CHAPTER 3

MATERIALS AND METHOD

3.1 Chemicals

1. Absolute Ethanol (Analytical Grade, Lab Scan, Ireland)
2. Acetone (Analytical Grade, Lab Scan, Ireland)
3. Acetonitrile (HPLC Grade, Lab Scan, Ireland)
4. n-Butanol (Analytical Grade, Lab Scan, Ireland)
5. Chloroform (Analytical Grade, Lab Scan, Ireland)
6. Dimethyl sulfoxide (Analytical Grade, Lab Scan, Ireland)
7. Ethyl acetate (Analytical Grade, Lab Scan, Ireland)
8. n-Hexane (Analytical Grade, Lab Scan, Ireland)
9. Methanol (Analytical Grade, Lab Scan, Ireland)
10. Methanol (HPLC Grade, Lab Scan, Ireland)
11. O-phosphoric acid (Analytical Grade, Lab Scan, Ireland)
12. Hydrochloric acid (Analytical Grade, Lab Scan, Ireland)
13. Sulfuric acid (Analytical Grade, Lab Scan, Ireland)
14. Glacial acetic acid (Analytical Grade, Lab Scan, Ireland)
15. Ferric chloride (Merck, Germany)
16. Ferrous chloride (Merck, Germany)
17. Potassium acetate (Merck, Germany)
18. Potassium persulfate (Merck, Germany)
19. Sodium carbonate (Merck, Germany)

20. Sodium acetate (Merck, Germany)
21. Propylene glycol (Union Science, Thailand)
22. Tween 80 (Union Science, Thailand)
23. 2,2'-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS) (Sigma, Germany)
24. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) (Fluka, USA)
25. 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) (Sigma, Germany)
26. 6-Hydroxyl-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (Aldrich, Germany)
27. Folin-Cioculteu' reagent (Merck, Germany)
28. β-Carotene (Fluka, USA)
39. Gallic acid (Fluka, Spain)
40. Quercetin dehydrate (Sigma, Switzerland)
41. Vitamin E (Sigma, Switzerland)
42. Linoleic acid (Aldrich, Gernany)

3.2 Apparatus and Instruments

1. Appendroffs
2. 96 well plate flat
3. Nylon filter membrane o.45 µm
4. Wathman filter paper No.1
5. Sonicator (Elma, Transsonic T460/H, Germany)
6. Hot air oven (Memmert, BEC, Germany)
7. Analytical balance 2 position (Sartorious AC210 S, Germany)

8. Analytical balance 4 position (Sartorious AC210 S, Germany)
9. pH meter (pH Level 2, Inolab, Germany)
10. Microplate UV-Vis Spectrophotometer (Model 680, BIO RAD, Japan)
11. UV-Vis Spectrophotometer (UV-2450, Shimadzu, Japan)
12. HPLC (Hewlett Packard series 1100, Agilent Technologies, U.S.A)
13. Magnetic stirrer

3.3 Plant extracts

3.3.1 Extracts from the company

Five medicinal plant extracts were obtained from S&J International Enterprises Public Company Limited, Bangkok, Thailand. The list of plants and their part used is shown in Table 3.1.

Table 3.1 Details of plant extracts from S&J Company for used in this study

Common Name	Scientific Name	Part Used	Code
Bua Phut	*Rafflesia kerrii* Meijer	Flower	RM
Damask rose	*Rosa damascena*	Flower	DR
Torch ginger	*Etlingera elatior (Jack)* R.M.Smith	Flower	EE
Cheburic myrobalan	*Terminalia chebula* Retz.	Fruit	MB
Beleric myrobalan	*Terminalia bellirica* (Gaertn.) Roxb.	Fruit	BM

The RM, DR, EE, MB, and BM extracts mentioned in this section were prepared from the company. The RM, DR, and EE extracts were prepared by maceration of dried powder of the respectively flowers in 50% hydroglycol

(propylene glycol + DI water). The MB and BM extracts were prepared by maceration of dried powder of the respectively fruits in 70% hydroglycol. After 3 days maceration, the solutions were filtered through Whatman No. 1 filter paper. The filtrates were kept at 4 °C until further use.

