International Food Research Journal 24(4): 1490-1499 (August 2017)
Journal homepage: *http://www.ifrj.upm.edu.my*

Anti-stress effects of *Apium graveolens* on rats subjected to immobilization

[1]Wongtawatchai, T., [2]Sarsutham, K., [3]Sukketsiri, W., [4]Tipmanee, V. and [2*]Chonpathompikunlert, P.

[1]Department of Anatomy, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, 90112, Thailand
[2]Department of Physiology, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, 90112, Thailand
[3]Department of Pharmacology, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, 90112, Thailand
[4]Department of Biomedical Sciences, Faculty of Medicine, Prince of Songkla University, Hat Yai, Songkhla, 90112, Thailand

Article history

Received: 7 June 2016
Received in revised form: 28 July 2016
Accepted: 3 August 2016

Keywords

Apium graveolens *Linn.*
Stress
Cognitive deficit
Rat

Abstract

Apium graveolens is a potent antioxidant and effective agent on neurological disease. However, the neuroprotective effect against stress still unclear. The present study investigates the anxiolytic effects of a methanol exact of *A. graveolens* in immobilization stressed rats. The five groups of rats used were control, vehicle, positive controls (diazepam 2 mg/kg) and treated groups with 125 and 250 mg/kg of *A. graveolens* for 21 days. On day 15, the rats were placed in the restrainer (12 h daily for 7 days). Restraint stress-induced anxiety-like behavior were assessed using the open field, hole-board and elevate plus maze test. For biochemical parameters, corticosterone, monoamine oxidase enzyme-A (MAO-A) activity, malondialdehyde (MDA), percentage of inhibition of superoxide anion (O_{-2}) and glutathione peroxidase (GPx) were measured. Administration of *A. graveolens* showed a significant increase in the frequency of head dips in the hole board, line crossings and rearing in the open field, time spent in open arm in elevate plus maze, the discrimination index in object recognition and an decrease in escape latency time in the Morris water maze test compared with vehicle. Moreover, results of the biochemical parameter were represented by treated versus vehicle group. Corticosterone level (250 mg/kg) was 9.76 ± 1.87 versus 27.75 ± 5.90 ng/mL. In cortex and striatum, MAO-A and MDA were significantly decreased while GPx and % inhibition of O_{-2} were significantly increased. These results indicated that *A. graveolens* has anxiolytic potential to prevent stress without cognitive deficit, whereas diazepam can cause cognitive deficit.

Introduction

Stress is defined as a condition in the life of an organism that results from the action of one or more stressors that may be of either external or internal origin (Von Borell, 2001). Stress is one of the basic contributing factors to the development of any disease and has been shown to be associated with altered homeostasis that may lead to an oxidant-antioxidant imbalance (Nadeem *et al*., 2006). Numerous reports have revealed that immobilization stress can affect the central nervous system functions by producing neurochemical and hormonal abnormalities associated with an imbalance of antioxidant status that results in odd neurological behavior including anxiety-like behavior, motor alteration and changes of biochemical parameters (Liu *et al*., 1996; Marzatico *et al*., 1998; Nishimura *et al*., 1999; Belanoff *et al*., 2001; Lupien *et al*., 2001; Şahin and Gümüşlü, 2004; Sevgi *et al*., 2006; Goyal and Kumar, 2007; Nooshinfar *et al*., 2011). It is well known that an intensive stress response results in creation of reactive oxygen species (ROS) that causes lipid peroxidation, especially in membranes and can play an important role in tissue injury (Kovacs *et al*., 1996). It has been suggested that chronic stress and high levels of glucocorticoids, the adrenal steroids secreted during stress, affect diverse processes involving ROS with an increase of ROS by approximate 10% above the base (McIntosh and Sapolsky, 1996). The membrane injury causes disruption of the tissue integrity (Cochrane, 1999). It is reported that a disturbance of the balance of the free radical processes was observed with maximal changes in the brain after immobilization stress

*Corresponding author.
Email: *pennapa.ch@psu.ac.th*
Tel: +66-7428-8215; Fax: +66-7444-6680

(Voronych and Lemel, 1994). In order to neutralize ROS, the body uses intracellular antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and also glutathione peroxidase (GPx).

In addition, stress causes several neurobehavioral deficits on a variety of cognitive tasks (Nagata *et al*., 2009) that are also associated with oxidative damage. On the other hand, studies with short term exposure to stress have been shown to facilitate learning and memory performance both in animals (Oitzl *et al*., 2001) and humans (Cahill *et al*., 2003). This increase or decrease in memory performance has been attributed to the hormones and neurotransmitters released under stress conditions. The acute stress may activate the sympathetic nervous system and the hypothalamic-pituitary-adrenal (HPA) axis causes a surge of arousal that is believed to result in memory consolidation following exposure to stress conditions (Joels *et al*., 2006). In contrast, chronic stress may activate the hippocampal glucocorticoid receptors (GRs) which to a great extent are responsible for cognitive deficits (Pavlides *et al*., 1995; McEven, 2000; Lupien *et al*., 2001; Roozendaal *et al*., 2003). The central nervous system has traditionally been considered to be a target site for free radical damage because the brain contains abundant amounts of polyunsaturated fatty acids, which are particularly vulnerable to free radical attacks (Gutteridge, 1995) and consumes high amounts of oxygen (Halliwell and Gutteridge, 1985).

