***Original article***

# *Centella asiatica* decrease bax expression In prefrontal cortex of rat models

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**Abstract**

***Introduction**: Centella asiatica* is a herb, which has neuroprotective properties, thus, it can be used to prevent neural damage caused by stress and increased glucocorticoid level. *Madecassoside, asiaticoside, asiatic acid,* and *madecassic acid* that are contained inside *Centellaasiatica* extract have antioxidant and neuroprotective properties. ***Method:*** This research is an experimental research using an animal model with post test-only control group design. We used samples from biologically stored product, in the form of 25 rat-brain tissue paraffin blocks. These brain tissues originated from 25 rat models that were divided into groups P1, P2, P3, P4, and P5. Group P1 was intervened with restrain stress.Group P2, P3, and P4 was intervened with restrain stress and ethanolic extract of *Centella asiatica*at 150, 300, and 600 mg/kgBW/days, respectively. Group P5 was intervened with stress and fluoxetinat 10 mg/kgBW/days. On day 23, brain tissues were terminated, perfused, and extracted. Brain tissues were then made into paraffin blocks, turned into preparation pieces, histochemically stained, and cells that expressed caspace-bax were counted. ***Results**:* There was a statistically significant difference of Bax expression between P1 group and P3, P4, and P5 (p=0,01). Bax expression in group P1 was significantly higher than P2, P3, P4, and P5. There was no statistically significant difference of Bax expression between group P1 and P2 (p=0,08). ***Conclusion**:* Ethanolic extract of *Centella asiatica* was able to decrease Bax expression in prefrontal cortex of chronic restraint stress induced *Spraque Dawley* rat models.

**Keyword:** *Centella asiatica;* prefrontal cortex; bax; *chronic restraint stress.*

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## Introduction

*Centella asiatica* is an herb that has neuroprotective properties, thus it can be used to prevent neural damage caused by stress and increased glucocorticoid level. *Madecassoside, asiaticoside, asiatic acid,* and *madecassic acid* contained inside *Centella asiatica* extract have antioxidant and neuroprotective properties[1]. *Centella asiatica* extract that were given to rat models in the dosage of 150 and 300 mg/kgBW was proven to improve memory, inhibit oxidative stress by decreasing *malondialdehyde* (MDA) and nitrate concentration as well as increasing antioxidant activities such as gluthatione-s-transferase*, superoxide dismutase,* and catalase[2]. *Centella asiatica* is also able to enhance dendrite length and branch of the amygdala neurons[3]. *Centella asiatica* can increase neuron proliferation in the CA3 and CA4 region of the hippocampus[4].

The medial prefrontal cortex is an area of the human brain that is importantfor cognitive integration, emotion, and concentration focus of received information. It has a high number of glucocorticoid receptors, thus it is influenced by stress. *Repeated stress* caused 16% decrease of *apical dendritic spine density* in the neurons of the medial prefrontal cortex. Repeated stress is also thought to cause a one third loss of total axons and apical dendrite synapse of pyramidal neurons in the medial prefrontal cortex. This condition may also be due to neuroplasticity failure caused by stress exposure[5].

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*Single-prolonged stress* (SPS) caused an increase of Bax expression, decrease of Bcl-2 expression, and increase of Bax/Bcl-2 ration in Wistar rat amygdala. SPS increased the number of apoptosis in amygdala cells analyzed with flow cytometry. The morphology of apoptotic cells could also be seen in amygdala in which there were chromatin condensation, chromatin crescents, nucleus fragmentation, andnucleolus disappearance. SPS increased the number of cells expressing positive TUNEL in the nucleus[6].Stress increased synaptosomal polysialic neural cell adhesion molecule (PSA-NCAM) in prefrontal cortex[7].

The objective of this research is to determine the antiapoptotic effect of *Centella asiatica* extract through decreased bax expression in *Sprague dawley* rat prefrontal cortex intervened with chronic restraint stress.

**Materials and Methods**

This research is an experimental research, which uses animal model with post test only control group design. It was conducted in the pathology anatomy laboratory of RSUP DR. Sardjito Yogyakarta and in the anatomy laboratory of Universitas Islam Indonesia between February and August 2015. This research has gotten ethical clearance from Ethics Committee of Medical and Health Research Faculty of Medicine Universitas Islam Indonesia number 03/ Ketua/70/KEFKUII/III/2015.

