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IOP Conf. Series: Earth and Environmental Science **305** (2019) 012011 doi:10.1088/1755-1315/305/1/012011

Antioxidant Effect of *Clerodendrum* sp and *Acanthus illicifolius* Methanol Extraction on Blood Profile of Male Mice Induced by Benzo(α)pyrene

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Abstract. Benzo(α)pyrene is one of carcinogenic PAH substances which commonly produced by many different sources of polutant. The study was conducted in order to determine the effect of methanol extraction of *Clerodendrum* sp and *Acanthus illicifolius* which known to be two of common mangrove plants on the blood profile of male mice induced by benzo(α)pyrene (one of polycyclic aromatic hydrocarbon/PAH). Thirty male mice (*Mus musculus*) of *ddy* strain from Lampung Vetenary Agency were used to conduct the study. Completely randomized design was assigned for this study. Mice were grouped into 5 different group treated with PAH injection, one groups used as control without any injection, one as postitif group with PAH injection but not given with any methanol extraction and the last three group were given with methanol extraction of *Clerodendrum* and *Acanthus* and taurine prior PAH injection. All the animal study were kept in room temperature with 12:12 light cycle and fed and water *ad libitum*. Observation of their blood cells and protein profile was made after 14 days. Data was analyzed by *One-Way* Anova at 5% followed by Fisher's at level of $p<0.05$. The study showed that methanol extraction of *Clerodendrum*, *Acanthus* and taurine were able to return back either the number of redblood or whiteblood cells. However, the protein profile of the blood was not able to show any differences among treatment groups from which 14 different bands were observed under SDS-PAGE method.

1. Introduction

One of the destruction made on the tissues by procarcinogenic substances, such as polycyclic aromatic hydrocarbon (PAH) is demaging the DNA structure leading to abnormality forms of the tissue cells. The carcinogenic substance is able to form some free radicals which then interupted with any cell activity, including producing expressing some of proteins, namely p53 or p21. These two proteins are responsible to cell regulation and are influenced by most of free radicals inside the cells. High numbers of free radicals then will attenuate inbalancing between oxidant and antioxidant molecules formed in the body, leading to oxidative stress [1] and causing oxidative damage which presumably able to lengthen aging process and initiate degenerative ailments by losing of regulatory of homeostasis of cell cycle including cancer [2]. In order to protect the exceeding of the free radicals action, antioxidant should be released and trap these free radicals leading to stabilizing and by far of

recovering the cells. These antioxidant molecules then can be released from the cell levels through cell membrane and extracellular compartment and/or from diet to balance this high numbers of free radicals [3].

Phytopharmacological studies have been explored in many different plants, especially from those of extreme habitat, namely mangrove. They are considered to be significant sources for new drugs which due to their effectiveness with lack of side effects. One of extreme habitats is mangrove, in which two among other mangrove plants are *Clerodendrum* sp and *Acanthus ilicifolius*. Alot of studies had been done related to utilize of herbs as medicine. Yet, it is important to identify some of substances produced by plants from which they are able to reduce the number of free radicals. It is known that phenols and/or flavonoids are substances which can reduce free radicals. As one of mangrove flowering plants *Clerodendrum* sp., has been used for traditional medicine in many parts of the world, especially in India, Thailand, China, Korea and Japan. *Clerodendrum* was used to treat cold, hyperpyrexia, asthma, furunculosis, hypertension, rheumatism, dysentery, mammitis, toothache, anorexia, leucoderma, leprosy, arthrophlogosis, and other inflammatory disease [4], beside their commonly known for its pesticidal properties [5]. Diterpenoids, flavonoids, phenylethanoid glycosides, and steroids are abundant and major bioactive values of the *Clerodendrum* genus [4].

In order to elucidate the effect of these two mangrove plants, we compared them with taurine (2-*aminoethanesulfonic acid*) which is known to be one of free amino acid with high antioxidant activity in our previous study [8]. Taurine is known also affecting lipid absorption, stabilizing and maintaining cell membranes including neurons, improving nutritional supply throughout the body, regulating heart rate up to boosting the cell growth. Therefore, it is believed that taurine plays an important role in protecting oxidative tension as well as in regulating biochemical shifting due to aging and/or cell damaging related to excessive of free radicals [6–9].

