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Anti-Stress Effects of *Kaempferia parviflora* in Immobilization Subjected Rats

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ABSTRACT

It is well known that intense and prolonged stress can produce hippocampal neuronal damage and cognitive impairments, few studies have investigated possible ways to prevent its deleterious effects. Here, we investigated the neuroprotective effects of a Thai traditional herb, *Kaempferia Parviflora* (KP) extract, commonly known as Kra-Chai-Dum, on learning and memory loss and the induction of neurodegeneration in the hippocampus by chronic stress. Rats were orally administered KP extract (100, 200 and 300 mg kg^{-1}) or vehicle over a period of 21 days while being exposed to chronic restraint stress (6 h day^{-1}). Investigated learning and memory using Morris water maze test after 7, 14 and 21 days of treatment and then the rats were sacrificed for determining the densities of survival and cholinergic neurons in the all regions of hippocampus. Treatment with KP extract at a dose of 200 mg kg^{-1} blocked the ability of chronic stress to impair spatial learning and memory retention and enhanced both neuron densities as mention earlier, in all areas of the hippocampus. Present study highlights the modest activity of KP extract against chronic restraint stress induced modification. Thus, using these substances may be useful in neuroprotective strategy in the treatment of stress.

Keywords:*Kaempferia Parviflora*, Cognitive Impairment, Neuroprotective Effects, Spatial Memory, Traditional Herb, Neurodegeneration, Stress

1. INTRODUCTION

Chronic stress is known to play a causal role in several psychological disorders associated with increased risk for illness and changes in cognition. It is well reported that that stress causes several neurobehavioral deficits on a variety of cognitive tasks (Silakova *et al*., 2004) that are associated with oxidative damage i.e., free radical damage (Sherki *et al*., 2001). Chronic restraint stress stimulates numerous cellular cascade that lead to increase ROS production (Liu *et al*., 1996). Therefore, free radical scavengers and antioxidants have been proposed as agents that may delay or inhibit the progression of chronic stress on cognitive functions.

Up to date, close attention was paid to studies of protecting effects of natural potential ingredients on stress induced injury. The drugs of plant origin are gaining increasing popularity and are being investigated for remedies of a number of disorders including anti-stress activity (Edzard, 1998).

Kaempferia parviflora wall. Ex. Baker (K. parviflora; KP) is a native plant of Thailand and belongs to the Zingiberaceae family. Its rhizomes, known as Thai ginseng, have been widely used in traditional medicine

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as a health promoting herb and according to Thai folk medicine; KP has been used in the treatment of many diseases including heart disease (Tewtrakul *et al*., 2008). Recently, the ethanolic extract of this plant, which is known to contain a variety of flavones and phenolic compounds (Yenjai *et al*., 2004), has been shown to increase blood flow to the testis (Chaturapanich *et al*., 2008) and enhance Nitric Oxide (NO) production in human umbilical vein endothelial cells (Tewtrakul and Subhadhirasakul, 2008). However, this exact status in stressful conditions is still not clear so far.

With this background, the present study was designed to investigate protective effect of KP extract in chronic restraint induced certain cognitive impairment and neurodegeneration in rats.

2. MATERIALS AND METHODS

2.1. Animals

A total of 50 Male Wistar rats, weighing (180-220 g) were obtained from National Laboratory Animal Center, Salaya, Nakorn Pathom. They were maintained on a 12 h light/dark cycle under constant temperature 22±2°C and 70% humidity and housed five per a cage in wire mesh cages, except during testing when they were housed in plexiglass boxes with wood shavings as bedding. The procedures were conducted between 8:00 and 13:00 h. 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.

There were 5 experimental groups of animals (10 in each group). Group I: Vehicle (2% SCMC), which used as vehicle to a desired concentration. Group II: Vehicle (2% SCMC) + stress. Group III-V: Rats were treated with different doses of the alcoholic extract of KP (100, 200 and 300 mg kg^{-1}). The doses of KP were selected on the basis of our previous studies conducted in laboratory (Phachonpai *et al*., 2012).

2.2. 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}).

KP extract suspended in 2% SCMC (Sodium carboxymethylcellulose) and given orally by a gavage at the dose of 100-300 mg kg^{-1}once daily for 21 days, in a volume of 2 mg kg^{-1}, 30 min before stress procedure.