This research used samples from biologically stored product, in the form of 25 rat-brain tissue paraffin blocks. These brain tissues originated from 25 rat models that were divided into groups P1, P2, P3, P4, and P5.Group P1 was intervened with restrain stress. Group P2 was intervened with stress and ethanolic extract of *Centella asiatica* at 150 mg/ kgBW/days. Group P3 was intervened with stress and ethanolic extract of *Centellaasiatica* at 300 mg/ kgBW/days. Group P4 was intervened with stress and ethanolic extract of *Centella asiatica* at 600 mg/ kgBW/days. Finally,group P5 was intervened with stress and fluoxetinat 10 mg/kgBW/days. Stress was given as restraint stress, in which1 rat was placed inside a transparent acrylic tube (15 x 5.5 cm) for 6 hours every day overa period of 21 days. This intervention prevented the rat from moving freely, thus causing psychological stress. The ethanolic extract of *Centella asiatica* and fluoxetin was given 30 minutes before stress-induction begun.On day 23, termination, perfusion, and extraction of brain tissues was undertaken. Following this, the brain tissues were made into paraffin blocks.

*Centella asiatica* was obtained from Merapi Farma Herbal (commercial herbs producer) in the form of simplicia. Obtained *Centella asiatica* was identified and determined in Laboratorium Sistematik Tumbuhan Fakultas Biologi UGM with No. 0354/S. Tb/VI/2012.

*Centella asiatica* extract was non-specifically standardised by measuring water content and specifically standardised by identifying extract active ingredient with asiaticoside marker using thin layer chromatography (TLC). Obtained *Centella asiatica* extract contained 4,16±0,51% asiaticoside.

*Centella asiatica* extract was made into three types of concentration, which was 30 mg/ml, 60 mg/ ml, and 120 mg/ml, respectively. The administered *C.asiatica* dosage was150 mg/kgBW,300 mg/kg BW, and 600 mg/kg BW, respectively. *Centella asiatica* extract in the concentration of 30 mg/ml was used for 150 mg/kg BW dosage, concentration 60 mg/ml was used for 300 mg/kg BW dosage, and concentration 120 mg/ ml was used for 600 mg/kg BW dosage. The dilution of extract was undertaken in the Pharmacology Laboratory of Faculty of Medicine Gadjah Mada University every 3 days. The diluted extract was stored in a refrigerator. To determine the volume of *Centella asiatica* extract used for 1 rat, dosing formula was:

Volume = dosage (mg/kg BW) X body weight (kg)/ concentration (mg/ml).

Paraffin blocks of rat brain tissues were dissected to 4 μm thickness. Dissection was undertaken in the prelimbic, infralimbic, and cortex cynguli areas, which was in Bregma 3,72 mm to 2,52 mm according to Paxinos& Watson atlas (Paxinos and Watson, 2007).One dissection of one paraffin block of rat brain tissue was taken as a sample.

Immunohistochemistry (IHC) staining using bax antibody (santacruz) was conducted in the Pathology Anatomy laboratory of RSUP DR. Sardjito Yogyakarta. Initial staining was done by deparaffinization using xylol and alcohol with decreased concentration. Tissues were then incubated with $H_2O_2$ 3% in 10% methanol for 20 minutes. They were subsequently washed with aquadest 3 times followed byphosphate buffer saline (PBS) 3 times. The next process was antigen retrievalusing pH 6 citrate buffer inside the microwave. For approximately 10 minutes, the section was heated in a high temperature *(*$100^0$C) environment, continued with med-low temperature heating for 20 minutes. It was subsequently re-chilled and rewashed using PBS three more times.

Furthermore, the section was blocked with protein background snipper for 10 minutes. Without rewashing the tissues, they were added to bax primer antibody (Ab) and incubated overnight in $4^0$C temperature. Following this, they were washed in PBS three times and incubated with Trekkie Universal Link for 10 minutes. Washed with PBS three times and continued with incubation using horseradish peroxidase conjugated Streptavidin (SA-HRP complex) for 10 minutes. Finally, it was rewashed with PBS three more times.

The identification of pyramidal cell labelled bax was undertaken using 3,3'-diaminobenzidin ( 1 : 100) for 5 minutes. The tissues were then washed with aquadest 5 times, continued with counterstaining using Mayer's haematoxylin for 1 minute, then washed with running water for 2 minutes. They were then dehydrated with ethanol at 70%, 80%, 90%, 95%, and 100%, respectively, for 1 minute each and cleaned withxylene and cover slipped with Canada balsam.

