The Functional Effect of Kaempferia Parviflora on Ischemic Stroke in Rats

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Abstract: Problem statement: Oxidative stress has been implicated as important factors in the pathogenesis of cerebral ischemia-induced behavioral deficits particularly memory. Growing attention is being paid to traditional medicinal plants possessing antioxidant activity, as they have been proved therapeutically/prophylactically fruitful against stroke condition. Present study provides evidence of the neroprotective effects of a Thai traditional herb, *Kaempferia Parviflora* (KP) extract, against oxidative stressrelated brain damage and memory deficit induced by focal cerebral ischemia in rats. **Approach:** Male Wistar rats were orally given the ethanolic extract of KP at doses of 100, 200 and 300 mg kg^{-1} BW once daily continually for 14 days before and 7 days after right Middle Cerebral Artery occlusion (MCAO). Spatial memory was evaluated using the Morris water maze test at 7 days after MCAO and then rats were sacrificed for determining the survival neurons and biochemical markers of lipid peroxidation in the hippocampus. **Results:** Only KP at 200 mg kg^{-1} BW could ameliorate oxidative injuries induced by cerebral ischemia by decreasing the lipid peroxidation leading to the enhanced neuron densities in all areas of hippocampus, which in turn resulted in the improved spatial memory impairment. **Conclusion:** Overall, KP supplementation may be advantageous to the reduction of oxidative damage and for the improvement of memory deterioration in stroke or vascular dementia.

Key words: *Kaempferia Parviflora*, spatial memory, traditional medicines, lipid peroxidation, cerebral ischemia, Middle Cerebral Artery (MCAO)

INTRODUCTION

Cerebral ischemia is a major cause of mortality and morbidity, results from a permanent or transient reduction in cerebral blood flow in a major brain artery (Dirnagl *et al*., 1999). Reduction of flow is leading to neuronal damage and related behavioral deficits particularly memory (Hacke *et al*., 1996). Cumulative evidences show that oxidative stress such as generation of damaging reactive oxygen species plays a key role in the pathogenesis of ischemic brain damage (Traystman *et al*., 1991). Therefore, inhibition of production and enhanced degradation of reactive oxygen species with pharmacological agents have been found to limit the extent of brain damage under ischemic condition (Calapai *et al*., 2000) and antioxidants have been the focus of studies for developing neuroprotective agents to be used in the management of cerebral ischemia therapy.

During this decade, abundant concentration has been paid toward flavonoids, the substances found in fruits, vegetables and beverages. The flavonoids have aroused considerable interest recently because of their potential beneficial effects on human health. They have been reported to have antiviral, anti-allergic, anti-inflammatory, anti-tumor and antioxidant activities (Bors and Saran, 1987; Robak and Gryglewski, 1988).

Kaempferia Parviflora Wall. ex Baker (KP), commonly known as Krachai Dam and belongs to Family Zingiberaceae is used in Thai traditional medicine for the treatment of anti-mycobacterial (Yenjai *et al*., 2004), anti-allergic (Tewtrakul *et al*., 2008), sexual dysfunction and ulcer (Rujjanawate *et al*., 2005). Moreover, it is also used as longevity promoting substance and as nerve tonic. Furthermore, it has been reported that the alcoholic extract of KP rhizomes

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contained numerous flavonoids (Sutthanut *et al*., 2007) which previously reported to possess antioxidant activities, neuroprotective and cognitive enhancing effects (Spencer, 2009; Tong-Un *et al*., 2010). From the point of view of "preventive medicine" or "self-medication" and the numerous beneficial effects of KP, we hypothesized that KP extract might be able to protect against neuronal damage and memory impairments induced by focal cerebral ischemia via reduction of oxidative stress. The objective of this study is to determine the possible effects of KP extract on the cognitive deficit, histopathological changes and oxidative stress marker after occlusion of the right Middle Cerebral Artery (MCAO) in rats.

MATERIALS AND METHODS

Plant material and preparation of the crude extract: *Kaempferia Parviflora* rhizomes (Rom gloa variation) were collected from Tombon Boh-Park, Charttrakarn, Phitsanulok, Thailand. The herbarium was authenticated by Associate Professor Bungorn Sripanidkulchai and deposited as voucher specimen (KP-CRD 10D) at Center for Research and Development of Herbal Health Product, Faculty of Pharmaceutical Sciences, Khon Kaen University, Thailand. The dried plant rhizome powder was macerated in 95% ethanol for 4 days (1 kg L^{-1}) with occasionally stirring. After filtration, the residual was further repeat macerated with 95% ethanol and then the filtrate were combined and dried by freeze dryer. The percent yield of the final product was 4.82%. The extract contained total flavonoids content approximate 40.37 mg g^{-1} dried powder consisting 2 main constituents 5, 7-dimethoxyflavone (8.789 mg g^{-1}) and 3,5,7,3',4'- pentamethoxyflavone (9.858 mg g^{-1}).