2.3. Restraint Stress Procedure

Restraint was performed by daily placing animals in 20×7 cm^2 plastic tubes for 2 h for 21 days (Magarinos *et al*., 1997). There were several 3 mm holes at the far end of the tubes for breathing. They allowed ample air but animals were unable to move.

2.4. Morris Water Maze Test

Spatial 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. Spatial memory was assessed at 7, 14 and 21 day of treatment. Later on, the KP at dose produced optimum changes on learning memory was selected for further evaluation of the survival and cholinergic neurons densities in all regions of hippocampus.

2.5. Histological Procedure

Following anesthesia with sodium pentobarbital (60 mg kg^{-1}), the rats were transcardially perfused with fixative containing 4% paraformaldehyde in 0.1M phosphate buffer pH 7.3. The brains were removed and stored over night in the same fixative. They were infiltrated with 30% sucrose solution and kept at 4°C. The specimens were frozen rapidly and 30 μM thick sections were cut on a cryostat. The sections were rinsed in phosphate buffer and picked up on slides coated with 0.01% of poly L-lysine.

2.6. Cresyl Violet 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.

2.7. Choline Acetyltransferase and Immunohistochemistry

A series of section containing hippocampus from each groups were reacted in parallel experiments using a mouse monoclonal antibody detected against Choline Acetyltransferase (ChAT) (Chemicon Internation, Inc., CA, USA) and a modification of a previously described protocol employing the DAKO Strept ABC Complex/HRP duet kit. In brief, the sections were eliminated endogenous peroxidase activity by 0.5% H2O2 in methanol. Sections were washed in running tap water and distilled water for 1 min each, then rinsed in KPBS and KPBS-BT for 5 min per each process. Excess was removed and then incubated for 30 min in a blocking solution composed of 5% normal horse serum in KPBS-BT. The sections were then incubated in mouse primary antibody against ChAT diluted 1:100 in KPBS-BT at room temperature for 2 h and then incubated at 4°C for 48 h. The tissue was rinsed in KPBS-BT (two washes 7 min), incubated for 4 h in biotinylated goat antimouse IgG antibody, rinsed in KPBS-BT (two washes 7 min) and then incubated in Strep ABC Complex/HRP for 4 h. In preparation for visualization step, sections were rinsed in KPBS-BT (1 min) and KPBS (two washes 10 min). ChAT immunoreactivity was visualized using 0.025% 3, 3' Diaminobenzidine (DAB, Sigma) and 0.01% H2O2. Finally, sections were rinsed in running tap water, air dried and cover-slipped using permount.

2.8. Morphological Analysis

Five coronal sections from each rat in each group were studied quantitatively. Neuronal counts were performed by eye using a 40x 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.

2.9. Statistical Analysis

Data were presented as mean ± Standard Error of Mean (SEM). One-way Analysis Of Variance (ANOVA), followed by Post hoc test. A probability level less than 0.05 were accepted as significance.

3. RESULTS

3.1. Improvement of *Kaempferia Parviflora* on Learning Memory

One way ANOVA of both escape latency and retention time in all trials in Morris water maze yielded significant differences ($p<0.05$). Post hoc comparisons showed that the vehicle plus stress treated group markedly decreased spatial learning ability as indicated by both escape latency and retention time as compared to vehicle treated group ($p<0.05$; **Fig. 1 and 2**).

It is clear that the memory deficit was significantly developed in the vehicle + stress treated group. In contrast, only KP at dose of 200 mg kg^{-1}plus stress significantly decreased in the escape latency while increased in the retention time as compared to vehicle+ stress treated group ($p<0.05$).

