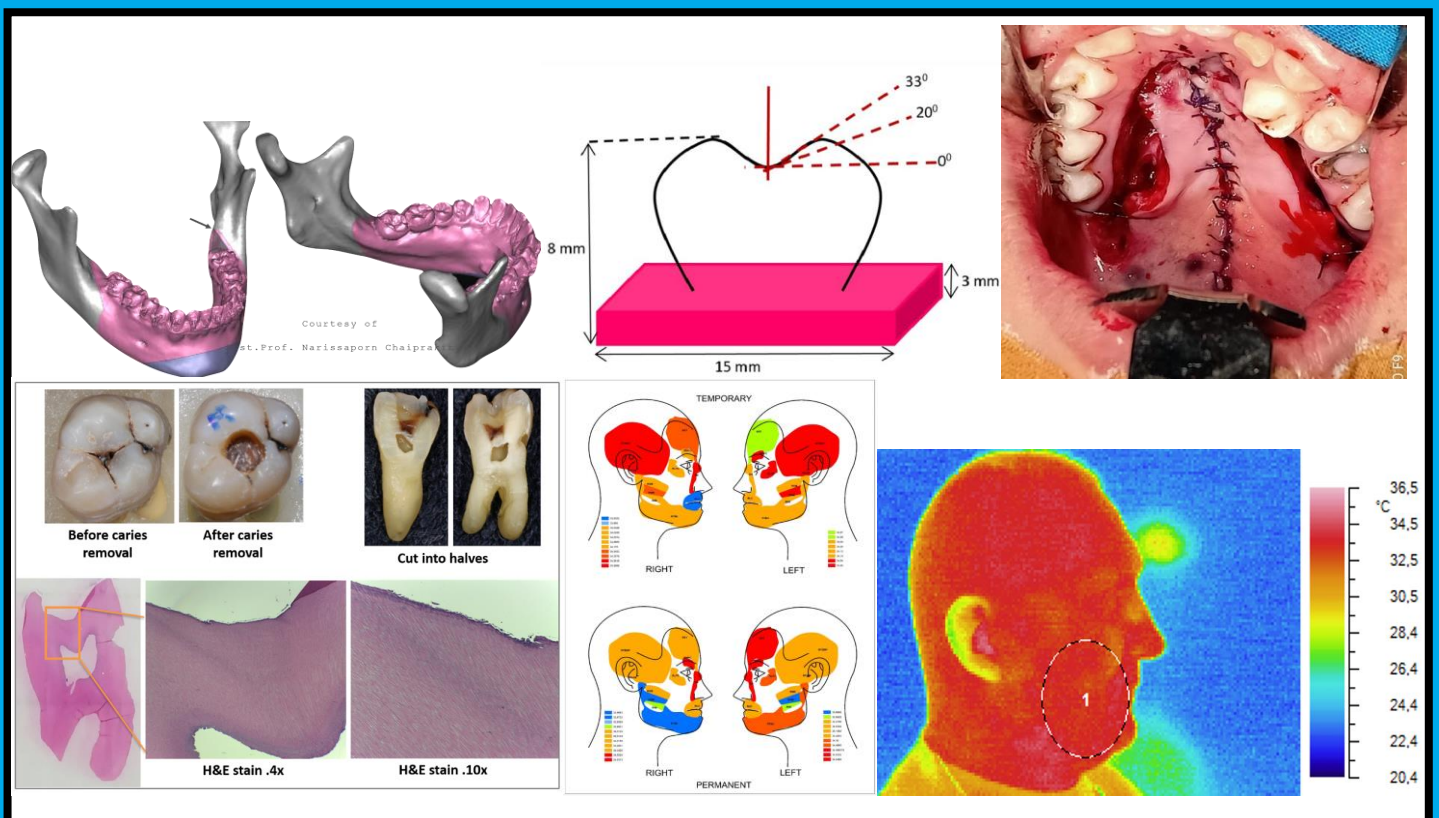


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Mechanism of *Centella asiatica* extract in Increasing Alveolar Macrophages Apoptosis in Rat Tuberculosis Models

