American Journal of Agricultural and Biological Sciences 8 (2): 126-134, 2013
ISSN: 1557-4989

doi:10.3844/ajabssp.2013.126.134 Published Online 8 (2) 2013 (http://www.thescipub.com/ajabs.toc)

Evaluation of Acute Toxicity and Neuropharmacological Activities of PM-52

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Received 2012-04-22, Revised 2012-05-05; Accepted 2013-05-27

ABSTRACT

The neuropsychological disorders are increasing their importance nowadays. Unfortunately, the therapeutic efficacy is still not in satisfaction level. Therefore, the novel agents that are effective, safe, cheap and easy to approach are in required. Therefore, we aimed to determine the neuropharmacological activities of PM52, the novel herbal supplement. We had developed the PM52, a novel product comprising of *Cissampelas pariera* and *Anethum graveolens* at aconcentarion of 1: 5. Then, it was determined acute toxicity and the neuropharmacological activities of PM52. Male Wistar rats, weighing 180-220 g, were orally given PM52 up to the dose of 5000 mg.kg^{-1} BW and observed acute toxicity. The neuropharmacological activities of PM52 were also determined. Various doses of PM52 ranging from 2, 10 and 50 mg.kg^{-1} BW were orally given once daily to the animals at a period of 14 days. Anxiolytic, anti-depression and cognitive enhancing effects were evaluated after single administration and every 7 days throughout 14-day experimental period using elevated plus maze, forced swimming test and Morris water maze tests respectively. PM 52 was safe up to dose of 5000 mg kg^{-1} BW. Moreover, it also showed anxiolytic-like activity and cognitive enhancing effect. Moreover, it also enhanced climbing activity in forced swimming test. PM 52 is the potential novel food supplement to enhance cognitive function and decrease anxiety. However, further researches on subchronic toxicity, possible active gradient and shelf life of product are also necessary before moving forward to clinical trial study.

Keywords: World Health Organization (WHO), Central Nervous System (CNS), Standard Error of Mean (SEM), Organization Economic Cooperation and Development (OECD)

1. INTRODUCTION

To date, the neuropsychological disorders are continually increasing their importance. Recent report of World Health Organization (WHO) has demonstrated that the global prevalence of mental and brain disorders, particularly anxiety, depression and dementia are dramatically increased. Approximate 450 million people worldwide had suffered from mental problems especially mood disorders and dementia (Brundtland, 2001). Though, they are very important, the therapeutic efficacies of available drugs are not in satisfaction level due to their side effects and some drugs are expensive. Therefore, the novel agents that are effective, safe, cheap and easy to approach are in required.

Herbal medicines have been long term recognized as popular remedies for diseases used by a vast majority of world population. In addition, herbal

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formulations are attained widespread acceptability as therapeutic agents. Herbs are relatively cheap and available. They also represent untapped reservoir of drugs. Therefore, the development of novel therapeutic agent against neuropsychological disorders from herbs has gained very much attention.

Numerous herbs are used for treating neurological disorders including *Cissampelos pariera* and *Anethum graveolen*. *C.pariera* has been shown to enhance sense of well being and memory (Kulkani *et al*., 2011) while *A.graveolen* has been reported to possess anti-amnesia and anti-stress (Koppula and Choi, 2011). Based on the reputation of both plants on Central Nervous System (CNS), we hypothesized that the combination of both plants at appropriate ration might provide wide spread activities on the CNS. To date, no scientific data were available. Therefore, we aimed to determine the neuropharmacological activities of PM 52, a combined extract of *C.pariera* and *A.graveolens*. In addition the acute toxicity of this novel agent was also determined to assure the consumption safety

2. MATERIALS AND METHODS

2.1. Preparation of PM52

The dried herbs of *Cissampelos pariera* and *Anethum graveolen* were purchased from organic farms of Srithat District, Udorn Thani province. They were identified morphologically, histologically and authenticated by Associate Professor Panee Sirisa-ard, Faculty of Pharmacy, Chiangmai University. Voucher specimens were kept at Integrated Complimentary Alternative Medicine Research and Development Group, Khon Kaen University.

