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ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITIES FROM ETHANOL EXTRACT OF *Eucheuma cottonii* FROM LEMUKUTAN ISLAND WATERS WEST KALIMANTAN

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ABSTRACT

Free radicals contribute to human health problem resulting in various human diseases, including central nervous system injury, cancer, inflammations, and the decrease of organ function related to oxidation. This condition has encouraged the effort of finding new natural antioxidant and anti-inflammatory sources. Macroalgae act as excellent natural resources due to their bioactive potential with diverse applications in various fields. Eucheuma cottonii belonging to Rhodophyceae grow abundantly along Lemukutan Island waters, however, their existence has not been exploited. This study aims to evaluate antioxidant and anti-inflammatory activities of ethanol extract of E. cottonii from Lemukutan Island waters, West Kalimantan. The observation of antioxidant activity was done using the method of DPPH (1,1-diphenyl-2-picrylhydrazyl) with UV-vis spectrophotometer, while anti-inflammatory acyivity activity was determined using the RBCs membrane stability method. The ethanolic extract of E. cottonii had potential antioxidant activity with IC50 of 127.75 ppm and was classified as moderate category. Extracts showed also anti-inflammatory acyivity activity with the concentration of 219.83 ppm. Red macroalgae E. cottonii can be used as potential natural antioxidant and anti-inflammatory agent.

Keywords: antioxidant; anti-inflammatory; ethanol; Eucheuma cottonii; Lemukutan Island

INTRODUCTION

Free radicals contribute to human health problem, bother cellular metabolism, and make alterations to the major target like DNA, proteins, and lipids (Young and Woodside, 2001). Furthermore, free radicals also cause depletion of antioxidants that play an important role in the immune system, changes gene expression and induces abnormal protein (Lan *et al*., 2007). Damage to protein cause an oxidative protein denaturation while DNA damage resulting in gene mutation and formation of cancer cells (Sivanandham, 2011). It has consequences on diverse diseases, namely atherosclerosis, arthritis, gastritis, ischemia, central nervous system injury, cancer, and joints inflammation (Abdolghaffari, 2010; Rohman *et al*., 2010).

The oxidation process is one of the most important pathways for the formation of free radicals in food, medicine, and even in living systems. Basically, antioxidants are present in the human body and sufficient amount of antioxidants is an attempt to prevent the adverse effects of ROS (Reactive Oxygen Species) on body tissues. When there are too many free radicals in the body, external antioxidants are needed by consuming foods or supplements containing antioxidant compounds (Fernández-Sánchez *et al*., 2011). Antioxidants play crucial preventive role in several diseases linked with oxidative stress (Bungau *et al*., 2019; Sunkara and Raizner, 2019; Bakir *et al*., 2020). Natural antioxidants can be derived from various plants, including marine macroalgae (Caleja *et al*., 2017; Sofiana *et al*., 2020) because they generally contain bioactive constituents such as alkaloids, flavonoids, steroids, terpenoids (Rickert *et al*., 2016) which are potential as antioxidant compounds.

Macroalgae is one of the photosynthetic organisms, found abundantly in marine waters (Setyobudiandi *et al*., 2009; Simatupang *et al*., 2018), and plays a crucial role in the primary productivity (Tait and Schiel, 2010; Runcie and Riddle, 2012; Sudhakar *et al*., 2018). In marine ecosystem, macroalgae act as an excellent natural resources due to their bioactive potential with diverse applications in various fields (Leandro *et al*., 2020), such as human health and alternative source of nutrients (Afonso *et al*., 2019; Corsetto *et al*., 2020; Kulshreshtha *et al*., 2020; Garcia-Oliveira *et al*., 2021; Carpena *et al*., 2021), medicine (Pooja, 2014), therapeutic (Ismail et al., 2020), cosmeceutical and nutraceutical (Garcia-Vaquero *et al*., 2020; Ruslan *et al*., 2021), therapeutic and pharmaceutical (Abdelwahab, 2017).

