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. 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. Quercetin 1mg and tomato extract 1mg.

   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
Folin ciocateau	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 with UV-spectrophotometer (Pharmacia LKB-Biochrom4060)

Calculation of total phenolic contents

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

$$\mathbf{X= (Y-0.0066)/0.0009}$$

$$\mathbf{R^2= 0.9991}$$

X= total phenolic compound of Quercetin and tomato extract loaded polymer matrix,
Y= O.D of Quercetin and tomato extract loaded polymer matrix

Reference

Waterhouse AL. Determination of Total Phenolics. ***Current Prots in Food Analy Chem*** 2002: I1-I8.

APPENDIX B

DETERMINATION OF DPPH RADICAL SCAVENGING ACTIVITY

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 room temperature.
2. Quercetin and tomato extract loaded polymer matrix, Asiaticoside, Quercetin and Tomato extract
   Prepare series concentration of Quercetin and Tomato extract loaded polymer matrix, Asiaticoside, Quercetin and Tomato extract in methanol.
3. L-ascorbic acid standard
   Dissolve 2.5 mg L-ascorbic acid with methanol 5 ml. Prepare series concentration containing 1, 2, 3, 5 and 10 µg/ml.

Procedures

1. Pipette the following reagents into labeled glass tube:

	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)

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): 303-311.

APPENDIX C

DETERMINATION OF FRAP ACTIVITY

DETERMINATION OF FRAP ACTIVITY

Reagents

A 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.

B 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.

C $FeCl_3 \cdot 6H_2O$ 20 mM

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

Standard L-ascorbic acid with concentration 100, 250, 500 and 1000 µg/ml

Quercetin and tomato extract loaded polymer matrix, Asiaticoside, Quercetin and Tomato extract

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:

	Volume (µl)		
	Blank	**Sample**	**Standard**
FRAP reagent	1,500	1,450	1,450
Quercetin loaded polymer matrix, Tomato extract loaded polymer matrix , Asiaticoside, Quercetin, Tomato extract	-	50	-
L-ascorbic acid	-	-	50

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 Quercetin and Tomato extract loaded polymer matrix, Asiaticoside, Quercetin and Tomato extract was calculated from the following equation:

$$\mathbf{Y = 0.0018X + 0.1028}$$

$$\mathbf{R^2 = 0.9988}$$

X= FRAP activity equivalent of Quercetin and Tomato extract loaded polymer matrix, Asiaticoside, Quercetin and tomato extract,

Y = O.D of Quercetin and tomato extract loaded polymer matrix, Asiaticoside, Quercetin and tomato 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): 303-311.

APPENDIX D

PHOSPHATE BUFFER SALINE SOLUTION

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	32 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 pH with NaOH
4. Make up the final volume to 4,000 ml

Reference

Merrante F, Raha S, Reod JK, Protean G. The Sikultaneuos isolation of RNA and DNA from tissue and cultured cells. In: Harwood AJ, editor. ***Methods in Molecular Biology: Basic DNA and RNA Protocols***. London: Humma press; 1996. p. 3-9.

APPENDIX E

PREPARATION OF TISSUE SECTIONS

PREPARATION OF TISSUE SECTIONS

Procedures

Modified Paraffin embedding method for muscle and nerve

1. Animals were transcardially perfused with 10% formaldehyde in 0.1 M phosphate buffer pH 7.4.
2. Following the perfusion, the sciatic nerve were removed and postfixed with 10% formaldehyde in 0.1 M phosphate buffer.
3. Following the fixation, fix tissue at least overnight. Cut cross sections into 3-5 mm pieces and continue fixing until ready to process overnight.

3.1 Processing schedule:

Washing runing tap water

70% EtOH 1 hr

3X 95% EtOH 1 hr each

3X 100% EtOH 1 hr each

4X XYLENE 1 hr each

3X Paraffin 1 hr each and then overnight.

The infiltrated tissues are then embedded into wax blocks.