The reducing anxiety-like behavior associated with protecting side effect such as amnesia may be alleviated using therapeutic strategies involving medicinal and dietary phyto-antioxidants. Many of nutracelticals such as *Bacopa monniera* and Hesperidin have been found to ameliorate the immobilization-stress-induced oxidative-stress, biochemical alterations, and associated neurobehavioral alterations (Rai *et al*., 2003; Sheikh *et al*., 2007; Viswanatha *et al*., 2012). Moreover, one such neutraceutical is *Apium graveolens* Linn. that possesses very good antioxidant properties (Popovic *et al*., 2006; Jain *et al*., 2009; Yao and Ren, 2011; Iswantini *et al*., 2012) and it has been proved to be very effective in treating various neurological diseases (Chen *et al*., 2008; Park *et al*., 2009; Peng *et al*., 2010; Feng *et al*., 2012). Moreover, many of nutracelticals such as *Bacopa monniera* and Hesperidin has been found to ameliorate the immobilization-stress-induced oxidative-stress, biochemical alterations, and associated neurobehavioral alterations (Rai *et al*., 2003; Naila *et al*., 2007; Viswanatha *et al*., 2012).

With this background, the present study was designed to investigate the possible neuroprotective effect of *A. graveolens* against immobilization-stress-induced anxiety-like behavior and associated oxidative damage in a rat.

Materials and Methods

Drugs and chemicals

Diazepam (2 mg/tablet) (Government Pharmaceutical Organization) was used as standard drugs. All chemical substances used in this study were analytical grade.

Preparation of A. graveolens *crude extract*

A. graveolens was collected from Lumpang, Thailand. Identification (BKF No. 188856) was made and deposited at the Forest Herbarium, Royal Forest Department, Ministry of Agriculture and Cooperatives, Bangkok, Thailand. The powdered dried whole plant (2 kg) was extracted with 70% methanol for 3 days at room temperature and filtered. Then, the solvent was evaporated to dryness under reduced pressure (Buchi, Switzerland). *A. graveolens* methanol extracts were prepared and analyzed as our previous described (Choosri *et al*., 2016).

Animals

Adult male Wistar rats (250 ± 50 g, 10 weeks old) were obtained from the Southern animal unit, Prince of Songkla University, Songkhla, Thailand. They were randomly housed 6 per cage and maintained in a clean room at a temperature of 26 ± 1ºC, with a 12 h light-dark cycle and a relative humidity of 50%. Rats were housed in metabolic cages and supplied with filtered pathogen-free air with access to food and water *ad libitum*. All administration activities in this study were performed once daily between 8:00-9:00 a.m. The experimental procedures were performed in accordance with the principles of animal care outlined by the Faculty of science, Prince of Songkla University, Songkhla, Thailand (MOE 0521.11/462).

Immobilization stress

On the 15th-21st day all the animals (except naive control) were immobilized for 12 h (6:00 p.m.-6:00 a.m.) by putting them in plexiglass tubes. In the unstressed group, the rats were kept in an animal cage with soft bedding in the experimental room. Following each stress session, animals were returned to their home cages and had free access to food and water for the remainder of the experiment.

Experimental procedures

The *A. graveolens* extract suspension and diazepam in distilled water was freshly prepared before being administered. All the treatments were

given once daily for three weeks and the animals were subjected to immobilization stress on the 15th to 21st day. All the animals were randomly divided into five groups with six animals in each group. The following protocol was used: Group I: unstressed animals (control, daily oral administration of 0.5 mL distilled water. Group II: stressed animals, daily oral administration of 0.5 mL distilled water. Group III-IV: stressed animals, with daily oral administration of *A. graveolens* extract at doses of 125 and 250 mg/kg, respectively. Group V: stressed animals, daily oral administration of diazepam 2 mg/kg.

Behavioral assessments

The rats were acclimatized for 5 min on each behavioral test apparatus consecutively for 3 days before initiating the actual experimental protocol, except for the Morris water maze test, were acclimatized for 1 min (5 times/day) consecutively for 5 days before initiating the actual experimental protocol. It was necessary to acclimatize the animals to the test apparatus in the behavioral studies to avoid any potentially conflicting effects induced by the novelty of the testing apparatus that in turn reduced the variations in the experimental data. Thereafter, the rats were subjected to immobilization stress and the battery of behavioral tests was performed with the sequence of the elevated plus maze, the hole board, the open field, the object recognition and the Morris water maze. All behavioral test equipments were cleaned after each test with alcohol and water. At the end of behavioral tests, blood was withdrawn from the heart for the estimation of corticosterone. The corticosterone levels were at their highest levels in the morning; therefore, all the experiments were performed during 8:00 a.m.-12:00 a.m.

Open field test

The open field has been considered to be a non-conditioned anxiety test based on the creation of a conflict between the exploratory drive of the rat and its innate fear to exposure in an open area (Angrini *et al*., 1998). The open field test has been employed to assess the spontaneous activity, general exploration and ambulation of the rodents. Each rat was placed individually in the center of the apparatus and observed for 5 min to record its locomotor activity (the number of line crossings), exploratory activity (indicated by frequency of rearing) and time spent in the center (Manchanda *et al*., 2011).

Elevated plus maze test

Anxiety was evaluated in an elevated plus maze. The experimental procedure was similar to that described by Pellow *et al*. (1985). Immediately after the pretest exposure rats were placed in the center of the elevated plus maze facing one of the open arms. During the 5 min test period the number of entries into the open arms and the time spent there were measured manually. An entry was defined as all four feet into one arm. An increase in open arms entries and increase in time spent in open arms were accepted as the measures of potential anxiolytic activity.

Hole board test

The hole board apparatus was used to determine a high-anxiety-like state in rats. Each animal was placed in the hole board and allowed to freely explore the apparatus for 5 min. The numbers of head dips by the rats were counted (Takeda *et al*., 1998).

