CHAPTER III

MATERIALS AND METHODS

The experiments were divided into five parts:

1. Preparation of crude extracts from fresh roots of *Raphanus sativus* L. locally grown in Thailand (Freeze-dried water extract and Methanol extract)
2. Quantitative analysis of *Raphanus sativus* L. extracts
3. Determination of anti-tyrosinase enzyme activity of *Raphanus sativus* L. extracts
4. Determination of antioxidant and free radical scavenging activities of *Raphanus sativus* L. extracts
5. Cytotoxicity test of *Raphanus sativus* L. extracts on normal human fibroblast cell line by LDH assay

Materials

1. Methanol AR grade, Labscan, Asia Ltd., Ireland
2. Absolute ethanol AR grade, E. Merck, Germany
3. 2,2 -Diphenyl-1-picryl-hydrazyl stable radical (DPPH), Sigma-Aldrich, Inc., USA
4. Sodium phosphate monobasic, Ajax Finechem, Labscan Ltd., Australia
5. Sodium phosphate dibasic, Ajax Finechem, Labscan Ltd., Australia
6. L-3, 4-dihydroxyphenylalanine (L-DOPA) Fluka Chemie GmbH, Germany
7. Mushroom tyrosinase (F-3824), Sigma-Aldrich, Inc., USA
8. Propylene glycol, Srichand United Dispensary Co.,Ltd., Thailand
9. Disodium ethylene diamine tetra-acetic acid (EDTA), Ajax Finechem, Labscan Ltd., Australia
10. Riboflavin (Vitamin B2) Fluka Chemie GmbH, Germany
11. L-methionine (2-Amino-4-(methylmercapto) butyric acid), Fluka Chemie GmbH, Germany
12. Nitro blue tetrazolium (NBT), Sigma-Aldrich Co., USA
13. Hydrogen peroxide solution (30%w/v), VWR International Ltd., UK

14. Sodium hypochlorite (6.3 %w/v), E. Merck, Germany
15. *N,N*-dimethyl-*p*-nitrosoaniline, Sigma-Aldrich, Inc., USA
16. Histidine, Sigma-Aldrich, Inc., USA
17. Aluminium chloride ($AlCl_3$), , E. Merck, Germany
18. Potassium acetate (CH_3COOK), May & Baker Ltd., England
19. Folin-Denis' reagent, Fluka Chemie GmbH, Germany
20. Sodium bicarbonate B.P., Srichand United Dispensary Co., Ltd., Thailand
21. Sinapic acid 98% titration, Sigma-Aldrich, Inc., USA
22. Quercetin dihydrate, Sigma-Aldrich, Inc., USA
23. Iodine, E. Merck, Germany
24. Potassium iodide, E. Merck, Germany
25. Starch, R&B Supply Co., Ltd., Thailand
26. In vitro toxicology assay kit, Lactic dehydrogenase based (Tox-7), Sigma-Aldrich, Inc., USA

Reference antioxidants

1. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox®) (250.3 g/mol, 97.0% purity), Calbiochem, Inc., USA
2. L-ascorbic acid (176.13 g/mol, 99.75% purity), DSM Nutritional Product Co.,Ltd., Switzerland

Reference anti-tyrosinase

1. Licorice extract (PT-40), Maruzen Pharmaceutical Co., Ltd., Japan
2. L-ascorbic acid (176.13 g/mol, 99.75% purity), DSM Nutritional Product Co.,Ltd., Switzerland

Apparatus

1. Analytical balance, AG 285, Mettler Toledo, Switzerland
2. Fluorescent lamp, PL 9W, Sylvania, Thailand
3. Rotary evaporator, Rotavapor RE-120, Buchi, Switzerland
4. Sonicator (Transonic digitals), Elma, Germany
5. Microplate reader, VICTOR® , multilabel counter, Perkin Elmer Ltd., USA
6. pH meter, Model 420A, Orion, USA

7. UV-Visible spectrophotometer, UV-1601, Shimadzu, Japan
8. Vortex mixer, Vortex Genie-2, Scientific Industries, Inc., USA
9. Micropipette, Gilson, France
10. Multi-channel micropipette, Gilson, France
11. Lyophilizer, Dura-Dry II MP, FTS Systems, Inc., USA
12. CO_2 incubator, Forma scientific, Thermo cooperation, USA

Others

1. Quartz cell, Starna, UK
2. 96-well microplates, Nunc, Denmark
3. Parafilm, American National Can TM, USA
4. Whatman filter paper No.1, 150 mm, Whatman International Ltd., UK
5. Normal human fibroblast cell line (CC 2511)

Methods

Part 1. Preparation of crude extracts from fresh roots of *Raphanus sativus* L. locally grown in Thailand

In this experiment, fresh roots of *R. sativus* were purchased from a market by the trade name "Doctor". The crude extracts were prepared by two methods. The details were as follows:

1.1. Freeze - dried water extract

Fresh roots (1.1 kg) of *R. sativus* were squeezed and their residue was filtered out using No.1 filter paper. The juice was then frozen at -40°C and dried in a freeze dryer apparatus. The powder was collected in a well-closed container with silica gel at -20°C for further studies.

