

## Effect of Dietary *Kaempferia parviflora* on Ischemic Brain Injury in the rat

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**Abstract: Problem statement:** Stroke often causes irreversible neuronal damage and related behavioral deficits. The lack of effective and widely applicable pharmacological treatments for ischemic stroke patients may explain a growing interest in traditional plant medicines. We investigated the ability of consuming *Kaempferia parviflora* extract, a Thai medicinal plant reputed for neuroprotective effects against brain damage, reduced infarct volume and improve neurological outcome in rats with permanent right Middle Cerebral Artery Occlusion (MCAO). **Approach:** Male adult Wistar rats were administrated an alcoholic extract of KP orally once daily at a period of 14 days before and 7 days after MCAO. Neurological function assessment was performed at 7 days after MCAO using the 6-points postural reflex test. The brain infarct volume and the densities of survival neurons in hippocampus were also determined at the end of experiment. **Results:** Oral administration with KP at a dose of 200 mg kg<sup>-1</sup> BW significantly improved the neurological behavior performances and reduced the infarct volume when compared to the vehicle treated group. The reductions of survival neurons densities were also mitigated. **Conclusion:** The consumption of KP extract may have the possibility of protective effect against neurological disorders such as brain ischemia, while further investigations about precise underlying the defense mechanism against cerebral ischemia are still required.

**Key words:** *Kaempferia parviflora*, neuroprotective, traditional plant medicine, cerebral ischemia, neurological behavior deficit, Middle Cerebral Artery Occlusion (MCAO)

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### INTRODUCTION

Ischemic stroke is a leading cause of death and severe disability and place a large burden on healthcare and social service resources in most countries (Plaisier *et al.*, 2008). Therapeutic strategies to limit infarct size and improve functional outcome after stroke injury are aimed at rescuing this potential reversible ischemic region (Wang *et al.*, 2008). To date, the available efficacies of drugs treatment for stroke condition are still very limited due to their adverse side effects.

Accumulating lines of evidence reported that oxidative stresses such as generation of damaging reactive oxygen species will lead to cell death under

ischemic condition (Gilgun-Sherki *et al.*, 2002). Furthermore, oxidative stress involves redox signaling to molecular mediators of inflammation pathways, which induce further cell damage (Chang *et al.*, 2005; Ritz *et al.*, 2008). Increasing evidence has indicated that ischemia, which occurs due to blockage of the Middle Cerebral Artery or due to recirculation of blood flow, causes oxidative stress that may potentiate ischemic injury (Jittiwat *et al.*, 2009). In recent times, growing evidences support participation of phenolic antioxidants from traditional plant medicines to possess as neuroprotective agents against cerebral ischemia (Rice *et al.*, 1981; Calapai *et al.*, 2000; Phachonpai *et al.*, 2010).

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Traditional plant medicines are useful alternative source of novel drugs containing various pharmacological activities. Zingiberaceae, the Ginger family, includes many important medicinal plants and is a rich source of biologically active compounds (Ojewole, 2006; Stoilova *et al.*, 2007). In Thailand, a member of the Zingiberaceae family, *Kaempferia parviflora* (Krachaidum; KP), is used as a folk medicine. The rhizomes of KP are applied as a medicine for a treatment of hypertension (Tewtrakul *et al.*, 2008), anti-male impotence (Sudwan *et al.*, 2006) and anti-gastric ulcer activity (Rujjanawate *et al.*, 2005). Furthermore, the extracts and flavone derivatives from the rhizome of KP could exhibit anti-inflammatory and antioxidant activities (Tewtrakul *et al.*, 2009; Sakong *et al.*, 2011). Moreover, administration of KP extract has been proven to reduce the brain damage, improve memory impairment (Spencer, 2009) and induce vasorelaxation activity (Tep-Areenan *et al.*, 2010).

Based on the flavonoid ingredients, antioxidant activity and biological effects of KP rhizome extract, the present study therefore sought to confirm the usefulness of KP extract against the neuronal damage, brain infarct and neurological deficits in rats with permanent right Middle Cerebral Artery Occlusion (MCAO) model.

## 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 ( $1 \text{ kg L}^{-1}$ ) 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  $40.37 \text{ mg g}^{-1}$  dried powder consisting 2 main constituents 5, 7-dimethoxyflavone ( $8.789 \text{ mg g}^{-1}$ ) and 3,5,7,3',4'-pentamethoxyflavone ( $9.858 \text{ mg g}^{-1}$ ).

