

## The Potency of Monoterpenes Contained in Essential Oils of Canary Sap (*Canarium indicum* L.) as Anti-inflammatory Agent on Asthmatic Rats (*Rattus norvegicus*)

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Received 12 January 2018; Revised 3 April 2018; Accepted 6 July 2018

### ABSTRACT

The aim of this study is to determine the potency of EO canary therapy towards level of malondialdehyde (MDA) and lung histopathology of asthmatic rats. The asthmatic rats were prepared by sensitization of allergen conducted by intraperitoneal injection and nebulized of ovalbumin (OVA) also intrasulcular injection of lipopolysaccharide from *Porphyromonas gingivalis* bacteria. Five groups of rats (*Rattus norvegicus*) were used in this research; i.e. the control group, the asthmatic group, and three other groups with therapy of EO canary with the dose of 25, 50, and 100 mg/kg body weight for 7 days. The EO canary contents were analyzed by gas chromatography–mass spectrometry (GC-MS). The MDA levels were measured using the thiobarbituric acid (TBA) reagent and histopathology of bronchial stained using Hematoxylin-Eosin (HE) and observed microscopically. The results showed that EO canary significantly ( $p < 0.01$ ) decrease MDA levels and repair lung histopathology of asthmatic rats. The analysis of EO canary performed by GC-MS composed of thujene,  $\alpha$ -pinene, camphene, sabinene, 2- $\beta$ -pinene,  $\beta$ -myrcene, 1-phellandrene, p-cymene,  $\gamma$ -terpinene, cis-Sabinene hydrate,  $\alpha$ -terpinolene, camphor, limonene dioxide, sabiny acetate, piperitone oxide, ascaridole,  $\alpha$ -cubene,  $\alpha$ -copaene, trans-caryophyllene, and 1,8-cineol. It can be concluded that EO canary has potency as anti-inflammatory agent of asthmatic condition. The most effective dose therapy was obtained as high as 100 mg/kg body weight that decreased 46.56 % level of MDA.

Key word: Ovalbumin (OVA), *Porphyromonas gingivalis*, Canary (*Canarium indicum* L) sap, Essential oil (EO), and malondialdehyde (MDA)

### INTRODUCTION

Asthma is a chronic disease of the airways that is characterized by exacerbations of significant bronchospasm and marked airway inflammation. Bronchospasm can also present as a cough, chest pain, shortness of breath, and fatigue with exertion [1, 2]. Asthma is also a dangerous global health problem affecting at least 300 million people. According to WHO estimation, approximately 250.000 people die prematurely each year from asthma [3]. According to GINA (2011) [4], asthmatic patients have significantly increased worldwide to 300 million people. The number of patients due to asthma in 2025 was approximately 400 million people. In Indonesia, there are 18 provinces that have asthma disease exceeding national numbers. One of them is East Nusa Tenggara (ENT) province that had a high prevalence of asthma in 2007. It was observed that the prevalence of 2007 to 2013 increased

The journal homepage [www.jpacr.ub.ac.id](http://www.jpacr.ub.ac.id)  
p-ISSN : 2302 – 4690 | e-ISSN : 2541 – 0733

with 1 % patients with asthma [4]. People with asthma have an allergic trigger (e.g. house dust mite, pollen). In one study, non-allergic triggers such as air pollution, cigarette smoke, perfume, stress, negative emotions or physical activity may also trigger asthma symptoms [5].

The rats as an experimental model for the asthmatic disease can be induced by OVA sensitization followed by an OVA exposure and use alum as an adjuvants [6], and also use *Porphyromonas gingivalis* as an oral infection [7]. Lipopolysaccharide (LPS) in *Porphyromonas gingivalis* is an endotoxin that can stimulate the response of inflammatory mediator cells [8]. Inflammation is a complex process mediated by the activation of various immune cells. Macrophages play a central role in mediating many different immunopathological phenomena during inflammation, including the overproduction of pro-inflammatory cytokines and inflammatory mediator [9]. The severity of asthma is determined by the presence of inflammation and infection. Asthma is not only characterized by episodes of acute inflammation but may also be related to the development of airway remodeling. Traits of remodeling histologic condition in asthma include the presence of subepithelial fibrosis and smooth muscle hypertrophy/hyperplasia. The process of inflammation in cells will trigger the emerge of cytokines which is Interleukin-1 (IL-1) [10, 11]. IL-1 is a cytokine group that plays an important role in the inflammatory response to pathogenic bacteria and other harmful agents [12] that damage lung due to oxidative stress and trigger inflammation due to free radical increasing. The free radicals formed lead to oxidative stress and oxidative damage then caused lipid peroxidation [12,13,14], which cell membrane damage is characterized by elevated levels of MDA as an indicator of lipid peroxidation. Some studies showed that MDA involves several diseases include early breast cancer, oral cancer, cancer-bearing dogs, complex regional pain syndrome, alcohol dependent, atherosclerosis patients with familial Mediterranean fever, glaucoma, chronic obstructive pulmonary disease, Alzheimer's disease, cardiovascular, diabetes, liver disease, allergic asthma, and Parkinson disease [14, 15].