Powder of the *C.pariera* and *A.graveolen* were mixed at a ratio of 1:5 and extracted in water: Alcohol (50:50). Then, the extract was freeze dried to powder with evaporator.

2.2. Animals

Healthy male Wistar rats (180-220 g, 8 weeks old) were obtained from National Laboratory Animal Center, Salaya, Nakorn Pathom. They were housed in group of 4 per cage in standard metal cages at 22±2°C on 12:12 h light-dark cycle. All animals were given access to food and water ad labium. The 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). The experimental protocols were approved by the Institutional Animal Care and Use Committee (AE006/54).

2.3. Drugs

Diazepam (2 mg/tablet), Fluoxetine (20 mg/tablet), Donepezil (10 mg/tablet) (Government Pharmaceutical Organization) were used as standard drugs in this study. All drugs and PM52 were dissolved in distill water which used as vehicle to a desired concentration. Then, they were filtered through gauze and given to the animals via the intragastric feeding tube. All administered substances including the PM52 suspension were freshly prepared.

2.4. Acute Toxicity Study

Adult male Wistar rats were acclimatized to conditions in the laboratory (room temperature, 60-80% relative humidity, day night cycle) for 10 days prior to the commencement of the treatment, during which they received food and tap water ad libitum. Acute oral toxicity was done according to OECD guidelines. The extract of PM52 was administrated per os to rats of groups 10 in a single dose/day of 5000 $mg.kg^{-1}$ BW. The control group received an equal volume of vehicle.

Observations of acute toxic symptoms were made and recorded systematically 1, 2, 4, 6 and 24 h. after administration of the extract. The number of rats that survived were noted after 24 h and then maintained for further 3 days with daily observations. This visual observation included skin changes, mobility and aggressiveness, sensitivity to sound and pain, as well as respiratory movements. The acute toxic effects of the extract were assessed on the basis of mortality, which was expressed as LD_{50}.

2.5. Experimental Protocol

All rats were randomly divided into 6 groups. Each group contained 8 rats:

Group I: Naïve intact control rats.
Group II: Vehicle treated group. The animals in this group were treated with distill water
Group III: Positive control treated group. In each test, the positive control group was treated with the standard drugs used for treating the related disorders. In the determination of anxiolytic effect, the animals were treated with Diazepam (2 mg kg^{-1} BW). In addition, the positive

control treated group was treated with Fluoxetine (20 mg kg^{-1} BW) in the determination of anti-depressant effect while during the determination of cognitive function, the positive control group was treated with Donepezil (1 $mg.kg^{-1}$ BW).

Group IV-VI: PM 52 treated group. The animals in group IV-VI were treated with the alcoholic extract of PM 52 at various doses ranging from 2, 10 and 50 $mg.kg^{-1}$ BW. respectively via oral route for 2 weeks once daily throughout the experimental period.

The animals in all groups were assessed all behavioral tasks except that in the assessment of spontaneous locomotor behaviors, there was no positive control treated group.

2.6. Behaviors Evaluation

The rats were divided into various groups as mentioned earlier. The behavioral profiles were assessed both after the single dose and after the repetitive administrations of PM52 (7 and 14 days). Each animal was subjected to the following behavior taskforces (a) Elevated plus maze test (b) Forced swimming test (c) Morris water maze test (d) Stereotype behaviors.

2.7. Elevated Plus Maze Test

The elevated plus maze for rat consisted of opened arms (50×10 cm) and two enclosed arms (50×10 cm) with 40 cm high walls, extending from a central platform (10×10 cm). The arms were connected with a central square, 10×10 cm, to give the apparatus a plus sign appearance. The maze was raised to a height of 50 cm above floor. The maze floor and walls were constructed from dark opaque wood. Each rat was placed on the center of the platform facing an enclosed arm. Animals were recorded the time spent in the opened arms within 5 min. The maze was cleaned following each trial to remove any residue or odors. Each rat was assessed individually 30 min after the treatment.

