CHAPTER III
EVALUATION OF THE ANTI-STRESS POTENTIAL OF *MORINGA OLEIFERA* LEAVES, *ANACARDIUM OCCIDENTALE* LEAVES AND *NELUMBO NUCIFERA* FLOWERS

3.1 Introduction

Stress exposure is increased in the current daily life styles. Since stress produces numerous stress related disorders ranging from psychiatric, endocrine and sexual dysfunction to cognitive deficit (Bhattacharya *et al.*, 2000). It has been reported that the generation of free radical is increased during stressful situation (Bhaumik *et al.*, 1995; Kondo *et al.*, 1993). Under normal circumstance, the free radicals are buffered by the body's defense system. To enhance the body to combat with the oxidative stress by using various supplementation including herbal supplement is reported to be a successful strategy to protect stress deleterious effects (Brekhman and Dardymov, 1969). Since *Moringa oleifera*, *Anacardium occidentale* and *Nelumbo nucifera* are reputed for adaptogenic effect which can increase body dense against stress (Kulkarni and Juvekar, 2008) and antioxidant activity, the anti-stress potential of these plants are focused.. Previous findings showed that oxidative stress and dopamine played the crucial roles on both memory deficit and sexual dysfunction and acetylcholine played an important role on learning and memory whereas phosphodiesterase type-5 played a crucial role on penile tumescence. Therefore, this study aimed to determine the anti-stress potential of the mentioned plants by focusing on the antioxidant effect and the suppression effect of acetylcholinesterase (AChE), monoamine oxidase type B and phosphodiesterase type-5.

3.2 Materials and methods

3.2.1 Plant materials and preparation

Moringa oleifera Lam leaves and *Nelumbo nucifera* Gaertn flowers, the local indigenous plants were collected from the organic farm of Khon Kaen

province during September 2011 and December 2012, respectively. *Anacardium occidentale* Linn leaves were collected from Phuket province. Both *M.oleifera* and *A.occidentale* were authenticated by Associate Professor Panee Sirisa-ard, Faculty of Pharmacy, Chiang Mai University whereas *Nelumbo nucifera* was authenticated by Dr. Nopachai Chansilp, Rajamangala University of Technology Tawan-ok. The leaves of both *M.oleifera*, and *A.occidentale* and flowers of *Nelumbo nucifera* were cleaned, cut into small pieces and dried at 60 °C. They were grounded to fine powder using a homogenizer. Both *M.oleifera* leaves and *N.nucifera* flowers were extracted with 50% hydro-ethanol whereas *A.occidentale* leaves were extracted with 95% ethanol. The extracts were filtered by Whatman filter paper no.1. Then, the extracts were evaporized by using rotary evaporator. Percent yield of *M. oleifera*, *A. occidenale* and *N.nucifera* extract were 17.49%, 17.32% and 10.23% respectively. The plant extracts were kept at -20 ºC for further use. Voucher specimens were kept at Integrative Complementary Alternative Medicine Research and Development Center, Khon Kaen University.

3.2.2 Determination of the contents of total phenolics, flavonoids and tannin

The Folin–Ciocalteau method was used for determining of total phenolic amount in the extracts. The sample aliquots (200 µl) was mixed with the mixture containing 1.0 ml of Folin–Ciocalteau reagent and 800 µl of 7.5% sodium carbonate and mixed thoroughly by vortex. The absorbance at 765 nm was measured after standing for 30 min. Gallic acid was prepared as standard calibration curve and the total phenolic content was expressed as gallic acid equivalent (GAE) (Matkowski and Piotrowska, 2006).

Total flavonoid content was measured by aluminum chloride colorimetric method (Pourmorad *et al.*, 2006). Plant extracts (0.5 ml) were mixed with 1.5 ml of 50% alcohol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled Water and incubated for 45 minutes at room temperature. Then, the absorbance was read by spectrophotometer at 415 nm. The total flavonoid content was expressed as quercetin equivalent (QE) which was prepared as the standard calibration curve.

Tannin was determined by Folin and Ciocalteu method (Tamilselvi *et al.*, 2012). The plant sample (0.1 ml) was added to the mixture containing 7.5 ml of distilled water, 0.5 ml Folin Phenol reagent and 1 ml of 35% sodium carbonate solution. The, the solution was diluted to 10 ml with distilled water. The mixtures were shaken and read the absorbance by spectrophotometer at 725 nm after the incubation for 30 minute at room temperature. Standard tannic acid solution was prepared and served as standard calibration curve.

