

## The effects of *Eucheuma cottonii* on alveolar macrophages and malondialdehyde levels in bronchoalveolar lavage fluid in chronically particulate matter 10 coal dust-exposed rats

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### ABSTRACT

**Objective(s):** To investigate the effect of *Eucheuma cottonii* on alveolar macrophages (AM) and malondialdehyde (MDA) levels in bronchoalveolar lavage fluids (BALF) in particulate matter 10 (PM<sub>10</sub>) coal dust-exposed rats.

**Materials and Methods:** Ten groups, including a non exposed group and groups exposed to coal dust at doses of 6.25 (CD<sub>6.25</sub>), 12.5 (CD<sub>12.5</sub>), or 25 mg/m<sup>3</sup> (CD<sub>25</sub>) an hour daily for 6 months with or without supplementation of ethanolic extract of *E. cottonii* at doses of 150 (EC<sub>150</sub>) or 300 mg/kg BW (EC<sub>300</sub>). The number of macrophages was determined using a light microscope. MDA levels were measured by TBARS assay.

**Results:** EC<sub>150</sub> insignificantly ( $P > 0.05$ ) reduces the AM in CD groups compared to non treatment groups. EC<sub>150</sub> and EC<sub>300</sub> significantly ( $P < 0.05$ ) decreased MDA levels in CD<sub>12.5</sub> and CD<sub>25</sub> groups relative to non treatment groups.

**Conclusion:** *E. cottonii* attenuated oxidative stress in chronic exposure of PM<sub>10</sub> coal dust.

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### Introduction

Coal dust exposure can induce an acute alveolar and interstitial inflammation leading to chronic pulmonary diseases. The essential mechanism of coal dust-induced lung damage is likely to be mediated by macrophage activation and the recruitment of polymorphonuclear cells (1). The increase in lung macrophages has been reported to be the primary lung response to air pollutants in animal and human. Alveolar macrophages are cells first encountering inhaled pollutants in the lower respiratory tract. Their phagocytic capacity provides an efficient nonspecific defense against inhaled particles. Macrophages and other phagocytic cells produce reactive oxygen species (ROS) during phagocytosis or stimulation with a wide variety of agents (2). ROS can randomly react with lipids, proteins, and nucleic

acids. When ROS target lipids, they can initiate the lipid peroxidation, a chain reaction that produces multiple breakdown molecules such as malondialdehyde (MDA) (3).

The cultivated edible red seaweed, *Eucheuma cottonii* (*Kappaphycus alvarezii*), contains vitamin C,  $\alpha$ -tocopherol,  $\beta$ -carotene, zinc, selenium, phenols, and sterol. These compounds have antioxidant and anti-inflammatory properties, and thus directly or indirectly contribute to the inhibition or suppression of free radical generation (4). Previous studies also showed that the seaweed suppresses thiobarbituric acid reactive substances (TBARS) formation of H<sub>2</sub>O<sub>2</sub>-induced lipid peroxidation in red blood cells (5). Moreover, consumption of the seaweed is already proved not exert any damage to the liver, heart, kidney, brain, spleen, and eyes in normal rats (6).

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The present study aimed to investigate the effect of *E. cottonii* on the absolute number of bronchoalveolar lavage fluids (BALF) alveolar macrophages and BALF MDA levels in rats chronically exposed to particulate matter (PM)<sub>10</sub> coal dust.

## Materials and Methods

### Preparation and extraction of *E. cottonii*

*E. cottonii* was harvested from the coastal areas of Tamiang, Kotabaru (South Kalimantan, Indonesia). The preparation and extraction of the seaweed were performed according to the method of Fard *et al* (7).

### ABTS reducing activity *in vitro*

2, 2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS<sup>•+</sup>)-reducing activity was determined according to the previous method (8).

### Animal

A total of 40 male Wistar rats were randomly divided into ten groups. One group was a negative control group (without any treatment). Three groups were positive control groups exposed to coal dust at doses of 6.25 (CD<sub>6.25</sub>), 12.5 (CD<sub>12.5</sub>), or 25 mg/m<sup>3</sup> (CD<sub>25</sub>) one hr per day for 6 months. Six groups were treatment groups exposed to coal dust with supplementation of ethanolic extract of *E. cottonii* at doses of 150 (EC<sub>150</sub>) or 300 mg/kg BW (EC<sub>300</sub>).