1.2. Methanol extract

The extraction method was modified from Yoshiaki et al.(2003). Briefly, fresh roots (2.4 kg) of *R. sativus* were cut into small pieces and macerated three times with methanol at room temperature. This procedure was done within 7 days. The resultant methanol solution was evaporated in a rotary evaporator at 35°C. After methanol was evaporated, the residue which still contained some water was further dried in the freeze dryer apparatus to obtain powder. The powder was collected in a well-closed container with silica gel at -20°C for further studies.

Part 2. Quantitative analysis of *Raphanus sativus* L. extracts: Freeze-dried water extract and methanol extract.

Over the past 10 years, researchers have become increasingly interested in polyphenols. The chief reason for this interest is the recognition of the antioxidant property of polyphenols, their great abundance in our diet, and their probable role in the prevention of various diseases associated with oxidative stress. Furthermore, polyphenols constitute the active substances found in many medicinal plants (Middleton, Kandaswami and Theoharides, 2000).

2.1. Analysis of total phenolics content in *R. sativus* extracts

Folin-Denis method is the most widely used assay for the quantification of total phenolics in plant materials and beverages. This colorimetric procedure replies upon a reduction-oxidation in which the phenolate ion of phenolic compounds becomes oxidized whilst the phosphomolybdic-phosphotungstic acid (Folin-Denis) reagent is reduced in the presence of an alkali (sodium bicarbonate) turning into a blue-colored solution (Folin and Denis, 1912). In the plant family Crucifereae sinapic acid is a dominant phenolic acid identified. Thus, the content of total phenolics was expressed as sinapic acid equivalents. This method was modified from the method of Swin and Hills (1959).

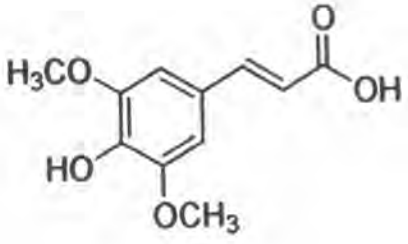


Figure 7. Structure of sinapic acid

2.1.1. Instrument

UV-visible spectrophotometer

2.1.2. Reagents

Folin-Denis reagent

Saturated sodium bicarbonate

Test solution

2.1.3. Standard and test preparations

A stock solution of sinapic acid was prepared by accurately weighed 10 mg of sinapic acid into 100 ml volumetric flask, then dissolved and adjusted to volume with absolute ethanol. This standard solution had a final concentration of 100 μg/ml. Different quantities (0.5, 1, 2, 4, 6, and 8 ml) of this solution were pipetted into 10 ml volumetric flasks, diluted and adjusted to volume with the absolute ethanol. The final concentrations of sinapic acid were 5, 10, 20, 40, 60, and 80 μg/ml, respectively. Then the standard solutions were analyzed using UV-spectrophotometer at 725 nm. The standard curve between sinapic acid concentration and absorbance was plotted. The content of phenolics was expressed as sinapic acid equivalents. The absorbances of the extract solutions (freeze-dried water extract and methanol extract) at 2 mg/ml concentration in absolute ethanol were interpolated, using the calibration curve to determine the concentration of the analyte in terms of sinapic acid equivalents.

2.1.4. Procedure of Folin-Denis method

Each 0.5 ml of test sample or standard was mixed with 7 ml distilled water. To this, 0.5 ml Folin-Denis reagent was added. After 3 minute standing, 1 ml saturated sodium bicarbonate solution was added, and the mixture was made up to 10 ml with distilled water and mixed well. The absorbance was measured at 725 nm after 1 hour standing at room temperature. A mixture of absolute ethanol, water and reagents was used as a blank.

2.1.5. Validation of UV spectrophotometric method

2.1.5.1 Linearity

Three sets of six standard solutions of sinapic acid ranging from 5 to 80 μg/ml were prepared and analyzed. Linear regression analysis of absorbances versus their concentrations was performed. The linearity was determined from the coefficient of determination (R^2).

2.1.5.2 Accuracy

The accuracy of an analytical method is the closeness of test results

obtained by that method to the true value. The accuracy of the method was determined from the percentage of recovery. Five sets of three concentrations at 16, 36 and 64 μg/ml were prepared and analyzed. The percentage of recovery of each concentration was calculated from the ratio of inversely estimated concentration to the known concentration multiplied by 100.

2.1.5.3 Precision

a) Within-run precision

The within-run precision was determined by analyzing five sets of three concentrations at 16, 36 and 64 μg/ml in the same day. The coefficient of variation (%CV) of sinapic acid at each concentration was determined.

b) Between-run precision

The between-run precision was determined by analyzing three concentrations at 16, 36 and 64 μg/ml on five different days. The percent coefficient of variation (%CV) of sinapic acid at each concentration was determined.

Acceptance criteria:

For accuracy, the percentage of recovery should be within 98-102% of each nominal concentration, whereas the percent coefficient of variation for both within run precision and between run precision should be less than 2%.