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Abstract

Mycobacterium tuberculosis has the capacity to manipulate host defense pathway, particularly the ability to inhibit apoptosis of infected host cells. *Centella asiatica* is a medicinal plant which is able to reduce growth of *Mycobacterium tuberculosis* and enhance the cellular immune response and apoptosis in cancer cells. The research is to determine effect and mechanism of the extract of *Centella asiatica* in enhancing macrophages apoptosis from rat tuberculosis models. Rat tuberculosis models were divided randomly into 4 groups. Group 1, 2, and 3 were given extracts of *Centella asiatica* at 375mg/kgbw, 750mg/kgbw, and 1500mg/kgbw daily for 14 days. The 4 group is a control group. Apoptosis, expression of Bcl 2, Bax, Caspase 8 were performed by immunohistochemistry.

The study shows reduce expression of Bcl-2, increase expression of Bax and Caspase 8 also enhance the macrophages apoptosis. Ethanol extract of *Centella asiatica* have active ingredients to enhance of apoptosis alveolar macrophages from rat lung tissue that were infected by *Mycobacterium tuberculosis* through the increase of Caspase 8's expression, Bax'expression and decreasing of Bcl-2's expression.

Experimental article J Int Dent Med Res 2021; 14(2): 812-819

Keywords: Apoptosis, *Centella asiatica*, Macrophage, Rat tuberculosis models.

Received date: 14 March 2021

Accept date: 09 May 2021

Introduction

Immune response plays a fundamental role for outcome of tuberculosis bacterial infection. It is believed that the failure in treating tuberculosis is because of the failure of patients' immune response¹⁻⁴. In tuberculosis infection, there is a macrophages anti-apoptotic phenomenon which is characterized by an increase of various anti-apoptotic protein such as Bcl-2 protein and a decrease of pro-apoptotic protein namely Bax⁵⁻¹¹. *Mycobacterium tuberculosis* can multiply in macrophages by various process. Therefore, inhibition process of apoptosis in macrophages by *Mycobacterium tuberculosis* is a virulence factor for bacteria¹²⁻¹⁵.

Mechanism of tuberculosis immune

pathogenesis as mentioned above become the foundation in conducting a research on a more appropriate method of tuberculosis treatment¹⁶. New treatment of tuberculosis should also enhance immune response, especially to increase apoptosis in infected macrophages. The advantage of this apoptosis is to increase the elimination of bacteria without any intracellular material that comes out into extracellular so that bacteria cannot spread out of the cell. In addition, this apoptotic process will minimize inflammatory reaction that damage surrounding tissue¹⁷⁻¹⁹.

Centella asiatica is a plant that is widely available in several countries including Indonesia and used as folk medicine as antimicrobial, anti-inflammation and wound healing agent. The biggest compounds of this plant are *madecasic acid*, *Asiatic acid*, *madecasoside* and *asiaticoside*. Other Ingredients are *asiatiquercetin-3-glycoside*, *kämpferol-3-glycoside*^{20,21}. Ethanol extract of *Centella asiatica* inhibits the growth of *Mycobacterium tuberculosis in vitro* and increases immune response²²⁻²⁴. Water extract and ethanol extract of *Centella asiatica* increase

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levels of *Tumor Necrosis Factor- α* (TNF- α) in healthy macrophage cell cultures²⁵. Those extracts also increase cell expression of *Cluster of Differentiation* (CD) 4, CD8, macrophages and production of cytokines TNF- α , *interferon- γ* (IFN- γ) and inhibit transforming growth factor- β (TGF- β) in macrophage cells^{26,27}. *Asiatic acid*, one of the chemical compounds in *Centella asiatica*, induces macrophage apoptosis through the increase of cytochrome-c and caspase-3 protein²⁸. Numerous studies have shown that extracts of both *Centella asiatica* and its derivative, *Asiatic acid*, induce apoptosis in various cancer cells²⁷⁻³¹. This study aimed to determine the effect of ethanol extract of *Centella asiatica* on protein expression of Bax, Bcl-2, Caspase 8, and apoptosis of alveolar macrophages in rat tuberculosis models.

Materials and methods

Preparation of extract of *Centella asiatica*

Centella asiatica plant was used for this study and it was taken from Balai Materia Medika, Batu, Malang after determination was performed in Purwodadi Botanical Garden, Pasuruan. The extraction was conducted using maceration by soaking *Centella asiatica* in 70% ethanol.

