CHAPTER 3

EXPERIMENTAL

3.1 Chemicals

Table 3.1 All chemicals used in this study were analytical grades and listed as following:

Chemicals	Companies (City, Country)
DPPH	Sigma-Aldrich (MO, USA)
ABTS	Sigma-Aldrich (MO, USA)
Trolox	Aldrich (Steinheim, Germany)
Potassium persulfate	BDH Chemicals (England)
Ethanol	Merck (Darmstadt, Germany)
Methanol	Merck (Darmstadt, Germany)
n-Butanol	Merck (Darmstadt, Germany)
Ethyl acetate	Merck (Darmstadt, Germany)
n-Hexane	Merck (Darmstadt, Germany)
Chloroform	Merck (Darmstadt, Germany)
β-Carotene	Fluka (Buchs, Switzerland)
Linoleic acid	Fluka (Buchs, Switzerland)
Tween 20	Numsiang (Thailand)

Table 3.1 (continued)

Chemicals	Companies (City, Country)
TPTZ	Fluka (Buchs, Switzerland)
HCl	Lab-Scan (Ireland)
$FeCl_3 \cdot 6H_2O$	Sigma (MO, USA)
Sodium acetate	BDH Chemicals (England)
Acetic acid	Lab-Scan (Ireland)
$FeSO_4 \cdot 7H_2O$	Sigma (MO, USA)
Gallic acid	Sigma (MO, USA)
BHT	Sigma (MO, USA)
Cosmosil 75µm C_{18}-OPN	Nacalai Tesque (USA)
Sephadex LH-20	GE Healthcare (Sweden)
Silica gel 60 (230-400 mesh)	Merck (Darmstadt, Germany)
Toyopearl HW-40C	TOSOH (Tokyo, Japan)
MCI-gel	Mitsubishi (Tokyo, Japan)
Sulfuric acid	Lab-Scan (Ireland)

3.2 Instruments

Table 3.2 Principal instruments used in this study and listed as following:

Instrument	Company (City, Country)
FT/IR-230 spectrophotometer	JASCO (Tokyo, Japan)
NMR/JNM-α600 spectrometer	JEOL (Tokyo, Japan)
Melting point apparatus	Yanako (Tokyo, Japan)
Microtiter plate reader	Biorad 680 (USA)
Vacuum rotary evaporator	EYELA®, N-100 (Tokyo, Japan)
Mass spectrometer	JEOL (Tokyo, Japan)
Hot air oven	(Thailand)
Miller	(Thailand)