Drug and chemicals: Vitamin C or ascorbic acid, Thiobarbituric Acid (TBA), Triphenyltetrazolium Chloride (TTC; 1, 1, 3, 3 tetraethoxypropane, reduced form of NADH, 2% SCMC (Sodium carboxymethylcellulose), 4% paraformaldehyde were purchased from Sigma Aldrich (St. Louis, MO, USA). Stock solutions of all administered substances were made fresh on the day of experiment.

Animals: Healthy male Wistar rats weighing (180-220 g) were obtained from National Laboratory Animal Center, Salaya, Nakorn Pathom. They were housed in group of 5 per cage in standard metal cages at 22±2°C on 12:12 h lightdark cycle. All animals were given access to food and water ad libitum. Experiments were performed to minimize animal suffering in accordance

with the internationally accepted principles for laboratory use and care of European Community (EEC directive of 1986; 86/609/EEC) and approved by the Ethical Committee of the Khon Kaen University.

Experimental protocol: All rats were randomly divided into 6 groups (8 in each group). Group I: sham-operated group. Group II: Vehicle (2% SCMC), which used as vehicle to a desired concentration. Group III: Vitamin C, a well-known antioxidant showing memory improvement, was orally administered at dose of 250 mg kg^{-1} BW and served as positive control. Group IV-VI: Rats were treated with different doses of the alcoholic extract of KP (100, 200 and 300 mg kg^{-1} BW), respectively. The doses of KP were selected on the basis of previous studies conducted in laboratory and those reported in literature.

All rats were orally assigned substances via the intragastric feeding tube once daily for 14 days before and 7 days after MCAO. Spatial memory was assessed at 7th day after MCAO. Later on, the KP at dose produced optimum changes on learning memory was selected for further evaluation of Malondialdehyde (MDA) levels and the densities of survival neurons in hippocampus.

Focal cerebral ischemia induction: Anesthesia was induced with intraperitoneal injection of thiopental sodium at dose of 50 mg kg^{-1} BW. Focal cerebral ischemia was performed according to modified method of Longa *et al*. (1989). Briefly, the right common carotid artery and the right external carotid artery were exposed through a ventral midline neck incision and were ligated proximally. A silicone coated nylon monofilament (4-0) suture (USS DGTM sutures; Tyco Healthcare group LP, Connecticut, USA) with its tip rounded by heating near a flame was inserted through an arteriectomy in the common carotid artery just below the carotid bifurcation and then advanced into the internal carotid artery approximately 17-18 mm distal to the carotid bifurcation until a mild resistance was felt. Occlusion of the origins of the anterior cerebral artery, the middle cerebral artery and the posterior communicating artery was thereby achieved. Then, the wound was sutured, the rats were returned to their cages with free access to food and water. The incision sites were infiltrated with 10% povidone-iodine solution for anti-septic postoperative care.

Morris water maze test: patial learning and memory of animals were tested in Morris water maze (Morris, 1984). It consisted of a circular water tank (170 cm diameter and 60 cm height) that was partially filled with water (25±2°C, 40 cm deep). A non-toxic paint

was used to render the water opaque. The pool was divided virtually into four equal quadrants, labeled North-South-East-West by two imaginary lines crossing the center of the pool.

An escape platform (10 cm in diameter) was hidden 2 cm below the surface of the water on a fixed location in one of the four quadrants of the pool. The platform remained in the same quadrant during the entire experiment.

Before the training started, rats were allowed to swim freely into the pool for 60 s without platform. They were given four trials (once from each starting position) per session for 5 days, each trial having a ceiling time of 60 s and a trial interval of approximately 30 s. After climbing on to the platform, the animal remained there for 30 s before the commencement of the next trial. If rat failed to reach the escape platform within the maximum allowed time of 60 s, it was gently placed on the platform and allowed to remain there for the same interval of time. The time to reach the hidden platform was recorded as escape latency. In addition to the acquisition test, the determination of retention memory was carried out on the next day. According to this test, the platform was removed and the rats were placed into the water maze for 60 s. 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.