The aims of this study were to clarify the effect of both mangrove plants (*Cloredendrum* sp. and *Acanthus ilicifolius*) as antioxidant compared with taurine on blood cells (namely erythrocytes and leucocytes) and protein of sera of mice stimulated by benzo(α)pyrene (one of procarsinogenic substances [10].

2. Method

In this study, prior applying treatments to the animals, preparation of plant extraction was made. Two annual flowering mangrove plants were used in this study namely *Clerodendrum* sp and *Acanthus ilicifolius*. Extraction of plant leaves was adopted from Indriani [11] as followed. After sorting and washing with tap running water, leaves were drying in oven at 30 -35^{o}C for a week followed by well mixed using a blender. Maceration of mixed leaves used methanol in ratio of 1:10 ume followed by evaporation by using rotary evaporator at 50 ^{o}C. Heating process in a oven was applied in for 5 hours to obtain well paste. This extraction then was diluted into 1% CMC used for food [12] prior given to treated mice. Phytochemical identification was also applied for leave extraction.

2.1. Benzo(α)pyrene induction

Male mice (*Mus musculus*) with *ddy* strain were used in this study and obtained from the Veterinary Investigation Center of Lampung Province, Indonesia (they were in 2-3 months with average 30-35 g in weight). All animal samples were accustomed in separate containers within air-conditioned room with exhaust at 25°C and light control (12:12). These mice as treated animal were fed with standard diet having 16.04% protein; 3.63% fat; 4.10% fibers; and metabolic energy of 0.012 MJ, and water *ad libitum*. Prior the study, animal samples were adapted in laboratory condition for 7 days. All animal samples were permitted by the University Ethics Committee on the laboratory animal procedure and the study were executed fitting to the Committee standards.

Benzo(α)pyrene (BAP) induction was given for 10 days in sub-cutaneous injection with dosage of 0.3 mg/bw diluted in 0.2 ml of corn oil. Once induction of benzo(α)pyrene, male mice indicated some oedema, nodules with fluid inside, in their necks. At the 10th day of injection, each group of animal

was administered for 15 days with different plant extract, namely *Clerodendrum* extract with dose of 10.5 mg/bw/day, modified from Renju [13], *Acanthus* extract with dose of 17.5 mg/bw/day, modified from Vijayaray [14] and taurine with dose of 15.6 mg/bw/day [6].

2.2. Blood sample collection

After 25 days of BAP induction, blood samples of treated mice were collected by euthanizing mice using pentobarbital sodium. For determining protein profile analysis of treated mice, collected blood were kept at -20 °C. Some of the blood was used to define the number of blood cells (erythrocyte and leucocyte) employing haemocytometer.

2.3. Treated animals and experimental design

Random assignment was delivered to thirty male mice to 5 experimental groups as follow (Table 1).

Table 1. List of Experimental Groups

No.	Groups	treated with
1	K1	Control group
2	K2	Benzo(α)pyrene induction only
3	K3	Benzo(α)pyrene induction and *Clerodendrum* sp extract (10.5 mg/bw/day)
4	K4	Benzo(α)pyrene induction and *Acanthus ilicifolius* extract (17.5 mg/bw/day)
5	K5	Benzo(α)pyrene induction followed by taurine (15.6 mg/bw/day)

2.4. Determinating and analyzing of blood cells number

Haemocytometer was used to determine eritrocytes and leucocytes cells as described in our previous study [15]. For determining leucocyte numbers, 0.5 µL blood was diluted with 101 µL of Hayem' solution making 200 times dilution, while for determining erythrocyte numbers 0.5 µL of blood was diluted with 11 µL of Turk's solution making 20 times dilution. Blood dilution then was homogenized well and was dripped into haemocytometer at the edge of the cover glass. Counting was made on the second drop of blood dilution. The erytrocyte cells was recorded from 5 small boxes of the middle larger box of haemocytometer and the leucocyte cells was recorded from 4 boxes of the edge. Both blood cell numbers were calculated as follows,

$$\text{The total erytrocyte and leucocyte cells/mm}^3 = N \times p \times 50$$

N is total erythrocyte/leucocyte cells in all counting boxes of haemocytometer, p: degree of dilution