Morris water maze test

In order to determine higher cognitive functions such as spatial memory which is believed to be involved with hippocampal functions, one of the most common behavioral paradigms for evaluating the rodents is the Morris water maze test. Animals were watched in a round polyvinyl water pool (120 cm diameter, 50 cm height) filled with water (25°C). Additional powder was used to provide the opaque water. The pool was divided equally into four quadrants: labeled N-S-E-W. A platform (10 cm diameter) was placed in one of the four quadrants (the target quadrant) and submerged 1.5 cm below the water surface. For animals, the location of the platform was invisible and it remained there throughout the training. The animals must memorize the environmental cues to locate the platform. Each animal was placed in the water in the starting quadrant and allowed to freely swim in the pool for 60 s or until it found and climbed onto the platform. During the training session, the mice were gently placed on the platform by the experimenter when it could not reach the platform in 60 s. In either case, the subject was left on the platform for 30 s and removed from the pool. The time for animals to climb onto the hidden platform was recorded as escape latency or acquisition time. In each trial, the animal was quickly dried with towel before being returned to the cage (Morris *et al*., 1984).

Object recognition test

The object recognition test was performed to determine the non-spatial memory of shape, color and texture. The apparatus consisted of a circular area 100 cm in diameter with a 40 cm high wall. The open field and the objects were cleaned between each trial using 70% ethanol to avoid odor trails. Before the experimental day, the animals were allowed to

acclimatize to the experimental environment. During habituation, the animals were allowed to freely explore the apparatus without objects for 5 min, once a day for three consecutive days before testing. On the experimental day, animals were submitted to two spaced trials. During the first trial (T1), animals were placed in the area containing the same two identical objects for an amount of time necessary to spend 15 s exploring these two objects in a limit of 3 min. Any mice which did not explore the objects for 15 s within the 3 min period were excluded from the experiments. 1 h after exposing to the first trial, the animal was exposed to the second trial (T2). According to this trial, one of the objects present in the first trial was replaced by a novel object. Animals were placed back in the area for 3 min, the total times which the animals spent to explore or directed their nose within 2 cm of the object while looking at, sniffing, or touching the novel object were recorded and recognized as total exploration time on the novel object or the time of approach to both objects were recorded and calculated according to the equation (Antunes and Biala, 2012).

Discrimination index = (A-B) / (A+B)

A = Time of approach to object 2
B = Time of approach to object 1

Biochemical parameters

At the end of the experimental periods, each rat was deeply anesthetized with sodium thiopental 50 mg/kg BW intraperitonelly. Whole blood was immediately withdrawn from the heart and collected in heparin-coated test tubes and centrifuged at 1500 g for 10 min to separate plasma from the erythrocytes. All the animals were then perfused by intracardiac perfusion with 500 ml 0.9% normal saline solution for 30 minutes and immediately separated from the brain area. The protein content from brain tissue was measured according to the method of Lowry *et al*. (1951) using bovine serum albumin as a standard.

Plasma corticosterone measurement

Post-stress corticosterone was measured in the plasma by an enzyme-linked immunosorbant assay using the corticosterone ELISA kits in accordance with the manufacturer's protocol (Enzo life sciences Inc, USA.). The resulting concentration of plasma corticosterone was expressed as ng/mL using prepared corticosterone standards.

Assay for monoamine oxidase (MAO-A) activity

The MAO-A activity from the supernatant of the brain tissues were determined by spectrophotometric measurement using the method of Elliott *et al*. (1991) with a slight modification (Holt *et al*., 1991). Brain tissue homogenates were incubated with the mixed substrate and 500 µM tyramine plus 500 nM pargyline to inhibit MAO-B. The chromogenic solution contained vanillic acid (1 mM), 4-aminoantipyrine (500 mM) and peroxidase (4 U/mL) in potassium phosphate buffer (0.2 M, pH 7.6). The assay mixture contains 50 µL of supernatant, 50 µL chromogenic solution and 100 µL of 100 mM amine substrate plus 1% H_2O_2 and the optical density (OD) was measured at 490 nm. The MAO-A activity was designated as µmol/min/ g tissue.

Lipid peroxidation assay

The quantitative measurement of lipid peroxidation in the brain tissues were assessed as per the modified method of Wills (1966). The amount of malondialdehyde (MDA) formed was measured by the reaction with thiobarbituric acid at 532 nm using a microplate reader. The results were expressed as micromole of malondialdehyde per mg protein.

Superoxide anion (O_{-2}) assay

The O_{-2} level was determined by a spectrophotometric measurement. The method was based on a xanthine/xanthine oxidase (XO) system that converted nitro blue tetrazolium (NBT)-yellow color to a formazan-blue color. The reagent mix was prepared with EDTA, NBT, xanthine and XO, mixed with a sample and measured at 560 nm compared with the standard curve of TEMPOL. The data was expressed as % inhibition which was calculated following the equation (Toda *et al*., 1991).

% inhibition = (A-B)/A x 100

A= OD of reagent only
B = OD of sample

Assay for GPx activity

The GPx activity was determined by the method of Wendel (1980), which measures the activity indirectly by a coupled reaction with glutathione reductase. The oxidation of NADPH to $NADP^+$ was accompanied by a decrease in absorbance at 340 nm. The rate of decrease in the A_{340nm} level was directly proportional to the GPx activity. The final 1 mL of the system mixture contained 48 mM sodium phosphate, 0.38 mM EDTA, 0.12 mM β-NADPH, 0.95 mM sodium azide, 3.2 units of glutathione reductase, 1 mM glutathione (GSH), 0.02 mM DL-dithiothreitol,