Preparation of Bacteria

Mycobacterium tuberculosis strain H₃₇ RV (ATCC27294) obtained from tuberculosis laboratory of Tropical Disease Institute Universitas Airlangga was used. The bacterial suspension was made by taking bacterial colonies which were then put into glass tubes, calibrated before being used containing 2 drops of 0.05% tween 80. Afterwards, \pm 6 ml aquadest was added and then vortexed. The suspension was compared to Mc Farland standard (1 Mc Farland is equal to 10⁸/ ml *Mycobacterium tuberculosis*^{32,33}).

Chemicals

The chemicals used in this study were ketamine HCl injection (Hameln Pharmaceuticals), ether (EMS catalogue no. 1180), absolute alcohol, alcohol 96%, alcohol 80%, alcohol 70% (Brataco), aqua destilata (Brataco), carboxy methyl cellulose sodium (Brataco), Kieselgel 60F 254 (Merck, catalog no. 0345629), xylol, n-Hexana, ethylacetate, anisaldehyde sulfuric acid, Hematoxylin Eosin staining (EMS catalogue no. 14653), *Phosphate Buffered Saline* (PBS) (Brataco), formaldehyde

(Brataco), Ziehl-Neelsen staining (EMS catalogue no. 484k), Middlebrook 7H9 and 7H10 medium (BD Biosciens catalogue no. 295964), fungizone (Sigma, catalog no. A2942), Apoptag detection kit (Millipore corporation, catalog no. S1701), primary antibody for Bcl-2 was *anti mouse monoclonal antibody Bcl-2* (Bioworld Technology Inc., catalog no. BS1511), primary antibody for Bax which was *rabbit Ig G Bax antibody (species reactivity to rat and mouse)* (Novus Biologicals, catalog no. NB120 -7977), primary antibody for Caspase-8 was *rabbit IgG caspase 8 antibody* (Novus Biologicals, catalog no. NB120-15552), and *Immunohistochemistry Kit* (secondary antibody, streptavidim-HRP, DAB from Dako LSAB catalog No. K0673).

Rats Tuberculosis Models

The Experimental animals used were male rats (*Rattus norvegicus* Wistar strain) at 2-3 months of age with body weight between 125-200 gram obtained from the animal unit of Universitas Gadjah Mada. Rats were kept in BSL cabinet in Faculty of veterinary Medicine Universitas Airlangga and were given ad libitum access to food and water. Ketamine were given for anesthesia to all rats before they were infected with *M.tuberculosis*. 50 μ l *Mycobacterium tuberculosis* suspension (1 ml contains 10⁸ *Mycobacterium tuberculosis*) was injected into the trachea^{7,34,35}. After injection, the rats were kept and given food and drink ad libitum for up to 28 days. On the 29th day, the rats were randomly sacrificed and their lungs were taken for culture to prove that they were infected with *M.tuberculosis* while other rats will be used for rat tuberculosis models.

Treatment

Rat tuberculosis models were divided into 4 group and began to receive ethanol extract of *Centella asiatica*. Group 1 was given a dose of 375 mg/kgbw of ethanol extract of *Centella asiatica*, group 2 was given a dose of 750 mg/kgbw of ethanol extract of *Centella asiatica*, group 3 was given a dose of 1,500 mg/kgbw of ethanol extract of *Centella asiatica*. The extract was administered orally using sonde once a day for 14 days. Group 4, a control group, was given a suspension of 1% CMC Na in aquadest orally using sonde once a day for 14 days. On the 15th day after treatment, rats from both treatment groups and control group were sacrificed. The right lung tissue was put in 10% buffered formalin for histopathological examination using

Immunohistochemistry. The method used for examination of macrophage apoptosis in this study was immunohistochemical staining using *Terminal dUTP Nick-End Staining Labeling* (TUNEL) assay.

Data Analysis

Data were assayed for normality and homogeneity of variance. For comparison, ANOVA was used for comparative test, while *Spearman* correlation and path analysis was used for correlation test.

