25.07	27.04	27.86	28.18	35.69	499.11
n-hexane extract	22.31	24.24	24.60	25.27	25.58	1474.44

Table 1. Classification of Antioxidant Activity Levels Based on IC50 Value (Molyneux, 2004)

Antioxidant activity	Sample test
Very strong (IC50< 50ppm)	Ascorbic ac
Strong (50ppm <IC50> 100ppm)	Methanol extract
Medium (100ppm <IC50> 150ppm)	-
Weak (150ppm <IC50> 200ppm)	-
Very weak (IC50> 200ppm)	Ethyl acetate extract n-hexane extract

The strong antioxidant activity in methanol extract (IC50: 84.46 ppm) can be due to the ability of methanol to dissolve all polar compounds such as tannins and flavonoids, which are generally bioactive components with strong antioxidant activity. The amount of antioxidant activity is also influenced by the type and amount of bioactive components of the extract used. Based on the test results of bioactive components using the phytochemical method in Table 1, it is known that methanol extract contains more types of bioactive components than extracts from other solvent types, (Fadly et al., 2020; Masriani et al., 2020). In this study, the ability of *M. meretrix* methanol extract as an antioxidant namely alkaloids, saponins, and flavonoids. The amount of antioxidant activity in methanol extract is strongly suspected to be due to the presence of flavonoid bioactive components, which are only found in methanol extract. According to (Mierziak et al., 2014) flavonoids are antioxidant compounds, and (Cook and Samman, 1996) also state that flavonoids are one of the potential bioactive components as antioxidants.

Antioxidants work effectively to neutralize the DPPH, which is triggered by the capability of antioxidant substances in donating H+ to those free radicals

was still lower than ascorbic acid (vitamin C) as a positive control. Ascorbic acid was chosen as a comparison because

it is a proven antioxidant and is widely used as a mixture of packaged beverage products, health, and cosmetics. Based on the test results in Table 2, ascorbic acid has the lowest IC50 value compared to all test samples, namely 4.38 ppm, and is classified as a very strong antioxidant. The high antioxidant activity of ascorbic acid compared to the test sample can be attributed to the fact that the ascorbic acid used is a pure compound, while the test sample is still a crude extract containing certain multi-components and not all of them have antioxidant activity.

CONCLUSION

Based on the results obtained in this study, it shows that the methanol extract of *M meretrix* has the greatest yield, with more bioactive compounds, including alkaloids, steroids, flavonoids, and saponins. Methanol extract also had the greatest antioxidant activity (IC50 = 84.46 ppm) compared to ethyl acetate extract and n-hexane extract.

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