Ethanol Extracts of *Centella asiatica* (L.) Urb. Leaf Increase Superoxide Dismutase-2 (SOD-2) Expression on Rat Cerebellar Purkinje Cells After Chronic Stress

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ABSTRAK

Beberapa penelitian menunjukkan bahwa stres kronis meningkatkan produksi *reactive oxygen species* (ROS), dan / atau menekan mekanisme pertahanan antioksidan. Efek neuroprotektif dari *Centella asiatica* (L.). Urb telah dilaporkan dapat melindungi neuron dari kerusakan oksidatif. Tujuan dari penelitian ini adalah untuk mengetahui efek ekstrak etanol *C. asiatica leaf* terhadap ekspresi superoxide dismutase-2 sel (SOD- 2) pada sel Purkinje serebelum tikus setelah diberikan kejutan kronis di kaki. Sebanyak 25 tikus Sprague Dawley jantan dewasa muda diacak ke dalam lima kelompok. Kelompok kontrol negatif terdiri dari tikus yang tidak stres; kelompok kontrol stres menerima aquadest; dan kelompok lain diobati dengan dosis yang berbeda (mg / kg berat badan / hari, p.o.) ekstrak etanol daun C. asiatica: 150, 300 dan 600, masing-masing diikuti oleh pemberian kejutan kronis di kaki selama dua puluh delapan hari. Ekspresi SOD-2 dari lapisan sel Purkinje diukur menggunakan metode imunohistokimia. Data dianalisis dengan one-way ANOVA ($p < 0,05$). Kami menemukan bahwa ekspresi SOD-2 (%) dari lapisan sel Purkinje untuk kelompok kontrol negatif, kelompok kontrol stres, CeA150, CeA300 dan CeA600 kelompok adalah $22,38 \pm 9,73$, $9,81 \pm 2,21$, $10,29 \pm 3,60$, $14,72 \pm 6,65$, dan $22,75 \pm 10,93$, masing-masing ($p < 0,05$). Analisis post-hoc menunjukkan perbedaan yang signifikan antara kelompok kontrol negatif dan kelompok kontrol stres ($p < 0,05$). Ada juga perbedaan yang signifikan antara kelompok kontrol stres dan kelompok CeA600 ($p < 0,05$), tetapi tidak ada perbedaan yang signifikan antara kelompok perlakuan ($p > 0,05$). Penelitian ini menunjukkan bahwa ekstrak etanol daun C. asiatica meningkatkan ekspresi SOD-2 pada sel Purkinje cerebellar tikus setelah stres kronis.

Keywords: *Centellaasiatica*,Cerebellum, Chronic Stress, SOD-2

Centella asiatica (L.) Urb. or 'Pegagan' is a tropical medicinal herb plant flourishing extensively in swampy areas of Asian countries (Figure 1). The primary active constituents of *C. asiatica* that have important roles in medicinal application are triterpenoids and flavonoids.[1] These triterpenesaponosides, which include asiatic acid and asiaticoside, exert a significant neuroprotective effect on rat brain against oxidative damage.[2] It is believed to be due to its high antioxidant activity and mediated by decreasing blood-brain barrier permeability.[3] In addition to neuroprotective effect of *C. asiatica,* it claimed to possess a wide range of pharmacological effects for human health such as wound healing, anti-inflammatory, antipsoriatic, antiulcer, hepatoprotective, anticonvulsant, sedative, immunostimulant, cardioprotective, antidiabetic, cytotoxic and antitumor, antiviral, antibacterial, insecticidal, antifungal, antioxidant, and for lepraand venous deficiency treatments.[4]

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Systematic classification (Taxonomy)

Kingdom	*Eukaryota*
Subkingdom	*Embryophyta*
Division	*Spermatophyta*
Subdivision	*Angiospermae*
Class	*Dicotyledoneae*
Subclass	*Rosidae*
Superorder	*Aralianae*
Order	*Araliales (Umbelliflorae)*
Family	*Apiaceae*or*Umbelliferae*
Subfamily	*Hydrocotyle*
Genus	*Centella*
Species	*Centella asiatica*

Figure 1. Morphological features of *Centella asiatica* (L.).Urb. A. Leaves; B. Stem; C. Flower[2]

The brain is the central organ for stress adaptation, and is also a target of stress. Chronic stress may result in abnormal changes in brain plasticity; including dendritic retraction, neuronal toxicity, and suppression of neurogenesis and axospinous synaptic plasticity.[5] Repetitive stress exposure will gradually change the electrical characteristic, morphology and proliferative capacity of neurons.[6] Several studies on animals have now examined the involvement of oxidative status in the adaptive response to stress. Physical or psychological stressors cause oxidative damage by inducing an imbalance between the pro-oxidant and antioxidant status.[7]

