# CHAPTER III

# MATERIALS AND METHODS

## MATERIALS

### CHEMICALS

1. Gallic acid (Sigma-Aldrich, Inc., USA)
2. Trolox (Sigma-Aldrich, Inc., USA)
3. Genistein (Sigma-Aldrich, Inc., USA)
4. Daidzein (Sigma-Aldrich, Inc., USA)
5. Estradiol (Sigma-Aldrich, Inc., USA)
6. Glutamic acid (Sigma-Aldrich, Inc., USA)
7. Hexane analytical grade (Labscan Asia Co., Ltd., Thailand and Fisher Scientific, USA)
8. Methanol analytical grade (Labscan Asia Co., Ltd., Thailand and Fisher Scientific, USA)
9. Methanol HPLC grade (Labscan Asia Co., Ltd., Thailand and Fisher Scientific, USA)
10. Ethyl acetate analytical grade (Labscan Asia Co., Ltd., Thailand and Fisher Scientific, USA)
11. Ethanol (Labscan Asia Co., Ltd., Thailand and Fisher Scientific, USA)
12. Folin-Ciocalteu reagent (Sigma-Aldrich, Inc., USA)
13. Sodium carbonate (Sigma-Aldrich, Inc., USA)
14. 2',7'-dichlorofluorescin diacetate (Sigma-Aldrich, Inc., USA))
15. 2, 2'-azobis-2-amidinopropane-dihydrochloride (Waco Chemical, USA)

16. 2,2-Diphenyl-l-picrylhydrazyl (Sigma-Aldrich, Inc., USA)
17. ICI 182,780 (Tocris Bioscience, UK)
18. Sulfrhodamine B (Sigma-Aldrich, Inc., USA)
19. Glacial acetic acid (Fluka Chemical, USA)
20. Trisbase (Fluka Chemical, USA)
21. Phosphate buffer saline (Sigma-Aldrich, Inc., USA)
22. Acetone analytical grade (Fluka Chemical, USA)
23. Fluorosave TM mounting (Calbiochem, USA)
24. Dulbecco's modified eagle medium (Gibco, Invitrogen, USA)
25. Fetal bovine serum (Atlanta Biologicals, USA)
26. Gentamycin (Hyclone, Thermo Fisher Scientific, Inc., USA)
27. Trypsin-EDTA (Gibco, Invitrogen, USA)
28. Anti-trypsin inhibitor (Gibco, Invitrogen, USA)
29. Hydrogen peroxide (Sigma-Aldrich, Inc., USA)
30. Tryptan blue (Fluka Chemical, USA)
31. Neutral red dye (Sigma-Aldrich, Inc., USA)
32. Trichloroacetic acid (Fluka Chemical, USA)

**PLANT MATERIAL**

Dried powder sample of the tuberous root of *Pueraria mirifica* (Lot No. 111103), Khao La Or Laboratories, Ltd, Bangkok, Thailand.

**CELLS**

HT-22 neuronal cells obtained from Dr. David Schubert at the Salk Institue (San Diego, USA).

**EQUIPMENT**

1. Rotary evaporator (Buchi, R-200, Switzerland)
2. High performance liquid chromatography system (Rabbit-HP, Rainin instrument , Dynamic software) with UV-VIS detector: RT, Knaver waroable wavelength monitor
3. HPLC Column: C18 reverse phase, Inertsil ODS-3®, 5 µm stainless steel column, 4.6 x 250 mm (GL sciences Inc, Japan)
4. Ultracentrifugation (Beckman, USA)
5. Fluorescence microscope (Olympus IX 51, Japan)
6. Fluorescent plate reader ( Biotek, FL 600, USA)
7. Microplate reader (Anthos htl, model A-5022, Austria)
8. Analytical balance (Satorius basic BA 210S and MC 1, USA)
9. Heating bath (Buchi B-490, Switzerland)
10. Magnetic stirrer (VELP Scientifica, model ARE, Italy)
11. pH meter (Consort, model C832T, Belgium)
12. Silica gel 60 thin layer chromatography (TLC) aluminum sheets (precoated, Merck, Germany)
13. UV-VIS light detector, 254 and 366 nm (Spectroline, model CM-10, USA)
14. Semi-automatic TLC sampler (Camag, Linomat 5, Switzerland)