Figure 3.1 Hot air oven

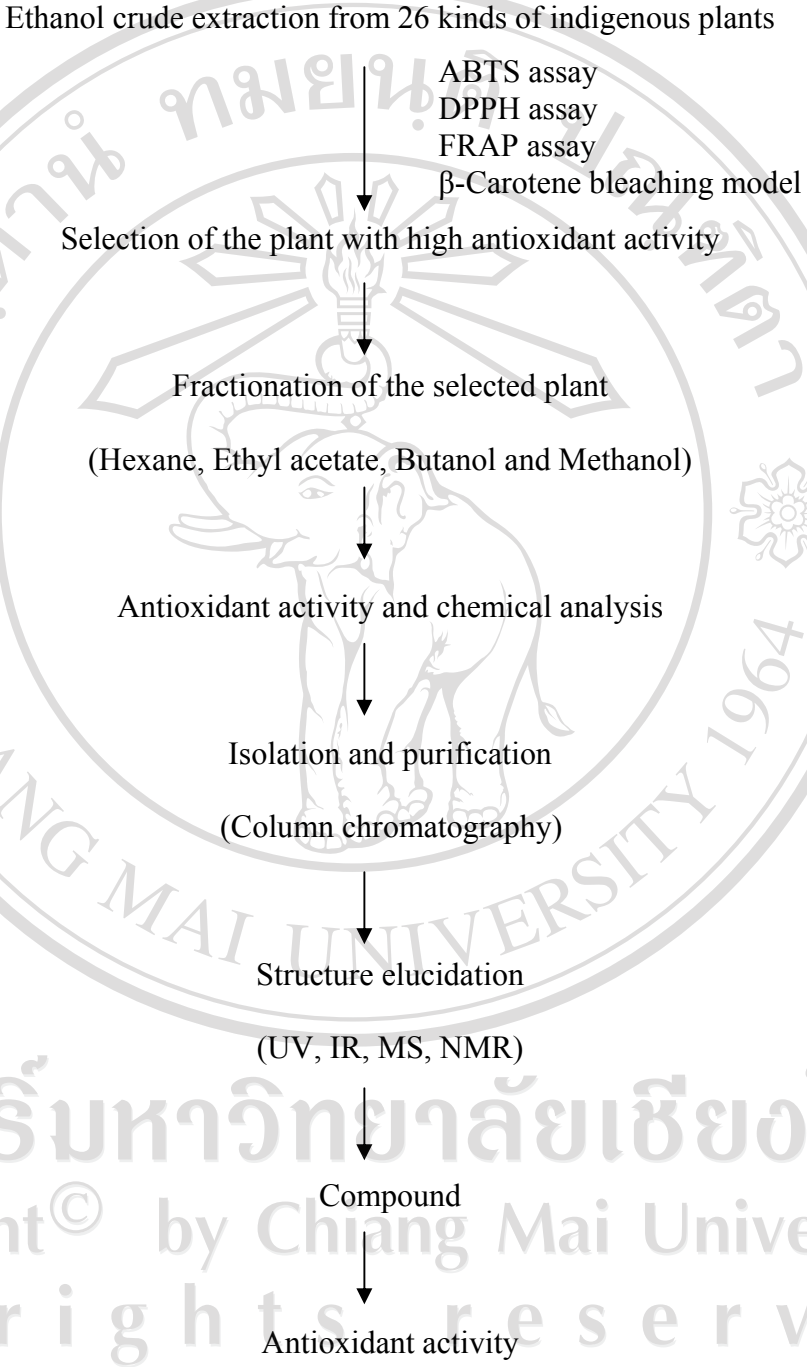
Figure 3.2 Miller

Figure 3.3 Rotary evaporator

Figure 3.4 Microtiter plate reader

3.3 Research designs

Scheme 3.1 Research designs



3.4 Plants materials

Twenty six plant species were collected during April and September 2004 from the northern region of Thailand. The criteria for choosing all these plants for the study are concluded as follows: a) they present the pharmacological property from the literature revision i.e. hypoglycemic effect of *Piper sarmentosum*[47], anticancer, antimicrobial and anti-inflammatory of *Andrographis paniculata*[48,49], antibacterial of Lemon-grass oil, *Hyptis suaveolens* and *Alpinia galanga*[50,51], antifungal of *Ocimum* species[52], antimicrobial and antiviral of *Cocos nucifera*[53], b) they produce a lot of waste products after the consumption i.e. peels from *Hylocereus undatus*, seeds from *Euphoria longan*, aerial part from *Hyptis suaveolens*, peels, seeds and leaves from *Punica granatum*, leaves from *Psidium guajava* and etc., c) they are indigenous vegetables, and d) they can easily be grown in northern Thailand and have a short life cycle. The collection sources of plant samples were different. Some were harvested from the plant gardens, the others were purchased from local markets. The names and part of used from certain plants were shown in Table 3.3. Voucher specimens of certain plants were deposited in the Herbarium of Faculty of Pharmacy, Chiang Mai University. The interesting parts of the plant sample were carefully separated and cut into small thin pieces and dried at 50 °C for a day. The dried plant samples were ground into fine powder and stored in a vacuum desiccator for further study.

3.5 Preparation of extracts

3.5.1 Ethanol crude extracts

The dried powder of each plant sample was extracted by maceration with ethanol (100 g dried power sample/ 500 ml 95% ethanol) for 48 hours (3 times) at room temperature. The filtrate of each time was pooled together and the solvent was removed under vacuum at 45 °C using rotary evaporator. The obtained crude extracts were stored in a desiccator.

3.5.2 Fractionation extracts

The comparison of dried powder plant sample whose ethanol crude extract showed the highest potential of antioxidant and safety was selected and macerated in hexane for 48 hours (3 times) at room temperature. The residue after the third filtration was dried at room temperature for 24 hours to ensure hexane was completely removed. The dried residue was further macerated in another three solvent as followed; ethyl acetate, butanol and methanol, respectively, in the same manner as hexane. The filtrate of the same solvent was pooled together. The solvent was removed under vacuum at 45 °C using rotary evaporator. The extracts obtained from each solvent were kept in a desiccator for further study.