**Animals:** Healthy male Wistar rats weighing 300-350 gm and aging 8 weeks were obtained from National

Laboratory Animal Center, Salaya, Nakorn Pathom. They were housed in group of 5 per cage in standard metal cages at  $2 \pm 22^\circ\text{C}$  on 12:12 h light-dark 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 assigned to 6 groups ( $n = 8$  in each group). Group I: Naïve intact control rats. Group II: sham-operated group. Group III: Vehicle (2% SCMC), which used as vehicle to a desired concentration. Group IV-VI: Rats were treated with different doses of the alcoholic extract of KP (100, 200 and  $300 \text{ mg kg}^{-1}\text{BW}$ ), 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. Neurological evaluation was determined at 7th day after MCAO. Later on, the dosage of KP that produced the improvement of neurological score was selected for further evaluation the infarct volume and the densities of survival neurons in hippocampus.

**Focal cerebral ischemia induction:** Anesthesia was induced with intraperitoneal injection of thiopental sodium at dose of  $50 \text{ mg kg}^{-1}\text{BW}$ . 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 DG<sup>TM</sup> sutures; Tyco Healthcare group LP, Connecticut, USA) with its tip rounded by heating near a flame was inserted through an arteriotomy 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.

**Assessment of neurological deficit:** All animals were subjected to neurological evaluation by using the 6-points postural reflex test according to the method of Bederson *et al.* (1986) and Schmid-Elsaesser *et al.* (1998). The deficit was graded from 0-5 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.

**Determination of infarct volume:** The infarct volume was assessed with 2% 2, 3, 5-Triphenyl Tetrazolium Chloride (TTC) solution in saline for 30 min at 37°C and photographed. These images were analyzed using a computerized image analysis system (Optimas 6.1; Media Cybernetics, Silver Springs, MD, USA) to measure the infarct area (Shimamura *et al.*, 2006). 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.

**Histological procedure:** As noted above, after behavioral tests, rats were killed. The brains were dissected out and placed in 4% paraformaldehyde solution as fixative. Histopathological sections were made using Nissl staining to identify the survival neurons in hippocampus. Five coronal sections of each rat in each group were studied quantitatively. Neuronal counts in hippocampus were performed by eye using a 40x objective with final field 225  $\mu\text{m}^2$  according to the following stereotaxic coordinates: AP-4.8 mm, lateral  $\pm 2.4$ -6 mm and 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  $\mu\text{m}^2$ . All data are represented as number of neurons per 225  $\mu\text{m}^2$ .

**Statistical analysis:** All analyses were performed using SPSS for Windows version 13 software (SPSS Inc., Chicago, IL, USA). Results are expressed as the mean  $\pm$  S.E.M. Statistical differences among the experimental groups were tested using one way Analysis of Variance (ANOVA), followed by Dunnett's t-test. The p-value < 0.05 was considered statistically significant.

## RESULTS

**Improvement of *Kaempferia parviflora* on neurological function:** There was no neurobehavioral dysfunction symptom in rats of the sham-operation group.

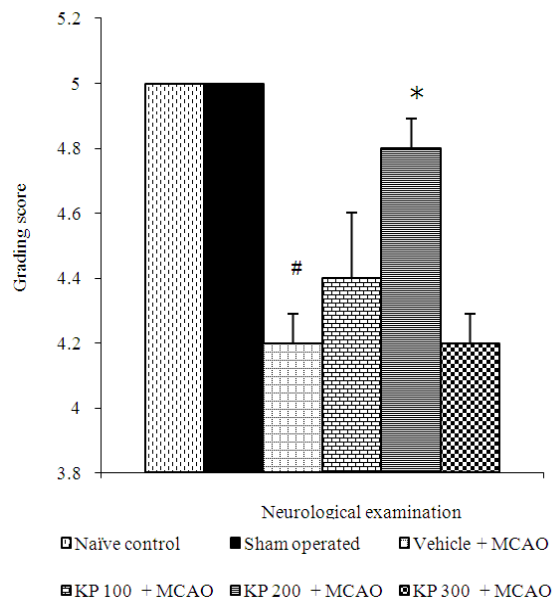


Fig. 1: Effect of KP extracts at dose of 100, 200 and 300 mg  $\text{kg}^{-1}$  BW on the neurological score deficits. Values given are the mean  $\pm$  S.E.M. (n = 8). # p<0.05 as compared to sham operation treated group, \*p<0.05 as compared to vehicle treated group

The Bederson's scores in the vehicle treated were lower than that in the sham-operation group (p-value <0.05). Interestingly, treatment with KP at a dose of 200 mg  $\text{kg}^{-1}$  BW, the neurological deficit score was significantly improved compared with vehicle treated group (p-value <0.05) while the low and high doses of KP extract did not produce the significant changes on this parameter (Fig. 1).

