CHAPTER 2

Experimental

2.1 Apparatus and chemicals

2.1.1 Apparatus

1. Gas chromatograph-mass spectrometer, Agilent 6850 Series GC system coupled to HP 5973 mass selective detector, manufactured by Agilent technologies, USA
2. A modified Likens-Nickerson simultaneous distillation and extraction (SDE) apparatus, manufactured by Becthai, Thailand
3. Freeze-dryer, Snijders scientific type 2040, Snijders Scientific B.V., Netherland
4. GC column HP5-MS column (30 m x 0.25 mm x 0.25 µm film thickness), Agilent technologies, USA
5. Shaker, Model SK-101, HL Instrument, Thailand
6. Vacuum rotary evaporator, Model B-490, Buchi, Switzerland
7. Water purification system, Model Milli-Q system, Millipore, USA
8. Filter unit, Model 11250, Millipore, USA
9. HPLC column, Wakosil-II 5C18 HG (5 µm, 0.3 x 250 mm), Wako Pure Chemical Industries, Ltd., Japan
10. HPLC column, VertiSep UPS C18 HPLC (5 µm, 4.6 × 250 mm), Vertical Chromatography Co., Ltd., Thailand
11. HPLC guard column, Zarbax RX-C8 (5 µm, 4.6 x 12.5 mm), Agilent Technologies, USA
12. Nylon membrane filter, 0.45 µm, Filtrex Technologies, India
13. Syringe membrane fitter, 0.45 µm, SiliCycle Inc., Canada

14. High performance liquid chromatography (HPLC), (Model HP 1100, Agilent Technologies, USA) system equipped with

A. Manual sample injector

B. Quaternary pump, HP 1100

C. Degasser, HP 1100

D. UV-Visible

15. Glass syringes, 1, 2, 5, 10 µL, Hamilton, USA
16. Ultrasonicator, Model 8891, Cole Parmer, USA
17. Vacuum pump, Gast, USA
18. Microsyringe 250 µL, SGE Analytical science, UK
19. Analytical balance, Mettle Toledo, USA
20. UV-Vis CE 1021 series 1000, Cecil Instruments Ltd., UK
21. pH meter, Model 713, Metrohm, Herisau, Switzerland
22. Blender, Otto king glass, Thailand

2.1.2 Chemicals

1. Dichloromethane (CH_2Cl_2), AR grade, QRëC, New Zealand
2. Methanol (CH_3OH), AR grade, QRëC, New Zealand
3. Ethanol (C_2H_5OH), AR grade, Merck, Germany
4. Acetone (C_3H_6O), AR grade, QRëC, New Zealand
5. Acetic acid , HPLC, Fisher scientific, Canada
6. Ethyl acetate ($C_4H_8O_2$), HPLC grade, RCL Labsan, Thailand
7. Methanol, HPLC grade, RCL Labsan, Thailand
8. *Ortho*-phosphoric acid (H_3PO_4, 85%), Merck, Germany
9. Helium gas, 99.99% HP grade, TIG, Thailand
10. Standard alkanes (C_7-C_{30}), Sima-Aldirich chemie, Germany
11. DPPH (2,2-diphenyl-1-picrylhydrazyl, 98%), Sigma chemical Co., USA
12. Caffeine was separated and purified from green tea.
13. Folin-Ciocalteu's reagent, Loba Chemie, India
14. Sodium sulphate anhydrous (Na_2SO_4, min 99%), AR grade, Loba Chemie, India

15.Sodium carbonate (Na_2CO_3, min 99.9%), QRëC, New Zealand

16.Distilled water (DI water)

17.Mill-Q water

18.Ferric chloride anhydrous ($FeCl_3.6H_2O$), Unilab, Philippines

19.Potassium hexacyanoferrate (III) ($K_3[Fe(CN)_6]$, 99%), Sima-Aldirich chemie, Germany

20.Sodium dihydrogen phosphate ($NaH_2PO_4 \cdot 2H_2O$, 99%), Fisher scientific, Canada