### *E. cottonii* administration

The ethanolic extract of *E. cottonii* was given orally using a gavage (gastric tube), and started together with coal dust exposure. Doses of *E. cottonii* and coal dust were based on previous study (7).

### Coal dust exposure

Coal dust exposure was performed according our previous study (9). The PM<sub>10</sub> coal dust exposure was conducted using equipment (0.5 x 0.5 x 0.5 m) that was designed by and available in Laboratory of Pharmacology, Faculty of Medicine, Brawijaya University, Malang. This equipment provided an ambient environment containing coal dust for inhalation by the animal. The airstream of the apparatus was set at 1.5-2 l/min mimicking an environmental airstream. To prevent hypoxia and discomfort, we also provided oxygen supply in the chamber. The non-exposure group was only exposed to filtered air in laboratory. In order to provide coal dust aerosolization, we put weighed coal dust in bottom hole of equipment; then the coal dust will circulate and enter the chamber again via upper hole. This aerosol will inhaled by rats in plastic chamber.

### Ethics

Animal care and experimental procedures were approved by the Institutional Research Ethics

Committee of Faculty of Medicine, Lambung Mangkurat University, Banjarmasin, Indonesia.

### Isolation of bronchoalveolar lavage fluids

The rats were euthanized by anesthetizing with ether inhalation and exsanguinated by cardiac puncture. Briefly, the thorax was opened by a median incision, the left bronchus was clamped with forceps, and a small incision was made in the the right bronchus and a plastic catheter attached to a 5-mL syringe was inserted. Bronchoalveolar lavage was performed a total of two times by repeated flushing with 5 ml ice-cold PBS, with gentle massage of the lungs. The bronchoalveolar lavage fluids were pooled and stored on ice until further use (10).

### Analysis of the number and viability of BALF alveolar macrophages

BALF were centrifuged at 1000 rpm for 10 min at 4°C to obtain the cells and supernatants. The total number of alveolar macrophages was determined using a light microscope by counting the number of cells in at least five squares of a hemocytometer after excluding dead cells using Trypan blue staining (10).

### Analysis of BALF malondialdehyde levels

The supernatants obtained after centrifugation of BALFs were measured for the MDA levels (11). The principle of the method is the spectrophotometric measurement of the color generated by the reaction of thiobarbituric acid (TBA) with MDA. For this purpose, 250 µl of trichloroacetic acid solution was added to 100 µl of supernatant in a test tube. Next, 100 µl of TBA solution, 200 µl of HCl, and 0.5 ml of aquabidest were respectively added to the mixture. The tube was placed in a boiling water-bath for 15 min and then cooled in tap water. The absorbance of the colored product was read at 532 nm using a spectrophotometer. The values obtained were compared with a series of MDA tetrabutylammonium salt (Sigma-Aldrich, St Louis, MO, USA) standard solutions.

### Statistical analysis

Data are presented as mean ± SD. The collected data were analyzed using Kruskal-Wallis test. Probability value of  $P < 0.05$  indicated a significant difference between groups and later subjected to Mann-Whitney *Post hoc* test. All statistical analyses were performed using SPSS software 17.0 for Windows.

## Results

### ABTS reducing activity

*In vitro*, the ethanolic extract of *E. cottonii* at concentration of 5 µg/ml was a weak scavenger of the ABTS radical (8.43%) compared to quercetin at the same concentration (94.30%). This finding means *E. cottonii* exhibited only weak antioxidant effect.