Treatment of asthma uses many synthetic drugs such as corticosteroid (aspirin) and salbutamol that act as an anti-inflammatory and reduces symptoms, but long-term use of synthetic drugs causes osteopenia, slow wound healing, hyperglycemia, hypertension, cataracts and debilitating systemic nerve function [16]. Therefore it is necessary to explore an alternative treatment of asthma using herbs [17], such as EO from canary sap (*Canarium indicum*).

*Canarium indicum* L. belongs to the family of Burseraceae, considered one of the most important herbs. Canary sap (*Canarium indicum* L.) was collected by means of tapping [18]. Canary sap belongs to oleoresin that is used as an ointment for the stomach and expectorant (cough medicine). Its skin is used for treating chest tightness, arthritis, while the oleoresin tree can treat stomach ulcers, as a stimulant, anti-rheumatic [19], bronchial disease, asthma, epilepsy, chronic skin disease, syphilis, hernia, and complications [20]. Canary sap is potentially used as an anti-inflammatory agent in rheumatic rats [19]. Phytochemical screening of canary resin shown that it contains glycoside compounds, saponins, tannins, alkaloids and polyphenol compounds [21]. The EO of the canary sap was reported to be composed mainly of monoterpenes. The major compounds reported from the same genus of *Canarium album* were  $\beta$ -pinene,  $\alpha$ -terpinene,  $\gamma$ -terpinene, terpinen-4-ol, p-cymene, limonene and  $\alpha$ -terpineol,  $\alpha$ -pinene [22, 23]. The potency of EO as an anti-inflammatory agent is higher than aspirin [23]. The content of EO was regarded to be important as a potential natural source for medical compounds because of anti-inflammatory activities.

This study aimed to determine the potency of EO canary sap (*Canarium indicum* L.) towards the level of MDA and histopathology of lung asthmatic rats (*Rattus norvegicus*) that induced by OVA and LPS [6, 24, 25].

## EXPERIMENT

### Chemicals and instrumentation

The chemicals used in this study include ovalbumin (Sigma-Aldrich, 950512), Aluminium hydroxide ( $\text{Al}(\text{OH})_3$ ), Phosphate Buffer Saline (PBS), 10 % paraformaldehyde (PFA), hematoxylin and eosin (HE), 0.9 % NaCl Physiological, LPS<sub>1435/1449</sub> of the *Porphyromonas gingivalis* (Astarte Biologics), Ketamine-A100, MDA standard, 10 % TCA, HCl, 1 % Na-Thio, and water was purified using distillation technique.

The instruments that were used in this research include water bath 100 °C, syringe (Terumo), autoclave (All American Model 20×), microscope (Olympus B×53), centrifuge (Kitman), pH meter (WTW inoLab), spectrophotometer UV-Vis (Thermo scientific Genesys 20), vortex (Maxi Mix Termolyne), ultrasonic cleaner (Branson 2000), set of cleverger apparatus which were applied into steam distillation, nebulizer (Omron CompAir Compressor), analytical balance (Sartorius), and GC-MS (Shimadzu QP2010 Ultra).

### Plant Material Procedure

*Canarium indicum* L. sap was collected from the wounded trunk of trees in Ende, Flores, Indonesia. The EO canary from the oleoresin (150 g) was collected using steam distillation method approximately for four hours. The result obtained the yellow oil which was dried using anhydrous magnesium sulfate. The EO canary was stored in glass vials covered with aluminum foil at -4 °C. The yield of the EO was 2.45 % with a density of 0.97 g/mL.

