Research Article

Neuroprotective effect of *Cleistocalyx nervosum* var. *paniala* extract in a rat model of ischemic stroke

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Abstract

Stroke is the most common cerebrovascular disease leading to cause of neurological damage, neurological impairment and memory deficits. Oxidative stress plays an important role in brain damage after stroke. *Cleistocalyx nervosum* var. *paniala* (*C. nervosum*), Ma-kiang, is a local plant in northern region of Thailand presenting high antioxidant activity. However, the potential of this extract as the neuroprotection has not been properly investigated. The present study using the rat model of middle cerebral artery occlusion (MCAO), it was found that *C. nervosum* extract at a dose of 500 mg/kg, administered once daily for 14 days, had markedly improved the neurological behavior performances, decreased the infarct volume, reduced neuronal damage, and improved cognitive impairment. Overall, *C. nervosum* extract supplementation may be advantageous to act as neuroprotective agent in ischemic stroke in rat.

Keywords: Stroke, antioxidant, *Cleistocalyx nervosum*, Makiang, neuroprotective effect

Introduction

Acute ischemic strokes result from vascular occlusion secondary to thromboembolic disease. Ischemia causes cell hypoxia and depletion of cellular adenosine triphosphate (ATP). Without ATP, there is no longer the energy to maintain ionic gradients across the cell membrane and cell depolarization. Influx of sodium and calcium ions and passive inflow of water into the cell lead to cytotoxic edema. [1-3] The cerebral occlusion during stroke initiates a cascade of deleterious process that contributes to brain injury. Increased generation of free oxygen radicals is one such process that leads to the activation of pro-apoptotic downstream signals. [4] Ischemia induced mitochondrial injury and subsequent activation of proapoptotic proteins in mitochondria like cytochrome c (cyt c) and apoptosis-inducing factor (AIF), may enhance neuronal apoptosis death after ischemia [4,5] Pharmacological agents with antioxidant properties may reduce brain damage during focal cerebral ischemia in mice. [6] It is possible that the anthocyanins, including cyanidine-3-glycoside, are strong antioxidant and also possess anti-inflammatory property. [7]

Cleistocalyx nervosum var. *paniala* family Myrtaceae is a native plant found in Northern Thailand, with an orange-red fruit that is commonly consumed either fresh fruit or food product. The previous studies reported that caynidin-3-glycosides were found in the ripe fruit of *C.* nervosum. [8]

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The aqueous extract of *C. nervosum* might show biphasic effect; low dose exhibited the pro-oxidant effect while high dose reduced oxidative stress in rat liver. [9] The study aimed at the evaluation of *in vitro* neuroprotective effect of *C. nervosum* extract by means of rat model of right middle cerebral artery occlusion (MCAO).

Material and Method

The fruits of *C. nervosum* were collected from Chiangmai horticulture research center, Lampang province. The pulp was manually separated, dried, ad extracted with distilled water. The ground mixture was filtrated, and concentrated under evaporator and lyophilizer to obtain the crude extract which was stored at minus 20°C for further analysis.

The experimental protocol was approved by the animal ethics committee, university of Phayao.

Male Wistar rats (weighing 180 to 220 g, national laboratory animal center, Nakornpathom province, Thailand) were used as subjects. After behavioral training, the focal cerebral ischemia was induced by right MCAO as previously described. [10]

All rats were housed in group of 5 per cage, and were randomly assigned to one of three groups (8 in each group): 1) MCAO + vehicle (distilled water), which served as vehicle to suspend the C. nervosum extract; 2) MCAO + Vitamin C 250 mg/kg; and 3) MCAO + C. nervosum 500 mg/kg. By the way, the dose of C. nervosum was selected on the dose dependent results of previous studies conducted in our laboratory. All rats were orally assigned substances via the intragastric feeding tube once daily for 14 days after MCAO. Spatial memory was assessed weekly on 7- and 14- day.

The spatial memory cognition of animals was tested in Morris water maze. [11] The measure of learning was escape latency, which was the time it took to find the platform. In addition to the acquisition test, the determination of retention memory was carried out on the next day. The time spent in the target quadrant, which had previously contained the hidden platform was recorded. The time spent in the target quadrant indicated the degree of memory consolidation taken place after learning. Any enhancement of cognition would be reflected by a decrease in escape latency and increase in retention time.