Histological procedure: Following anesthesia with of thiopental sodium (50 mg kg^{-1} BW), fixation of the brain was carried out by transcardial perfusion with fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.3. The brains were removed after perfusion and stored over night in a fixative solution that used for perfusion. Then, they were infiltrated with 30% sucrose solution approximately 4°C. The specimens were frozen rapidly and 25 μM thick sections were cut on cryostat. They were rinsed in the phosphate buffer and picked up on slides coated with 0.01% of poly L-lysine.

Nissl staining: Coronal sections of the brains were stained with 0.75% cresyl violet, dehydrated through graded alcohols (70, 95, 100% 2×) and xylene and cover slipped using DPX mountant.

Morphological analysis: Five coronal sections from each rat in each group were studied quantitatively.

Neuronal counts were performed by eye using a 40× objective with final field 225 μm^2 and bregma coordination according to the following stereotaxic coordinates: AP -4.8 mm, lateral ± 2.4-6 mm, depth 3-8 mm. The observer was blinded to the treatment at the time of analysis. Viable stained neurons were identified on the basis of a stained soma with at least two visible processes. Counts were made in five adjacent fields and the mean number extrapolated to give the total number of neurons per 225 μm^2. All data are represented as number of neurons per 225 μm^2.

Measurement of lipid peroxidation: MDA, which is a measure of lipid peroxidation, was measured spectrophotometrically (Ohkawa *et al.*, 1979).
Briefly, hippocampal areas were homogenized with 10 times (w/v) 0.1 M sodium phosphate buffer (pH 7.4).

The reagents acetic acid 1.5 mL (20%) pH 3.5, 1.5 ml thiobarbituric acid (0.8%) and 0.2 mL sodium dodecyl sulfate (8.1%) were added to 0.1 mL of processed tissue sample. The mixture was then heated at 100°C for 60 min. The mixture was cooled with tap water and 5 mL of n-butanol: pyridine (15:1% v/v), 1 mL of distilled water was added. The mixture was shaken vigorously. After centrifugation at 4000 rpm for 10 min, the organic layer was withdrawn and absorbance was measured at 532 nm using a spectrophotometer.

Statistical analysis: Results are expressed as mean ± S.E.M. Statistical analysis of the data was done by applying the Analysis Of Variance (ANOVA), followed by Tukey's test for biochemical parameters and Mann-Whitney-test for learning and memory observations. The p-value<0.05 was considered statistically significant.

RESULTS

Improvement of *Kaempferia parviflora* on learning memory: To investigate the effect of KP on cognitive enhancing effect following MCAO, rats were trained and tested on the Morris water maze, a valid test which is sensitive to the spatial learning memory ability or hippocampus-dependent memory (Liu *et al.*, 2003). Following vehicle treated group, rats exhibited markedly decreased spatial learning ability as indicated by both escape latency and retention time as compared to sham treated group (p-value<0.05; Fig. 1 and 2). It is clear that the memory deficit was significantly developed in the vehicle treated group. In contrast, only KP at dose of 200 mg kg^{-1} BW and Vitamin C treated groups significantly decrease in the escape latency as compared to vehicle treated group (p-value <0.05; Fig. 2).
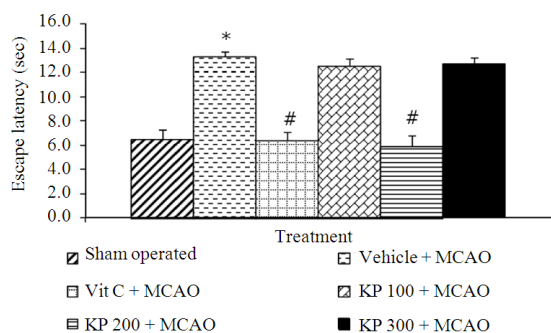
Fig. 1: Effect of Vitamin C and KP (100, 200 and 300 mg kg^{-1} BW) extract on escape latency time in Morris water maze test. Data were presented as mean ± SEM (n=8). *, #p-value<0.05 compared with sham and vehicle treated group respectively
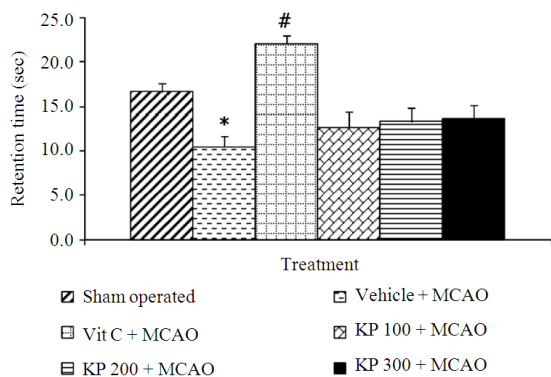