3.2.3 Determination of the 1,1-Diphenyl-2-picrylhydrazyl radicals

Stable free radical scavenging activity was determined by using DPPH method. 40 μl of plant extract was added to 2.96 ml of 0.1 mM solution of DPPH in methanol. After incubating for 30 min at 37°C the optical density was measured at 517 nm. L-ascorbic acid was used as control. The radical scavenging activity (percent inhibition) was expressed as percentage of DPPH radical elimination calculated according to the following equation:

Percentage Inhibition (%) = (Acontrol-Asample/Acontrol) × 100 Where Acontrol is the absorbance of the control (Lascorbic acid) and Asample is the absorbance of reaction mixture (in the presence of sample). All tests were run in triplicates (n = 3), and the average values were calculated (Djeridane *et al.*, 2006; Surveswaran *et al.*, 2007; Velioglu *et al.*, 1998).

3.2.4 Ferric reducing antioxidant power (FRAP) assay

A modified FRAP method of Pulido and coworkers (Pulido *et al.*, 2000). was performed. In brief, FRAP solution containing 300 mM sodium acetate buffer, pH 3.6, 10 mM TPTZ solution in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H2O$ solution was freshly prepared. Then, 2.85 ml of working FRAP was mixed with 150 μl of the extract and incubated at 37 °C for 10 minutes. The absorbance was recorded at 593 nm . Standard curve of ascorbic acid was prepared. The results were expressed as μmol ascorbic acid /mg extract sample.

3.2.5 Acetylcholinesterase (AChE) inhibition activity

A modified method of Ellman and coworkers was used for measuring AChE activity via microplate reader (Ellman and Courtney, 1961). According to this method, acetylthiocholine iodide (ATCI) was used as substrate and this substance was hydrolyzed by AChE and generated the product thiocholine which reacted with thiol

with 5, 5-dithiobis-2-nitrobenzoate (DTNB) ion giving rise to the production of yellow anion of 5-thio-2-nitro-benzoic acid. Which can be detected at 412 nm. In brief, 25 µl of 15 mM ATCI, 125 µl of 3 mM DTNB and 50 µl of 50 mM Tris-HCl, pH 8.0, containing 0.1 % bovine serum albumin (BSA) and 25 µl of the extracts were added to the 96 well plates and incubated for 5 minutes at room temperature. The absorbance at 405 nm was measured. Then, 25 µl of 0.22 U/mL of AChE from electric gel (Sigma-Aldrich Corporation, St. Louis, MO, USA) was added, the absorbance was measured again after 5 minutes of incubation at room temperature.. Percentage of inhibition was calculated by comparing the rate of sample hydrolysis by AChE with that of blank (50 % aqueous methanol in buffer). Donepezil (AChE-I) was used as a reference standard. All determinations were carried out at least 5 times and in triplicate at each concentration..

3.2.6 Monoamine oxidase type B suppression activity

The MAO type B activity was assessed using microplate reader method (Xu *et al.*, 2005). Rats were decapitated and brains were rapidly removed and frozen on dried ice. Then, they were homogenized in sodium phosphate buffer (0.1 M, pH 7.4) and centrifuged at 15,000×g for 20 min. Supernatant was removed and used for the estimation of MAO-B activity. The brain supernatant 150 µl was added in to the solution containing 2.5 ml Tris buffer (0.1 M, pH 7.4) and 100 µl of 0.1 M benzylamine. Then, the absorbance was measured at 340 nm for 30 min against the blank containing Tris buffer and 5-hydroxytryptamine.