2.8. Forced Swimming Test

In order to assess the anti-depression like behavior of the plant extract, the modified test was conducted. The apparatus used in this study is the cylinder glass aquarium (22 cm diameter× 40 cm high) filled to the depth of 20 cm with fresh water at 25°C. After 30 min of drug administration, each animal was placed individually into the cylinder for 5 min-test and observed for swimming (movement throughout the swimming chamber), climbing and immobility (by keeping the head of the animals above water in the way that animal made no further attempts to escape) by blind observer who has been trained for the observation. Upon removal from the water, rats were towel-dried and finally returned to their home cage.

2.9. Morris Water Maze Test

The water maze consisted of a metal pool (170 cm in diameter ×58 cm tall) filled with tap water (25°C, 40 cm deep). In the center of one quadrant was a removable escape platform below the water level and covered with a nontoxic milk powder. The pool was divided into four quadrants (NE, NW, SE and SW) by two imaginary lines crossing the center of the pool. For each animal, the location of invisible platform was placed at the center of one quadrant and remained there throughout training. The rats must memorize the platform location in relation to various environmental cues because there was nothing directly shows the location of the escape platform in and outside the pool. Therefore, the placement of the water tank and platform were the same in all acquisition trials. Each rat was gently placed in the water facing the wall of the pool from one of the four starting points (N, E, S, or W) along the perimeter of the pool and the animal was allowed to swim until it found and climbed onto the platform. During training session, the subject was gently placed on the platform by the experimenter when it could not reach the platform in 60 sec. In either case, the subject was left on the platform for 15 sec and removed from the pool. The time for animals to climb on the hidden platform was recorded as escape latency or acquisition time. 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 animals were placed into the water maze for 60 sec. The retention of memory or the time that the animal spent to swim around the previous location of platform before removing the platform on the test occurring in the next day was also recorded. It has been postulated that if the spatial memory of the rat for the trained platform location is accurate, the rat will swim to the platform location and search around the exact location. Therefore, the more accurate the spatial memory is, the greater the number of times rat swim across the trained platform. In each trial, the animal was

quickly dried with towel before being returned to the cage. All tests in Morris water maze tests were carried out within 30 min after the plant extract administration of the substances. Any enhancement of cognition would be reflected by a decrease in escape latency time but increase in retention time.

2.10. Stereotype Behaviors

In order to assure that anxiolytic, anti-depression like behaviors and cognitive enhancing effect which determined by various tests just mentioned earlier were not false positive due to the effect of PM52 on motor behaviors, we also determined the effect of PM52 on the spontaneous locomotor activities. Rats were individually placed in the central area of open field apparatus and locomotor activities including the number of grooming, licking and rearing were recorded within 5 min.

2.11. Statistical Analysis

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

3. RESULTS

3.1. Acute Toxicity

It was found to be safe at 5000 mg kg^{-1} body weight in rats. Further dosing to estimate LD_{50} of the drug was not performed. According to Organization Economic Cooperation and Development (OECD) guidelines for acute oral toxicity, an LD_{50} dose of 5000 mg kg^{-1} BW and above are categorized as unclassified and hence the substance is found to be safe.

3.2. Anxiolytic-Like Activity

The anxiolytic like behavior of PM52 was determined using elevated plus maze and the results were shown in **Fig. 1**. It was clearly demonstrated that oral administration of DW (distill water) or vehicle produced no significant changes in time spent in the opened arms after single and repetitive doses throughout 14-day experimental period. Diazepam, a standard drug used for the treatment of anxiety, which used as positive control in this study significantly increased time spent in the opened arms at all durations of treatment ($p<0.05$, 0.05 and 0.01 respectively; compared to vehicle treated groups). Rats subjected to PM52 treatment at dose of 50 mg kg^{-1} BW significantly enhanced time spent in the opened arm after single administration ($p<0.001$; compared to vehicle treated groups) and this significance was still presented at 7 and 14 days of treatment ($p<0.05$ all; compared to vehicle treated groups). The medium dose of PM52 (10 mg kg^{-1} BW) could enhance time spent in opened arm only after single dose administration and at 7 days after treatment ($p<0.05$ all; compared to vehicle treated groups).