Eucheuma cottonii is classified to red macroalgae (Rhodophyceae), characterized by the presence of phycoerythrin pigment, phlorotannins (Biris-Dorhoi *et al*., 2020), carotenoids compound (Suryaningrum *et al*., 2006), and well known as carrageenan producer (Anggadiredja *et al*., 2006). In addition, this species is able to synthesize significant amount of bioactive compounds which are potential to be developed. Previous studies have reported the valuable constituents of *E. cottonii* with a wide range of biological activities, such as antidiabetic (Prasasty *et al*., 2019), antibacterial (Bhuyar *et al*., 2020; Sukmawati, 2021), antioxidant to protect various diseases and stress (Muawanah *et al*., 2016; Yanuarti *et al*., 2017; Wulandari *et al*., 2018; Bangngalino and Badai, 2018; Sambodo, 2019; Ak and Türker, 2019). Moreover, macroalgae with phlorotannin content have been discovered to possess anti-inflammatory properties (Brown *et al*., 2014; Sudirman *et al*., 2018; Gutiérrez-Rodríguez *et al*., 2018).

In the medical sector, inflammation is one of the most complex problem conditions, triggered by many factors such as environmental pollution (Pratiwi and Lingkungan, 2010), infection from various bacteria, intoxication of chemical (Sornsiri *et al*., 2018), poisons and extreme temperature changes (Selawa *et al*, 2013). These factors lead to injury or cells death. Inflammatory reactions are the body's way of fighting these conditions and stimulation by proinflammatory agents such as cytokines and bacterial lipopolysaccharide (LPS) will activate immune cells through various reseptor (Zhang and Ghosh, 2000). The release of histamine, bradykinin, prostaglandins, cell migration, and repair of body tissues indicate an inflammatory response as the body's Defense (Chippada *et al*., 2011). Anti-inflammatory activity is closely related to antioxidant activity. These antioxidants can prevent oxidative stress that affects the stability of red blood cells (RBCs) (Kumar *et al*., 2011).

Marine macroalgae *E. cottonii* grow abundantly along Lemukutan Island waters, however, their existence has not been utilized optimally by the community. In the effort of finding new natural biological activities, this study aims to evaluate antioxidant and anti-inflammatory activities of ethanol extract of *E. cottonii* represent rich in bioactive compounds from Lemukutan Island waters, West Kalimantan.

RESEARCH METHODS

Samples Collection and Identification

Marine macroalgae *E. cottonii* samples were obtained from Lemuutan Island waters, West Kalimantan, Indonesia. Identification, extraction, antioxidant determination and the testing of anti-inflammatory activity had been carried out in the Laboratory of Marine Science, Faculty of Mathematics and Natural Sciences, Universitas Tanjungpura.

Figure 1. Macroalgae *E. cottonii* from Lemukutan Island

Samples Preparation

The preparation of *E. cottonii* sample has been carried out by referring to the method by Sofiana *et al*. (2020) and Warsidah *et al*. (2020). In order to reduce water content, samples were washed and dried at room temperature for 3-5 days. Furthermore, samples were chopped to a size of 0.5 cm, then weighed as much as 500 g, followed by the extraction using a maceration method with ethanol 70% solvent during 2x24 hours. The filtrate was separated and remaceration process was done on the extract dregs for 2x24 hours. The next step, the filtrate was collected and evaporated with a rotary evaporator at a boiling temperature of 70 °C. The concentrated extract was then tested for antioxidant and anti-inflammatory activities.

Antioxidant Activity Test

The test of antioxidant was specified using DPPH (2,2-diphenyl-1-picrylhydrazyl) method as a free radical and Vitamin C as a positive control solution. The DPPH method is based on the presence of a bond between the hydrogen atom of the antioxidant compound in the sample and the free electrons of the DPPH radical compound which causes a change from a free radical (diphenylpicrylhydrazyl) to a non-radical compound (diphenylpicrylhydrazine) (Banerjee *et al*., 2005). The preparation of concentration series of 50 ppm, 100 ppm, 150 ppm, and 200 ppm were done before starting the test. Then, reagent of 0.1 mM DPPH was prepared by dissolving 0,002 g of DPPH in 50 mL of 96% ethanol. As much as 3 mL (1:3 v/v) DPPH solution was added to each sample. Hereinafter, samples and DPPH were homogenized using vortex for 1 minute and incubated in dark place for 30 minutes at room temperature. The measurement of absorbance was conducted using a Spectrophotometer U-1240 Shimadzu MiniUV at a wavelength of 517 nm, and methanol was used as blank solution. The inhibition percentage was determined by the following formula:

$$\% \text{ inhibition} = \frac{\text{blank absorbance} - \text{sample absorbance}}{\text{blank absorbance}} x\ 100\% \quad (1)$$

Determination of Anti-inflammatory Activity

Solution Preparation

The solution of phosphate buffer (pH 7.4) was prepared by dissolving of 2.671 g disodium hydrogen phosphate ($Na_2HPO_4.2H_2O$) in distilled water to a volume of 100 mL. Then, as much as 2 g sodium dihydrogen phosphate ($NaH_2PO_4.H_2O$) was also dissolved in distilled water up to a volume of 100 mL. The mixing of the solution was carried out at room temperature by adding 81 mL of $Na_2HPO_4.2H_2O$ with 19 mL of $NaH_2PO_4.H_2O$, respectively. Furthermore, the preparation of isosaline solution was conducted at room temperature by dissolving of 85 g NaCl in a solution of phosphate buffer solution pH 7.4 up to reach a volume of 100 mL. In other hand, the preparation of hyposaline solution was done by dissolving of 0.25 g NaCl into a phosphate buffer solution of pH 7.4, then adjusted to a final volume of 100 mL (Oyedapo *et al*., 2010). Moreover, acetylsalicylic acid solution of 100 µg/mL was made by as much as 5 mg acetylsalicylic acid dissolved into an isosaline solution up to reach a volume of 50 mL.

Preparation of Red Blood Cells (RBCs) (10% v/v)

As much as 3 mL of RBCs were taken from female experimental rabbits, placed into the EDTA tube, followed by centrifugation with a speed of 3000 rpm for 15 minutes at room temperature. The supernatant was separated, then the residue was transferred into the centrifugation tube and added isosaline solution, and and re-centrifuged. In order to get a clear isosaline solution, the process was repeated three times (Oyedapo *et al*., 2010). Further, the preparation of 10% RBCs suspension was caried out by mixing of 2 mL RBCs with 18 mL of isosaline solution (Saleem *et al*., 2011).

Anti-inflammatory Activity Test

Anti-inflammatory activity was determined using the RBCs membrane stability method with various solutions, such as test solution, control test solution, and standard control solution. The test solution consisted of 1 mL ethanol extract solution of *E. cottonii*, 2 mL hyposaline, 1 mL of 0.15 M sodium phosphate buffer (pH 7.4), and 0.5 mL (10% v/v) RBCs suspension in isosaline solution. The test control solution consisted of 2 mL hyposaline; 1 mL of 0.15 M sodium phosphate buffer (pH 7.4); 1 mL of isosaline and 0.5 mL (10% v/v) of RBCs suspension in isosaline. The standard test solution consisted of 2 mL hyposaline; 1 mL of 0.15 M sodium phosphate buffer (pH 7.4); 1 mL acetylsalicylic acid (100 µg/mL) and 0.5 mL (10% v/v) suspension of RBCs in isosaline. Each of the solutions was then incubated in a water bath at the temperature of 56 ºC for 30 minutes. Then the solution was centrifuged at 3000 rpm for 15 minutes. The absorbance of the solution was measured using a UV-Vis spectrophotometer at a wavelength of 560 nm (Juvekar *et al*., 2009). The percentage of hemolysis inhibition calculated using the following formula (Leelaprakash and Dass, 2011).

$$\%\ \text{hemolysis inhibitory} = 100\ x\ \frac{A1 - A2}{A1} \quad \text{..... (2)}$$

where A1 is absorbance of test control solution and A2 is absorbance of test solution or standard test solution.