15. Illumination unit (Camag, Reprostar 3, Switzerland) with Digistore Documentation system with CCD camera (Camag, DigiStore 2, Switzerland)
16. Microscope with camera (Nikon, model 84000, Japan)
17. $CO_2$ incubator (Fisher Scientific, Isotemp, USA)

**Methods**

1. Preparation of *P. mirifica* extracts

Dried powder (200 g) obtained from the tuberous root of *P. mirifica* was macerated (24 h) in 500 ml of hexane for three times. The supernatant parts were collected, pooled and evaporated using rota-evaporator until dryness. This hexane extract was called PMH. The rest of the powder was further macerated (24 h) in 500 ml of ethyl acetate for three times. The ethyl acetate filtrate (Whatman filter paper No. 1) were collected, pooled and evaporated, then further dissolved with methanol (20 ml). The methanol insoluble part were collected, pooled and evaporated and was called PME, whereas, the methanol filtrate was evaporated followed by dissolving in water (10 ml). The water soluble part was collected and dried. This water extract was called as PMW. The water-insoluble sediment was dried and was called as PMM. All extracts were further characterized by HPLC.

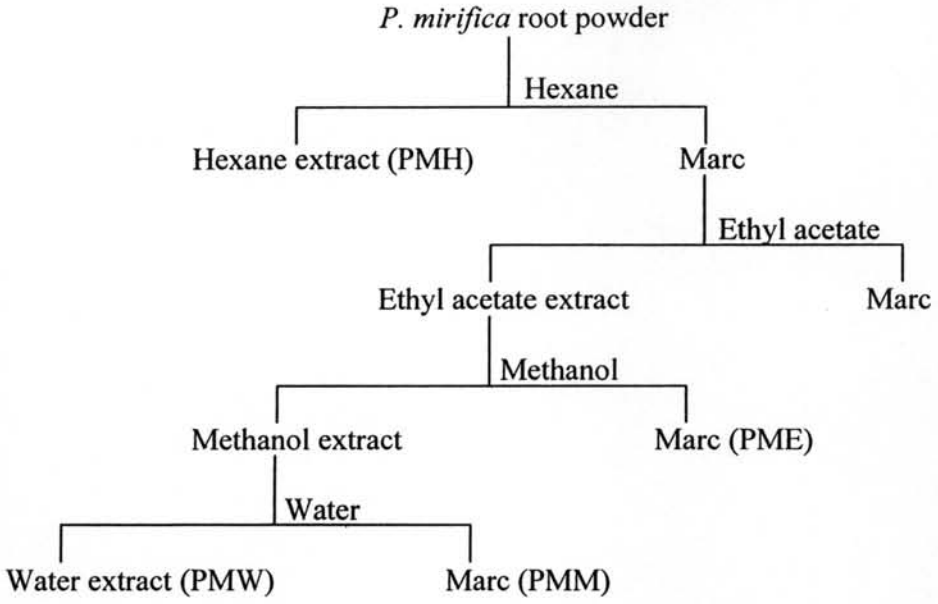


Figure 2 Schematic diagram of *P. mirifica* root powder extraction procedure