Fig. 2: Effect of Vitamin C and KP (100, 200 and 300 mg kg^{-1}BW) extract on retention time in Morris water maze test. Data were presented as mean ± SEM (n = 8). *, #p-value<0.05 compared with sham and vehicle treated group respectively

Moreover, rats exposed to KP at dose of 200 mg kg^{-1} BW intervention after MCAO not only performed significantly better than rats receiving vehicle treatment after MCAO, but performed similarly to rats receiving sham treated group. Unfortunately, our results show that there were no significant differences in retention time. However, these findings indicate that spatial learning impairments observed following brain ischemia are reversed by KP supplementation.

***Kaempferia parviflora* induces the survival neurons in various areas of hippocampus:** Based on the previous findings about the crucial role of hippocampus on spatial memory, we also determined the effect of KP on the densities of survival neurons in various areas of hippocampus.
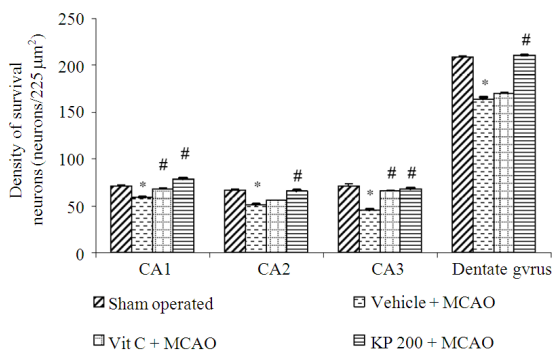

Fig. 3: Effect of Vitamin C and KP at dose of 200 mg kg^{-1} BW on the densities of survival neurons in hippocampus. Data were presented as mean ± SEM (n = 8). *, #p-value <0.05 compared with sham and vehicle treated group respectively
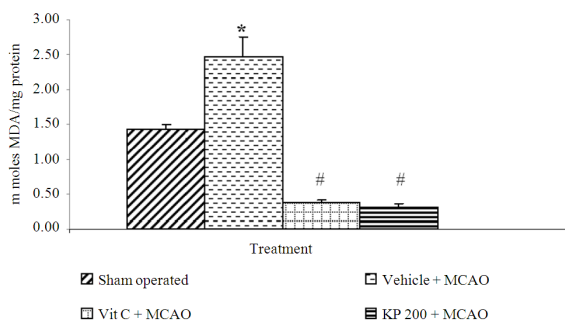

Fig. 4: Effect of Vitamin C and KP at dose of 200 mg kg^{-1} BW on the level of Malondialdehyde (MDA), a product of lipid peroxidation in hippocampus. Data were presented as mean ± SEM (n = 8). *, #p-value <0.05 compared with sham and vehicle treated group respectively

In order to elucidate of issue, the KP at dose of 200 mg kg^{-1} BW which produced optimum changes on learning memory was selected for further determination the densities of survival neurons in hippocampus.

The current data showed that Vitamin C significantly increased the survival neurons densities both in CA1 and CA3 as compared to vehicle treated group. Interestingly, in KP at dose of 200 mg kg^{-1} BW treated group, the rats showed significantly improved the densities of survival neurons in all areas of hippocampus as compared to vehicle treated group (p-value <0.05; Fig. 3).

***Kaempferia parviflora* suppresses lipid peroxidation:** Previous studies demonstrated that oxidative stress contributes the important role on the neurodegeneration

induced by cerebral ischemia. Therefore, this study also aimed to determine the effect of KP extract on the level of Malondialdehyde (MDA), an indirect marker of the oxidative damage. MDA level in hippocampus was significantly elevated in rats receiving vehicle treatment after MCAO as compared to sham treated group (p-value <0.05; Fig. 4). KP at dose of 200 mg kg^{-1} BW significantly decreased the elevated MDA level hippocampus as compared to vehicle treated group. Likewise, Vitamin C treatment significantly suppressed the increasing in lipid peroxidation or MDA level in hippocampus as compared to vehicle treated group.

DISCUSSION

In the present study, we investigated the neuroprotective effects of *Kaempferia Parviflora* (KP) against ischemia following Middle Cerebral Artery Occlusion (MCAO) in rats. The major findings of our study were that *Kaempferia Parviflora* (KP) extract significantly improved spatial memory, suppressed the accumulation of lipid peroxidation products and mitigate the degeneration of survival neurons in hippocampus.

