

APPENDICES

APPENDIX A

Determination of Total Phenolic Compounds

DETERMINATION OF TOTAL PHENOLIC COMPOUNDS

Reagents

1. Folin Ciocateau solution

Freshly mix 2 ml of Folin ciocateau solution with distilled water 4 ml.

2. 7.5% Sodium carbonate (Na_2CO_3)

Dissolve 7.5 g of sodium carbonate with distilled water 100 ml, stir with heat on magnetic stirrer.

3. Gallic acid standard

Dissolve Gallic acid 5 mg with distilled water 10 ml. Standard was then diluted into series of concentration (0, 50, 100, 150, 250 and 500 mg/L).

4. Plants extract 5mg/ml

Dissolve 0.1 g of extract with distilled water 20 ml, stir and centrifuge at 4,000 g for 10 minutes.

Procedures

1. Pipette solution into glass tube as follow:

Reagents	Volume (μl)		
	Blank	Sample	Standard
Sample	-	200	-
Gallic acid	-	-	200
Distilled water	200	-	-
Folin Ciocateau	1000	1000	1000

2. Mix and leave at room temperature for 8 minutes.
3. Add sodium carbonate 800 μl to all tubes.
4. Mix and leave at room temperature for 30 minutes.
5. Read the absorbance at 765 nm with UV-spectrophotometer (Pharmacia LKBBiochrom4060) Calculation of total phenolic contents.

The O.D from standard Gallic acid was plotted against concentration and the amount of total phenolic in plants extract was calculated from the following equation:

$$X = (Y - 0.0014) / 0.0007$$

$$R^2 = 0.9998$$

X = total phenolic compound of plants extract, Y = O.D of plants extract

Reference

- Quettier, D.C., Gressier, B., Vasseur, J., Thierry, D., Claude, B., Michel, L. et al. Phenolic compounds and antioxidant activities of buckwheat (*Fagopyrum esculentum* Moench) hulls and flour. **Journal of Ethnopharmacology** 2000; (72), 35–42.
- Prior, R.L., Wu, X., Schaich, K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. **Journal of Agricultural and Food Chemistry** 2005; 53(10), 4290-02.

APPENDIX B

Determination of Flavonoid Content

DETERMINATION OF FLAVONOIDS CONTENT

Reagents

1. 1 M Potassium acetate (KCH₃COO)
2. 10% AlCl₃ (1:10 w/v)
3. 50% alcohol
4. Distilled water

Standard Quercitin or Rutin with concentration 1, 5, 10, 20, 30 and 50 mg/ml

Plants extract containing 1 mg/ml

Procedures

1. Pipette the following reagents into the labeled glass tube:

Reagents	Volume (μl)		
	Blank	Sample	Standard
KCH ₃ COO	100	100	100
AlCl ₃	100	100	100
50% Alcohol	1500	1500	1500
Plant extract	-	500	-
Quercitin, Rutin	-	-	500
Distilled water	3300	2800	2800

2. Incubate at roomtemperature for 45 minutes.
3. Read the absorbance at 415 nm with UV-spectrophotometer (Pharmacia LKB - Biochrom4060).

Calculation of Flavonoid content

The O.D from standard Quercitin or Rutin was plotted against concentration and the Flavnoid content of Plants extract was calculated from the following equation:

Quercitin:

$$Y = 0.0898X + 0.3396$$

$$R^2 = 0.9908$$

Rutin:

$$Y = 0.0526X + 0.5652$$

$$R^2 = 0.9904$$

X = Flavonoid content equivalent of plants, Y = O.D of plants extract

Reference

Quettier, D.C., Gressier, B., Vasseur, J., Thierry, D., Claude, B., Michel, L. et al.

Phenolic compounds and antioxidant activities of buckwheat (*Fagopyrum esculentum* Moench) hulls and flour. **Journal of Ethnopharmacology** 2000; (72), 35–42.

Prior, R.L., Wu, X., Schaich, K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. **Journal of Agricultural and Food Chemistry** 2005; 53(10), 4290-02.

APPENDIX C
Determination of Tannin

DETERMINATION OF TANNIN

Reagents

1. Folin Ciocateau solution

2. 35% Sodium carbonate (Na_2CO_3)

Dissolve 35 g of sodium carbonate with distilled water 100 ml, stir with heat on magnetic stirrer.

3. Tannic acid standard

Dissolve Tannic acid 5 mg with distilled water 10 ml. Standard was then diluted into series of concentration (0, 5, 10, 25, 50, 100, 250, 250 , 500 and 1000 $\mu\text{g}/\text{mL}$).

4. Plants extract 5mg/ml

Dissolve 0.1 g of extract with distilled water 20 ml, stir and centrifuge at 4,000 g for 10 minutes.

Procedures

1. Pipette solution into glass tube as follow:

Reagents	Volume (μl)		
	Blank	Sample	Standard
Sample	-	100	-
Gallic acid	-	-	100
Distilled water	8500	8400	8400
Folin Ciocateau	500	500	500
Sodium Carbonate Solution	1000	1000	1000

2. Incubate at room temperature for 30 minutes.

3. Read the absorbance at 725 nm with UV-spectrophotometer (Pharmacia LKB - Biochrom4060).

Calculation of Tannin.

The O.D from standard Gallic acid was plotted against concentration and the amount of total phenolic in plants extract was calculated from the following equation:

$$X = (Y-0.0072)/0.0007$$

$$R^2 = 0.9961$$

X = total phenolic compound of plants extract, Y = O.D of plants extract

Reference

Tamilselvi N, Krishnamoorthy P, Dhamotharan R, Arumugam P and Sagadevan E.

Analysis of total phenols, total tannins and screening of phytochemicals in *Indigofera aspalathoides* (Shivanar Vembu) Vahl EX DC. *Journal of Chemical and Pharmaceutical Research* 2012; 4(6):3259-3262.

APPENDIX D
Determination of FRAP Activity

DETERMINATION OF FRAP ACTIVITY

Reagents

1. Acetate buffer 300 mM, pH 3.6

Dissolve 3.1 g sodium acetate with distilled water 900 ml and concentration acetic acid 16 ml is added. Add distilled water until the final volume is 1,000 ml. The solution can be prepared and kept at 4 °C.