Table 3.3 The name and part of use from certain plants

Scientific name	Thai name	Common name	Family name	Used parts
1. *Andrographis paniculata* (Borm. f.) Nees	ฟ้าทะลายโจร	-	Acanthaceae	Leaves, Stems
2. *Clausena lansium*	มะไฟจีน	-	Rutaceae	Leaves
3. *Citrus hystrix* D.C.	มะกรูด	Lime	Rutaceae	Leaves, Stems, Peels
4. *Cocos nucifera* Linn.	มะพร้าว	Coconut	Palmae	Peels (white fiber)
5. *Cymbopogon citratus* (DC.) Stapf	ตะไคร้	Lemon grass	Gramineae	Leaves, Stems, Rhizomes
6. *Dregea volubilis* (L.f.) Bth.ex Hk.f.	ฮ้วนหมู	Kra-Tung Maba	Asclepiadaceae	Leaves and stems
7. *Euphoria longan*	ลำไย	Longan	Sapindaceae	Seeds
8. *Garcinia mangostana*	มังคุด	Mangosteen	Guttiferae	Peels
9. *Gymnema inodorum* Decne.	เชียงดา	-	Asclepiadaceae	Leaves, Stems
10. *Hylocereus undatus* Britt.	แก้วมังกร	Dragon fruit	Cactaceae	Peels
11. *Hyptis suaveolens* (L.) Poit.	แมงลักคา	Wild spikenard	Labiatae	Leaves and stems
12. *Lansium domesticum*	ลองกอง	Lansium	Meliaceae	Peels

Table 3.3 (continued)

Scientific name	Thai name	Common name	Family name	Used parts
15. *Mentha cordifolia* Opiz	สะระแหน่	Kitchen mint	Labiatae	Leaves, Stems
16. *Musa sapientum*	กล้วย	Banana	Musaceae	Ripe peels, Green peels
17. *Nephelium lappaceum* Linn.	เงาะ	Rambutan	Sapindaceae	Peels
18. *Ocimum basilicum* Linn.	โหระพา	Basil	Labiatae	Leaves, Stems
19. *Ocimum gratissimum* Linn.	ยี่หร่า	Tree basil	Labiatae	Leaves
20. *Ocimum sanctum* Linn.	กระเพรา	-	Labiatae	Leaves, Stems
21. *Oroxylum indicum* (L.) vent.	เพกา	Damocles tree	Bignoniaceae	Pods
22. *Passiflora foetida* Linn.	เสาวรส	Passion fruit	Passifloraceae	Peels
23. *Piper sarmentosum* Roxb.ex Hunter	ชะพลู	-	Piperaceae	Leaves
24. *Psidium guajava*	ฝรั่ง	Guava	Myrtaceae	Leaves, Stems, Fruits
25. *Punica granatum* Linn.	ทับทิม	Pomegranate	Punicaceae	Peels, Seeds
26. *Thunbergia laurifolia* Lindl.	รางจืด	-	Thunbergiaceae	Leaves

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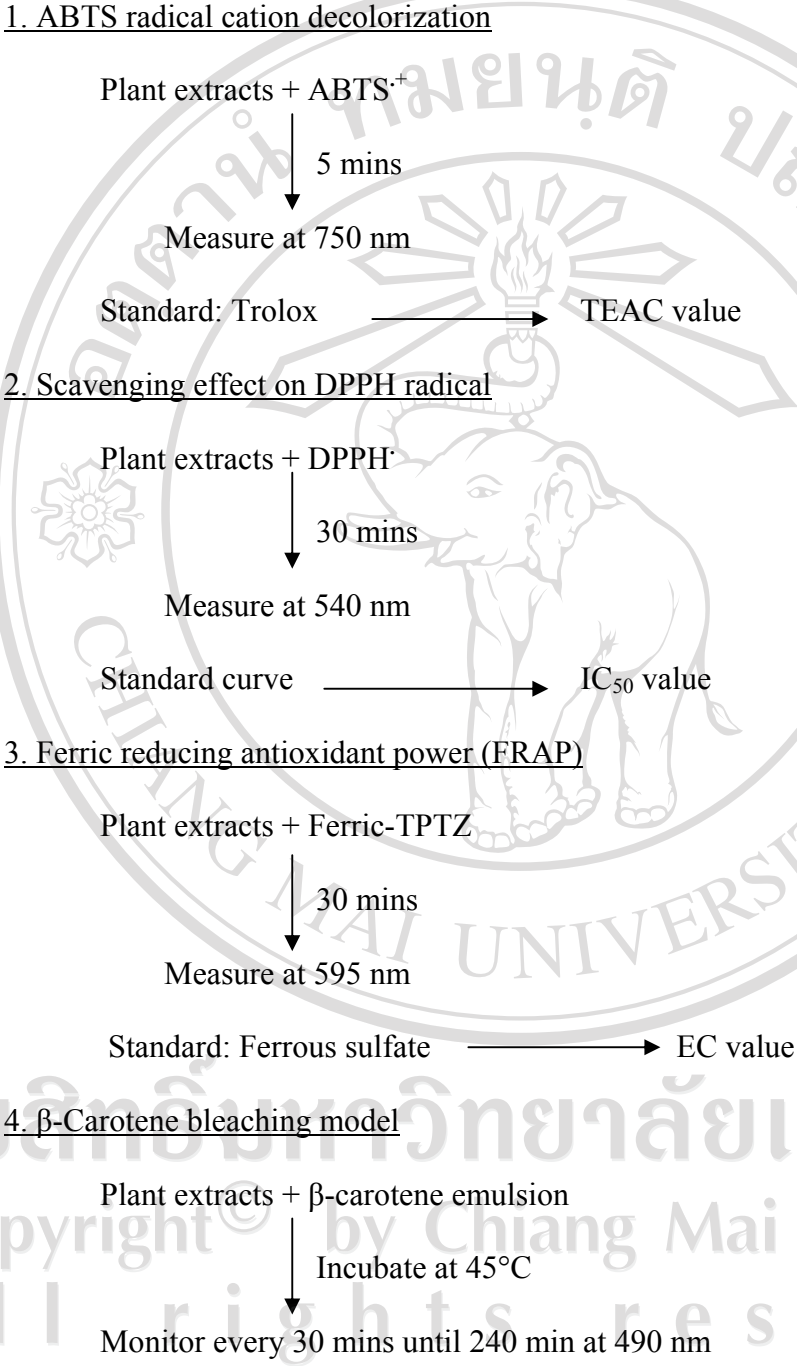
3.6 Antioxidant activity methods