Among the brain region, the cerebellum is known to be severely affected by oxidative damage associated with glucocorticoid levels. Several reasons might contribute to explain its high vulnerability: a large quantity of oxidizable lipids; a high iron content; and a relatively low level of antioxidant defense molecules, mainly glutathione and vitamin E[7]. Chronic stress has been shown to increase production of reactive oxygen species (ROS), and suppression of antioxidant defense mechanisms including superoxide dismutase-2 (SOD-2).A robust increase in basal cerebellar oxidative stress causes abnormal changes such as poor dendritic arborization of Purkinje cells, alteration of cellular organelles and even neuronal death.[8,9]In the present study we have investigated the neuroprotective effect of *C. asiatica*, as a potent antioxidant, on rat cerebellar Purkinje cell SOD-2 expression following chronic foot shock stress.

METHODS

Animals

Twenty-five young-adult (8 weeks old) male Sprague Dawley rats weighing 100-150 g were randomly assigned into five experimental groups with five rats per group. Negative control group consisted of non-stressed rats; stress control group received aquadest; and other groups were treated with different doses (mg/kg body weight/day,p.o.) of *C. asiatica* leaf ethanol extracts: 150 (CeA150), 300 (CeA300) and 600 (CeA600), respectively followed by chronic foot shock stress for twenty-eight days.Two animals were placed in the same house with food and water available *ad libitum* and maintained on a standard laboratory condition: 25-30^{o}C; 50%-60% humidity; 12 hr. light and 12 hr. dark cycles. All experimental procedures described were approved by the Medical and Health Research Ethics Committee, Faculty of Medicine, Gadjah Mada University (Ref. KE/FK/657/EC).

Administration of Ethanol Extracts of *Centellaasiatica* Leaf

Ethanol extracts of *C. asiatica* were obtained using maceration methods from Integrated Testing and Research Institute, Gadjah Mada University. The fresh leaves were cleaned, air-dried, and made into powder. The powder was soaked in 70% ethanol solution in a shaking incubator for 2 days. The extracted solution was filtered through filter paper and concentrated. The concentrate was evaporated

through a vacuum rotary evaporator at 70°C followed by a water bath. The filtrate from this process was weighed and dissolved in sterile aquadest to make the various dose-dependent preparations (150, 300 and 600 mg/kgbody weight/ day). Ethanol extracts of *C. asiatica* leaf were administrated orally using oral gastric tube for 28 consecutive days with weekly weight-adjusted dose.

Stress Procedure

Thirty minutes after the oral administration of *C. asiatica*, each rat was given foot shock stress. The plexiglas rodent shock box (TW-0313) consisted of a box containing an animal space positioned on a metallic grid floor connected to a shock generator. Test rats were placed in a box, and received 10 min/ day inescapable foot shock stress (0.8 mA/50V electrical foot shock in intensity and 10 s in duration with 15 s interval). Foot shock stress was given chronically for 28 consecutive days.

Measurement of Superoxide Dismutase-2 (SOD-2) Expression

On completion of the experimental period, the animals were sacrificed by decapitation. Brain tissue was dissected immediately over an ice pack, and cerebellar tissue was separated manually from other brain region. A piece of cerebellar hemisphere tissue was processed to get the section in coronal plane, and the paraffin embedding was done for block formation. Five µm thick sections were cut, stained with a rabbit-anti-SOD-2 polyclonal antibody (Bioss®; Cat. No. bs-1080R). The SOD-2 expression from Purkinje cells layer was measured using the immunohistochemistry method.Numbers of both negative and positive cells were counted manually in each image under light microscope with a 40x magnification level. For further calculation, the resulting data were calculated as follows: % positive cells = positive cells nucleiD total cells nuclei x 100%. The index was expressed as a percentage of positive cells per total number of cells.

Statistical Analysis

Data were presented as mean ± standard deviation. Differences among groups were calculated by the parametric one-way ANOVA test using the SPSS v 16.0 software. Post hoc analysis was done when it was needed. P values less than 0.05 were considered statistically significant.

RESULTS

Mean SOD-2 expression (%) of Purkinje cells layer for the negative control, stress control, CeA150, CeA300 and CeA600 groups were 22,38±9,73, 9,81±2,21, 10,29±3,60, 14,72±6,65, and 22,75±10,93, respectively ($p<0,05$). Post-hoc analysis showed a significant difference between negative control and stress control group ($p<0,05$). There was also a significant difference between stress control and CeA600 group ($p< 0,05$), but there was no significant difference among the treatment groups ($p> 0,05$) (Figure 2).