21. Disodium hydrogen phosphate ($Na_2HPO_4 \cdot 2H_2O$), Carlo erba, France

22.Trichloroacetic acid (CCl_3COOH, TCA), Carlo erba, France

23.Gallic acid hydrate ($C_7H_6O_5 \cdot H_2O$, ≥ 98%), Sigma-Aldrich, USA

24.Ascorbic acid (Vitamin C, min 99.7%), Merck Chemical, USA

25.(−)-epicatechingallate (ECG) from green tea, ≥ 98% (HPLC), Sigma-Aldrich, USA

26. (−)-gallocatechingallate (GCG), ≥90% (HPLC), Sigma-Aldrich, USA

27.(−)-epigallocatechin (EGC), ≥90% (HPLC), Sigma-Aldrich, USA

28. (−)-Gallocatechin (GC), ≥98% (HPLC), Sigma-Aldrich, USA

29. (−)-epigallocatechingallate (EGCG) from green tea, ≥80% (HPLC), Sigma-Aldrich, USA

30. (+)-catechin hydrate (C), ≥98% (HPLC), Sigma-Aldrich, USA

31.(−)-epicatechin (EC) from green tea, ≥98% (HPLC), Sigma-Aldrich, USA

2.2 Materials

Seventeen samples from Assam tea (*Camellia sinensis* var. *assamica*), including fresh, steamed, fermented leaves (15, 30, 45, 60, 90, 120 and 150 days), steamed and fermented water (15, 30, 45, 60, 90, 120 and 150 days) were obtained from Khuntan district, Chiang Rai, Thailand during May to November in 2015. Each sample was freeze – dried for 72 hours, then the leave samples were ground in a blender and kept at 4° C before subjecting to extraction.

2.3 Extraction of volatile constituents by simultaneous distillation extraction

The extraction was carried out in a modified Likens-Nickerson simultaneous distillation and extraction (SDE) apparatus illustrated in Figure 2.1. Thirty grams of each blended sample leaf was put in 500 mL round bottom flask and then 250 mL of distilled water was added. Dichloromethane (50 mL) was added to another 250 mL round bottom flask. Both flasks were connected to the apparatus, and more dichloromethane and distilled water were added to the central arm. The flask containing dichloromethane was heated by using a heating mantle at 50 °C, whilst the flask containing sample and distilled water were heated using a heating mantle at 150 °C. The extraction was submitted to steam distillation for 5 hours. The solution of the extracted oil obtained was dried over a layer of anhydrous sodium sulphate, filtered, concentrated using a rotary evaporator and stored in a freezer prior to analysis.

Figure 2.1 A modified Likens-Nickerson apparatus

2.4 Percentage of extraction yield

The extraction yield is a measure of the solvent efficiency to extract specific components from the sample and it was defined as the amount of extract recovered in mass compared with the initial amount of dry sample. All sample extracts obtained were evaporated to dryness by a rotary evaporator to obtain crude extracts. The crude extracts were weighed, and then the extraction yield was calculated and expressed as the percentage of the weight of the crude extract to the raw material. For each dried product, the oil extraction yield (%w/w) was calculated using:

$$\text{Extraction yield}\,(\%) = \frac{\text{Weight}_{\text{extract}}\,(g)}{\text{Weight}_{\text{sample}}\,(g)} \times 100 \qquad (2.1)$$

2.5 Analysis of volatile constituents by GC-MS

The volatile constituents of samples was analyzed by GC-MS using HP5-MS column (30 m x 0.25 mm x 0.25 µm film thickness) interfaced to a mass selective detector (HP5973, Hewlett Packard). The oven temperature was held at 60°C and then increased by 2°C/min to 250°C. Helium was used as a carrier gas at the flow of 1 mL/min. An injection volume of 1 µL with split ratio of 1:5 was used. Injector and detector temperature were set at 270 and 280° C, respectively. The mass spectrometer was operated in electron impact (EI) mode with an electron energy at 70 eV. Mass range was from *m/z* 50 - 550 amu. The ion source and quadrupole temperatures were set at 230 and 150° C, respectively. The relative percentage amounts of the separated compounds were calculated on the basis of people ours.