Scheme 3.2 Four complementary methods for antioxidant activity test



3.6.1 ABTS assay

This method was done by using ABTS free radical decolorization assay developed by Re et al. (1999)[54] with some modification. Briefly, the pre-formed radical monocation of ABTS was generated by reacting ABTS solution (7 mM) with 2.45 mM potassium persulfate ($K_2S_2O_8$). The mixture was allowed to stand for 15 hours in the dark at room temperature. The solution was diluted with ethanol to obtain the absorbance of 0.7 ± 0.2 units at 750 nm. The plant extracts were separately dissolved in ethanol to yield a certain concentration of 1 mg/ml. An aliquot of 20 µl ethanolic test solution of each sample was added to 180 µl ABTS free radical cation solution. The absorbance, monitored for 5 min, was measured spectrophotometrically at 750 nm using a microtiter plate reader. All measurements were performed in triplicate. The free radical scavenging activity of each sample was expressed as trolox equivalent antioxidant capacity (TEAC), which was obtained by comparing the absorbance change at 750 nm in a reaction mixture containing a sample of plant extract or test material with that containing trolox. This index is defined as the millimolar concentration of a trolox solution which antioxidant capacity is equivalent to 1.0 mg of the extract[55].

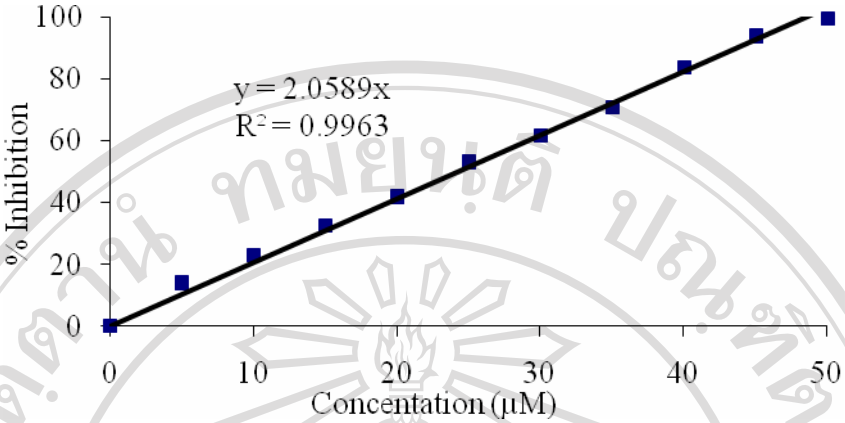


Figure 3.5 Relation of %inhibition and concentration of Trolox standard

3.6.2 DPPH method

The scavenging activity on DPPH radical of all extracts was determined by modifying the methods of Gamez et al. (1998)[56] and Brand-Williams et al. (1995)[57]. The extracts were mixed with ethanol to prepare an ethanolic test solution of different concentrations (0.1-1.0 mg/ml). DPPH was dissolved in ethanol and mixed with certain amount of the ethanolic test solution. The solution was adjusted to a final DPPH concentration of 100 µM. The mixture was shaken vigorously and left to stand for 30 minutes in the darkness at room temperature. The amount of DPPH remaining in each period of stand was determined spectrophotometrically at 540 nm using a microtiter plate reader. All measurements were performed in triplicate. The radical scavenging activity was calculated as % inhibition from the following equation;

$$\% \text{ Inhibition} = \frac{(OD_{blank} - OD_{sample})}{OD_{blank}} \times 100$$