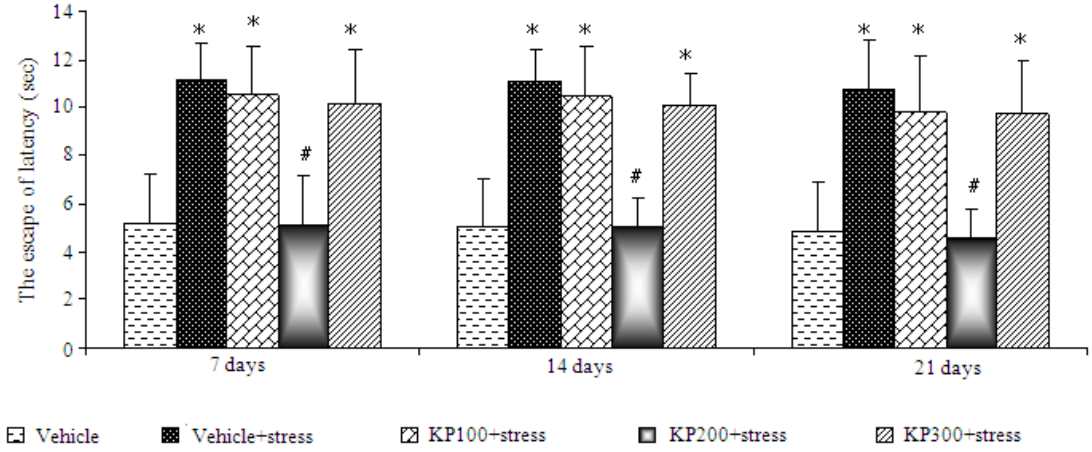

Fig. 1. Effect of KP (100, 200 and 300 mg kg^{-1}) extract on escape latency time in Morris water maze test. Data were presented as mean ± SEM (n = 10). *p-value < 0.05 compared with vehicle treated group, # p-value < 0.05 compared with vehicle + stress treated group

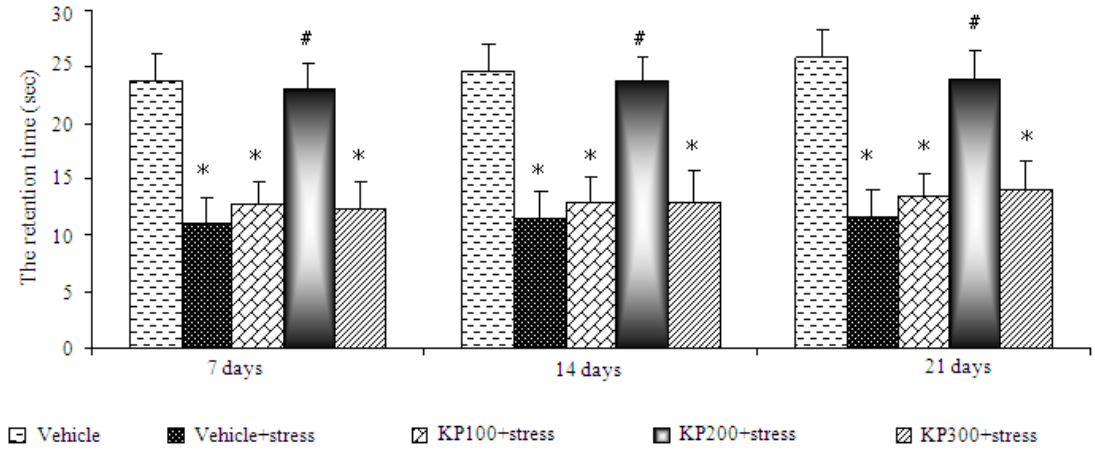

Fig. 2. Effect of KP (100, 200 and 300 mg kg^{-1}) extract on retention time in Morris water maze test. Data were presented as mean ± SEM (n = 10). *p-value < 0.05 compared with vehicle treated group, # p-value < 0.05 compared with vehicle + stress treated group

These phenomenon were still observed when the treatment was prolonged further to 21 days ($p<0.05$ all; compared with vehicle+stress treated group). These findings indicate that spatial learning impairments observed following restraint immobilization reversed by KP extract supplementation.

3.2. *Kaempferia Parviflora* Induces the Survival and Cholinergic Neurons Densities in all Regions 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 and cholinergic neurons in various areas of hippocampus. In order to elucidate of issue, the KP at dose of 200 mg kg^{-1} which produced optimum changes on learning memory was selected for further determination of both neurons in areas as mention previously.

The current results showed that KP at a dose of 200 mg kg^{-1} treated group, the rats showed significantly improved the densities of survival and cholinergic neurons only in CA3 and dentate gyrus of hippocampus as compared to vehicle + stress treated group ($p<0.05$; **Fig. 3 and 4**).