### Animals Treatment

Wistar male rats (*Rattus norvegicus*) (150–200 g) were used for the asthmatic models and were acclimated for 7 days. The animals were kept in cages at  $25 \pm 1^\circ\text{C}$  and fed with food and water ad libitum. All rats were acquired after being approved by Unit Pengembangan Hewan Percobaan (UPHP) UGM Yogyakarta. The rats were divided into five treatment groups: control group (A), asthma group (B), dosage of therapy group 25 mg/kg body weight (C), dosage of therapy group 50 mg/kg body weight (D) and dosage of therapy 100 mg/kg body weight (E). At the end, all groups of rats were euthanized by cervical dislocation. The lungs were fixed in PBS solution for MDA assay and the rest were preserved in PFA solution for histological analysis. The use of experimental rats in this study had been approved by the Research Ethics Committee of Brawijaya University, No. 815-KEP-UB.

### GC-MS Analysis

The EO canary (1  $\mu\text{L}$ ) was injected into GC-MS (Shimadzu QP2010 Ultra) with the specification of column oven temperature under 60 °C, injection temperature at 250 °C, split injection mode, pressure flow control mode 80 kPa, total flow 134.8 mL/min, column flow 1.30 mL/min, linear velocity 41.7 cm/sec, purge flow 3 mL/min, and split ratio 100.

### OVA Sensitization and LPS Injection

Rats were sensitized on day 1 with an intraperitoneal (*i.p.*) injection containing 10  $\mu\text{g}$  of OVA suspended in 10 mg of  $\text{Al}(\text{OH})_3$  and 200  $\mu\text{L}$  PBS. All rats were injected with the same dose of OVA and  $\text{Al}(\text{OH})_3$  on day 14. Then, inhalation of OVA was assessed using Omron

*CompAir Compressor Nebulizer* on day 21, performed by dissolving OVA in 1 mg/mL 0.9 % NaCl for 30 minutes.

Rats were anesthetized by ketamine *i.p* injection. Rats were injected of LPS with the dose of 1 µg/mL on the left upper rat molar gingiva mucus. The LPS used is LPS<sub>1435/1450</sub> from *Porphyromonas gingivalis* (Astarte Biologics) which acts as an oral cavity infection agent and modulates the immune response. The injection was conducted on days 10 and 11 [7].

### EO Dosage

The dosage of essential oil (EO) based on the previous study [19], *i.e.* used a dose of therapy 25, 50, and 100 mg/kg body weight. The EO has dissolved corn oil up to 1.5 mL and was given for 7 days on day 21.

### MDA Assay

The lung (0.5 g) was grilled by a cold mortar in 0.9 % NaCl and it was moved into a microtube. Then it was sonicated for 10 minutes and was centrifuged for 20 minutes temperature at 25 °C 8000 rpm to separate supernatant and sediment. Then 100 µL of lung supernatant was added by 550 µL of distilled water, 100 µL of 10 % TCA, 250 µL of 1 N HCl and 100 µL of 1 % Na-Thio. Homogenized it with a vortex mixer. The mixture was incubated in water bath at 100 °C for 30 minutes and left at room temperature. Furthermore, the mixture was centrifuged at 25 °C at 500 rpm for 10 minutes. The samples were measured at 530 nm in order to determine MDA concentration for TBA test by spectrophotometer UV-Vis (Thermo scientific Genesys 20) [13, 26].

### Histological Analysis of Lung Tissues

The lungs were washed using NaCl 0.9%, and fixed in PFA 10%, then embedded by paraffin. Sections of the lung were stained using HE (Hematoxylin-Eosin) for quantification of inflammatory cells. The sections of the lung were deparaffinized using xylol and rehydrated using gradual ethanol for 5 minutes. Then, soaked in distilled water for 5 minutes. The sections were dyed using Hematoxylin and were incubated for 10 minutes. Furthermore, they were washed by using flowing water for 30 minutes and were rinsed by using the distilled water. Next, the sections were dyed by using eosin with ethanol for 5 minutes. The last steps involved dehydrated using gradual ethanol, clearing by xylol and then drying. The dried and stained sections of the lungs were mounted by using entellan and were observed at 400 × by optical microscopy (Olympus BX53).

### Statistical Analysis

The effect of treatment of each group was analyzed by ANOVA using Tukey test, with a *p*-value threshold of < 0.01. The results were considered to be significant.

