CHAPTER III

MATERIALS AND METHODS

Source and Authentication of the Plant Materials

The rhizomes of *K. parviflora* were obtained from Chiangdao District, Chiangmai province in February, 2005. The herbarium voucher specimens (No. 009724) were identified and deposited at Herbarium of Faculty of Pharmacy, Chaingmai University, Chiangmai, Thailand.

General Techniques

1. Solvents

Commercial grade organic solvents were redistilled prior to use for extraction, as eluents for thin layer chromatography and column chromatography. Organic solvents used for antioxidant activity assessment were analytical grade including TPTZ (2,4,6-tripyridyl-s-triazine), ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), were purchased from Sigma-Aldrich Chemical Co. (St. Louis, USA). Ferric Chloride ($FeCl_3.6H_2O$), Potassium persulfate ($K_2S_2O_8$) were obtained from UNILAB (AU). Acetic acid (glacial, p.a.), Sodium acetate were purchased from BDH Chemical Ltd. (UK). Absolute ethanol (99%) was purchased from Merck (Damstadt, Germany). Ascorbic acid was purchased from Fisher Scientific (UK). Quercetin was purchased from ACROS (New Jersey, USA). Ferrous sulfate ($FeSO_4.7H_20$) was obtained from and all other reagents used were of analytical grade. Methanol, HPLC grade obtained from Fisher (Italy) was used for High Performance Liquid Chromatography.

2. Chromatography

2.1 Analytical Thin Layer Chromatography: Silica gel 60GF precoated aluminium plate (0.25 mm) and silica gel RP18 F-254 precoated aluminium plate (E. Merck) were used. Detection of spots was carried out by irradiation of UV lamp (254 and 365 nm).

2.2 Column Chromatography: Open Column Chromatography was carried out using Silica gel 60G (E. Merck), Silica gel 60GF (E. Merck) for Quick column chromatography, Sephadex LH-20 (E. Merck, Damstadt, Germany) for Gel Filtration Chromatography. Reverse Phase Column Chromatography was carried out with Peristatic pump (Pharmacia Fine Chemical, Sweden) and Rheodyne injector (7125, California, USA). Chromatographic separation was performed using Lichroprep® RP-18 lobar column, 40-63 μm, 240 x 10 mm (E. Merck, Damstadt, Germany).

2.3 Liquid Chromatography-Mass Spectrometry: Liquid Chromatography-Mass Spectrometry (LC-MS) was performed on Shimazu LC-MS. Chromatographic separation was achieved using Nova-Pak® C_{18} column, 3.9 x 390 mm (Waters, USA) using MeOH : H_2O : CH_3COOH (80:20:1) as mobile phase. The mass detector was ion-trap mass spectrometer equipped with and electrospray ionization (ESI) system. The heated capillary and voltage were maintained at 350 °c and 4Kv, respectively. The nebulizer pressure and flow rate of nitrogen were 65.0 psi and 111 / min, respectively. Mass scan (MS) was measured from 100 au to m/z 1500 collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas, with voltage ramping cycles from 0.3 up to 2 V. Mass spectrometry data were acquired in positive modes.

3. Identification

3.1 **Melting point:** Melting point of the isolated compounds were determined by Electrothermal SMP3 melting point apparatus, Stuart Scientific, UK. The temperatures were recorded in degree celcius.

3.2 **Spectroscopy**

3.2.1 **Mass spectra (MS):** The electron impact mass spectra (EIMS) of the isolated compounds were obtained on a JEOL JMS-HX110 spectrophotometer.

3.2.2 **Ultraviolet absorption spectra (UV):** The UV-spectra were measured on the UV-Visible Spectrophotometer model SPECAL-1200, Jena Analytic, Germany.

3.2.3 **Infrared absorption spectra (IR):** The IR-spectra were obtained by a NEXUS-465, Thermo Nicolet Electron Corporation FT-IR spectrophotometer. Solid samples were examined as potassium bromide disc (KBr).

3.2.4 **Proton and Carbon nuclear magnetic resonance spectra ^{1}H-NMR and ^{13}C-NMR** : ^{1}H-NMR and ^{13}C-NMR spectra were measured on INOVA-500 MHz spectrometer; 500 MHz for ^{1}H-NMR and 125 MHz ^{13}C-NMR in deuterated chloroform ($CDCl_3$). The chemical shifts were record in ppm with reference to TMS signal.

4. Antioxidant activity Assessment

The determination of antioxidant activity of crude extracts and isolated compounds were performed by methods as following:

4.1. Free Radical Decolorization Assay by ABTS

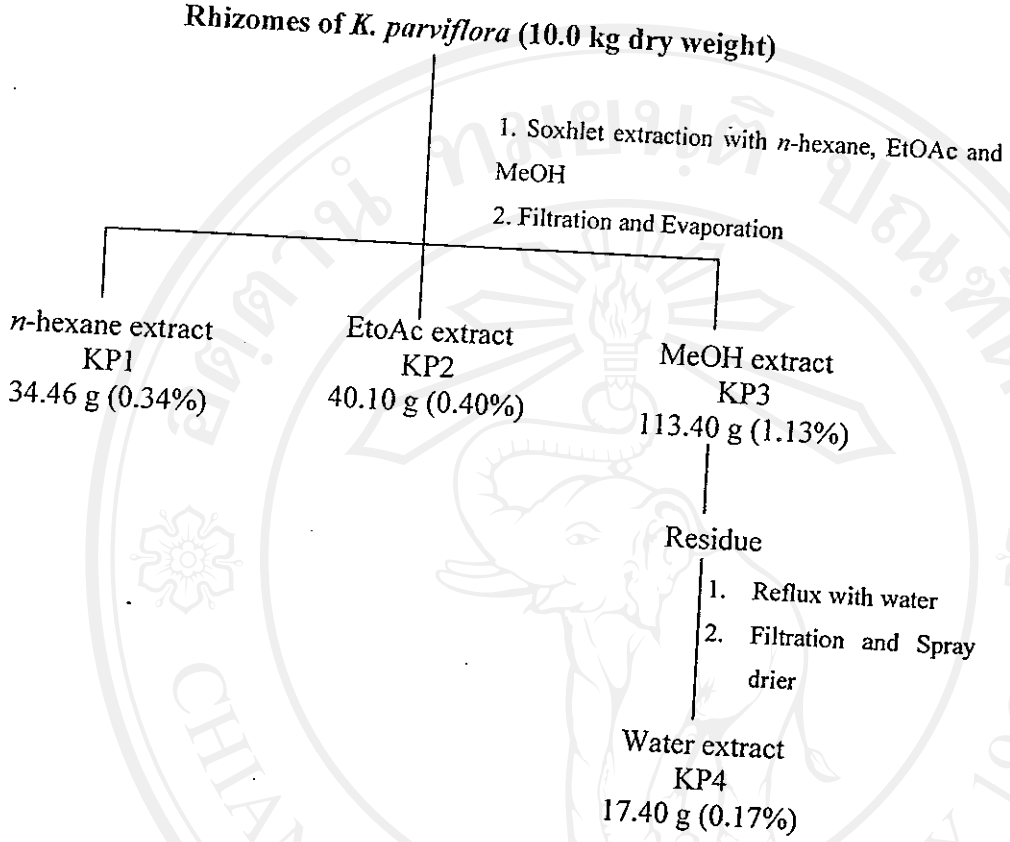
The determination of antioxidant activity by ABTS (2,2-azino-bis (3-ethyl-benz-thiazoline-6-sulfonic acid) assay was adapted from Miller et al. (1993). ABTS was dissolved in water to a 7 mM concentration and then ABTS cation radical($ABTS^{O+}$) was produced by reaction ABTS stock solution with 2.45 mM potassium persulfate (ratio 1 : 0.5 mole/mole) and allowing the mixture stand in dark at room temperature for 12-16 hrs. before use. The solution was diluted in water to give an absorbance at 734 nm approximately 0.700 (0.700-0.900). Then 2 ml of $ABTS^{O+}$ solution was added to 20 µl of standard (quercetin, ascorbic acid and pyrogallol) or sample and adjusted to 2.10 ml by deionized water and then vortexed. The test sample and reagent blank were incubated in dark at room temperature for 4 mins. At the end of incubation, the absorbance readings were taken immediately at 734 nm, using spectrophotometer (Genesys 2, USA). Quercetin, pyrogallol and ascorbic acid were used to prepare standard solution. Relative activities were calculated from the standard curve of standard solution under the same experiment conditions and expressed as mg standard per gram dry weight materials. All measurements were done in triplicate.

4.2 Ferric Reducing Ability Power Assay

The assay used was the FRAP procedure (58), the FRAP reagent was made by follows: 25 ml of 0.1 mol/L acetate buffer (pH 3.6) were mixed with 2.5 ml of 10 mol\L TPTZ and 2.5 ml of 20 mmol\L $FeCl_3$ (ratio 100 : 10 : 10 v/v). FRAP reagent, freshly prepared, 600 µl was mixed with 80 µl of deionized water and 20 µl of test sample or absolute ethanol. The test sample and reagent blank were incubated in dark at room temperature for 4 mins. At the end of incubation, the absorbance reading were taken immediately at 593 nm, using spectrophotometer (Genesys 2, USA). Aqueous solution of known Fe(II) concentration, ranging from 50 to 1000 µmol/L ($Fe_2SO_4.7H_20$) was used for the preparation of standard solution. Relative activities were calculated from the standard curve of standard solution under the same experimental conditions and expressed as mg standard per gram dry weight materials. All measurements were done in triplicate.

5. Extraction

Fresh rhizomes of *K. parviflora* (70.0 kg) were washed with running tap water before being chopped into pieces. They were oven-dried at 50 °c until dry and ground to powder then stored in high density plastic bags until used. The dried powder of tuberous roots (10.0 kg) was extracted exhaustively with *n*-hexane (5 liters), ethyl acetate (5 liters) and methanol (10 liters) by soxhlet apparatus, respectively. Each filtrate was evaporated under reduce pressure to give *n*-hexane extract (34.46 g, 0.34%), ethyl acetate extract (40.10 g, 0.40%) and methanolic extract (113.40 g, 1.13%), respectively. The residue of methanol extract was heated under reflux for 2 hours. The filtrate was removed by using spray-dried technique to yield 17.40 g, 0.17%. Then, each crude extract was evaluated antioxidant activity. The extraction sequence was shown in scheme 3.1.