2. 2,4,6-tripyridyl-striazine (TPTZ) 10 mM in 40 mM HCl

Freshly prepare TPTZ on a day of assay. Dissolve 31.2 mg TPTZ with 40 mM HCl 10 ml at 50 °C in water bath.

3. FeCl₃·6H₂O 20 mM

Freshly prepare on a day of assay. Dissolve 54.06 mg with distilled water 10 ml.

Standard L-ascorbic acid with concentration 10, 25, 50, 100, 250, 500 and 1,000 µg/ml

Plants extract containing 5, 10, 25, 50, 100, 250, 500 and 1,000 µg/ml

Procedures

1. Prepare FRAP reagent by mixing reagent A: B: C with the following proportion 10: 1: 1
2. Pipette the following reagents into the labeled glass tube:

Reagents	Volume (µl)		
	Blank	Sample	Standard
FRAP reagent	3,000	2,850	2,850
Plant extract	-	150	-
L-ascorbic acid	-	-	150

3. Incubate at 37 °C in water bath for 10 minutes.
4. Read the absorbance at 593 nm with UV-spectrophotometer (Pharmacia LKB-Biochrom4060).

Calculation of FRAP activity

The O.D from standard L-ascorbic acid was plotted against concentration and the FRAP activity of Plants extract was calculated from the following equation:

$$Y = 0.0018X + 0.1028$$

$$R^2 = 0.9988$$

X = FRAP activity equivalent of plants, Y = O.D of plants extract

Reference

Sreelatha S, Padma PR. Antioxidant activity and total phenolic content of *Moringa oleifera* leaves in two stages of maturity. Plant Foods Hum Nutr 2009; 64(1): 303-11.

APPENDIX E

Determination of DPPH Radical Scavenging Activity

DETERMINATION OF DPPH RADICAL SCAVENGING ACTIVITY

Reagents

- 0.1 mM DPPH (2, 2-diphenyl-1-picrylhydrazyl)

Dissolve 4.0 mg of DPPH with methanol 100 ml. Keep in dark room at room temperature.

- Plants extract

Prepare series concentration of plants extract containing 5, 10, 25, 50, 100, 250, 500 and 1000 $\mu\text{g/ml}$ in methanol.

- L-ascorbic acid standard

Dissolve 2.5 mg L-ascorbic acid with methanol 5 ml. Prepare series concentration containing 0, 1, 2, 3, 5 and 10 $\mu\text{g/ml}$.

Procedures

- Pipette the following reagents into labeled glass tube:

Reagents	Volume (ml)			
	Blank	Control	Sample	Standard
Extract	-	-	0.04	-
L-ascorbic acid	-	-	-	0.04
DPPH	-	2.96	2.96	2.96
Methanol	3.0	0.04	-	-
Total	3.0	3.0	3.0	3.0

- Shake all tube vigorously and incubate in dark room at room temperature for 30 minutes
- Read the absorbance at 517 nm with UV-spectrophotometer (Pharmacia LKB-Biochrom 4060)

Calculation of the half maximal inhibitory concentration (IC₅₀)

The percentage of inhibition of each concentration is calculated by the following

Formula;

$$\% \text{Inhibition} = [1 - (A_{\text{extract}}/A_{\text{control}})] \times 100$$

The percentage of inhibition of each substance is plotted against concentration and the IC₅₀ is calculated

Standard is calculated from the following equation;

$$\text{L-ascorbic acid; } Y = 13.034X + 30.787$$

$$R^2 = 0.9988$$

$$Y = 50\% \text{ inhibition, } X = \text{EC}_{50}$$

Reference

Sreelatha, S. & Padma, P.R. Antioxidant activity and total phenolic content of *Moringa oleifera* leaves in two stages of maturity. **Plant Foods Hum Nutr** 2009; 64(1): 303-11.

APPENDIX F

Determination of in Vitro Acetyl Cholinesterase (AChE) Inhibition

DETERMINATION OF ACETYLCHOLINESTERASE (AChE) INHIBITION

Reagents

1. 15 mM Acetylthiocholine iodide (ATCI)
2. 3 mM 5,5-dithiobis-2-nitrobenzoate (DTNB)
3. 50 mM Tris-HCl, pH 8.0 containing 0.1% bovine serum albumin (BSA)
4. 0.22 U/ml of Acetylcholinesterase (AChE)

Standard Donepezil with concentration 5, 10, 25, 50, 100, 250, 500 and 1000 $\mu\text{g/ml}$

Plants extract containing 5, 10, 25, 50, 100, 250, 500 and 1000 $\mu\text{g/ml}$

Procedures

1. Pipette the following reagents into the labeled glass tube:

Reagents	Volume (μl)		
	Blank	Sample	Standard
50% aqueous methanol in buffer	25	-	-
ATCI	25	25	25
DTNB	125	125	125
Tris-HCl	50	50	50
Plant extract	-	25	-
Donepezil	-	-	50

2. Incubate at 25 °C for 5 minutes and read the absorbance at 415 nm with microplate reader
3. Add Acetylcholinesterase 25 μl to all tube
4. Read the absorbance at 415 nm with microplate reader

Calculation of Acetylcholinestearse

The percentage of inhibition of each concentration is calculated by the following formula;

$$\% \text{Inhibition} = [1 - (A_{\text{extract}}/A_{\text{control}})] \times 100$$

The percentage of inhibition of each substance is plotted against concentration and the IC50 is calculated

Standard is calculated from the following equation;

$$\text{Donepezil; } Y = 0.6904X + 36.41$$

$$R^2 = 0.9916$$

$$Y = 50\% \text{ inhibition, } X = \text{IC50}$$

Reference

- Ellman, G.L., Courtney, K.D., Andres, V.J. & Feath-erstone, R.M. A new and rapid Colorimetric determination of acetylcholinesterase activity. **Bio-chem. Pharmacol** 1961; (7), 88-95.
- Feitosa, C.M., Freitas, R.M., Luz, N.N.N., Bezerra, M.Z.B., Trevisan, M.T.S. acetylcholinesterase inhibition by some promising Brazilian medicinal plants **Braz. J Biol** 2011; 71(3), 783-9.