One Way ANOVA (*Analysis of Variance*) followed by Fisher's test at 5% level of significant (SPSS 16.0 for Windows program; SPSS Inc., Chicago, Illinois, USA) was applied to analyze all the collecting data.

2.5. Determination of blood protein profile

Collected sera from all treated mice were centrifuged in 13.000 rpm for 10 minutes, then supernatants were collected from which 1 µl of supernatant was diluted with sodium chloride physiologic salt as much as 50 µl. 10 µl of this supernatant was added with 10 µl *loading buffer* (Laemmli Buffer Sample and β-Mercaptoethanol 19:1). TGX Stain-Free™ FastCast™ Acrylamide Kit 12% was applied in order to run 12% electrophoresis. The acrylamide kit contained of *resolver stacker* gel. APS 10% and TEMED was used followed by employing the electrophoresis comb for 30 minutes and was sited in *electrophoresis chamber*. Buffer solution (10 x Tris/Glycine/SDS Electrophoresis Buffer) was added into chamber. *Marker protein* of 10 Kda up to 250 Kda from Bio-Rad Precision Plus Protein™ Standards was used, by adding 10 µl of the marker into each wheel, and each serum sample (20 µl) was filled in each wheel. The set of electrophoresis then was run in 100 t.

Note: Before filling in the blood plasma to each wheels, estimation of the protein contain of each sample was applied by *ELIZA* using *bradford* method. To run the gel, commasie blue was used and placed in shaker (made of Ultra Rocker Bio-Rad) with shaking rate of 40 rpm for an hour. To dispose excessive *commasie blue* from the gel, destainer was applied and stopped when the bands observed. The electrophoresis gel, then, was retained in refrigerator within distilled water. In order to assess the protein profile showed in the electrophoresis gel, the *Retention factor* (RF) was determined.

2.6. Estimating molecular weight of protein from mice blood sample

BioMed MW Converter©- Molecular Weight Conversion Tool (*copyright* - Didik T. Subekti 2018) was used to calculate protein band molecular weight produced by SDS-PAGE. The Rf To determine the standard curve, RF values from marker bands was calculated by determining the band gap from the wheel mouth divided by total migration of the sample (as x axis) and the molecular weight of the marker bands (as y ordinate), finally this calculation used Regresi Power ($y = ax^b$) method. The RF then was applied to estimate the protein molecular weight of the mice blood samples. The data of this protein profile then was descriptively evaluated by detecting composition protein of mice blood plasma (existing and thickening of protein bands).

2.7. Statistical analysis

Statistical analysis was conducted to analysis the blood cells data using *ANOVA* and Fisher's (statistic was run by SPSS 13.0 for Windows program; SPSS Inc., Chicago, Illinois, USA) at 5% of significant level and values were expressed in mean ± standard error mean (SEM), while the protein profile was descriptively analyzed.

3. Results and Discussion

3.1. Phytochemical screening

Phytochemical screening of *Clerodendrum* sp and *Acanthus ilicifolium* can be seen in Table 2. Mostly these two plants contained of flavonoid, saponin and tannin.

Table 2. Phytochemical test for *Clerodendrum* and *Acanthus*

Phytochemical Test	***Clerodendrum* sp**	***Acanthus ilicifolium***	**Source of Method**
Flavonoid	+	+	Sangi *et al*, 2008
Saponin	+	+	Darwis, 2000
Steroid	-	+	Kadarisman, 2000
Terpenoid	+	-	Kadarisman, 2000
Tannin	+	+	Kadarisman, 2000
Alkaloid	-	+	Darwis, 2000

Flavonoid has been known as antioxidant. This flavonoid was expected able to reduce the free radicals produced by PAH injection which lead to destruction of the tissues, namely in this study was the bloods.