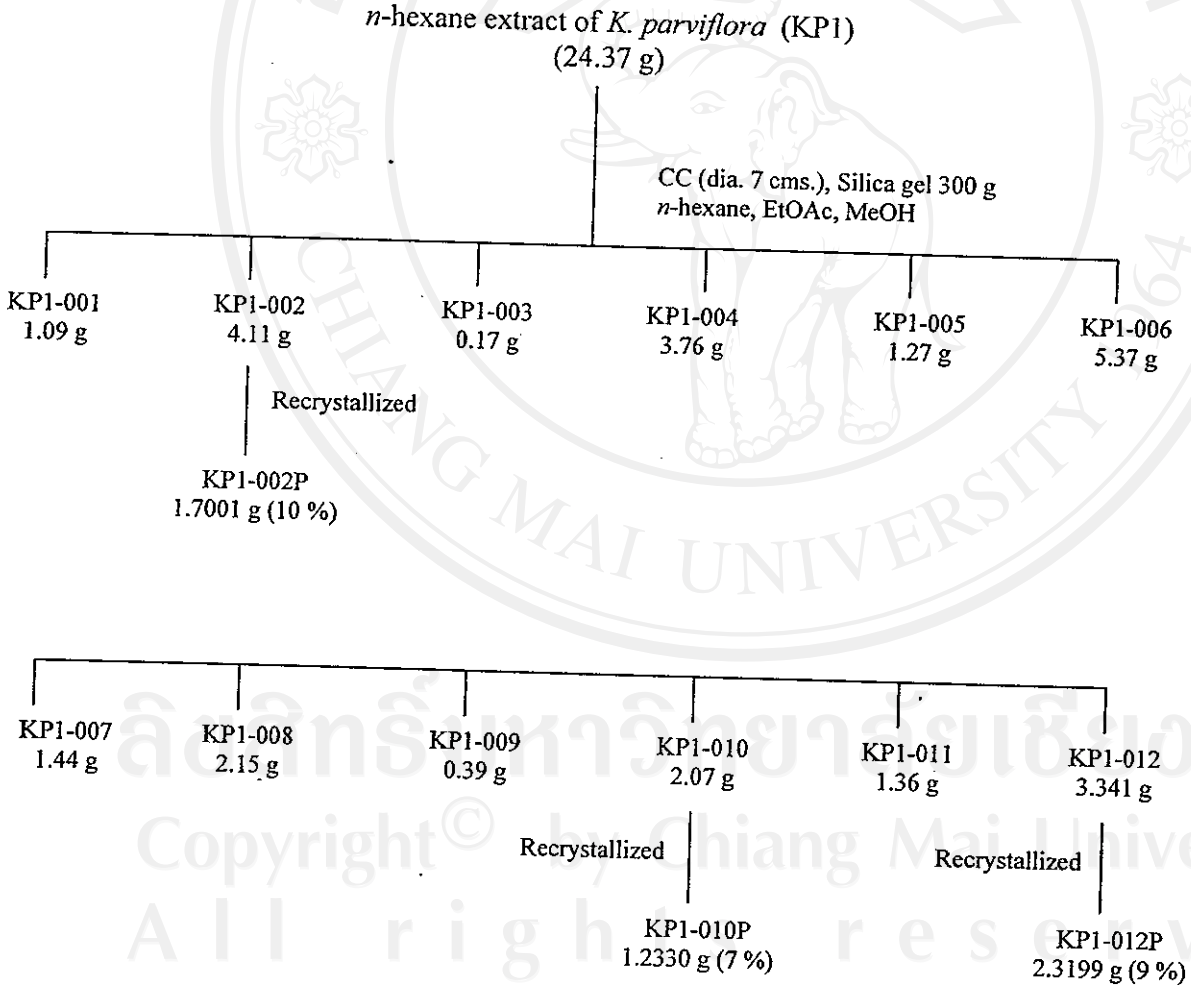


Scheme 3.1 Extraction of Rhizomes of *K. parviflora*

All crude extracts of *K. parviflora* were subjected to phytochemical screening test (52-53). The results revealed that *n*-hexane and ethyl acetate extracts supposed to consist of flavones whereas anthocyanin, flavones and other phenolic compounds were detected in methanol extract. Then, each extract was evaluated for antioxidant activity. The methanol extract gave the highest activity followed by ethyl acetate, *n*-hexane and water extract, respectively. The *n*-hexane extract was selected first to isolate for studying an antioxidant activity.

6. Isolation the *n*-hexane extract

The *n*-hexane extract, KP1 (24.37 g), was initiated by open column chromatography (diameter 7 cms) on Silica gel 60 (300 g) using the following solvents system of *n*-hexane, ethyl acetate and methanol. Ninety fractions of eluate (200 ml each) were collected and evaluated by TLC analysis patterns giving 12 fractions, KP1-001 to KP1-012.



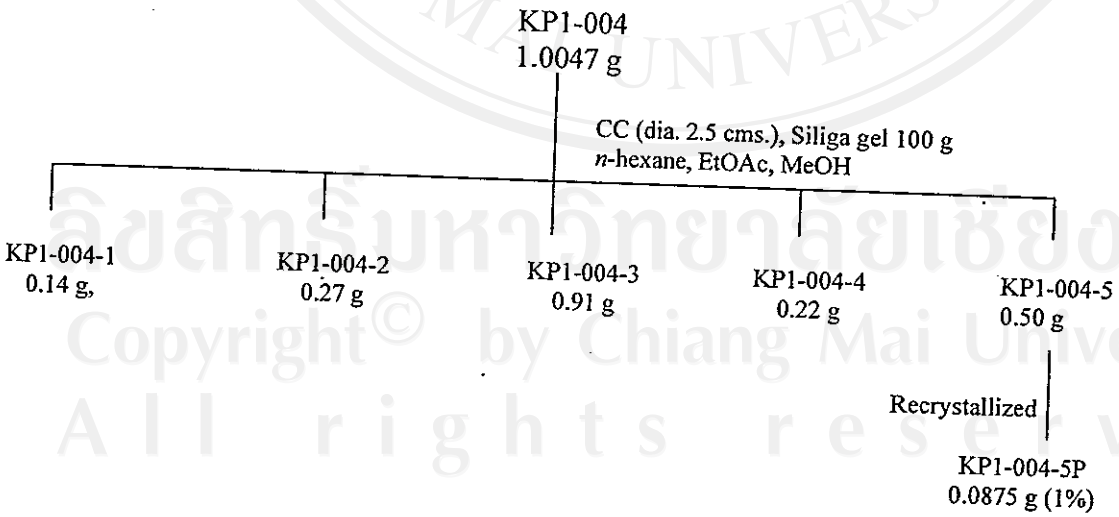
Scheme 3.2 Isolation scheme of *n*-hexane extract of *K. parviflora* (KP1)

Isolation of KP1-002P

A portion of fraction KP1-002 (2.6363 g) was further purified by crystallization to yield yellow needle 1.7001 g (10% by weight based on *n*-hexane extract), mp 167.6-168.2 °c.