4. After embedding, 4 µm thick of specimen were sectioned using a microtome.
5. Place the slides with paraffin sections on the warming block in a 65°C oven for 20 minutes to bond the tissue to the glass. Slides can be stored at room temperature.

Reference

Kiernan JA (2008) Histological and Histochemical Methods: Theory and Practice. 4th ed. Bloxham, UK: Scion.

Lillie RD, Pizzolato P, Donaldson PT Nuclear stains with soluble metachrome mordant lake dyes. The effect of chemical endgroup blocking reactions and the artificial introduction of acid groups into tissues. Histochemistry 1976 49: 23-35.

APPENDIX F

TOLUIDINE BLUE STAINNING FOR NERVE AXON

TOLUIDINE BLUE STAINNING FOR AXON DENSITY

The section were stained with toluidine blue O. The toluidine blue is a blue cationic (basic) dry used in histology to determined densities and total number of axonal.

Staining solution:

1. Stock solution :
   Toluidine blue O 1gm.
   70% Alcohol 100ml
   Mix Solution
2. 1% Sodium chloride:
   Sodium chloride 0.5 gm
   Distilled water 50 ml
   Make fresh.

Working Solution

Toluidine blue, stock 5.0 ml
1% Sodium chloride 45.0ml

Procedures:

1. 3X XYLENE 10min each
2. Dehydrate quickly through 100%, 95%, 70%, 50%, 30% and 100% alchohol. (2min each)
3. 0.85%NaCl 2min
4. 1X PBS 2min
5. Stain sections in toluidine blue working solution for 1-2 minutes.
6. Wash in distilled water, 3 changes.
7. Dehydrate quickly through 95% and 2 changes of 100% alchohol (10 dips each since stain fades quickly in alcohol).
8. Clear in xylene or xylene substitute, 3 changes, 3 minutes.
9. Coverslip with resinous mounting medium.
   Results: Axonal: Blue

Reference

Sheehan D, Hrapchak B, Theory and practice of Histotechnology, 2nd Ed, 1980, pp282, Battelle Press, Ohio

Luna L, Manual Of Histologic Staining Methods from the AFIP, 3rd Ed, 1968, pp 162-163, McGraw-Hill, NY

Crookham J, Dapson R, Hazardous Chemicals in the Histopathology Laboratory, 2nd ED, 1991, Anatech

APPENDIX G

MASSON TRICHROME STAINING FOR SKIN COLLAGEN

MASSON TRICHROME STAINING

REAGENTS NEEDED:

- Sigma Accustain Trichrome Stain Kit (Catalog #HT15) contains:

Biebrich Scarlet-Acid Fuchsin Solution
(# HT15-1, 0.9% biebrich scarlet, 0.1% acid fuchsin, 1%acetic acid)
Phosphotungstic Acid Solution (#HT15-2, 10% phosphotungstic acid)
Phosphomolybdic Acid Solution (#HT15-3, 10% phosphomolybdic acid)
Aniline Blue Solution (#HT15-4, 2.4% aniline blue, 2% acetic acid)

Procedures

Modified Masson trichrome staining method for skin.

1. Deparaffinize slides and rehydrate sections:
2. 3 x 3´ Xylene (blot excess xylene before going into ethanol)
3. 3 x 3´ 100% ethanol
4. 1 x 3´ 95% ethanol
5. 1 x 3´ 80% ethanol
6. 1 x 5´ deionized H2O
7. Wash slides in running tap water to remove yellow color from sections. Rinse briefly indistilled water.
5. Stain in Working Weigert's Iron Hematoxylin Solution for 5 minutes. Make Hematoxylin
Solution fresh by adding equal volumes of Solution A (1% Hematoxylin in 95% EtOH) and Solution B (1.2% Ferric Chloride and 1% Acetic Acid in distilled water).
6. Wash in running tap water for 5 minutes. Rinse in deionized water.
7. Stain in Biebrich Scarlet-Acid Fuchsin for 5 minutes.
8. Rinse in deionized/distilled water.
9. Place the slides in Phosphomolybdic/Phosphotungstic Acid Solution for 5-10 minutes.