The observation of preparatory was undertaken in the Anatomy laboratory of FK UII. Preparatory was observed using Olympus CX 22 microscope, which was connected with an optilab camera. Counting of bax expressing cells were conducted on the medial part of prefrontal cortex, including the prelimbic, infralimbic, andcingulate cortex areas. Preparate was seen using light microscope in 400 magnifications. Cell count was done in 10 field of view. The percentage of bax expressing cells was the amount of bax expressing cells compared to total cell count times 100%.

**Ethical clearance**: This research proposal was accepted by the Ethics Committee of Faculty of Medicine Universitas Islam Indonesia.

**Result**

Data gained was in the form of cell percentage, analysedusing *one way Anova* to compare the mean of bax expressing cells in the neurons of each group. After that, *post-hoc test* analysis was conductedto determine the difference between groups.

In P1 group, the average amount of bax expressing cells was 72,54%. Bax expression in this group was higher than other groups, which were intervened with restraint stress and treated with *centella asiatica* orfluoxetin (P2, P3, P4, and P5 group). The presentationof bax expressing cellsand the results of cell count canbe seen in Table 1. Statistical analysis using one way ANOVA showed statistically significant differences (p = 0,01). Results of *post hoc* analysis showed significant differences between group P1 andP3, P4, and P5, respectively. There were no statistically significant differences between groups P3, P4, and P5. Bax expression in group P1 and P2 were also not significant different (p= 0,08). Lowest bax expression was found in group P5, which was intervened with stress and fluoxetine treatment.

**Table 1. Mean of bax expression in prefrontal cortex**

| | Intervention group | | | | | P |
|---|---|---|---|---|---|---|
| | P1 (n=5) | P2 (n=5) | P3 (n=5) | P4 (n=5) | P5 (n=5) | |
| Bax Expression | 72,54 | 44,60 | 28,11 | 28,97 | 7,76 | 0.010 |

P1: stress; P2: stress + *Centella asiatica* extract 150 mg/kgBW/day; P3:stress + *Centella asiatica* extract 300 mg/kgBW/day;P4: stress + *Centella asiatica* extract 600 mg/kgBW/day; P5: stress + fluoxetin 10 mg/kgBW/day.

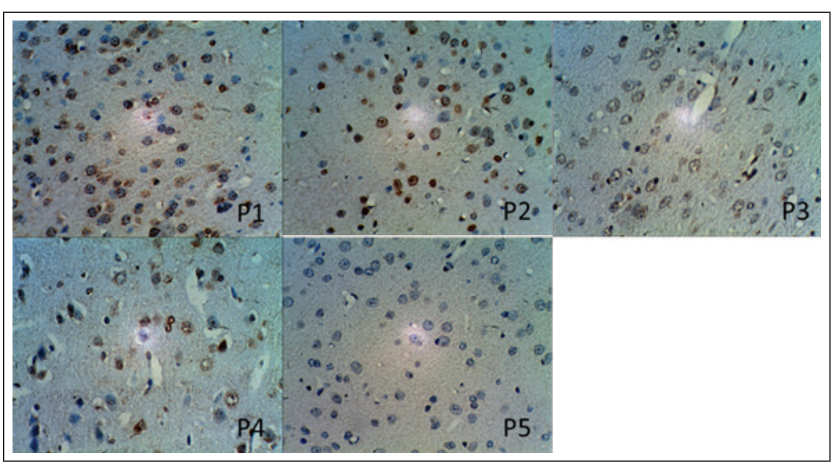


Figure 1. Bax expression in group P1, P2, P3, P4, and P5

**Discussion**

In this research, bax expression was found to be higher in stress group (P1) compared to groups which were intervened with *Centella asiatica* extract (group P3 and P4). This result indicated that the administration of *Centella asiatica* ethanolic extract in the dosage of300 and 600 mg/kgBW/day for 21 dayscoulddecrease bax expression in the prefrontal cortex, thus decreasing the amount of cells undergoing apoptosis. Bax is a proapoptotic protein that contributes in the intrinsic apoptosis pathway. The increase of bax expression and/or the decrease of antiapoptosis protein Bcl-2 can disturb the bax and Bcl-2 balance, causing bax to activate and form homo-oligomeron the outer membrane of mitochondria and increase mitochondria membrane permeability. Thus, cytocrome C would detach from mitochondria intermembrane space to the cytoplasm and induce apoptosis[8].

