

# THE N-HEXANE FRACTION OF KESUM (*Polygonum Minus L.*) INDUCE APOPTOSIS THE LUNG EPITHELIAL CELLS OF THE *RATUS NOVERGICUS* THAT EXPOSED BY BENZOPYRENE

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## ABSTRACT

Kesum (*Polygonum minus L.*) is a popular plant and is often used as additives in many typical dishes in the West Kalimantan. These plants belong to the family Polygonaceae, and potential to use in the prevention and treatment of lung cancer. This research aimed at studying the effect of giving the n-hexane fraction of the kesum to induces of apoptosis in epithelial cells of lung tissue of the animal models that exposed by benzopyrene. research was conducted on three groups of animal models: i) the healthy group, ii) the group treated by benzopyrene 200 mg/kg, and iii) the therapy group by n-hexane fraction 100 mg/kg. The results of the study then carried measurements of lung epithelial cell apoptosis index and measurements of lung ROS levels. The results showed that n-hexane fraction able to reduce of lung ROS levels of the animal models that exposed by benzopyrene with MDA levels respectively are  $5.09 \pm 0.76$  ppm for the healthy group,  $8.44 \pm 1.89$  ppm for the group treated by benzopyrene, and  $5.47 \pm 1.76$  ppm for the therapy group. The n-hexane fraction also able to induce apoptosis of the lung epithelial cells of the animal models that exposed by benzopyrene with apoptosis index values respectively are  $2.61 \pm 1.98$  for the healthy group,  $7.41 \pm 2.26$  for the group treated by benzopyrene, and  $16.31 \pm 5.73$  for the therapy group.

**Key words:** apoptosis, kesum, the fraction of n-hexane, *Polygonum minus L.*

## INTRODUCTION

Apoptosis or programmed cell death is an important mechanism in the control of cell number and as part of normal growth in multicellular organisms as a result of DNA damage (Ghobrial *et al.*, 2005). DNA damage is a common one in life and requires continuous improvement. If the cells fail to repair DNA damage, the cells will be instructed to undergo apoptosis. Dysregulation of apoptosis in some cells with damaged DNA will result in a higher risk of various diseases, such as lung cancer (Janne *et al.*, 2005; Karakas *et al.*, 2006; Riely *et al.*, 2009).

Carcinogenic compounds can induce Dysregulation of apoptotic. One is benzopyrene compounds in cigarette smoke that can induce dysregulation of apoptotic and the occurrence of lung cancer (Lin *et al.*, 2003; Aoki *et al.*, 2007). The ability of benzopyrene to induce lung cancer and dysregulation of apoptotic because benzopyrene is able to induce an increase in ROS (reactive oxygen species) (Benerjee *et al.*, 2005). The Increasing of the ROS production would induce the oxidation of DNA that resulting in DNA damage and genetic mutations in vital genes that controlling the cell cycle. This will encourage the lung cells to proliferate and suppress apoptosis (Ambs

*et al.*, 1997; Palackal *et al.*, 2002; Belous *et al.*, 2007; Leavitt *et al.*, 2008).

Kesum (*Polygonum minus L.*) is a plant widely used as an ingredient in various food recipes typical of the West-Borneo peoples and a potential cure for cancer. The kesum extract is cytotoxic to cancer cells but not on normal cells so that potentially be used to prevent cancer, such as lung cancer. The Potential of kesum in preventing cancer was demonstrated by cytotoxic activity of kesum extract against cancer cells. The ethanolic extract of kesum can kill HeLa cells with LC50: 30  $\mu$ g/ml (Mackeen *et al.*, 1997). On the other hand, the extracts of kesum is not cytotoxic to normal lung fibroblast cells (Hs888Lu) in both water and methanol extract (Qader *et al.*, 2011). In the in-vivo tests, the n-hexane fractions of kesum leaf extract can inhibit the proliferation of lung epithelial cells previously increase by benzopyrene exposure (Wibowo *et al.*, 2010). Wibowo *et al.* (2011) also states that the n-hexane fraction of kesum able to inhibit the destruction of lung tissue previously damaged by benzopyrene exposure.

Some researchers say that the cytotoxic properties of kesum are due to his activities as an antioxidant. Huda-Faujan *et al.* (2007), on their research that examined the

antioxidant activity of kesum aqueous extracts, were states that extracts water from kesum are powerful antioxidants. This was confirmed by research Qader *et al.* (2011) which states that the both methanol and water extract of the kesum have strong antioxidant activity. Kesum showed antioxidant activity on the in-vivo tests. Wibowo *et al.* (2011) on the in-vivo study states that the fraction of n-hexane extract of the kesum leaves able to suppress ROS production on the lung tissue of animal model that previous increased as a result of benzopyrene exposure.