RESULTS AND DISCUSSION

Antioxidant Activity

Antioxidant activity was described as the capability of a compound to impede oxidation reactions, and commonly expressed by the percentage of inhibition. IC_{50} was the 50% inhibitory concentration used to assess the effectiveness of an extract in resisting exposure to DPPH free radicals (Chakraborty and Joseph, 2016). Our finding result of ethanolic extract of *E. cottonii* exhibited a potent antioxidant activity with IC_{50} of 127.75 ppm (Fig. 1). This value was classified as moderate antioxidant category (Molyneux, 2004). As a comparison, the use of vitamin C as positive control showed very strong antioxidant activity (IC_{50} = 7.14 ppm). This corresponds that vitamin C is commercial antioxidants that found in food ingredients (Rodwell *et al*., 2018).

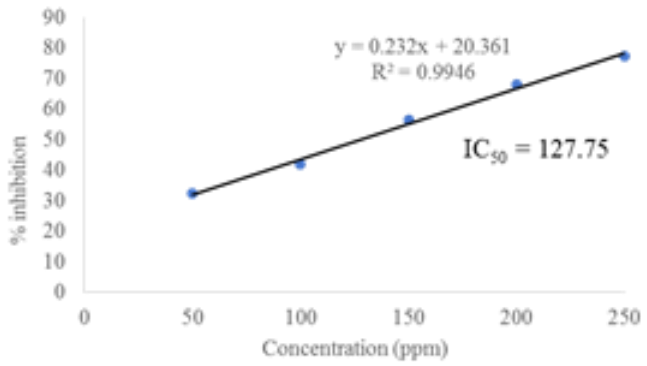

Figure 2. Antioxidant Activity of Ethanol Extract of *E. cottonii*

The result of previous studies showed that ethanol extracts from red seaweed generally contain flavonoid, steroid, tannin, saponin as well as sulfated polysaccharide compounds (Imran *et al*., 2021; Widowati *et al*., 2021) which have a major role in increasing the immune system through inhibition of free radicals or as antioxidants (Abdalla and Shigidi, 2019). Flavonoid is one of the main phenolic compounds in plants which can inhibit cyclooxygenase enzymes and arachidonic acid, thereby inhibiting the synthesis of prostaglandin (Murningsih and Ahmad, 2016). Meanwhile, saponins and tannins play a role in binding cations, therefore the stability of the red blood cell membrane is maintained (Oyedapo *et al*., 2010).

In biological systems, antioxidants had several functions, including preventing oxidative damage. Some studies have demonstrated antioxidant activity from *E. cottonii*. Polysaccharide crude extract of this species showed relatively strong antioxidant activity with IC_{50} of 72,49 ppm (Muawanah *et al*., 2016). The ethanolic extraction from *Eucheuma* species was assessed to produce antioxidant potential properties (Bhuyar *et al*., 2020), and ethanol solvent exhibited the strongest value of antioxidant activity compared to distilled water (Prasasty *et al*., 2019). Furthermore, the extraction of *E. cottonii* using an alkaline solution had IC_{50} value of 39,926 ppm, and was classified as very strong antioxidant activity (Wulandari *et al*., 2018).

Antioxidant activity in macroalgae is closely associated with the content of total phenolic compounds (Fernando *et al*., 2018; Ak and Türker, 2019; Arunkumar *et al*., 2020). It is in accordance with previous studies that phenolic is presently dominantly in macroalga and this compound extracts exhibited a wide range of biological activities, including anti-inflammatory activity (Catarino *et al*., 2017; Neto *et al*., 2018) and as the most antioxidant potential source (Cox *et al*., 2010; Machu *et al*., 2015). Besides, the IC50 value was also determined by the type of solvent during the extraction process (Bangngalino and Badai, 2018).