The result of this research is in line with previous research by Priyantiningrum, which showed that the administration of *Centella asiatica* ethanolic extract in the dosage of 300 mg/kgBW/days for 21 days in rat models induced with chronic stress, were able to increase the number of neurons in the prefrontal cortex[9]. This increase is probably due to *Centella asiatica* capability of inhibiting apoptosis. Previous research has supported this theory, in which the administration of *Centella asiatica* ethanolic extract in the dosage of 150, 300 and 600 mg/kgBW/days were able to increase the expression of anti-apoptosis Bcl-2 protein and inhibit the expression of active caspase-3 in the prefrontal cortex of rat models induced with chronic restraint stress for 21 days[10].

The result of this research could enhance our understanding about the neuroprotective effect of *Centella asiatica.* Many other studies have also shown the neuroprotective effect of *Centella asiatica* and its active ingredients. Treatment using *Centella asiatica* extract in the dose of 500 mg/kg BW in rats who were induced with 6 hours of restraint stress per week for 6 weeks can increase the number of branch of apical and basal dendrites as well as elongate apical and basal dendrites in the CA3 area of the hippocampus. These effects can happen through various mechanism, for instance, *Centella asiatica* contains neurostimulant and nerve growth factor (NGF), which stimulates new dendrite formation. In addition, it induces neurogenesis in the hippocampus, and has neuroprotection and antioxidant properties[11]. The administration of *Centella asiatica* lowers the concentration of calcium and intracellular ROS in response to β- amyloid of SH-SY5Y and MC65 cell cultures**,** induces the expression of antioxidantresponse genes NFE2L2, and inhibits the decrease of mitochondrial DNA expression. The exposure of β-amyloid in SH-SY5Y cell cultures will decrease the level of adenosine tri phosphate (ATP). *Centella asiatica* prevents the decrease of ATP, increases basal oxygen consumption, inhibits mitochondria respiration reduction, and inhibits metabolic rate deceleration. *Caffeoylquinic acids* contained in *Centella asiatica* showed effect towards antioxidant gene and mitochondrial gene expression in the same neuroblastoma cells[12].

Asiatic acid in the dosage of 75 mg/kg, given intravenously at the 6th, 9$^{th}$, and 12th hour after ischaemia, can decrease brain infarct volume. When given in the 6th and 9th hour post-ischaemia, asiatic acid decrease infarct volume was 53,1% and 52,3%. Asiatic acid significantly reduce brain infarct volume until 12 hours post-ischaemia. Asiatic acid therapy reduced motoric and sensoric deficits as well as restoring reflex on days 1, 3, 7, and 14 after ischaemia.Asiatic acid can maintain the integrity of blood brain barrier by inhibiting the activity of MMP-9 in the brain[13]. Asiatic acid in the dosage of 75 mg/kg, given intravenously 3 hours after middle carotid artery occlusion (MCAO), significantly reduced infarct volume and improved neurological deficits. The combination of asiatic acid and low dose tissue plasminogen activator (t-PA) can decrease infarct volume and improve neurological deficit compared to placebo and low dose t-PA (2.5 mg/kg). Ischaemia caused mitochondrial dysfunction and triggered apoptosis of the neuron. Ischaemia can also cause a decrease in the level of cytochrome Cand AIF (*apoptosis inducing factor*) inside mitochondria. Asiatic acid inhibits the detachment of cytochrome C as much as 36% and AIF as much as 30% from the mitochondria. Cytochrome C and AIF triggers apoptosis through caspase dependent and caspase independent pathways. In the caspase dependent pathway, the detachment of cytochrome C would activate caspase cascade thus causing DNA damage and cell death. In the caspase independent pathway, apoptosis occurred through calpain or poly (ADP-ribose) polymerase-1, which would induce the release of AIF from the mitochondria[14].