2.2. Analysis of total flavonoid content in *R. sativus* extracts

The total flavonoid content was determined by spectrophotometric assay with aluminum chloride complex, based on the priniciple that aluminium chloride forms acid stable complexes with the C-4 keto group and either the C-3 or the C-5 hydroxyl group of flavones and flavonols. In addition, aluminum chloride forms acid labile complexes with the ortho-dihydroxyl groups in the A or B ring of flavonoids. The absorbance of the reaction mixture upon the development of a pink color was measured at 415 nm. This method was modified from the method of Chang et al. (2002).

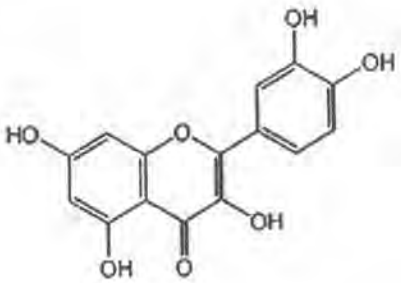


Figure 8. Structure of quercetin

2.2.1. Instrument

UV-visible spectrophotometer

2.2.2. Reagents

10% Aluminum chloride (w/v)

1M Potassium acetate

95% Ethanol (v/v)

Distilled water

Test solution

2.2.3. Standard and test preparations

A stock solution of quercetin was prepared by accurately weighed 20 mg of quercetin into 100 ml volumetric flask, then dissolved and adjusted to volume with absolute ethanol. This standard solution had a final concentration of 200 µg/ml. After 0.5, 1, 2, 3, 4, and 5 ml of this solution were pipetted into 10 ml volumetric flasks, they were diluted and adjusted to volume with the absolute ethanol. The final concentrations of quercetin were 10, 20, 40, 60, 80, and 100 µg/ml, respectively. Then the standard solutions were analyzed using UV-spectrophotometer at 415 nm. The standard curve between quercetin concentration and absorbance was plotted. The content of flavonoids was expressed as quercetin equivalents. The absorbances of the extract solutions (freeze-dried water extract and methanol extract) at 30 mg/ml concentration in absolute ethanol were interpolated from the calibration curve to determine the concentration of the analyte in terms of quercetin equivalents.

2.2.4. Determination of flavonoids

Each 0.5 ml of test sample or standard was mixed with 1.5 ml of 95% ethanol (v/v) , 0.1 ml of 10% aluminum chloride (w/v), 0.1 ml of 1M potassium acetate and 2.8 ml water. The volume of 10% aluminum chloride (w/v) and test/standard was substituted by the same volume of distilled water in blank. After incubation at room temperature for 30 minutes, the absorbance of the reaction mixture was measured at 415 nm.

2.2.5. Validation of UV spectrophotometric method

2.2.5.1 Linearity

Three sets of six standard solutions of quercetin ranging from 10 to 100 µg/ml were prepared and analyzed. Linear regression analysis of absorbance versus their concentrations was performed. The linearity was determined from the coefficient of determination (R^2).

2.2.5.2 Accuracy

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. The accuracy of the method was determined from the percentage of recovery. Five sets of three concentrations at 25, 50 and 75 µg/ml were prepared and analyzed. The percentage of recovery of each concentration was calculated from the ratio of inversely estimated concentration to known concentration multiplied by 100.

2.2.5.3 Precision

a) Within-run precision

The within-run precision was determined by analyzing five sets of three concentrations at 25, 50 and 75 µg/ml in the same day. The percentage of variation (%CV) of quercetin at each concentration was determined.

b) Between-run precision

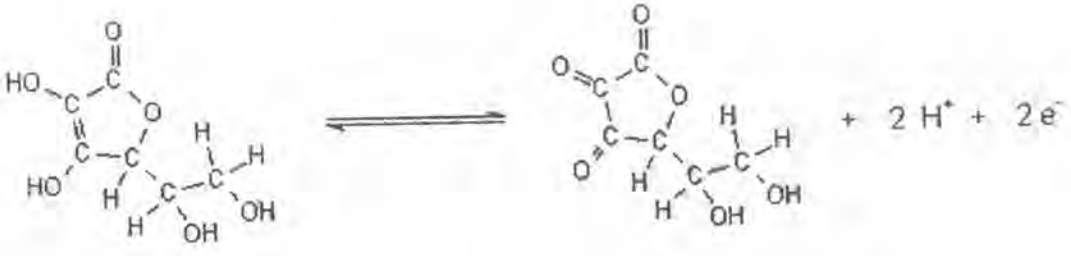
The between –run precision was determined by analyzing three concentrations at 25, 50 and 75 µg/ml on five different days. The percent coefficient of variation (%CV) of quercetin at each concentration was determined.

Acceptance criteria:

For accuracy, the percentage of recovery should be within 98-102% of each nominal concentration, whereas the percent coefficient of variation for both within run precision and between run precision should be less than 2%.

2.3. Analysis of Vitamin C content in *R. sativus* extracts using direct titration

Vitamin C or ascorbic acid is a water-soluble vitamin, which is present in fresh roots of *R. sativus* ranging from 17.95-27.86 mg% (Bulinski and Zhinda, 1962). It is involved in wound healing and tyrosinase metabolism. In addition, vitamin C shows antioxidant effects and under certain conditions can protect against oxidative stress induced DNA damage (Sweetmann, Strain and Mckelvey-Martin, 1997). Determination of vitamin C can be performed by various methods. One of the most commonly used analysis is the direct titration method. The method based on the oxidation of ascorbic acid to dehydroascorbic acid by iodine was used for the determination of vitamin C content in the test samples.