3.3.2 *R. kerrii* extracts preparation

The dried powder of *R. kerrii* flowers was obtained from S&J Company. The powder then devided into two parts, each for extraction as following. For the first part, 30 g of dried powder was macerated with 100 ml of 95% ethanol for 48 hours × 3 times at room temperature. The ethanolic solution was then filtered through Whatman No. 1 filter paper, after that the solvent was removed by using rotary evaporator under vacuum at 45 °C to obtained the crude ethanolic extract code RME. For the second part, 50 g of dried powder was extracted by maceration with different polar solvent in orderly. First, it was macerated with n-hexane for 48 hours × 3 times at room temperature. The residue after the third extraction was dried at room temperature for 24 hours in order to remove n-hexane. After that, the dried residue was further macerated with ethyl acetate, n-butanol and methanol, respectively, in the same procedure as n-hexane. The filtrates of each solvent were collected and removed solvent by using a rotary evaporator under vacuum. The extracts from n-hexane (RMH), ethyl acetate (RMA), n-butanol (RMB), and methanol (RMM) including RME were stored at 4°C for further study.

3.3.3 Characterizations of the extracts

The characterizations of all extracts were done by observation of the outer appearance including color, odor, turbidity, and precipitation by visualization.

3.4 Determination of antioxidant activity

In this study, determination of antioxidant activity was carried out using 4 methods, i.e. ABTS, DPPH, FRAP, and β-carotene bleaching assays.

3.4.1 ABTS assay [38]

3.4.1.1 $ABTS^{\bullet+}$ working solution preparation

The ABTS 0.0376 g was dissolved and adjusted in DI water to the final volume of 10 ml (7 mM ABTS). Potassium persulphate ($K_2S_2O_8$) 0.0166 g was dissolved and adjusted in DI water to the final volume of 25 ml (2.45 mM potassium persulfate). The pre-formed radical monocation of ABTS was generated by reacting 7 mM ABTS 8 ml with 2.45 mM $K_2S_2O_8$ 12 ml. The mixture was allowed to stand for 16 hours in the dark at room temperature. The $ABTS^{\bullet+}$ working solution was diluted with absolute ethanol to obtain the absorbance of 0.7-0.2 units at 750 nm before used.

3.4.1.2 Sample preparation

The extracts were dissolved and diluted appropriately concentration with absolute ethanol.

3.4.1.3 Antioxidant activity testing

The testing antioxidant activity by ABTS assay was conducted in the 96 well plate. The positive control solution was the mixture of 20 µl absolute ethanol and 180 µl $ABTS^{\bullet+}$ working solution, and the negative control solution was the absolute ethanol 200 µl. Whereas, the sample test solution was the mixture of 20 µl sample solution and 180 µl $ABTS^{\bullet+}$ working solution, and blank solution was the mixture of 20 µl sample solution and 180 µl absolute ethanol. The entire solutions were measured for their visible absorbance by using microtitre plate reader at 750 nm for 5 min. All measurements were carried out in triplicate. Standardized trolox dissolved in absolute ethanol at a final concentration from 0 to 50 mM was used as the standard calibration curve (Appendix A). The results were expressed as mM concentration of a trolox solution whose antioxidant capacity is equivalent to 1 mg of extract (TEAC). The radical scavenging activity was calculated as percentage inhibition (% inhibition) from the following equation:

$$\% \text{ Inhibition} = \frac{Abs_{control} - Abs_{sample} \times 100}{Abs_{control}}$$

$$Abs_{control} = Abs_{positive\ control} - Abs_{negative\ control}$$

$$Abs_{sample} = Abs_{sample\ solution} - Abs_{blank\ solution}$$

The % inhibition of sample was compared with standard calibration curve of Trolox equivalent by replaced % inhibition/mg is y in linear equation to give x, which is TEAC value.

3.4.2 DPPH assay [39]

3.4.2.1 DPPH$^{\bullet}$ working solution preparation

The DPPH 0.0049 g was dissolved in absolute ethanol and adjusted to the final volume of 100 ml (100 µM DPPH$^{\bullet}$ working solution).

3.4.2.2 Sample preparation

The extracts were dissolved with absolute ethanol. Each sample was took the serial dilution (twofold dilution) to 7 appropriately concentrations with absolute ethanol.

