# CHAPTER III

# MATERIALS AND METHODS

The experiments were divided into five main parts:

1. Preparation of crude extracts from fruit juice of *Phyllanthus emblica* locally grown in Thailand
2. Evaluation of different *Phyllanthus emblica* extracts for antioxidant activities
3. Evaluation of different *Phyllanthus emblica* extracts for anti-collagenase activity
4. Evaluation of different *Phyllanthus emblica* extracts for anti-tyrosinase activity
5. Preliminary stability evaluation of *Phyllanthus emblica* extracts

## Crude drugs

1. Fruit juice of *Phyllanthus emblica* collected from Kanjanaburi province, Thailand, spray-dried at Rangsit University.

## Materials

1. Acetone AR grade, Lot no. 04101109, Labscan, Asia Ltd., Ireland
2. Absolute ethanol AR grade, Lot no. V569 A22D51, Labscan, Asia Ltd., Mexico
3. 1,1-Diphenyl-2-picryl-hydrazine stable radical (DPPH), Lot no. 51K1419, Sigma-Aldrich, Inc., USA
4. 2-Deoxy-D-ribose, D5899, Lot no. 084K0971, Sigma-Aldrich, Inc., USA
5. Disodium ethylene diamine tetra-acetic acid (EDTA), Lot no. A663, Ajax Finechem, Labscan Ltd., Australia
6. Ethyl acetate AR grade, Lot no. 03110166, Labscan, Asia Ltd., Ireland
7. EnzChek® Gelatinase/Collagenase assay kit, Lot no. 36686A, Molecular Probes, Inc., USA,
8. Hydrogen peroxide solution (30%w/v), VWR International Ltd., UK
9. Iron (III) chloride-6-hydrate, VWR International Ltd., UK

10. L-3,4-dihydroxyphenylalanine (L-DOPA), Lot no. 446073/1, Fluka Chemie GmbH, Germany
11. Mushroom tyrosinase (T-7755), Lot no.124K7038 , Sigma-Aldrich, Inc., USA
12. Propylene glycol, U.S.P. grade, Srichand United Dispensary Co., Ltd., Thailand
13. Sodium phosphate monobasic, Lot no. A471, Ajax Finechem, Labscan Ltd., Australia
14. Sodium phosphate dibasic, Lot no. A621, Ajax Finechem, Labscan Ltd., Australia
15. Thiobarbituric acid, Lot no. T5500, Sigma-Aldrich, Inc., USA
16. Tricholoacetic acid, Lot no. T6399, Sigma-Aldrich, Inc., USA

## Reference antioxidants

1. (-)-Epigallocatechin gallate (EGCG) (458.4 g/mol, 90.0% purity), Lot no. 123K1375, Sigma-Aldrich, Inc., USA
2. 6-Hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox®) (250.3 g/mol, 97.0% purity), Lot no. B64311, Calbiochem, Inc., USA
3. L-ascorbic acid (176.13 g/mol, 99.75% purity), Lot no. TL00411042, DSM Nutritional Product Co., Ltd., Switzerland
4. *Phyllanthus emblica* extract, 130165 Lot no. CA0403001, Merck, Ltd., Thailand

## Reference anti-collagenase agent

1. 1, 10-phenanthroline, (198.22 g/mol), Molecular Probes, Inc., USA, Lot no. 36686A

## Reference anti-tyrosinase agent

1. Licorice extract (PT-40), Lot no. 50421066, Maruzen Pharmaceutical Co., Ltd., Japan

## Apparatus

1. Soxhlet extractor apparatus, N.K. Joshi & Co., India

2. Heater, Barnstead/Electrothermal, England
3. Cooler, Boss Tech., Scientific Instruments, Thailand
4. Rotary vacuum evaporator
   - Rotavapor R-114, BÜCHI., Switzerland
   - Vaccum pump Vao® V-500, BÜCHI., Switzerland
   - Water bath B-480, BÜCHI., Switzerland
5. Vortex mixer, Vortex Genie-2, Scientific Industries, Inc. USA
6. Electronic balance, BA2105, Sartorius Basic, Scientific Promotion, co., Ltd., Thailand
7. Electronic balance, Precision Plus TP 2000, Mettler-Toledo, Switzerland
8. UV-visible spectrophotometer, UV-160A, Shimadzu, Japan
9. Micropipette, Gilson, France
10. Multi-channel micropipette, Gilson, France
11. Microplate reader, VICTOR®, multilabel counter, Perkin Elmer Ltd., USA.
12. pH meter Ø 50 pH meter, Beckman instruments, Inc., USA
13. Sonicator (Transonic digitals), Elma, Germany
14. Water bath, DT 2 CB22-20e, Heto-Holten A/S, Denmark

## Others

1. Extraction cellulose thimbles 41*123 mm, Whatman International Ltd., UK
2. Quartz cell, Starna, UK
3. 96-well microplates, Costar® 3912, Corning, Inc., USA
4. Parafilm, American National Can TM, USA

## Methods

## Part 1. Preparation of crude extracts from fruit juice of *Phyllanthus emblica* locally grown in Thailand

Extraction method was developed using Soxhlet apparatus and three kinds of solvent that have been previously employed to extract *Phyllanthus emblica* (Ghosal, 1996; Kumar and Muller, 1999; Mahattanapokai, 2004).

### 1.1 Plant material

Fresh fruits of *P. emblica* were purchased from Kanjanaburi province, Thailand. Then, 70 kg of *P. emblica* fruits were squeezed and 80 Liter of juice dried by spray dry apparatus at Rangsit University, giving 1.15 kg of spray-dried powder (1.64 % w/w yield). The powder of spray-dried *P. emblica* was directly and successively extracted with ethyl acetate, acetone and ethanol to provide five extracts according to the diagram shown in Figure 13, using Soxhlet extraction apparatus.

### 1.2 Extraction

Soxhlet extraction device is an ingenious invention for the continuous extraction of solids with a fresh warm volatile solvent that does not contain the extract. In this method the sample was dried, ground into small particles and placed in a porous cellulose thimble. The thimble was placed in an extraction chamber (2), which was suspended above a flask containing the solvent (1) and below a condenser (3) that is shown in Figure 14.

