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2 Antioxidant effect of taurine and macroalgae (*Sargassum* sp. and *Gracilaria* sp.) extraction on numbers of blood cells and protein profile of mice induced by benzo(α)piren

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23 **Abstract.** The aims of the study was to elucidate the antioxidant effect of taurine and two macro-algae (*Sargassum* sp. and *Gracilaria* sp.) methanol extraction on the number of blood cells (RBCs and leucocytes) and blood protein profile of mice induced by benzo(α)piren. Six experimental groups were applied to this study, they were control group (K-), benzo(α)piren injection only group (K+), benzo(α)piren injection then followed by *Sargassum* extraction (T1), benzo(α)piren injection then followed by *Sargassum* extraction and taurine (T2), benzo(α)piren injection then followed by *Gracillaria* extraction (T3) and benzo(α)piren injection then followed by *Gracillaria* extraction and taurine (T4). The amount of benzo(α)piren applied by sub-cutaneous injection was 0.3 mg/bw benzo(α)piren for 10 days, while the amount given of *Sargassum* sp. and *Gracillaria* sp. extraction each was 8 mg/bw and the amount given of taurine was 15.6 mg/bw. All the extracts and taurine was given to male mice prior benzo(α)piren injection. One way ANOVA was used followed by LSD at α 5% to analyze the collecting data. The results indicated that taurine and both macro-algae were able to normalize back the numbers of red blood cells and leucocytes affected by benzo(α)piren induction. While the blood protein profiles (from the SDS-Page) indicated that there was an increase in expression of 16KDa, expression of protein with 27 KDa (presumably BCl-2 in which its band of 26 KD was phosphorylized) and expression of 41KDa (which presumably α -1-Acid glycoprotein, the protein produced in response to cell damage). Yet the 41 KDa protein was not expressed in groups of both macro-algae combined with taurine.

1. Introduction

3 Proliferation of ROS (Reactive Oxygen Species) molecules and the oxidation of NADPH (Nicotinamide Adenine Dinucleotide Phosphate) is required for redox reactions in metabolic processes [1,2,3]. The ROS formed then will bind to polyunsaturated fatty acids which are abundant in cell membrane, causing oxidative damage to various organs, including bloods. The abundant of these free radicals will affect the oxidant and antioxidant balancing produced the body, called oxidative stress [4]. This oxidative stress which followed by oxidative damage then will prolong the process of aging and initiate degenerative diseases including cancer [5]. To prevent this oxidative stress, antioxidant is released through the level of cells, membrane and extracellular of the body and/or from intake food to compensate the excessive of free radicals [6]. Plants, from many different habitat, are considered an important source for novel drugs due to the potent efficacy with few side effects. From which many studies had been done to explore natural potential of antioxidant, some of which were from marine



products, such as macroalgae or seaweeds which found abundantly in Indonesia marine [7]. *Sargassum* sp., one of macroalgae (Phaeophyceae), is known to produce secondary metabolite in form of phenol and its derivatives which also believed as one of major component for antioxidant [8]. *Sargassum* sp., consisted of protein, lipid, carbohydrate, alginate, vitamins, minerals including iodine, polyphenol (flavonoid and phlorotannin) and fucoxanthin which play role as antioxidant to counteract free radicals [9,10]. Another seaweed which commonly fed by coastal community as medicine is *Gracilaria* sp., one of red algae. *Gracilaria* sp., is source of good fibers which contained of 66.5% fibers and three times longer compared to others algae [11]. As well as those in *Sargassum* sp., *Gracilaria* sp. also contained of protein, lipid, alkaloid, flavonoid, saponin, triterpenoid and tannin [12]. The total phenolic content of *Gracilaria* sp. was as much as 31.38 mg/g in extract, producing antioxidant activity as much as 24.37 ± 1.63 [13].