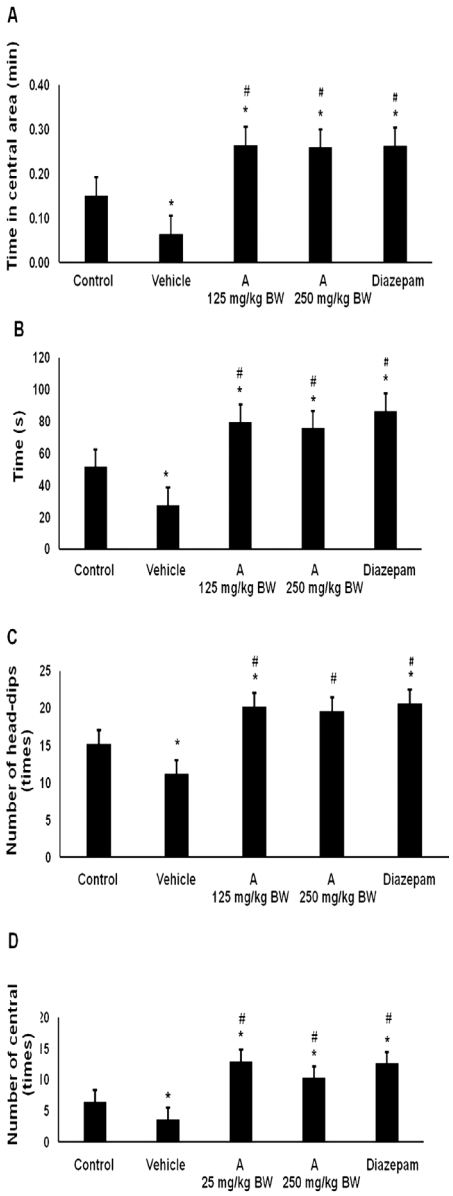

Figure 1. Stress indices after *A. graveolens* (A) Time spent in central (open field) (B) Time spent in open arms (elevated plus maze) (C) Number of head-dips (hole broad test) (D) Number of line crossing of the central area Data are presented as the mean ± standard error of the mean. *P<0.05 compared with the control; #P<0.05 compared with the vehicle

0.0007% H_2O_2, and the standard enzyme GPx solution or supernatant from the brain tissue sample. The GPx solution was used to represent the standard enzyme activity. The standard curve was plotted as the rate of A_{340nm} per min against the GPx activity. One unit of activity was defined as the amount of enzyme necessary to catalyze the oxidation by H_2O_2 of 1 µmol of GSH to GSSG per min at pH 7 and 25°C. The data were reported in units of GPx per g protein.

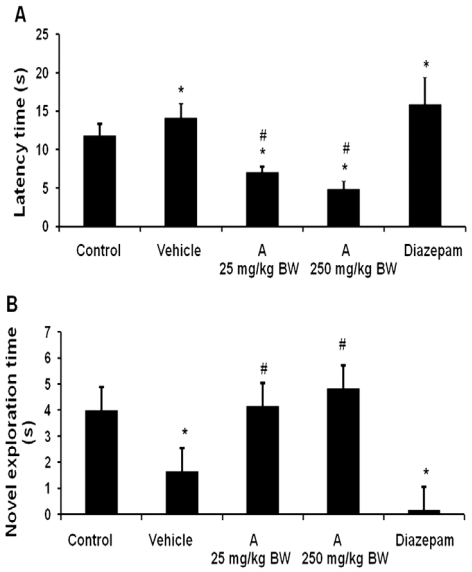

Figure 2. Morris water maze test and object recognition test were used to assess the effects of *A. graveolens* on learning and memory (A) Latency time of the Morris water maze test after stress. (B) Novel exploration time of the object recognition test after stress. Data are presented as the mean ± standard error of the mean. *P<0.05 compared with the control; #P<0.05 compared with the vehicle.

Statistical analysis

All data are presented as mean values ± standard error of the mean (SEM). Statistical analysis using Statistical Package for the Social Sciences (SPSS) was performed by one-way analysis of variance (ANOVA), followed by the Least Significant Difference (LSD's post-hoc test). A probability value of less than 0.05 was considered significant.

Results

Behavioral estimations

The control animals showed consistent and stable locomotor activity, exploratory activity and anxiety-like behavior. The immobilization stress had significantly reduced the locomotor activity and exploratory activity (as indicated by the decreased number of line crossings and frequency of rearing in the open field test) and induced anxiety-like behavior (decreased time spent in central area in the open field test, decreased frequency of head dips in the hole board test and decreased time spent in an open arm in the elevated plus maze test) as compared to the unstressed control group (P<0.05) (Figure 1). Twenty-one days of *A. graveolens* extract (both 125 and 250 mg/kg) treatment had significantly inhibited the anxiety-like behavior (increased time spent in the open arm (P<0.05 and P<0.05), frequency of head dips) (P<0.05 and P<0.05), improved locomotor

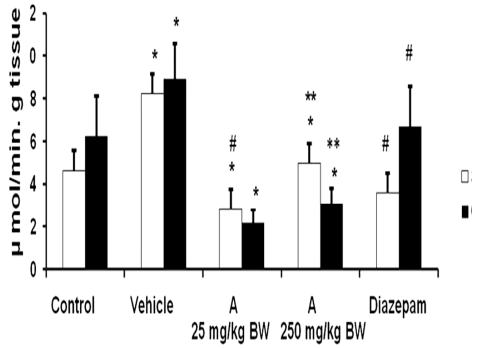

Figure 3. Post-stress corticosterone level in the plasma. Data are presented as the mean ± standard error of the mean. *P<0.05 compared with the control; #P<0.05 compared with the vehicle.

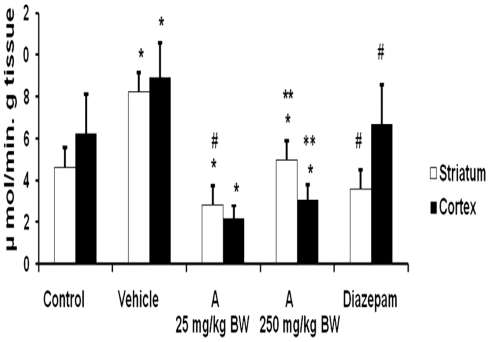

Figure 4. The MAO-A activity in striatum and cortex after stress. Data are presented as the mean ± standard error of the mean. *P<0.05 compared with the control; #P<0.05 compared with the vehicle; ** compared with diazepam.

activity (increased number of line crossings) (P<0.05 and P<0.05) and exploratory activity (increased frequency of rearing (data not shown)) as compared to the vehicle (restraint stress) group (P<0.05 and P<0.05). Diazepam also showed the same pattern with *A. graveolens* extract but was more potent in increasing the time spent in the opened arm of the elevated plus maze test than both concentration of *A. graveolens* extract (Figure 1). In the Morris water maze test, stress led to a prolongation of the average escape latency, which indicated a spatial learning deficit. Treatment with *A. graveolens* extract prior to stress prevented this spatial deficit but diazepam showed more spatial deficit (Figure 2). This is consistent with the object recognition test, stress decreased novel exploration time when compared to the vehicle group. Administration of *A. graveolens* extract also significantly increased the novel exploration time as compared to the vehicle group (P<0.05) but diazepam was better at reducing the novel exploration time (Figure 2).