Isolation of KP1-004-5P

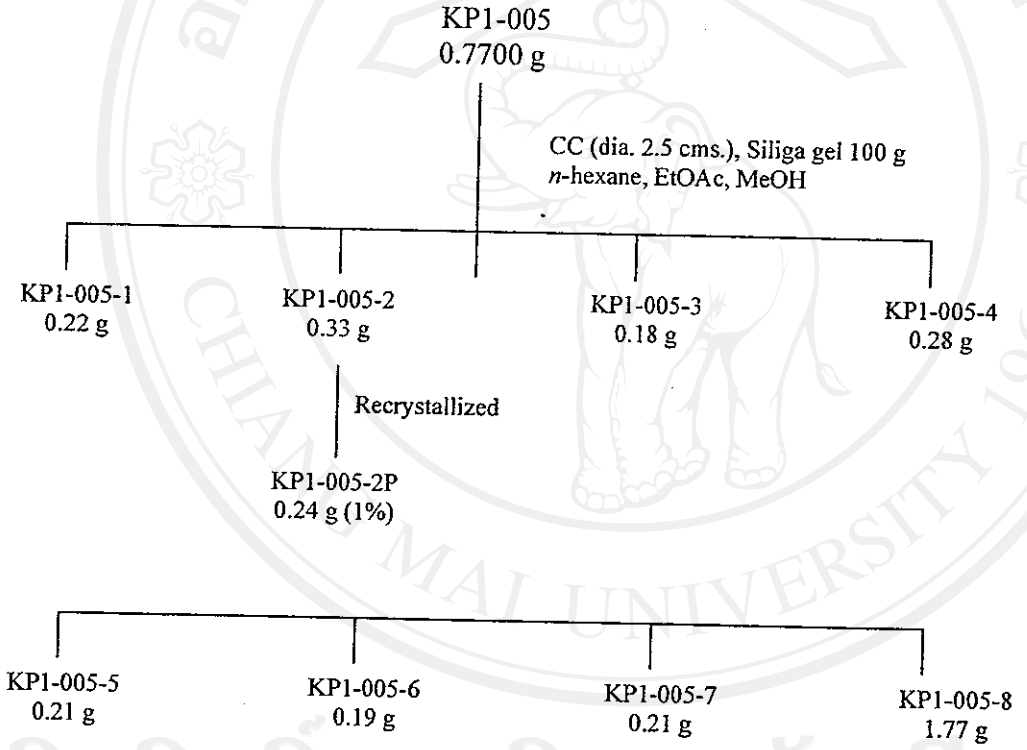
The fraction KP1-004 (1.0047 g) was repeatedly purified by Silica gel 60 (100 g) eluting with *n*-hexane, ethyl acetate and methanol, respectively. Each 25 ml fraction was collected and combined according to their TLC patterns giving 5 fractions, KP1-004-1 to KP1-004-5 as shown in scheme 3.2. Among these, a portion of KP1-004-5 was recrystallized with *n*-hexane and ethyl acetate to obtain KP1-004-5P 0.0875 g (1% by weight based on *n*-hexane extract) as yellow needle, mp 145.4-145.6°c.



Scheme 3.3 Isolation scheme of compound KP1-004-5P

Isolation of KP1-005-2P

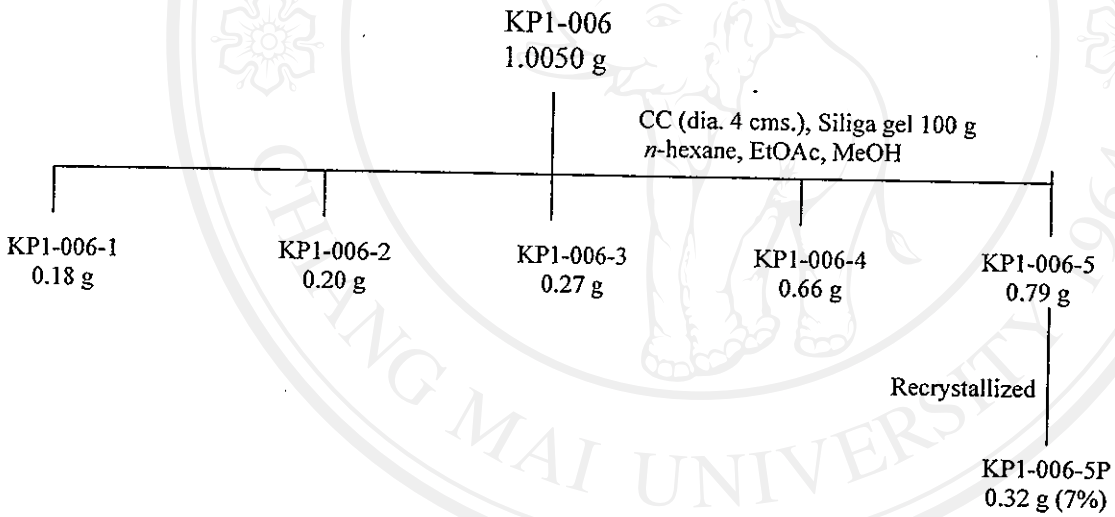
The fraction KP1-005 (0.7700 g) was rechromatographed with silica gel to give 8 fractions, KP1-005-1 to KP1-005-8, as shown in scheme 3.3. Among these, a portion of KP1-005-2 was further recrystallized with *n*-hexane and ethyl acetate to obtain KP1-005-2P 0.2350 g (1% by weight based on *n*-hexane extract) as yellow needle, mp. 166.0-167.2 °c.