APPENDIX G

Determination of in Vitro Monoamine Oxidase-B (MAO-B) Inhibition

DETERMINATION OF MONOAMIN OXIDASE-B INHIBITION

Reagents:

1. 0.2 M Potassium phosphate saline buffer
2. 50 μ M Clorgyline
3. 2.5 mM P-Tyramine
4. Chromogenic solution (1 mM Vanillic acid, 0.5 mM 4-aminoantipyrine, 4 U/ml peroxidase and PBS)
Standard Selegiline with concentration 5, 10, 25, 50, 100, 250, 500 and 1000 μ g/ml

Procedures:

1. Pipette (in micrometers) the following reagents into cuvettes:

	Volume (μ l)		
	Blank	Standard	Unknown
Distilled water	40	-	-
Selegiline	-	40	-
Sample	-	-	40
MAO enzymes	40	40	40
Clorgyline	40	40	40

2. Incubate the mixture at 37 °C, 30 minutes
3. Add Tyramine 160 μ l (except blank)
4. Add chromogenic solution 40 μ l
5. Incubate the mixture at 37 °C, 30 minutes
6. Read endpoint absorbance at 490 nm

Calculation of Monoamine oxidase-B

The percentage of inhibition of each concentration is calculated by the following formula;

$$\% \text{Inhibition} = [1 - (A_{\text{extract}}/A_{\text{control}})] \times 100$$

The percentage of inhibition of each substance is plotted against concentration and the IC50 is calculated

Standard is calculated from the following equation;

Selegiline: $Y = 9.034X + 4.933$

$$R^2 = 0.964$$

$$Y = 50\% \text{ inhibition, } X = \text{IC50}$$

References:

Holt A, Sharman DF, Baker GB, Palcic MM. A continuous spectrophotometric assay for monoamine oxidase and related enzymes in tissue homogenates. *Analytical biochemistry* 1997; 244: 384-392.

APPENDIX H

Determination of in Vitro Phosphodiesterase-5 (PDE-5) Inhibition

DETERMINATION OF PHOSPHODIESTERASE-5 INHIBITION

Reagents:

PDE-Glo™ Phosphodiesterase Assay kit (Promega)

1. Active PDE in 1X PDE-Glo™ Reaction Buffer
2. 20μM cGMP solution
3. PDE-Glo™ Termination Buffer
4. PDE-Glo™ Detection Solution
5. Kinase-Glo® Reagent

Standard Sildenafil with concentration 5, 10, 50, 100, 500 1,000, 5,000 and 10000 μg/ml

Procedures:

1. Pipette (in micrometers) the following reagents into cuvettes:

	Volume (μl)		
	Blank	Standard	Unknown
Sample	-	-	6.0
Deionized water	6.0	-	-
Standard	-	6.0	-
Active PDE in 1X PDE-Glo™ Reaction Buffer	6.5	6.5	6.5
20μM cGMP solution	12.5	12.5	12.5

2. Incubate the mixture for 10 minutes
3. Add PDE-Glo™ Termination Buffer 12.5 μl to all well for 10 minutes
4. Add PDE-Glo™ Detection Solution 12.5 μl to all well for 20 minutes
5. Add Kinase-Glo® Reagent 50.0 μl to all well for 10 minutes
6. Read with luminometer

Calculation of Phosphodiesterase-5 activity

The percentage of inhibition of each concentration is calculated by the following formula;

$$\% \text{Inhibition} = [1 - (A_{\text{extract}}/A_{\text{control}})] \times 100$$

The percentage of inhibition of each substance is plotted against concentration and the IC50 is calculated

Standard is calculated from the following equation;

$$\text{Sildenafil; } Y = 7.061X - 8.726$$

$$R^2 = 0.999$$

$$Y = 50\% \text{ inhibition, } X = \text{IC50}$$

References:

Protocol CZ. PDE-Glo™ Phosphodiesterase Assay

APPENDIX I

Preparation of Phosphate Buffer Saline Solution

PREPARATION OF PHOSPHATE BUFFER SALINE SOLUTION

0.1 M phosphate buffer saline

Reagents

1. KCl 0.8 g.
2. KH_2PO_4 0.8 g.
3. NaCl 3.2 g.
4. Na_2HPO_4 4.6 g.

Procedures:

1. Add KCl, KH_2PO_4 , NaCl and Na_2HPO_4 in 3,800 ml of distilled water.
2. Dissolve these chemicals on magnetic stirrer.
3. Adjust to pH 7.4 with NaOH.
4. Make up the final volume to 4,000 ml.

Reference

Merrante, F., Raha, S., Reod, J.K. & Proteau, G. The simultaneous Isolation of RNA and

DNA from tissues and Cultured Cells. In: Harwood AJ, editor. Methods in Molecular Biology: Basic DNA and RNA Protocols. London: Humana Press, 1996. 3-9.

APPENDIX J
Preparation of Tissue Sections

PREPARATION OF TISSUE SECTIONS

Procedures:

1. The brains of the animals were perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.3.
2. Following the perfusion, the brains removed and post fixed with 4% paraformaldehyde in 0.1 M phosphate buffer overnight at 4 °C.
3. Tissues were rinsed with phosphate buffer and infiltrated with 30% sucrose solution in order to provide cryoprotection.
4. The specimens were frozen rapidly with deep freeze at -25 °C in cryostat (model JUNG FRIGOCUT 2800E).
5. After freezing, 30 µm thick of specimens were cut on cryostat.
6. Sections were stored in phosphate buffer and they were picked up on slides coated with 0.3% gelatin.

Reference

Krill, J.J., Halliday, G.M., Svoboda, M.D. & Cartwright, H. The cerebral cortex damaged in chronic alcoholic. **Neuroscience** 1997; 79(7), 983-98.

APPENDIX K
Cresyl Violet Staining For Nissl Substance

CRESYL VIOLET STAINING FOR NISSL SUBSTANCE

Cresyl violet can be used to demonstrate Nissl substance. The rationale of the technique is simple acid-base reaction, where the cationic dyes bond with the anionic RNA of the Nissl substance, plus the DNA and RNA of cell nuclei.

