APPENDICES

APPENDIX A

Determination of Total Phenolic Compounds

Reagents

1. FolinCiocateau solution

   Freshly mix 2 ml of Folinciocateau solution with distilled water 4 ml.

2. 20% Sodium carbonate (Na_2CO_3)

   Dissolve 20 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	-	20	-
Gallic acid	-	-	20
Distilled water	1600	1580	1580
FolinCiocateau	100	100	100

2. Mix and leave at room temperature for 8 minutes.
3. Add sodium carbonate 300 µl to all tubes.
4. Mix and leave at room temperature for 2 hours.
5. Read the absorbance at 765 nm withUV-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 DC, Gressier B, Vasseur J, Thierry D, Claude B, Michel L. et al. Phenolic compounds and antioxidant activities of buckwheat (Fagopyrum esculentumMoench) hulls and flour. **Journal of Ethnopharmacology** 2000; 72: 35-42.

Prior RL, Wu X, Schaich K. Standardized methods for the determination of antioxidant capacity and phenolicsinfoods and dietary supplements. **Journal of Agricultural and Food Chemistry** 2005; 53: 10: 4290-02.

APPENDIX B

Determination of Flavonoid Content

Reagents

1. $NaNO_2$(1:20 w/v)
2. $AlCl_3$(1:10 w/v)
3. 1 M NaOH
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
$NaNO_2$	300	300	300
$AlCl_3$	500	500	500
NaOH	2000	2000	2000
Distilled water	2400	1900	1900
Plant extract	-	500	-
Quercitin, Rutin	-	-	500

2. Incubate at 25 ˚C for 15 minutes.
3. Read the absorbance at 510 nm withUV-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 = Flavnoid content equivalent of plants, Y = O.D of plants extract

Reference

Quettier DC, Gressier B, Vasseur J, Thierry D, Claude B., Michel L. et al. Phenolic compounds and antioxidant activities of buckwheat (FagopyrumesculentumMoench) hulls and flour. **Journal of Ethnopharmacology** 2000; 72: 35-42.

Prior RL, Wu X, Schaich K. Standardized methods for the determination of antioxidant capacity and phenolics infoods and dietary supplements. **Journal of Agricultural and Food Chemistry** 2005; 53: 10, 4290-02.

APPENDIX C

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 mMHCl

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. $FeCl_3 \cdot 6H_2O$ 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	1,500	1,450	1,450
Plant extract	-	50	-
L-ascorbic acid	-	-	50

3. Incubate at 37 ˚C in water bath for 10 minutes.

4. Read the absorbance at 593 nm withUV-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 *Moringaoleifera*leaves in two stages of maturity. **Plant Foods Hum Nutr** 2009; 64(1): 303-11.

APPENDIX D

Determination of DPPH Radical Scavenging Activity

Reagents

1. 0.15 mM DPPH (2,2-diphenyl-1-picrylhydrazyl)

Dissolve 5.1 mg of DPPH with methanol 100 ml. Keep in dark room at roomtemperature.

2. Plants extract

Prepare series concentration of plants extract containing 5, 10, 25, 50, 100, 250,
500 and 1000 µg/ml in methanol.

3. 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 µg/ml.

Procedures

1. Pipette the following reagents into labeled glass tube:

Reagents	Volume (ml)			
	Blank	Control	Sample	Standard
Extract	1	-	1	-
L-ascorbic acid	-	-	-	1
DPPH	-	0.5	0.5	0.5
Methanol	0.5	1	-	-
Total	1.5	1.5	1.5	1.5

2. Shake all tube vigorously and incubate in dark room at room temperature for 30 minutes

3. Read the absorbance at 517 nm with UV-spectrophotometer (Pharmacia LKB-Biochrom4060)

Calculation of the half maximal inhibitory concentration (IC_{50})

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

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

The percentage of inhibition of each substance is plotted against concentration

and the IC50is calculated from the following equation;

L-ascorbic acid $Y = 13.034X + 30.787$

$R^2 = 0.9988$

Plants $Y = 0.244X + 34.645$

$R^2 = 0.9935$

Y = 50% inhibition, X = EC50

Reference

Sreelatha S. and Padma PR. Antioxidantactivity and total phenolic content of *Moringaoleifera*leaves in two stages of maturity. **Plant Foods Hum Nutr** 2009; 64: 1: 303-11.

APPENDIX E

Determination of in Vitro Acetyl Cholinesterase (AChE) Inhibition

Reagents

1. 15 mMAcetylthiocholine iodide (ATCI)
2. 3 mM 5,5-dithiobis-2-nitrobenzoate (DTNB)
3. 50 mMTris–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 µg/ml

Plants extract containing 5, 10, 25, 50, 100, 250, 500 and 1000 µg/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	75	75	75
Tris–HCl	50	50	50
Acetylcholinesterase	25	25	25
Plant extract	-	25	-
Donepezil	-	-	50

2. Incubate at 25 ˚C for 5 minutes.
3. 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;

%Inhibition = % IA = [(Cc*Ce)/Cc] X100

The percentage of inhibition of each substance is plotted against concentration and the IC50is calculated from the following equation;

Donepezil Y = 0.6904X + 36.41

R^2= 0.9916

Y = 50% inhibition, X = EC50

Reference

Ellman GL, Courtney KD, Andres VJ. and Feath-erstone RM. A new and rapid Colorimetric determination ofacetylcholinesterase activity. **Bio-chem. Pharmacol** 1961; 7: 88-95.

