APPENDICES

APPENDIX A

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. Plants extract 5 mg/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
Folin ciocateau	100	100	100

2. Mix and leave at room temperature for 5 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 M. oleifera* extract was calculated from the following equation:

$$\mathbf{X= (Y-0.004)/0.001}$$

$$\mathbf{R^2= 0.9998}$$

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

Reference

Quettier-Deleu C, Gressier B, Vasseur J, Dine T, Brunet C, Luyckx M, et al. Phenolic compounds and antioxidant activities of buckwheat (Fagopyrum esculentum Moench) hulls and flour. *J Ethnopharmacol* 2000; 72: 35-42.

APPENDIX B

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. Plants extract
   Prepare series concentration of plants extract containing 0.1, 0.5, 1,5,10 and 20 g/ml in hydroalcoholic 50%.
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 (µl)			
	Blank (Control)	**Blank (Sample)**	**Blank (Extract)**	**Sample**	**Standard**
Methanol	1500	1375	1475	1350	1350
DPPH	-	125	25	125	125
Extract (vary dose)	-	-	-	25	-
STD	-	-	-	-	25
Total	1500	1500	1500	1500	1500

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 efficient concentration (EC_{50})

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

% DPPH scavenging effect = [(A control - A extract)/A control] x 100

The percentage of effective of each substance is plotted against concentration and the EC_{50} is calculated from the following equation;

L-ascorbic acid $Y= 3.081X + 6.289$, $R^2=0.989$

Plants extract $Y= 24.59X + 0.235$, $R^2=0.993$

Y= 50% effective concentration, X= EC_{50}

Reference

Tai A, Sawano T, Yazama F, Ito H. Evaluation of antioxidant activity of vanillin by using multiple antioxidant assays. *Biochim Biophys Acta* 2011; 1810: 170-7.

APPENDIX C

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 µM

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
Extract	-	50	-
L-ascorbic acid	-	-	50

3. Read the absorbance at 593 nm with UV-spectrophotometer (Pharmacia LKB Biochrom4060) before incubate.
4. Incubate at 37 ˚C in water bath for 10 minutes.
5. 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:

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

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

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

Reference

Benzie IF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal Biochem* 1996; 239: 70-6.

APPENDIX D

Determination of Flavanoids Content

Reagents

2% $AlCl_3$

Mix 200 µl of $AlCl_3$ with ethanol 9800 µl

Standard quercetin with concentration 1, 5, 10, 20, 30 and 50 mg/ml

Procedures

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

	Volume (µl)		
	Blank	**Sample**	**Standard**
Ethanol	100	-	-
Plants extract	-	100	-
Quercetin	-	-	100
2% $AlCl_3$	100	100	100

2. Mixed and shake on shaker for 15 minutes.
3. The absorbance was measured at 415 nm by with a UV-spectrophotometer (Pharmacia LKB-Biochrom 4060).

APPENDIX E

Determination of Anthocyanin Contents

Reagents

Two types of buffers were prepared.

1. 0.025 M KCl (pH =1)

   Buffer was prepared by dissolving 1.86g of KCl in 980 mL distilled water and adjust pH =1

2. Sodium acetate (pH=4.5)

   Buffer was prepared by dissolving 54.43g of sodium acetate in 970 mL distilled water and adjusted pH=4.5

Procedures

3 ml of extract was diluted in 5 ml of two different buffers; 0.025 M potassium chloride pH = 1.0 and 0.4 M sodium acetate pH = 4.5, respectively. After 30 minutes of incubation at room temperature, absorption (A) was measured at wavelength of 510 and 700 nm respectively. The absorbance of the diluted sample (A) was calculated as follows:

$$A = (A510 – A700)\ pH\ 1.0 – (A510 – A700)\ pH\ 4.5$$

The monomeric anthocyanin pigment concentration in the original sample using the following formula:

$$\text{Monomeric anthocyanin pigment (mg/L)} = (A \times MW \times DF \times 1000)/(\varepsilon x1)$$

MW is the molecular weight, DF is the dilution factor, and ε is the molar absorptivity, MW = 449.2 and ε= 26,900 l $mol^{-1}cm^{-1}$

Reference

Giusti M, Wrolstad R. Characterization and measurement of anthocyanins by uv-visible spectroscopy. In; 2005. p. p. 19-31. .

APPENDIX F

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; 3-9.

APPENDIX G

Hematoxilin and Eosin Staining

Staining solution:

1. Harris Alum Heamatoxylin

Haematoxylin (Cl 75290)	2.5	g
Absolute alcohol	25	ml
Distilled water	500	ml
Ammonium or potassium alum	50	g
Mercuric oxide (yellow)	1.25	g

Filter before us

Dissolve the haematoxylin in the absolute alcohol. Dissolve the alum in water, using heat if necessary. Mix the two solutions, rapidly bring to the boil and carefully add the mercuric oxide a little at a time. Cool rapidly by immersing the flask into iced water. The addition of 20 ml of glacial acetic acid is optional but gives sharper nuclear staining; this must be added just before use and the stain filtered. The solution is ready for staining as soon as it is cool.