3.3. Anti-Depression Like Activity

The effect of PM52 on anti-depression like behavior was determined using forced swimming test, a valid tool using for the screening the effect of substances possessing anti-depressant activity and the neurobilogical mechanism related to depression. The results in **Fig. 2-4** demonstrated that rats subjected to vehicle administration did not produce significant changes in immobility time, climbing and swimming times in forced swimming test throughout the observation time, while the rats treated with Fluoxetine significantly increased climbing time throughout 14-day experimental period ($p<0.001$ all; compared to vehicle treated group) . It was found that these rats also decreased immobility time at 7 and 14 days of treatment ($p<0.001$ all; compared to vehicle treated group). However, they failed to show the significant changes of swimming time throughout the observation period. Rats exposed to PM52 at dose of 50 mg kg^{-1} BW also showed the elevation of climbing time at 7 and 14 days after treatment ($p<0.01$ all; compared to vehicle treated groups). However, no other changes were observed.

3.4. Cognitive Enhancing Effect

The effect of PM52 on spatial memory was assessed using a Morris water maze test, a valid tool for assessing spatial memory, a hippocampal dependent memory. The results in **Fig. 5-6** demonstrated that vehicle treated group produced no changes in both escape latency and retention time compared to vehicle treated group. Donepezil treated rats showed the decreased escape latencies ($p<0.05$ all; compared to vehicle treated group) throughout 14-day experimental period. However, the elevation of retention times were observed at 7 and 14 days after treatment ($p<0.05$ and 0.01 respectively; compared to vehicle treated group). Rats which received PM 52 at doses of 2 and 10 mg.kg^{-1} BW significantly decreased escape latencies ($p<0.01$ all; compared to vehicle treated group) throughout experimental period while the animals which received the high dose of PM52 showed the significant reduction of escape latency only at 14 days of treatment ($p<0.05$; compared to vehicle treated group).

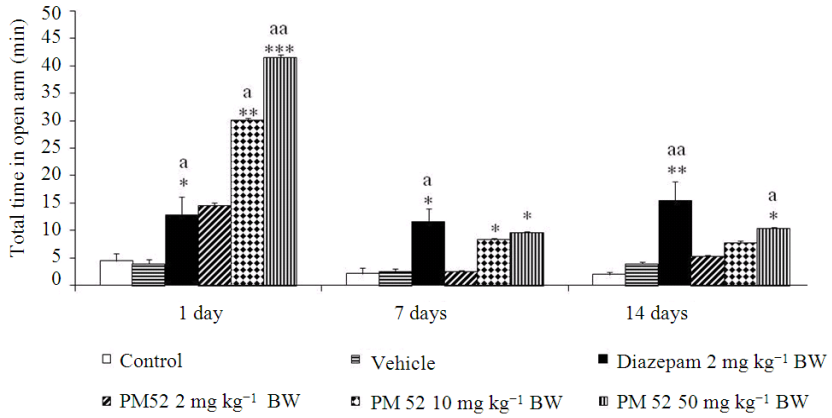

Fig. 1. Effect of Diazepam and PM52 (2, 10 and 50 mg kg^{-1} BW) on the time spent in opened arms in elevated plus maze test. Data were presented as mean ± SEM (n = 8 group^{-1}). $^{a;aa}$ p<0.05; 0.01, *;**;*** P<0.05; 0.01; 0.001 compared with control and vehicle treated group respectively