Table 1. Effects of *A. graveolens* extract following immobilization stress on the MDA levels, % inhibition of O_{-2} and antioxidant enzyme activity.

Groups	MDA		O_2^-		GPx	
	striatum	cortex	striatum	cortex	striatum	cortex
Control	0.08 ± 0.02	0.08 ± 0.03	49.99 ± 8.03	40.16 ± 10.94	21.29 ± 9.01	10.34 ± 2.52
Vehicle	0.13 ± 0.03*	0.12 ± 0.04*	19.36 ± 13.71*	14.67 ± 7.86*	5.52 ± 2.34*	5.57 ± 2.41*
A 125 mg/kg	0.10 ± 0.02#	0.08 ± 0.02#	42.50 ± 16.63#	41.42 ± 8.65#	9.44 ± 1.40#	9.77 ± 1.87#
A 250 mg/kg	0.07 ± 0.02#	0.08 ± 0.07#	49.16 ± 10.94#	46.75 ± 7.62#	10.63 ± 3.42#	12.20 ±3.74#

Data are presented as the mean ± standard error of the mean. *P<0.05 compared with the control; #P<0.05 compared with the vehicle.

Corticosterone level

Figure 3 shows the plasma corticosterone level in all the experimental groups and indicated the significant differences among the groups. Comparison of the control group and vehicle group indicated that the immobilization stress significantly increased plasma corticosterone (P<0.05). In contrast, the corticosterone level in the *A. graveolens* extract groups (125 and 250 mg/kg) were significantly reduced after treatment with *A. graveolens* extract (P<0.05). These finding indicated that *A. graveolens* extract did significantly prevent stress-induced elevation of corticosterone levels.

Monoamine oxidase-A (MAO-A) activity

The vehicle (immobilization stress) group exhibited a significant higher level of brain MAO-A activity as compared to the control (unstressed rats) in both the cortex and striatum of the brain (P<0.05 and P<0.05). *A. graveolens* extract (125 and 250 mg/kg) and diazepam (2 mg/kg) administered for 21 days significantly reduced brain MAO-A activity as compared to the vehicle group (P<0.05 and P<0.05) (Figure 4).

Brain lipid peroxidation and antioxidant enzyme activity

Effects of *A. graveolens* extract following immobilization stress on the MDA levels, % inhibition of O_{-2} and antioxidant enzyme activity are shown in Table 1. Levels of MDA were significantly increased in the immobilization stress group as compared to the unstressed group; while administration of *A. graveolens extract* (125 and 250 mg/kg) significantly lowered the levels of MDA (P<0.05). A significant decrease in levels of GPx and the % inhibition of O_{-2} were observed after immobilization stress as

compared to the unstressed group ($P<0.05$), that indicated induction of stress in the rats. *A. graveolens* extract (125 and 250 mg/kg) produced a significant rise in the levels of the % inhibition of the O_{-2}, GPx activity and markedly decrease MDA as compared to the vehicle group ($P<0.05$).

Discussion

Stress is able to activate the HPA axis and change the catecholamine, GABA and serotonin levels. Stress thereby influences several neurological functions at both the central and peripheral levels (Von Borell, 2001), and exposure to chronic immobilization stress in animals and psychological stress in humans has implicated the pathophysiology of anxiety and mood disorder (Walesiuk *et al*., 2006). Immobilization stress, both acute and chronic, has affected motor activity, anxiety-like behavior, and depression-like behavior in animals (Sevgi *et al*., 2006). Furthermore chronic immobilization stress has been reported to influence cognitive impairment (Nishimura *et al*., 1999; Sevgi *et al*., 2006). In the present study, immobilization stress for 12 h daily for 7 days indicated similar results to previous reports along with neurobehavioral alterations due to acute and chronic stress. In this study the behavioral changes observed in the experiments might be due to alterations in the brain regions that control motor activity and anxiety-like behavior. *A. graveolens* extract (125, 250 mg/kg, p.o.) provided significant protection against immobilization stress. The neuroprotective effect against stressful conditions occurred via locomotor activity and anxiety-like behavior, oxidative damage control, monoaminergic system changes and elevation of corticosterone levels.

The HPA axis activity can be regulated by monoamines (Garabadu *et al*., 2011; Krishnamurthy *et al*., 2013). Generally the monoamieric level varies among brain regions such as the hippocampus, hypothalamus, prefrontal cortex and amygdale in stress conditions (Greenwood *et al*., 2001; Garabadu *et al*., 2011; Krishnamurthy *et al*., 2013). In this study *A. graveolens* did cause changes in the monoaminergic system in both the cortex and striatum regions as shown by the decrease of the MAO-A enzyme. Moreover, a corticotrophin-releasing factor (CRF) was considered to be part of the extra-hypothalamic stress system. Due to the abundance of CRF terminals and receptors, Amygdala becomes an important neuroanatomical area for the extra-hypothalamic stress system (Jankord and Herman, 2008). Prior to *A. graveolens* treatment, changes in the monoaminergic system induced by immobilization stress in rats could be due to decreased corticosterone levels. A similar result for corticosterone was also observed in our diazepam treated group, however the mechanism seems to be different from the case of the *A. graveolens* treated group (Kalman *et al*., 1997).