Beside these two macroalgae, taurine (*2-aminoethanesulfonic acid*), also known has highly antioxidant activity [14], can be found in beans and nuts and also used to absorb lipid and lipid solved vitamins, to enhance cell growth particularly in muscle tissue, to help nutritional distribution in the body, to control heart rate, to stabilize cell membranes and to maintain neurons. Taurine is also considered to play role in controlling biochemical changes due to aging and cell damage caused by free radicals as well protecting in oxidative stress [15,16,17].

This study, therefore, was to elucidate the antioxidant effect of both macroalgae (*Sargassum* sp. and *Gracillaria* sp.) and taurine on the number of blood cells (erythrocytes and leucocytes) and plasma protein profile of mice blood induced by benzo(a)pyrene, which is known as PAH (*Polycyclic Aromatic Hydrocarbon*), one of strong procarcinogenics [18].

2. Materials and Methods

2.1 Plant material

The plants which consisted of two different macroalgae for the present investigation was collected from the Teluk Lampung seawater for *Sargassum* sp. and *Gracillaria* sp. was collected from West Borneo seawater in June – August 2017.

2.2 Preparation of the extract

Both of macroalgae were sorted and rinsed with tap running water. They were dried in oven at 30 - 35°C temperature for a week before crushed and blended well by using a food processor. The fine two macroalgae each then were macerated by using methanol (in 1:10 volume). The macerated macroalgae then was evaporated by using rotary evaporator at 50°C followed by heated process in the oven for 4 – 5 hours until the very fine paste was obtained [19]. The evaporated final content was used for the phytochemical work and animal treatment. Prior given to the treated animals, each of the extract was diluted into 1% CMC used for food [20].

2.3 Animals and benzo(a)pyrene induction

All experimental animals were approved by the University Ethics Committee on the use of laboratory animals and experiments were performed according to the Committee guidelines. Thirty male mice (*Mus musculus*) were purchased from the Veterinary Investigation Center (BPPV), Lampung, Indonesia (age 2-3 months and weight 30-35 g). Mice were conditioned in polyurethane boxes, placed in an air-conditioned environment at 25°C with controlled lighting and exhaust, and received standard laboratory diet (protein, 16.04%; fat, 3.63%; fiber, 4.10%; and metabolic energy, 0.012 MJ) and water *ad libitum*. All experimental animals were acclimatized in laboratory condition for 7 days.

Induction of benzo(a)pyrene was assigned for 10 days in sub-cutaneous injection with dosage of 0.3 mg/bw diluted in 0.2 ml of corn oil. After 10 days of injection, each groups of animal were

treated with macroalgae extraction with and without taurine (15.6 mg/bw) administration. Mice induced with benzo(α)pyrene showed oedema in their necks in form of nodules which consisted of yellowish fluid.

2.4 Collection of blood samples

Mice were euthanized using pentobarbital sodium after 25 days of treatment. Blood samples were taken and harvested sera stored at -20°C for biochemical analysis and some was used to determine the number of blood cells (erythrocyte and leucocyte number) by using haemocytometer. For the protein profile analysis, the nodule fluid was also determined. Animals were randomly assigned to 6 experimental treatments as shown in **Table 1**.

Table 1. Experimental design

Treatments	
K -	Normal group
K+	Benzo(α)pyrene induction without any macroalgae extract nor taurine
T1	Benzo(α)pyrene induction with <i>Sargassum</i> sp. extract (8 mg/bw)
T2	Benzo(α)pyrene induction with <i>Sargassum</i> sp. extract (8 mg/bw) + taurine (15.6mg/bw)
T3	Benzo(α)pyrene induction with <i>Gracillaria</i> sp. extract (8 mg/bw)
T4	Benzo(α)pyrene induction with <i>Gracillaria</i> sp. extract (8 mg/bw) and taurine (15.6 mg/bw)

