CHAPTER IV

RESULTS AND DISCUSSION

The dried powder of rhizomes (10.0 kg) was exhaustively extracted for 10 hours by soxhlet apparatus using *n*-hexane, ethyl acetate and methanol, respectively. The extract solutions were filtered and then removed the solvents by evaporation to yield the extracts as shown in Table 4.1. The water extract was obtained by reflux method and removed the solvent by freeze-dryer.

Table 4.1 The amount and percentage yield of extracts from *K. parviflora*

Fraction	**Weight (g)**	**% yield ***
n-hexane extract	34.46	0.33
Ethyl acetate extract	40.10	0.40
Methanol extract	113.40	1.13
Water extract	17.40	0.17

* the percentage yield was calculated on fresh weight basis

The extraction dependent upon the polarity of solvents (*n*-hexane, ethyl acetate, methanol and water) were assessed for antioxidant activity using the modified ABTS radical cation decolourization assay (ref 72-73, 75), the results are shown as following:

Table 4.2 The antioxidant activity of crude extracts of *K. parviflora*

Fraction	**Code**	**ABTS scavenging activity, %**
n-hexane extract	KP1	38.437
Ethyl acetate extract	KP2	74.164
Methanol extract	KP3	93.993
Water extract	KP4	29.577

From Table 4.2, the results showed that the highest antioxidant activity was found in methanol extract followed by ethyl acetate, *n*-hexane and water extract, respectively. On our preliminary study, the chemical compositions from *n*-hexane and ethyl acetate extract were found to be flavones whereas anthocyanin, flavones and other phenolic compounds were detected in alcohol extract. Each extracts was separated by chromatographic techniques and then obtained fractions were examined antioxidant activity. Trial for the isolation of any active components from these fractions successfully afforded KP3-005 (from methanol extract) with strong activity. Unfortunately, the totally isolated yield was insufficiency for further study. Examination of n-hexane extract and ethyl acetate extract led to the identification of a number of known flavonoids which had been isolated from the same plant (4-5,7).

Structure elucidation of the isolated compounds

As described in Chapter III, five pure compounds were successfully isolated from *n*-hexane and ethyl acetate extract. One active fraction was separated from methanol extract which was obtained using chromatographic techniques according to and activity-guided separation. The structure elucidations of the isolated compounds were discussed as follow.

1. Structure elucidation of KP1-002P, KP1-004-5P and KP1-010P

KP1-002P, KP1-004-5P and KP1-010P were obtained as yellow needle from fraction KP1-002, KP1-004 and KP1-010 of *n*-hexane extract (scheme 3.2 and 3.3) by chromatographic techniques using silica gel column to yield 3.0201 g (18%). Its molecular formula was proposed to be $C_{17}H_{14}O_5$ by EIMS which was exhibited the molecular ion peak at m/z 298. The UV absorption bands at λ_{max} 270 nm (logε 4.4) and 325 nm (logε 4.3) showed the characteristic of flavone chromophore (53-55). The IR spectrum showed an OH absorption (ν_{max} 3150 cm^{-1}) and C=O absorption (ν_{max} 1659 cm^{-1}).

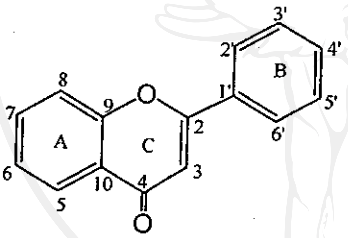


Figure 4.1 The flavone skeleton and its numbering system.

Seven aromatic protons and two signals due to methoxy groups were observed in the ^{1}H-NMR ($CDCl_3$). Di-substituted in A-ring was deduced by the appearance of a singlet signal of OH at δ 12.58 and OCH_3 at δ 3.87. Moreover, the AM (meta) system coupling at δ 6.36 (1H, d, J= 1.6 Hz, H-8) and 6.46 (1H, d, J= 1.6 Hz, H-6) were also observed. In B-ring, two signals of five aromatic protons were observed at δ 7.52 (3H, m) and δ 8.07 (2H, dd, J= 4.0 and 2.0 Hz). A singlet signal of OCH_3 at δ 3.88 (H-3) was also observed. Regarding to these spectral data and the previous report, compound KP1-004-5P was assignable to be a flavone, 5-hydroxy-3,7-dimethoxyflavone which has also been found in the same species(4,7).