Staining solution:

0.5% aqueous solution of cresyl violet 100 ml

10% acetic acid 7 ml

Add 10% acetic acid 7 ml in 0.5% aqueous solution of cresyl violet 100 ml and adjust pH to 3.5-3.8. Stand the solution at room temperature for 24-48 hours. The solution should be heated gently and filtrated before used.

Procedures:

1. Immerse slides into xylene solution for 2 times. Approximate 2-3 minutes each.
2. Hydrate the sections in serial concentration of alcohol; absolutes, 95% and 70% alcohol approximate 3 minutes per each process.
3. Wash the sections in distilled water.
4. Stain the sections in cresyl violet solution for 3-5 minutes, Nissl body should be violet.
5. Immerse the sections in the serial concentration of alcohol; 70%, 95% and absolute alcohol for 1 minute or longer per each process until the background is relatively clear.
6. Clear the sections in xylene solution for 2-3 minutes.
7. Mount the slides and coverslipped with DPX permount.

Results: Nissl body: Violet

Reference

Paxinos, G. & Chorles, W. **Cresyl Violet**. In: Paxinos G, Chores W, editors. The rat brain

in stereotaxic coordinates London: Academic Press; 1981.

APPENDIX L

Hematoxylin and Eosin Staining for Testis

HEMATOXYLIN AND EOSIN STAINING FOR TESTIS

Reagents:

Hematoxylin; Iron Hematoxylin (Weigert's hematoxylin)

- 1% Hematoxylin solution in absolute alcohol
- Iron solution (30% of ferric chloride 4 ml, Hydrochloric acid 1 ml, Distilled water 95 ml)

Eosin

- 0.5% Eosin Y in 95% alcohol

Procedures:

1. Immerse slides into xylene solution for 2 times. Approximate 2-3 minutes each.
2. Hydrate the sections in serial concentration of alcohol; absolutes, 95% and 70% alcohol approximate 3 minutes per each process.
3. Wash the sections in distilled water.
4. Stain the sections in Hematoxylin solution for 15 minutes, nucleic acid should be blue.
5. Stain the sections in Eosin solution for 45 seconds, cytoplasm should be pink.
6. Immerse the sections in the serial concentration of alcohol; 70%, 95% and absolute alcohol for 1 minute or longer per each process until the background is relatively clear.
6. Clear the sections in xylene solution for 2-3 minutes.
7. Mount the slides and coverslipped with DPX permount.

Reference

Turner TT, Tung KS, Tomomasa H, Wilson LW. Acute testicular ischemia results in germ cell-specific apoptosis in the rat. **Biol Reprod** 1997; 57(6): 1267-1274.

APPENDIX M

Immunohistochemical Study of Tyrosine Hydroxylase (TH) Enzyme

IMMUNOHISTOCHEMICAL STUDY OF TYROSINE HYDROXYLASE (TH) ENZYME

Reagents:

1. KPBS-BT (Kreb phosphate buffer saline containing bovine serum albumin and triton x-100).
2. 0.05 M Tris-HCL buffer pH 7.6.
3. 0.5% H₂O₂ in methanol.
4. Primary antibody against tyrosine hydroxylase (TH) dilute 1:400.
5. DAKO Strept ABC Complex/HRP duet kit. This kit consist of reagent

A: Streptavidin

B: biotinylated horseradish

C: biotinylated goat antibody to mouse immunoglobulin

Working solution of biotinylated goat antibody to mouse immunoglobulin: Add reagent C 10 µl in 1 ml of KPBS-BT.

Working solution of Strept AB Complex/HRP: Add 10 µl of reagent A and B into 1 ml of KPBS-BT.

6. 0.4% H₂O₂ and diaminobenzidine in 0.05 M Tris-HCL.
7. 5% normal horse serum in KPBS-BT.

Procedures:

1. Inhibit endogenous peroxidase activity by incubating in 0.5% H₂O₂ in methanol for 30 minutes.
2. Wash slides in running tap water for 1 minute than was slides again in distilled water for 1 minute.
3. Wash slides in KPBS and KPBS-BT for 5 minutes per each process.
4. Remove excess buffer, then apply the 5% normal goat serum in KPBS-BT to the sections and incubate in moist chamber for 30 minutes in order to minimize background staining.
5. Drain off excess normal goat serum.

6. Incubate sections in mouse primary antibody against TH dilute 1:400 in KPBS-BT at room temperature for 2 hours and then incubate at 4°C for 48 hours (This step is omitted in control slide).
7. Wash off excess antiserum and wash slides in KPBS-BT for two 7 minutes changes.
8. Drain off excess buffer and incubate slides with 100 µl of working solution of biotinylated goat antibody to mouse for 4 hours at room temperature.
9. Wash slides in KPBS-BT for two 7 minutes changes.
10. Drain off excess buffer and incubate slides with 100 µl of working solution of Strept AB Complex/HRP for 4 hours at room temperature.
11. Wash slides in KPBS-BT for 1 minute and then was slides again with KPBS for 10 minutes two times.
12. React for peroxidase activity in KPBS-BT containing 0.025% diaminobenzidine (DAB) and 0.01% H₂O₂ for 48 hours at room temperature.
13. Wash in running tap water, let dry and mount section in DPX permount.

Reference

Wood GS, Warnke R. Suppression of endogenous avidin-binding activity in tissues and

its relevance to biodin-avidin detection systems. **J Histochem Cytochem** 1981; 29(10): 1196-1204.

APPENDIX N

Preparation of Tissue Homogenates

PREPARATION OF TISSUE HOMOGENATES

After the last orally conventional feeding, all animals were anesthetized with intraperitoneal injection of pentobarbital sodium (Nembutal®) at dose 50 mg/kg BW. Brains and penis were isolated and kept cool in ice buckets. Then brain tissues were homogenized in 5 volumes of PBS with a glass Potter-Elvehjem homogenizer. While, penis was homogenized in 5 volumes of lysate buffer.

Reference

Marzel, P. General principle and procedure for drugs metabolism in vitro.

In: La Du BN, Mandel HG, Way EL, editors. Fundamentals of drugs metabolism and drug disposition. Newyork: Krieger Publishing Company; 1979. 527-52.