Scheme 3.4 Isolation scheme of compound KP1-005-2P

Isolation of KP1-006-5P

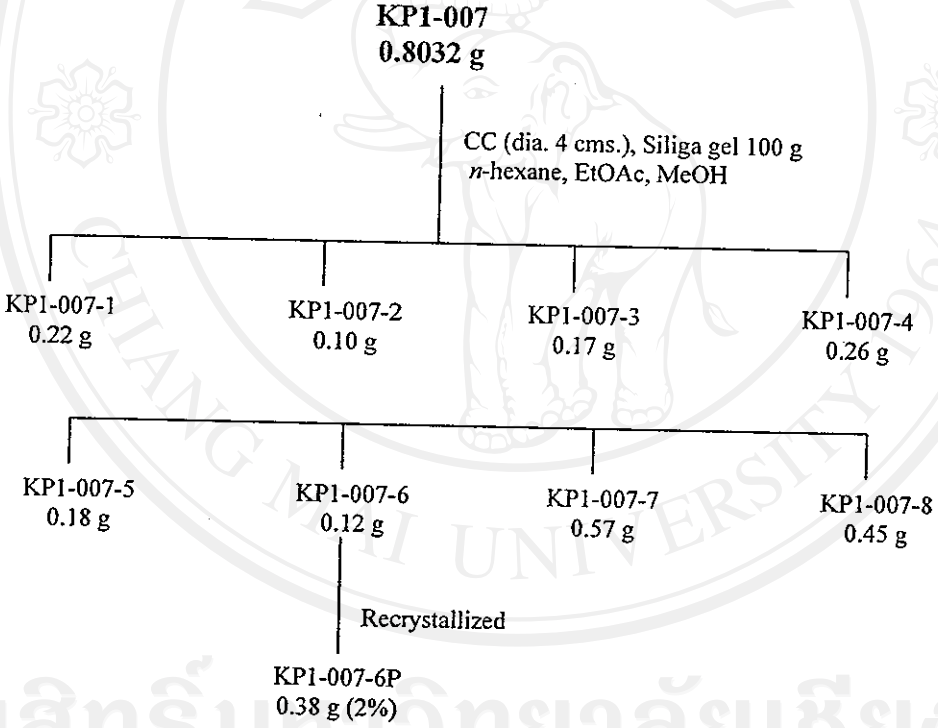
The fraction KP1-006 (1.0050 g) was isolated with Silica gel 60 column chromatography using *n*-hexane, ethyl acetate and methanol as eluents. Each 50 ml fraction was collected and combined according to their TLC patterns giving 5 fractions, KP1-006-1 to KP1-006-5. Then a portion of KP1-006-5 was further recrystallized in *n*-hexane and ethyl acetate to obtain KP1-006-5P, 1.3166 g (7% by weight based on *n*-hexane extract) as yellow needles, mp. 136.3-137.2 °c as shown in scheme 3.5.



Scheme 3.5 Isolation scheme of compound KP1-006-5P

Isolation of KP1-007-6P

A portion of fraction KP1-007 (0.8032 g) was fractionated by Silica gel 60 column chromatography as shown in scheme 3.6. Each 25 ml fraction was collected and combined according to their TLC patterns giving 8 fractions, KP1-007-1 to KP1-007-8. A portion of KP1-007-6 was further recrystallized in *n*-hexane and ethyl acetate to obtain KP1-007-6P 0.38 g (2% by weight based on *n*-hexane extract) as yellow needles, mp 168.3-169.6 °c as shown in scheme 3.6.



Scheme 3.6 Isolation scheme of compound KP1-007-6P

Isolation of KP1-010P

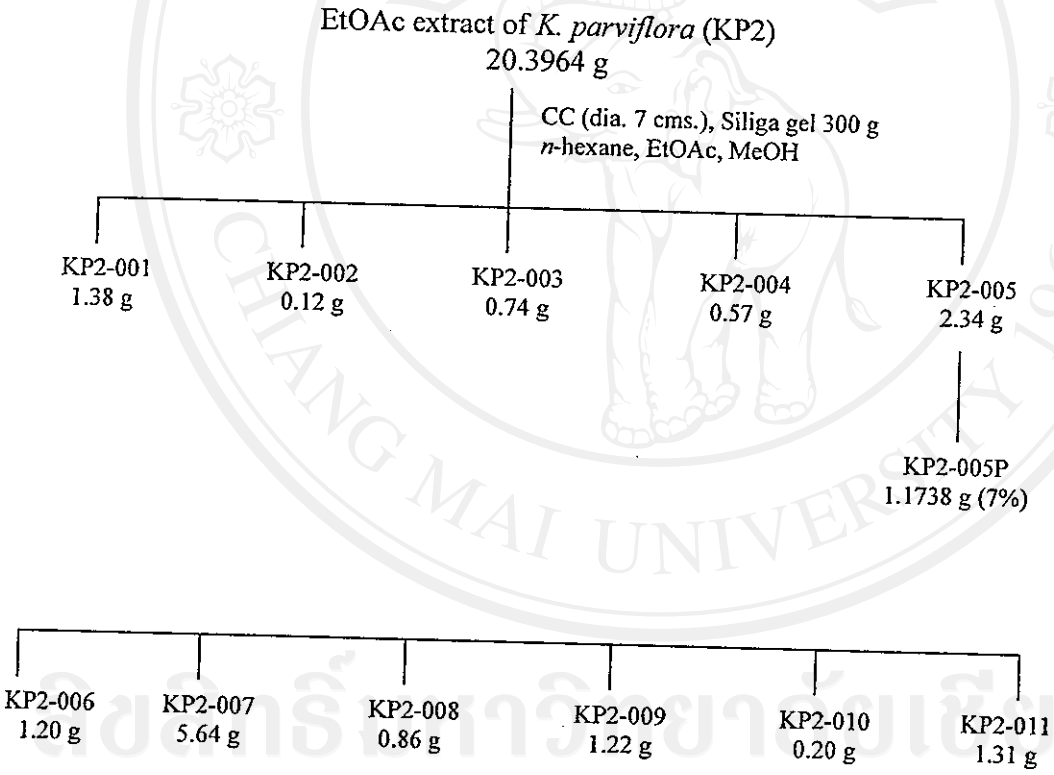
A portion of fraction KP1-010 was recrystallized in ethyl acetate giving pale yellow crystals of the compound KP1-010P 1.2330 g (7% by weight based on *n*-hexane extract), mp 144.4-145.4 °c.

