Original Article

Evaluation of Total Phenolic Compound, Antioxidant Effect. and Neuropharmacological Activities of *Moringa oleifera* Lam. Leaves Extract.

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Abstract

Introduction: Despite the increasing their importance, the therapeutic efficacy of neuropsychological disorders are not in satisfaction level. Based on the AChEI and MAOI activities of *Moringa oleifera* and the health benefits of toal phenolic compound and antioxidant effect, we hypothesized that the leaves extract of *M.,oleifera* could exhibit some neuropharmacological activities.

Objective: To determine total phenolic compound, antioxidant and neuropharmacological activities of *M.oleifera* Lam leaves extract.

Materials and Methods: The total phenolic compound was determined using Folin-Ciocalteu reagent whereas the antioxidant activity was determined using 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) assay and the ferric reducing/antioxidant potential (FRAP) activity. The extract was also further screened for the neuropharmacological activities. In order to determine the neuropharmacological activities of *M.oleifera* leaves extract, male Wistar rats, weighing 250-300g had been orally given the extract at doses of 100, 200 and 400 mg kg BW^{-1} for 14 days. Rats were determined the cognitive enhancing effect, anxiolytic and anti-depression like activities after the single dose of administration and every 7days throughout the 14-day experimental period.

Results: The extract contained total phenolic compound at concentration of 68.16±0.71 mg/L Gallic acid equivalent/mg extract. It also exhibited antioxidant activity determined by DPPH and FRAP assays. In addition, all doses of the extract decreased escape latency at 7-day treatment whereas only the medium and high doses of extract enhanced the retention time only after single dose administration., Rats subjected to the low and medium doses showed the increased %change of time spent in the open arm after the single dose of administration but at 14-day treatment, only the high dose treatment showed the significant change. Therefore, our data suggested the cognitive enhancing effect and anxiolytic effect of *M.oleifera* leaves extract.

Conclusion: *M oleifera* is the potential food supplement to provide health benefits for the brain function both as cognitive enhancer and as anxiolytic agent. However, more researches are still essential to determine the possible active ingredient and precise underlying mechanism before move forward to clinical trial study.

Key words: *M. oleifera*, cognitive enhancing, antioxidant, neuropharmacological profiles

Introduction

At present, the neuropsychological disorders are affecting more than 450 million people globally. It has been reported that they account for four out of the six leading causes of years lived with disability[1]. Therefore, they are the important problems that should be concerned. Drugs currently used in treatment of these disorders have massive side effects or posses unfavourable drug-drug/drug-food interactions. Therefore, the novel therapeutic strategy is required. Based in the accumulative lines of evidence which point out that oxidative stress play the crucial role on the pathophysiology of neurological and psychiatric disorders[2], the searching for the novel therapeutic agent from plants possessing antioxidant has gained much attention.

Moringa oleifera Lam. or Drumstick tree or Ma-rum is belonging to Moringaceae family. It has been long-term used both as food and as medicine in traditional folklore. According to the traditional folklore, it has been claimed for the treatment of numerous ailments including asthma, gout, lumbago, rheumatism, enlarged spleen or liver, etc. Recent studies have shown that the leaves extract of *M.oleifera* can regulate the thyroid hormone status[3]. In addition, it also possesses radioprotective[4] and hypocholestrolemic effects[5]. Moreover, it has also suppressed the activities of both acetylcholinesterase (AChE) and monoamine oxidase (MAO) (Sutalanga et al., 2011), the important neurotransmitters of brain function.

Therefore this study was conducted to determine the neuropharmacological effects of *M. oleifera* leaves extract. Since previous findings have demonstrated that the health benefits of medicinal plants including the benefit for the brain function are associated with the phenolic compounds and antioxidant effect, we also determined the total phenolic compounds and antioxidant activity of the mentioned medicinal plant.