2. Eosin Y, 1 % aqueous (store at room temperature)

Eosin Y dye	1	g
95% ethanol	100	ml

3. Bluing Reagents for H&E Staining

0.1% Sodium Bicarbonate:

Sodium bicarbonate	1	g
Distilled water	1000	ml

Mix to dissolve. The pH was around 8.0 Store this solution at room temperature.

Procedures:

1. 95% Ethanol (brought from Cryostat) - Dip until clear
2. Running tap water (gentle stream of water running into a bowl or appropriate container) –Dip until clear
3. Distilled water - Dip until clear
4. Hematoxylin 2 (Richard Allen Scientific) - 30 seconds
5. Running tap water - Until clear
6. Bluing Reagent (Richard Allen Scientific) - Dip 3 - 4 times
7. Running tap water - 30 seconds
8. 95% Ethanol - 3 - 4 dips
9. Eosin - Y (Richard Allen Scientific) - 1 - 2 dips
10. 95% Ethanol - 2 changes - 5 - 10 dips
11. 100% Ethanol - 3 changes - 5 - 10 dips
12. Xylene - 2 changes - 5 - 10 dips
13. Mount with resinous medium (cover slip)

APPENDIX H

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 (1:10)	-	-	10
Solution C	200	200	200

2. Mixed and shake on shaker for 10 minutes.
3. Solution D 20 µl was added to all wells and let stand at room temperature for 1 hour.
4. The absorbance was measured at 655 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 I

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 (µl)		
	Standard	**Unknown**	**Blank =DW**
Sample (1:50)	-	100	
8.1% SDS	100	100	
20% Acetic acid (pH3.5)	750	750	
0.8% TBA	750	750	
TMP stock standard	0.1	-	

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 J

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	200	40	-	-	-
Test 1	200	20	20	-	-
Test 2 Std SOD	200	-	20	20	-
Test 2 Sample	200	-	20	-	20

4. Immediately mix by inversion and record the increase in A_{415nm} for 5 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. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). **J Biol Chem** 1969; 244: 6049-6055.

Sun Y, Oberley L, Li Y. A simple method for clinical assay of superoxide dismutase. **Clinical Chemistry** 1988; 34: 497-500

APPENDIX K

Determination of Glutathione Peroxidase

Reagents

A 50 mM Sodium Phosphate Buffer, pH 7
Prepare in deionized water using Sodium Phosphate, Monobasic, Anhydrous

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

C 200 mM Glutathione, Reduced
Prepare in deionized water using Glutathione, Reduced Form.

D 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

E 5,5'-dithiobis-2-nitrobenzoic acid (DTNB)
Prepare in deionized water using 5,5'-dithiobis-2-nitrobenzoic acid

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

Procedures

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

	Volume (µl)		
	Blank	**Standard**	**Unknown**
Deionized water	30	-	-
STD	-	-	20
Sample	-	20	-
Solution D	10	10	10
Solution B	100	100	100
Solution C	-	10	10
Solution F	100	100	100

2. Mixed and shake on shaker for 10 minutes.
3. Solution E 10 µl was added to all wells and Mixed and shake on shaker**.**
4. The absorbance was measured at 412 nm by with a UV-spectrophotometer (Pharmacia LKB-Biochrom 4060).
5. Report and graph the enzyme activity and it is expressed in U/mg.protein

Reference

Rotruck JT, Pope AL, Ganther HE, Swanson AB, Hafeman DG, Hoekstra WG. Selenium: biochemical role as a component of glutathione peroxidase. **Science** 1973; 179: 588-90.

APPENDIX L

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.

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

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

Reference

Goth L. A simple method for determination of serum catalase activity and revision of reference range. **Clin Chim Acta** 1991; 196: 143-51.

APPENDIX M

Determination of Aldose Reductase Activity

Reagents

1. 0.1 M phosphate buffer solution pH=6.2
2. 10 mM DL-glyceraldehyde

   Dissolve 0.009 g DL-glyceraldehyde with 0.1 M PBS (pH =6.2) 100 ml
3. 0.15 mM NADPH

   Dissolve 0.0125 g NADPH with 0.1 M PBS (pH =6.2) 100 ml

Procedures

1. Add the following substances in the table into the series of the labeled glass tube.

	Volume (µl)			
	Blank	**Control**	**STD**	**Sample**
0.1 M PBS (pH=6.2)	900	400	300	300
Aldose reductase (lens or nerve homogenate)	-	100	100	100
Plants (vary dose)	-	-	-	100
STD (Quercetin)	-	-	100	-
DL-glyceraldehyde	-	300	300	300
NADPH	-	100	100	100

2. Shake and quick read the absorbance reader at 390 nm with UV-spectrophotometer (Pharmacia LKB-Biochrom4060) after mix solution.
3. Read the absorbance at 390 nm with UV-spectrophotometer (Pharmacia LKB-Biochrom4060) at 4 minutes.

Calculation of aldose reductase activity

$$\% \text{ inhibition of AR} = [(\text{OD control} - \text{OD sample})/\text{OD control}]*100$$

Reference

Patel M, Mishra S. Aldose reductase inhibitory activity and anticataract potential of some traditionally acclaimed antidiabetic medicinal plants. **Oriental Pharmacy and Experimental Medicine** 2009; 9: 245-251