Isolation of KP1-012P

Fraction KP1-012 was dissolved in ethyl acetate-chloroform. The mother liquor was removed and the precipitate then purified by crystallization in ethyl acetate-chloroform to yield a colorless crystal of KP1-012P, 2.3199 g (9% by weight based on *n*-hexane extract), mp. 200.5-200.6 °c.

7. Isolation the Ethyl acetate extract

The ethyl acetate extract, KP2 (20.3964 g), was fractionated on column chromatography of Silica gel 60 (300 g), with diameter 7 cms, using *n*-hexane, ethyl acetate and methanol as eluting solvent. Thirty seven fractions (200 ml each) were obtained and combined according to their TLC analysis patterns giving 11 fractions, KP2-001 to KP2-011.



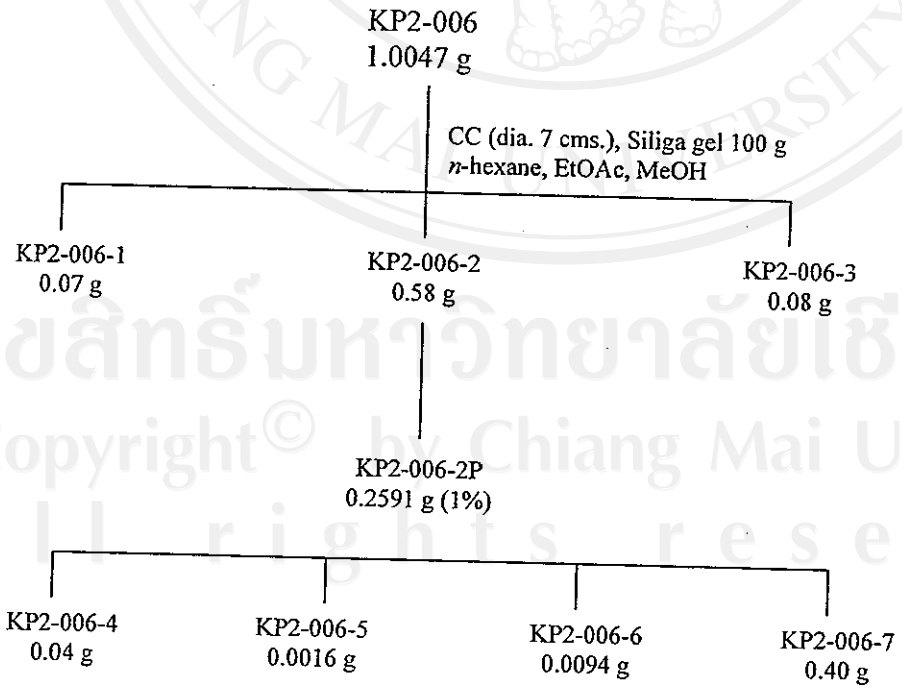
Scheme 3.7 Isolation scheme of Ethyl acetate extract of *K. parviflora* (KP2)

Isolation of KP2-005P

A portion of fraction of KP2-005 was recrystallized with a mixture of ethyl acetate and chloroform to obtained white crystal of KP2-005P (1.1738 g, 7% by weight based on ethyl acetate extract), mp 147.9-150.5 °c.

Isolation of KP2-006-2P

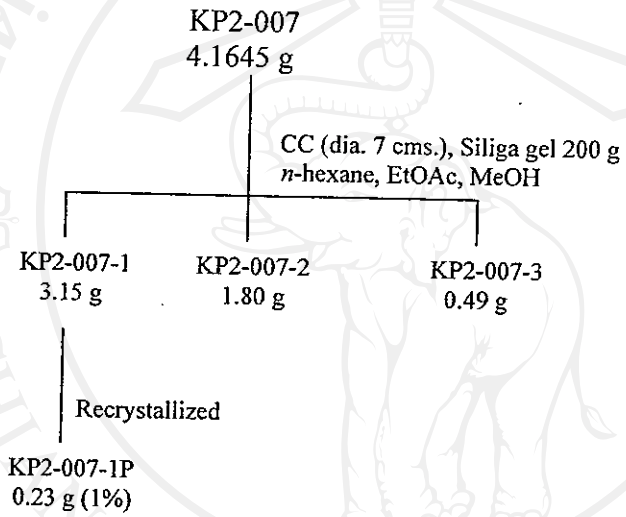
The fraction KP2-006 (1.0047 g) was rechromatographed with Silica gel 60 (100 g, diameter 3 cms) column chromatography, using ethyl acetate, ethanol and methanol as eluents. Each 50 ml fraction was collected and combined according to their TLC patterns giving 7 fractions, KP2-006-1 to KP2-006-7. Then KP2-006-2 was further recrystallized obtaining a white crystal of KP2-006-2P, 0.2591 g (1% by weight based on ethyl acetate extract), as presented in scheme 3.8.



Scheme 3.8 Isolation scheme of compound KP2-006-2P

Isolation of KP2-007-1P

A portion of fraction KP2-007(4.1645 g) was fractionated by column chromatography of Silica gel 60, diameter 4 cms. Each 50 ml fraction was collected and combined according to their TLC patterns giving 3 fractions, KP2-007-1 to KP2-007-3.