Identification of the volatile constituent was performed by comparison of their mass spectra with those of the database using NIST 98, W8N08 and Wiley7 mass spectra libraries and these volatile constituents were confirmed by comparison of their Kovát retention indices (KI, with respect to homologous series of C_7-C_{30} *n*-alkane under the same experimental conditions). Kovát retention index was calculated as shown in equation 2.2

$$I_X = 100Z + 100\Delta Z\left(\frac{\log t_x - \log t_n}{\log t_{n+1} - \log t_n}\right) \quad (2.2)$$

Where t_n and t_{n+1} are retention times of the reference *n*-alkane hydrocarbons eluting immediately before and after chemical compound X; t_x is the retention time of compound X. Z is the number of carbon atoms of the *n*-alkane eluting before compound X. ΔZ is the deference carbon atom number of the *n*-alkane eluting before and after peak compound X. The obtained I_x was comparative to those obtained in the Adams's reference[75] which retains the I_x of most components in various essential oils.

2.6 Optimization of solvent extraction

In most of previous reports, solvent extraction has been the most commonly used method for extraction of phenolic compounds. The extraction could be improved by more polar solvents such as methanol. Binary mixture of methanol : water has been used as an extraction solvent, which has been shown to improve the extraction upon solvent acidification. In this study, thirteen solvents were used for comparison of extraction efficiency, including acetone : water (25%, 50%, 80%, and 100%), ethanol : water (25%, 50%, 80%, and 100%), methanol : water (25%, 50%, 80%, and 100%) and water. One gram of fresh leaves was extracted with 20 mL of solvent. The mixture was shaken for 2 hours. The supernatant was filtered through a filter paper (Whatman No.1) and evaporated under vacuum at 40 °C. The dried residue was redissolved in 100% methanol for antioxidant activities and HPLC analysis.

2.7 Determination of total phenolic content

2.7.1 Preparation of solutions

Sodium carbonate solution (7.5% w/v) was prepared daily by dissolving 7.5 g Na_2CO_3 in 100 mL with DI water.

Folin-Ciocalteu reagent solution (10% v/v) was diluted at a volume ratio of 1:10 with DI water.

Gallic acid as a standard solution of 1000 ppm was prepared daily by dissolving 10.0 mg in 10.00 mL of methanol and diluted to 400, 300, 200, 100, 50, 25, 13, 6 and 3 ppm, respectively

2.7.2 Protocol for total phenolic content

The total phenolic content (TPC) was estimated by the Folin-Ciocalteau method, modified as described by Kanhkonen *et.al*.[76] as follows: 300 µL of each sample extract was mixed with 1.5 mL Folin-Cicalteu's reagent and 1.2 mL of Na_2CO_3 (7.5% w/v). The tubes were then allowed to stand at room temperature for 30 min (shown in Figure 2.2) before absorbance at 765 nm was measured. The standard curve of gallic acid was prepared by 3-400 µg/mL. Total phenolic contents in the extracts were compared with standard curve of gallic acid. The results were expressed as mg gallic acid equivalents (GAE) per grams of extract.

Figure 2.2 Various concentration of gallic acid standard for TPC measurement

2.8 DPPH radical scavenging assay

2.8.1 Preparation of solutions

The DPPH stock solution was prepared by dissolving 0.0295 g of 2,2-diphenyl-1-picrylhydrazyl (1.5 mM) in methanol and made up to the final volume of 500.00 mL with methanol.

An ascorbic acid as standard solution of 1000 ppm was prepared daily by dissolving 10.0 mg of ascorbic acid in 10.00 mL of methanol and diluted to 500, 250, 125, 62.50, 31.25, 15.63 and 7.81 ppm, respectively.

Stock solution of samples was prepared daily by dissolving 10.0 mg of crude extract in 10.00 mL of methanol to give a concentration of 1000 ppm and dilute to 500, 250, 125, 62.50, 31.25, 15.63 and 7.81 ppm, respectively.

2.8.2 Protocol for DPPH radical scavenging activity

DPPH radical scavenging assay was determined according to the method of Chan *et al.*[77] with some modifications. The solution of DPPH in methanol (1.5 mM) was prepared daily before measured on a UV-Visible spectrophotometer. A 0.5 mL of various concentrations of sample extract was mixed with 1 mL DPPH solution as shown in Figure 2.3 and allowed to stand for 30 min in the dark at room temperature. The absorbance was measured at 517 nm. The antioxidant activity was calculated by the equation:

$$\%\ \text{radical scavenging} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100 \qquad (2.5)$$

Where $\text{Abs}_{\text{control}}$ is the absorbance of the control reaction (blank with 0.5 mL methanol and DPPH) and $\text{Abs}_{\text{sample}}$ is the absorbance of the sample reaction (0.5 mL sample in methanol and DPPH). The results were expressed as IC_{50} values.