## RESULT AND DISCUSSION

### EO Isolation and Analysis Using GC-MS

Isolation of EO made using steam distillation method resulted in a yield of 2.45%. The EO obtained is yellow with a distinctive aroma of sharp exudate and has a density of 0.97 g / mL. The anti-inflammatory of canary sap (*Canarium indicum*) EO by GC-MS contained monoterpenes including 1-phellandrene, p-cymene, γ-terpinene, α-terpinolene (linalool), camphor, limonene, and 1,8-cineole as shown in Table 1. Results obtained are in agreement with the most of the previous works who reported that canary is characterized by the presence of monoterpenes as major component [27].

The Table 1. shows that the abundant concentration unit that decreased inflammation is 1-phellandrene (31.612 %). According to the previous study, 1-phellandrene is able to decrease mast cells degranulation and production of pro-inflammatory cytokines, such as IL-6, TNF- $\alpha$ , histamine, prostaglandins, leukotrienes, and some chemotactic cytokines, including IL-8. These mediators contribute to an allergic disease [28]. *p*-cymene and  $\gamma$ -terpinene exhibit strong anti-inflammation.  $\gamma$ -terpinene have a higher selective inhibitor for COX-2 activity than aspirin. This component is able to relieve the production of TNF- $\alpha$ , IL-1 $\beta$ , IL-8, IL-10, and PGE2 by LPS-activated human peripheral blood monocytes. Linalool is an anti-inflammatory agent in hypersensitivity mechanism. Limonene inhibits mast cell activation and degranulation. The previous study reported that limonene exerts an anti-inflammatory agent by inhibiting NF- $\kappa$ B decreasing the production of NO and PGE2. Thereby, decreasing the expression of iNOS and COX-2 protein thus cytokine production of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 levels in macrophages stimulated by LPS [9]. Camphor and phellandrene are able to inhibit leukotriene production for asthma, chronic bronchitis, and allergic rhinitis. 1,8-cineole has an immunomodulatory effect, anti-asthmatic bronchial and anti-allergic effect as in type I allergic that can inhibit histamine release from rat peritoneal mast cell, relieve rhinitis symptoms [23].

**Table 1.** The monoterpenes composition of EO identified by GC–MS

No.	Name	RT	MW	SI	%
1.	Thujene	6.2560	136	94	0.3290
2.	$\alpha$ -Pinene	6.4380	136	96	1.2540
3.	Camphene	6.7600	136	92	0.1240
4.	Sabinene	7.3690	136	95	0.2120
5.	2- $\beta$ -Pinene	7.4560	136	94	0.1410
6.	$\beta$ -Myrcene	7.8510	136	94	0.5910
7.	1-Phellandrene	8.2610	136	93	31.612
8.	<i>p</i> -Cymene	8.6130	134	96	4.6050
9.	$\gamma$ -Terpinene	9.3760	136	94	0.3220
10.	Cis-Sabinene hydrate	9.4550	154	91	0.6890
11.	$\alpha$ -Terpinolene	10.022	136	95	2.2710
12.	Camphor	10.817	152	95	1.7530
13.	Limonene dioxide	12.592	168	85	2.9320
14.	Sabinyol acetate	12.006	194	94	1.6140
15.	Piperitone oxide	14.164	168	86	0.0640
16.	Ascaridole	14.565	168	84	0.0460
17.	$\alpha$ -Cubene	14.725	204	92	0.1170
18.	$\alpha$ -Copaene	15.133	204	92	0.2730
19.	trans-Caryophyllene	15.743	204	85	0.0280
20.	1,8-Cineole	15.837	154	79	0.0230

Note : Retention Time (RT), Molecular Weight (MW), Similarity Index (SI), and area percentage (%). SI abbreviated to the Similarity Index and determined by the comparison library from the names of these obtained constituents. The percent area shows the different abundance of constituents.

### MDA Assay

This study observes oxidative stress in the lung of asthmatic rats by measuring MDA. The average MDA levels of control group were applied to determine the increase in the MDA levels of other groups. The MDA level of the control group was the lowest ( $0.70 \pm 0.05$   $\mu$ g/mL). MDA levels on group with asthmatic showed increasing MDA levels to be  $1.33 \pm$

0.01 µg/mL. It was 89.54% increased from the control group. The EO canary showed decreasing MDA levels to be 21.68%; 36.46 %; and 46.56% with dose therapy of 25 mg/kg body weight; 50 mg/kg body weight and 100 mg/kg body weight, respectively. Statistical analysis also showed a significant different between groups ( $p < 0.01$ ) (Table 2).