3.6.3 Determination of the reducing power

The reducing power was determined by using a ferric reducing ability of plasma (FRAP) assay described by Benzie and Strain (1996)[58] with some modification. Briefly, the FRAP reagent contained 2.5 ml of 10 mM TPTZ solution in 40 mM HCl plus 2.5 ml of 20 mM $FeCl_3$ and 25 ml of 0.3 M acetate buffer, pH 3.6 was freshly prepared. The extracts were dissolved in ethanol to a certain concentration of 1 mg/ml. An aliquot of 20 µl test solution was mixed with 180 µl FRAP reagent. The absorption of the reaction mixture was measured at 595 nm by microtiter plate reader. The ethanolic solutions of known Fe (II) concentration in the range of 5-50 µM ($FeSO_4$) were used as calibration curve. The reducing power was expressed as equivalent concentration (EC). This parameter was defined as the concentration of antioxidant having a ferric reducing ability equivalent to that of 1 mM $FeSO_4$.

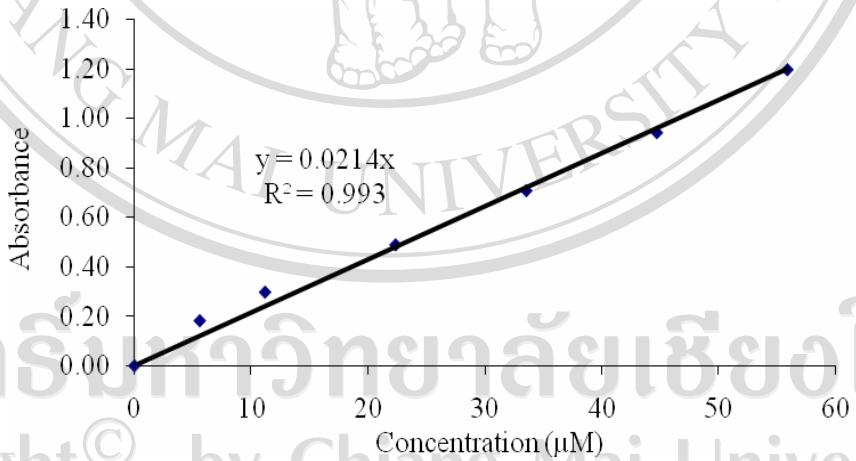


Figure 3.6 Relation of absorbance and concentration of $FeSO_4$ standard

3.6.4 β-Carotene bleaching model

The percentage of antioxidant activity was determined by using a β-carotene bleaching model assay described by Jayaprakasha et al. (2001)[59] with some modification. Briefly, the β-carotene emulsion contained the dried mixtures of 2 ml of β-carotene 1mg/ml in chloroform and linoleic acid 50 mg and Tween 20 50 mg and water (saturated by oxygen) was prepared. The extracts were dissolved in ethanol to a certain concentration of 1 mg/ml. An aliquot of 20 µl test solution was mixed with 180 µl β-carotene emulsion. The absorption of the reaction mixture was measured at 490 nm at started reaction time and 4 hrs by microtiter plate reader. The percentage of antioxidant activity was expressed as

$$\% \text{ Antioxidant activity} = 100 \times 1 - \frac{(\text{Absorbance}_{to} - \text{Absorbance}_{t240}) \text{ of sample}}{(\text{Absorbance}_{to} - \text{Absorbance}_{t240}) \text{ of control}}$$

3.7 Determination of total phenolic content

Total phenolic content was analyzed using the Folin-Ciocalteu reagent method described by Sato et al. (1996)[60] with some modification. Briefly, the plant extract was dispersed in ethanol to yield 1 mg/ml test solution. An aliquot of 1 ml test solution was diluted with 9 ml distilled water. Afterwards, 200 µl Folin-Ciocalteu reagent and 600 µl of 2% sodium carbonate were added. The mixture was allowed to stand for 30 minutes at room temperature before the absorbance was measured spectrophotometrically at 750 nm. Gallic acid was used as the standard for the

calibration curve. Total phenolic content of the sample was expressed as gallic acid equivalent concentration (mg/ml).