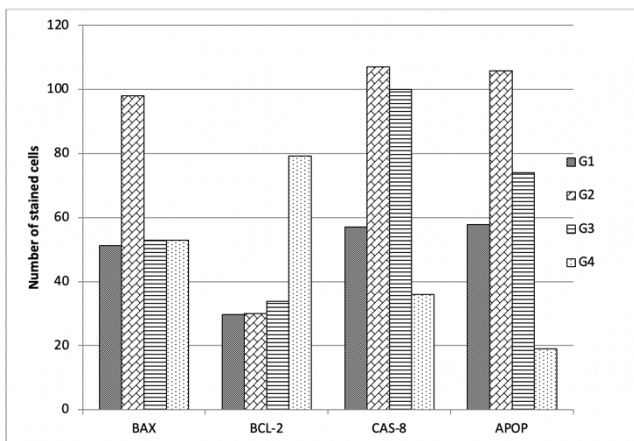
Ethical Approval

This study was ethically approved by Ethics Committee of Faculty of Veterinary Medicine, Universitas Airlangga (Ethical Clearance No. 129-KE) before conducting the study.

Results

The effect of *Centella asiatica* ethanol extract on Bcl-2 protein expression in alveolar macrophage

The results showed a significant difference between treatment groups and control group. Highest mean of Bcl-2 protein expression in alveolar macrophage was found in the control group (Table 1) (Figure 1.)



G1= 375mg/kgbw; G2 = 750mg/kgbw; G3 = 1500mg/kgbw; G4 = control group* p < 0.05

Table 1. The average of Expression of Bax, Bcl-2, Cas-8 protein and apoptosis of alveolar macrophages of rat lung tissue.

The effect of *Centella asiatica* ethanol extract on Bax protein expression in alveolar macrophage

The results showed a significant difference between treatment groups and control group. Highest mean of Bax protein expression

of alveolar macrophages was found in the second group (Table 1) (Figure 2).

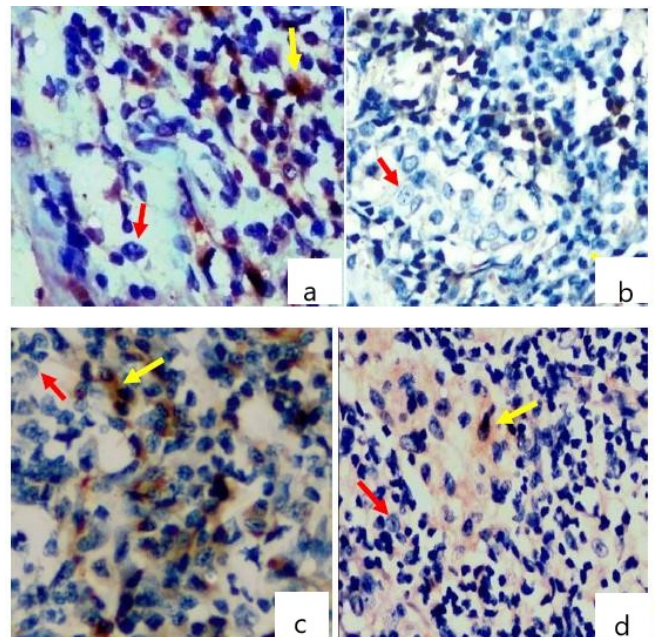


Figure 1. Expression of Bcl-2 protein from alveolar macrophages in rat lung tissue. The image above shows the effect of ethanol extract of *Centella asiatica* of 375 mg/kgbw (a), 750 mg/kgbw (b), 1500 mg/kgbw (c), and control group (d) on expression of Bcl-2. Examination was conducted by using immunohistochemistry. Brown cytoplasm shows positive expression of Bcl-2 protein (yellow arrow) whereas colorless cytoplasm shows negative result (red arrow). The amount of Bcl-2 protein expression is the highest in the control group. 400x magnification.