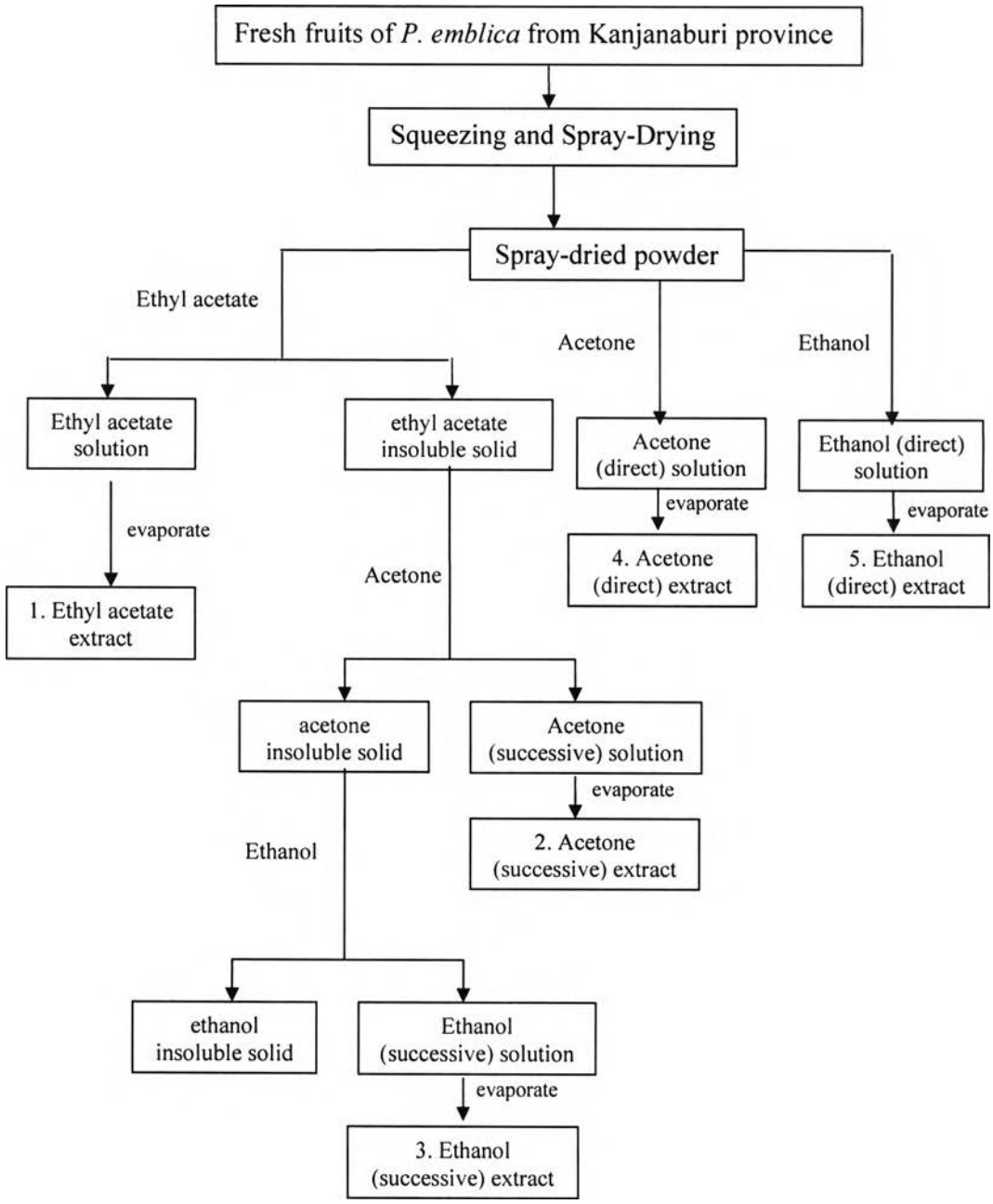



Figure 13. Diagram of the extraction process of *P. emblica* fruits

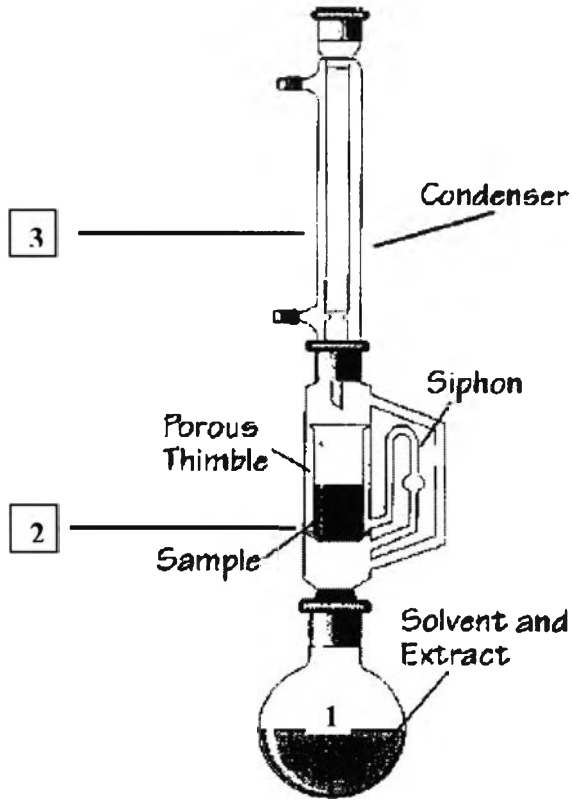



Figure 14. Soxhlet extractor.

The flask and extraction solvent were heated and the vapor rises up the outer sidearm until it reach the water-cooled condenser where it was converted into a liquid (condensed solvent). The condensed solvent drips into the extraction chamber and onto a porous cellulose thimble containing the material to be extracted. When the level of the condensate in the extractor rose to the top of the inner U-shaped sidearm, it automatically siphoned the solvent and dissolved material back down into the lower flask. The solvent was re-boiled, and the cycle was repeated until the sample was completely extracted, as observed by the solvent in the cellulose porous thimble becoming clear. The extract was now in the lower flask.

The process was started with direct extraction of spray-dried powder of *P. emblica* using ethyl acetate, acetone and ethanol as the solvent. Each of the extracted solution was dried using a rotary evaporator to obtain the direct extracts of ethyl acetate, acetone and ethanol, respectively. In addition, after extraction with ethyl acetate, the remaining solid (ethyl acetate insoluble part) was further extracted with

two successive solvents of increasing polarity, namely acetone and ethanol, to yield the acetone (successive) and the ethanol (successive) extracts, respectively, as depicted in diagram of Figure 13.