bw : body weight

2.5 Determination and analyzing of the blood cells number

The numbers of erythrocytes as well as leucocytes was done by using haemocytometer. As much as 0.5 μL of blood was drawn into pipet and diluted with 101 μL of Hayem' solution to make 200x dilution. For leucocytes number, 0.5 μL of blood was drawn into pipet and diluted with Turk's solution as much as 11 μL to make 20x dilution. Blood suspension was shook well until it became homogenized suspension. Each of the suspension blood was dripped into haemocytometer covered by a cover glass. The first drip was discharged then the second one was used and dripped at the edge of the cover glass within haemocytometer. The number of erythrocytes was counted from 5 small boxes of the middle larger box of haemocytometer, while the leucocytes was counted from 4 box of the edge. Calculation of the both blood cell numbers was based the following formula:

The total number of erythrocytes/ $\text{mm}^3 = N \times p \times 50$

The total number of leucocytes/ $\text{mm}^3 = N \times p \times 50$

Note : N : Total number of erythrocytes/leucocytes in all counting chambers, p : degree of dilution

All the collecting data was tested by using *One Way ANOVA (Analysis of Variance)* followed by *Dunnnett's test* at 5% level of significant (SPSS 16.0 for Windows program; SPSS Inc., Chicago, Illinois, USA).

2.6 Determination of blood protein profile

For protein profile of the blood, all the collected sera and the nodule fluid was centrifuged in 13.000 rpm for 10 minutes, then supernatants were collected. One (1) μl of supernatant was taken and diluted with NaCl physiologic salt 50 μl (for 50x dilution), then 10 μl of the plasma dilution was added with 10 μl loadingbuffer (Laemmli Buffer Sample and β -Mercaptoethanol). TGX Stain-Free™ FastCast™ Acrylamide Kit 12% was used to run 12% gel electrophoresis, in which it contained of

resolverstacker gel. APS 10% and TEMED was filled into the electrophoresis glass followed by placing the electrophoresis comb for 30 minutes or after the thickened gel formed. This gel then was placed in *electrophoresis chamber* and added with buffer solution (10x Tris/Glycine/SDS Electrophoresis Buffer). Each of the wheel was added with 10 μ l *marker protein* (Bio-Rad Precision Plus Protein™ Standard with mw of 10 KDa up to 250 KDa), and each sample in each wheel as much as 20 μ l. Electrophoresis was run in 100 Volt.

Commasie blue was used to run the gel and placed in Ultra Rocker Bio-Rad with shaking rate of 40 rpm for an hour. Destainer from higher to lower concentration was applied to dispose of excessive *commasie blue* from the gel until the bands were visible. Electrophoresis gel then was placed in aquades and refrigerator. Analysis of the electrophoresis gel showing the protein profile was done and calculated for their Rf (*Retention factor*).

2.7 Molecular weight determination of protein profile of the mice blood sample

Molecular weight of the protein bands was calculated by using BioMed MW Converter®- Molecular Weight Conversion Tool [21]. The standard curve was made by calculating the Rf (*Retention Factor*) values from marker bands, from which the distance of band from the wheel mouth divided the total migration of the sample as x axis and the molecular weight of the marker bands as y as ordinate. The value of axis and ordinate was used to make standard curve with Regresi Power ($y = ax^b$) method and this formula then used to determine the molecular weight of profile protein in the mice blood samples. All the data in protein profile of the mice bloods was descriptively analyzed by observing the protein composition of mice blood plasma, such as existing and thickening of protein bands.

2.8 Statistical analysis

Biochemical and blood cells number data were compared using one-way analysis of variance (ANOVA) followed by Post Hoc Dunnett's test (SPSS 13.0 for Windows program; SPSS Inc., Chicago, Illinois, USA). The p value of <0.05 was considered statistically significant and values were expressed as the mean \pm standard deviation of the mean (SDM).