Materials and Methods

Plant Materials and extraction: *M.oleifera* leaves were collected from Khonkaen province, Thailand between November and January period. The plant was identified by Assoc. Prof. Dr. Panee Sirisa-ard (Department of Pharmaceutical science, Faculty of Pharmacy, Chiang Mai University) and the voucher specimen has been kept at Integrative Complimentary Alternative Medicine Research and

Development Research and Development Group, Faculty of Medicine under the voucher specimen ICAM Mo1. *M. oleifera* leaves and its stalk were dried and blended into a powder. 100 g of *M. oleifera* leaves powder was twice extracted with 500 ml of 50% aqueous alcohol for 3 days and the resulting extracts were filtered using Whatman filter paper No. 1. The filtrates were then pooled and evaporated to get rid of alcohol by rotary evaporator at 60°C, 600 mmHg. The pure liquid extraction was lyophilized and the extract powder was kept in -20 freezers until use. The percentage yield of leaves extract was 17.30%.

Determination of total phenolic compounds: Total phenolic compound was determined as using Folin Ciocalteau method and the result was expressed as mg/L Gallic acid equivalent[7]. In brief, 20 μl of extract was added to 1.58 ml of distilled water and followed 100 μl of Folin Ciocalteu reagent. The mixtures were mixed and kept in dark room for 8 minutes. Then, 300 μl of 20% sodium carbonate was added and shake to mix again. The mixtures were leaved in dark room for 2 hours and absorbance was measured at 765 nm with a UV-spectrophotometer (Pharmacia LKB-Biochrom4060). Gallic acid at the concentrations range between 50-600 mg/L was used as standard calibration curve and distilled water was used as blank calibration.

DPPH Radical Scavenging Activity Assay: The free radical scavenging activity of *M. oleifera* was measured according to method described by Sreelatha and Padma[8]. 1 ml of the extract solutions (5-250 μg/ml) was added to 0.5 ml of 0.15 mM DPPH in methanol and mixed. After incubated in dark room for 30 minutes, the solutions were read at 517 nm with UV-spectrophotometer (Pharmacia LKB-Biochrom4060). L-Ascorbic acid (1-10 μg/ml) was used as standard antioxidant whereas *M. oleifera* extract 250 μg/ml and methanol were used as blank and control, respectively. The DPPH radical scavenging activity was calculated using the following equation:

$$\text{Scavenging of DPPH radical (\%)} = [1-(A_{extract}/A_{DPPH})]x100$$

The result was expressed as IC_{50} value (concentration provides 50% DPPH radical scavenge).

Ferric Reducing Antioxidant Power (FRAP) Assay: The ferric reducing antioxidant power of *M. oleifera* extract was studied following by the method of Benzie and Strain[9] with slightly modified. FRAP reagent was freshly prepared which consisting of A: 300 mM Acetate buffer pH 3.6, B: 2,4,6-tripyridyl-striazine (TPTZ) 10 mM in 40 mM HCl and C: Ferric chloride 20 mM. The FRAP solution was mixed with ratio A: B: C; 10:1:1, respectively and kept in water bath at 37°C. Then 50 μl of extract solution (5 mg/ml) was added to 1.45 ml of FRAP solution and mixed well. The mixtures were incubated in water bath at 37°C for 10 minutes and the absorbance was measured with spectrophotometer at 593 nm. FRAP reagent and L-Ascorbic acid (100-1000 μM) were used as blank and standard calibration, respectively. The result was expressed as μM L-ascorbic acid equivalent.

Experimental Animal: Male Wistar rats (250-300g) were purchased from National Animal Center, Salaya and used as an experimental animals. Rats were housed 6 per cage and maintained in 12:12, light:dark cycle and given access to food and water ad libitum. The experimental protocols have been approved by animal ethics committee of KhonKaen University, based on the ethic of animal experimentation of national research council of Thailand (record no. AEKKU 51/2553). Rats were divided into 8 groups, 6 rats per group as following

Group I: Rats were orally given with carboxymethylcellulose which was used as vehicle to dissolve all administered substances.

Group II-IV: *M. oleifera* extract treated groups which were orally given the leaves extract of *M,oleifera* at doses of 100, 200 and 400 mg BW^{-1} respectively.