Freshly prepare Working Phosphotungstic/Phosphomolybdic Acid Solution by mixing 1volume of Phosphotungstic Acid Solution and 1 volume of Phosphomolybdic Acid Solution with 2 volumes of distilled water. Discard after one use. Formation of a precipitate in Phosphomolybdic Acid Solution does not affect performance.

10. Stain sections in Aniline Blue Solution for 5 minutes.
10. Rinse slides briefly in distilled water.
11. Place slides in 1% acetic acid solution for 3-5 minutes.
12. Dehydrate to xylene.
13. 2 x 3´ 95% ethanol
14. 2 x 3´ 100% ethanol (blot excess ethanol before going into xylene)
15. 3 x 5´ Xylene
16. Coverslip slides using Permount or Polymount (xylene based).

- Dry overnight in the hood.

Results:

Hematoxylin stains nuclei blue-black.

Beibrich scarlet-acid fuchsin stains cytoplasm and muscle red.

Aniline blue stains collagen blue.

Reference

Rosen lab website http://www.bcm.edu/rosenlab

Last modified August 29, 2005 (Michelle's protocol, 9/01)

APPENDIX H

MAYER'S HEMATOXYLIN AND EOSIN STAINNING FOR MUSCLE

MAYER'S HEMATOXYLIN AND EOSIN STAINNING FOR MUSCLE

Procedures:

1. 3X XYLENE 10min each
2. Dehydrate quickly through 100%, 95%, 70%, 50%, 30% and 100% alchohol. (2min each)
3. DW 5 min
4. Stain sections in Hematoxylin 5min
5. Wash in distilled water, 3 changes.
6. 1% HCL 3 Dips
7. Litium carbonate solution 3 Dips
8. DW 5 min
9. 70 % ethanol 3min
10. Stain sections in Eosin 1 min
11. Dehydrate quickly through 95% and 2 changes of 100% alchohol (10 dips each since stain fades quickly in alcohol).
12. Clear in xylene or xylene substitute, 3 changes, 3 minutes.
13. Coverslip with resinous mounting medium.

Results

Nuclei should be blue, cytoplasm pink to red.

Reference

Llewellyn BD Nuclear staining with alum-hematoxylin. Biotech. Histochem. 2009, 84: 159-177.

Puchtler H, Meloan SN, Waldrop FS Application of current chemical concepts to metal-haematein and -brazilein stains. Histochemistry 1986, 85: 353-364.

APPENDIX I

PREPARATION OF TISSUE HOMOGENATES

PREAPARTION OF TISSUE HOMOGENATES

After the last feeding with various substance, all animals were anesthetized with intraperitoneal injection of thiopental sodium at dose 60 mg/kg BW. After transcardially perfusion, sciatic nerve was removed and it was homogenized in 500 µl of 1.15% KCl with a glass Potter-Elvehjem homogenizer. All procedures were performed and cooled in ice buckets.

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. p.527-52.

APPENDIX J

DETERMINATION OF PROTEIN

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 bovin serum albumin (BSA) 100 mg with 5 ml. Standard protein was diluted in to series of concentration (2,4,6,8,10 and 20 mg/ml).

Procedures

1. 96 wells plate was used and solution was pipetted into each well as follows:

	Volume (µl)		
	Blank	**Standard**	**Unknown**
Distilled water	10	-	-
Standard BSA	-	10	-
Sample (1:50)	-	-	10
Solution C	200	200	200

2. Mixed and shake on shaker for 10 minutes.
3. Solution D was added to all wells and let stand at room temperature for 1 hour.
4. The absorbance was measured at 650 nm by with a UV-spectrophotometer (Pharmacia LKB-Biochrom4060).
5. The O.D of standard protein was plotted against concentration and the protein concentration was calculated from the following equation

$$x=(y+0.0599)/0.1704$$

x= protein concentration of unknown, y=O.D of each unknown

Reference

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. ***J Biol Chem*** 1951 Nov; 193(1): 265-75.