Ascorbic acid

Dehydroascorbic acid

$$I_2 + 2e^- \longrightarrow 2I^-$$

The titration end point is reached when a slight excess of iodine is added to ascorbic acid solution. This method was modified from the method of Suntornsuk et al. (2002).

2.3.1. Instrument

Buret

Erlenmeyer flask

2.3.2. Reagents

0.1N Iodine

Starch T.S.

2N Sulfuric acid

Distilled water

Test solution

2.3.3. Determination of ascorbic acid

Eight hundred mg of each test sample (freeze-dried water extract and methanol extract) were diluted with 25 ml of distilled water and the solution was transferred to a 250 ml Erlenmeyer flask. Twenty-five ml of 2N sulfuric acid was added, mixed, diluted with 50 ml of water and 3 ml of starch T.S. was added to the mixture as an indicator. The solution was directly titrated with 0.1 N iodine. A blank titration was performed prior to the titration of each sample (n=5). Each ml of 0.1N iodine is equivalent to 8.806 mg ascorbic acid.

2.3.4. Validation of titration method

2.3.4.1 Linearity

The linearity of the method was determined by adding standard ascorbic acid at 10, 20, 30, 40, and 50 fold of the ascorbic acid amount originally found in each 200 mg of test samples (freeze-dried water extract and methanol extract) to each solution. Triplicate titrations were made for each standard added solution. The linear regression line was plotted between the amount of standard ascorbic acid found and the amount of standard ascorbic acid added. The regression equation and the coefficient of determination (R^2) values were obtained.

2.3.4.2 Accuracy

The accuracy calculated from percentages of bias and recovery was determined using standard addition method. Standard ascorbic acid at 10, 20, 30, 40 and 50 fold of the ascorbic acid amount originally found in each 200 mg of the test samples (freeze-dried water extract and methanol extract) was added to the test solution. Triplicate titrations were made for each standard added solution. The percentage of recoveries were calculated as follows:

$$\% \text{ Recovery} = \frac{\text{Amount of standard recovered}}{\text{Amount of standard added}} \times 100$$

* Amount of standard recovered was calculated from total amount measured-known ascorbic acid amount in sample.

2.3.4.3 Precision

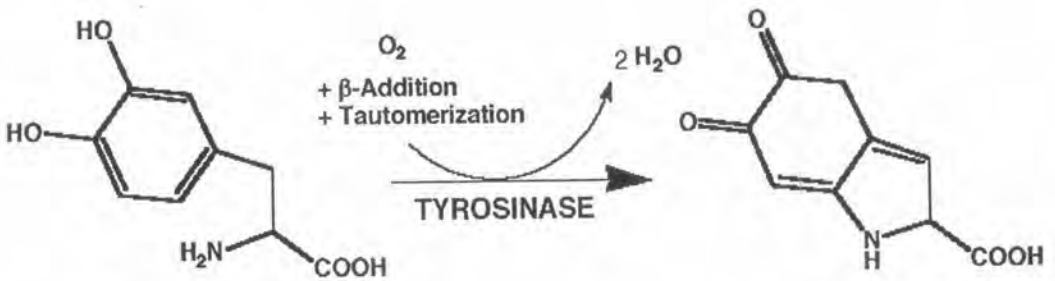
The within-run and between-run precision was studied by determining vitamin C content in 800 mg of test samples (freeze-dried water extract and methanol extract) by the same analyst on the same and different days. Five titrations (n=5) were made for each sample. Precision was expressed as % coefficient of variation (%CV).

2.3.4.4 Limit of detection and quantitation

The limit of detection (LOD) was determined by decreasing the concentration of standard ascorbic acid 10 fold each time. The amount of standard ascorbic acid which could be detected by observation of the end-point was considered to be limit of detection. The lowest amount of standard ascorbic acid that could be quantified with reasonable precision and accuracy was considered the limit of quantitation (LOQ).

Part 3. Determination of anti-tyrosinase activity of *Raphanus sativus* L. extracts

Tyrosinase inhibitory activity was determined by DOPAchrome method using L-DOPA as a substrate. DOPAchrome is one of the intermediate substances in the melanin biosynthesis. The red color of DOPAchrome can be detected by visible light. In this experiment a 96-well microplate reader (VICTOR®) with 492 nm interference filter was used for detection.



3,4-Dihydroxy-L-Phenylalanine
L-DOPA

Dopachrome

The potential tyrosinase inhibitory activity of the test substance would show minimal DOPAchrome absorption. This method was modified from the methods of Sritularak (1998) and Shin et al. (1998).

Figure 9. The VICTOR$^{3®}$ multilable plate reader

3.1. Instrument

96-well Microplate reader (VICTOR®)

3.2. Reagents

Phosphate buffer pH 6.8 (20 mM)

L-DOPA (0.85 mM)

Mushroom tyrosinase (480 units/ml)

Test solution

3.3. Preparation of the reaction mixtures

3.3.1 L-DOPA (0.85 mM)

0.8 mg of L-DOPA was dissolved in 5 ml of 20 mM phosphate buffer (pH 6.8).