Based on the potential of the kesum extracts especially the n-hexane fraction of kesum in inhibiting the growth of lung cancer, so this study will be discussed about the ability of the n-hexane fraction of kesum to inducing apoptosis in lung epithelial cells and suppressing ROS production in the animal models that exposed by benzopyrene.

## MATERIALS AND METHODS

### Research material

The kesum (*Polygonum minus* L) that used in this study was collected from Kota Baru, Pontianak-Indonesia. This species was determined in the Laboratory of Plant Taxonomy and Structure Development, Department of Biology, Brawijaya University and the specimen stored there. The n-hexane fraction of kesum that used in this study was prepared by the method of extraction. The first step, the leaves of kesum was cleaned, dried, and crushed. After the leaves of kesum crushed, it was macerated by methanol solvent for  $2 \times 24$  hours. Furthermore, the macerate was fractionated by n-hexane solvent and evaporated to get the n-hexane fraction. The n-hexane fraction that obtained was used as a therapy material in this research.

### Animals handling

The using of the animal models in the study was approved by the Research Ethics Committee of University of Brawijaya, Malang-Indonesia. The first time, it were chosen the fifteen of the male mice (*Ratus norvegicus*) that have aged 2–3 months and body weight (BW) 200–250 grams. The mice was adapted for 7 days before treatment. The animal experiments were grouped into three treatment groups: the T0 group (healthy control mice), the T1 group (benzopyren exposed mice), and the T2 group (benzopyren treated mice continued by provision of n-hexane fraction). The T0 group is a group of healthy experimental animals were given by distilled water. The T1 group is a group that exposed by benzopyren (200 mg/kg BW for four times, by intraperitoneal) and incubated for thirty days. The T2 Group is the animal models that exposed by benzopyren (200 mg/kg

BW for four times, by intraperitoneal) and incubated for thirty days. After exposed by benzopyrene, the T2 group were treated by the n-hexane fraction of kesum (100 mg/kg BW for one week) and incubated for seven days. At the end of the treatment, all of the animal models were sacrificed and collected the lung organs.

### Measuring the malondialdehyde (MDA) content

It were crushed 0.45 g of the lungs tissue, added by 0.9% sodium chloride (1 mL), and centrifuged at a speed of 8000 rpm for 20 minutes to obtain supernatant. Against supernatant were then taken as many as 100  $\mu$ L, put into small tubes, and it were added successively by aquades (550  $\mu$ L), trichloroacetic acid (100  $\mu$ L, 10%), HCl (250  $\mu$ L, 1 N), and Na-thiobarbiturat (100  $\mu$ L, 1%), and then incubated in a water bath at a temperature of 100° C for 30 minutes. The results obtained and then centrifuged at 500 rpm for 10 minutes to obtain supernatant. The supernatant were measured by spectrophotometer at 533 nm.

### Examination of apoptosis by TUNEL staining

Examinations of the apoptotic were done with Immunohistochemistry method by using the Apo-BRDU IHC Kit (K403-50). The slides (the paraffin-embedded tissue sections of the lung) put into the multilevel of xylene (1–3) respectively for 5 minutes. Furthermore, the slide were rehydrated in the graded ethanol from ethanol 100% ( $2 \times 5$  min), ethanol 90 (5 min), ethanol 80 (5 min), and 70% ethanol (5 minutes). The slides were washed with PBS (pH 7.4) for 10 minutes of, applied with proteinase K for 20 min, washed with PBS (pH 7.4), applied with 3%  $H_2O_2$  for 5 min, washed with PBS (pH 7.4) for 5 min, and cover by reaction buffer for 30 minute. Applied by the complete labeling reaction mixture and incubated at 37° C for 1.5 hours. The slide were washed by PBS for 5 minutes, and covered by blocking buffer for 10 minutes. Incubate with the antibody solution in the dark for 1.5 hours at the room temperature. Rinse slide with PBS and cover the entire specimen with the blocking buffer. Apply the conjugate solution to the slide for 30 min. Slide were washed with PBS once, incubated with DAB solution for 15 min, washed with  $H_2O$ , and dried. The specimen were covered by Mayer hematoxilen for 10 min, washed by the tap water for 5 minute, and dried by the wind. The specimen were mounted by entellan, and then covered by cover glass.

### Data analysis

The analysis of apoptosis performed on twenty fields of view under the microscope at a magnification of 400 times by three observers, with a blind test technique.