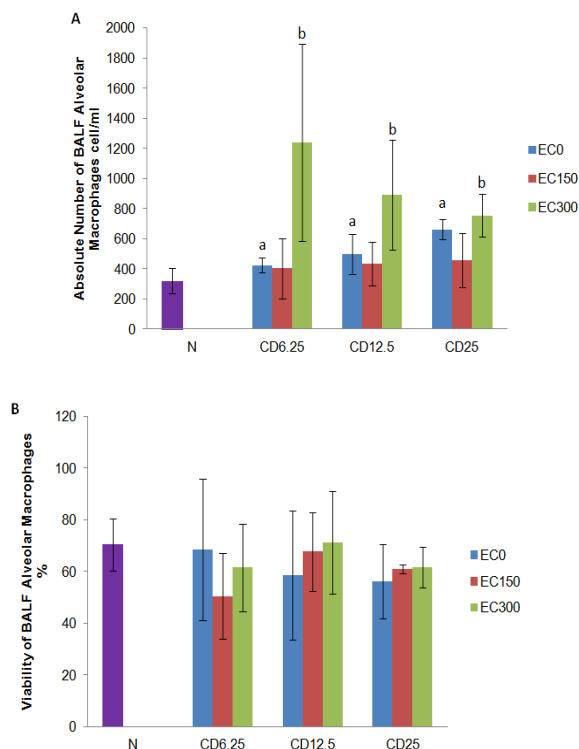
### Analysis of BALF alveolar macrophages

Figure 1 (A) showed that coal dust exposure at all doses significantly ( $P < 0.05$ ) increased the absolute number of BALF alveolar macrophages compared to the negative control group. The supplementation of *E. cottonii* had fluctuative effect on the absolute number of BALF alveolar macrophages. The administration of EC<sub>150</sub> insignificantly ( $P > 0.05$ ) decreased the number of BALF alveolar macrophages, whereas EC<sub>300</sub> significantly ( $P < 0.05$ ) increased the number of BALF alveolar macrophages in coal dust-exposed groups compared to the positive control groups.

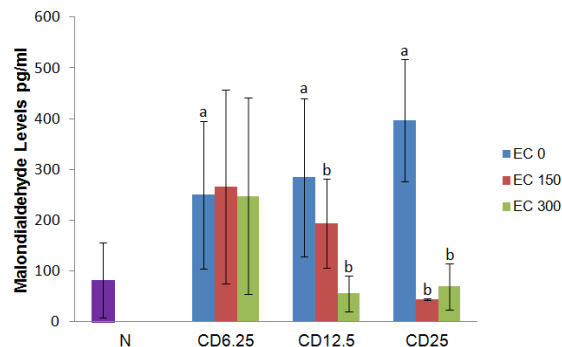
Figure 1 (B) showed that coal dust exposure at all doses insignificantly ( $P > 0.05$ ) increased the viability of BALF alveolar macrophages compared to the negative control group. The administration of EC<sub>150</sub> and EC<sub>300</sub> insignificantly ( $P > 0.05$ ) increased the viability of BALF alveolar macrophages in CD<sub>12.5</sub> and CD<sub>25</sub> groups compared to the positive control groups.

### Analysis of BALF malondialdehyde levels

Figure 2 showed that coal dust exposure at all doses significantly ( $P < 0.05$ ) increased the BALF MDA



**Figure 1.** (A) The number of bronchoalveolar lavage fluids alveolar macrophages. (B) The viability of broncho alveolar lavage fluid alveolar macrophages. Data are presented as mean  $\pm$  SD (n = 4 each group). <sup>a</sup> $P < 0.05$  in comparison with negative control group, <sup>b</sup> $P < 0.05$  in comparison with its positive control group. Negative control group (N); group exposed to coal dust at dose of 6.25 (CD<sub>6.25</sub>), 12.5 (CD<sub>12.5</sub>), or 25 mg/m<sup>3</sup> (CD<sub>25</sub>); group supplemented with the ethanolic extract of *Eucheuma cottonii* at dose of 150 (EC<sub>150</sub>) or 300 mg/kg BW (EC<sub>300</sub>); non-supplemented group (EC<sub>0</sub>)



**Figure 2.** The bronchoalveolar lavage fluids malondialdehyde levels. Data are presented as mean  $\pm$  SD (n = 4 each group). <sup>a</sup> $P < 0.05$  in comparison with negative control group, <sup>b</sup> $P < 0.05$  in comparison with its positive control group. Negative control group (N); group exposed to coal dust at dose of 6.25 (CD<sub>6.25</sub>), 12.5 (CD<sub>12.5</sub>), or 25 mg/m<sup>3</sup> (CD<sub>25</sub>); group supplemented with the ethanolic extract of *Eucheuma cottonii* at dose of 150 (EC<sub>150</sub>) or 300 mg/kg BW (EC<sub>300</sub>); non-supplemented group (EC<sub>0</sub>)

### Analysis of BALF malondialdehyde levels

Figure 2 showed that coal dust exposure at all doses significantly ( $P < 0.05$ ) increased the BALF MDA levels compared to the negative control group. The administration of EC<sub>150</sub> and EC<sub>300</sub> significantly ( $P < 0.05$ ) decreased the BALF MDA levels in CD<sub>12.5</sub> and CD<sub>25</sub> groups compared to the positive control groups.