3.3.2 Tyrosinase (480 units/ml)

1.13 mg of tyrosinase (2130 units/mg) was dissolved in 5.4 ml of 20 mM phosphate buffer (pH 6.8).

3.3.3 Test solution

All the test samples were diluted with 20% v/v propylene glycol in water prior to analysis for tyrosinase inhibitory activity. The radish extract solutions were prepared with initial concentration of 60 mg/ml. For IC_{50} analysis, the solution was added in different volumes to the reaction mixture to give six final concentrations of 40, 20, 10, 5, 2.5 and 1 mg/ml. Assay was carried out in triplicate.

3.4. Measurement of activity

The reaction mixture (total volume of 200 µl) was measured in four wells designated as A, B, C and D. In each well, the substance was added in the order of mixing, as shown below:

A (control)	40 µl of mushroom tyrosinase solution (480 units/ml)
	80 µl of 20 mM phosphate buffer (pH 6.8)
	40 µl of solvent (20% v/v propylene glycol in water)
B (blank of A)	120 µl of 20 mM phosphate buffer (pH 6.8)
	40 µl of solvent

C (test)	40 µl of mushroom tyrosinase solution (480 units/ml)
	80 µl of 20 mM phosphate buffer (pH 6.8)
	40 µl of test solution in solvent
D (blank of C)	120 µl of 20 mM phosphate buffer (pH 6.8)
	40 µl of test solution in solvent

After each well was mixed and pre-incubated at ambient temperature for further 10 minutes, 40 µl of 0.85 mM L-DOPA was added and incubated at ambient temperature for 10 minutes. The absorbance of each well was measured at 492 nm with the microplate reader after incubation. Licorice extract and L-ascorbic acid were used as anti-tyrosinase. The assay mixture of each concentration was performed and measured in triplicate.

3.5. Calculation of the percent inhibition of tyrosinase enzyme

The percent inhibition of tyrosinase reaction was calculated according to the following equation:

$$\% \text{ tyrosinase inhibition} = \left(\frac{(A - B) - (C - D)}{A - B} \right) \times 100$$

Where A: The absorbance after incubation at 492 nm without test sample(only enzyme and substrate)

B: The absorbance after incubation at 492 nm without test sample and enzyme (blank of A)

C: The absorbance after incubation at 492 nm with test sample (enzyme plus substrate and test substance)

D: The absorbance after incubation at 492 nm with test sample, but without enzyme (blank of C)

3.6. Calculation at 50 % tyrosinase inhibition (IC_{50})

After the percent tyrosinase inhibition of the test solution at each concentration was obtained, a graph showing concentration versus % tyrosinase inhibition was plotted. The IC_{50} of each sample was then obtained from the graph.

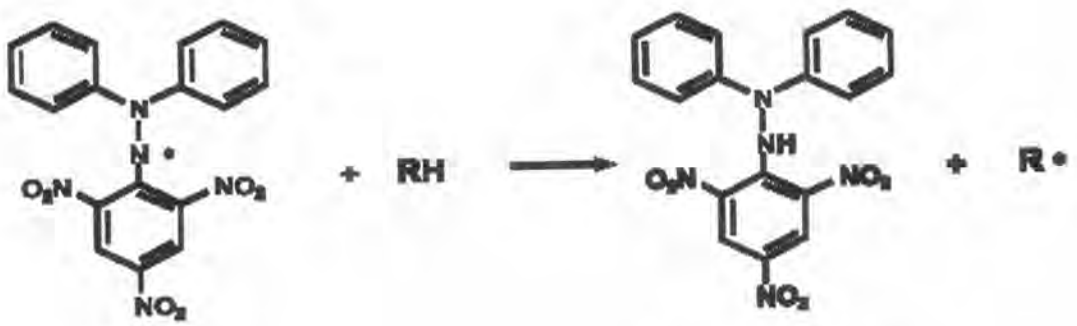
3.7. Statistical analysis

All experiments were carried out in triplicate (n=3). The data were calculated as mean ± SD.

Part 4. Determination of antioxidant and free radical scavenging activities of *Raphanus sativus* L. extracts

4.1 Determination of DPPH free radical scavenging activity

Scavenging activity has been determined by measuring the remaining concentration of DPPH radical which was used as the substrate. The violet color of DPPH radical can be detected by UV-visible spectrophotometer at 517 nm. The presence of any potential DPPH radical scavenger would decrease the absorbance of DPPH in the reaction mixture. This method followed that of Haraguchi et al. (1998), which was modified from Blois (1958).



DPPH· (purple) **DPPH-H (yellow)**

Figure10. Structure of DPPH and reaction with an antioxidant

4.1.1. Instrument

UV-visible spectrophotometer

4.1.2. Reagents

2,2 -diphenyl-1-picrylhydrazyl (DPPH)(150μM)

Absolute ethanol AR grade

Test solution

4.1.3. Preparation of the reaction mixture

4.1.3.1 Preparation of DPPH solution (150μM)

5.91 mg of DPPH (MW 394.3) was dissolved in 100 ml of absolute ethanol and the solution was subsequently stirred for 15 min.