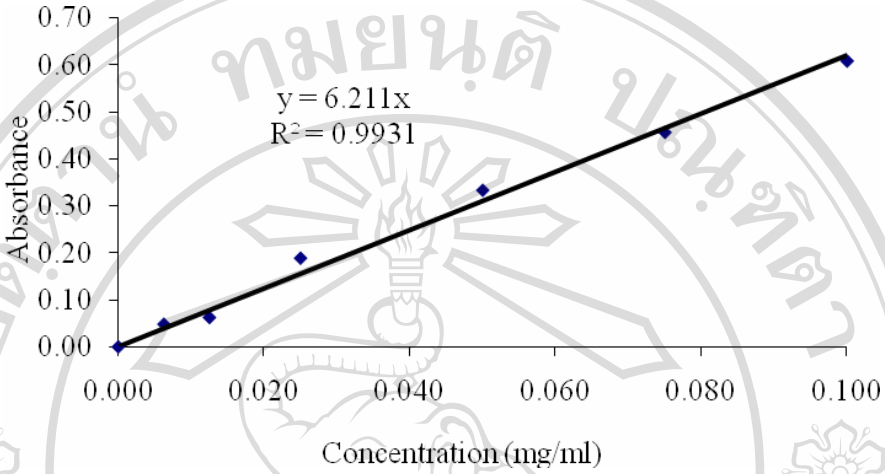


Figure 3.7 Relation of absorbance and concentration of gallic acid

3.8 Chemical screening test [61]

3.8.1 Phenolic and Tannin test

Procedure: A sample of 100 mg was weighted and 25 ml of hot water was added, this was followed by stirring until the mixture turned cold. After, 10% NaCl 1 ml was added to the mixture. Upon filtration, the filtrate was brought into the following:

Tube 1: Sample + nothing = control

Tube 2: Sample + gelatin 1% 5 drops

Tube 3: Sample + $FeCl_3$ 1% 5 drops

Tube 4: Sample + Bromine water 5 drops

Tube 5: Sample + Lime water 5 drops

Tube 6: Sample + Vanillin-HCl

Interpretation:

1. If no reaction with $FeCl_3$, it means no tannin or phenolic compounds.
2. If color is blue-green or black-green with $FeCl_3$ and sediment with gelatin, it means tannin (catechol group) is existing in sample.
3. If color is navy blue with $FeCl_3$ and sediment by gelatin, it means tannin (pyrogallol or gallic tannin) is existing in sample.
4. If color is green or navy blue with $FeCl_3$ and not sediment by gelatin, it means no tannin but contains other phenolic.
5. If negative result with gelatin and no reaction with $FeCl_3$, it means no tannin and polyphenol.
6. If positive result to gelatin and green color to $FeCl_3$ and positive result to bromine water, vanillin-HCl test but negative result to lime water, it means condensed tannin.
7. If positive result to gelatin and navy blue to navy blue-black color to $FeCl_3$ and negative result to bromine water, vanillin-HCl test and positive result to lime water, it means hydrolyzable tannin is existing.
8. If positive test every tube and get navy blue-green to $FeCl_3$, it means condensed tannin and hydrolyzable tannin exist.

3.8.2 Flavonoid test

3.8.2.1 Cyanidin reaction

Procedure: A sample of 100 mg was dissolved in 10 ml of ethanol. To 1 ml of this solution, add magnesium plate 2-3 pieces and drop concentration HCl into tube and observe for 1-2 minutes.

Interpretation:

1. If the sample contains Flavones, it will change orange to orange-red color.
2. If the sample contains Flavonols, it will change to a red color.
3. If the sample contains Flavanones, it will change red to violet color.
4. If the sample contains Flavanonols, it will change to a dark color.

3.8.2.2 Pew test (test for flavanonols or flavonol-3-glycoside)

Procedure: A sample of 100 mg was dissolved in 10 ml of ethanol. To 1 ml of this solution, add 0.5 g zinc of dust and 2 drops of 2N HCl, mix for 1 minute and add 10 drops of concentration HCl.

Interpretation: Red color will appear within 2-5 minutes, if there is flavanonols or flavonol-3-glycoside.

3.8.2.3 Chalcones and Aurones

This test is used for sample which appearing the negative results in cyaniding test.

Procedure: 1-2 drops of concentration HCl into the test sample.

Interpretation: If the sample contains flavonoids (chalcones or aurones), it will change to a red color.

3.8.2.4 Base reaction

Procedure: In to a sample of 1 ml of ethanol, add ammonia TS and observe.