The administration of madecassoside, isolated from Centella asiatica, in the dosage of 6, 12 and 24 mg/kg BW i.v, in SD rat models intervened with ischemic reperfusion injuryin the cerebral neuron, showed decreased neurological deficits and infarct volume;

madecassoside protects the brain from damage due to ischemic reperfusion injury. Cell depiction with haematoxylin eosin (HE) staining was relatively clean, the cell structure looked intact,and there was minimal cell swelling and interstitial oedema as well as decreased necrotic cells. Madecassoside could decrease the number of cells undergoing apoptosis as seen in TUNEL assay. Madecassoside can inhibit oxidative stress in the neuron by decreasing free radicals like malondialdehyde (MDA), increasing the activity of superoxide dismutase (SOD) and serum level of gluthation(GSH). Madecassoside has anti-inflammatory effects by increasing the expression of interleukin-1β, interleukin-6, and tumour necrosis factor-α. In addition, madecassoside decreased the expression of p65 subunit of NF-κβ in infarct area. NF-κβ is an inflammation and apoptosis mediator, which regulates the expression of pro- and anti-oxidant enzymes. The activation of NF-κβ can be triggered by TNF-α and IL-1β as well as increase reactive oxygen species (ROS). NF-κβ contributes tooxidative stress and post ischemic reperfusion injury neuron inflammation mechanism[15].

In this study, groups that received fluoxetin 10 mg/kg BW/day (group P5) showed lowest bax expression. Fluoxetin is a *selective serotonin reuptake inhibitor* (SSRI) antidepressant drug that has neuroprotective effects. The administration of 12,5 mg/kg BW fluoxetinin rat models increased the concentration of brain derived neurotropic factor (BDNF) in the prefrontal cortex and striatum. The combination of fluoxetin and olanzapine increased the expression of BDNFin the prefrontal cortex, hippocampus, and striatum. Fluoxetin treatment in the dosage of 12,5 and 25 mg/kg BW increased the level of protein kinase B in the prefrontal cortex of adult Wistar rat models. A combination of olanzapine 6 mg/kg BW and fluoxetin 25 mg/kgBW increases the level of protein kinase B in the prefrontal cortex of adult Wistar rat models. In the hippocampus and striatum, the combination of olanzapine 3mg/kg BW or 6 mg/kg BW and fluoxetin 12,5 or 25 mg/kg BW increases the level of protein kinase B. The administration of olanzapine, fluoxetine, and the combination of both, increase the level of cAMP response element binding (CREB) in the prefrontal cortex, hippocampus, and striatum. CREB and BDNF is a neurotropic protein that controls the expression of proapoptosis and antiapoptosis protein[16].

The expression of Bcl-2 protein in the prefrontal cortex increased in the administration of olanzapine 3 mg/kg BW and 6 mg/kg BW. The administration of fluoxetin alone did not increase Bcl-2 expression in the prefrontal cortex. The combination of olanzapine 3 mg/kg BW or 6 mg/kg BW and fluoxetin 12,5 mg/kg BW or 25 mg/kg BW increased the expression of Bcl-2in the prefrontal cortex. In the hippocampus, the administration of olanzapine, fluoxetin, and their combination, increase the expression of Bcl-2. In the striatum, olanzapine 6 mg/kg BW and fluoxetin 12,5 mg/kg BW as well as their combination, increased the expression of Bcl-2. The expression of Bcl-2 associated death promoter (BAD) protein in the prefrontal cortex increased with the administration of olanzapine 3 mg/kg BW. The combination of olanzapine 3 mg/kg BW and fluoxetin 12,5 or25 mg/kg BW increased the expression of BAD. In the hippocampus, the expression of BAD protein increased with the administration of olanzapine or fluoxetin alone, as well as the combination of both. In the striatum, the administration of olanzapine 3 mg/kg BW did not affect BAD expression. In the dosage of6 mg/kg BW, olanzapine decreases the expression of BAD. Fluoxetin in the dosage of 12,5 and 25 mg/kg BW as well as its combination with olanzapine 6 mg/kg BW did not affect the expression of BAD. The combination of olanzapine 3 mg/kg BW and fluoxetin 12,5 or 25 mg/kg BW increased the expression of BAD[16].

## Conclusion

According to this study, it can be concluded that the ethanolic extract of *Centella asiatica* decreases the expression of bax in the prefrontal cortex of *Sprague Dawley* rat models induced with chronic restraint stress.

## Recommendation

To improve study results, the amount of preparate section should be increased to 6-10 sections for 1 research subject, so that more samples canbe obtained. Observations should be conducted in all fields of view to enhance view area. In addition, further research needs to be undertaken to determine the number of cells undergoing apoptosis using various examinations like the *TUNEL marker.*

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**Conflict of interest:** None declared.

**Author's Contributions**:

Data gathering and idea owner of this study: Kuswati and Nanang Wiyono ( BK )

Study design: Kuswati

Data gathering: Kuswati

Data analysis and consultation: Kuswati

Writing and submitting manuscript: Syaefudin Ali Akhmad

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