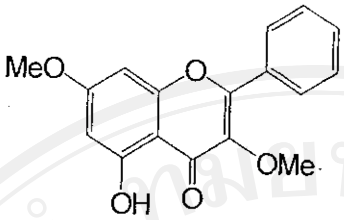


Figure 4.2 The chemical structure of compound KP1-002P, KP1-004-5P and KP1-010P

2. Structure elucidation of KP1-005-2P and KP1-007-6P

KP1-005-2P and KP1-007-6P were obtained as yellow needle from fraction KP1-005 and fraction KP1-007 of *n*-hexane extract (scheme 3.4 and 3.6) by chromatographic techniques using silica gel column to yield 0.6200 g (3%). Its molecular formula was proposed to be $C_{17}H_{14}O_5$ by EIMS which was exhibited the molecular ion peak at m/z 298. The UV absorption bands at λ_{max} 265 nm (logε 4.5) and 310 nm (logε 4.2) showed the characteristic of flavone chromophore as same as KP1-004-5P. The IR spectrum showed an OH absorption (ν_{max} 3200 cm^{-1}) and C=O absorption (ν_{max} 1663 cm^{-1}).

Seven aromatic protons and two signals due to methoxy groups were observed in the ^{1}H-NMR ($CDCl_3$). The meta-disubstitution on ring A was indicated by the presence of singlet signal of OH at δ 12.80 and OCH_3 at δ 3.88. The AM (meta) system coupling of H-8 at δ 6.47 (1H, d, $J = 2.5$ Hz) and H-6 at δ 6.35 (1H, d, $J = 2.5$ Hz) were also observed. Two signals of four aromatic protons were observed at δ 7.82 (2H, d, $J = 9.0$ Hz, H-2' and H-3') and δ 7.01 (2H, d, $J = 9.0$ Hz, H-5' and H-6') on B-ring. Moreover, the para-monosubstitution of OCH_3 on ring B was also observed at δ 3.87 (H-4'). Regarding to these spectral data and the previous report, compound KP1-005-2 was assigned to be 5-hydroxy-7,4'-dimethoxyflavone which has also been found in this plant (4,7).

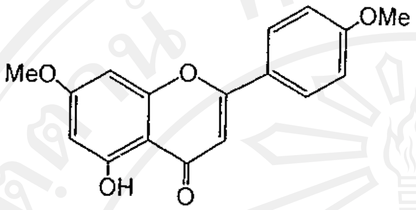


Figure 4.3 The chemical structure of compound KP1-005-2P and KP1-007-6P

3. Structure elucidation of KP1-006-5P and KP2-007-2-02P

KP1-006-5P and KP2-007-2-02P were obtained as yellow plates from fraction KP1-006-5 of *n*-hexane extract (scheme 3.5) and fraction KP2-007-2-02 of ethyl acetate extract (scheme 3.10) by chromatographic techniques using silica gel column to yield 0.34 g (7.15%). Its molecular formula was proposed to be $C_{16}H_{12}O_4$ by EIMS which was exhibited the molecular ion peak at m/z 268. The UV absorption bands showed the characteristic of flavone chromophore as same as two compounds (KP1-004-5P and KP1-005-2P) at λ_{max} 267 nm (logε 4.7) and 310 nm (logε 4.4). The IR spectrum showed an OH absorption (ν_{max} 3150 cm^{-1}) and C=O absorption (ν_{max} 1668 cm^{-1}).