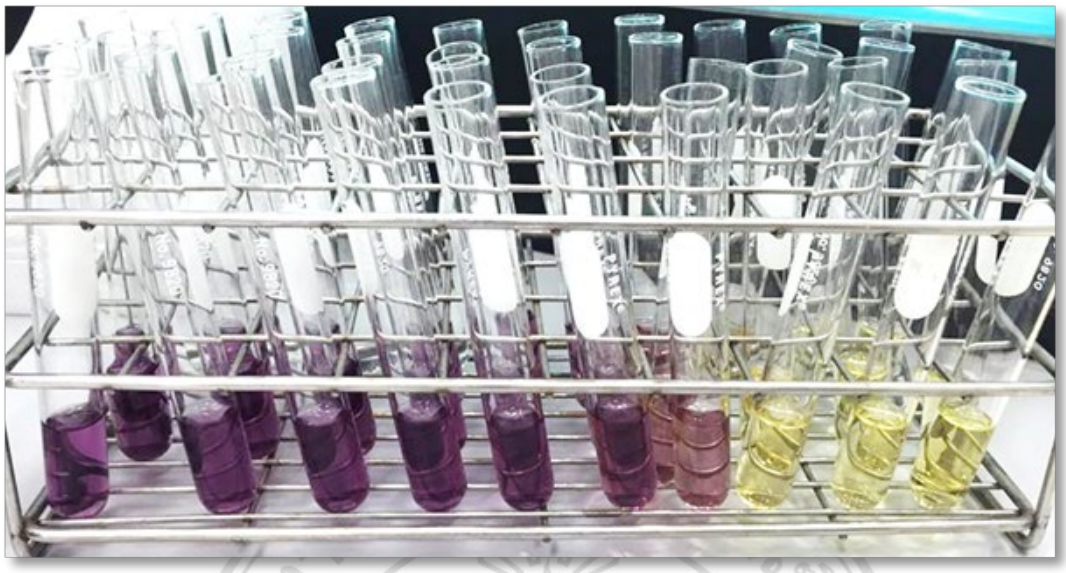

Figure 2.3 The samples at various concentrations in DPPH solution

2.9 FRAP assay

2.9.1 Preparation of reagents

To prepare the phosphate working buffer solution, 7.80 g of $NaH_2PO_4 \cdot 2H_2O$ was dissolved in water and diluted to 250 mL with DI water to give final concentration of 0.2 M: 8.89 g of $Na_2HPO_4 \cdot 2H_2O$ was dissolved in DI water and diluted to 250 mL to give its final concentration of 0.2 M. A volume of 68.5 mL of the $NaH_2PO_4 \cdot 2H_2O$ solution was mixed with 31.5 mL of the $Na_2HPO_4 \cdot 2H_2O$ solution to prepare the 0.2 M phosphate buffer at pH 6.6 using pH meter.

Potassium ferricyanide solution (1%, w/v) was prepared daily by dissolving 1.0 g $K_3Fe(CN)_6$ in 1 mL of 1 M HCl and diluted to 100 mL with DI water.

Ferric chloride solution (0.1%, w/v) was prepared daily by dissolving 0.1 g of $FeCl_3 \cdot 6H_2O$ in 1 mL of 1 M HCl and diluted to 100 mL with DI water.

Trichloroacetic acid (TCA) solution (10%, w/v) was prepared by dissolving 10.0 g of TCA in water and diluted to 100 mL with DI water.

Gallic acid as a standard solution of 1000 ppm was prepared daily by dissolving 10.0 mg in 10.00 mL of methanol and diluted to 400, 300, 200, 100, 50, 25, 13, 6 and 3 ppm, respectively.