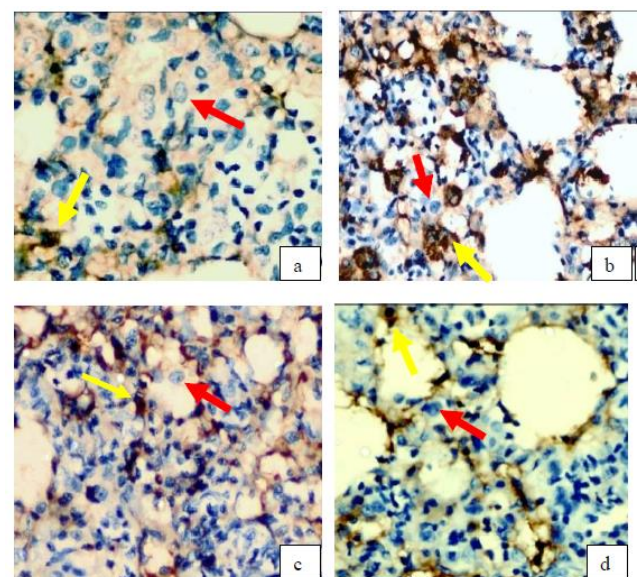


Figure 2. Expression of Bax protein of alveolar macrophages of rat lung tissue. The image

above shows the effect of ethanol extract of *Centella asiatica* at a dose of 375 mg/kgbw (a), 750 mg/kgbw (b), 1500 mg/kgbw (c), and control group (d) on expression of Bax. Examination was conducted by using immunohistochemistry. Brown cytoplasm shows positive expression of Bax protein (yellow arrow) whereas colorless cytoplasm shows negative result (red arrow). The amount of Bax protein expression is the highest in the group that received ethanol extract of *Centella asiatic* of 750 mg/kgbw. 400x magnification.

The effect of *Centella asiatica* ethanol extract on Caspase-8 expression of alveolar macrophage

The results showed a difference between treatment groups and control group. Highest mean of Caspase-8 protein expression of alveolar macrophages was found in group 2 (Table 1) (Figure 3).

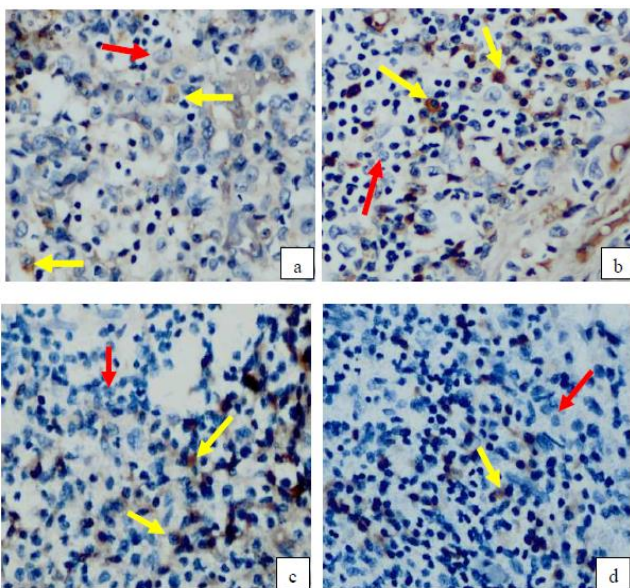


Figure 3. Expression of Caspase-8 protein of alveolar macrophages of rat lung tissue. The image above shows the effect of ethanol extract of *Centella asiatica* at a dose of 375 mg/kgbw (a), 750 mg/kgbw (b), 1500 mg/kgbw (c), and control group (d) on expression of Caspase-8 protein. Examination was conducted by using immunohistochemistry. Brown cytoplasm shows positive expression of Caspase-8 protein (yellow arrow) whereas colorless cytoplasm shows negative result (red arrow). The amount of Caspase-8 protein expression is the highest in the group that received ethanol extract of *Centella 815siatic* at a dose of 750 mg/kgbw. 400x magnification.

The effect of *Centella asiatica* ethanol extract in alveolar macrophage apoptosis

The effect of ethanol extract of *Centella asiatica* on apoptosis of alveolar macrophage in *Mycobacterium tuberculosis* - infected rat lung tissue showed a significant difference between treatment groups and control group. Highest mean of alveolar macrophage apoptosis was found in group 2 (Table 1) (figure 4).