APPENDIX O
Determination of Protein

DETERMINATION OF PROTEIN

Reagents:

1. Solution A: Alkaline tartate reagent

0.1 gm of sodium tartate ($\text{Na}_2\text{C}_4\text{H}_4\text{O}_6 \cdot 2\text{H}_2\text{O}$), 10 gm of sodium carbonate (Na_2CO_3) and 1.2 gm of sodium hydroxide (NaOH). Dissolve the chemicals in distilled water to make 500 ml.

2. Solution B

Dissolve 0.5 gm of copper sulfate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) with distilled water 100 ml.

3. Solution C

Freshly mix 50 ml of solution A and 1 ml of solution B.

4. Solution D

Dilute commercial 2.0 N Folin phenol reagent with distilled water 1:1 and use immediately.

5. Standard protein

Dissolve bovine serum albumin (BSA) 60 mg to 100 mg with distilled water.

Procedures

1. Pipette solution into each tube as follows:

Reagents	Volume (ml)		
	Blank	Standard	Unknown
Distilled water	0.2	0.1	0.1
Standard BSA	-	0.1	-
Sample	-	-	0.1
Solution C	5.0	5.0	5.0
Solution D	0.5	0.5	0.5

2. Mix and let stand at room temperature for 10 minutes.

3. Mix and let stand at room temperature for 1 hour.

4. Read OD at 650 nm by spectronic 20 against the reagent blank.

Calculation

$$\text{Protein concentration (g \%)} = \frac{\text{O.D. unknown} \times \text{concentration of standard}}{\text{O.D. standard}}$$

Reference

Lowry, O.H., Roseburgh, N.J., Farr, A.L. & Randall, R.J. Protein measurement with the folin phenol reagent. **J Biol Chem** 1951; 193: 263.

APPENDIX P

Determination of Testosterone and Corticosterone

DETERMINATION OF TESTOSTERONE AND CORTICOSTERONE

Reagents

1. Testosterone levels were measured using a radioimmunoassay (RIA) Kit (TESTO-CT2, Cisbio International, France)
2. Corticosterone levels were measured using a Corticosterone Double Antibody Radioimmunoassay Kit (MP Biomedicals)

Serum preparation

Venous blood of each animal was prepared as serum by the centrifugation at 2000g, 4 °C for 15 minutes and stored at -80 °C until used.

Procedures

Reagent	Testosterone (µl)		Corticosterone (µl)	
Standard	-	25	-	10
Sample	25	-	10	-
¹²⁵ I-Testosterone	500	500	-	-
¹²⁵ I-Corticosterone	-	-	500	500

1. Mixed and incubated at 37 °C for 1 hour
2. Aspired liquid from each tubes
3. Measured the remaining radioactivity bound to the tube with a Gamma scintillation counter
4. Testosterone and corticosterone levels were expressed ng/ml

APPENDIX Q
Determination of Lipid Peroxidation Contents

DETERMINATION OF LIPID PEROXIDATION CONTENTS

Reagents

1. 8.1% SDS (sodium dodecyl sulfate).
2. 20% acetic acid solution adjust to pH 3.5 with NaOH.
3. 0.8% TBA (Triobarbituric acid).
4. TMP (1, 1, 3, 3- tetramethoxy propane) or malondialdehyde bis (dimethyl acetal) solution was used as an external standard, and the level of lipid peroxide was expressed as nmol of MDA (malondialdehyde).

Procedures:

1. Add the following substances in the table into the series of glass tubes with screw capped.

Reagents	Volume (ml)		
	Blank	Standard	Unknown
Sample*	-	-	0.2
8.1% SDS	0.2	0.2	0.2
20% acetic acid	1.5	1.5	1.5
0.8% TBA	1.5	1.5	1.5
TMP stock standard	-	0.2	-
Distilled water	0.8	0.6	0.6

* sample dilution 1:50 for brain

2. Heated the tube in the water-bath at 95 °C for 60 minutes.
3. After cooling with tap water, 1.0 ml of distilled water and 5.0 ml of the mixture of n-butanol and pyridine (15:1, v/v) are added and shaken vigorously.
4. After centrifugation at 4,000 rpm for 10 minutes, the organic layer is taken and its absorbance at 412 nm is measured.
5. The content of lipid peroxide is expressed in term of nmol MDA/100 mg protein.

Calibration Curve

1. Prepare a series of tubes containing TMP stock standard in water in the following concentrations: 2.0 nmol/0.2ml, 4.0 nmol/0.2 ml, 6.0 nmol/0.2 ml, 8.0 nmol/0.2 ml, 10.0 nmol/0.2 ml.
2. Perform the procedure as in step 2.
3. Determine the absorbance at 532 nm. The O.D. was plotted against concentration of MDA which expressed as nmol MDA/100 mg protein.

Reference

Ohkawa, H., Ohishi, N. & Yagi, K. Assay for lipid peroxide in animal tissues by thiobarbituric acid reaction. **Analytical biochemistry** 1979; 95, 351-8.

APPENDIX R
Determination of Catalase Activity

DETERMINATION OF CATALASE

Reagents:

1. 50 mM Potassium Phosphate Buffer, pH 7 at 25 °C
Prepare in deionized water using Potassium Phosphate, Monobasic, Anhydrous.
2. 0.005 N Potassium Permanganate Solution (KMnO₄)
Prepare in deionized water using Potassium Permanganate.
3. 5 N Sulfuric Acid Solution (H₂SO₄)
Prepare in deionized water using Sulfuric Acid.
4. 0.01 N Hydrogen Peroxide (H₂O₂)
Prepare in the buffer using Hydrogen Peroxide, 30% (w/w) Solution.
5. Standard Catalase Enzyme Solution (Std CAT)
Immediately before use, prepare a solution containing 20, 40, 60, 80, and 100 units/ml of Catalase in cold buffer.

Prepare brain homogenates:

Weight a brain sample and diluted in the cold buffer (25 mg brain tissue/ml). Homogenize this solution until the brain is uniformly dispersed in the buffer.