**Table 2.** MDA level on treated rats

Treatment Group	MDA
	Mean $\pm$ SD (µg/mL)*
Control (A)	0.70 $\pm$ 0.05 <sup>a</sup>
Asthma (B)	1.33 $\pm$ 0.01 <sup>b</sup>
Dose of 25 mg/kg body weight (C)	1.04 $\pm$ 0.02 <sup>c</sup>
Dose of 50 mg/kg body weight (D)	0.85 $\pm$ 0.02 <sup>d</sup>
Dose of 100 mg/kg body weight (E)	0.71 $\pm$ 0.02 <sup>a</sup>

\*different notations of a, b, c, d, and e show a significantly different effect in each group ( $p < 0.01$ )

Table 2. MDA level on treated rats and elevated levels of MDA in asthmatic rats are due to oxidative stress and inflammation in the animal model, caused by ovalbumin induction intraperitoneally and inhaled allergens and LPS that exacerbate oxidative stress and inflammation. OVA sensitization caused an acute inflammatory response in the airways. Induction of LPS will activate inflammatory cells and activate macrophages to produce and release cytokines such as IL-1 and TNF- $\alpha$  thus causing tissue damage. The consequences of uncontrolled oxidative stress on the tissues give rise to lipid peroxidation. Lipid peroxidation in the cell membrane produces an aldehyde product such as MDA [29]. Lipid peroxidation will cause damage to epithelial cells of the bronchioles and alveoli in the lung. Induction of OVA and LPS will decrease HDL levels in the blood resulting in decreased activity of lipoprotein lipase enzyme (LPL) [30].

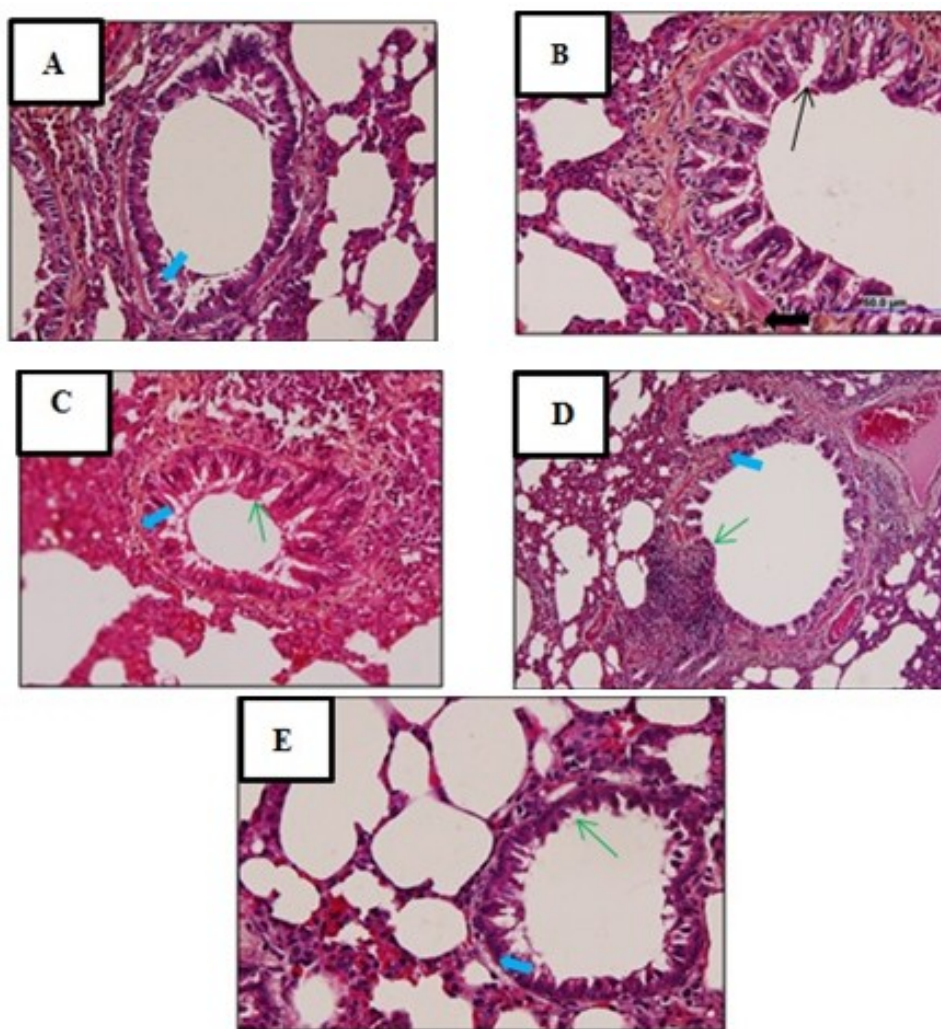
EO canary as an antioxidant and anti-inflammatory can stabilize the free radical of asthma. The content of the canary sap is a monoterpene compound of essential oils in which the  $\gamma$ -terpinene compounds have the potential of anti-inflammatory by inhibiting COX-2 enzyme activity. This enzyme is only formed during inflammation that produces prostaglandins and leukotriene [23]. Decreased levels of MDA due to the essential oil contains monoterpene compounds that are able to inhibit the COX enzyme that may be produced by OVA and LPS.