Hippocampal areas are heavily engaged in cognitive processes and are very sensitive to corticosteroid damage (McEwen, 2000, 2001; Belanoff *et al*., 2001), partly because of their high density of mineralcorticoids (MRs) and GRs. The activation of the hippocampal GRs may, to a great extent, be responsible for the behavioral deficits in animal and humans with hypercortisolemia (Pavlides *et al*., 1995; McEven, 2000; Lupien *et al*., 2001; Roozendaal *et al*., 2003). The result from our study was consistent with other reported studies that immobilization stress in rats increased corticosterone secretion and decreased memory (Nooshinfar *et al*., 2011) whereas the ability of *A. graveolens* extracts provided an effect that was in contrast to the corticosterone level and memory. In another way, gamma-aminobutyric acid (GABA) is the principal inhibitory neurotransmitter of the brain and its main actions are mediated by fast-acting GABA-A receptors. Binding of benzodiazepines at the benzodiazepine site of GABA-A receptors resulted in a potentiation of inhibitory neurotransmission with consequent behavioral changes. Their capacity to elicit sedative, anxiolytic, myorelaxant, hypnotic and antiepileptic effects has made benzodiazepines the drugs widely used in clinical practice. However, they may also induce some effects, such as amnesia (McNamara and Skelton, 1991; Nakamura and Roelke, 1997; Stewart, 2005; Sasaki *et al*., 2013). Our result correspond to previous findings that showed that the escape latency of spatial memory in the Morris water maze test and the discrimination index in the object recognition test were impaired in the diazepam-treated group but not in the *A. graveolens*-treated rats. We thus provide further evidence for the potential usefulness of *A. graveolens* as an anxiolytic without causing amnesia. Furthermore, *A. graveolens* (125, 250 mg/kg) displayed a similar efficacy to diazepam in the suppression anxiety in immobilization stressed rats.

Apart from the mentioned immobilization stress that contributed to a drastic increase in the reactive oxygen species and consequent oxidative damage with a proportionate decrease in the intracellular antioxidant defense (Şahin and Gümüşlü, 2004). Oxidative stress leads to cellular damage and accelerates neuronal death by inducing ROS via oxidizing vital cellular components such as lipids, proteins and DNA (Voronych and Lemel, 1994;

Marzatico *et al*., 1998; Şahin and Gümüşlü, 2004). In our study, immobilized stress for 12 h daily for 7 days caused significant oxidative damage as indicated by the rise in MDA, GPx activity and a decrease of the % inhibition of the O_{-2}. Pretreatment with *A. graveolens* extracts significantly attenuated the MDA concentration, decreased the GPx activity and increased the % inhibition of the superoxide anion and this indicated its antioxidant-like effect. In support of our study, a clinical trial also indicated a raised level of MDA in patients with affective disorders (Ozcan *et al*., 2004). In accordance with the above findings, in the present study, *A. graveolens* elevated the antioxidant enzyme defense system against immobilization stress-induced oxidative damage. The possible mechanism behind the protective effect of *A. graveolens* is inhibition of synthesis of the O_{-2} in the brain.

Conclusion

Our finding has proposed that an *A. graveolens* extract can ameliorate the immobilization-stress-induced oxidative stress, biochemical alterations and associated anxiety-like behavior in the rat, as well as its involvement of the neurotransmitter pathway in the protective effect of the *A. graveolens* extract against immobilization-stress-induced anxiety-like behavior without memory loss. In summary *A. graveolens* could serve as a novel approach for anxiety treatment.

Acknowledgements

This project received financial support from Development and Promotion of Science and Technology Talents Project, General project and invention of Prince of Songkla University (SCI570371S), Thailand and BRAND'S Brain Research Centre, Thailand. Thanks to Dr Brian Hodgson for assistance with the English.

References

Angrini, M., Leslie, J.C. and Shephard, R.A. 1998. Effects of propranolol, buspirone, pCPA, reserpine, and chlordiazepoxide on open-field behavior. Pharmacology Biochemistry and Behavior 59(2): 387-397.

Antunes, M. and Biala, G. 2012. The novel object recognition memory: neurobiology, test procedure, and its modifications. Cognitive Processing 13(2): 93-110.

Belanoff, J.K., Gross, K., Yager, A. and Schatzberg, A.F. 2001. Corticosteroids and cognition. Journal of Psychiatric Research 35(3): 127-145.

Cahill, L., Gorski, L. and Le K. 2003. Enhanced human memory consolidation with post-learning stress: interaction with the degree of arousal at encoding. Learning and Memory 10(4): 270-274.

Chen, H.Q., Jin, Z.Y., Wang, X.J., Xu, X.M., Deng, L. and Zhao, J.W. 2008. Luteolin protects dopaminergic neurons from inflammation-induced injury through inhibition of microglial activation. Neuroscience Letters 448(2): 175-179.

Choosri, N., Tanasawet, S., Chonpathompikunlert, P. and Sukketsiri, W. *Apium graveolens* extract attenuates adjuvant induced arthritis by reducing oxidative stress. Journal of Food Biochemistry (online) doi:10.1111/jfbc.12276.

Cochrane, C.G. 1999. Mechanisms of oxidant injury of cells. Molecular Aspects of Medicine 12: 137-147.

Elliott, J., Fowden, A.L., Callingham, B.A., Sharman, D.F. and Silver, M. 1991. Physiological and pathological influences on sheep blood plasma amine oxidase: effect of pregnancy and experimental alloxan-induced diabetes mellitus. Research in Veterinary Science 50(3): 334-339.

Feng, X., Peng, Y., Liu, M. and Cui, Y. 2012. DL-3-n-Butylphthalide extends survival by attenuating glial activation in a mouse model of amyotrophic lateral sclerosis. Neuropharmacology 62(2): 1004-1010.