3.4.2.3 Antioxidant activity testing

The testing antioxidant activity by DPPH assay was conducted in the 96 well plate. The positive control solution was the mixture of 20 µl absolute ethanol and 180 µl DPPH$^{\bullet}$ working solution, and the negative control solution was the absolute ethanol 200 µl. Whereas, the sample test solution was the mixture of 20 µl sample solution and 180 µl DPPH$^{\bullet}$ working solution, and blank solution was the mixture of 20 µl sample solution and 180 µl absolute ethanol. The entire solutions were left to stand for 30 min in the dark at room temperature and measured for their visible absorbance by using microtitre plate reader at 540 nm. All measurements were carried out in triplicate. The radical scavenging activity was calculated as % inhibition from the following equation:

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

$Abs_{control}$ = $Abs_{positive\ control} - Abs_{negative\ control}$

Abs_{sample} = $Abs_{sample\ solution} - Abs_{blank\ solution}$

The graph between concentration and % inhibition of each sample was plotted to obtained linear equation (y = ax + b). The results were expressed as concentration of extracts which obtained 50 % Inhibition (IC_{50}).

3.4.3 Ferric reducing antioxidant power (FRAP assay) [42]

3.4.3.1 FRAP working solution preparation

The TPTZ 0.0309 g was dissolved and adjusted to the final volume of 10 ml with 40 mM hydrochloric acid (HCl) to obtained 10 mM TPTZ. The 40 mM HCl was prepared from 340 µl HCl in DI water 100 ml. The ferric chloride ($FeCl_3.6H_2O$) 0.0540 g was dissolved and adjusted to the final volume of 10 ml with DI water to obtained 20 mM $FeCl_3.6H_2O$. The 0.3 M acetate buffer (pH 3.6) was prepared by dissolved sodium acetate ($CH_3COONa.3H_2O$) 1.55 g in acetic acid 8.0 ml following by the addition of water to the final volume of 100 ml. The FRAP working solution was freshly prepared by mixing 10 mM TPTZ, 20 mM $FeCl_3.6H_2O$, and 0.3 M acetate buffer (pH 3.6) in a ratio of 1:1:10.

3.4.3.2 Sample preparation

The extracts were dissolved and diluted appropriately concentration with absolute ethanol.

3.4.3.3 Antioxidant activity testing

The testing antioxidant activity by FRAP assay was conducted in the 96 well plate. The sample test solution was the mixture of 20 µl sample solution and 180 µl FRAP working solution, and blank solution was the mixture of 20 µl sample solution and 180 µl of 0.3 M acetate buffer (pH 3.6). The entire solutions were measured for their visible absorbance by using microtitre plate reader at 595 nm for 5 min. All measurements were carried out in triplicate. The ethanolic of ferrous sulphate ($FeSO_4.7H_20$) was used as the standard calibration curve at the final concentration from 50 – 600 µM (Appendix A). The results were expressed as equivalent concentration (EC), defined as mM concentration of antioxidant having a ferric-TPTZ reducing ability equivalent to that of a 1 mM concentration of $FeSO_4 \cdot 7H_2O$. The EC value was calculated by replacing absorbance/mg extract is y in linear equation of $FeSO_4.7H_20$ standard calibration curve to give x, which is EC value.

3.4.4 β-carotene bleaching assay [44-45]

3.4.4.1 β-carotene emulsion (working solution) and blank emulsion preparation

The β-carotene emulsion, working solution was carried out by mixed 20 mg linoleic acid, 200 mg tween 80, and 0.3 mg β-carotene in 1 ml chloroform into round-bottom flask. The mixture was then evaporated to remove chloroform at 40 °C for 10 minutes by using rotary evaporator under vacuum. Immediately after, the mixture was diluted with 50 ml oxygenated DI water generated by bubbling air, which was added slowly to the mixture with vigorous agitation to form and emulsion. Whereas, blank emulsion was prepared the same procedure, but there is without β-carotene.

3.4.4.2 Sample preparation

The extracts were dissolved and diluted appropriately concentration with absolute ethanol.