Feitosa CM, Freitas RM, Luz NNN, Bezerra MZB, Trevisan MTS. acetylcholinesterase inhibition by some promising Brazilian medicinal plants Braz. **J Biol** 2011; 71: 3: 783-9.

APPENDIX F

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_4in 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 JK. and Proteau G. The sikiltaneous Isolation of RNA and DNA from tissues and Cultured Cells. In: Harwood AJ, editor. **Methods in Molecular Biology: Basic DNA and RNA Protocals.** London: Hamma Press, 1996: 3-9.

APPENDIX G

Preparation of Tissue Sections

Procedures:

1. The brains of the animals were perfusedtranscardially with 4% paraformaldehydein 0.1 M phosphate buffer pH 7.3.
2. Following the perfusion, the brains removed and post fixed with 4% paraformaldehydein 0.1 M phosphate buffer overnight at 4 ^{o}C.
3. Tissues were rinsed with phosphate buffer and infiltrated with 30% sucrose solutionin order to provide cryoprotection.
4. The specimens were frozen rapidly with deep freeze at -25 ^{o}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 JJ, Halliday GM, Svoboda MD. and Cartwright H. The cerebral cortex damaged in chronic alcoholic. **Neuroscience** 1997; 79: 7: 983-98.

APPENDIX H

Cresyl Violet Staining For Nissl Substance

Cresyl violet can be used to demonstrate Nissl substance. The rational 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% aqueoussolution 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 and Chorles W. Cresyl Violet. In: Paxinos G, Chores W, editors. **The rat brain in stereotaxic coordinates.** London: Academic Press; 1981.

APPENDIX I

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% H_2O_2in 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 regent 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% $H_2O_2$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% H_2O_2in methanol for 30 minutes.
2. Wash slides in running tap water for 1 minute than was slidesagain 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 washslides 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% $H_2O_2$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 HistochemCytochem** 1981; 29: 10: 1196-1204.

APPENDIX J

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 were isolated and kept cool in ice buckets. Then these tissues were homogenized in 4 volumes of 1.15% KCl with a glass Potter-Elvehjem homogenizer.

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 K

Determination of Protein

Reagents:

1. Solution A: Alkaline tartate reagent

0.1 gm of sodium tartate ($Na_2C_4H_4O_6.2H_2O$), 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 ($CuSO_4.5H_2O$) 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

* sample dilution 1:50 for brain

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 OH, Roseburgh NJ, Farr AL and Randall RJ. Protein measurement with thefolin phenol reagent. **J BiolChem** 1951; 193: 263.

APPENDIX L

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) ormalondialdehydebis (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 ^{o}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 asnmol MDA/100 mg protein.

Reference

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

APPENDIX M

Determination of Catalase Activity

Reagents:

1. 50 mM Potassium Phosphate Buffer, pH 7 at 25 ^{o}C

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

2. 0.005 N Potassium Permanganate Solution ($KMnO_4$)

Prepare in deionized water using Potassium Permanganate.

3. 5 N Sulfuric Acid Solution (H_2SO_4)

Prepare in deionized water using Sulfuric Acid.

4. 0.01 N Hydrogen Peroxide (H_2O_2)

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, A515nm, by using 1 ml of the buffer as the blank.

2. Pipette 300 µl of H_2O_2(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 H_2SO_4(Reagent 3) and mix.

4. Add 600 µl of $KMnO_4$(Reagent 2) and mix.

5. Read the absorbance, A515nm.

6. Repeat step 2-5 for the different concentrations of Std CAT.

7. Pipette 300 µl of H_2O_2(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 A515nmagainst Std CAT (units/ml) and determine the linear equation of the curve.

10. Calculate the activity of catalase in samples using A515nmand the linear equation of the standard curve.

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

Reference:

Goldblith SA and Proctor BE. Photometric determination of catalase activity. **J Biol Chem** 1950; 187: 2: 705-709.

APPENDIX N

Determination of Superoxide Dismutase Activity

Reagents:

1. 216 mM Potassium Phosphate Buffer, pH 7.8 at 25 ^{o}C

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

2. 10.7 mMEthylenediaminetetraacetic 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 ^{o}C. Monitor the absorbance, A550nm, 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 A550nmfor 3 minutes. Obtain the rate of change (ΔA_{550}nm/minute) for both Test 1 (Uninhibited) and Test 2 (Inhibited).