### 1.2.1 Ethyl acetate extraction

The powder of spray-dried *P. emblica* 50 g was extracted continuously in the Soxhlet apparatus using ethyl acetate at 50-60°C. The process was allowed to proceed until the sample was completely extracted giving clear solution in the extraction chamber. The extracted solution was concentrated under reduced pressure at 50°C using a rotary evaporator to get a viscous residue. This residue was allowed to stand overnight at room temperature in a desiccator to obtain a solvent free extract and a constant weight. The percentage yield was also calculated.

### 1.2.2 Acetone (successive) extraction

The remaining solid that had not been extracted with ethyl acetate was further extracted continuously in the Soxhlet apparatus using acetone at 50-60°C. The process was allowed to proceed until the sample was completely extracted with clear solution in the extraction chamber. The extracted solution was concentrated under reduced pressure at 50°C using a rotary evaporator to get a viscous residue. This residue was allowed to stand overnight at room temperature in a desiccator to obtain a solvent free extract and a constant weight. The percentage yield was subsequently calculated.

### 1.2.3 Ethanol (successive) extraction

The remaining solid that had not been extracted with acetone was further extracted continuously in the Soxhlet apparatus using ethanol at 50-60°C. The process was allowed to proceed until the sample was completely extracted. The extracted solution was concentrated under reduced pressure at 50°C using a rotary evaporator to get a viscous residue. This residue was allowed to stand overnight at room temperature in a desiccator to obtain a solvent free extract and a constant weight. The percentage yield was similarly calculated.

### 1.2.4 Acetone (direct) extraction

Spray-dried *P. emblica* powder was extracted directly and continuously in the Soxhlet apparatus using acetone at 50-60°C. The process was allowed to proceed until the sample was completely extracted. The extracted solution was concentrated under reduced pressure at 50°C using a rotary evaporator to get a viscous residue. This residue was allowed to stand overnight at room temperature in a desiccator to obtain a solvent free extract and a constant weight.

### 1.2.5 Ethanol (direct) extraction

Spray-dried *P. emblica* powder was extracted directly and continuously in the Soxhlet apparatus using ethanol at 50-60°C. The process was allowed to proceed until the sample was completely extracted. The extracted solution was concentrated under reduced pressure at 50°C using a rotary evaporator to get a viscous residue. This residue was allowed to stand overnight at room temperature in a desiccator to obtain a solvent free extract and a constant weight.

## Part 2. Evaluation of different *Phyllanthus emblica* extracts for antioxidant activities

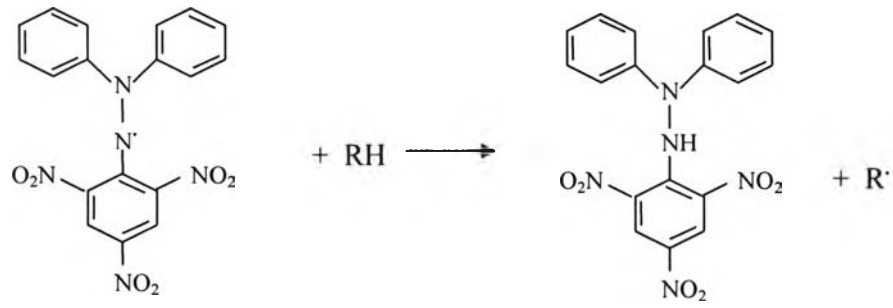
Various evaluation methods for antioxidant activity have been used to monitor and compare the antioxidant activity of plants. In recent years, oxygen radical absorbance capacity assays have been used to evaluate antioxidant activity.

Various anti-oxidative properties of *P. emblica* extract were determined using 3 different methods as follows:

### 2.1 Hydrogen-donating activity (DPPH radical scavenging activity)

Hydrogen-donating activity of the spray-dried *P. emblica* powder, five fractions of *P. emblica* extract, commercial *P. emblica* powder, L-ascorbic acid, Trolox® and (-)-epigallocatechin gallate (EGCG) was determined using the stable 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical. Hydrogen-donating activity of an antioxidant was determined based on its ability to donate hydrogen to DPPH radical (Hsu, Coupar and Ng, 2005).

The diphenylpicrylhydrazyl (DPPH) method is a simple colorimetric assay (Prakash, 2001) of antioxidant activity based on the decrease in absorbance at 517 nm of the DPPH radical (deep purple) after the addition of an antioxidant compound or a hydrogen donor in an ethanolic solution (Chaudhuri, 2002). The addition of an antioxidant (RH) results in the changing of this color to yellow. The structure of DPPH and its reduction product by an antioxidant are shown as follows:




DPPH· (purple) DPPH-H (yellow)

DPPH· = 1,1-diphenyl-2-picrylhydrazyl free radical

RH = antioxidant or hydrogen donor

Figure 15. Structure of DPPH and reaction with an antioxidant

The odd electron in the DPPH free radical gives a strong absorption maximum at 517 nm and is purple in color. The color turns from purple to yellow when the odd electron of DPPH radical becomes paired with hydrogen from a free radical scavenging antioxidant to form the reduced DPPH-H. The resulting decolorization is stoichiometric with respect to the number of electrons captured. The DPPH method has also been used to quantify antioxidants in complex biological systems in recent years. This method can be used for solid or liquid samples and is not specific to any particular antioxidant component, but applies to the overall antioxidant capacity of the sample (Prakash, 2001).

### 2.1.1 Instrument

a) UV-visible spectrophotometer

### 2.1.2 Reagents

a) 1,1-diphenyl-2-picryhydrazyl (DPPH) (0.1 mM)

b) Absolute ethanol AR grade

c) Test samples

### 2.1.3 Preparation of the reaction mixture

a) Preparation of 0.1 mM DPPH radical solution

1.9715 mg of DPPH (M.W. = 394.3) was dissolved in 50 mL of absolute ethanol.

b) Preparation of the test sample

The test samples were prepared as an ethanolic solution with initial concentration of 200 μg/mL. The stock solution was diluted with absolute ethanol until a suitable range of concentration (μg/mL) was obtained including 100, 40, 20, 15, 10, 5, 2 and 1 μg/mL. The DPPH solution (1 mL) was added to the test sample solution (1 mL) to make the total volume of 2.0 mL. The final concentration was calculated by the formula below.

$$C_1V_1 = C_2V_2$$

$C_1$ = Beginning concentration (μg/mL)

$V_1$ = Beginning volume (mL)

$C_2$ = Final concentration (μg/mL)