3.2. Eritrocytes and Leucocytes Numbers

The study indicated that induction of benzo(α)pyrene affected blood cells of every experimental groups (Table 3). Related to the K1 (control group), the erythrocytes numbers for all treated groups was declining in response to benzo(α)pyrene induction. However, erythrocyte numbers in treated group given *Acanthus'* extract as well as taurine showed ability to closely return back their erythrocytes (RBC's). In contrast, leucocytes increased by induction of benzo(α)pyrene.

Table 3. Numbers of mice blood cells induced by benzo(α)pyrene (at 25days treatment)

Treatment Group	Erytocytes (10^6 cells/µL)	Leucocytes (10^3 cells/µL)
K1	14.45 + 1.62[a]	14.60 + 1.68[a]
K2	8.43 + 0.85[b]	51.30 + 8.87[b]
K3	8.17 + 1.57[b]	17.00 + 3.39[ab]
K4	9.60 + 0.71[b]	21.52 + 1.52[ab]
K5	11.52 + 0.85[ab]	4.15 + 2.83[ab]

[a,b] Alphabetical notation in the same column indicated significant different at $p<0.05$

Normally, erythrocyte number of male mice blood was in range of 6.5 -10.1 x 10^6 cells/µL of blood [16], while leucocyte number was in range of 5,000 – 10,000 cell/µL[17]. In our earlier experiment [15], benzo(α)pyrene induction likewise reduced the number of erythrocyte, reaching to 5.57 x 10^6 cells/µL but increased in the number of leucocyte, reaching to 17.94 x 10^3cells/µL. These change in numbers of blood cells also indicated [18,19]. The increasing in number of leucocytes was likely as a result of either both of cellular and humoral responses of treated male mice to benzo(α)pyrene (PAH) one of procarcinogenic substances [20].This response also can be seen in differential leucocyte (Table 4).

Table 4. The differential number of leucocytes from male mice induced by benzo(α)pyrene

Treatment Group	Differential Leucocyte Number (in every 100 cells counted)				
	Neutrophil	Eosinophil	Basophil	Monocyte	Lymphocyte
K1	57.20 + 1.69[a]	3.40 + 1.92	5.40 + 1.92	12.20 + 1.88	21.80 + 3.21[a]
K2	12.00 + 1.45[b]	3.40 + 1.21	2.80 + 1.36	8.60 + 0.64	73.20 + 2.23[b]
K3	47.20 + 11.19[a]	5.00 + 1.90	6.40 + 3.00	11.20 + 3.61	30.60 + 6.95[a]
K4	45.80 + 9.28[a]	2.20 + 0.98	7.40 + 0.81	7.80 + 3.25	35.40 + 7.32[a]
K5	58.40 + 4.60[a]	2.80 + 0.67	2.20 + 0.74	13.00 + 2.31	23.60 + 2.66[a]

Differential leucocytes was determined from 100 leucocytes counted: Notation ([ab]) on each column indicated significant different ($p<0.05$)

From Table 4, it was showed that methanol extraction of *Clerodendrum* sp. and *Acanthus ilicifolius* as well as taurine was able to return leucocyte number by increasing in number of neutrophil and lymphocyte presented by K1 (control group). The capability of both mangrove plants and taurine to normally gain back mice blood cells was probably because of flavonoid antioxidant ability contained in those plants and taurine. We assumed that free radicals produced by induction of benzo(α)pyrene was reduced by flavonoid and free amino acid (*β-aa*) taurine. The taurine capability as antioxidant also presented in rat while protecting erythrocytes from Cadmium (Cd) toxicity [21]. Other studies [18][19] also indicated that free amino acid taurine was able to restore blood cells by defending as well as accumulating and restoring capability of lymphocytes [22].