All rats were subjected to neurological evaluation by using the 6-points postural reflex test according to the method of Bederson *et al* [12] and Schmid-Elsaesser *et al*. [13] The deficit was graded from 0 to 5 as follow: grade 0 no spontaneous activity; grade 1 spontaneous circling; grade 2 circling if pulled by tail; grade 3 lowered resistance to lateral push without circling; grade 4 contra lateral forelimb flexion; grade 5 no apparent deficit.

Fourteen days after MCAO, rats were euthanized, and infract volumes were determined by 2% 2, 3, 5-triphenyltetrazolium chloride (TTC) solution in saline for 30 min at 37°C and photographed. [14] The infarct area in each slice was calculated by subtracting the normal ipsilateral area from that of the contralateral hemisphere to reduce errors due to cerebral edema and was presented as a percentage relative to the area of the contralateral hemisphere.

Coronal sections of the brains were stained, dehydrated, and manual counting neurons. Counts were made in five adjacent fields and the mean number extrapolated to give the total number of neurons per 255 μm^2.

The neuroprotective effects of *C. nervosum* were elucidated by measuring the survival of hippocampal neurons densities in all areas of hippocampus including Cornu Ammonis 1 (CA1), CA2, CA3 and dentate gyrus (DG), 14 days after ischemia.

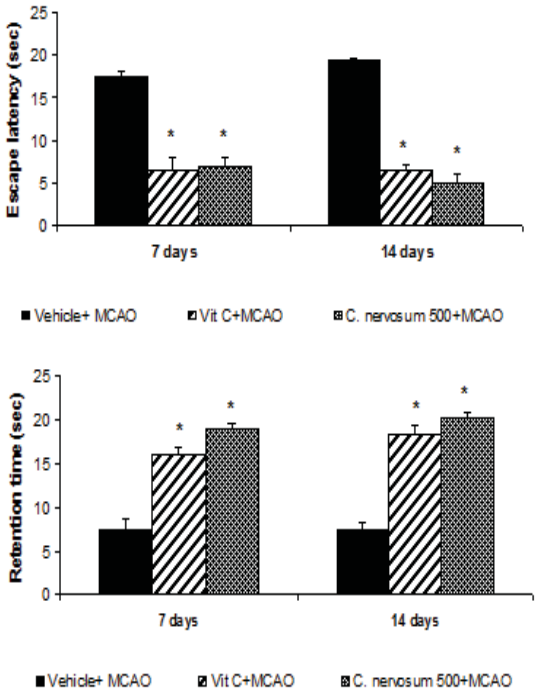
The data were expressed as mean ± standard error of mean (SEM). Statistical differences among

the experimental groups were tested using one way analysis of variance (ANOVA), followed by Dunnett's t-test. The level of statistical significance was set at $p<0.05$.

Results

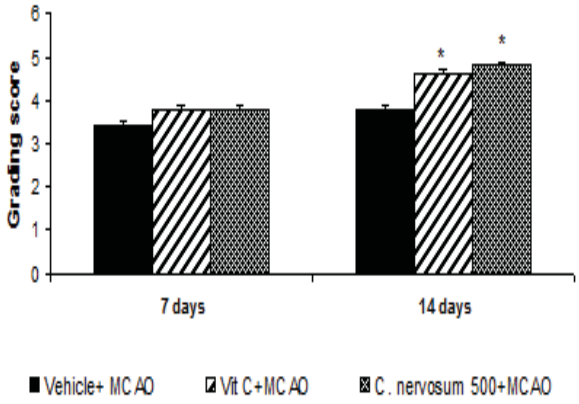
The rats of vehicle treated group exhibited markedly decreased spatial learning ability as indicated by both escape latency and retention time. In contrast, both MCAO + Vitamin C and MCAO + 500 mg/kg *C. nervosum* treated groups significantly decreased in the escape latency but increased the retention time as compared to MCAO + vehicle treated group ($p<0.05$), as demonstrated in Figure 1.

The Bederson's score in the MCAO+500 mg/kg *C. nervosum* administration were higher than that in the MCAO + vehicle treated group ($p<0.05$) as shown in Figure 2.