$V_2$ = Final volume (mL)

Table 6. The initial and final concentrations (μg/mL) of the test sample

| Initial concentration (μg/mL) | 200.0 | 100.0 | 40.0 | 20.0 | 15.0 | 10.0 | 5.0 | 2.0 | 1.0 |
|---|---|---|---|---|---|---|---|---|---|
| Final concentration (μg/mL) | 100.0 | 50.0 | 20.0 | 10.0 | 7.5 | 5.0 | 2.5 | 1.0 | 0.5 |

### 2.1.4 Measurement of activity

The assay mixture contained 1 mL of 0.1 mM DPPH radical solution and 1mL of test sample solution. The solution was rapidly mixed and after standing for 30 min, the absorbance of the mixture was measured at 517 nm using UV-

spectrophotometer (Sakanaka, Tachibana and Okada, 2005). The absolute ethanol was used instead of the test sample to serve as a control (0 µg/mL). Commercial *P. emblica* powder, L- ascorbic acid, water soluble form of vitamin E (Trolox®) and (-)-epigallocatechin gallate (EGCG) were used as reference antioxidants. The assay mixture of each concentration was prepared and measured in triplicate.

The decrease in absorbance of the DPPH radical (deep purple) after the addition of an antioxidant was used to determine the antioxidant activity.

### 2.1.5 Calculation of the percentage of antioxidant activity

The percentage of antioxidant activity (%inhibition) was calculated as the following equation (Dasgupta and De, 2004):

$$\% \text{ inhibition} = \frac{(\text{Abs. control} - \text{Abs. sample})}{\text{Abs. control}} \times 100$$

Abs. control: The absorbance of mixture containing 1mL of 0.1mM DPPH radical solution and 1mL of absolute ethanol without test sample

Abs. sample: The absorbance of mixture containing 1mL of 0.1mM DPPH radical solution and 1mL of test sample solution

### 2.1.6 Calculation of $IC_{50}$

After calculation of % inhibition for each concentration of the test sample, a graph showing concentration versus % inhibition was plotted. The concentration at 50% inhibition ($IC_{50}$) of each sample was then obtained from the equation of polynomial regression of the initial portion of the graph. The mean values were obtained from triplicate experiments. A lower $IC_{50}$ value indicates greater antioxidant activity.

### 2.1.7 Statistical analysis

All experiments were carried out in triplicate (n = 3) and the data were calculated as means ± SD. Statistical comparison of the $IC_{50}$ values among different *P. emblica* extracts and other antioxidants was made using one-way ANOVA and Tukey's test at $\alpha = 0.05$, where appropriate.

## 2.2 Hydroxyl radical scavenging activity

The hydroxyl radical scavenging ability of an antioxidant was determined based on its ability to inhibit deoxyribose degradation. The degradation of deoxyribose by hydroxyl radical generated through Fenton reaction [reaction of an iron-EDTA complex with $H_2O_2$ in the presence of ascorbic acid (reaction 1-2)] is measured colorimetrically in the presence and absence of a scavenger. The hydroxyl radicals attack deoxyribose to form products (reaction 3). If the resulting complex mixture of products is heated under acid conditions, malondialdehyde (MDA) is formed and may be detected by its ability to react with thiobarbituric acid (TBA) to form a pink chromogen (reaction 4). Addition of hydroxyl radical "scavengers" competes with deoxyribose for the hydroxyl radical produced and diminishes chromogen formation (Halliwell et al., 1987; Kunchandy and Rao, 1990; Aruoma, 1994).

$$Fe^{3+}\text{-EDTA} + \text{ascorbate} \longrightarrow Fe^{2+}\text{-EDTA} + \text{oxidized ascorbate} \quad (1)$$

$$Fe^{2+}\text{-EDTA} + H_2O_2 \longrightarrow Fe^{3+}\text{-EDTA} + OH^{\cdot} + OH^{-} \quad (2)$$

$$OH^{\cdot} + \text{deoxyribose} \longrightarrow \text{fragment} \xrightarrow[\text{acid condition}]{\text{Heat under}} \text{MDA} \quad (3)$$

$$\text{TBA} + \text{MDA} \longrightarrow \text{pink chromogen} \quad (4)$$

### 2.2.1 Instrument

a) UV-visible spectrophotometer

b) Water bath

### 2.2.2 Reagents

a) Phosphate buffer pH 7.4

b) 2-deoxy-D-ribose (37.5 mM)

c) Hydrogen peroxide ($H_2O_2$) (10 mM)

d) EDTA (1 mM)

e) $FeCl_3.6H_2O$ (1 mM)

f) L-ascorbic acid (1 mM)

g) Thiobarbituric acid (1 %)

h) Trichloroacetic acid (2.8 %)

i) Test samples

### 2.2.3 Preparation of the reaction mixture

a) Preparation of phosphate buffer (pH 7.4)

Solution A : 20 mM $NaH_2PO_4.2H_2O$ (312.0 mg) was dissolved in 100 mL of $H_2O$

Solution B : 20 mM $Na_2HPO_4$ (567.8 mg) was dissolved in 200 mL of $H_2O$

Then, solutions A (66 mL) and B (184 mL) were mixed to provide phosphate buffer pH 7.4.

b) Preparation of 37.5 mM 2-deoxy-D-ribose (deoxyribose)

Deoxyribose (M.W. = 134.13) (25.15 mg) was dissolved in 5 mL of phosphate buffer pH 7.4.

c) Preparation of 10 mM hydrogen peroxide ($H_2O_2$)

$H_2O_2$ (30 % w/v, M.W.= 34.01) (11.3 µL) was dissolved phosphate buffer pH 7.4 and volume adjusting to 10 mL.

d) Preparation of 1 mM EDTA

EDTA (M.W. = 292.25) (2.92 mg) was dissolved in 10 mL of phosphate buffer pH 7.4.

e) Preparation of 1mM $FeCl_3.6H_2O$

$FeCl_3.6H_2O$ (M.W. = 270.33) (2.70 mg) was dissolved in 10 mL of phosphate buffer pH 7.4.

f) Preparation of 1mM L-ascorbic acid

L-ascorbic acid (M.W. = 176.13) (0.8807 mg) was dissolved in 5 mL of phosphate buffer pH 7.4.

g) Preparation of 1% thiobarbituric acid

Thiobarbituric acid (0.50000 mg) was dissolved in 50 mL of DI-water.