3.3. Blood Protein Profile

Figure 1 indicated marker protein as model curve which employed to define mice blood protein profile from each treatment groups. Its regression value was $\boldsymbol{y = 1{,}3797x^2 + 21{,}568x - 18{,}465.}$ From Figure 1, protein profile of mice blood was determined and can be seen in Figure 2. From the SDS-PAGE (Figure 2), it can be seen that there were 11 protein bands which occured in all treatment groups including control group. They were 14 kDa, 26 kDa, 38 kDa, 45 kDa, 51 kDa, 52 kDa, 67 kDa, 85 kDa, 100 kDa, 168 kDa, 226 kDa molecular weight.

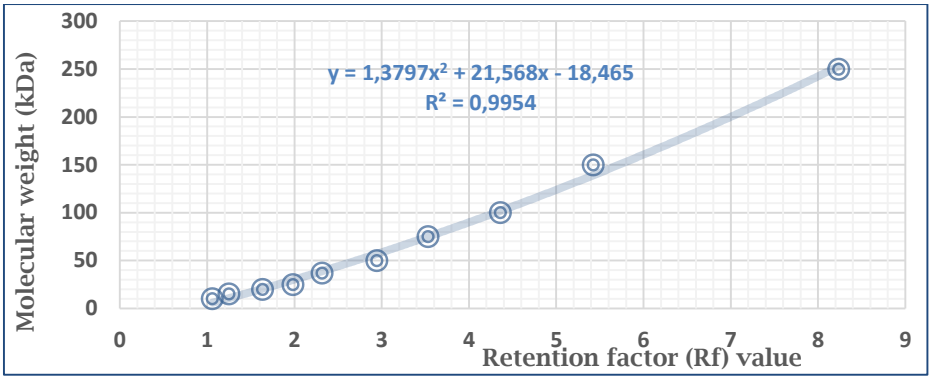
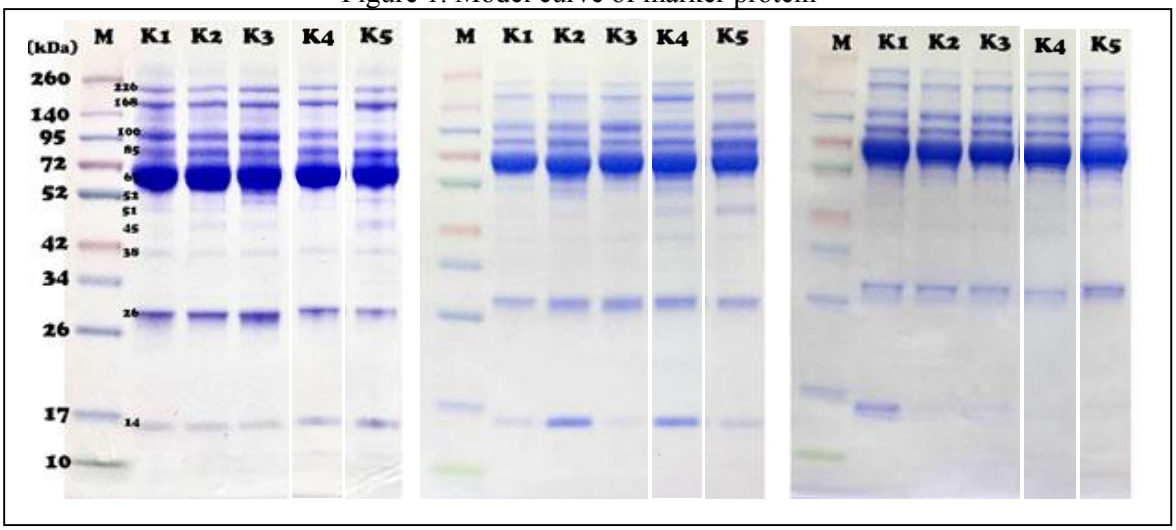


Figure 1. Model curve of marker protein



Note: M : marker; K1 : control; K2 : Benzo; K3 : Benzo + *Clerodendrum*; K4 : Benzo + *Acanthus*; K5 : Benzo + Taurine

Figure 2. Protein of blood plasma of treated male mice in SDS-Page gel

In this Figure 2, the level of protein concentration in the blood plasma of treated mice was indicated by the band width seen in SDS-PAGE [23]. It can be seen that the thickest protein band was 64 Kda which known to be albumin and it is the highest concentration protein in blood plasma consisted of 55% to 60%. This albumin functioned in preserving blood osmotic pressure [24]. Most of rodent has shown albumin protein band in very thick and laid from 60 to 69 KDa [25][26].