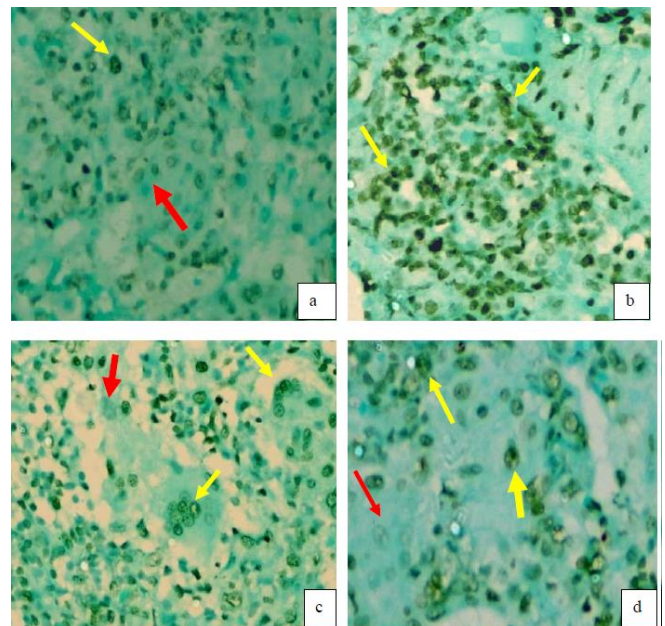


Figure 4. Apoptosis of alveolar macrophage cell of rat lung tissue. The image above shows the effect of ethanol extract of *Centella asiatica* of 375 mg/kgbw (a), 750 mg/kgbw (b), 1500 mg/kgbw (c), and control group (d) on expression of Caspase-8 protein. Examination was conducted by using *Tunnel assay (Apoptag detection kit)*. Brown nucleus shows positive result (yellow arrow) whereas colorless nucleus shows negative result (red arrow). The amount of apoptosis is the highest in the group receiving ethanol extract of *Centella asiatic* at a dose of 750 mg/kgbw. 400x magnification.

Pathway analysis was conducted to determine the correlation between ethanol extract of *Centella asiatica* and Bcl 2 protein expression, Bax protein expression, caspase-8 protein expression, apoptosis and the number of bacteria. The results of pathway analysis showed that ethanol extract of *Centella asiatica* significantly decreased Bcl-2 protein expression of alveolar macrophage ($p = 0.044$), increased Bax protein expression significantly ($p = 0.001$),

as well the increase of Caspase-8 protein ($p = 0.000$). the significant increase of Caspase 8 protein expression affected the increase of Bax protein expression ($p = 0.000$) and the increase of apoptosis of alveolar macrophage ($p = 0.000$). Ethanol extract of *Centella asiatica* increased the apoptosis of alveolar macrophage ($p = 0.000$).

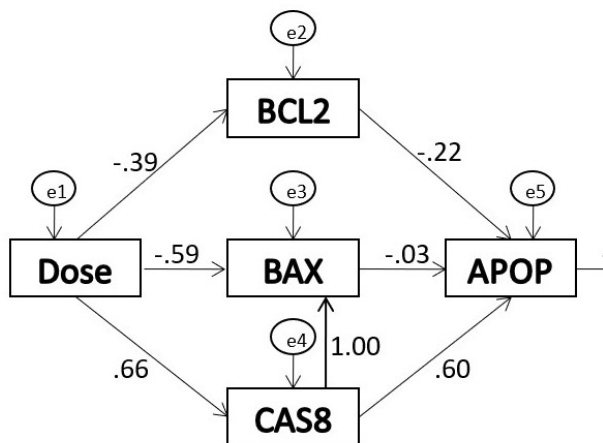


Figure 5. Results of Path Analysis.

Discussion

The results of this study showed that ethanol extract of *Centella asiatica* decreased the Bcl-2 protein expression in rat tuberculosis models. The ethanol extract of *Centella asiatica* did not linearly decrease Bcl-2 protein expression of alveolar macrophages cell of rat lung tissue with increasing given doses. The ethanol extract of *Centella asiatica* at a dose of 375 mg/kgbw, 750 mg/kgbw and 1500 mg/kgbw showed a significant difference in the decrease of Bcl-2 protein expression of alveolar macrophage when compared to the control group. However, there was no significant difference between treatment groups.