Garabadu, D., Shah, A., Ahmad, A., Joshi, V.B., Saxena, B., Palit, G. and Krishnamurthy, S. 2011. Eugenol as an anti-stress agent: modulation of hypothalamic-pituitary-adrenal axis and brain monoaminergic systems in a rat model of stress. Stress 14(2): 145-155.

Goyal, R. and Kumar, A. 2007. Protective effect of alprazolam in acute immobilization stress-induced certain behavioral and biochemical alterations in mice. Pharmacological Reports 59(3): 284-290.

Greenwood-Van Meerveld, B., Gibson, M., Gunter, W., Shepard, J., Foreman, R. and Myers, D. 2001. Stereotaxic delivery of corticosterone to the amygdale modulates colonic sensitivity in rats. Brain Research 893(1-2): 135-142.

Gutteridge, J.M. 1995. Lipid peroxidation and antioxidants as biomarkers of tissue damage. Clinical Chemistry 41(12): 1819-1828.

Halliwell, B. and Gutteridge, J.M.C. 1985. Oxygen radicals and the nervous system. Trends in Neuroscience 8: 22-26.

Holt, A., Sharman, D.F., Baker, G.B. and Palcic, M.M. 1997. A continuous spectrophotometric assay for monoamine oxidase and related enzymes in tissue homogenates. Analytical Biochemistry 244(2): 384-392.

Iswantini, D., Ramdhani, T.H. and Darusman, .L.K. 2012. *In vitro* inhibition of celery (*Apium graveolens* L.) extract on the activity of xanthine oxidase and determination of its active compound. Indonesian Journal of Chemistry 12(3): 247-254.

Jain, G.C., Pareek, H., Khajja, B.S., Kusum J., Jhalani, S., Agarwal, S. and Sameer, S. 2009. Modulation of di (2 ethylhexyl) phthalate induced hepatic toxicity by

Apium graveolens L. seeds extract in rats. African Journal of Biochemistry Research V 3(5): 222-225.

Jankord, R. and Herman, J.P. 2008. Limbic regulation of hypothalamo-pituitary-adrenocortical function during acute and chronic stress. Annals of the New York Academy of Sciences 1148: 64-73.

Joëls, M., Pu, Z., Wiegert, O., Oitzl, M.S. and Krugers, H.J. 2006. Learning under stress: how does it work? Trends in Cognitive Sciences 10(4): 152-158.

Kalman, B.A., Kim, P.J., Cole, M.A., Chi, M.S. and Spencer, R.L. 1997. Diazepam attenuation of restraint stress-induced corticosterone levels is enhanced by prior exposure to repeated restraint. Psychoneuroendocrinology 22(5): 349-360.

Kovacs, P., Juranek, I., Stankovicova, T. and Svec, P. 1996. Lipid peroxidation during acute stress. Die Pharmazie 51(1): 51-53.

Krishnamurthy, S., Garabadu, D. and Reddy, N.R. 2013. *Asparagus racemosus* modulates the hypothalamic-pituitary-adrenal axis and brain monoaminergic systems in rats. Nutritional Neuroscience 16(6): 255-261.

Liu, J., Wang, X., Shigenaga, M.K., Yeo, H.C., Mori, A. and Ames, B.N. 1996. Immobilization stress causes oxidative damage to lipid, protein, and DNA in the brain of rats. Federation of American Societies for Experimental Biology 10(13): 1532-1538.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. The Journal of Biological Chemistry 193: 265-75.

Lupien, S.J. and Lepage, M. 2001. Stress, memory, and the hippocampus: can't live with it, can't live without it. Behavioral Brain Research 127(1-2): 137-158.

Manchanda, R.K., Jaggi, A.S. and Singh, N. 2011. Ameliorative potential of sodium cromoglycate and diethyldithiocarbamic acid in restraint stress-induced behavioral alterations in rats. Pharmacological Reports 63(1): 54-63.

Marzatico, F., Bertorelli, L., Pansarasa, O., Paola, G., Carla, T. and Giuseppe, B. 1998. Brain oxidative damage following acute immobilization and mild emotional stress. International Journal of Stress Management 5(4): 223-236.

McEwen, B.S. 2000. The neurobiology of stress: from serendipity to clinical relevance. Brain Research 886(1-2): 172-189.

McEwen, B.S. 2001. Stress, sex, hippocampal plasticity: relevance to psychiatric disorders. Clinical Neuroscience Research 1(1-2):19-34.

McIntosh, L.J. and Sapolsky, R.M. 1996. Glucocorticoids increase the accumulation of reactive oxygen species and enhance adriamycin-induced toxicity in neuronal culture. Experimental Neurology 141(2): 201-206.

McNamara, R.K. and Skelton, R.W. 1991. Diazepam impairs acquisition but not performance in the Morris water maze. Pharmacology Biochemistry and Behavior 38(3): 651-658.

Morris, R. 1984. Developments of a water-maze procedure for studying spatial learning in the rat. Journal of Neuroscience Methods 11(1): 47-60.

Nadeem, A., Masood, A., Masood, N., Gilani, R.A. and Shah, Z.A. 2006. Immobilization stress causes extra-cellular oxidant-antioxidant imbalance in rats: restoration by L-NAME and vitamin E. European Neuropsychopharmacology 16(4): 260-267.

Nagata, K., Nakashima-Kamimura, N., Mikami, T., Ohsawa, I. and Ohta, S. 2009. Consumption of molecular hydrogen prevents the stress-induced impairments in hippocampus-dependent learning tasks during chronic physical restraint in mice. Neuropsychopharmacology 34(2): 501-508.

Nakamura-Palacios, M. and Roelke, C.E. 1997. Effects of acute or daily administration of diazepam on spatial learning and working memory. Drug and Alcohol Dependence 46(3): 181-190.

Nishimura, J., Endo, Y. and Kimura F. 1999. A long-term stress exposure impairs maze learning performance in rats. Neuroscience Letters 273(2): 125-128.