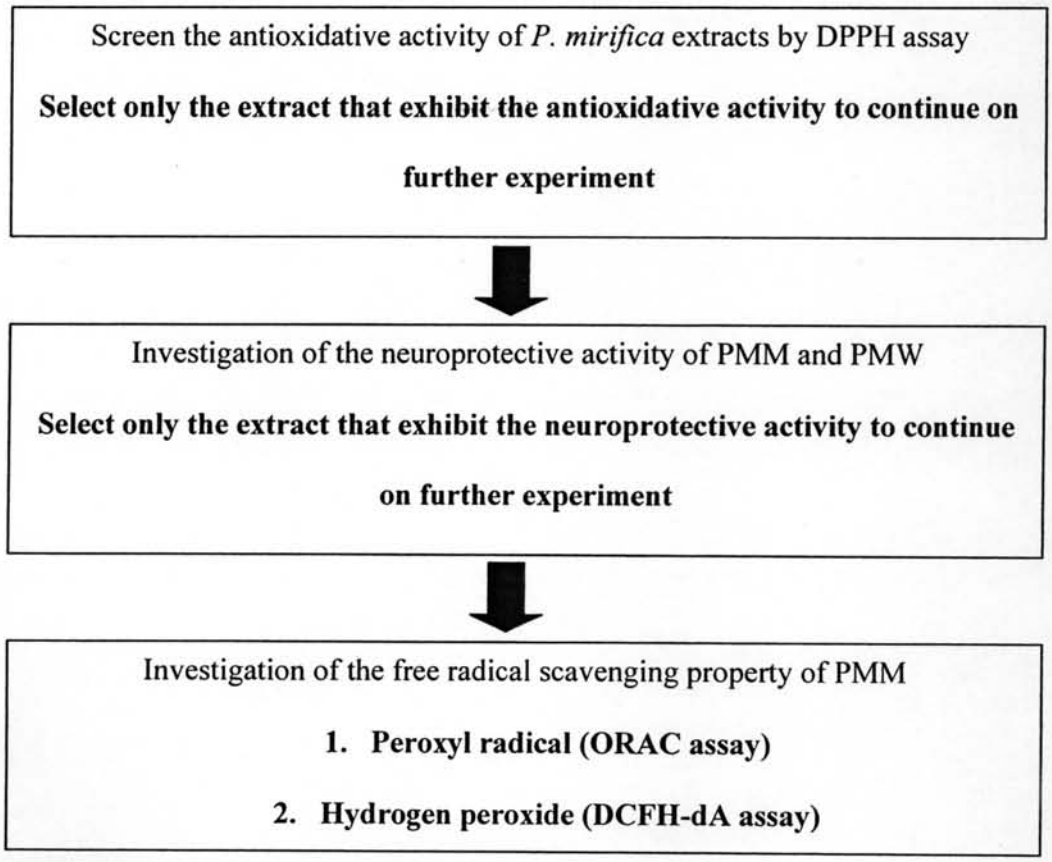


Figure 3 Schematic diagram of methodology process

2. Scavenging of diphenyl-picrylhydrazyl (DPPH) radicals

Radical scavenging potential was determined photometrically by reacting with 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals in a microplate format at 515 nm. Various dilutions of PME, PMM, PMW, PMH, Trolox, genistein and daidzein (in 100% ethanol) were each treated with a solution of 100 μM of DPPH in ethanol for 30 min at 37 °C. Scavenging potential was compared with the solvent control (0% radical scavenging). The half-maximal scavenging concentration was calculated.

For the spot-DPPH assay, various amount of PMM, PMW and the standards were spotted on the thin layer chromatography (TLC) plate then sprayed with the 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution (100 μM).

For TLC-DPPH assay, PMM, PMW and standards were streaked on to a TLC plate (aluminium sheet silica gel 60 F254, precoated, Merck). The plate was then developed using chloroform:methanol (9.2:0.8) as the mobile phase. TLC chromatograms were recorded under the utlraviolet light at 254 and 365 nm. After that the DPPH solution was sprayed evenly through the plate. The pictures were recorded in visible light after 30 min.