Group V-VI: Piracetam and Vitamin C treated grousp were used as positive control group for cognitive enhancing activity due to their ability to enhance cognitive function by increasing cerebral blood flow and antioxidant effect, respectively. Rats were orally given either Piracetam or Vitamin C at the dose of 250 mg BW^{-1}

In order to determine anxiolytic and anti-depression like behavior, we divided the animals in to the group I-IV as mentioned earlier. In Group V or positive control group, the animals were treated with diazepam (2 mg BW^{-1}) in the determination of anxiolytic activity whereas they were

treated with fluoxetine (20 mg BW^{-1}) in the determination with anti-depression like behavior.

Rats had been treated with the assigned substance for 14 days and the determination of neuropharmacological activities including cognitive enhancing effect, anxiolytic and antidepressant activities were performed after single dose administration and every 7 days throughout the experimental period.

Morris water maze test: The water maze test was used to measure cognitive performance according to the study of Wattanathorn and coworker[11]. Briefly, rats were trained for 5 days to remember the location of platform which place in the center of 1 quadrant related to the various environmental cues. On test day, rats were gently placed into quadrant opposite to platform, an escape latency was recorded as a time when rat climb onto the platform. The retention memory was performed 24 hours later. Time which each rat spent to swim around the previous location of platform after removing for 60 seconds was recorded and regarded as retention time.

Elevated plus maze test: This apparatus was used to test anxiety like behavior in rat. It was a wooden plus shape with 2 open and 2 closed arms, elevated 40 cm from the floor. On test day, rats were placed into the center of an apparatus facing one of the open arms. During 5 minutes test period, time spent in open arm was recorded and expressed as %change from baseline of the time spent in open arm.

The force swimming test: This test is used for measuring the antidepressant activity which mimics depression like behavior in rat. Rats were placed into a glass cylinder water-filled tank. The water was filled into tank high enough to prevent rat reach the bottom of tank and low enough from the edge of tank to prevent rat escape from the tank. In the experiment, rats were placed into water tank for 5 minutes and immobility time which indicated depression like behavior in rat was recorded.

Statistical analysis: All data were presented as mean±SEM value. Statistical analysis used one way analysis of variance (ANOVA) followed by LSD post hoc test for multiple comparison using SPSS® (v. 17.0 for Window®). The statistical significant level was set at p-value <0.05.

RESULTS

Determination of total phenolic compounds and antioxidant activity: Since the phenolic compounds and antioxidant effect have been reported to be important for health benefits, we had determined the total phenolic compounds content using Folin Cicalteau method and determined antioxidant activity using DPPH and FRAP assays.. The results showed that the total phenolic compound of *M. oleifera* extract was 68.16±0.71 mg/L Gallic acid equivalent/ mg extract. Antioxidant activities of *M. oleifera* extract via DPPH and FRAP asssays were 96.49±0.87 μg/ml and 171.54±1.21 μM L-ascorbic acid equivalent/ mg extract, respectively.

Determination of cognitive enhancing effect of *M. oleifera* leaves extract: Cognitive performance was assessed using escape latency time and retention time as indices. Figure 1A showed that at 7 and 14 days of treatment, rats subjected to Piracetam treatment significantly decreased escape latency (p-value<.01 and .05 respectively; compared with vehicle treated group) while rats subjected to Vitamin C showed significant reduction of escape latency only at 7 days of treatment (p-value<.01; compared with vehicle treated group). Rats which received *M.oleifera* extract at doses of 100, 200 and 400 mg kg BW^{-1} also showed the significant reduction of escape latency at 7 days of treatment (p-value<.01, .05 and .05 respectively; compared with vehicle treated group). It was found that both rats treated with Piracetam and Vitamin C

Produced the significant elevation of retention time (p-value<,05 all; compared with vehicle treated group). Only rats which received the extract at doses if 200 and 400 mg $kgBW^{-1}$ significantly enhanced the retention time (p-value<.05, .01 respectively; compared with vehicle treated group).