Procedures:

1. Prepare a spectrophotometer to monitor the absorbance, A_{515nm}, by using 1 ml of the buffer as the blank.
2. Pipette 300 µl of H₂O₂ (Reagent 4) and 50 µl of a Std CAT (Reagent 5) into a cuvette.
Immediately mix by inversion and leave the reaction goes on for exact 60 seconds.
3. Then, immediately add 150 µl of H₂SO₄ (Reagent 3) and mix.
4. Add 600 µl of KMnO₄ (Reagent 2) and mix.
5. Read the absorbance, A_{515nm}.
6. Repeat step 2-5 for the different concentrations of Std CAT.
7. Pipette 300 µl of H₂O₂ (Reagent 4) and 50 µl of a sample homogenate into a cuvette.
Immediately mix by inversion and leave the reaction goes on for exact 60 seconds.
8. Repeat step 3-5 for the different samples.

9. Plot the CAT standard curve by plotting A515nm against Std CAT (units/ml) and determine the linear equation of the curve.
10. Calculate the activity of catalase in samples using A515nm and the linear equation of the standard curve.
11. Report and graph the enzyme activity and it is expressed in units/mg protein.

Reference:

Goldblith, S.A. & Proctor, B.E. Photometric determination of catalase activity. **J Biol Chem** 1950; 187(2), 705-709.

APPENDIX S

Determination of Superoxide Dismutase Activity

DETERMINATION OF SUPEROXIDE DISMUTASE ACTIVITY

Reagents:

1. 216 mM Potassium Phosphate Buffer, pH 7.8 at 25 °C

Prepare in deionized water using Potassium Phosphate, Monobasic, Anhydrous.

2. 10.7 mM Ethylenediaminetetraacetic Acid Solution (EDTA)

Prepare in deionized water using Potassium Ethylenediaminetetraacetic Acid, Disodium Salt, Dihydrate.

3. 1.1 mM Cytochrome c Solution (Cyt c)

Prepare in deionized water using Potassium Cytochrome c.

4. 0.540 mM Xanthine Solution (Xanthine), pH 7.4

Prepare in deionized water using Xanthine.

5. Xanthine Oxidase Enzyme Solution (XOD)

Immediately before use, prepare a solution containing 0.5 units/ml of Xanthine Oxidase in cold deionized water.

6. Standard Superoxide Dismutase Enzyme Solution (Std SOD)

Immediately before use, prepare a solution containing 1, 5, 10, 50, 100, 500, and 1000 units/ml of Superoxide Dismutase in cold buffer.

Prepare brain homogenates:

Weight a brain sample and diluted in the cold buffer (5 mg brain tissue/ml). Homogenize this solution until the brain is uniformly dispersed in the buffer.

Procedures:

1. Prepare a reaction cocktail by pipetting the following reagents into a container:

- A. Buffer 25 ml
- B. EDTA 1 ml
- C. Cyt c 1 ml
- D. Xanthine 50 ml

2. Mix and adjust to pH 7.8, 25 °C. Monitor the absorbance, A_{550nm}, of the reaction cocktail until constant using a spectrophotometer.

3. Pipette (in micrometers) the following reagents into cuvettes:

	Cocktail	DI water	XOD	Std SOD	Sample
Blank	900	100	-	-	-
Test 1	900	50	50	-	-
Test 2 Std SOD	900	-	50	50	-
Test 2 Sample	900	-	50	-	50

4. Immediately mix by inversion and record the increase in A_{550nm} for 3 minutes. Obtain the rate of change ($\Delta A_{550nm}/\text{minute}$) for both Test 1 (Uninhibited) and Test 2 (Inhibited).

5. Calculate %inhibition as following:

$$\% \text{inhibition} = \frac{(\Delta A_{550nm}/\text{min Test 1}) - (\Delta A_{550nm}/\text{min Test 2})}{(\Delta A_{550nm}/\text{min Test 1})} \times 100$$

6. Plot the SOD standard curve by plotting %inhibition against Std SOD (units/ml) and determine the linear equation of the curve.

7. Calculate the activity of superoxide dismutase in samples using %inhibition and the linear equation of the standard curve.

8. Report and graph the enzyme activity and it is expressed in units/mg protein.

Reference:

McCord, J.M. & Fridovich, I. Superoxide dismutase. An enzymic function for erythrocyte (hemocuprein). **J Biol Chem** 1969; 244: 6049-55.

APPENDIX T

Determination of Glutathione Peroxidase Activity

DETERMINATION OF GLUTATHIONE PEROXIDASE ACTIVITY

Reagents:

1. 50 mM Sodium Phosphate Buffer with 0.40 mM EDTA, pH 7 at 25 °C
Prepare in deionized water using Sodium Phosphate, Monobasic, Anhydrous and Ethylenediaminetetraacetic Acid, Tetrasodium Salt.
2. 1 mM Sodium Azide Solution (Buffer w/Azide)
Prepare Sodium Azide in reagent 1.
3. β -Nicotinamide Adenine Dinucleotide Phosphate, Reduced Form (β -NADPH)
Use 5 mg vial of β -NADPH (Reduced Form).
4. Glutathione Reductase Enzyme Solution (GR)
Immediately before use, prepare a solution containing 100 units/ml of Glutathione Reductase in cold deionized water.
5. 200 mM Glutathione, Reduced (GSH)
Prepare in deionized water using Glutathione, Reduced Form.
6. 10 mM Sodium Phosphate Buffer with 1 mM Dithiothreitol, pH 7 (Buffer w/DTT)
Prepare in deionized water using Sodium Phosphate, Monobasic, Anhydrous and DL- Dithiothreitol.
7. Standard Glutathione Peroxidase Enzyme Solution (Std GSH-Px)
Immediately before use, prepare a solution containing 1.5, 2, 2.5, 3, and 5 units/ml of Glutathione Peroxidase in cold buffer w/DTT (Reagent 6).
8. 0.042% (w/w) Hydrogen Peroxide (H₂O₂)
Prepare in deionized water using Hydrogen Peroxide, 30% (w/w) Solution.

Prepare brain homogenates:

Weight a brain sample and diluted in the cold buffer w/DTT (Reagent 6, 25 mg brain tissue/ml). Homogenize this solution until the brain is uniformly dispersed in the buffer.