3.4.4.3 Antioxidant activity testing

The testing antioxidant activity by β-carotene bleaching assay was conducted in the 96 well plate. The positive control solution was the mixture of 20 µl absolute ethanol and 180 µl β-carotene emulsion, and the negative control solution was the mixture of 20 µl absolute ethanol and 180 µl blank emulsion. The sample test solution was the mixture of 20 µl sample solution and 180 µl β-carotene emulsion, and blank solution was the mixture of 20 µl sample solution and 180 µl blank emulsion. The entire solutions were gently mixed and incubated at 50 °C for 2 hours. Measurements for their visible absorbance were carried out at zero time and 30 minutes intervals by using microtitre plate reader at 450 nm. All measurements were carried out in triplicate. Antioxidant activity, defined as lipid peroxidation inhibition was calculated using the following equation:

$$\% \text{ Inhibition} = \frac{\Delta Abs_{control} - \Delta Abs_{sample} \times 100}{\Delta Abs_{control}}$$

$$\Delta Abs_{control} = (Abs_{positive\ control} - Abs_{negative\ control})_{0\ min} - (Abs_{positive\ control} - Abs_{negative\ control})_{120\ min}$$

$$\Delta Abs_{sample} = (Abs_{sample\ solution} - Abs_{blank\ solution})_{0\ min} - (Abs_{sample\ solution} - Abs_{blank\ solution})_{120\ min}$$

3.5 Determination of phenolic compounds

In this study, determination of total phenolic content was carried out by using folin-ciocalteu assay, whereas, total flavonoid content carried out by using aluminium chloride colorimetric assay.

3.5.1 Total phenolic content by folin-ciocalteu assay [46]

3.5.1.1 Reagent preparation

The 2% sodium carbonate ($NaCO_3$), which prepared from 2 g $NaCO_3$ disolved and adjusted in DI water 100 ml and folin-ciocalteu reagent 100 ml were used as working solution.

3.5.1.2 Sample preparation

The extracts were dissolved and diluted appropriately concentration with absolute ethanol.

3.5.1.3 Total phenolic content testing

The determination of total plenolic content by folin-ciocalteu assay was conducted in 96 well plate. The sample test solution, 45 µl folin-ciocalteu reagent was added in 20 µl sample solution, left it for 3 minutes, 135 µl of 2% $NaCO_3$ was added into the mixture, and blank solution was the mixture of 20 µl sample solution and 180 µl 2% $NaCO_3$. The entire solutions were placed at room temperature for 2 hours. Measurements for their visible absorbance were carried out by using microtitre plate reader at 750 nm. All measurements were carried out in triplicate. Gallic acid was

used as the standard calibration curve (Appendix A). The results were expressed as GAE value, was defined as gallic acid equivalent concentration (mg/g extract).

3.5.2 Total flavonoid content by aluminium chloride colorimetric assay [47-48]

3.5.2.1 Reagent preparation

The 10% aluminium chloride ($AlCl_3$) was prepared from 5 g $AlCl_3$ disolved and adjusted in DI water to final volume at 50 ml. The 1 M potassium acetate (CH_3COOK) was prepared from 4.9075 g CH_3COOK dissolved and adjusted in DI water to final volume at 250 ml.

3.5.2.2 Sample preparation

The extracts were dissolved and diluted appropriately concentration with absolute ethanol.

3.5.2.3 Total flavonoid content testing

The determination of total flavonoid content by aluminium chloride colorimetric assay was conducted in figure 3.6. The 100 µl sample solution was mixed with 20 µl 10% $AlCl_3$, 20 µl 1 M CH_3COOK, and 860 µl DI water. The mixture was incubated at room temperature for 30 minutes. Measurements for their visible absorbance were carried out by using UV-Visible spectrophotometer at 415 nm. All measurements were carried out in triplicate. Quercetin was used as the standard calibration curve (Appendix A). The results were expressed as QE value, was defined as quercetin equivalent concentration (mg/g extract).