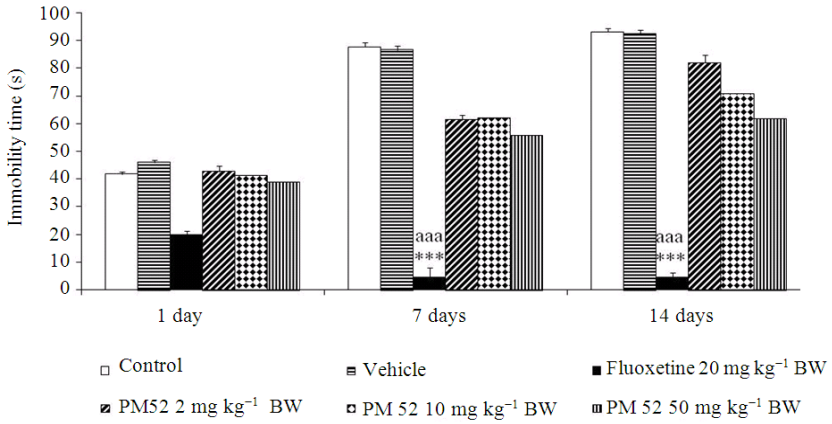

Fig. 2. Effect of Fluoxetine and PM52 (2, 10 and 50 mg kg^{-1} BW) on immobility time in forced swimming test. Data were presented as mean ± SEM (n = 8 group^{-1}). ^{aaa}, *** p<0.001 compared with control and vehicle treated group respectively

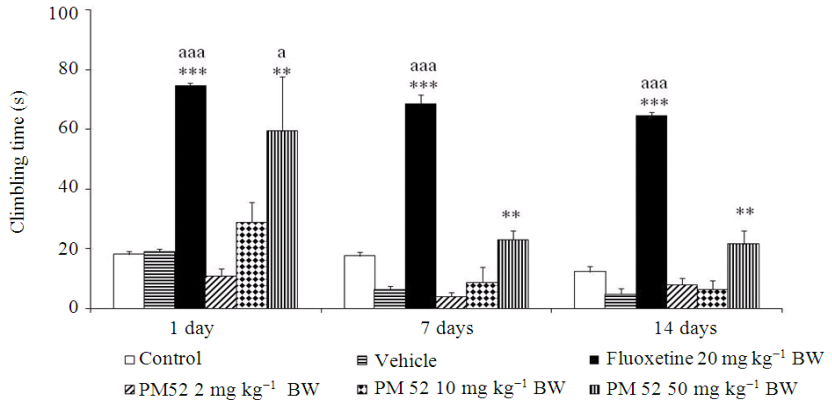

Fig. 3. Effect of Fluoxetine and PM52 (2, 10 and 50 mg kg^{-1} BW) on climbing time in forced swimming test. Data were presented as mean ± SEM (n = 8 group^{-1}). $^{a;aaa}$ p<0.05; 0.001, **;*** p<0.01; 0.001 compared with control and vehicle treated group respectively

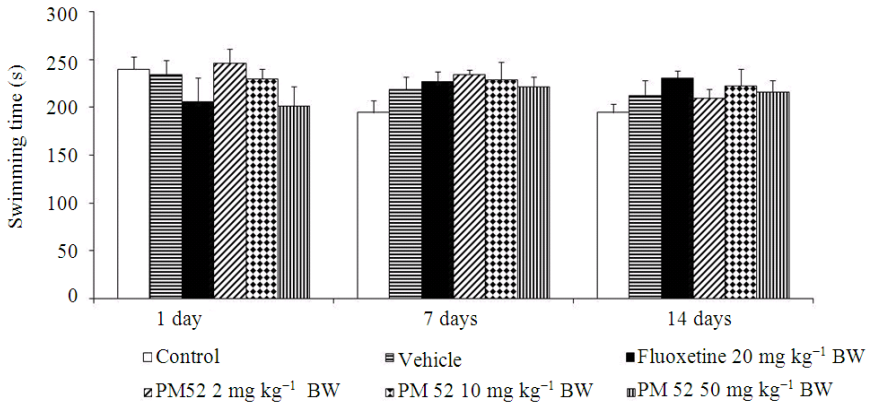

Fig. 4. Effect of Fluoxetine and PM52 (2, 10 and 50 mg kg^{-1} BW) on swimming time in forced swimming test. Data were presented as mean ± SEM (n = 8 $group^{-1}$)