3.2.7 Phosphodiesterase type 5 inhibition activity

Phosphodiesterases (PDE-5) are a superfamily of enzymes that degrade cyclic guanosine monophosphate (cGMP). The level of PDE-5 was determined using a PDE-Glo™ Phosphodiesterase Assay kit (Promega) (Z Factor, 2007). The testis was used as PDE-5 source. Testis was washed with PBS, cut into small pieces and homogenized by using lysate RIPA buffer. The testicular sample was centrifuged at 14,000 ×g at 4 °C for 20 min. The supernatant was separated and served for the determination of PDE-5 activity. To determine the inhibition of PDE-5 activity, PDE-5 was incubated with various concentrations of *M.oleifera, A.occidentale* and *N.nucifera* extract according to the guidelines of the kit. In brief, plants and PDE-5 were incubated with cGMP substrate and the reaction was terminated by PDE-Glo™

Termination Buffer and detected by PDE Detection Solution containing ATP and protein kinase A (PKA). The amount of ATP consumed by this reaction was directly correlated with the cGMP level and was evaluated using the luciferase-based Kinase-Glo Reagent. RLU was measured by using a SpectraMax L microplate luminometer (MSD AT (US) Inc). Sildenafil citrate (PDE 5-I) was used as the positive control. Percent inhibition was calculated and expressed as IC50.

3.2.8 Sample analysis

The analysis of sample was carried out via gradient high performance liquid chromatography (HPLC) system consisting of 515 HPLC pump and 2998 Photodiode array detector of Waters company, USA. Chromatographic separation was performed using Purospher®STAR,C-18 encapped (5µm), LiChroCART®250-4.6 and HPLC-Cartridge, Sorbet Lot No. HX255346 (Merk, Germany). Two mobile phases consisting of methanol and 2.5%acetic acid in deionized (DI) water were used to induce gradient elution. The injection volume was 20 µL and the flow rate was 1.0 mL/min. During HPLC analysis the solvent gradient was programmed as shown in Table 3-1 and data analysis was performed using Empower^{TM} 3.

Table 3-1 Gradient program of HPLC analysis

Times (minutes)	Solvents (%)	
	A (Methanol)	B (2.5% acetic acid)
0	10	90
17	70	30
18	100	-
20	100	-
20.5	10	90
25	10	90

3.2.9 Acute toxicity

Healthy young adult male and female Wistar rats, weighing 200-250 g, were purchased from National Laboratory Animal Center, Salaya, Nakorn Pathom, Thailand. The animals were maintained and treated in accordance with the guideline and approval of the Ethical Committee on Animals Experiments of Khon Kaen University (AEKKU 43/2554). Lighting was controlled to supply 12 h of light and 12

h of dark for each 24-h period. They were maintained at room temperature approximately 23 °C with constant humidity and they were allowed to acclimatize to laboratory conditions for a week before starting the experiment. They were given water ad libitum throughout the study period. Acute oral toxicity test was carried out according to the Organization for Economic Co-operation and Development (OECD) guidelines (Organization for Economic Co-operation and Development (OECD), 2012) (Chemicals, 2005; Combes *et al.*, 2004). by using fixed dose test. And used a dose of 5000 mg/kg as limit test.

3.3 Results

3.3.1 Total phenolic content and flavonoid contents and tannin

Table 3-2 showed the concentrations of total phenolic contents, flavonoid contents and tannin of selected Thai medicinal plants. The results showed that among 3 types of the selected plants, *N.nucifera* extract showed the highest total phenolic contents at the concentration of 152.963±0.009 mg GAE/g extract. *A.occidentale* and *M.oleifera* extracts contained total phenolic contents at concentrations of 102.963±0.006 and 62.333±0.008 mg GAE/g extract, respectively. It was found that *M. oleifera* extract contained the highest concentration of total flavonoid contents at the concentration of 29.900±0.001 mg QE/g extract followed by *N.nucifera* and *A.occidentale* extracts which showed the total flavonoid contents at the concentrations of 4.305±0.009 and 0.625±0.013 mg QE/g extract, respectively. Moreover, *A.occidentale* extract contained the highest concentration of tannin at the concentration of 136.952±0.020 mg TAE/g extract followed by *N.nucifera* and *M.oleifera* extract which showed the tannin at the concentrations of 133.524±0.014 and 85.048±0.017 mg TAE/g extract, respectively.