3. Characterization of *P. mirifica* extracts

The optimal concentrations of PMM, PMW and the standards daidzein and genistein were all analyzed by HPLC under the following conditions:

Apparatus: Rabbit-HP, Rainin instrument, Dynamic software

Column: C18 reverse phase, Inertsil ODS-3®, 5 μm stainless steel column, 4.6 x 250 mm (GL sciences Inc, Japan)

UV detector: RT, Knaver waroable wavelength monitor

Wavelength: 280 nm

Flow rate : 1 ml/min

Mobile phase : Water: Methanol

(10:90 -95:5: 0-60 min, 95:5- 10:90: 60.01-85 min)

Retention time : 39 min for daidzein

43 min for genistein

The stock solution of daidzein and genistein used as standards were prepared and analyzed by HPLC method under the same conditions. The calibration curves were used to quantify the amount of daidzein and genistein in the extracts.

4. Cell Culture

HT-22 rat neuronal cells were cultured using standard Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum and gentamycin. The cells were maintained at 37°C in a humidified 5% $CO_2$ atmosphere until they were confluent. The cells were washed three times with 7 ml of PBS solution, and trypsinized with 3 ml of trypsin/EDTA until they were slightly rounded-up. Trypsin with debris of dead cells were removed. An additional 3 ml aliquot of trypsin was added and incubated (about 2-3 min), until cells were detached from the bottom of the flask. Trypsin inhibitor (7 ml) was then added, and the cells were collected after centrifugation. The supernatant part was discarded and the cells were resuspended with 5 ml of 10% FBS in DMEM to completely inhibit the trypsin reaction. The cell suspension was centrifuged and the pellets were resuspended with 5 ml of 10% FBS in DMEM. The cells were separated from each other by repeatedly drawing cells through the 23-guage needle 10 times. Fifty microliters of the cell suspension was then mixed with

50 µl of trypan blue to stain the dead cells. The mixture was transferred to a haemocytometer. Numbers of cells in 4 square areas of haemocytometer were counted and calculated by the following equation (Baserga, 1988).

$$\text{No. of cell in 5 ml} = \frac{\text{No. of cell counted} \times 2 \times 10^4 \times 5}{4} \qquad \text{Eq. 16}$$

After calculation, the cell suspension was centrifuged. The supernatant was removed. The pellets were then diluted in DMEM with 10% FBS to the desired concentration. The cells were passed through a 23-guage needle 10 times before adding into wells. The cells were plated into 96-well plates at a density of $1 \times 10^4$ cells/ $cm^2$.

5. Cell proliferation experiments

HT-22 neuronal cell, HT-22 cells were treated with various concentrations of PMM and PMW (0-100 µg/ml) for 24 h. The cell number was determined by the SRB assay (see SRB assay, section 11).

6. Determination of glutamate toxicity to HT-22 cells

6.1 Effect of solvent used to cell number of HT-22 cells

HT-22 cells were treated with various concentrations (0, 0.5 and 1 mM) of glutamate in the presence and absence of 1% ethanol for 24 h to determine the effect of solvent on cell number. The cell number was determined by SRB assay (see SRB assay, section 11).

6.2 Glutamate toxicity

HT-22 cells were treated with various concentrations (0, 0.5, 1, 3, 3.5, 4 and 5 mM) of glutamate in 1% ethanol for 24 h. The cell number was determined by SRB assay (see SRB assay, section 11). The 50% killing dose of glutamate was calculated.

7. Neuroprotective activity of PMM and PMW

HT-22 cells were treated with different concentrations of PMM and PMW (0-100 μg/ml), Trolox (13.2, 26.4, 132 and 264 μg/ml) and genistein (0-2.7 μg/ml) and daidzein (0-0.25 μg/ml) in the presence of 3.5 mM of glutamate for 24 h. The morphology of cells were examined by phase contrast microscopy.

To examine cell viability and cell apoptosis of HT-22 cells treated with PMM and Trolox against glutamate toxicity, the cell and the nuclei of cells were stained with neutral red dye (see NR assay, section 10) and DAPI (see DAPI staining, section 12), respectively.