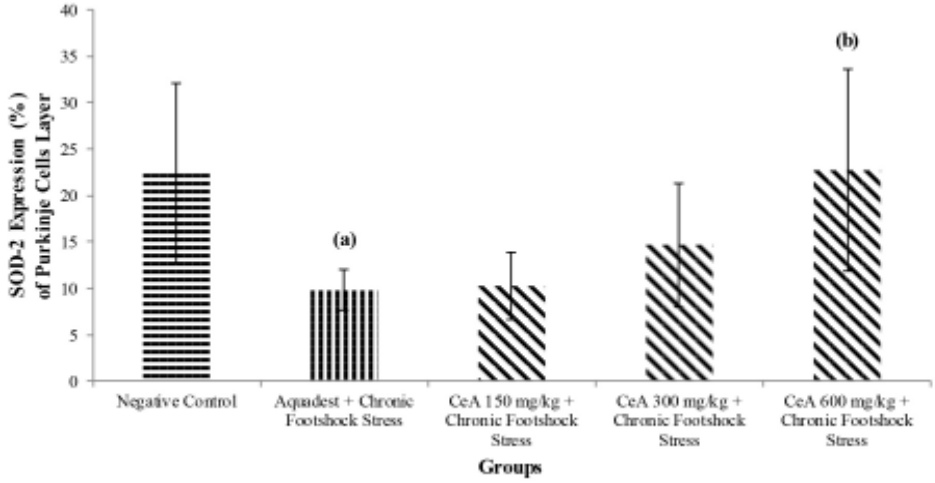

Figure 2. Rats SOD-2 expression of Purkinje cells layer after the administration of *C. asiatica* followed by chronic footshock stress. (a) $p<0,05$ v.s. negative control group; (b) $p<0,05$ v.s. stress control group.

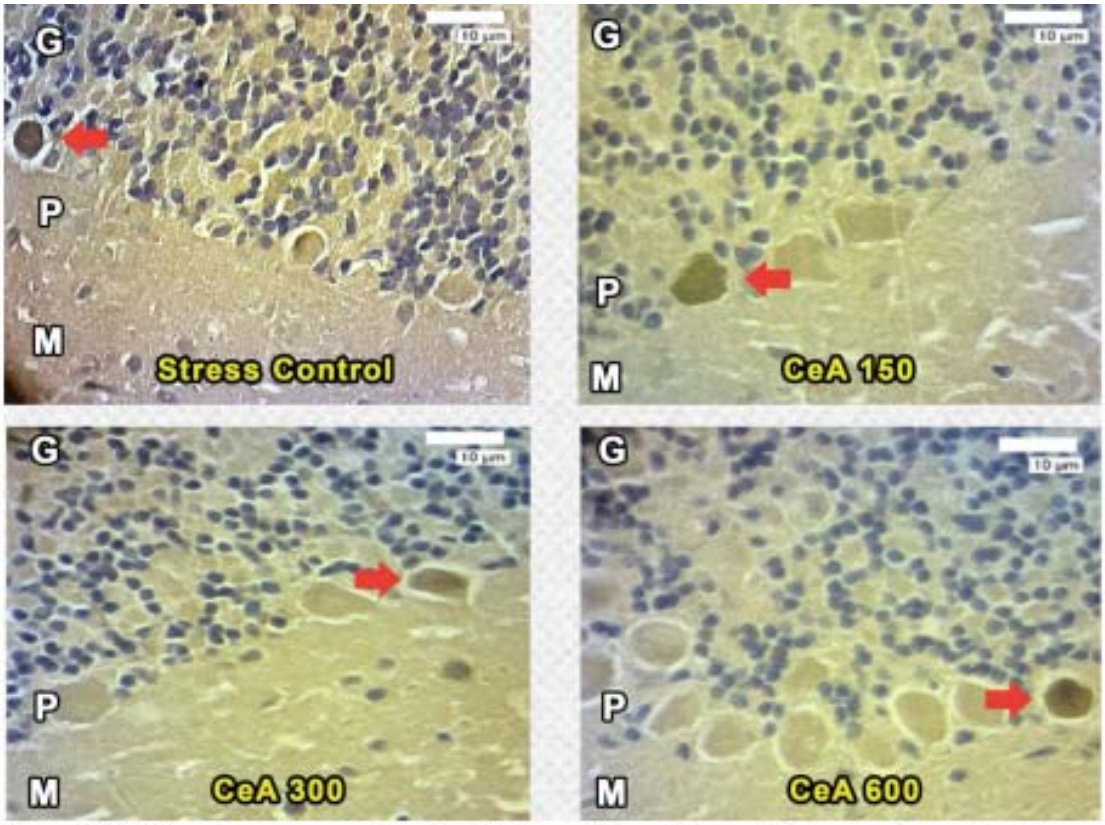

Figure 3.SOD-2 protein is expressed in the Purkinje cell layer of the cerebellar cortex hemisphere. Tissues were stained with rabbit anti-SOD 2 polyclonal antibody (Bioss®; No. Cat. Bs-1080R), and observed under a light microscope with a 40x magnification level. (G) granular cell layer; (P) Purkinje cell layer; (M) molecular cell layer; Red mark displayed SOD 2 expression.