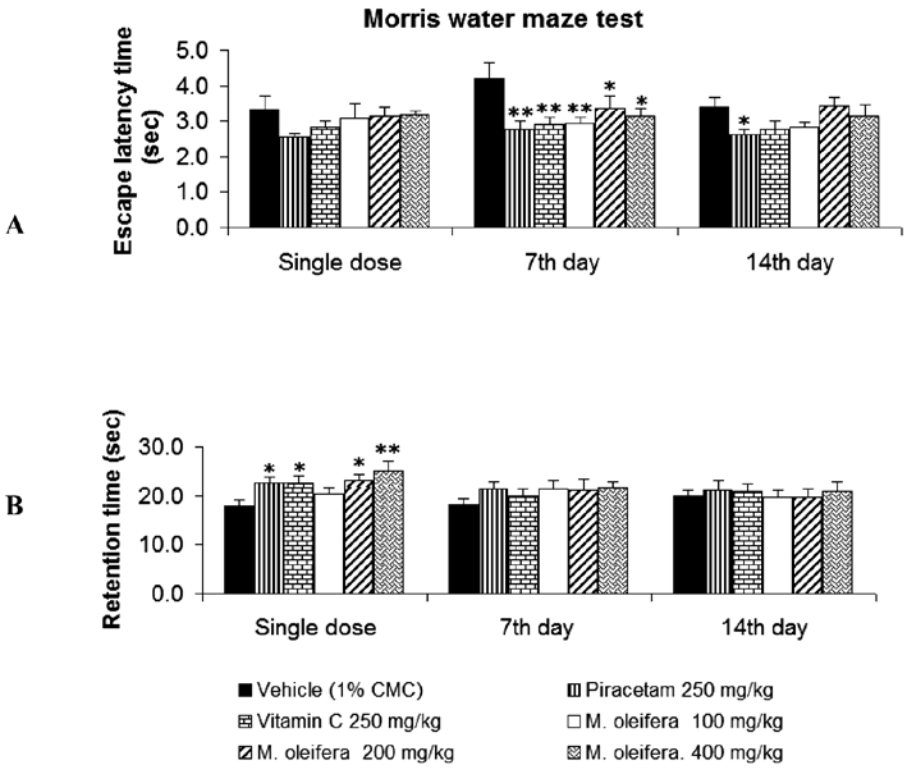

Figure 1 The effect of *M. oleifera* extract on cognitive function evaluated using Morris water maze test. (A) Escape latency time after single dose of administration, 7th and 14th day of administration (B) Retention time after single dose of administration, 7th and 14th day of administration. Data are presented as mean±SEM (n=6/group).

* Compare with vehicle treated group: *p*-value< 0.05

** Compare with vehicle treated group: *p*-value<0.01

Determination of anxiolytic activity of *M. oleifera*: The anxiety like behavior was determined using elevated plus maze test. Figure 2 demonstrated that rats subjected to diazepam, a positive control treated group, increased %change of time spent in open arm after single dose administration and at 14th day of treatment (p-value<,.05 and ,01 respectively; compared with vehicle treated group). After single administration,

it was found that rats exposed to *M.oleifera* extract at doses if 100 and 200 mg kgBW^{-1} produced significant elevation in %change of time spent in open arm (p-value<.05 and ,01 respectively; compared with vehicle treated group). However, at 14 -day experimental period, only rats subjected to the extract at dose of 200 mg kg BW^{-1} produced significant elevation in %change of time spent in open arm (p-value<.05; compared with vehicle treated group).