3. Results and Discussion

3.1 Erythrocytes and Leucocytes Numbers

The effect of benzo(α)pyrene on the number of blood cells from different treatment groups can be seen in **Figure 1**. All of the treatment groups decreased in their erythrocytes number as the response to benzo(α)pyrene induction. Yet giving the extraction of *Sargassum* sp., *Gracillaria* sp. With/without taurine were able to increase in their erythrocytes numbers, almost reaching the erythrocytes numbers of control group. Contrary to the leucocytes, induction of benzo(α)pyrene was able to increase the number of leucocytes for all the treatment groups.

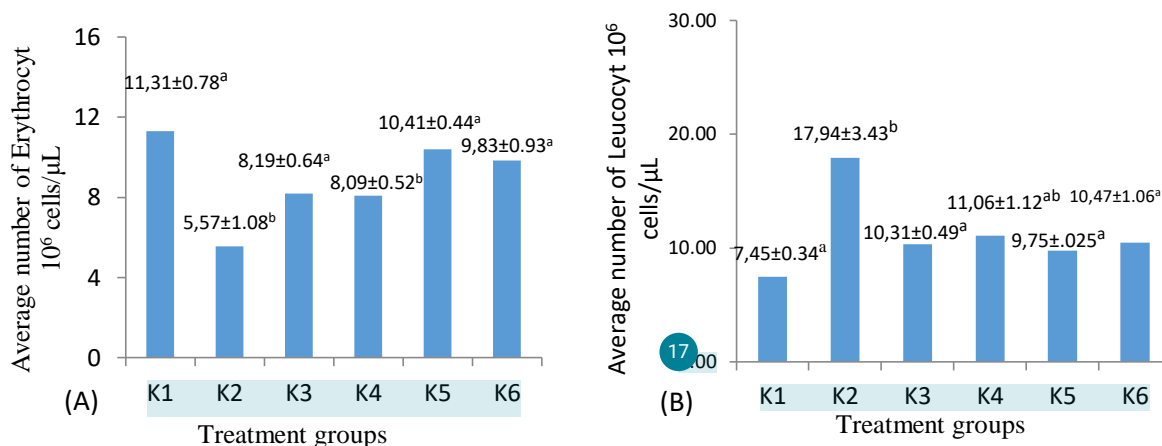


Figure 1. (A) Average number of mice erythrocytes (millions cells/μL), (B) Average number of mice leucocytes (millions cells/μL). The average numbers followed by the alphabetic notation indicated significantly different at $\alpha = 5\%$

The normal number of erythrocytes was $6.50-10.10 \times 10^6$ cells/μL [22], while the normal number of leucocytes was $5,000 - 10,000$ cell/μl blood [23]. In previous study induction of benzo(α)pyrene also indicated in decreasing in erythrocytes number (up to 3.60×10^6 cells/μL) and increasing in leucocytes number (up to 14.17×10^3 cells/μL) [24,25]. The increase in leucocyte number presumably was due to humoral and cellular responses of the mice to benzo(α)pyrene as carcinogenic substance [26].

The data also indicated that methanol extraction of *Sargassum* sp. and *Gracillaria* sp. with/without taurine was able to return both blood cells close to normal number as those shown by the control group. This ability of both macroalgae with/without taurine to return the blood cells number was due to antioxidant content in form of flavonoid in both macroalgae and antioxidant ability of taurine as free amino acid (β -aa) to free radicals produced by benzo(α)pyrene induction. Antioxidant ability of taurine to protect rat erythrocytes from Cadmium (Cd) toxicity also shown by Sinha [27]. Both Maysa and Marlinda [24,25] indicated that taurine effectively restored blood cells back to normal levels by protecting as well as increasing number and viability of the lymphocytes [28].

3.2 Protein Profile

Figure 2 was the standard curve of marker protein used to determine the protein profile of mice blood in each treatment groups, by regresi of $y = 11.786x^{1.4628}$.