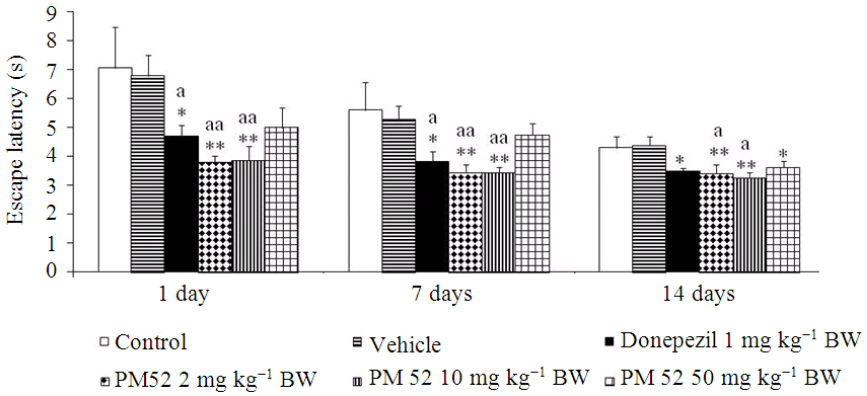

Fig. 5. The cognitive enhancing effect of Donepezil and PM52 (2, 10 and 50 mg kg^{-1} BW) on escape latency time in Morris water maze test. Data were presented as mean ± SEM (n = 8 $group^{-1}$). $^{a;aa}$ p<0.05; 0.01, *;** p<0.05; 0.01 compared with control and vehicle treated group respectively

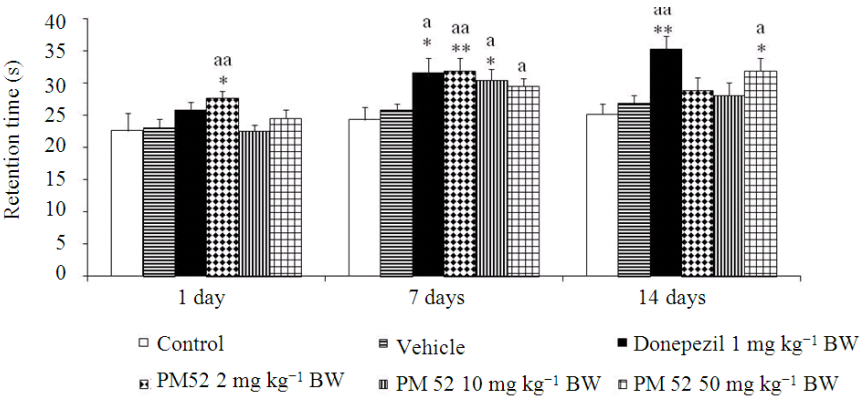

Fig. 6. The cognitive enhancing effect of Donepezil and PM52 (2, 10 and 50 mg kg^{-1} BW) on retention time in Morris water maze test. Data were presented as mean ± SEM (n = 8 $group^{-1}$). $^{a;aa}$ p<0.05; 0.01, *;** p<0.05; 0.01 compared with control and vehicle treated group respectively