h) Preparation of 2.8% tricholoacetic acid

Tricholoacetic acid (1.40000 mg) was dissolved in 50 mL of DI-water.

i) Preparation of the test sample

The stock solutions containing the spray-dried *P. emblica* extract (15 mg/mL), the commercial *P. emblica* extract (15 mg/mL), Trolox® (4 mg/mL) and EGCG (4 mg/mL) were prepared by directly dissolving and sonicating the extracts in phosphate buffer pH 7.4, whereas the ethyl acetate, acetone (successive and direct) and ethanol (successive and direct) extracts were first dissolved in varying concentration of alkaline disodium hydrogen phosphate (50-600 mM) to aid dissolution in order to obtain stock solutions with concentration of 15 mg/mL. The stock solutions were diluted with phosphate buffer pH 7.4 until a suitable range of concentrations (mg/mL) was obtained including 10, 5, 3 and 1 mg/mL for *P. emblica* extracts. As for, Trolox®, its stock solution was diluted to 3, 2.5, 2, 1 mg/mL and, for EGCG, its stock solution was diluted to 3, 2, 1 mg/mL. Final concentration for *P. emblica* extract was observed at various concentrations (0, 0.1, 0.3, 0.5, 1.0, 1.5, 2.0, 3.0 mg/mL), whereas

Trolox® and EGCG could be observed at maximum concentration of 2.0 mg/mL because their solubility in phosphate buffer and the volume in the reaction mixture was limited.

### 2.2.4 Measurement of activity

Reaction mixtures contained, in a final volume of 1.0 mL, the following reagents at final concentrations stated: deoxyribose, 3.75 mM; $H_2O_2$, 1 mM, $FeCl_3$, 100 μM; EDTA, 100 μM; and test sample in phosphate buffer (pH 7.4) (Halliwell, Gutteridge and Aruoma, 1987; Singh and Rajinia, 2004; Mathew and Abraham, 2006). Where indicated, the iron salts (ferric chloride solutions) were pre-mixed with EDTA solution at the ratio of 1:1 before addition to the reaction system. Reaction was started by adding ascorbic acid to a final concentration of 100 μM (Laughton et al., 1989; Aruoma, 1994; Hagerman et al., 1998). Solutions of $FeCl_3$ and ascorbic acid were made up immediately before use (Li and Xie, 2000) in phosphate buffer. The reaction mixture in a final volume of 1.0 mL is shown in Table 7.

Table 7. Reaction mixture (final volume 1.0 mL) for hydroxyl scavenging activity

| | Phosphate buffer pH 7.4 | $H_2O_2$ | Deoxy-ribose | Test sample | EDTA | $FeCl_3$ | Ascorbic acid |
|---|---|---|---|---|---|---|---|
| Control (A) | 500µL | 100µL | 100µL | - | *100µL | *100µL | 100µL |
| Blank of reaction mixture (B) | 600µL | 100µL | 100µL | - | 100µL | - | 100µL |
| Sample (C) *P. emblica* | 400µL (for 1.5, 1, 0.5, 0.3, 0.1mg/mL) | 100µL | 100µL | 100µL | *100µL | *100µL | 100µL |
| | 300µL (for 3, 2 mg/mL) | 100µL | 100µL | 200µL | *100µL | *100µL | 100µL |
| Trolox® | 200µL (for 0.75 mg/mL) | 100µL | 100µL | 300µL | *100µL | *100µL | 100µL |
| Trolox®, EGCG | - (for 2, 1.5, 1, 0.5 mg/mL) | 100µL | 100µL | 500µL | *100µL | *100µL | 100µL |
| | 400µL (for 0.3, 0.1 mg/mL) | 100µL | 100µL | 100µL | *100µL | *100µL | 100µL |
| Blank of sample (D) *P. emblica* | 700µL (for 1.5, 1, 0.5, 0.3, 0.1mg/mL) | - | - | 100µL | *100µL | *100µL | - |
| | 600µL (for 3, 2 mg/mL) | - | - | 200µL | *100µL | *100µL | - |
| Trolox® | 500µL** (for 0.75 mg/mL) | - | - | 300µL | *100µL | *100µL | - |
| Trolox®, EGCG | 300µL (for 2, 1.5, 1, 0.5 mg/mL) | - | - | 500µL | *100µL | *100µL | - |
| | 700µL (for 0.3, 0.1 mg/mL) | - | - | 100µL | *100µL | *100µL | - |

* premixed with each other before adding to the reaction mixture

After incubation at room temperature for 1 hr, the color was developed by adding 1 mL of 1% TBA (w/v) and 1 mL of 2.8% tricholoroacetic acid (TCA) (w/v). The mixture was then heated in a water bath at 100 °C for 20 min. Samples were allowed to cool and the absorbance of the resulting solution was measured spectrophotometrically at 532 nm (Halliwell et al., 1987; Yen, et al., 1997; Hagerman et al., 1998). Scavenging activity of the test sample was determined by measuring the absorbance of the reaction mixture in comparison with the control samples having no hydroxyl radical scavenger.

Commercial *P. emblica* powder, Trolox® and EGCG were used as reference antioxidants. The assay mixture of each concentration was prepared and measured in triplicate.

### 2.2.5 Calculation of the percentage of antioxidant activity

The percentage of antioxidant activity (%inhibition) was calculated using the following equation

$$\% \text{ inhibition} = \frac{[(A-B)-(C-(B+D))] \times 100}{(A-B)}$$

A : The absorbance of the control reaction without test sample

B : The absorbance of blank of reaction mixture

C : The absorbance of test sample

D : The absorbance of blank of test sample

Since phenolic compounds tend to form violet color with ferric chloride (USP 28), thus the absorbance interference from this interaction (D) must be subtracted from the overall absorbance of the test sample reaction mixture (C). This reaction (C) also contains other reagents that might interfere with its absorbance in addition to its blank of reaction mixture (B). Therefore, the absorbance of blank (B) was also subtracted from C in order to measure the actual absorbance from the inhibition against hydroxyl radical, which should exhibit the decrease in deoxyribose degradation and thus reduce the absorbance at 532 nm in comparison with the control (A-B).

### 2.2.6 Calculation of $IC_{50}$

After the percentage of antioxidant activity of the test sample solution at each concentration was obtained, a graph showing concentration versus % inhibition was plotted. The concentration at 50% inhibition ($IC_{50}$) of each sample was then obtained from the equation of polynomial regression of the graph. The mean values were obtained from triplicate experiments. A lower $IC_{50}$ value indicates greater antioxidant activity, i.e. greater extent of radical inhibition.