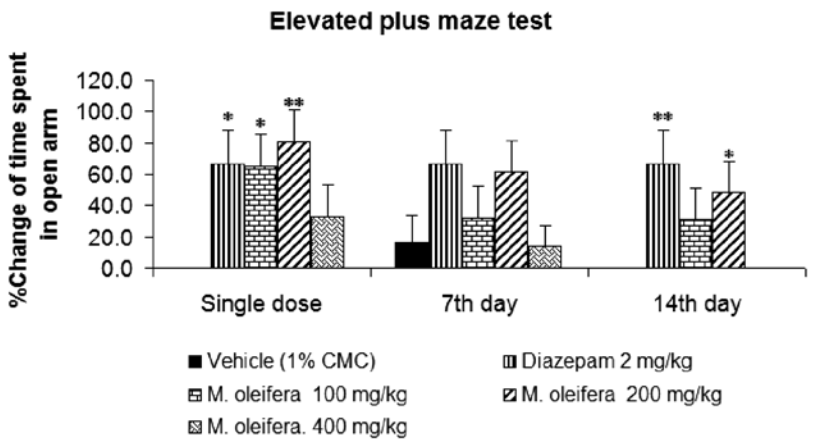

Figure 2 Effect of Diazepam and various doses of *M. oleifera* extract on %change of time spent in the open arm of elevated plus maze test. Data are presented as mean±SEM (n=6/group).

* Compare with vehicle treated group: *p*-value< 0.05

** Compare with vehicle treated group: *p*-value<0.01

Anti-depression like activity of *M. oleifera*: The depression like behavior was determined using force swimming test and data were shown in figure 3. The current data showed that rats which obtained fluoxetine (20 mg/kg BW^{-1}) significantly decreased immobility time after single administration, 7 and 14 days of treatment (p-value<.05, ,01 and .01 respectively; compared with vehicle treated group). Unfortunately, no significant decrease in immobility time was observed in rats subjected to all doses of *M,oleifera* leaves extract.

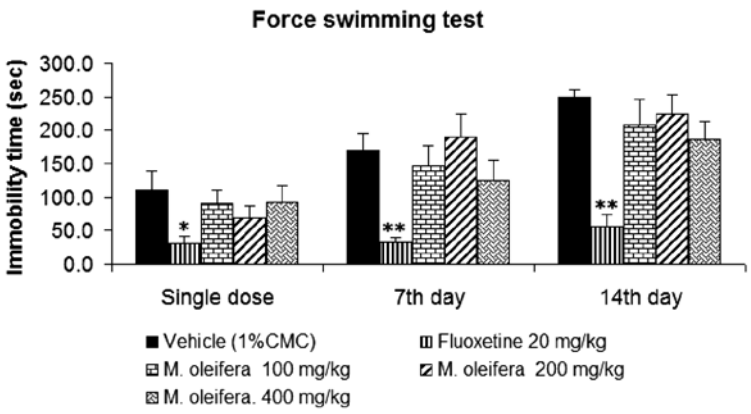

Figure 3 Effect of Fluoxetine and various doses of *M. oleifera* extract on the immobility time in force swimming test. Data are presented as mean±SEM (n=6/group).

* Compare with vehicle treated group: p-value< 0.05

** Compare with vehicle treated group: p-value<0.01

Discussion

Our results have demonstrated that the extract used in this study contained phenolic compounds and antioxidant activity. Moreover, it also possesses cognitive enhancing effect and anxiolytic activity.

Since free radicals are the important cause of numerous diseases including neuropsychological disorders, abundant attention has been focused in order to develop the novel strategy for preventing and treating the mentioned diseases from antioxidant. Our data obtained from both DPPH and FRAP have demonstrated that *M.oleifera* extract showed antioxidant activity. This was corresponding with previous studies[9,12]. Since plant phenolics are a major group of compounds acting as primary antioxidants or free radical scavengers[13], the antioxidant activity observed in this study might be associated with the phenolic compounds presented in the extract.

It is of interest to note that *M.oleifera* leaves extract also enhanced spatial memory and decreased anxiety like behaviors. Previous findings revealed that the suppression of monoamine oxidase (MAO) could improve anxiety[14] and

memory[15]. Since previous study demonstrates that *M.oleifera* extract can suppress the activities of both MAO and AChE, the neurotransmitters playing the important role on learning and memory, we do suggest that the cognitive enhancing effect of *M.oleifera* may occur partly via the suppression of MAO and AChE whereas the anxiolytic effect of the plant extract may occur artly via the suppression of MAO.The current results failed to show the dose dependent manner. The possible explanation may be due to the masking effects of some other ingredients when the concentration of the crude extract was increased.