Interpretation:

1. If the sample contains Flavones, Flavonol, or Xanthone, it will change to a yellow color.
2. If the sample contains Flavonone, it will change to a orange red color.
3. If the sample contains Chalcones and Aurones, it will change to a immediate red-purple color.
4. If the sample contains Flavanonols, it will change to a orange-brown color.

3.8.2.5 Anthocyanins test

Procedure: In to a sample of 1 ml of ethanol, add 1 drop of 2N HCl. This solution will change to a red color and then slowly add ammonia TS and observe.

Interpretation: If the sample contains Anthocyanins, it will change red to navy blue color.

3.8.2.6 Leucoanthocyanins test

Procedure: In to a sample of 1 ml of ethanol, add 2 ml of 2N HCl and boiling on water bath.

Interpretation: If the sample contains catechin, it will change to a yellow-brown color.

: If the sample contains leucoanthocyanins, it will change to a red color.

3.9 Cytotoxicity

3.9.1 Caco-2 cell culture

The Caco-2 cells (ATCC, Manassas, VA) were routinely cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 1% nonessential amino acids, 100 units/ml penicillin, and 0.1 mg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37 ˚C. The cells were used from passage 64 to 75. The culture medium was renewed on alternate days. When they had reached confluence, the cells were passaged at a split ratio of one to four by trypsinizing with 0.1% trypsin and 0.02% EDTA in phosphate-buffered saline (PBS). The cytotoxicity study was performed when the cells grew up to 80-90% confluence and were seeded into the 96-well plated at a density of 8×10^3 cells/well.

3.9.2 Preparation of PBMC

Blood samples from healthy volunteers were collected by venepuncture and transferred into 15 ml heparin coated test tubes. Blood was diluted at 1:1 ratio with PBS and layered onto Histopaque®-1077 at a volume ratio of 3:1 and centrifuged at 1,000 rpm for 30 minutes. During centrifugation the PBMCs move from the plasma and are suspended in the density gradient isolating from erythrocytes and granulocytes. The PBMC layer was removed and then washed twice with PBS. The supernatant was then removed and the cells were resuspended in RPMI1640 culture medium supplemented with 10% FBS, 100 U/ml of penicillin, 0.1 mg/ml of streptomycin and an appropriate amount of sodium bicarbonate. Cell viability was

determined by using trypan-blue dye exclusion method. The cell density of PBMC used in the cytotoxicity study was 1 x 10^5 cells/well of the 96-well tissue culture plate.

3.9.3 Measurement of cytotoxicity activity

A standard MTT assay[62] was performed by plating Caco-2 cells or PBMCs in 96 well plates, in 100 µl medium per well, and incubating before crude plant extract treatment at 37°C for 48 hours for Caco-2 and 24 hours for PBMCs. After the incubation, various concentrations of crude plant extracts (5-100 µg/ml) in a medium (100 µl) were added to the cells and incubated for another 48 hours. Each extracted concentration was added into three separated wells. The metabolic activity in each well was determined by the MTT assay and compared to untreated cells. Briefly, after removal of 100 µl medium, MTT stock dye solution (5 mg/ml MTT dye in PBS) was added (15 µl/100 µl medium) to each well, and the plate was incubated at 37°C in 5% CO_2 atmosphere. After 4 hours, supernatant was removed, followed by an addition of DMSO (200 µl) to each well and mixed thoroughly to dissolve the dye crystals. The absorbance was measured by using ELISA plate reader at 570 nm with a reference wavelength of 630 nm. The experiment was done in triplicate. The cell viability was determined by the following formula.

$$\% \text{ Cell viability} = \frac{\text{Mean absorbance in test wells}}{\text{Mean absorbance in control wells}} \times 100$$

Dose-response curves between percentage of cell viability and concentrations of the extracts were constructed. The 50% inhibitory concentration (ID_{50}), in case of cell

inhibition or effective concentration (ED_{50}), in case of cell stimulation was determined from the plotted curve.

3.10 Isolation of bioactive compound from guava leaves extract

The methanol extract (20 g) was subjected to silica gel column chromatography (Cosmosil C_{18}-OPN, 75 µm, 6.0 cm i.d. x 40 cm) and eluted stepwise with MeOH-aqueous mixtures of different polarity (5 to 100% aqueous MeOH). About 90 fractions, measuring 100 ml, were collected and concentrated by rotary evaporator. After analysis by TLC (methanol : water; 1:1), some were pooled together to give 6 fractions (A, B, C, D, E, F) and evaluated for the DPPH scavenging effect.