Table 3-2 Total phenolic contents, total flavonoid contents and tannin of *M.oleifera* leaves, *A.occidentale* leaves and *N.nucifera* flowers extract

Plants extract	Total Phenolic contents (mg GAE/g extract)	Total flavonoid contents (mg QE/g extract)	Tannin (mg TAE/g extract)
M.oleifera leaves	62.333±0.008	29.900±0.001	85.048±0.017
A.occidentale leaves	102.963±0.006	0.625±0.013	136.952±0.020
N.nucifera flowers	152.963±0.009	4.305±0.009	133.524±0.014

3.3.2 Antioxidant activity

Table 3-3 showed the antioxidant activity via DPPH and FRAP assays of the selected medicinal plants. The results showed that IC50 via DPPH radical scavenging activity of *N.nucifera* extract showed the lowest IC50 at concentration of 1.39±.0017 mg/ml., followed by *A.occidentale* and *M.oleifera* which showed IC50 of DPPH at the concentrations of 1.70±0.019 and 8.27±0.023 mg/ml, respectively. Ascorbic acid which was used as the standard control showed the IC50 of DPPH at the concentration of 0.12±0.009 mg/ml. It was found that FRAP activities of *N.nucifera*, *A.occidentale* and *M.oleifera* extracts were 1048.33±0.016, 898.33±0.018 and 339.00±0.010 µM AAE/mg extract. *N.nucifera* showed the highest FRAP activity.

Table 3-3 Antioxidant activity of *M.oleifera* leaves, *A.occidentale* leaves and *N.nucifera* flowers extract

Drug and plants extract	IC_{50} of DPPH assay (mg/ml)	FRAP activity (µM AAE/mg extract)
Ascorbic acid	0.12±0.009	-
M.oleifera leaves	8.27±0.023	339.00±0.010
A.occidentale leaves	1.70±0.019	898.33±0.018
N.nucifera flowers	1.39± 0.017	1048.33±0.016

3.3.3 Acetylcholinesterase inhibition activity

The AChE inhibition activity of the selected medicinal plants were shown in table 3-4. It was found that IC50 values of AChEI of *M. oleifera, A.occidentale* and *N.nucifera* extracts were 922.0±0.800, 651.0±0.731 and 438.0±0.868 µg/ml respectively. *N.nucifera* showed the lowest IC50 of AChE-I.

Donepezil is an AChE-I was used as the standard control showed the IC50 of AChE-I at the concentration of 0.700±0.001 μg/ml.

Table 3-4 Acetylcholinesterase inhibition (IC50) of *M.oleifera* leaves, *A.occidentale* leaves and *N.nucifera* flowers extract

Drug and plants extract	IC_{50} (μg/ml)
Donepezil (AChE-I)	0.700±0.001
M.oleifera leaves	922.0±0.800
A.occidenale leaves	651.0±0.731
N.nucifera flowers	438.0±0.868

3.3.4 Monoamine oxidase B suppression activity

The MAO-B suppression of *M. oleifera, A.occidentale* and *N.nucifera* extracts were shown in table 3-5. It was found that IC50 values of MAO-B-I of *M. oleifera, A.occidentale* and *N.nucifera* extracts were 0.255±0.008, 0.064±0.007 and0.140±0.008 mg/ml respectively. *A.occidentale* showed the lowest IC50 of MAO-B-I. Interestingly, Seligeline, a MAO-B inhibitor,was used as the standard control and showed the IC50 of MAO-B-I at the concentration of 0.005±0.001 mg/ml.

Table 3-5 Monoamine oxidase B suppression (IC50) of *M.oleifera* leaves, *A.occidentale* leaves and *N.nucifera* flowers extract.

Drug and plants extarct	IC_{50} (mg/ml)
Selegiline (MAO-B-I)	0.005±0.001
M.oleifera leaves	0.255±0.008
A.occidenale leaves	0.064±0.007
N.nucifera flowers	0.140±0.008

3.3.5 Phosphodiesterase 5 inhibition activity

The PDE5-I of *M. oleifera, A.occidentale* and *N.nucifera* extract were shown in table 3-6. It was found that IC50 values of PDE5-I of *M. oleifera, A.occidentale* and *N.nucifera* extracts were 2.104±0.008, 7.627±0.005 and 20.000±0.008 mg/ml respectively. *M.oleifera* showed the lowest IC50 of PDE5-I. Sildenafil citrate, a PDE5-I, was used as the standard control showed the IC50 of PDE5-I at the concentration of 0.001±0.001 mg/ml.

Table 3-6 Phosphodiesterase 5 inhibition (IC50) of *M.oleifera* leaves, *A.occidentale* leaves and *N.nucifera* flowers extract.