### 2.2.7 Statistical analysis

All experiments were carried out in triplicate (n = 3) and the data were calculated as means ± SD. Statistical comparison of the $IC_{50}$ values of the various *P. emblica* extracts in comparison with the reference antioxidants was made using one-way ANOVA and Tukey's test at $\alpha = 0.05$, where appropriate.

## 2. 3 Pro-oxidant activity

Compounds which act as pro-oxidants are thought to be detrimental since they may enhance oxidative damage. Some compounds are pro-oxidants because they are able to redox cycle the metal ion required for OH generation, such as $Fe^{2+}$ and $Cu^{+}$ and thus increase the radical concentration. Since ascorbic acid in the deoxyribose method serves as a pro-oxidant by reducing $Fe^{3+}$ to form more reactive $Fe^{2+}$ iron, modification of this method by omitting ascorbic acid can be used to evaluate the potential of an antioxidant to behave as a pro-oxidant (Hagerman et al., 1998; Li and Xie, 2000). In this modification, the pro-oxidant substitutes for ascorbic acid in the Fenton reaction to regenerate $Fe^{2+}$ and increase the color formation over the ascorbic acid-free control (Hagerman et al., 1998). Therefore, a decrease in color formation should be observed for compounds which are free from pro-oxidant activity.

### 2.3.1 Instrument

- As in 2.2.1

### 2.3.2 Reagents

- As in 2.2.2

### 2.3.3 Preparation of the reaction mixture

- As in 2.2.3 but for the preparation of the test sample, the stock solution was diluted and the final concentration was observed at various concentrations (1.0, 1.5, 2.0, 3.0 mg/mL). EGCG were dissolved in phosphate buffer pH 7.4 to prepare 5 mg/mL stock solution, of which the final concentration in the reaction mixture was

3.0 mg/mL, whereas Trolox® could not be prepared the stock solution for the final concentration of 3.0 mg/mL because its solubility in phosphate buffer pH 7.4 was limited. In this study, the pro-oxidant activity of Trolox® was observed at the final concentration of 0.05, 0.1, 0.3, 0.5, 0.75, 1.0, 1.5 and 2.0 mg/mL

### 2.3.4 Measurement of activity

Reaction mixtures contained, in a final volume of 1.0 mL, the following reagents at final concentrations stated: deoxyribose, 3.75 mM; $H_2O_2$, 1 mM; $FeCl_3$, 100 μM; EDTA, 100 μM; and the test sample in sodium phosphate buffer (pH 7.4). The iron salt (ferric chloride solution) was also pre-mixed with EDTA before addition to the reaction system (Laughton et al., 1989; Aruoma, 1994). This assay was performed without ascorbic acid to assess the pro-oxidant action. The reaction was started by addition of the test compound (Hagerman et al., 1998; Li and Xie, 2000).

Table 8. Reaction mixture (final volume of 1.0 mL) for pro-oxidant activity.

| | Phosphate buffer pH 7.4 | $H_2O_2$ | Deoxy-ribose | EDTA | $FeCl_3$ | Test sample |
|---|---|---|---|---|---|---|
| Control | 600µL | 100µL | 100µL | *100µL | *100µL | - |
| sample *P. emblica* | 500µL (for 1.5, 1mg/mL) | 100µL | 100µL | *100µL | *100µL | 100µL |
| | 400µL (for 3, 2mg/mL) | 100µL | 100µL | *100µL | *100µL | 200µL |
| EGCG | - (for 3 mg/mL) | 100µL | 100µL | *100µL | *100µL | 600µL |
| | 100µL (for 2, 1.5 , 1 mg/mL) | 100µL | 100µL | *100µL | *100µL | 500µL |
| Trolox® | 100µL (for 2, 1.5, 1, 0.5 mg/mL) | 100µL | 100µL | *100µL | *100µL | 500µL |
| | 300µL (for 0.75 mg/mL) | 100µL | 100µL | *100µL | *100µL | 300µL |
| | 500µL (for 0.3, 0.1, 0.05 mg/mL) | 100µL | 100µL | *100µL | *100µL | 100µL |
| Blank of sample *P. emblica* | 700µL (for 1.5, 1mg/mL) | - | - | *100µL | *100µL | 100µL |
| | 600µL (for 3, 2mg/mL) | - | - | *100µL | *100µL | 200µL |
| EGCG | 200µL (for 3 mg/mL) | - | - | *100µL | *100µL | 600µL |
| | 300µL (for 2, 1.5, 1 mg/mL) | - | - | *100µL | *100µL | 500µL |
| Trolox® | 300µL (for 2, 1.5, 1, 0.5 mg/mL) | - | - | *100µL | *100µL | 500µL |
| | 500µL (for 0.75 mg/mL) | - | - | *100µL | *100µL | 300µL |
| | 700µL (for 0.3, 0.1, 0.05 mg/mL) | - | - | *100µL | *100µL | 100µL |
| Ascorbic acid 0.1mM | 500µL | 100µL | 100µL | *100µL | *100µL | 100µL |

* premixed with each other before adding to the reaction mixture

After incubation at room temperature for 1 hr, the color was developed by adding 1 mL of 1% TBA (w/v) and 1 mL of 2.8% tricholoroacetic acid (TCA) (w/v). The mixture was then heated in a water bath at 100 °C for 20 min. Samples

were allowed to cool and the absorbance of the resulting solution was measured spectrophotometrically at 532 nm. The absorbance was compared to the control sample where no test sample was added and also compared to the reaction mixture where ascorbic acid had been added to stimulate the deoxyribose degradation.

Commercial *P. emblica* powder, Trolox® and EGCG were used as reference antioxidants. The assay mixture of each substance was measured in triplicate at each concentration.

### 2.3.5 Statistical analysis

All experiments were carried out in triplicate (n = 3) and the data were calculated as means ± SD. Statistical comparison of the absorbance values at each concentration of test samples to their corresponding control was made using one-way ANOVA and Dunnett's test at $\alpha = 0.05$, where appropriate.