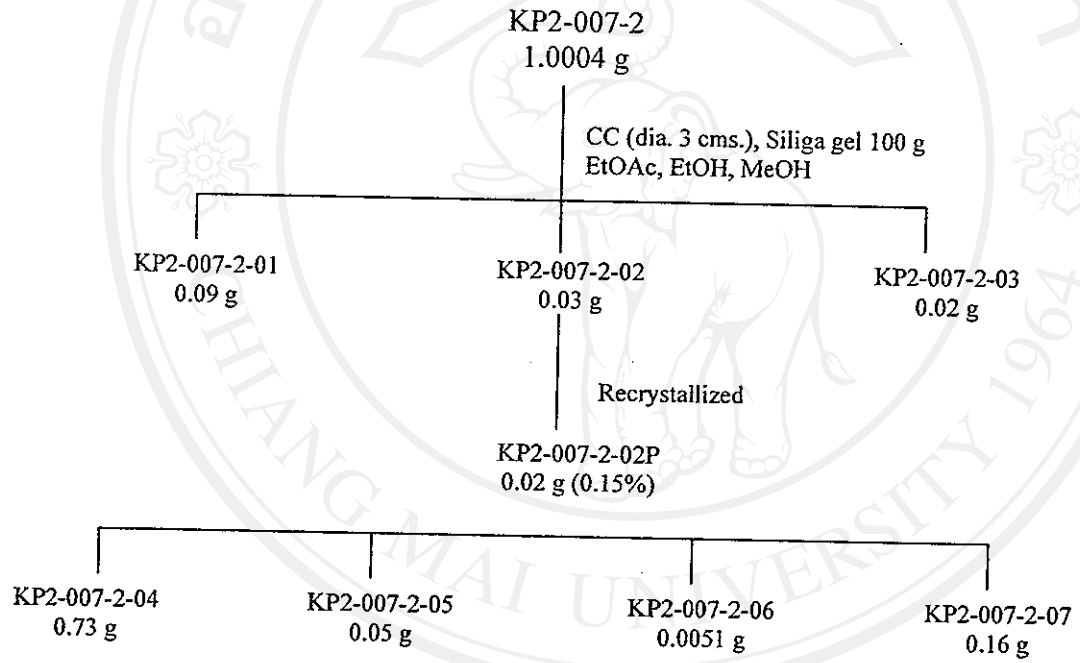


Scheme 3.9 Isolation scheme of compound KP2-007-1P

Compound KP2-007-1 was further recrystallized in ethyl acetate to yield KP2-007-1P (0.23 g, 1% by weight based on ethyl acetate extract) mp 147.9-150.5 °c, as pale yellow needles as shown in scheme 3.9.

Isolation of KP2-007-2-02P

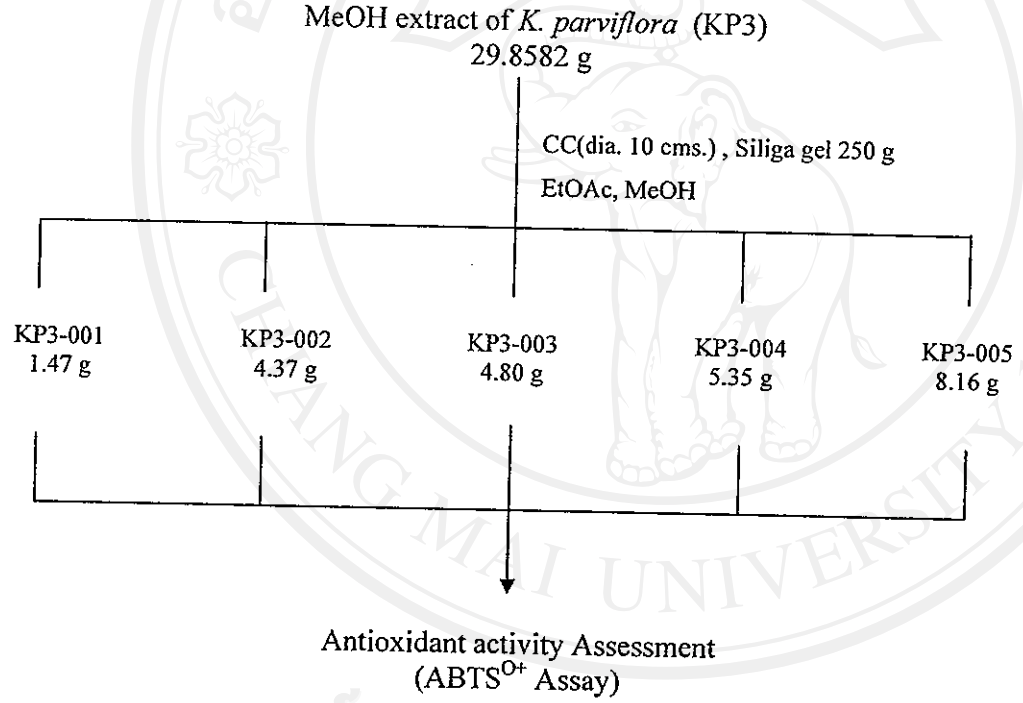
A portion of the fraction KP2-007-2, 1.0004 g, was further fractionated by using column chromatography of Silica gel 60, diameter 3 cms. Each 50 ml fraction was collected and combined according to their TLC patterns giving 7 fractions, KP2-007-2-01 to KP2-007-2-07. Compound KP2-007-2-02P was recrystallized with *n*-hexane-ethyl acetate and gave a pale yellow needle as shown in scheme 3.10.



Scheme 3.10 Isolation scheme of compound KP2-007-2-02P

3. Isolation the Methanol extract

The methanol extract, KP3 (29.8582 g), was fractionated on quick column chromatography of Silica gel 60G (250 g, diameter 10 cms) using ethyl acetate, ethanol and methanol as eluting solvent. Twenty five fractions (200 ml each) were obtained and combined according to their TLC analysis patterns giving 5 fractions, KP3-001 - KP3-005, as performed in scheme 3.11.