## Discussion

In this study, we found that the number of BALF alveolar macrophages significantly increased after chronic PM<sub>10</sub> coal dust exposure. This is consistent and extended with previous study that alveolar macrophages increased after acute coal dust exposure by intratracheal instillation (1). The increasing may be due to the accumulation of coal dust particles in the alveoli leading to increased number of alveolar macrophages to engulf the particles and activation of alveolar macrophages and neutrophils. Coal dust exposure causes acute inflammation in lung and recruitment of macrophages and neutrophils to the site of the injury. This study also showed that the viability of alveolar macrophages decreased after coal dust exposure. This is consistent with previous study that coal dust significantly decreased the viability of alveolar macrophages due to apoptosis compared to untreated controls (9, 12, 13).

The administration of EC<sub>150</sub> insignificantly decreased number of BALF alveolar macrophages, otherwise EC<sub>300</sub> significantly increased number of BALF alveolar macrophages in coal dust-exposed groups compared to positive control group. The increased number of BALF alveolar macrophages in coal dust-exposed groups due to the administration of EC<sub>300</sub> may be caused by the increase in antioxidant levels. Previous studies showed that supplementation of 0.3% (0.187 mg/kg feed) of vitamin C has no effect on the activity of neutrophils and monocytes, but at dose of 0.6% (375 mg/kg feed) there is an increase in

neutrophils and monocytes (14). Otherwise, the increased alveolar macrophages in this study may be also due to the method of anesthesia conducted using ether.

Coal dust exposure significantly decreased the viability of BALF alveolar macrophages compared to the negative control group. The reduction in cellular viability may be as a consequence of the coal dust-induced apoptosis of macrophages. Previous study found that coal dust increases pulmonary apoptosis and increases the expression of the pro-apoptotic mediator Bax (15). The administration of *E. cottonii* tended to increase the viability of BALF alveolar macrophages but not reach significant level in coal dust-exposed groups compared to the positive control groups. This finding indicated that *E. cottonii* inhibit apoptosis pathway of macrophage.

This study showed that coal dust exposure significantly increased the BALF MDA levels, an index of lipid peroxidation of the lung tissue. Previous study also showed the same result that coal dust exposure for 60 days caused an increase in lipid peroxidation in rats. Inorganic components of coal dust such as iron, which can stimulate hydroxyl radical formation, and also chromium, nickel, manganese, and vanadium, have oxidative properties able to induce lipid peroxidation. The higher concentration of these active components in coal dust may result in higher oxidative stress (1, 9). This study also indicated that *E. cottonii* was able to significantly reduce BALF MDA levels in coal-dust exposed rats. This is consistent with previous study that *E. cottonii* significantly reduces plasma lipid peroxidation in rats with high-cholesterol diet (15). According to the result of ABTS reducing activity *in vitro*, *E. cottonii* was a weak scavenger of the ABTS radical compared to quercetin. However, the decreased BALF MDA levels in the present study may be still due to inhibition of lipid peroxidation via free radicals scavenging mechanism or metals chelating mechanisms. The antioxidant contents of *E. cottonii* such as  $\alpha$ -tocopherol,  $\beta$ -carotene, ascorbic acid, and polyphenols are able to scavenge the hydroxyl radical and superoxide anions. Phenolic compounds also have a metal chelating potency depending upon their unique phenolic structure as well as the number and location of the hydroxyl groups.

## Conclusion

The present study showed that chronic coal dust exposure increases oxidative stress and the absolute number of alveolar macrophages in rat BALFs. The ethanolic extract of *E. cottonii* is able to significantly decrease oxidative stress but not the inflammatory cells.

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