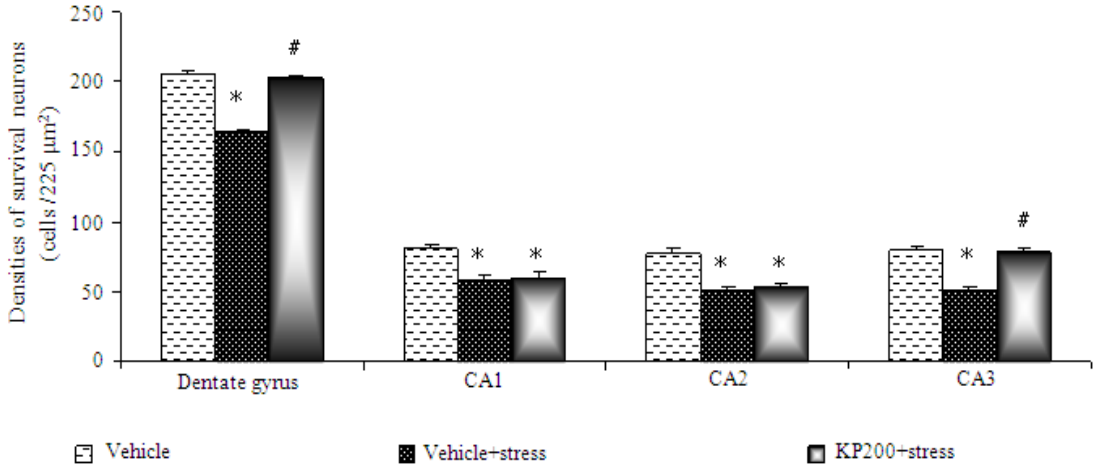

Fig. 3. Effect of KP at a dose of 200 mg kg^{-1} extract on the densities of survival neurons in all regions of hippocampus. Data were presented as mean ± SEM (n = 10). *p-value < 0.05 compared with vehicle treated group, # p-value < 0.05 compared with vehicle + stress treated group

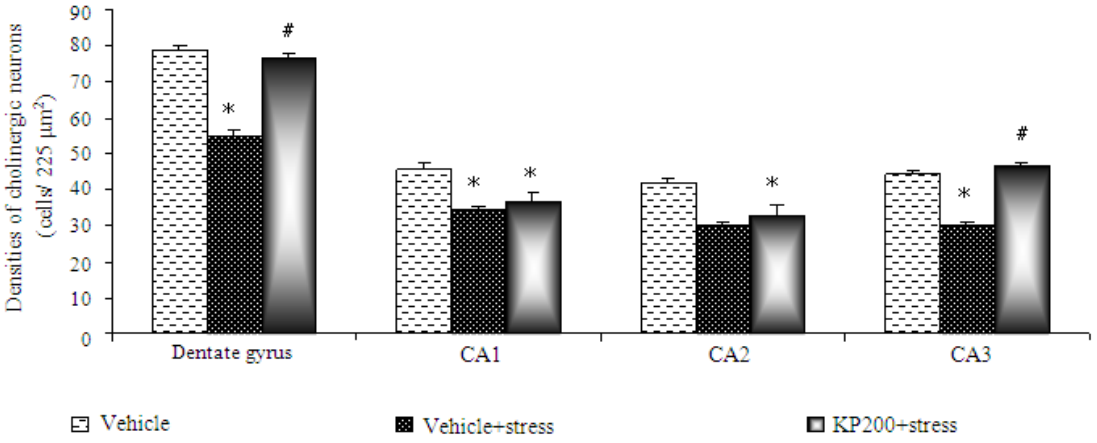

Fig. 4. Effect of KP at a dose of 200 mg kg^{-1} extract on the densities of cholinergic neurons in all regions of hippocampus. Data were presented as mean ± SEM (n = 10). *p-value < 0.05 compared with vehicle treated group, # p-value < 0.05 compared with vehicle + stress treated group

4. DISCUSSION

Up to date, there are no previous reports on the protective effect of KP extract in chronic immobilization stress. The core findings of the present study are that chronic restraint stress impairs spatial learning and memory and induces neuronal damage in all regions of hippocampus. These harmful effects of chronic stress can be prevented by KP extract pretreatment, suggesting that this herb extract has potential therapeutic applications protecting against the detrimental effects of chronic stress on cognitive functions.