DISCUSSION

Previous studies have reported that various stress models are associated with enhance free radical generation and altered antioxidant enzyme activities. Stress response may result in creation of ROS, such as hydrogen peroxide (H_2O_2), hydroxylradical ($HO^{\bullet}$) and superoxide anion radical ($O_2^{\bullet-}$) that cause lipid peroxidation in membranes andcan play an important role in tissue injury.[10,11] The membrane injury causes disruption of the tissue integrity and enhance cell death induced by oxidative stressors. Several studies on animals have now examined that foot shock stress is an easy method to induce both psychological and physical stress resulting in fear and aggression.[11]This study suggests that the increased level of glucocorticoids during stress induced an imbalance between the pro-oxidant and antioxidant status and may affect the whole antioxidant capacity of the brain, especially superoxide dismutase (SOD-2).SOD-2 represents the front line of defense against oxidative damage. Their rolesas protective enzymes are well known and have been investigated extensively in animal model systems.

Brain is the target for different stressors because of its high sensitivity to stress-induced degenerative conditions.[5,6] It is believed to be due to the large amounts of polyunsaturated fatty acids, which are particularly vulnerable to freer adical attacks. ROS damage proteins, nucleic acids and lipid membranes, thereby disrupting cellular functions and integrity. The cerebellum is known to be severely affected by oxidative stress because its high levels of glucocorticoid receptors localized in the external granular layer.[9] The decline of antioxidant enzyme (SOD-2) expressions on stress control group compared with non-stressed group suggested that chronic foot shock stress decreased SOD-2 production on rat cerebellar Purkinje cells (Table 1). The decreased SOD-2 expressions may be explained

by two mechanisms. The first mechanism is the possible impairing effect of glucocorticoid on brain antioxidant capacity. Glucocorticoid enhanced cerebellar neurotoxicity of ROS and decreased the expression of SOD-2.[12] The second mechanism isincreasing of intracellular calcium ion concentration (Ca^{2+}) during oxidative stress in the neuronal cells. This increased intracellular Ca^{2+}induce the irreversible conversion of xanthine dehydrogenase to xanthine oxidase, which in turn catalyzes the oxidation of xanthine to provide a source of $O_2^{\bullet}$".[13] Inaddition, auto-oxidation of dopamine in brain could also serve as a source of superoxide anion. These mechanisms could be the chief possibilities for the reduction in the expression of SOD-2 in stressed rats leading to an overload of oxygen radicals.

This study showed that ethanol extracts of *C. asiatica* leaf increased SOD-2 expression on rat cerebellar Purkinje cells after chronic foot shock stress.It is believed to be due to the antioxidant present that may have different functional properties, such as ROS scavenging (quercetin and catechins), inhibition of the generation of free radical sand chain-breaking activity (coumaricacids).[14] SOD-2 by catalyzing the removal of superoxide radical protects biological membranes and associated structures from free radicals.[14] Phenolic compounds of *C. asiatica* (tocopherols, flavonoids, cinnamic acid, and phosphatidic) are an effective hydrogen donating radical scavengers by scavenging lipid alkoxyl andperoxyl radical.[14]Previous studies have reported thatphenolic compound of *C. asiatica* also exhibit another antioxidant activity, such ascatalase, glutathione peroxidase, vitamin C and vitamin E.[15,16]Although the mechanism were still unclear, we have added biochemical evidence that *C. asiatica* has a role as a neuroprotective agent in the brain, mainly in cerebellum.The consumption of *C. asiatica* was useful to protect the cells from oxidative damage, to destroy excess free radicals, and keep the oxidative stress state in balance.[2] These effects may be attributed to the anti oxidant compounds present in *C. asiatica,* that are known to be potent anti oxidants.

CONCLUSION

Ethanol extracts of *C. asiatica* leaf increase SOD-2 expression on rat cerebellar Purkinje cells after chronic stress. Further studies on the other biological markers of oxidative stress are necessary in order to conclude a role of *C. asiatica* as a neuroprotective agent on oxidative damage.

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