There was an increase of Bcl-2 expression in alveolar macrophage by 65% when compared to treatment group. This finding was consistent with previous study by Adolfo (2006) which stated that *Mycobacterium tuberculosis* has the ability to increase the expression of Bcl-2 protein in the alveolar macrophages cell. Bcl-2 protein expression in alveolar macrophage increases from 12.5% in the first days after infection to 32% at day 28 after infection³⁶. One of the active ingredients of ethanol extract of *Centella asiatica* is *Asiatic acid*. This ingredient is believed to has a significant role to decrease Bcl-

2 protein expression in alveolar macrophage. Bcl-2 is a member of Bcl protein family that has a very important role in apoptosis inhibition of macrophage cell by *Mycobacterium tuberculosis*^{6,11,37}. One possible mechanism of *Mycobacterium tuberculosis* to increase Bcl-2 protein expression in alveolar macrophage is through the increase of cytokines interleukin-10 secretion⁶. However, this ability can be inhibited by IFN- γ ^{38,39}.

Bcl-2 protein is a competitive inhibitor against Bax protein receptor, BH3 domain. Bcl-2 protein bonding makes Bax protein unable to bind BH3 domain, which makes Bax protein is not activated and this leads to the inhibition of the apoptotic process¹¹. BH3-only protein has a function to receive stimuli originated from the outside cell such as drug or radiation. These stimuli activate BH3-only protein and work directly in releasing bond the between Bax and Bcl-2 protein and decrease Bcl-2 expression^{9,10}. In this study, ethanol extract of *Centella asiatica* at a dose of 375 mg/kgbw has not reached the therapeutic dose required to increase Bax protein expression while at a dose of 1500 mg/kgbw there may have been a *down-regulation* or desensitization of the receptor. Hence, the effective dose of ethanol extract of *Centella asiatica* to increase Bax protein expression is 750 mg/kgbw.

Asiatic acid, one of the active ingredient of *Centella asiatica* ethanol extract, plays an important role in the increase of Bax protein expression in alveolar macrophage. Previous study showed that *Asiatic acid* increases apoptosis of melanoma cell through increased Bax protein expression³⁰. The results of path analysis showed that ethanol extract of *Centella asiatica* had a strong correlation with a decrease of Bcl-2 protein expression of alveolar macrophage and an increase of Bax protein expression of alveolar macrophage. However, correlation between these two proteins and apoptotic proteins was weak, meaning that the two did not directly increase apoptosis of alveolar macrophages but through other pathways which was not examined in this study(Figure 5).

In normal condition without any stimulation, Bax protein presents in cytosol cell in an inert state. Appropriate stimulus can change protein structure so that Bax protein can perform insertions on the target membrane, which are at the outer membrane of mitochondria and

endoplasmic reticulum. Interactions between Bax protein and any protein mentioned afore occur in BH3 domain and trigger multilevel changes of Bax protein and this phase is an early stage of apoptotic process through intrinsic pathway. BH3-only proteins receive stimuli originated from outside cell such as drug or radiation. These stimuli make BH3-only proteins activated and work directly in releasing the bond between Bax and Bcl-2 protein and decrease Bcl-2 expression. Stimuli from outside such as medicinal materials, irradiation and stress can affect interactions between pro-apoptosis proteins and anti-apoptosis proteins^{40,41}.

The graphic between *Centella asiatica* ethanol extract doses and caspase-8 protein expression of alveolar macrophage showed an inverted U-shaped curve with highest peak in dose of 750mg/kgbw. Ethanol extract of *Centella asiatica* at a dose of 375 mg/kgbw is not reach the therapeutic dose require to increase caspase-8 protein expression whereas down-regulation or desensitization of the receptor probably already happens at a dose of 1500 mg/kgbw. Caspase-8, is an active form from procaspase-8. Activation of procaspase-8 into caspase-8 occurs through extrinsic pathway involving *Death receptor* transduction signal. The *Death receptor* will bind to appropriate ligands to form a death domain which has a significant role in transmitting death signal from cell surface to intracellular. *Death domains* to have been identified included FasL/FasR, TNF- α /TNFR1, Apo3L/DR3, Apo2L/DR4 and Apo2L / DR5^{40,42,43}.