Many studies have reported that chronic stress may induce spatial memory deficits (Nishimura *et al*., 1999). Several physical or psychological stressful animal models have been established to induce stress responses. Among them, restraint stress has been well accepted as an experimental model in rodents which evokes unconditioned and unavoidable neuroendocrine responses (Howland and Cazakoff, 2010). Therefore, we selected the chronic restraint stress procedure induced memory impaired rats. In addition, it is well known that the Morris water maze test which is the best tool to determine spatial learning and memory in rodents

(Yonemori *et al*., 1996). In this respect, we choose this test to investigate the cognitive enhancing effect of KP extract.

Cumulative evidences show that the spatial memory impairment or hippocampal dependent memory was tightly associated with the neurodegeneration in all areas of hippocampus, which in turn depended on the densities of various neurons in this area (Taylor and Crack, 2004). Therefore, our study had determined the survival and cholinergic neuron densities in all regions of hippocampus.

With a chronic restraint stress procedure, our results show that in the vehicle intervention plus restraint treated rats there was the reduction of survival and cholinergic neurons densities in all areas of hippocampus leading to impairment of learning and memory as evidenced by significantly increased the escape latency while decreased the retention time. These findings are in agreement with previous studies showing similar deficits in spatial learning and memory following chronic restraint stress (Abidin *et al*., 2004; Moosavi *et al*., 2007).

Oxidative stress could be one of the mechanisms by which chronic stress or glucocorticoids negatively affect learning and memory (Abidin *et al*., 2004) and induce neuronal damage (Patel *et al*., 2002). This implication has led to the notion that antioxidant defense mechanisms in the brain are not sufficient to prevent oxidative neurons damage and that dietary intake of a variety of antioxidants might be beneficial for improving brain damage and cognitive function.

In our present study revealed that only prophylactic treatment with KP extract at a dose of 200 mg kg^{-1}could mitigate memory impairment following chronic restraint stress whereas the low and high doses of plant 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 a low dose might possibly fail to raise the concentration of active ingredients to reach the therapeutic level. On the other hand, a high dose of the 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 cognitive enhancing effect of active ingredients. This finding is in accordance with the previous report of KP extract effect on cognitive enhancement in rats (Hawiset *et al*., 2011; Phachonpai *et al*., 2012).

However, the animal model to induce the memory impairment, the dose of KP extract and the duration of treatment of the plant extract were different.

Interestingly, our results found that KP extract supplementation significantly reduced the degeneration of survival and cholinergic neurons densities only in CA3 and dentate gyrus of hippocampus. One possible explanation for the selective vulnerability in different hippocampal areas might be associated with various types of neuronal, internal antioxidant defense system, trophic factor particularly Nerve Growth Factor (NGF) and Brain Derived Growth Factor (BDNF) distribution in each area of hippocampus.

Numerous previous studies had demonstrated that polyphenolic compounds from various medicinal plants could exert the neuroprotective effects via the antioxidant activity (Mandel and Youdim, 2004). Earlier studies have shown that KP rhizome extract contain many of polyphenolic antioxidants compound. Thus, it was possible that the neuroprotective and cognitive enhancing effects of KP extract observed in our study might occur partly via its antioxidant effect which in turn gave rise to the decreased of neuronal cells damage.

Recent studies have demonstrated that Nitric Oxide (NO) levels are associated directly with the development of neuronal damage in chronic stress condition (Gulati *et al*., 2009). NO can be neuroprotective or neurotoxic products, depending on the Nitric Oxide Synthase (NOS) isoform involved. eNOS produces NO with beneficial effects (vasodilation), whereas NO overproduction by nNOS or iNOS 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 (Wattanapitayakul *et al*., 2007; Sae-Wong *et al*., 2011).

Taken all data together, we believe that the neuroprotective and cognitive enhancing effects observed in our study may be mediated through one of these KP mechanisms.

5. CONCLUSION

Our study illustrated that KP extract is effective at ameliorating immobilization stress induced behavioral alterations and neurodegeneration. This ability of KP extract makes it a suitable candidate for consideration as a dietary supplement or functional food to reduce neuronal damage and may also provide beneficial effect as neuroprotectant in the treatment of chronic stress. However, further researches about possible active ingredient and the precise underlying mechanism are still essential before move forward to clinical trial study to confirm this advantage.

6. ACKNOWLEDGEMENT

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