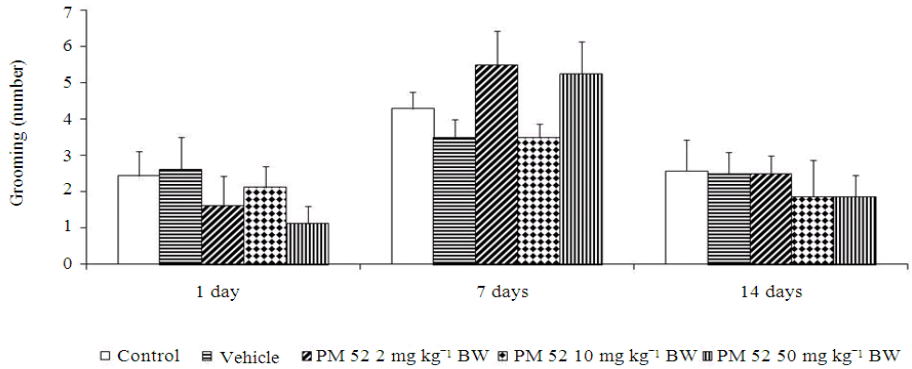

Fig. 7. Effect of PM52 (2, 10 and 50 mg kg^{-1} BW) on grooming behavior. Rats were treated with either vehicle or PM52 at various doses ranging from 2, 10 and 50 mg kg^{-1} BW via intragastric route for 2 weeks, then they were determined the number of grooming behavior. Data were presented as mean ± SEM (n = 8 $group^{-1}$)

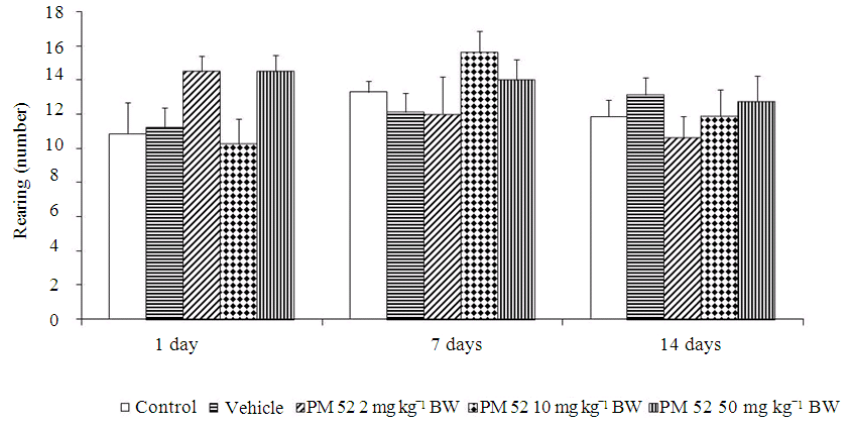

Fig. 8. Effect of PM52 (2, 10 and 50 mg kg^{-1} BW) on rearing behavior. Rats were treated with either vehicle or PM52 at various doses ranging from 2, 10 and 50 mg kg^{-1} BW via intragastric route for 2 weeks, then they were determined the number of rearing behavior. Data were presented as mean ± SEM (n = 8 $group^{-1}$)

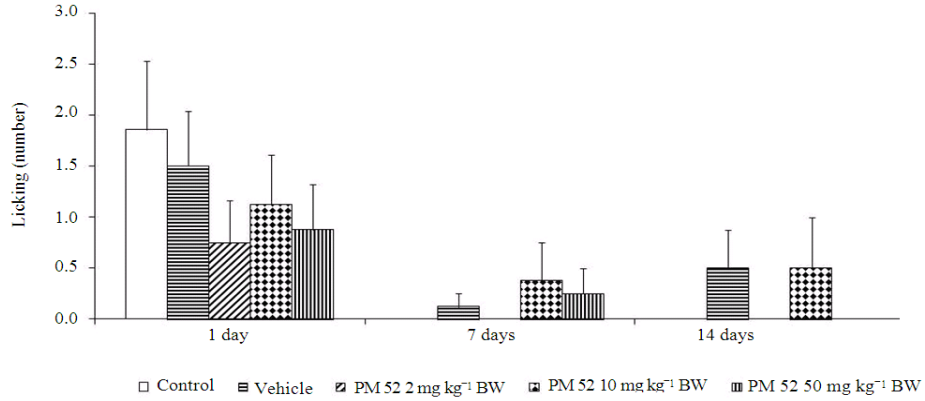

Fig. 9. Effect of PM52 (2, 10 and 50 mg kg^{-1} BW) on licking behavior. Rats were treated with either vehicle or PM52 at various doses ranging from 2, 10 and 50 mg kg^{-1} BW via intragastric route for 2 weeks, then they were determined the number of licking behavior. Data were presented as mean ± SEM (n = 8 $group^{-1}$)