Many studies have demonstrated that cerebral ischemia can develop cognitive deficit and neuronal damage (Nunn and Hodges, 1994; Wattanathorn *et al*., 2011). According to clinical observation, the patients of stroke are generally accompanied with cerebral ischemia and memory impairment (Hacke *et al*., 1996; Hodges *et al*., 1997). Middle Cerebral Artery Occlusion (MCAO) is most commonly model used to induce experimental focal cerebral ischemia (Longa *et al*., 1989). In addition, it had been reported to induce an excessive free radicals leading to neuronal damage in the hippocampus resulted in cognitive deficit (Rice *et al*., 1981). Thus, we selected the animal model of permanent occlusion of Middle Cerebral Artery (MCAO) to mimic the events of human cerebral ischemia.

It is well known that the hippocampus is the brain area that plays an important role in spatial memory (Block and Schwarz, 1997; Cain and Boon, 2003; Phachonpai *et al*., 2010). Therefore, we determined the cognitive performance of the rats using the Morris water maze test which is the best tool to determine spatial learning and emory in rodents (Yonemori *et al*., 1996).

With an MCAO injury paradigm, our results show that in the vehicle intervention after MCAO treated rats there was the reduction of survival neurons in all areas of hippocampus leading to impairment of learning and memory as evidenced by significantly induced the escape latency but reduced the retention time. These results are in conformity with other workers who have demonstrated cognitive impairment after MCAO in rats (Netto *et al*., 1993; Wattanathorn *et al*., 2011).

Oxidative stresses such as generation of damaging reactive oxygen species has been proven in relation to pathogenesis of cerebral ischemia (Yun *et al*., 2007; Jittiwat *et al*., 2009). When neuronal cells were attacked by excessive free radicals, the content of MDA, the by-product of lipid peroxidation, was increased leading to brain damage resulted in the memory impairment during cerebral ischemia (Reynolds *et al*., 2007). This implication has led to the notion that antioxidant defense mechanisms in the brain are not sufficient to prevent ischemic injury and that dietary intake of a variety of antioxidants might be beneficial for improving brain damage and cognitive function.

In our present study, the biochemical analysis result indicated that in the vehicle intervention after MCAO treated rats there was the induction of the MDA level in hippocampus, which ran parallel with the cognitive impairment and histopathological changes. Our study showed that Vitamin C, an effective antioxidant, markedly reduced the level of MDA and protection against neuronal damage both in CA1 and CA3 resulting in the improvement of spatial memory after MCAO in rats, in harmony with other studies (Wattanathorn *et al*., 2011). However, only prophylactic treatment with KP at dose of 200 mg kg^{-1} BW could mitigate memory impairment following ischemic stroke whereas the low and high doses of KP extract did not produce the significant changes on both escape latency and retention time. One possible explanation for this phenomenon might be due the KP extract at low dose might possibly fail to raise the concentration of active ingredient in the extract to the therapeutic level. On the other hand, the high dose of KP extract also failed to show a cognitive enhancing effect. This might occur because the KP extract used in this study was the crude extract; therefore, increasing the dose of the extract might also increase the concentration of some ingredients which masked the effect of the active ingredient. This finding is in accordance with the previous report of KP extract effect on cognitive enhancement in rats (Hawiset *et al*., 2011). However, the model to induce the oxidative damage, the dose of KP extract and the duration of treatment of the plant extract were different.

Recent findings reported that the spatial memory impairment or hippocampal dependent memory was tightly associated with the neurodegeneration in hippocampus, which in turn depended on the densities of survival neurons in this area (Taylor and Crack, 2004). Therefore, our study had determined the neuron densities in this area. The current data clearly

demonstrated that the plant extract at dose of 200 mg kg^{-1} BW significantly enhanced neuron densities in all investigated subregions of hippocampus. In accompany with these changes, we also observed the decreased MDA level in hippocampus. Taken all data together, KP extract suppressed the accumulation of lipid peroxidation products and mitigate the degeneration of survival neurons in hippocampus and resulting in the improved memory impairment. Although KP had the reputation to provide the beneficial affects more that Vitamin C because it could approach at more targeted sites, no significant difference on memory improvement was observed.

CONCLUSION

Our study illustrated for the first time that neuroprotective effect of KP oral administration against focal cerebral ischemia. This ability of KP extract makes it a suitable candidate for consideration as a dietary supplement or functional food to reduce ischemia injury and may also provide beneficial effect as neuroprotectant against stroke or vascular dementia. However, further researches about possible active ingredient, the precise underlying mechanism and safety range are still essential before move forward to clinical trial study to confirm this advantage.

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