2.9.2 Protocol for reducing power

The ferric-reducing antioxidant power (FRAP) of the extract was determined according to the method of Chu *et al.*[77] Briefly, the sample (1 mL) was mixed with 2.5 mL phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% w/v $K_3[Fe(CN)_6]$. The mixture was incubated at 50 °C for 20 min. The incubated mixture was allowed to cool at room temperature. The 2.5 mL of TCA solution (10% w/v) was added to the mixture to stop the reaction. The solution was thoroughly mixed, an aliquot of 2.5 mL was withdrawn, and 2.5 mL water followed by 0.5 mL of $FeCl_3 \cdot 6H_2O$ solution (0.1%) was added (shown in Figure 2.4). The absorbance was measured at 700 nm. The result was expressed as gallic acid equivalent (GAE) in mg/g extract. The concentrations of gallic acid was ranged from 3 – 400 µg/mL.

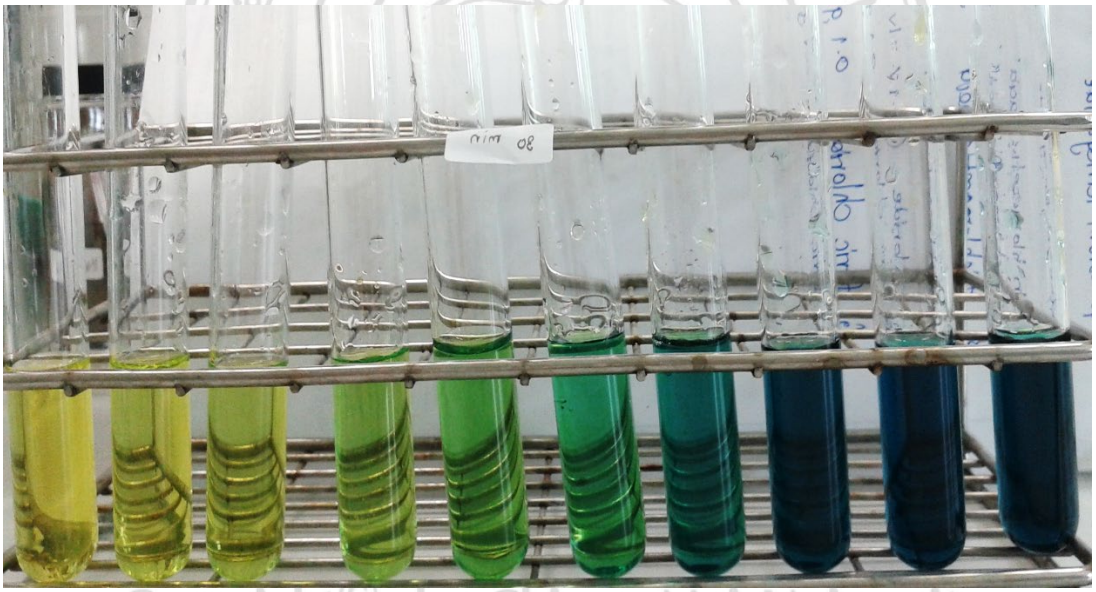

Figure 2.4 The gallic acid (standard) at various concentrations in FRAP

2.10 Quantitative analysis by HPLC

The determination of some phenolic compounds, including GA, GC, EGC, C, EC, EGCG, GCG and ECG, and caffeine in fresh, steamed, fermented leaves (25, 30, 45, 60, 90, 120 and 150 days), steamed, fermented water (25, 30, 45, 60, 90, 120 and 150 days) by HPLC was carried out as following.

2.10.1 Isolation of caffeine

The overall steps in the extraction and purification of caffeine from tea leaves are outlined in Figure 2.5 below. The tea is made under basic conditions to ionize mildly acidic flavonoids and tannins, causing them to be more water-soluble. In the liquid-liquid (DCM-water) extraction, caffeine will be separated from the water-soluble pigments. After removing trace amounts of water, the low-boiling solvent is evaporated to the crude organic extracts. Further purification by sublimation results in white crystals of caffeine.[78]

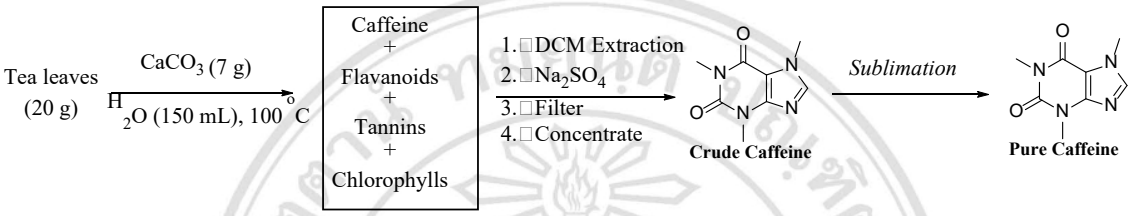

Figure 2.5 Isolation of caffeine from tea leaves

2.10.2 Preparation of standard stock solutions

Each standard stock solution (1000 ppm) was prepared by dissolving 5 mg of each of standard, including GA, C, EGCG, GCG, ECG and caffeine in 5 mL of methanol. 1 mg of EC, EGC and GC was dissolved 1 mL of methanol was added. Before use, the stock solution was further diluted to 100 ppm and desired concentrations.