However, induction of benzo(α)pyrene in this study did not show any protein bands expression of treated mice. Unlike in our previous study, we were able to detect protein band at 16 KDa, which was possibly as survivin. This survivin is known to be one of protein acted as apoptosis inhibitor (IAP) [27]. This protein was also detected in cancer cells 4T1 of mice breast [28]. In our study, such protein was not able to be detected which may due to physiological effects on each of treated animals with benzo(α)pyrene or PAH induction. Furthermore, 26 and 27 KDa protein here was obtained in very thin band from all benzo(α)pyrene treated animals. We presumed that this protein was associated to BCl-2 with 26 KDa and it does have anti-apoptosis effects by retaining mitochondria [29]. Meanwhile, some study indicated that protein with 27 KDa was BCl-2 (26 KDa) protein which was phosphorylated becoming phosphopeptide [30]. Additionally, this 27 KDa protein experienced immunoprecipitation by anti-BCl-2 antibody. This 27 KDa protein was also expressed in the *R6 cell line* in the role of *Flag-BCl-2* protein [31]. Since induction of benzo(α)pyrene was introduced, the

occurrence of BCl-2 protein which phosphorylazed was possible occurred. Other study on induction of benzo(α)pyrene into mice mucosal cells also indicated presence of BCl-2 protein [32]. However, very surprisingly, this protein band also indicated in control group. We were unable to explain the existing of this band in this control group.

Expression of 41 KDa protein which was known as α-1-Acid glycoprotein (AGP) did not shown in SDS-PAGE of this study. AGP was commonly produced by hepatocyte cells in chronic phase, such as through PAH induction, and secreted into blood. PAH or benzo(α)pyrene that was induced into treated mice was supposed to be easy and fast dispersed into many vital organ matters, such as liver, testis, kidney, esophagus, stomach, intestine and trachea [33]. PAH caused carcinogenesis by attacking cytochrome P450 enzyme and forming reactive epoxide metabolites, for instance BaP 7,8 diol-9,10-epoxide [33]. Based on phytochemical analysis (Table 2), *Clerodendrum* sp and *Acanthus ilicifolium* contained of flavonoid, saponin, tannin (for both), steroid, terpenoid and alkaloid which presumably acted by way of antioxidant in male mice in response to induction of benzo(α)pyrene, as already stated also that those compounds performed as antioxidant [34]. Particularly the flavonoid which known to perform important part in diminishing hydroxyl, peroxil and superoxide radicals in lipid peroxidation process [35].

Ability of taurine in this study to gain the blood cells back to normal was assuming of taurine played an important role as antioxidant protector, beside its physiological function as in neuromodulation, energy production and immunomodulation. In addition to all, it was believed that taurine proceeded defensively and therapeutic to benzo(α)pyrene affected on blood cells damage [19].

4. Conclusions

Clerodendrum sp and *Acanthus ilicifiolium* methanol extraction and free amino acid taurine was capable to gain leucocytes cells back of mice induced by benzo(α)pyrene, while protein profile in their blood plasma did not show expression of 41 KDa (α-1-Acid glycoprotein) nor protein with 21 KDa which yielded in response to cell injury and/or tissue infection.

Acknowledgements

The authors gratefully acknowledge that the present research is supported by the Directorate of Research and Community Development (DRCD or DRPM) of Ministry of Research Technology and Higher Education – Indonesia and the University of Lampung through Graduate Program Research Grant. Greatly appreciation was distributed to drh. Didik T. Subekti, MSc., of Lampung Vetenery for helping us setting up this manuscript.

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