**Effect of *Kaempferia parviflora* reduces the volume of infarction:** The infarct volume was significantly higher in the vehicle treated group than in the sham-operation group (p-value <0.05) while treatment with KP at a dose of 200 mg  $\text{kg}^{-1}$  BW could reduce the brain infarct volume only in the cortical whereas no significant changes in subcortical areas as compared with vehicle treated group (p-value <0.05; Fig. 2).

**Neuroprotective effect of *Kaempferia parviflora*:** The Nissl-stained slices of rat's brain showed intense, rich Nissl bodies in the sham-operated group whereas the vehicle treated group, Nissl bodies were significantly reduced in all areas of the hippocampus. Notably, KP at a dose of 200 mg  $\text{kg}^{-1}$  BW could mitigate the reduction of Nissl bodies after MCAO in all areas of the hippocampus (p <0.05 all; Fig. 3).

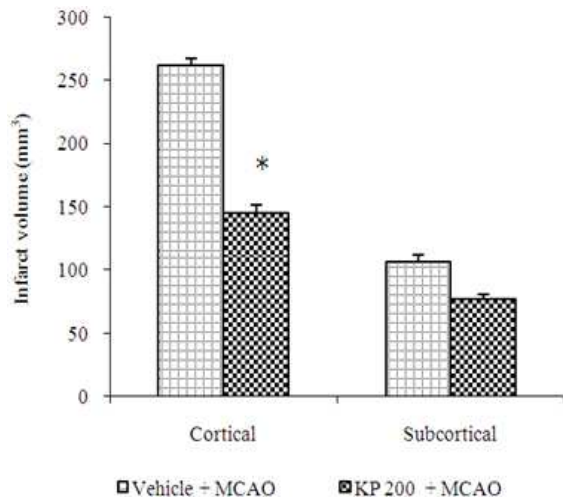


Fig. 2: Effect of KP extract at a dose of 200 mg kg<sup>-1</sup> BW on the infarct volume. Values given are the mean ± S.E.M. (n = 8). \*p<0.05 as compared to vehicle treated group

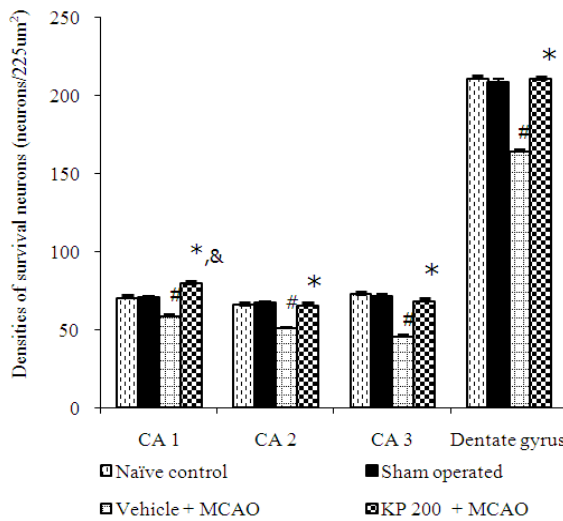


Fig. 3: Effect of KP extract at a dose of 200 mg kg<sup>-1</sup> BW on the densities of survival neurons in hippocampus. Values given are the mean ± S.E.M. (n = 8) and p<0.05 as compared to naïve control group, # p<0.05 as compared to sham operation treated group, \*p<0.05 as compared to vehicle treated group

### DISCUSSION

Despite the impacts of stroke on several million people worldwide every year, no treatment exists to

minimize the neurological damage. People who suffer stroke often experience persistent functional impairment. Accumulating lines of data reported that dietary enrichment with nutritional antioxidants could reduce brain damage and improve behavior functional deficits (Suk *et al.*, 2002; Koo *et al.*, 2004). Evidence suggests that antioxidant activities of plants used in traditional herbal medicine have been shown to be neuroprotective when given before or shortly after induction of focal cerebral ischemia (Hall *et al.*, 1988; Liu *et al.*, 1989). The results of the present study revealed that KP oral administration significantly ameliorated the neurological deficit, reduced the infarct volume and mitigated the degeneration of survival neurons in hippocampus within 7 days post-ischemia. These encouraging results may have future clinical importance because of the increase using of herbal supplements by the general population. However, this study did not investigate about the precise underlying the defense mechanism of KP extract could protect against cerebral ischemia; this is planned in future studies.