The measurement results of the both parameters were statistically analyzed by SPSS program using the One-way ANOVA method and the correlation analysis performed using the LSD (least significant difference) method.

## RESULTS

### MDA levels of the lung tissue of animal model

From the analysis of MDA to the three groups of the animals model, it showed that the *n*-hexane fraction be able to suppress the production of ROS, were previously increased by benzopyrene exposure. Benzopyrene exposure to the experimental animals increased the lung MDA levels of  $8.44 \pm 1.89$  ppm, which the control MDA is  $5.09 \pm 0.76$  ppm. It reveals that exposure of benzopyrene able to increase the ROS production in the lung tissue of the animal models. Therapy on animals models with *n*-hexane fraction kesum able to suppress the production of ROS in the lung tissues of the animal model ( $5.47 \pm 1.76$  ppm), which had previously increased due to exposure to benzopyrene. The ability of the *n*-hexane fraction of kesum to suppress the ROS production in the lung tissue shows that the *n*-hexane fraction is the good antioxidants for lung health.

The Analysis of the data by one way ANOVA followed by LSD test (least significant difference) showed a significant difference between the *n*-hexane therapy with the benzopyrene expose 0.011 ( $p < 0.05$ ). There was no significant difference between the *n*-hexane therapy with the control 0.704 ( $P > 0.05$ ).

### Apoptotic Index of the lung tissue of the animal model

Test the ability of the *n*-hexane fraction, its ability to induce apoptosis of the lung epithelial cells in the animal models that exposed by benzopyrene, provided that the *n*-hexane fraction of kesum able to induce the increasing of apoptosis of the lung epithelial cells of the animal models that exposed by benzopyrene. The ability of the *n*-hexane fraction of kesum to induces apoptosis in the lung epithelial cells of animal models that exposed by benzopyrene were indicated by increase the apoptosis of the lung epithelial cell on the TUNEL staining. Results the scoring of apoptosis index (IA) in each of the group of the animal models indicated that the *n*-hexane fraction has a higher IA value than the control group and the benzopyrene group. The ability of the *n*-hexane fraction to inducing apoptosis because the fraction is able to suppress the ROS production in the lung tissue of the animal models that exposed by benzopyrene. The Analysis of the IA data by one way ANOVA followed by LSD test (Least Significant

Difference) showed a significant difference between the *n*-hexane therapy with the benzopyrene expose 0.003 ( $p < 0.05$ ) and the control 0.000 ( $P > 0.05$ ).

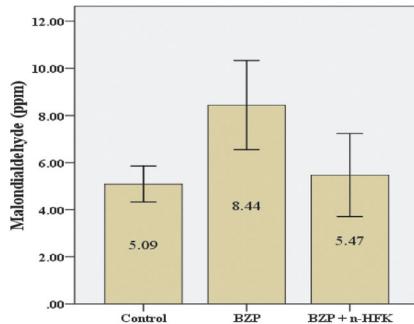
## DISCUSSION

The benzopyrene exposures to lead the lung epithelial cells become resistant to apoptosis. In this study, the benzopyrene exposure will lead to an increase in ROS levels in the lung of animal models. The ability of benzopyrene in improving of ROS production because it will undergo oxidation reactions that catalyzed by Cytochromes P450 (CYPs) become reactive metabolites such as (+)-benzopyrene-7,8-dihydrodiol [(+)-BP-7,8-diol], (-)-benzopyrene-7,8-dihydrodiol [(-)-BP-7,8-diol], N2-guanine-benzopyrene-7,8-diol-9,10-epoxide (BPDE) and radical cation (Kushman *et al.*, 2007). This reactive metabolite by the enzyme AKRs will be encouraged to induce ROS produced. Several studies have demonstrated the role of ROS in inducing DNA damage and mutations (Azad *et al.*, 2008).