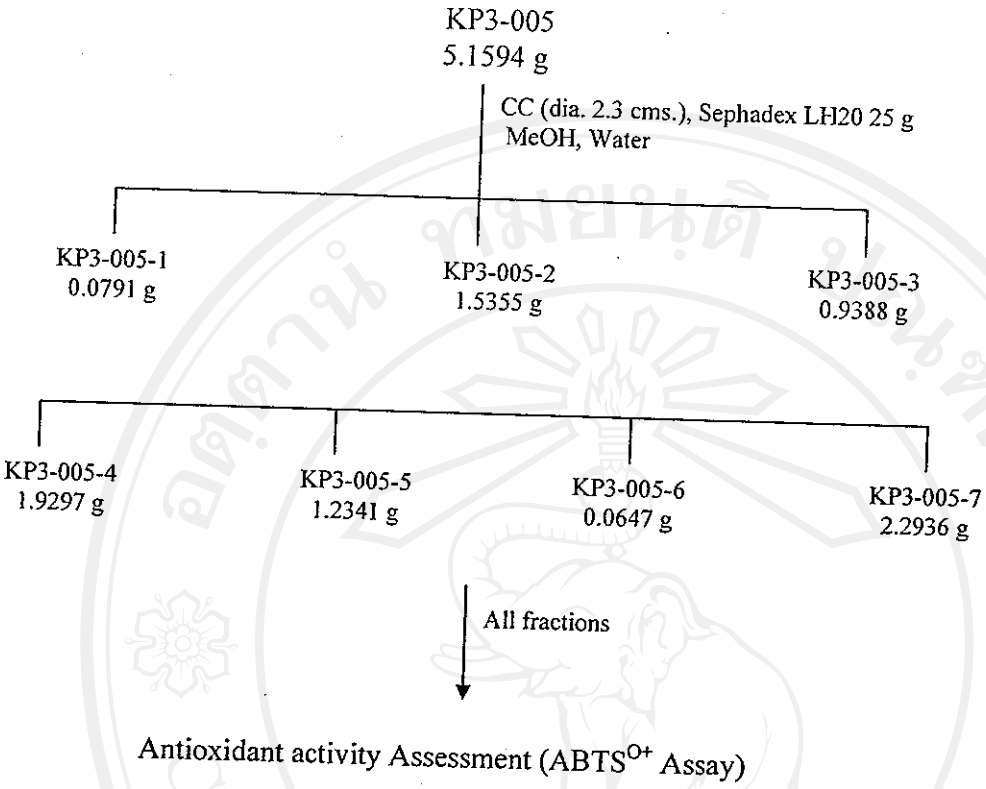
Scheme 3.11 Isolation scheme of Methanol extract of *K. parviflora* (KP3)

Then fraction KP3-001 to KP3-005 were assessed for antioxidant activity by $ABTS^{O+}$ assay (as mentioned in chapter II). The results of antioxidant activity was shown in Table 3.1

Table. 3.1 Antioxidant activity of fraction KP3-001 to KP3-005 by $ABTS^{O+}$ assay

Fraction	% Inhibition	Scavenging activity in mg ascorbic acid /g sample
KP-MeOH	74.08	2.280
KP3-001	29.40	0.808
KP3-002	47.63	1.493
KP3-003	45.22	1.351
KP3-004	45.68	1.318
KP3-005	53.10	1.635

As a result, fraction KP3-005 gave the strongest activity. So, it was selected first to isolate. A portion of KP3-005 (5.1594 g) was dissolved in a small volume of methanol. The mixture was fractioned on Gel Filtration Chromatography of Sephadex LH-20, eluting with gradient of methanol-water. Fourteenth fractions were obtained and combined according to their TLC analysis patterns giving 7 fractions, KP3-005-1 to KP3-005-7, as shown in scheme 3.12.



Scheme 3.12 Isolation scheme of compound KP3-005 from Methanol extract

Then, all of fractions, KP3-005-1 to KP3-005-7, were assessed for antioxidant activity by $ABTS^{O+}$ assay, the results as shown below.

Table 3.2 Antioxidant activity of KP3-005-1 to KP3-005-7 by $ABTS^{O+}$ assay

Fraction	% Inhibition	Scavenging activity in mg ascorbic acid /g sample
KP3-005	43.24	2.095
KP3-005-1	83.60	3.570
KP3-005-2	62.03	2.558
KP3-005-3	41.62	1.600
KP3-005-4	37.93	1.426
KP3-005-5	55.45	2.142
KP3-005-6	66.89	2.786
KP3-005-7	54.51	2.106

According to Table 3.2, fraction KP3-005-1 gave the highest activity but the yield of it was too low for further study. Therefore, fraction KP3-005-2 which exhibited high activity was selected to isolate. KP3-005-2 (1.0106 g) was dissolved in methanol and rechromatographed with Reverse Phase Column Chromatography (RP-CC) analysis using Lichroprep® RP-18 lobar column. Chromatographic separation was achieved under isocratic condition using MeOH : H_2O : CH_3COOH (80:20:1) as mobile phase with flow rate 1.5 ml/min.

Five fractions (KP3-005-2-01 to KP3-005-2-05) were obtained with limited amount. Trial of fraction KP3-005-2-02 was performed under Liquid Chromatography-Mass Spectrometry (LC-MS) using Nova-Pak® C_{18} column. Chromatographic separation was performed under the isocratic condition using MeOH : H_2O : CH_3COOH (80:20:1) as mobile phase with flow rate 0.5 ml/min. Chromatogram were recorded at 254 nm. The mass detector was ion-trap mass spectrometer equipped with and electrospray ionization (ESI) system.