Middle Cerebral Artery Occlusion (MCAO) is most commonly model used to induce experimental focal cerebral ischemia (Garcia *et al.*, 1995). The advantages of the MCAO model are its reproducibility and ease of reperfusion. Besides, the type of ischemic injury observed is similar to that found in human (Hacke *et al.*, 1996). Therefore, MCAO model has been employed in the present study.

The brain injury induced by MCAO leads to neurobehavioral impairment. We performed the modified by using 6-points postural reflex test to measure neurobehavioral changes. In this study, all rats in the operation (MCAO) groups showed a neurologic deficit after MCAO injury. Surprisingly, at 7 days after MCAO, the rats treated with KP at a dose of 200 mg kg<sup>-1</sup> BW were improved the neurological deficit scores compared to the vehicle treated group. Unfortunately, the low and high doses of KP extract did not produce the significant changes on this parameter. One possible explanation for this phenomenon might be due the KP extract at a low dose might possibly fail to raise the concentration of active ingredient in the extract to the therapeutic level. On the other hand, the increasing a dose of the crude extract of KP might also increase the concentration of some ingredients which masked the effect of the active ingredient. These findings are consistent with *in vivo* observations as well as previous studies (Hawiset *et al.*, 2011). However, the animal model to induce the lesion, the dose of KP extract and the duration of treatment of the plant extract were different.

Furthermore, the permanent MCAO in rat model cause cerebral cell death, resulting in local infarction. The infarct area represents the degree of brain injury (Ishibashi *et al.*, 2004). In the present study, rats were oral administered with KP extract at a dose of 200 mg kg<sup>-1</sup> BW for 14 days before and 7 days after MCAO showed markedly the reduction in infarction volume only in the cortex whereas no significant change in subcortical versus vehicle administered rats. As is the case in clinical stroke, infarct volume does not necessarily correlate with functional outcome in patients (Gladstone *et al.*, 2002). This observation needs further investigation in near future.

Previous study had demonstrated that the behavior impairments in stroke patients were associated with the severity of brain infarction, which in turn depended on the densities of survival neurons in the cortex, the hippocampus and the striatum (Leist and Jaattela, 2001). Our results clearly demonstrated that the KP extract at a dose of 200 mg kg<sup>-1</sup> BW significantly enhanced neuron densities in all areas as mention. Notably, KP extract could increase the densities of neurons in CA1 more than those in naive intact control. Therefore, the increased neuron densities in CA1 might involve its neurotrophic action. However, further researches particularly BrdU staining are needed to identify the active constituent of the KP extract responsible for its neurotrophic activity.

Recent studies have demonstrated that Nitric Oxide (NO) levels are associated directly with the development of brain injury in strokes and other neuropathological disorders in humans (Veltkamp *et al.*, 2002). NO can be neuroprotective or neurotoxic during cerebral ischemia, depending on the Nitric Oxide Synthase (NOS) isoform involved. eNOS produces NO with beneficial effects (vasodilation), whereas NO overproduction by nNOS or iNOS during ischemia is cytotoxic (Li *et al.*, 2002). Numerous studies of KP rhizomes extract have reported that they are the effective upregulate the expression of eNOS, iNOS express inhibition, free radical scavenger and antioxidant (Wattanapitayakul *et al.*, 2007; Sae-Wong *et al.*, 2011). We believe that the effects observed in our study may be mediated through one of these KP mechanisms.

To the best of our knowledge, this is the first report to show the neuroprotective effects of KP oral administration against focal cerebral ischemia. These results suggested that KP extract holds promise as a neuroprotectant agent for use as a food supplement or functional food to reduce ischemia injury. Further studies are warranted to pursue the interesting lead emerging from the present results to exploit the full therapeutic potential of KP in ischemic stroke.

## CONCLUSION

In conclusion, we have shown that the KP supplementation significantly prevents the development of ischemic injury in stroke condition. Recently, self-medication with dietary supplements has become increasingly popular for health-maintenance and prevention of life-style related diseases including cerebral ischemia. Our results may help the development of preventive medicine against the cerebrovascular diseases.

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