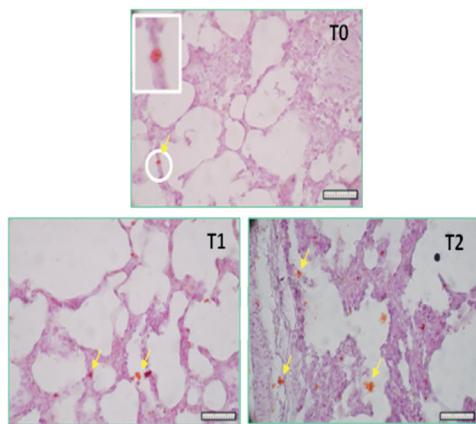
In our study, the high production of ROS will be inhibited apoptosis of the lung epithelial cell in the animal models that exposed by benzopyren. The ability of ROS to inhibit apoptosis due to its ability in activates Nf-KB (Morgan & Liu, 2011). NF-kappaB plays a critical role in blocking apoptosis because the active NF-kB plays a role in increasing production of enzymes iNOS and anti-apoptotic proteins (Katsuyama *et al.*, 1998; Chen *et al.*, 2001). Increased expression of iNOS in pulmonary organ will increase the production of NO radicals (Chen *et al.*, 2008). Increased production of NO will be inducing the expression of Bcl-2 and the mutations of p53 gene (Ambs *et al.*, 1997; Ferrer *et al.*, 2007). Increased production of anti-apoptotic protein Bcl-2 will be inhibit apoptosis of lung epithelial cells are intrinsically due to protein Bcl-2 plays a role in inhibiting the release of cytochrome C by mitochondria (Iyer *et al.*, 2008). Increased of p53 mutant will be inhibiting the induction of apoptosis by p53 gene, so that the cells remain lives (Ambs *et al.*, 1997). In this study, the ability of benzopyrene in inhibit apoptosis be indicated by the poor apoptotic events in the benzopyren exposure group compared to the *n*-hexane therapy group.

The results of this study indicate that the *n*-hexane fraction of kesum able to induce apoptosis of lung epithelial cells of the animal models that have been exposed by benzopyrene. The ability of the *n*-hexane fraction of kesum to induce apoptosis in the lung epithelial cells is due to its ability to suppress the ROS production of the lungs animal models that exposed by benzopyrene. Pressed the production

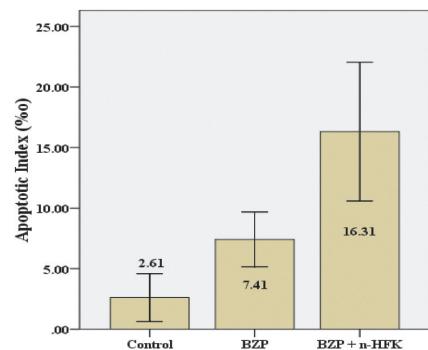
of ROS in lung organ would inhibit the activation of NF- $\kappa$ B. Pressed the production of ROS in lung organ would inhibit the activation of NF- $\kappa$ B, which means that the production of the enzyme iNOS and anti-apoptotic protein Bcl-2 will be suppressed (Chen *et al.*, 2001; Ramasamy *et al.*, 2011). Pressed the expression of iNOS may result in suppressed of Bcl-2 expression and p53 gene mutations, thereby increasing the release Cytochrome C by mitochondria. The release cytochrome C by mitochondria would mediate apoptosis of lung epithelial cell (Jiang & Wang 2004).



**Figure 1.** The malondialdehyde levels of the lung tissue. Therapy animal model with *n*-hexane fraction kesum leaf extract (BZP+n-HFK) can reduce lung malondialdehyde levels were increased due to previous exposure to benzopyrene (BZP). The malondialdehyde level respectively is  $5.09 \pm 0.76$  ppm for the controls,  $8.44 \pm 1.89$  ppm for the BZP, and  $5.47 \pm 1.76$  ppm for BZP+n-HFK. The malondialdehyde values were taken from the mean value with standard deviation ( $p = 0.005$ ).



**Figure 2.** Apoptosis the lung epithelial cells, by TUNEL staining. Therapy of the animal model by *n*-hexane fraction able to increase apoptosis of epithelial cells of lung tissue on the animal models that had been exposed by benzopyrene, it is demonstrated by the increase of apoptosis in the lung epithelial cells. T0 is a control group, T1 is a benzopyrene group, and T2 is an *n*-hexane therapy group. Yellow arrows indicate apoptosis events of the lung epithelial cell. (400 $\times$ )



**Figure 3.** Apoptotic index the lung epithelial cell. The animal models therapy by *n*-hexane fraction able to increase apoptosis the lung epithelial cells of the animal models that have been exposed by benzopyrene. The Apoptosis index for each group of animal models is  $2.61 \pm 1.98$  for the controls group,  $7.41 \pm 2.26$  for the benzopyrene group, and  $16.31 \pm 5.73$  for *n*-hexane therapy group. The values of Apoptotic Index is taken from the mean value with standard deviation ( $p = 0.005$ ).

From this study it can be concluded that the *n*-hexane fraction of kesum can improve apoptosis of the lung epithelial cell via suppressed of ROS production in the lung tissue of the animal models that exposed by benzopyrene. These results give hope about the use of *n*-hexane fraction of kesum in preventing lung cancer.

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