Procedures:

1. Prepare a reaction cocktail by pipetting the following reagents into 5 mg vial of Reagent 3 (β -NADPH):

B. Buffer w/Azide	46	ml
D. GR	0.5	ml
E. GSH	0.25	ml

2. Mix and adjust to pH 7, 25 °C.

3. Pipette (in micrometers) the following reagents into cuvettes:

	Cocktail	Buffer w/DTT	DI water	Std GSH-Px	Sample
Blank	-	-	1000	-	-
Test 1	1000	17	-	-	-
Test 2 Std GSH-Px	1000	-	-	17	-
Test 2 Sample	1000	-	-	-	17

4. Mix by inversion and monitor the absorbance, A_{340nm}, of the reaction cocktail until constant using a spectrophotometer.

5. Add (in micrometers) H₂O₂ (Reagent 8):

	H ₂ O ₂
Test 1	17
Test 2 Std GSH-Px	17
Test 2 Sample	17

Then, immediately mix by inversion and record the decrease in $A_{340\text{nm}}$ for 3 minutes. Obtain the rate of change ($\Delta A_{340\text{nm}}/\text{minute}$) for both Test 1 (Noreaction) and Test 2 (Reaction).

6. Calculate $\text{Diff}\Delta A_{340\text{nm}}/\text{minute}$ as following:

$$\text{Diff}\Delta A_{340\text{nm}}/\text{minute} = (\Delta A_{340\text{nm}}/\text{min Test 2}) - (\Delta A_{340\text{nm}}/\text{min Test 1})$$

7. Plot the GSH-Px standard curve by plotting $\text{Diff}\Delta A_{340\text{nm}}/\text{minute}$ against Std GSH-Px (units/ml) and determine the linear equation of the curve.

8. Calculate the activity of glutathione peroxidase in samples using $\text{Diff}\Delta A_{340\text{nm}}/\text{minute}$ and the linear equation of the standard curve.

9. Report and graph the enzyme activity and it is expressed in units/mg protein.

Reference:

Wendel A. Glutathione Peroxidase, in: W.B. Jakoby editor. Enzymatic Basis of Detoxication, Vol.2, Academic Press, New York; 1980.

APPENDIX U

Determination of Acetylcholinesterase

DETERMINATION OF ACETYLCHOLINESTERASE

Solutions:

1. 0.1 M phosphate buffer (PB), pH 8.0
2. 0.075 M acetylthiocholine iodide (ATCId)
3. 0.01 M dithiobisnitrobenzoate (DTNB)

Prepare brain homogenates:

1. Weight a sample (about 30 mg) and add 1 ml of PB/30 mg tissue (30 mg/ml). Homogenize this solution until the brain is uniformly dispersed in the buffer. Place the tube on ice.

2. Assay

- 2.1 Turn on the spectrophotometer and set at 412 nm. Let it warm up for at least

- 15 minutes prior to reading.

- 2.2 Label the assay tubes-four tubes (3 for the assay and one for a control).

- 2.3 Pipette 3 ml PB into each assay tube.

- 2.4 Using a pipette add 200 μ L of sample homogenate to each of the four labeled assay tubes. Vortex each tube and return it to the ice.

- 2.5 Zero the spec.20 without a tube by setting the needle to 0 transmittance.

- 2.6 Add 100 μ L DTNB to the first cortex tube, vortex, and place it in a test tube rack for five minutes. This allows the solution to reach room temperature.

- 2.7 Vortex and quickly wipe the outside of the tube with a kimwipe. Place the tube in the spectrophotometer. 20 and zero the spectrophotometer to 0 absorbance.

- This will be a baseline reading before measuring product formation.

- 2.8 Take the tube out of the spectrophotometer, quickly add 20 μ L ATCId add vortex.

- 2.9 Immediately return the tube to the spec.20. Note the time and take a zero reading of absorbance. Take readings at 30 sec, 60 sec, 2 min, and 3 min and record the data in a table.

- 2.10 Repeat this procedure (step 6-10) for the other sample homogenates.

Run the control through the same procedure except do not add substrate (ATCld) but add 20 μ L PB instead.

3. Calculate of the rate of the reaction:

3.1 Graph the data for the different brain regions-change in absorbance/min. against time. Are the graphs linear?

3.2 Calculate the rate of color change per minute for each reading and average the rates within each three minute run. Then average the rates between each run for each brain region, calculate the rate of the reaction according to the following equation:

$$R = \Delta A / 1.36 \times 10^4 \times 1 / (200/3320) C_0 = 1.22(10^{-3}) A / C_0$$

R = rate, in moles substrate hydrolyzed/min. g tissue

ΔA = change in absorbance/min.

C_0 = original concentration of tissue (mg/ml) 200/3320 are volume corrections
 $1.36(10^4)$ is the extinction coefficient of the yellow product

3.3 Make a bar graph to show the enzyme activity of each brain region

References:

Thompson, R.F. & Freeman, W.H. *The Brain: A Neuroscience Primer*. New York, 1993; 13.

Robertson, R.T., Holunann, C.F., Bruce, J.L. & Oyle, J.T.C. Neonatal enucleation reduces

specific activity of acetylcholinesterase in developing rat visual cortex. **Devel. Brain Res** 1988; 39: 298-02.

APPENDIX V

Determination of Monoamine Oxidase A and B Activity

DETERMINATION OF MONOAMIN OXIDASE-A AND B ACTIVITY

Reagents:

1. 0.2 M Potassium phosphate saline buffer
2. 50 μ M Clorgyline
3. 2.5 mM P-Tyramine
4. Chromogenic solution (1 mM Vanillic acid, 0.5 mM 4-aminoantipyrine, 4 U/ml peroxidase and PBS)

Standard H₂O₂ with concentration 0, 0.0004, 0.004, 0.04, 0.01, 0.2, 0.4 and 4.0 μ M

Procedures:

1. Pipette (in micrometers) the following reagents into cuvettes:

	Volume (μ l)		
	Blank	Standard	Unknown
PBS	200	-	-
H ₂ O ₂	-	40	-
Sample	-	-	40
Pargyline*	40	40	40
Clorgyline**			

*For MAO-A determination

**For MAO-B determination

2. Incubate the mixture at 37 °C, 30 minutes
3. Add Tyramine 160 μ l (except blank)
4. Add chromogenic solution 40 μ l
5. Incubate the mixture at 37 °C, 30 minutes
6. Read endpoint absorbance at 490 nm