Red blood cells are very vulnerable from exposure to free radicals, therefore antioxidant and anti-inflammatory active compounds are needed to protect cells. Antioxidant compounds play a role in inhibiting the oxidation process by binding free radicals (Corsetto *et al*., 2020), while anti-inflammatory compounds has the function in stabilizing cell membranes. The occurrence of oxidative stress in cells is caused by high exposure to free radicals that can not be neutralized by antioxidant compounds. Thus, there will be an imbalance between free radicals and antioxidant compounds (Halliwell and Whiteman, 2004). Excess radicals that could not be captured by antioxidants will then react with lipids, proteins and nucleic acids from cells and ultimately cause damage in the cell membrane or hemolysis (Kumar *et al*., 2011).

Anti-inflammatory activity on stability of RBCs membrane

In vitro anti-inflammatory observation on the ethanolic extract of *E. cottonii* was carried out using the RBCs membrane stability method, because these cells have similarities to the structure of the lysosomal membrane. Red blood cells were induced by adding a hypotonic solution which caused lysis of the cells. In addition, the sample solution and red blood cells were heated in a waterbath before the measurement of anti-inflammatory activity. The ability to inhibit the red blood cells lysis indicates the stability of the lysosomal membrane (Leelaprakash and Dass, 2011).

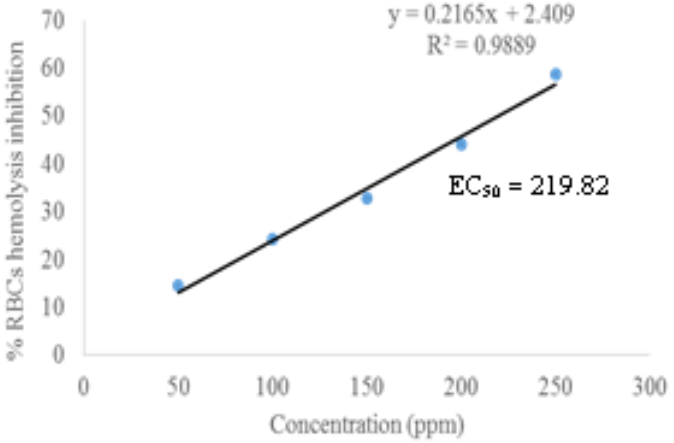

Figure 3. The Inhibition Curve of an Ethanol Extract Derived from *E. cottonii*

In this study, anti-inflammatory activity of ethanolic extract from *E. cottoni* was 219.83 ppm and as positive control, acetyl salicylic acid (aspirin) had percent inhibition of 160 ppm. Some studies reported anti-inflammatory activity from red macroalgae. Ethanolic extract of *E. cottonii* indicated protection to the colonic tissue damage and has potential apllication for the colitis disease treatment (Sudirman *et al*., 2018). The macroalgal extraction produces a rich bioactive potential compounds, such as carrageenan, fucoidan, and chondroitin which showed different effects (Biris-Dorhoi *et al*., 2020). Moreover, sulphate polysaccharides (fucoidans) extract was reported as potential anti-inflammatory and did not cause negative effects on human health (Gunerken *et al*., 2015). Anti-inflammatory activity of sulfated polysaccharide can be seen by the presence of inhibition of histamin and vascular permeability (Coura *et al*., 2015). Carrageenans play as an induction agent of anti-inflammatory biological activity in experimental animals (Morris, 2003).

Compounds that are able to stabilize red blood cell membranes allow them to have the ability to inhibit the initial process of the inflammatory phase. This process is characterized by the release of the enzyme phospholipase (A2), which can damage tissues thereby initiating the formation of free radicals. The role of phospholipase A2 in the cell membrane is to convert the phospholipids in the cell membrane into arachidonic acid, which is then converted by the cyclooxygenase enzyme into prostaglandin compounds. Prostaglandins are the mediators of the inflammatory reaction in cells (Kumar and Vivek, 2011).

CONCLUSION

The ethanolic extract of *E. cottonii* had potential antioxidant activity with IC_{50} of 127.75 ppm and was classified as moderate category. Extracts showed also anti-inflammatory acyivity with the concentration of 219.83 ppm. Red macroalgae *E. cottonii* can be used as potential natural antioxidant and anti-inflammatory agent.

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