In conclusion, *M. oleifera* possessed the antioxidant activity, cognitive enhancing effect and anxiolytic activity. Therefore, *M oleifera* is the potential food supplement to provide health benefits for the brain function both as cognitive enhancer and as anxiolytic agent. However, more researches are still essential to determine the possible active ingredient and precise underlying mechanism before move forward to clinical trial study.

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References

1. Mathers, C. D., A. D. Lopez, and C. J. L. Murray. "The Burden of Disease and Mortality by Condition: Data, Methods, and Results for 2001." In *Global Burden of Disease and Risk Factors,* eds. A. D. Lopez, C. D. Mathers, M. Ezzati, D. T. Jamison, and C. J. L. Murray. New York: Oxford University Press.
2. Kusano C, Ferrari B. Total antioxidant capacity: a biomarker inbiomedical and nutritional studies. J Cell Molec Biol. 2008; 7: 1-15.
3. Tahiliani P, Kar A. Role of Moringa oleifera leaf extract in the regulation of thyroid hormone status in adult male and female rats. Pharmacol. Res. 2000; 41: 319-23.

4. Rao A, Devi P, Kamath R. In vivo radioprotective effect of *Moringa oleifera* leaves. Indian J Exp Biol. 2001; 39 (9):858-63.
5. Ghasi S, Nwobodo E, Ofili JO. Hypocholesterolemic effects of crude extract of leaf of *Moringa oleifera* Lam in high-fat diet fed wistar rats**.** J Ethnopharmacol 2000; 69: 21-5.
6. Sutalanga C, Wattanathorn J, Muchinapura S, Wannnanond P. Screening the potential of selected Thai medicinal plants for treating Alzheimer's disease and Parkinson's disease. 2011 Poster session presented at the 5th International Conference on Polyphenols and Health (ICPH), Barcelona, Spain.
7. Waterhouse AL. Determination of Total Phenolics. Current Protocols in Food Analytical Chemistry 2002: I1-I8.
8. Srilatha S, Padma PR. Antioxidant activity and total phenolic content of *Moringa oleifera* leaves in two stages of maturity. Plant Foods Hum Nutr 2009; 64: 303-11.
9. Benzie IF, Strin JJ. The Ferric Reducing Ability of Plasma (FRAP) as a Measure of antioxidant power: The FRAP Assay. Anal Biochem. 1996; 239: 70-6.
10. Papandreou MA, Dimakopoulou A, Linardaki ZI, Cordopatis P, Klimis-Zacas D, Margarity M, et al. Effect of a polyphenol-rich wild blueberry extract on cognitive performance of mice, brain antioxidant markers and acetylcholinesterase activity. Behav Brain Res 2009 ; 198: 352-8.
11. Wattanathorn J, Jittiwat J, Tongun T, Muchimapura S, Ingkaninan K. *Zingiber officinale* mitigates brain damage and improves memory impairment in focal cerebral ischemic rat. Evid Based Complement Alternat Med 2011 ; 2011: 429505.
12. Mensor LL, Menezes FS, Leitao GG, Reis AS, dos Santos TC, Coube CS, et al. Screening of Brazilian plant extracts for antioxidant activity by the use of DPPH free radical method. Phytother Res 2001; 15: 127-30.
13. Kåhk÷nen MP, Hopia AI, Vuorela HJ, Rauha JP, Pihlaja K, Kujala TS, Heinonen M. Antioxidant activity of plant extracts containing phenolic compounds. J Agric Food Chem. 1999; 47(10): 3954-62.

14. Tyrer P, Shawcross C. Monoamine oxidase inhibitors in anxiety disorders. J Psychiatr Res. 1988;22 Suppl 1:87-98.
15. Wong FK, Lee SH, Atcha Z, Ong AB, Pemberton DJ, Chen WS. Rasagiline improves learning and memory in young healthy rats. Behav Pharmacol. 2010;21(4):278-82.
16. Ihalainen J, Sarajårvi T, Rasmusson D, Kemppainen S, Keski-Rahkonen P, Lehtonen M, et al. Effects of memantine and donepezil on cortical and hippocampal acetylcholine levels and object recognition memory in rats. Neuropharmacology. 2011; 61(5-6):891-9.