Calculation of Monoamine oxidase-A and B

The O.D from standard H₂O₂ was plotted against concentration and MAO-A activity was calculated from the following equation:

$$\text{H}_2\text{O}_2: Y = 0.085X + 0.132$$

$$R^2 = 0.987$$

X = Concentration of H₂O₂, Y = O.D of plants extract

The O.D from standard H₂O₂ was plotted against concentration and MAO-B activity was calculated from the following equation:

$$\text{H}_2\text{O}_2: Y = 0.095X + 0.007$$

$$R^2 = 0.995$$

X = Concentration of H₂O₂, Y = O.D of plants extract

References:

Holt A, Sharman DF, Baker GB, Palcic MM. A continuous spectrophotometric assay for monoamine oxidase and related enzymes in tissue homogenates. *Analytical biochemistry* 1997; 244: 384-392.

APPENDIX W

Determination of Phosphodiesterase-5 (PDE-5) Activity

DETERMINATION OF PHOSPHODIESTERASE-5 ACTIVITY

Reagents:

PDE-Glo™ Phosphodiesterase Assay kit (Promega)

1. Active PDE in 1X PDE-Glo™ Reaction Buffer
2. 20μM cGMP solution
3. PDE-Glo™ Termination Buffer
4. PDE-Glo™ Detection Solution
5. Kinase-Glo® Reagent

Procedures:

1. Pipette (in micrometers) the following reagents into cuvettes:

	Volume (μl)	
	Blank	Unknown
Sample	-	6.0
Deionized water	6.0	-
Standard	-	-
Active PDE in 1X PDE-Glo™ Reaction Buffer	6.5	6.5
20μM cGMP solution	12.5	12.5

2. Incubate the mixture for 10 minutes
3. Add PDE-Glo™ Termination Buffer 12.5 μl to all well for 10 minutes
4. Add PDE-Glo™ Detection Solution 12.5 μl to all well for 20 minutes
5. Add Kinase-Glo® Reagent 50.0 μl to all well for 10 minutes
6. Read with luminometer

Calculation of Phosphodiesterase-5 activity

The activity of PDE-5 was calculated. Data were expressed percent of control.

References:

Protocol CZ. PDE-Glo™ Phosphodiesterase Assay

APPENDIX X

Western Blotting Analysis of ki-67 and eNOS

WESTERN BLOTTING ANALYSIS OF Ki-67 AND eNOS

Sample preparation

1. Remove hippocampus and penis for determination of Ki-67 and eNOS respectively (store at -80°C).
2. Homogenate frozen tissues in ice cold RIPA buffer with protease inhibitors.
3. Centrifuge at 14,000 g for 30 min and collected the supernatant.
4. Determine protein concentrations using NANO drop Spectrophotometers.

Reagents

1. Electrophoresis buffer: 25 mM Tris, 192 mM glycine and 0.1% SDS (store at room temperature)

Electrophoresis buffer	
Tris	3.03 g
Glycine	14.42 g
SDS	1g
Distilled water up to	1000 ml

2. Transfer buffer: 20 mM Tris, 20% methanol 150 mM glycine (store at 4°C)

Electrophoresis buffer	
Tris	2.42 g
Glycine	12 g
Methanol	200 ml
Distilled water up to	1000 ml

3. Tris - buffer saline (TBS) : 20 mM Tris, 137 mM NaCl

Tris - buffer saline	
Tris	2.42 g
Glycine	8 g
pH adjust to	7.6
Distilled water up to	1000 ml

4. TBS-Tween: Tween 0.1% in TBS; 1 ml of tween 20 is diluted in 1000 ml of TBS (store at room temperature).

5. SDS – PAGE recipes for one gel:

10% separating gel	
Distilled water	1.393 ml
30% Acrylamide gel	1.65 ml
1.5 mM Tris-HCl, pH 8.8	1.875 ml
10% SDS	50 μ l
10% ammonium persulphate	50 μ l
TEMED	15 μ l

4% stacking gel	
Distilled water	2.165 ml
30% Acrylamide gel	400 μ l
0.5 mM Tris-HCl, pH 6.8	375 ml
10% SDS	30 μ l
10% ammonium persulphate	30 μ l
TEMED	10 μ l

Procedures

1. Separate equal amounts of protein (50 μ g for hippocampus and 100 μ g for penis) by SDS-PAGE on 10% SDS-polyacrylamide gel electrophoresis and transfer onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA).
2. Load prestained protein markers to assess completeness of electrophoretic transfer.
3. Incubate blots after electrophoretic transfer to nitrocellulose membrane with blocking buffer (5% skim milk in Tris-buffer saline with 0.05% Tween-20) for 1 h at room temperature.
4. Incubate blots overnight with using one of these antibodies:
Ki-67 (1:400, Sigma-Aldrich, Pte Ltd, Singapore)
eNOS (1:400, Sigma-Aldrich, Pte Ltd, Singapore).
5. Incubate membranes after several washing steps with HRP-linked secondary antibody (1:2,000) for 1 hr at room temperature.
6. Visualize signals by chemiluminescence using ECL kit (Pierce, ThermoScientific).

7. PVDF was probed with the beta actin antibody (1:2,000, Sigma-Aldrich, Pte Ltd, Singapore) as a loading control
8. Quantify images and band densities NIH-ImageJ (Version 1.48V; National Institutes of Health,USA).

Reference

Smith FS, Titheradge MA. Detection of NOS isoforms by western-blot analysis. *Methods Mol Biol* 1998;100: 171-180.

RESEARCH PUBLICATIONS

1. PAPER PUBLICATION

Prabsttroo T, Wattanathorn J, Iamsaard S, Somsapt P, Sritragool O, Thukhummee W, Muchimapura S. *Moringa oleifera* extract enhances sexual performance in stressed rats 2015. *Journal of Zhejiang University Science B*; 3(16):179-190.

2. PETIT PATENT

Wattanathorn J, **Prabsattroo T**, Muchimapura S, Thukham-mee W. Drink from *Moringa Power*. Thai patent request no. 1403001019.