4.1.3.2 Preparation of the test solution

The test samples (freeze-dried water extract and methanol extract) were prepared with initial concentration of 6 mg/ml in absolute ethanol. For IC_{50} analysis, serial dilution with absolute ethanol was performed to give eight concentrations (0, 0.05, 0.1, 0.5, 1, 2, 3 and 4 mg/ml). The reference samples (Trolox® and l-ascorbic acid) were prepared with initial concentration of 100 µg/ml in absolute ethanol. For IC_{50} analysis, the solution was added in different volumes to absolute ethanol to give eight concentrations 0, 0.5, 1.0, 2.5, 5, 10, 25 and 50 µg/ml. Assays were carried out in triplicate.

4.1.4. Measurement of activity

One ml of the test sample or reference antioxidant was added to 1 ml of DPPH solution. The reaction mixture was incubated at room temperature for 30 min and then the absorbance of each sample was measured at 517 nm. The absolute ethanol was used instead of test sample to serve as control (0 µg/ml). L-ascorbic acid and water soluble form of vitamin E (Trolox®) were used as reference antioxidants.

4.1.5. Calculation of percentage of free radical scavenging activity and IC_{50}

The percentage of scavenging activity was calculated from the following equation.

$$\% \text{ DPPH reduction} = \left(\frac{A - B}{A} \right) \times 100$$

A = The absorbance of control solution at 517 nm

B = The absorbance of reaction mixture at 517 nm

After the % DPPH reduction of the test sample at each concentration was

calculated, a plot of sample concentration versus % DPPH reduction was constructed. The IC_{50} of each test sample was then obtained from the graph. By definition, IC_{50} was the concentration at which 50% DPPH reduction was obtained.

4.1.6 Statistical analysis

All experiments were carried out in triplicate (n=3). The data were expressed as mean ±SD.

4.2 Superoxide anion scavenging activity (NBT test)

Riboflavin photo-oxidation is used for the determination of superoxide anion scavenging. In this method, the photo-excitation of riboflavin leads to the generation of riboflavin radical which then auto-oxidizes and generates superoxide anions (Geetha et al., 2004). Then, superoxide anion reduces nitroblue tetrazolium (NBT), resulting in the formation of blue formazan (Dasgupta and De, 2004)

The assay was based on the capacity of the sample to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) into formazan by superoxide anion in the presence of riboflavin-light-NBT system (Banerjee, Dasgupta, and De, 2005). The production of blue formazan was followed by measuring the absorbance at 560 nm after a 30 min illumination from a fluorescent lamp. This method was modified from the method of Dasgupta and De (2004) and Banerjee et al. (2005).

4.2.1.Instrument

UV-visible spectrophotometer

4.2.2.Reagents

Methionine (130 mM)

Riboflavin (200 μM)

EDTA (1 mM)

NBT (750 μM)

Phosphate buffer pH 7.4 (50 mM)

Test solution

4.2.3. Preparation of the reaction mixture

4.2.3.1 Preparation of methionine solution (130 mM)

1.94 g of methionine (MW 376.38) was dissolved in 100 ml of phosphate buffer and stirred until a clear solution was obtained.

4.2.3.2 Preparation of riboflavin solution (200 μM)

7.53 mg of riboflavin (MW 149.21) was dissolved in 100 ml of phosphate buffer and stirred until a clear solution was obtained.

4.2.3.3 Preparation of EDTA solution (1 mM)

37.22 mg of EDTA (MW 372.24) was dissolved in 100 ml of phosphate buffer and stirred until a clear solution was obtained.

4.2.3.4 Preparation of NBT solution (750 μM)

0.61 mg of NBT (MW 817.64) was dissolved in 1 ml of phosphate buffer and stirred until a clear solution was obtained.

4.2.3.5 Preparation of test solution

The test samples (freeze-dried water extract and methanol extract) were prepared with initial concentration of 10 mg/ml in phosphate buffer. For IC_{50} analysis, the solution was further diluted with phosphate buffer to give six concentrations of 0, 1.0, 2.0, 4.0, 6.0 and 8.0 mg/ml. The reference samples (Trolox® and l-ascorbic acid) were prepared with initial concentration of 400 μg/ml in phosphate buffer. For IC_{50} analysis, the solution was further diluted with phosphate buffer to give six concentrations of 0.0, 10.0, 20.0, 50.0, 100.0 and 200.0 μg/ml. Assays were carried out in triplicate.

4.2.4 Measurement of activity

In a test tube, 100 μl of methionine (130 mM), 100 μl of riboflavin (200 μM), 100 μl of EDTA (1 mM) and 100 μl of NBT (750 μM) were placed. Then, the test solution (500 μl) and phosphate buffer (100 μl) were added to give a total volume of 1.0 ml. The production of blue formazan was followed by measuring of absorbance at 560 nm after 30 min illumination from a fluorescent lamp (0.36 mW/cm^2). The entire reaction assembly was enclosed in a box lined with aluminium foil. Identical tubes with corresponding reaction mixtures were kept in the dark and served as respective blanks for the individual samples. The sample without test

solution (0 μg/ml) was used as a control. L-ascorbic acid and water-soluble form of vitamin E (Trolox®) were used as reference antioxidants.