### Lung Histopathology on Rats Model

The decreasing of MDA level was proven by improvement of lung histology. To characterize the changes in lung histology, we examined lung sections stained using HE. OVA induction (Figure 1B) caused cell infiltrate containing many lymphocytes, macrophages, and eosinophils. These led to the inflammatory and cellular infiltration into the airways. Airway epithelium becomes fragile and thick with increased mucus production. Mediator induced abnormalities in the parasympathetic, non-adrenergic and non-cholinergic nervous systems may also increase bronchial hyper-responsiveness [2].

Rat treated with EO had reduced inflammation and reduced eosinophil infiltration (Figure 1C, 1D, and 1E). The determination whether there were differences in lung inflammation between rats treated with EO and OVA, BSM (bronchioles smooth muscle) was evaluated. The number of BSM cells, which reflect the intensity of airway inflammation, was significantly reduced after EO treatment compared to OVA-treated group (Figure 1A).

Figure 1A is healthy rat histology images and there is no airways remodeling. BSM helps exhalation and organize the distribution of ventilation within the airways in mucus [31] Figure 1B showed bronchial remodeling such as abnormal epithelium and an increased bronchioles smooth muscle (BSM). The prominent of BSM is related to a decrease in lung function. Figure 1C, 1D and 1E show an improvement in bronchioles and MDA concentration after treatment with the EO for 7 days (Table 2).



**Figure 1.** Histopathology of lung rats at 400X magnification.

(A) Control; (B) Asthma; (A) Dose therapy of 25 mg/kg body weight; (B) Dose therapy of 50 mg/kg body weight; (E) Dose therapy of 100 mg/kg body weight. (↑) bronchioles epithelial damage; (↑) improvement of bronchioles epithelial; (→) prominent smooth muscle; (→) improvement of smooth muscle

## CONCLUSION

The EO canary samples obtained from Flores contained monoterpenes including 1-phellandrene, p-cymene,  $\gamma$ -terpinene,  $\alpha$ -terpinolene (linalool), camphor, limonene, and 1,8-cineole as anti-inflammatory agents. These monoterpenes had action through two different mechanisms, neutrophil migration inhibition and mast cell stabilization. Exposure to LPS with a dose of 1  $\mu$ g/mL can increase levels of MDA as a marker of inflammation in lung organs of asthma rats by 89%. The doses variety of EO (25, 50, and 100 mg/kg body weight)

for asthmatic rats significantly decrease MDA levels (respectively 21.68 %, 36.46 %, and 46.56 %) and improve histopathology of the lungs. Thus, monoterpenes (primarily 1-phellandrene) are anti-inflammatory agents with potential for treatment of inflammatory diseases such as asthma and allergic diseases.

## ACKNOWLEDGMENT

The authors are grateful to the Biochemistry and Molecular Biology Laboratory of Medical Faculty, Organic and Biochemistry Laboratory of Mathematics and Natural Sciences Faculty, and Bioscience Institute in Brawijaya University. We thank Wibi Riawan, S.Si., M.Biomed and Fitri Armania, S.Si for assisting with IHC technique.

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