It was also found that rats which obtained PM52 at dose of 2 mg kg^{-1} BW revealed the enhanced retention time after single dose administration and at 7 days of treatment ($p<0.05$, 01 respectively; compared to vehicle treated group) whereas the rats which received PM52 at doses of 10 and 50 mg kg^{-1} BW produced significant elevation of retention time at 7 and 14 days of treatment respectively ($p<0.05$ all; compared to vehicle treated group).

3.5. Spontaneous Locomotor Activities

The present results in **Fig. 7-9** showed that no significant changes on grooming, rearing and licking were observed in PM52 treated group. Therefore, our results suggested that PM52 exerted no effect on spontaneous motor activities.

4. DISCUSSION

Acute toxicity data have revealed that PM52 can be used safely up to 5000 mg kg^{-1} BW. PM52 failed to show significant changes on spontaneous locomotor activities indicating that the observed anxiolytic and cognitive enhancing effect in this study are not false positive.

Our results showed that fluoxetine treated group significantly decreased immobility time but increased climbing time. Therefore, this was in agreement with previous study who used fluoxetine from the same company (The Government Pharmaceutical Organization) (Hawiset *et al*., 2011). However, it was not corresponding with another group (Page *et al*., 1999) who used drug from different company and different strain of rat. The possible explanation for the disagreement might be due to the different quality of drug and different species of animals. PM52 could enhance climbing activity. Previous study had reported that climbing activity was reported to be associated with Norepinephrine (NE) function (Page *et al*., 1999). Therefore, PM52 particularly at high dose could enhance NE function.

The present data also demonstrated that after single administration PM52 at low dose could significantly decreased escape latency but increased retention time while the medium dose only produced the significant reduction of escape latency. In addition, after single administration, PM52 at medium and high dose also exhibited the anxiolytic like activity similar to that observed in the diazepam treated group. Since the anxiolytic activity was previously reported to be associated with GABA ergic function, the anxiolytic effect after single administration of PM52 might be related to the enhanced GABAergic function (Rago *et al*., 1988). When, the treatment was prolonged, it was still found that low dose of PM52 showed cognitive enhancing effect whereas the medium dose showed both cognitive enhancing effect and anxiolytic activity. In addition the high dose appeared to show the effect to enhance anxiolytic like activity, climbing behavior and cognitive enhancing effect. Taken all together, obtained data suggested that at low dose and early phase of response, PM52 selectively enhanced cholinergic function and resulting in the enhanced cognitive function. At medium dose, PM52 could enhance the function of both cholinergic and GABAergic system, therefore, the increased cognitive function and anxiolytic activity were observed. The increasing dose further to 50 mg kg^{-1} BW appeared to produce less specificity, the increased activities of cholinergic, GABAergic and noradrenergic were observed.

Our study failed to show dose dependent manner. The possible explanation might be due to the masking effect of other ingredients in PM52 which contained numerous ingredients both active and inactive ingredients. The interacting effect of various ingredients might also contribute the vital role on this phenomenon. Besides the mentioned effect, it was also possible that we couldn't found the dose dependent response of PM52 because it produced the effect on numerous chemical systems mentioned above and gave rise to the complex relationship between the concentration of PM52 and the observed behaviors.

5. CONCLUSION

In conclusion, PM52, a novel food supplement, is the potential food supplement to enhance memory and decrease anxiety with high safety. However, subchronic toxicity study and the assessment of active ingredient are still essential before moving forward to clinical trial study.

6. ACKNOWLEDGEMENT

This study was supported by the National Research Council of Thailand, The Royal Golden Jubilee, Ph.D Program, Thailand Research Fund, Research Division of Faculty of Medicine via Targeted Research and Integrative Complementary Alternative Medicine, Khon Kaen University, Thailand.

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