## Part 3. Evaluation of different *Phyllanthus emblica* extracts for anti-collagenase activity

In this study, collagenase inhibitory activity was performed in a 96-well microplate reader (VICTOR®) using EnzChek® Gelatinase/ Collagenase Assay kit (E-12055) (Molecular Probes) that has been previously used in the study of Chaudhuri et al. (2004). The substrate was DQ™ gelatin, which is gelatin labeled by fluorescein to such a degree that the fluorescence is quenched. The substrate typically exhibits less than 3% of the fluorescence of the corresponding free dyes. This substrate is efficiently digested by most gelatinases and collagenases to yield highly fluorescent peptides. Collagenase Type IV from *Clostridium histolyticum* was used as a control enzyme. The increase in fluorescence is proportional to its proteolytic activity and can be monitored with a fluorescence microplate reader. Therefore, to assay for a potential gelatinase/collagenase inhibitor, the decrease in fluorescence compared with the enzyme activity alone was observed. Fluorescence was measured using a fluorescence microplate reader set for excitation at 485 nm and emission detection at 535 nm (Molecular Probes, product information, 2001).

**3.1 Instrument**

3.1.1 Microplate reader VICTOR®, multilabel counter, Perkin Elmer Ltd., USA.

**3.2 Reagents**

3.2.1 EnzChek® Gelatinase/ Collagenase Assay kit (E-12055) consisting of

a) 10X Reaction buffer, 50mL of 0.5M Tris-HCL, 1.5 M NaCl, 50 mM $CaCl_2$, 2 mM sodium azide, pH 7.6

b) DQ gelatin from pig skin, fluorescein conjugate

c) Collagenase, Type IV from *Clostridium histolyticum*

d) 1,10-Phenanthroline, monohydrate

3.2.2 Test samples

**3.3 Preparation of the reaction mixture**

3.3.1 Preparation of 1X reaction buffer 100 mL

10X reaction buffer (10 mL) was diluted in 90 mL deionized water

3.3.2 Preparation of DQ gelatin solution

A DQ gelatin (1.0 mg/mL) stock solution was prepared by adding 1.0 mL of deionized $H_2O$ directly to one of the vials containing the lyophilized substrate. And then, a 125 µg/mL working solution was prepared by adding 1 mL of this stock solution to 7 mL of 1X reaction buffer prepared in step 3.3.1. 40 µL of this working solution was used for the assay (final volume of the reaction mixture = 200 µL per microplate well).

3.3.3 Preparation of *Clostridium* collagenase solution

A 1000 U/mL stock solution of the *Clostridium* collagenase was prepared by dissolving the contents of the vial in 0.5 mL deionized $H_2O$. And then, a 0.25 U/mL working solution was prepared by diluting the stock solution with the 1X reaction buffer prepared in step 3.3.1. 40 µL of this working solution was used for the assay (final volume of the reaction mixture = 200 µL per microplate well).

3.3.4 Preparation of 1,10-phenanthroline as a control inhibitor

1,10-phenanthroline (9.9 mg) was weighed in a plastic microtube and dissolved with 25 µL ethanol. A 10 mM working solution was prepared by adding 10 µL of this solution to 2 mL of 1X reaction buffer prepared in step 3.3.1.

3.3.5 Preparation of the test samples

The test compound (6.25 mg) was dissolved in 5 ml of 1X reaction buffer to prepare 1.25 mg/mL stock solution. The stock solution was further diluted with 1X reaction buffer until a suitable range of concentrations (µg/mL) was obtained. In addition, commercial *P. emblica* extract was prepared into 2 mg/mL working solution, in order to give a final concentration of 800 µg/mL. For each well, each concentration of the test solution (80 µL) was added to the reaction mixture to furnish the total volume of 200 µL. The final concentration was calculated by the formula shown below.

$$C_1V_1 = C_2V_2$$

$C_1$ = Beginning concentration (mg/mL)

$V_1$ = Beginning volume (µL)

$C_2$ = Final concentration (mg/mL)

$V_2$ = Final volume (µL)

For example,

Final concentration of sample solution (beginning conc. = 1.25 mg/mL) =1.25 mg/mL* 80 µL/200 µL
= 0.5 mg/mL

**3.4 Measurement of activity**

The absorbance of the reaction mixtures was measured in four wells (A, B, C and D). In each well, the substance was added in the order of mixing (final volume = 200 µL) as follows:

A (control) 120 µL of 1X reaction buffer (pH 7.6)
40 µL of collagenase, type IV solution (0.25 U/mL)
40 µL of DQ™ gelatin 125 µg/mL

B (blank of A) 160 µL of 1X reaction buffer (pH 7.6)
40 µL of DQ™ gelatin 125 µg/mL

C (sample) 40 µL of 1X reaction buffer (pH 7.6)
80 µL of sample solution in 1X reaction buffer (pH 7.6)
40 µL of collagenase, type IV solution (0.25units/mL)
40 µL of DQ™ gelatin 125 µg/mL

D (blank of C) 80 µL of 1X reaction buffer (pH 7.6)
80 µL of sample solution in 1X reaction buffer (pH 7.6)
40 µL of DQ™ gelatin 125 µg/mL

After each well was mixed, the mixture was incubated at room temperature for 90 minutes. The fluorescence intensity of each well was measured in a fluorescence microplate reader. Digested products from the DQ™ gelatin were detected at 485 nm of excitation and at 535 nm of fluorescence emission. 1,10-Phenanthroline, a general inhibitor of metalloproteinases, was used as a reference anti-collagenase substance. Assay of the mixture at each concentration was performed in triplicate.

**3.5 Calculation of the percent inhibition of collagenase enzyme**

The percent inhibition of collagenase reaction was calculated as follows:

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

A : The fluorescence after incubation without test sample (control)

B: The fluorescence after incubation without test sample and enzyme (blank of A)

C : The fluorescence after incubation with test sample

D : The fluorescence after incubation with test sample, but without enzyme (blank of C)

### 3.6 Calculation of $IC_{50}$

After the percent collagenase inhibition of the test solution at each concentration was obtained, a graph showing % collagenase inhibition versus concentration was plotted. The $IC_{50}$ (concentration at 50% collagenase inhibition) of each sample was then obtained from the graph. A lower $IC_{50}$ value indicates greater anti-collagenase activity.

### 3.7 Statistical analysis

All the experiments were carried out in triplicate (n = 3) and the data expressed as means ± SD. Statistical comparison of the $IC_{50}$ values of different solvent extracts of *P. emblica* and the commercial product was made using one-way ANOVA and Tukey's test at $\alpha = 0.05$, where appropriate.