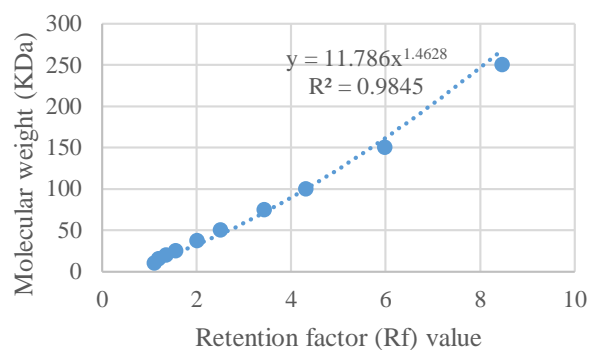


Figure 2. Standard curve of marker protein

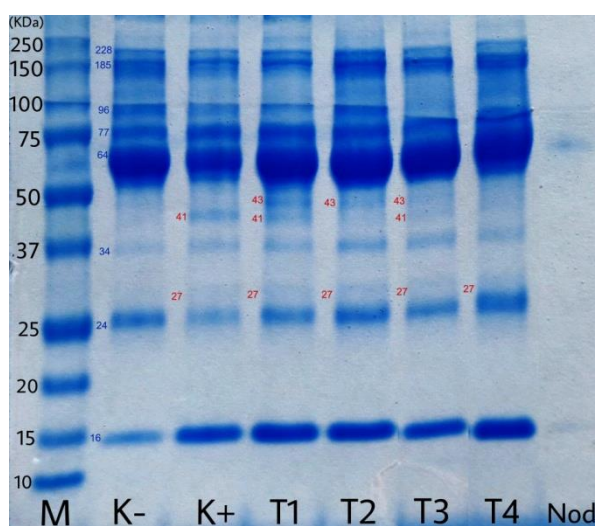


Figure 3. Protein profile of the blood plasma and nodule fluid of mice in gel SDS-Page (note: M : marker; K : control groups; T: treatment groups; Nod: nodule fluid)

From the standard curve the protein profile of each treatments then can be seen in **Figure 3**. The result indicated that 8 (eight) protein band was shown from each of treatment groups (K-, K+, T1, T2, T3 and T4), they were respectively with molecular weight of 16 KDa, 34 KDa, 64 KDa, 77 KDa, 96 KDa, 185 KDa, and 228 KDa. Meanwhile, the nodule fluid only shown 2 (two) bands, they were 16 KDa and 64 KDa.

Occurrence of nodule at the back neck of mice presumably existed as effect of sub-cutaneous injection of benzo(α)pyrene induction causing inflammation (edema), which most of edema caused by arteriole dilatation followed by increase in capillary permeability [29].

Figure 3 also indicated that there was a 27 KDa band which shown in all benzo(α)pyrene induction treatment groups (namely K+, T1, T2, T3 and T4) and a 41 KDa band which shown in K+, T1 and T3 groups. While those in T1, T2 and T3 treatment groups, there were also another band indicating 43 KDa molecular weight. However the three protein bands, 27 KDa, 41 KDa and 43 KDa did not shown in K- (normal) group.

In addition to existing protein bands, the thickness of the bands of SDS-PAGE electrophoresis indicated the level of protein concentration in the blood plasma [30]. The thicker protein band of the blood plasma was in 64 KDa, which was for albumin. Albumin is the biggest plasma protein in the body (55-60%), which functioning in maintaining the osmotic pressure of blood [31]. Albumin protein

band in blood plasma of many rodents always shown very thick and lied in molecular weight of 60-69 KDa [32,33].

Benzo(α)pyrene induction shown some affect in expression of protein bands of the mice blood plasma. The expression of bands was not only in they existence but also in the thickness of the bands. The thickness of each protein band from each treatment groups were different, such as 16 KDa which probably survivin. Survivin is one of the protein as inhibitor apoptosis (IAP) group which involved in cell division and blockade apoptosis [34]. Survivin protein band with molecular weight of 16 KDa also shown in western blot of mice breast cancer cells 4T1 [35]. In this study, the protein which probably survivin, shown much thicker in all the groups treated with benzo(α)pyrene induction compared to those in control/K- (normal) group. Furthermore, this protein (16KDa) also shown in nodule fluid with very thin band (**Figure 3**).