* $p<0.05$ as compared to MCAO + vehicle treated group

Figure 1. Escape latency and retention times of rats orally treated with vehicle, Vitamin C, C. nervosum at dose of 500 mg/kg body weight (BW). Values are mean ± SEM (n = 8 per group)



* $p<0.05$ as compared to MCAO + vehicle treated group

Figure 2. Effect of C. nervosum on the neurological score deficits. Values are mean ± SEM (n = 8 per group)

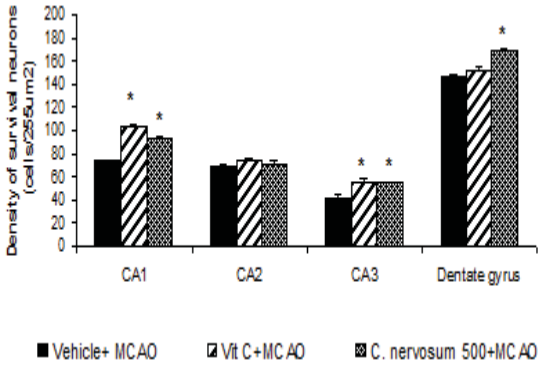
In the MCAO + vehicle treated group, the cerebral infarction was found on most of the cerebral cortex. While, the MCAO + 500 mg/kg BW dose of *C. nervosum* considerably reduced the cerebral infarction size. The proportion of the cerebral infarction volume of the MCAO + 500 mg/kg BW *C. nervosum* treated group exhibited a significant reduction of the cerebral infarction size compared to the MCAO + vehicle treated group ($p<0.05$), as shown in Table 1.

Table 1. Comparison the effect of vehicle and 500 mg/kg BW of *C. nervosum* after 14 days of treatment on the percentage of infarction volume after 24 hours of right MCAO

Treatment	Infarction volume; mm^3 (SEM)		
	Cortical	Subcortical	Total
MCAO + vehicle	261.12 (6.86)	105.80 (6.04)	369.92 (12.90)
MCAO + *C. nervosum* 500	144.84 (3.20)	76.65 (3.74)	221.48 (6.64)

* p<0.05 as compared to MCAO + vehicle treated group

The MCAO + Vitamin C significantly increased the neuronal densities both in CA1 and CA3 (p<0.05 all), compared with the MCAO + vehicle treated group as well as the MCAO + 500 mg/kg BW C. nervosum treated rats exhibited increases in neuronal densities in CA1, CA3 and DG (p<0.05 all), as shown in Figure 3.



* p<0.05 as compared to MCAO + vehicle treated group

Figure 3. Survival neurons in hippocampus of the rats which treated with vehicle, Vitamin C, *C. nervosum* at dose of 500 mg/kg BW. Values are mean ± SEM (n = 8 per group)

Discussion

C. nervosum extract had demonstrated the improvement of spatial learning ability, Bederson's score, cerebral infraction, and neuronal densities in hippocampus, compared with vehicle treated group. However, animal models of ischemic stroke are procedures inducing cerebral ischemia. It aims to study basic processes or potential therapeutic interventions in this disease, and the extension of the pathophysiological knowledge on and/or the improvement of medical treatment of human ischemic stroke. Nevertheless, ischemic stroke has a complex pathophysiology involving the interplay of many different cells and tissues such as neuron, glia, endothelial, and the immune system. These events cannot be mimicked satisfactorily *in vitro* yet. Thus a large portion of stroke research is conducted on animals.

Studies in humans and monkeys have identified structures in the medial temporal lobe essential for memory (the hippocampal region, i.e., the dentate gyrus, the hippocampus, and the subicular complex, and the adjacent perirhinal, entorhinal, and parahippocampal cortices). Additional work has revealed that for both species, damage limited to the hippocampal region produces less severe memory impairment than damage that includes additional structures within the medial temporal lobe. An important issue about ischemic damage is whether the damage identifiable in the histopathological examination provides an accurate estimate of direct neural damage or whether additional direct damage might be present that is sufficient to disrupt neuronal function in areas important for memory and sufficient to impair behavioral performance, but not sufficient to progress to cell death and to be detectable in conventional histopathology. [15]

Thus, the researchers encourage additional experimental work, especially in rats, that could

further illuminate how to evaluate the behavioral effects of ischemic lesions. In addition, the manual counts are not typically higher or lower than machine counts. However there is a clear improvement in precision when using flow cytometry. Thus, the machine counts produce faster, more consistent results. [16]

In conclusion, the *C. nervosum* extract supplementation may be advantageous to act as the neuroprotective agent in ischemia stroke in rat.

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