2.10.3 Preparation of mobile phase

The investigation for suitable mobile phase was initiated using four types of binary solvent systems where methanol and acetonitrile were mixed with two common acid solutions, i.e. phosphoric acid and acetic acid. Common acid solutions were prepared by dissolving the appropriate volume of each acid in Milli-Q water.

For example, the preparation of 0.05% (v/v) acetic acid solution was done by adding 0.5 mL of glacial acetic acid in 1000 mL of Milli-Q water. In the case of 0.1% (v/v) phosphoric acid solution was done by adding 1.0 mL of concentrate phosphoric acid in 1000 mL of Milli-Q water.

All mobile phase solutions were filtered using a filter unit with 0.45 μm filter membrane and vacuum pump. Finally, the prepared solutions were degassed for 20 minutes using ultrasonicator.

2.10.3 Optimization of separation conditions

Sample extracts obtained from experimental 2.6.1 were used for optimization of HPLC conditions for separation individual component in the sample extract. These conditions were mobile phase composition, column, detection wavelength and gradient profile of mobile phase.

1) Column type

HPLC optimization, two columns, namely VertiSep UPS C18 and Wakosil-II 5C18 HG were used for analysis of catechins and caffeine in sample extracts under condition as follows:

HPLC conditions:

Column	1. VertiSep UPS C18 (5 µm, 4.6 × 250 mm)
	2. Wakosil-II 5C18 HG (5 µm, 0.3 x 250 mm)
Mobile phase	A: methanol
	B: 0.05% acetic acid in water

Gradient profile[79]:

Time (minute)	% A
0	3
1	3
21	50
26	55
40	95

Detector	UV (wavelength 270 nm)
Injection volume	20 µL
Flow rate	0.5 mL/min

2) Mobile phase composition

Methanol, 1% ethyl acetate in methanol and acid in water were used for optimization of mobile phase composition. The conditions of HPLC used in the experiment were as follows:

HPLC conditions:

Column	Wakosil-II 5C18 HG (5 μm, 0.3 x 250 mm)
Detector	UV (wavelength 270 nm)
Injection volume	20 μL
Flow rate	0.45 mL/min

Gradient profile:

Time (minute)	% A
0	3
1	3
21	50
26	55
40	95

Mobile phase varying mobile phase compositions in series as follows:

Serie 1	A: methanol	3 %
	B: 0.1 % phosphoric acid in water	97 %
Serie 2	A: 1 % ethyl acetate in methanol	15 %
	B: 0.1 % phosphoric acid in water	85%

3) Gradient profile of mobile phase

The gradient profiles of mobile phase were optimized for component separation. The conditions of HPLC used in the experiment were as follows:

HPLC conditions:

Column	Wakosil-II 5C18 HG (5 μm, 0.3 x 250 mm)
Mobile phase	A: 1 % ethyl acetate in methanol
	B: 0.1 % phosphoric acid in water
Detector	UV (wavelength 270 nm)
Injection volume	20 μL
Flow rate	0.45 mL/min
Gradient profile:	varying compositions (%) in batch as follows:

	Time (minute)	% A
Batch 1	0	15
	60	15
	62	30
Batch 2	0	15
	60	15
	70	30
	75	30
	80	15

4) Detection wavelength

The absorption spectra of 5 ppm of each phenolic compound solution, including GA, GC, caffeine, EGC, C, EC, EGCG, GCG and ECG were examined in the wavelength range between 200 to 600 nm using a UV-VIS spectrophotometer. Maximum absorbance values were determined by injecting mixed standard into HPLC at different wavelengths (270 and 280 nm). The conditions of HPLC used in the experiment were as follows:

HPLC conditions:

Column	Wakosil-II 5C18 HG (5 μm, 0.3 x 250 mm)
Mobile phase	A: 1 % ethyl acetate in methanol
	B: 0.1 % phosphoric acid in water

Gradient profile:

Time (minute)	% A
0	15
60	15
70	30
75	30
80	15

Detector	UV varying wavelength 270 and 280 nm
Injection volume	20 μL
Flow rate	0.45 mL/min

2.10.4 Method validation of HPLC analysis

1) Detection limit

The standard solutions were prepared as shown Table 2.1 and 20 μL of each standard mixture was injected onto the HPLC column under the optimum condition. The detection limits at each compound can be calculated by the method reported by Miller and Miller, which was calculated from the linear regression line of the calibration curve.

Table 2.1 Concentration of standard mixtures for determination of detection limit

Standards	Concentration (ppm)			
	1	2	3	4
GA	0.60	0.90	1.20	1.50
GC	5.00	10.00	15.00	20.00
caffeine	0.10	0.15	0.20	0.25
EGC	1.60	2.40	3.20	4.00
C	1.00	1.50	2.00	2.50
EC	1.40	2.10	2.80	3.50
EGCG	1.20	1.80	2.40	3.00
GCG	1.30	1.95	2.60	3.25
ECG	0.90	1.35	1.80	2.25

2) Precision

The precision of a method is the degree of closeness of the results which is usually reported as a percent of relative standard deviation. The precision can be expressed as the standard deviation (SD) and the relative standard deviation (RSD), which can be calculated from the following equation.

$$\%\text{RSD} = \frac{\text{SD}}{\overline{\text{X}}} \times 100 \qquad (2.3)$$

Where %RSD = persent of relative standard deviation

SD = standard deviation

$\overline{\text{X}}$ = mean measured value

SD can be calculated from this equation

$$SD = \sqrt{\sum_{i=1}^{n} \frac{\left(x_i - \overline{X}\right)}{n-1}} \quad (2.4)$$

Where x_i = individual measured value

n = number of measurements

The standard mixture solution consisted of 5, 10, 2, 10, 10, 15, 10, 20 and 5 ppm for GA, GC, Caf, EGC, C, EC, EGCG, GCG and ECG, respectively. The repeatability was investigated using five injections of the standard mixture in the same day and the reproducibility was determined in different days for five injections under the optimum conditions.

3) Percent recovery

Recovery is the fraction of the analyte determined in blank test sample after spiking with a known quantity of the analyte. The percent recovery was calculated based on the external calibration curve and peak area. It could be calculated from the following equation.

$$\%\,Recovery = \left(\frac{C_{sample+added} - C_{sample}}{C_{added}} \right) \times 100 \quad (2.5)$$

Where C_{sample} = concentration of standard compound in sample

$C_{sample + added}$ = concentration of sample solution when added standard solution

C_{added} = concentration of standard solution

The sample of extraction was investigated by spiking mixed standard at concentrations 5LOQ and 10LOQ (shown in Table 2.2) into sample.

Table 2.2 Concentration of standard mixtures for determination of % recovery

Standards	Concentration (ppm)	
	5LOQ	10LOQ
GA	1.60	3.20
GC	40.95	81.90
caffeine	0.65	1.30
EGC	12.45	24.90
C	5.15	10.30
EC	8.25	16.50
EGCG	7.80	15.60
GCG	10.80	21.60
ECG	3.50	7.00

2.10.5 Calibration curves

The external standard was employed for the purpose of quantifying the sample. Calibration curves for each sample at concentrations of an appropriate range were constructed by plotting the peak areas obtained from standard mixture chromatograms against the corresponding concentrations.

2.10.6 Determination of phenolic compounds and caffeine in sample.

The sample extracts were decreases caffeine with CH_2Cl_2 by separatory funnel. The solute in aqueous phase concentrated using a rotary evaporator and dissolved with methanol. The solutions were filtered through a syringe membrane fitter 0.45 μm and analyzed under the optimum HPLC condition.

2.11 Statistical analysis

The results were expressed as the mean values ± standard deviation (SD) of triplicate determinations by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. Correlations between antioxidant activities and total phenolic contents were calculated using Pearson's correlation coefficient (r^2) at a significant level of 99% ($P<0.01$).