In addition to, the protein with molecular weight of 27 KDa was also expressed in very thin band from all benzo(α)pyrene induction groups. It was assumed that this protein was related to BCl-2 which also has molecular weight of 26 KDa and has anti-apoptosis effects by maintaining mitochondria [36]. [37] also indicated that protein with molecular weight of 27 KDa was BCl-2 (26 KDa) protein which undergone phosphorylation becoming phosphor-peptide. Furthermore, this protein was also undergone immune-precipitation by antibody of anti-BCl-2. Protein 27 KDa was also expressed in *western blot* of the R6 cell line, as *Flag-BCl-2* protein [38]. The presence of BCl-2 protein which phosphorized was possible since benzo(α)pyrene induction was performed. This presence of BCl-2 protein also shown in benzo(α)pyrene induction into mucosal cells of mice mouth [39].

The protein band with molecular weight of 41 Kda was only shown in 3 treatment groups, namely K+ (group with benzo(α)pyrene induction), T1 (group with *Sargassum* sp. extraction) and T3 (group with *Gracilaria* sp. extraction). [40] indicated that α -1-Acid glycoprotein (AGP) protein with molecular weight of 41 KDa was one of the protein produced by hepatocyte cells in chronic phase and secreted into the blood plasma as response to cell infection and damage. The presence of AGP was probably caused by induction of benzo(α)pyrene acted as hepatotoxic carcinogenic which easily absorbed into cells. Benzo(α)pyrene is also easily and fast distributed into many rat tissues including kidneys, intestine, trachea, stomach, testis, liver and esophagus. The metabolism product of benzo(α)pyrene with cytochrome P450 enzyme form some reactive epoxide metabolites, such as BaP 7,8 diol-9,10-epoxide, which was believed play role in carcinogenesis [41].

On the other hand, expression of 41 KDa (AGP) did not shown in groups with *Sargassum* sp. + taurine (T2) nor *Gracilaria* sp. + taurine (T4), as well as in those of normal (K-) group. It was expected that the antioxidant content in both *Sargassum* sp., *Gracilaria* sp. and taurine probably was able to reduce the infection and damage of cells, therefore, the AGP did not shown in mice blood of T2 and T4 groups. *Sargassum* sp. contains of polyphenol bioactive substance which is phlorotanin [42]. Phlorotanin contained in *Sargassum* sp. is 5-15% of its dry weight and derivate from phloroglucinol (1,3,5- trihydroxybenzine). Poliphenol containing in seaweed (macroalgae) has antioxidant activity, therefore, it can protect many kind of degenerative syndrome as well as any other oxidative stress syndrome [43].

Gracilaria sp. is also antioxidant source, such as caratenoid, pigment, poliphenol, enzyme and abundant of many polisaccaride. Based on phytochemical analysis, *Gracilaria* sp. is rich of flavonoid, terpene, steroid, tannin, alcaloid, phenol and glicoside which act as antioxidant [44]. [45] also stated that flavanoid as antioxidant play important role in reducing of hydroxyl radicals, superoxide and peroxil radicals in the process of lipid peroxidation caused by free radicals.

Taurine in this study also played in important role in physiological function, such as acts as neuromodulation in neuro system, energy production, immunomodulation and oxidation ptector [46]. Taurine acted not only preventively but also terapeucto blood cell damage caused by benzo(α)pyrene induction [25].

4. Conclusion

Methanol extraction of *Sargassum* sp. and *Gracilaria* sp. as well as addition of taurine was able to resume back the blood cells (erythrocytes and leucocytes) of mice affected by benzo(α)pyrene induction, while the plasma protein profile of treated mice with both macroalgae (*Sargassum* sp. and *Gracilaria* sp.) plus taurine did not show protein expression of 41 KDa (α -1-Acid glycoprotein) which produced in response to infection and/or cell damage.

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