4.2.5 Calculation of percentage of free radical scavenging activity and IC_{50}

The percentage of scavenging activity was calculated according to the following equation.

$$\% \text{ superoxide anion scavenging activity} = \left(\frac{(A - B) - (C - D)}{(A - B)} \right) \times 100$$

Where A: The absorbance of control solution at 560 nm

B: The absorbance of control solution in the dark at 560 nm (blank of A)

C: The absorbance of test solution at 560 nm

D: The absorbance of test solution in the dark at 560 nm (blank of C)

After the % superoxide anion scavenging activity of the test sample at each concentration was calculated, the data were plotted against the concentration for each substance. The IC_{50} of each substance was then obtained from the graph by regression analysis of its initial portion.

4.2.6 Statistical analysis

All experiments were carried out in triplicate (n=3). The data were expressed as mean ±SD.

4.3. Singlet oxygen ($^1O_2$) Assay

The production of singlet oxygen by sodium hypochloride (NaOCl) and hydrogen peroxide (H_2O_2) was determined by spectrophotometric method. *N,N*-dimethyl-*p*-nitrosoaniline was used as a selective scavenger of $^1O_2$ and histidine as a selective acceptor of $^1O_2$ (stabilizer of singlet oxygen). The extent of $^1O_2$ production was determined by measuring the decrease in absorbance of *N,N*-dimethyl-*p*-

nitrosoaniline at 440 nm. This method was modified from the method of Wang and Jiao (2000).

$$HOOH + OCl^- \rightarrow {}^1O_2^* + HOH + Cl^-$$

4.3.1. Instrument

UV-visible spectrophotometer

4.3.2. Reagents

Histidine (10 mM)

Hydrogen peroxide (10 mM)

Sodium hypochloride (10 mM)

N,N-Dimethyl-*p*-nitrosoaniline (50 µM)

Phosphate buffer pH 7.1 (45 mM)

Test solution

4.3.3. Preparation of the reaction mixture

4.3.3.1 Preparation of histidine solution (10 mM)

155.16 mg of histidine (MW 155.16) was dissolved in 100 ml of phosphate buffer pH 7.1 and stirred until a clear solution was obtained.

4.3.3.2 Preparation of *N,N*-dimethyl-*p*-nitrosoaniline solution (50 µM)

0.751 mg of *N,N*-dimethyl-*p*-nitrosoaniline (MW 150.18) was dissolved in 100 ml of absolute ethanol and stirred until a clear solution was obtained.

4.3.3.3 Preparation of hydrogen peroxide solution (10 mM H_2O_2)

Adding 11.3µl of H_2O_2 (30% w/v, MW 34.01) to 10 ml 45 mM phosphate buffer pH 7.1

4.3.3.4 Preparation of sodium hypochlorite solution (10 Mm NaOCl)

Adding 118.2 µl of NaOCl (6.3% w/v, MW 74.45) to 10 ml 45 mM phosphate buffer pH 7.1

4.3.4 Preparation of test solution

The test samples (freeze-dried water extract and methanol extract) were prepared with initial concentration of 6 mg/ml in phosphate buffer. For IC_{50} analysis, the solution was further diluted with phosphate buffer to give six concentrations of 0, 0.1, 0.5, 1.0, 2.0 and 4.0 mg/ml. The reference samples (Trolox® and l-ascorbic acid) were prepared with initial concentration of 200 µg /ml in phosphate buffer. For IC_{50} analysis, the solution was further diluted with phosphate buffer to give six concentrations of 0.0, 10.0, 25.0, 50.0, 75.0 and 100 µg/ml. Assays were carried out in triplicate.

4.3.5 Measurement of activity

In a test tube, 400 µl of histidine (10 mM), 400 µl of *N*, *N* - dimethyl-*p*-nitrosoaniline solution (50 µM), 400 µl of H_2O_2 (10 mM) and 400 µl of NaOCl (10 mM) were placed. Then, 200 µl of test solution and 200 µl of phosphate buffer were added to give a total volume of 2.0 ml. The production of singlet oxygen was followed by monitoring the decrease in absorbance at 440 nm after 40 min. The sample without test solution (0 µg/ml) was used as a control. L-ascorbic acid and water-soluble form of vitamin E (Trolox®) were used as reference antioxidants.

4.3.6 Calculation of percentage of free radical scavenging activity and IC_{50}

The percentage of scavenging activity was calculated according to the following equation.

$$\% \text{ singlet oxygen scavenging activity} = \left(\frac{(B - C)}{(A - C)} \right) \times 100$$

Where A: The absorbance of test solution at 440 nm (without H_2O_2 and NaOCl)

B: The absorbance of test solution at 440 nm (with H_2O_2 and NaOCl)

C: The absorbance of control solution at 440 nm (with H_2O_2 and NaOCl)

After the % singlet oxygen scavenging activity of the test sample at each concentration was calculated, the data were plotted against the concentration for each substance. The IC_{50} of each substance was then obtained by regression analysis of the initial portion of the graph.

4.3.7. Statistical analysis

All experiments were carried out in triplicate (n=3). The data were expressed as mean ±SD.