Nooshinfar, E., Akbarzadeh-Baghban, A. and Meisami, E. 2011. Effects of increasing durations of immobilization stress on plasma corticosterone level, learning and memory and hippocampal BDNF gene expression in rats. Neuroscience Letters 500(1): 63-66.

Oitzl, M.S., Reichardt, H.M., Joels, M. and de Kloet, E.R. 2001. Point mutation in the mouse glucocorticoid receptor preventing DNA binding impairs spatial memory. Proceedings of the National Academy of Sciences 98(22): 12790-12795.

Ozcan, M.E., Gulec, M., Ozero, l.E., Polat, R. and Akyoil, O. 2004. Antioxidant enzyme activities and oxidative stress in affective disorders. International Clinical Psychopharmacology 19(2): 89-95.

Park, S.Y., Kim, H.S., Hong, S.S., Donggeun, S., Kwang, W.H. and Dongho, L. 2009. The neuroprotective effects of traditional oriental herbal medicines against β-amyloid-induced toxicity. Pharmaceutical Biology 47(10): 976-981.

Pavlides, C., Watanabe, Y., Magarinos, A.M. and McEwen, B.S. 1995. Opposing roles of Type I and Type II adrenal steroid receptors in hippocampal long-term potentiation. Neuroscience 68(2): 387-394.

Pellow, S., Chopin, P. and Briley, M. 1985. Validation of open: closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. Journal of Neuroscience Methods 14(3): 149-167.

Peng, Y., Sun, J., Hon, S., Nylander, A.N., Xia, W., Feng, Y., Wang, X. and Lemere, C.A. 2010. L-3-n-Butylphthalide improves cognitive impairment and reduces amyloid-β in a transgenic model of Alzheimer's disease. The Journal of Neuroscience 30(24): 8180-8189.

PopoviÇ, M., KaurinoviÇ, B., TriviÇ, S., Mimica-Dukić, N. and Bursać, M. 2006. Effect of celery (*Apium graveolens*) extracts on some biochemical parameters of oxidative stress in mice treated with carbon tetrachloride. Phytotherapy Research 20(7): 531-537.

Rai, D., Bhatia, G., Palit, G., Pal, R., Singh, S., Singh, H.K. 2003. Adaptogenic effect of *Bacopa monniera* (Brahmi). Pharmcology Biochemistry Behaviour

75(4): 823–830.

Roozendaal, B., Griffith, Q.K., Buranday, J., De Quervain, D.J. and McGaugh, J.L. 2003. The hippocampus mediates glucocorticoid-induced impairment of spatial memory retrieval: dependence on the basolateral amygdale. Proceedings of the National Academy of Sciences 100(3): 1328-1333.

Şahin, E. and Gümüşlü, S. 2004. Alterations in brain antioxidant status, protein oxidation and lipid peroxidation in response to different stress models. Behavioural Brain Research 155(2): 241-248.

Sasaki-Hamada, S., Sacai, H., Sugiyama, A., Iijima, T., Saitoh, A., Inagaki, M., Yamada, M. and Oka, J. 2013. Riluzole does not affect hippocampal synaptic plasticity and spatial memory, which are impaired by diazepam in rats. Journal of Pharmacological Sciences 122(3): 232-236.

Sevgi, S., Özek, M. and Eroğlu, L. 2006. L-NAME prevents anxiety-like and depression-like behavior in rats exposed to restraint stress. Methods and Findings in Experimental and Clinical Pharmacology 28(2): 95-99.

Sheikh, N., Ahmad, A., Siripurapu, KB., Kuchibhotla, VK., Singh, S., Palit, G. 2007. Effect of *Bacopa monniera* on stress induced changes in plasma corticosterone and brain monoamines in rats. Journal of Ethnopharmacology 111 (3): 671–676.

Stewart, S.A. 2005. The effects of benzodiazepines on cognition. Journal of Clinical Psychiatry 66(2): 9-13.

Takeda, H., Tsuji, M. and Matsumiya, T. 1998. Changes in head-dipping behavior in the hole-board test reflect the anxiogenic and/or anxiolytic state in mice. European Journal of Pharmacology 350(1): 21-29.

Toda, S., Kumura, M. and Ohnishi, M. 1991. Effects of phenolcarboxylic acids on superoxide anion and lipid peroxidation induced by superoxide anion. Planta Medica 57(1): 8-10.

Viswanatha, GL., Shylaja, H., Sandeep, Rao KS., Santhosh, Kumar VR., Jagadeesh, M. 2012. Hesperidin ameliorates immobilization-stress-induced behavioral and biochemical alterations and mitochondrial dysfunction in mice by modulating nitrergic pathway. International Scholarly Research Network (online) doi: 10.5402/2012/479570

Von Borell, E.H. 2001. The biology of stress and its application to live-stock housing and transportation assessment. Journal of Animal Science 79: 260-267.

Voronych, N.M. and Lemel'ianenko, I.V. 1994. Lipid peroxidation and antioxidant system activity in the brain, stomach and heart tissues and blood serum of rats under stress. Fiziol Zh 40(5-6): 114-117.

Walesiuk, A., Trofimiuk, E. and Braszko, J.J. 2006. Ginkgo biloba normalizes stress-and corticosterone-induced impairment of recall in rats. Pharmacological Research 53(2): 123-128.

Wendel, A. 1980. Enzymatic basis of detoxification. In: Jacoby, W.B. (Eds). Glutathione Peroxidase, p.333-353. New York: Academic Press.

Wills, E.D. 1966. Mechanisms of lipid peroxide formation in animal tissues. Biochemical Journal 99(3): 667-676.

Yao, Y. and Ren, G. 2011. Effect of thermal treatment on phenolic composition and antioxidant activities of two celery cultivars. LWT-Food Science and Technology 44(1): 181-185.