## Part 4. Evaluation of different *Phyllanthus emblica* extracts for anti-tyrosinase activity

In this study, tyrosinase inhibitory activity was performed in a 96-well microplate reader (VICTOR®) with 492 nm interference filter used for detection. The *P. emblica* extracts were screened for *O*-diphenolase inhibitory activity of tyrosinase using L-DOPA as the substrate (Sritularak, 2002; Nerya et al., 2004). The reaction mixture and the enzyme reaction (control mixture) were monitored by measuring the change in absorbance at 492 nm due to the formation of the DOPAchrome for 10 min. The DOPAchrome is one of the intermediate substances in the melanin biosynthesis with its red color that can be detected by visible light. The potential tyrosinase inhibitor will cause a decrease in dopachrome absorption.

### 4.1 Instrument

4.1.1 Microplate reader VICTOR®, multilabel counter, Perkin Elmer Ltd., USA.

### 4.2 Reagents

4.2.1 Phosphate buffer pH 6.8

4.2.2 L-DOPA (0.85mM)

4.2.3 Mushroom tyrosinase (480 units/mL)

4.2.4 Test samples

**4.3 Preparation of the reaction mixture**

4.3.1 Preparation of phosphate buffer (pH 6.8)

Solution A : 20mM $NaH_2PO_4.2H_2O$ (312 mg) was dissolved in 100 mL of $H_2O$

Solution B : 20mM $Na_2HPO_4$ (284 mg) was dissolved in 100 mL of $H_2O$

Then, solutions A (70 mL) and B (30 mL) were mixed to provide phosphate buffer pH 6.8

4.3.2 Preparation of 0.85 mM L-DOPA

L-DOPA (0.80 mg) was dissolved in 5 mL of phosphate buffer (pH 6.8)

4.3.3 Preparation of 480 U/mL mushroom tyrosinase solution

Mushroom tyrosinase enzyme (0.7229 mg) (labeled activity 3320 U/mg) was dissolved in 5 mL of phosphate buffer (pH 6.8)

4.3.4 Preparation of the test sample

The test samples were prepared by sonicating the solid extracts with 60% PG in water for 10 minutes to make an initial concentration of 6 mg/mL, except the ethanol (successive) extract which was prepared as a 7 mg/mL stock solution. The stock solution was diluted with 60% PG until a suitable range of concentrations (mg/mL) was obtained. For each well, 80 µL of the test solution was added to the reaction mixture to furnish the total volume of 200 µL. The final concentration was calculated by the formula shown below.

$$C_1V_1 = C_2V_2$$

$C_1$ = Beginning concentration (mg/mL)

$V_1$ = Beginning volume (μL)

$C_2$ = Final concentration (mg/mL)

$V_2$ = Final volume (μL)

For example,

Final concentration of sample solution (beginning conc. = 6 mg/mL) = 6 mg/mL * 80 μL/200 μL
= 2.4 mg/mL

**4.4 Measurement of activity**

The absorbance of the reaction mixtures was measured in four wells (A, B, C and D). In each well, the substance was added in the order of mixing (final volume = 200 μL) as follows:

| | |
|---|---|
| A (control) | 40μL of phosphate buffer (pH 6.8) |
| | 80μL of 60%PG |
| | 40μL of mushroom tyrosinase solution (480 U/mL) |
| B (blank of A) | 80μL of phosphate buffer (pH 6.8) |
| | 80μL of 60%PG |
| C (sample) | 40μL of phosphate buffer (pH 6.8) |
| | 80μL of sample solution in 60%PG |
| | 40μL of mushroom tyrosinase solution (480 U/mL) |
| D (blank of C) | 80μL of phosphate buffer (pH 6.8) |
| | 80μL of sample solution in 60%PG |

After each well was mixed and preincubated at room temperature for 10 minutes, 40 μL of 0.85 μM L-DOPA was added, and the mixture was further incubated at room temperature for 10 minutes. The absorbance of each well was measured at 492 nm with the microplate reader. Licorice was used as a reference anti-tyrosinase agent. Assay of the mixture at each concentration was performed in triplicate.

**4.5 Calculation of the percent inhibition of tyrosinase enzyme**

The percent inhibition of tyrosinase activity was calculated as follows:

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

A : The absorbance after incubation at 492 nm without test sample

B: The absorbance after incubation at 492 nm without test sample and enzyme

C : The absorbance after incubation at 492 nm with test sample

D : The absorbance after incubation at 492 nm with test sample, but without enzyme

**4.6 Calculation of $IC_{50}$**

After the percent tyrosinase inhibition of the test solution at each concentration was obtained, a graph showing % tyrosinase inhibition versus concentration was plotted. The $IC_{50}$ (concentration at 50% tyrosinase inhibition) of each sample was then obtained from the graph. A lower $IC_{50}$ value indicates greater anti-tyrosinase activity.

**4.7. Statistical analysis**

All the experiments were carried out in triplicate (n = 3) and the data expressed as means ± SD. Statistical comparison of the $IC_{50}$ values of different solvent extracts of *P. emblica* and the commercial product was made using one-way ANOVA and Tukey's test at $\alpha = 0.05$, where appropriate

## Part 5. Preliminary stability evaluation of *Phyllanthus emblica* extracts

The preliminary stability of various solvent extracts of *P. emblica* was evaluated in terms of its antioxidant activity using DPPH radical as a testing model.

All of the *P. emblica* extracts were packed in close containers, protected from light and kept at ambient temperature for 9 months. The remaining antioxidant activity was investigated at various times (0, 6 and 9 months). Details of the reagents and assay procedure were as described in 2.1.1-2.1.5 with the exception of the following procedure for the test sample preparation.

The test samples were prepared as ethanolic solutions with initial concentration of 40 μg/mL, except the ethyl acetate extract which was prepared as a 100 μg/mL stock solution. The 40 μg/mL stock solution of the test sample was diluted with absolute ethanol to 20 μg/mL, whereas the 100 μg/mL stock solution of ethyl acetate extract was diluted to 40 and 20 μg/mL.

Comparison between the initial antioxidant activity and those remaining after 6 and 9 months was made to evaluate the preliminary stability of the various extracts of *P. emblica.*

The % relative antioxidant activity of each test sample was calculated using the following equation:

$$\% \text{ relative antioxidant activity} = [A/B]*100\%$$

Where A: = % antioxidant activity at the storage testing time (6 and 9 months)

B: = % antioxidant activity at the initial time (0 month)

Thus, the percentage of antioxidant activity remaining at any particular time-point was expressed in relative to the initial inhibitory activity at time zero.