Activation of procaspase-8 into caspase-8 in this study happened because ethanol extract of *Centella asiatica* improved the interaction between TNF- α ligand and transmembrane TNFR1 receptors. This finding was in accordance with previous study which stated that ethanol extract of *Centella asiatica* increases levels of TNF- α ^{25,27}. The increased levels of TNF- α causes the bond between TNF- α ligand to its receptors, TNFR1, to form *death domain* in cytoplasm cell. *Death domain* along with the *death effector domains* forms an adapter protein that is *TNF Receptor-Associated Death Domain* (TRADD). Part of the complex, *death effector domains*, binds to procaspase-8 to form a complex called *Death-Inducing Signaling Complex* (DISC). DISC complex makes activation of procaspase-8 become caspase-8 autolytical. When caspase-8 is activated, it will

trigger apoptosis of alveolar macrophages^{40,42}.

Mycobacterium tuberculosis has an ability to increase secretion of TNFR2 proteins that can inactivate the bond between TNF- α and TNFR2 so that TNF- α cannot bind to TNFR1 and form a *death domain*⁴³. Increased TNFR2 secretion occurs because of the induction of cytokine IL-10. Therefore, inhibition on IL-10 will decrease TNFR2 secretion. The results of path analysis showed that caspase-8 increased not only apoptosis but also Bax protein expression in intrinsic pathway. We suggest that there is a communication between extrinsic and intrinsic pathways on apoptosis.

There was a significant increase of apoptosis of alveolar macrophage in *Mycobacterium tuberculosis* - infected rat lung tissue in treatment groups compared to control group. However, the graphic between *Centella asiatica* ethanol extract doses and apoptosis of alveolar macrophage protein expression of alveolar macrophage showed an inverted U-shaped curve with highest peak in dose of 750mg/kg bw. We suggest that ethanol extract of *Centella asiatica* given at a dose of 375 mg/kgbw did not achieve a therapeutic dose required to increase apoptosis of alveolar macrophage whereas receptor desensitization or down-regulation probably already happens at a dose of 1500 mg/kgbw.

Each cell will respond to any stress or stimuli received through a variety of ways from activation of life signal to initiation of cell death. The purpose of this process is to maintain homeostasis so cells not needed will be eliminated. The mechanism depends on a variety of exogenous factors and ability of cells to cope with stress or stimulus⁴⁴. In this study, the stimulus was provided in the form of natural ingredient and this material showed an increased in apoptosis of alveolar macrophages. The results of path analysis showed that there was a strong correlation between ethanol extract of *Centella asiatica* and the increase of caspase-8 protein expression and there was also a strong correlation between the increase of caspase-8 expression and apoptosis of alveolar macrophage. This proved that the mechanism of increased apoptosis by ethanol extract of *Centella asiatica* was through the extrinsic pathway.

Mycobacterium tuberculosis death mechanism in apoptotic process is through an

increase in Nitric Oxide (NO) and through the process of phagosome fusion into phagolysosome^{39,45}. NO can kill intracellular *Mycobacterium tuberculosis* through a variety of mechanisms namely directly killing DNA of bacteria, affecting copper metabolism, virulence targets of microbacterial or serving as a second messenger. In addition, Nitric Oxide (NO) is known to have the ability to induce apoptosis in macrophages and NO production was induced by IFN- γ through the increase of expression of Nitric Oxide Synthetase 2 (NOS2)⁴⁶. Research conducted by Herbs in 2011 shows that NO induced by IFN- γ increases macrophage apoptosis infected with *Mycobacterium tuberculosis* and kills the bacteria. Previous study showed that ethanol extract of *Centella asiatica* increased IFN- γ production so that ethanol extract of *Centella asiatica* which, in turn, also increases NO production is alleged to cause death mechanism of intracellular *Mycobacterium tuberculosis*⁴⁶. Apoptotic process is suspected to have links with architectural change of host vesicular system causing fusion between phagosome and lysosome previously inhibited by *Mycobacterium tuberculosis*⁴⁷.

Conclusions

The present research clearly demonstrated that the ethanol extract of *Centella asiatica* have active ingredients to enhance of apoptosis alveolar macrophages from rat lung tissue that were infected by *Mycobacterium tuberculosis* through increasing of Caspase 8's expression, Bax'expression and decreasing of Bcl-2's expression.

Acknowledgements

We thank the Directorate of Research and Community Services, the Directorate General of Higher Education, and Ministry of Education and Culture in Indonesia for the financial support.

Declaration of Interest

The authors report no conflict of interest.

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