APPENDIX K

DETERMINATION OF LIPID PEROXIDE CONTENTS

DETERMINATION OF LIPID PEROXIDE 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 (Thiobarbituric acid)
4. TMP (1,1,3,3-tetramethoxy propane) or malondialdehyde bis (dimethyl acetate) solution is 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.

	Volume (ml)		
	Blank	**Standard**	**Unknown**
Sample (1:50)	-	-	0.1
8.1% SDS	0.1	0.1	0.1
20% Acetic acid (pH3.5)	0.75	0.75	0.75
0.8% TBA	0.75	0.75	0.75
TMP stock standard	-	0.1	-
Distilled water	400	300	300

2. Mix and heat the tube in water bath at 95 ˚C for 1 hour
3. After cooling with tab water, 0.5 ml of distilled water and 2.5 ml of mixture of n-butanol and pyridine (15:1) are added and shake.
4. After centrifugation at 4,000 rpm for 10 minutes, the organic layer is taken and the absorbance is measured at 412 nm.
5. The contents of lipid peroxide is expressed in term of nmol MDA/mg protein

Calibration Curve

1. Prepare a series of tubes containing TMP stock standard in water in the following concentrations: 2.0 nmol/0.2 ml, 4.0 nmol/0.2 ml, 6.0 nmol/0.2 ml, 8.0 nmol/0.2 ml, 1.0 nmol/ 0.2 ml.
2. Perform the procedure as in step2.
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: 351-358.

APPENDIX L

DETERMINATION OF SUPEROXIDE DISMUTASE ACTIVITY

DETERMINATION OF SUPEROXIDE DISMUTASE ACTIVITY

Reagents

A Solution A: 216 mM Potassium Phosphate Buffer pH 7.8

Dissolve 1.47 g monobasic potassium phosphate (KH_2PO_4) with 50 ml of deionized water. Adjust to pH 7.8 with NaOH.

B 10.7 mM Ethylenediaminetetraacetic Acid Solution (EDTA)

Dissolve 0.2 g EDTA with deionized water 50 ml.

C 1.1 mM Cytochrome C Solution (Cyt C)

Dissolve 30 mg potassium Cytochrome C with deionized water 2.2 ml.

D 0.540 mM Xanthine Solution (Xanthine), pH 7.4

Dissolve xantine 8.21 mg with 100 ml of deionized water, adjust to pH 7.4 with NaOH.

E Xanthine Oxidase Enzyme Solution (XOD)

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

F Standard Superoxide Dismutase Enzyme Solution (STD SOD)

Immediately before use, prepare a solution containing 1, 5, 10, 50, 100 and 500 units/ml of Superoxide Dismutase in cold 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, A_{415nm}, of the reaction cocktail until constant using a microplate reader.

3. Pipette the following reagents into 96 wells plate

	Volume (µl)				
	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_{415nm} for 3 minutes. Obtain the rate of change (ΔA_{415nm}/minute) for both Test 1 (Uninhibited) and Test 2 (Inhibited).
5. Calculate %inhibition as following:

$$\%\text{inhibition} = \frac{(\Delta A_{415nm}/\text{min Test 1}) - (\Delta A_{415nm}/\text{min Test 2})}{(\Delta A_{415nm}/\text{min Test 1})} \times 100$$

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 JM and Fridovich I. (1969). Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). ***J Biol Chem***. 244: 6049-6055.

APPENDIX M

DETERMINATION OF GLUTATHIONE PEROXIDASE

DETERMINATION OF GLUTATHIONE PEROXIDASE

Reagents

A 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.

B 1 mM Sodium Azide Solution (Buffer w/Azide)
Prepare Sodium Azide in reagent A.