Drug and plants	IC_{50} (mg/ml)
Sildenafil (PDE5-I)	0.001±0.001
M.oleifera leaves	2.104±0.008
A.occidenale leaves	7.627±0.005
N.nucifera flowers	20.000±0.008

3.3.6 Sample analysia of *M.oleifera* leaves, *A.occidentale* leaves and *N.nucifera* flowers extract

The finger prints of *M.oleifera* leaves, *A.occidentale* leaves and *N.nucifera* flowers extracts were shown in figure 3-1 – 3-3. The contents of gallic acid, ferulic acid and quercetin were expressed in table 3.7. It was found that *A.occidentale* extracts showed the gallic acid at the concentrations of 7.771±0.003. *M. oleifera* extracts showed the ferulic acid content at the concentrations of 0.003±0.0001 FAE/mg extract. Quercetin contents in *M. oleifera, A.occidentale* and *N.nucifera* extract were also determined and the concentrations of quercetin in *M. oleifera, A.occidentale* and *N.nucifera* extract were 0.444±0.0001, 0.617±0.0001 and 0.456±0.0001 mg QE/mg extract respectively.

Table 3-7 The contents of gallic acid, ferulic acid and quercetin of *M.oleifera* leaves, *A.occidentale* leaves and *N.nucifera* flowers extract by HPLC analysis

Plants	Gallic acid	Ferulic acid	Quercetin
	mg GAE/mg extract	mg FAE/mg extract	mg QE/mg extract
M.oleifera leaves	-	0.003±0.0001	0.444±0.0001
A.occidentale leaves	7.771±0.003	-	0.617±0.0001
N.nucifera flowers	-	-	0.456±0.0001

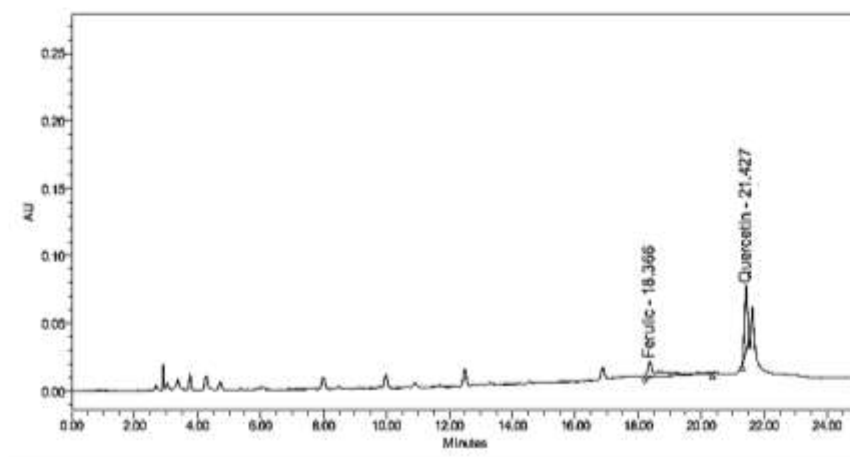

Figure 3-1 Finger print chromatogram of 50%hydro-alcoholic extract of *M.oleifera* leaves

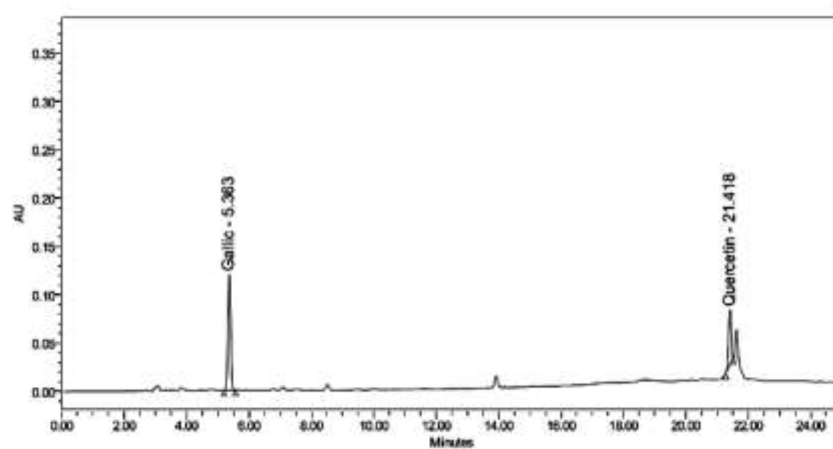

Figure 3-2 Finger print chromatogram of 95% hydro-alcoholic extract of *A.occidentale* leaves