Part 5. Cytotoxicity test of *Raphanus sativus* L. extracts on normal human fibroblast cell line by LDH assay

LDH is a cytoplasmic enzyme. Normally, it is not secreted outside the cells, but upon damage of cell membrane LDH leaks out. With LDH test, it is possible to measure the release of LDH from cells based on a colorimetric quantitation after an enzymatic reaction (Vihola et al., 2005). The assay is based on the reduction of NAD (nicotinamide adenine dinucleotide) by the action of LDH. The resulting reduced NAD (NADH) is utilized in the stoichiometric conversion of tetrazolium dye. LDH leakage was measured from normal human fibroblast cells by using TOX-7 Cytotoxicity Assay-kit. This method was modified from the methods of Issa et al.(2004).

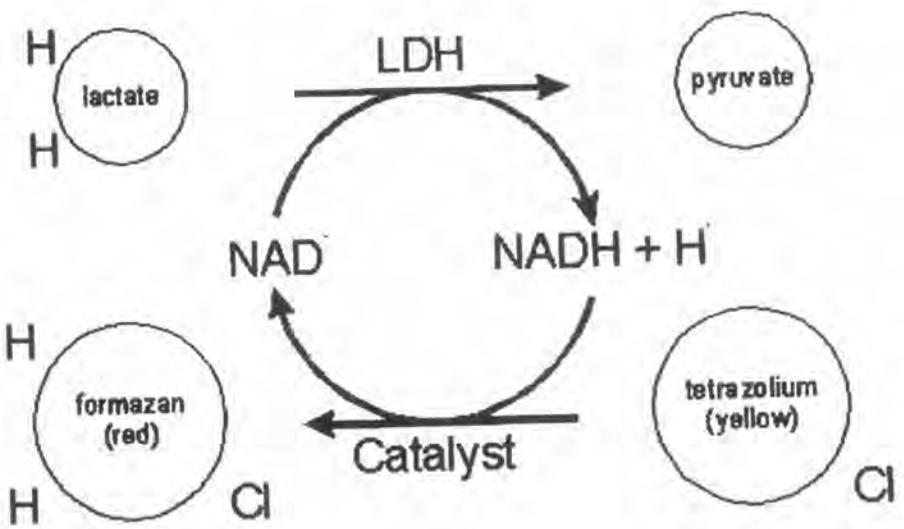


Figure 11. The principle of the cytotoxicity detection kit (LDH)

5.1. Instrument

96-Well Microplate reader (VICTOR®)

5.2. Reagents

5.2.1 LDH assay substrate solution

5.2.2 LDH assay cofactor preparation

5.2.3 LDH assay dye solution

5.2.4 LDH assay lysis solution

5.2.5 1N HCl

5.3. Preparation of the test solution

The test samples (freeze-dried water extract and methanol extract) were prepared with initial concentration of 40 mg/ml. For % LDH release, serial dilution was performed to give eight concentrations (0, 0.25, 0.5, 5.0, 10.0, 10.0, 15.0 and 20.0 mg/ml)

5.4. Preparation of the reaction mixture

5.4.1 Preparation of LDH assay cofactor

Preparation was made by adding 25 ml of tissue culture grade water to a bottle of lyophilized cofactors. The reconstituted cofactor preparation was stored at 0 °C.

5.4.2 Preparation of LDH assay mixture

Equal amounts of LDH assay substrate, cofactor and dye solution were mixed together at time of use.

5.5. LDH assay

Normal human fibroblasts were seeded in 96-well plates at $5x10^3$ cells per well in 200 µl DMEM medium and incubated for 48 hours.

a) Total LDH (100 % LDH release, no test extract added)

After 48 hr seeding, the cultures were removed from the incubator into laminar air flow hood and 20 µl of LDH assay lysis solution was added to each well. The plate was shaken and returned to incubator for 45 min to allow for precipitation. The supernatant was used for analysis (n=30 wells).

b) LDH release

After 48 hr seeding, 200 µl of various concentrations of the extracts (freeze-dried water extract and methanol extract) were added and incubated for 24 hr, the cultures were removed from the incubator into laminar air flow hood and the supernatant was used for analysis (n=3 wells/conc).

c) Enzymatic assay

Supernatant of each well 100 µl was moved to a new 96-well plate and 50 µl of LDH assay mixture added. The plates were then kept in a dark room at room temperature. After 30 min, the reaction could be determined by the addition of 15 µl of 1N HCl to each well and absorbance recorded at a wavelength of 490 nm. When 1N HCl was added, the color of the reaction mixture would change from yellow to red. Cytotoxic effects were calculated as a percentage of average reading of single treatment groups compared to that of total LDH.

5.6 Calculation of percentage loss of membrane integrity

The percentage loss of membrane integrity was calculated according to the following equation:

$$\% \text{ LDH release} = \left(\frac{A - B}{C - D} \right) \times 100$$

Where A: The absorbance for LDH release in the cells that were treated with test solutions

B: The absorbance of test solutions (blank of A)

C: The absorbance for maximum LDH release in lysed cells

D: The absorbance of medium (blank of C)

5.7. Statistical analysis

All experiments were carried out in triplicate (n=3). The data were expressed as mean ±SD.