3.10.1 General techniques

1. Thin layer chromatography (TLC)

Technique	:	one dimension, ascending
Adsorbent	:	RP-18 silica gel GF_{254}
Layer thickness	:	250 µm
Distance	:	5 cm
Temperature	:	25-30°C
Detection	:	Ultraviolet light at wavelength of 254 nm
	:	10% H_2SO_4 spraying reagent followed by heating

An aliquot of column fraction was spotted on a pre-coated RP-18 F_{254} (0.25 mm). The plates were developed in ascending direction with different proportions and

types of suitable mobile phase. The spots were detected by UV (254 nm) and by 10% H_2SO_4 spraying reagent followed by heating.

2. Column chromatography

1) Flash column chromatography

Adsorbent	:	Silica gel (No. 1.07734), particle size 0.063-0.200 mm (70230 mesh ASTM) RP18 silica gel (No. 1.10167), particle size 43-60 μm (E. Merck).
Packing method	:	The adsorbent was suspended in the eluent. The slurry of the adsorbent was poured into the column and then allowed to settle overnight.
Sample loading	:	The sample was dissolved in a small volume of the eluent and loaded on top of the column
Detection	:	Fractions were detected by TLC technique

2) Gel filtration chromatography

Gel filter	:	Sephadex LH-20, Toyopearl HW-40C and MCI-gel
Packing method	:	The gel was suspended in the eluent and left standing to swell overnight prior to use. It was then poured into column and allowed to set tightly
Sample loading	:	The sample was dissolved in a small volume of the eluent and loaded on top of the column
Detection	:	Fractions were detected by TLC techniques

3.10.2 Isolation of quercetin

Fraction C was firstly selected as it showed the highest DPPH radical scavenging activity (IC_{50} = 4.2 µg/ml). A portion of this fraction (1.5 g) was re-chromatographic over Sephadex LH-20 column chromatography (2.5 cm i.d. x 100 cm) and eluted with MeOH, acetone:MeOH (1:1) and acetone. After TLC analysis (methanol : water; 1:1), 4 fractions were obtained and subjected to DPPH assay. The most active fraction C II (48.8 mg) was then subjected to silica gel column chromatography (Silica gel 60, 230-400 mesh, 1.0 cm i.d. x 20 cm) and eluted with several kinds and proportions of solvents. Compound **1** (14.9 mg) was obtained from C-II-2 fraction.

3.10.3 Isolation of quercetin-3-*O*-glucopyranoside

Fraction E (1.2 g) was chromatographic over Cosmosil 75µm C18-OPN (2.3 i.d. x 13 cm) and eluted with 20-100% aqueous ethanol. The 30% ethanol eluate (691.3 mg) was re-chromatographic over Cosmosil 75µm C18-OPN (2.0 i.d. x 12 cm) and eluted with 25-100% aqueous ethanol. The 50% ethanol eluate (567.5 mg) was subjected to column chromatography over MCI-gel (1.0 i.d x 53 cm) and eluted with 30-100% MeOH to give four fractions. The 40% MeOH eluate (231.0 mg) was re-chromatographic over Toyopearl HW-40C and eluted with 50-100% aqueous methanol and 70% aqueous acetone. Compound **2** (33.7 mg) was obtained.

3.10.4 Isolation of morin

Fraction D (2.2 g) was chromatographic over Toyopearl HW-40C (1.0 i.d. x 70 cm) and eluted with 5-100% aqueous MeOH. The received fractions were treated with DPPH assay to search for the most active fraction. The 10% MeOH eluate (61.9 mg) was selected and subjected to Cosmosil 75μm C18-OPN (1.0 i.d. x 20 cm) and eluted with 5-100% MeOH. Compound **3** (4.0 mg) was obtained.

3.11 Identification of bioactive compounds[63, 64, 65]

The three isolated bioactive compounds **1**, **2**, and **3** were subjected for identification process as following. Melting points were determined on a Yanako melting point apparatus. IR spectra were recorded with a JASCO FT/IR-230 spectrophotometer. The IR spectra within the range of 4000–600 cm^{-1} were made on a FT–IR spectrophotometer. MS measurements were performed on a JEOL GCmate mass spectrometer equipped with an electrospray ionization source. The mass spectrometer was operated in the positive or negative ion mode. Samples were dissolved in methanol, and EI mass spectra were recorded on a JEOL GCmate. NMR spectra, ^{1}H and ^{13}C NMR spectra for the samples in CD_3OD were recorded on a JEOL JNM-α600 spectrometer (400 and 600 MHz for ^{1}H and 100 and 150 MHz for ^{13}C). Chemical shifts were shown as δ values, using tetramethylsilane (TMS) as an internal reference.