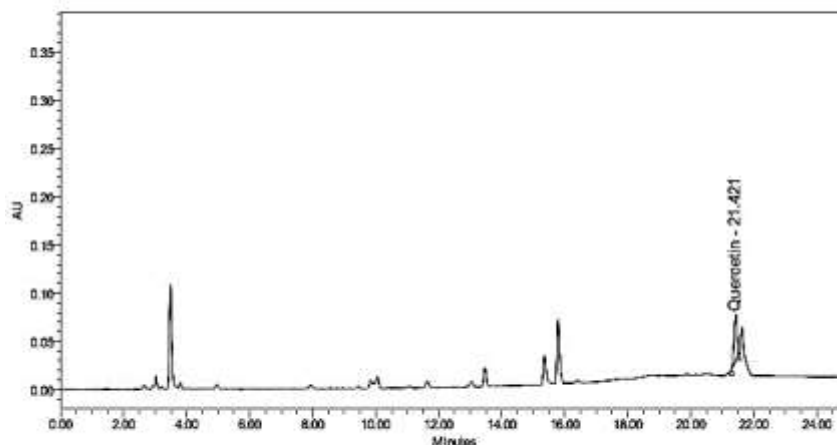

Figure 3-3 Finger print chromatogram of 50% hydro-alcoholic extract of *N.nucifera* flowers

3.3.7 Acute toxicity of *M.oleifera* leaves, *A.occidentale* leaves and *N.nucifera* flowers extract

The data obtained from the present study demonstrated that *M.oleifera* leaves, *A.occidentale* leaves and *N.nucifera* flowers extracts did not produce any mortality throughout the study period of 14 days. Our data also showed no signs of changes in the skin, fur, eyes mucous membrane, behavior patterns, tremors, salivation, and diarrhea of the rats were observed. The results also showed that no significant changes of hematology and clinical chemistry were observed in this study. In addition, no significant changes of gross morphology and histology were observed. According to the Organization of Economic Cooperation and Development (OECD) guidelines for acute oral toxicity, an LD50 of 2,000 mg/kg BW or above is categorized as unclassified and hence the extract is found to be safe. LD50 of *M.oleifera* leaves and *N.nucifera* flowers extracts should be more than 5,000 mg/kg BW while LD50 of *A.occidentale* leaves extract should be more than 2,000 mg/kg BW. Therefore, *M.oleifera* leaves, *A.occidentale* leaves and *N.nucifera* flowers extracts are safe especially for short duration application.

3.4 Discussion

The data obtained from this study showed that all selected plants exhibited the antioxidant activity. However, the IC50 values were more than ascorbic acid which was used as positive control. However, the daily allowance of ascorbic acid was not more than 2100 international unit or around 1.75 mg/kg BW. Therefore, it is not convenient to use vitamin C for protecting stress related disorder in practical. According to this study, the data clearly revealed that the IC50 values of antioxidant activity and IC50 of the suppression effect of AChE, MAO-B and PDE-5 are not too high. In addition, LD50 of all selected plants were more than 5000 mg/kg BW. Based on this data, it has been suggested that *M.oleifera* leaves, *A.occidentale* leaves and *N.nucifera* flowers extracts are the potential natural resources for developing health products protecting against stress related disorders. *A.occidentale* and *M.oleifera* leaves extracts appeared to show high potential to protect against sexual dysfunction because it showed the lowest IC50 of MAO-B and PDE-5 when compared to *N.nucifera. A.occidentale* leaves extract showed low IC50 of both AChE and MAO-B together with high antioxidant activity. Therefore, it showed high potential to be served as the protective health product against memory deficit induced by stress.

Based on the finger print chromatogram, quercetin might be responsible in part for the anti-stress effect of *M.oleifera* leaves extract whereas gallic acid might play the important role on the anti-stress effect of *A.occidentale* and *N.nucifera.* However, this requires further investigations.

3.5 Conclusion

Leaves of *M.oleifera* and *A.occidentale* and *N.nucifera* flowers are the potential natural resources for developing health products protecting against memory deficit and sexual dysfunction induced by stress. However, further research in animal model is necessary.