5. Calculate %inhibition as following:

$$\%\text{inhibition} = \frac{(\Delta A550\text{nm/min Test 1}) - (\Delta A_{550}\text{nm/min Test 2}) \times 100}{(\Delta A_{550}\text{nm/min Test 1})}$$

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 JM and Fridovich I. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). **J BiolChem** 1969; 244: 6049-55.

APPENDIX O

Determination of Glutathione Peroxidase Activity

Reagents:

1. 50 mM Sodium Phosphate Buffer with 0.40 mM EDTA, pH 7 at 25 ^{o}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 mMDithiothreitol, 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 ^{o}C.
3. Pipette (in micrometers) the following reagents into cuvettes:

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

4. Mix by inversion and monitor the absorbance, A340nm, of the reaction cocktail until constant using a spectrophotometer.
5. Add (in micrometers) H_2O_2(Reagent 8):

	H_2O_2
Test 1	17
Test 2 Std GSH-Px	17
Test 2 Sample	17

Then, immediately mix by inversion and record the decrease in A340nmfor 3 minutes. Obtain the rate of change (ΔA340nm/minute) for both Test 1 (Noreaction) and Test 2 (Reaction).

6. Calculate DiffΔA340nm/minute as following:

DiffΔA340nm/minute = (ΔA_{340}nm/min Test 2) - (ΔA_{340}nm/min Test 1)

7. Plot the GSH-Px standard curve by plotting DiffΔA_{340}nm/minute against Std GSH-Px (units/ml) and determine the linear equation of the curve.
8. Calculate the activity of glutathioneperoxidase in samples using DiffΔA_{340}nm/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 P

Determination of Acetylcholinesterase

Solutions:

1. 0.1 M phosphate buffer (PB), pH 8.0
2. 0.075 M acetylthiocholine iodide (ATCld)
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 µL of samplehomogenate 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 µL 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 20µL ATCld 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 µL 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:

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

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

ΔA = change in absorbance/min.

Co = 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 RF and Freeman WH. The Brain: **A Neuroscience Primer**. New York: 1993; 13.

Robertson RT, Holunann, CF, Bruce JL and Oyle JTC. Neonatal enucleation reduces specific activity of acetylcholinesterace in developing rat visual cortex. **Devel. Brain Res** 1988; 39: 298-02.

APPENDIX Q

Determination of Monoamine Oxidase B activity

Reagents:

1. 0.2 M Potassium phosphate saline buffer
2. 500 nMClorgyline
3. 2.5 mM P-Tyramine
4. Standard MAO – B (30% H_2O_2)

Immediately before use, prepare a solution containing 20, 40, 60, 80, and 100 nM

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

	Volume (µl)		
	Blank	Standard	Unknown
PBS	250	-	-
Standard H_2O_2	-	50	-
Sample	-	-	50
Clorgyline	50	50	50

2. Incubate the mixture to 37 °C, 30 minute
3. Add Tyramine 200 µl (except blank)
4. Read endpoint absorbance at 490 nm

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 R

Western Blotting Analysis of ERK 1/2

Sample preparation

1. Remove hippocampus (store at -80°C).
2. Homogenatefrozen tissues in ice cold RIPA buffer with protease inhibitors.
3. Centrifuge at 10,000 g for 30 min andcollectedthe supernatant.
4. Determineprotein concentrations using NANO drop Spectrophotometers.

Reagents

1. Electrophoresis buffer: 25 mMTris, 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 mMTris, 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 mMTris, 137 mMNaCl

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 mMTris-HCl, pH 8.8	1.875 ml
10%SDS	50 µl
10% ammonium persulphate	50 µl
TEMED	15 µl
4% stacking gel	
Distilled water	2.165 ml
30% Acrylamide gel	400 µl
0.5 mMTris-HCl, pH 6.8	375 ml
10%SDS	30 µl
10% ammonium persulphate	30 µl
TEMED	10 µl

Procedures

1. Separateequal amounts of protein (35 µg) by SDS-PAGE on 10% SDS-polyacrylamide gel electrophoresis and transfer onto a polyvinylidenedifluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA).
2. Loadprestained protein markers to assess completeness of electrophoretic transfer.
3. Incubate blots after electrophoretic transfer to nitrocellulose membranewith blocking buffer (5% skim milk in Tris-buffer saline with 0.05% Tween-20) for 1 h at room temperature.
4. Incubateblotsovernight with using one of these antibodies:
   Phospho-ERK1/2 (1:1,000, Cell Signaling Cell Signaling Technology,Inc., Boston, MA, USA),
   Total ERK1/2 (1:1,000, Cell Signaling Cell Signaling Technology,Inc., Boston, MA, USA).
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. Quantifyimages and band densities by ImageQuant LAS 4000 and ImageQuant TL (IQTL) software, GE healthcare.

Reference

Gong QH, Pan LL, Liu XH, Wang Q, Huang H, Zhu YZ. S-propargyl-cysteine (ZYZ-802), a sulphur-containing amino acid, attenuates beta-amyloid-induced cognitive deficits and pro-inflammatory response: involvement of ERK1/2 and NF-κB pathway in rats. **Amino acids** 2011; 40: 601-610.