3.6 Study on physicochemical properties of RM and *R. kerrii* extract containing the highest antioxidant activity

3.6.1 Solubility testing

The solubility testing was evaluated only the *R. kerrii* extract containing the highest antioxidant activity. It was carried out using the various solvents commonly used in laboratory. The solubility is expressed in terms of "parts", representing the number of ml of the solvent, in which 1 g of the extract is soluble. Descriptive terms are sometimes used to indicate the solubility of a substance, with the following meanings [62]:

Very soluble	less than 1 part
Freely soluble	1–10 parts
Soluble	10–30 parts
Sparingly soluble	30–100 parts
Slightly soluble	100–1000 parts
Very slightly soluble	1000–10000 parts
Practically insoluble	more than 10000 parts

3.6.2 UV-visible spectroscopy testing

The RM and *R. kerrii* extract containing the highest antioxidant activity were scanned for spectrum by UV-visible spectrophotometer at the wavelength range of 200 – 800 nm. The spectrum peak was expressed as the maximum wavelength (λ_{max}).

3.6.3 HPLC chromatogram testing

The HPLC chromatogram of the RM and *R. kerrii* extract containing the highest antioxidant activity were carried out using with the following condition that shown in table 3.2

Table 3.2 HPLC condition for HPLC chromatogram testing of RM and *R. kerrii* extract containing the highest antioxidant activity

Operating parameter	**Condition**
Stationary phase (column)	A reversed phase column ZORBAX SB-C18 (4.6 × 250 mm, i.d., 5 µm, Agilent, USA)
Mobile phase	(A) acetronotrile and (B) 0.3% v/v o-phosphoric acid in DI water
Detector	UV-visible detector at 280 nm
Flow rate	0.8 ml/minute
Injection volume	10 µl
Run time	15 minute

3.7 Phytochemical screening

3.7.1 Flavonoid glycoside testing [63-64]

The 3 ml of the ethanolic extract was added the 1 ml drops of the concentrated HCl. Then, one small thin piece of magnesium metal was put. When the reaction ended, the color of the solution was orange-red, it could be consider that the extract exist flavonoid glycoside.

3.7.2 Tannin testing [63-64]

The extract was dissolved in ethanol and divided into 2 parts. For the first part, 3 ml of solution was completely evaporated on the water bath. After that, 9 ml of DI water was added. Then, 2-3 drops of 10 % NaCl solution was added to salt out the other compounds. The mixture was filtered using a buchner funnel. The obtained clear solution was divided into 3 tests which contained 2 ml in each tube. The first tube was added with the 4-5 drops of 1 % gelatin solution second tube was added with gelatin salt, and the third tube was used as control. The precipitation in first and second tubes was detected as a positive result. For the second part, the solution of extract was divided into 4 tests which contained 1 ml in each tube. The first tube was added with 3-4 drops of 1 % ferric chloride solution. The solution was a blue-green or dark green as a positive result. The second tube was added with 3-4 drops of formalin-HCl and boiled to 2 minutes. The red precipitant was detected as positive result. The third tube was added with 3-4 drops of line water. The sleek gray precipitant was detected as positive result. The last tube was used as control.

For the result interpretation, if there was no reaction with 1 % gelatin solution and gelatin salt, it could be considered that the tannin compounds was not existed in the extract. Whereas, the extract solution was presented blue-green or dark green colors after adding the 1 % ferric chloride solution, precipitated after adding formalin-HCl, and not precipitated after adding lime water, it could be considered that the condensed tannins were existed in the extract. Final case, the extract solution was presented blue-green or dark green colors after adding the 1 % ferric chloride solution, precipitated after adding lime water, and not precipitated after adding

formalin-HCl, it could be considered that the hydrolysable tannins were existed in the extract.

3.8 Stability testing

The stability testing was used RM and *R. kerrii* extract containing the highest antioxidant activity. This extract was dissolved in 50% propylene glycol with the maximum concentrate can be freely soluble, similar solution of RM, for compared potential antioxidant activity when the extract was in different form (liquid and solid forms). The stability testing was stored the extracts in the amber vial with screw cap at 40 °C, 75% RH for 4 months. The extracts were collected at 0, 1, 2, 3, and 4 month to measuring antioxidant activity, total phenolic content, and total flavonoid content followed the assays as mentioned above.

3.9 Statistical analysis

All experiments were carried out in triplicate. The results were expressed as mean values ± SD. Analysis of variance (ANOVA) in a multiple comparison and Duncan's tests were performed to compare the data by using SPSS statistical software package v.17. *P* value < 0.05 was regarded as significant.