C β-Nicotinamide Adenine Dinucleotide Phosphate, Reduced Form (β-NADPH)
Use 5 mg vial of β-NADPH (Reduced Form).

D Glutathione Reductase Enzyme Solution (GR)
Immediately before use, prepare a solution containing 100 units/ml of Glutathione Reductase in cold deionized water.

E 200 mM Glutathione, Reduced (GSH)
Prepare in deionized water using Glutathione, Reduced Form.

F 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.

G Standard Glutathione Peroxidase Enzyme Solution (Std GPx)
Immediately before use, prepare a solution containing 1,5,10,20,50 and 100 units/ml of Glutathione Peroxidase in cold buffer w/DTT (Reagent F).

H 0.042% (w/w) Hydrogen Peroxide (H_2O_2)
Prepare in deionized water using Hydrogen Peroxide, 30% (w/w) Solution.

Procedures

1. Prepare a reaction cocktail by pipetting the following reagents into 5 mg vial of Reagent C (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	Std GPx	Sample
Blank	-	-	1000	-	-
Test 1	1000	17	-	-	-
Test 2 Std GPx	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) H_2O_2 (Reagent H):

	H_2O_2
Test 1	17
Test 2 Std GPx	17
Test 2 Sample	17

Then, immediately mix by inversion and record the decrease in A_{340nm} for 3 minutes. Obtain the rate of change (ΔA_{340nm}/minute) for both Test 1 (No reaction) and Test 2 (Reaction).

6. Calculate DiffΔA_{340nm}/minute as following:

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

7. Plot the GPx standard curve by plotting DiffΔA_{340nm}/minute against Std GPx (units/ml) and determine the linear equation of the curve.

8. Calculate the activity of glutathione peroxidase in samples using DiffΔA_{340nm}/minute and the linear equation of the standard curve ($y=0.1241x + 0.0334$, $R^2=0.9951$)

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

Reference

Wendel A. ***Enzymatic Basis of Detoxication.*** New York: Academic Press; 1980; Volume 2: 333-53.

APPENDIX N

DETERMINATION OF CATALASE ACTIVITY

DETERMINATION OF CATALASE ACTIVITY

Reagents

A. 50 mM Potassium Phosphate Buffer, pH 7 at 25 °C

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

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

Prepare in deionized water using Potassium Permanganate.

C. 5 N Sulfuric Acid Solution (H_2SO_4)

Prepare in deionized water using Sulfuric Acid.

D. 0.01 N Hydrogen Peroxide (H_2O_2)

Prepare in the buffer using Hydrogen Peroxide, 30% (w/w) Solution.

E. 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.

Procedures

1. Pipette the reagents into 96 wells plate as following;

	Volume (µl)		
	Blank	**Standard**	**Unknown**
DI	10	-	-
Standard	-	10	-
Sample	-	-	10
H_2O_2	50	50	50
H_2SO_4	25	25	25
$KMnO_4$	150	150	150

2. Mix and read the absorbance with microplate reader at 490 nm

3. Plot the CAT standard curve by plotting A_{490nm} against Std CAT (units/ml) and determine the linear equation of the curve.

10. Calculate the activity of catalase in samples using A_{515nm} and the linear equation of the standard curve ($y=0.1023x + 0.0806$, $R^2=0.9922$).

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 O

WESTERN BLOTTING ANALYSIS OF ERK 1/2

WESTERN BLOTTING ANALYSIS OF ERK 1/2

Sample preparation

1. Remove lesion nerve (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 µl
10% ammonium persulphate	50 µl
TEMED	15 µl

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

Procedures

1. Separate equal amounts of protein (70 µg) 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 (10% 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:

   Phospho-ERK 1/2 (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, Thermo Scientific).
7. Quantify images and band densities by ImageQuant LAS 4000 and ImageQuant TL (IQTL) software, GE healthcare.

Reference

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