To qauntitate the neuroprotection of PMM and Trolox against glutamate toxicity, the cell number was determined by using the SRB assay (see SRB assay, section 11).

8. ORAC assay (Ou, Hampsch-Woodill and Prior, 2001).

Each 20 microliters from different concentrations of Trolox and PMM or ethanol as blank was added into each well of a 96- black plastic well plates. The plates were incubated with 200 μl of fluorescein (75 nM) at 37°C. After 30 min of incubation, freshly prepared 75 μl of AAPH (60 mg/ml) was added in each well and the resulting fluorescence intensity of fluorescein was recorded every minute for 2.5 h by fluorescent plate reader. The temperature of the plate was maintained at 37°C

throughout the experiment. The antioxidative activity was observed as a preservation of the fluorescent signal. The protection was quantitated by calculating area under the curve (AUC) from the experimental sample. After subtracting the AUC from blank (Net AUC), the resultant difference was the protection conferred by the antioxidant compound being tested.

The final ORAC values were calculated by using a regression equation between the Trolox concentration and the net area under the fluorescence decay curve. The ORAC value was calculated as:

$[(AUC_{sample}-AUC_{blank})/(AUC_{Trolox}-AUC_{blank})]$ x (Conc. of Trolox/ Conc. of sample)

9. 2,7-Dichlorofluorescin diacetate (DCFH-dA) assay (Choi et al., 2002)

Scavenging ability for ROS in cells was assessed using the probe 2',7'-dichlorofluorescin diacetate (DCFH-dA). HT-22 cells were seeded into 96-well plates at a density of 5,000 cells per well one day prior to an experiment. On the day of the experiment, DCFH-dA loading medium was added at a final concentration of 50 μM. The cells were then incubated in 5% $CO_2$/95% air at 37 °C for 45 min. After removing DCFH-dA, cells were washed twice with PBS and incubated in phenol red-free DMEM medium containing 10% (v/v) FBS, with various combination of Trolox and PMM with hydrogen peroxide. The fluorescence of cells from each well was measured with a Bio-Tek microplate fluorescence reader FL-600. The excitation filter was set at 485±20 nm and emission filter was set at 530±25 nm.

10. Neutral Red (NR) assay (Modified from Ciapetti et al., 1996)

Cell viability was observed by neutral red dye retained by the living cells. HT-22 neuronal cells were incubated for 1 h with 200 µl of 1% neutral red diluted 1: 125 in DMEM with 10% FBS. Pictures were taken under the microscope with camera.

11. Sulfrhodamine B (SRB) assay (Papazisis et al, 1997)

Cell number was assessed using sulfrhodamine B (SRB) to stain the protein of cells. The medium was removed and the cells were fixed with 1 ml of 10% trichloroacetic acid at 4°C. After 10 min of fixing, cells were washed 4 times with distilled water then stained with 200 µl of 0.4% of SRB in 1% acetic acid for 30 min. The unbound dye was removed by washing the cells 4 times with 1% acetic acid. The plate was air-dried overnight. The bound dye was solubilized with 200 µl of 10 mM Trisbase. The absorbance was measured at 560 nm.

12. DAPI staining

HT-22 cells were plated in wells containing coverslips. Cells were allowed to attach for 24 h. Then cells were treated with different concentration of PMM and Trolox in the presence of glutamate for 24 h. After the end of treatment, cells were washed with PBS 3 times to remove the remaining media. Then cells were fixed with 1:1 of acetone and methanol at 4°C. After 10 minutes of fixing, cells were washed with PBS 5 times. Next, 300 nM of 4',6-diamidino-2-phenylindole (DAPI) was used to stain the nuclei for 5 min at room temperature. Cells were washed with distilled water for 5 times. Coverslip was mount with Fluorosave to protect degradation of fluorescence. The pictures of cells were taken by fluorescence microscope.

13. Statistical analyses

One-way analysis of variance (ANOVA) was used to test for overall statistical significance. A difference was considered significant at $p<.05$.