Eight aromatic protons and singlet signal of methoxy groups were observed in the ^{1}H-NMR ($CDCl_3$). The di-substitued on ring A was deduced by the appearance of a singlet signal of OH at δ 12.65 and OCH_3 at δ 3.81. An AM (meta) system coupling at δ 6.43 (1H, d, $J = 2.4$ Hz, H-8) and 6.30 (1H, d, $J = 2.4$ Hz, H-6) were also observed. Beside that, two signals of five aromatic protons were observed at δ 7.47 (3H, m, H-3', H-4', H-5') and δ 7.81 (2H, dd, $J = 6.4$ Hz, and 1.1 Hz, H-2' and H-6') on B-ring. Regarding to these spectral data and the previous report, compound KP1-005-2P was assigned to be 5-hydroxy-7-methoxyflavone which has also been found in this plant (4,7).

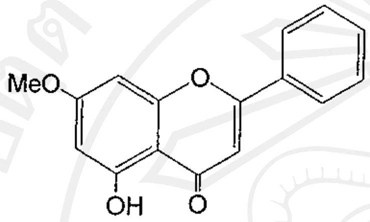


Figure 4.4 The chemical structure of compound KP1-006-5P and KP2-007-2-02P

4. Structure elucidation of KP1-012P and KP2-007-1P

KP1-012P and KP2-007-1P were obtained as yellow needle from fraction KP1-012 of *n*-hexane extract (scheme 3.2) and fraction KP2-007 of ethyl acetate extract (scheme 3.9) by chromatographic techniques using silica gel column to yield 2.5994 g (10%). Its molecular formula was proposed to be $C_{18}H_{16}O_5$ by EIMS which was exhibited the molecular ion peak at m/z 282. The UV absorption bands a showed the characteristic of flavone chromophore at λ_{max} 265 nm (logε 4.5) and 310 nm (logε 4.4). The IR spectrum showed an OH absorption (ν_{max} 3020 cm^{-1}) and C=O absorption (ν_{max} 1650 cm^{-1}).

Seven aromatic protons and two signals due to methoxy groups were observed in the ^{1}H-NMR ($CDCl_3$). The meta-disubstitution on ring A was indicated by the appearance of singlet signal at δ 3.96 (OCH_3 x 2) and the AM (meta) system coupling at δ 6.51 (1H, d, $J = 2.4$ Hz, H-8) and 6.35 (1H, d, $J = 2.4$

Hz, H-6) were also observed. Two signals of five aromatic protons were observed at δ 8.06 (3H, m, H-3', H-4' and H-5') and δ 7.48 (2H, dd, *J* = 6.4 Hz, and 1.1 Hz, H-2' and H-6') on B-ring. Moreover, a methoxy group at δ 3.96 (OCH_3, H-3) was also observed. Regarding to these spectral data and the previous report, compound KP1-012 was assigned to be 3,5,7-trimethoxyflavone which has also been found in this plant (4,7).

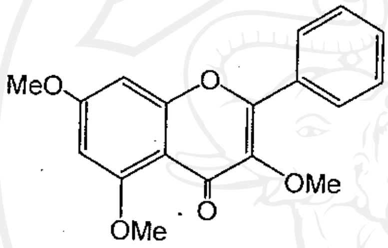


Figure 4.5 The chemical structure of compound KP1-012P and KP2-007-1P

5. Structure elucidation of KP2-005P and KP2-006-2P

KP2-005P and KP2-006-2P were obtained as white needle from fraction KP2-005 of ethyl acetate extract (scheme 3.7) and fraction KP2-006 of ethyl acetate extract (scheme 3.8) by chromatographic techniques using silica gel column to yield 1.4329 g (8%). Its molecular formula was proposed to be $C_{17}H_{14}O_4$ by EIMS which was exhibited the molecular ion peak at m/z 282. The UV absorption bands at λ_{max} 260 nm (logε 4.6) and 300 nm (logε 4.3) showed the characteristic of flavone chromophore. The IR spectrum showed an OH absorption (ν_{max} 3072 cm^{-1}) and C=O absorption (ν_{max} 1647 cm^{-1}).

Eight aromatic protons and two signals of methoxy groups were observed in the ^{1}H-NMR ($CDCl_3$). The meta-disubstitution on ring A was indicated by the singlet signal at δ 3.93 (OCH_3) and δ 3.88 (OCH_3), an AM (meta) system coupling at δ 6.55 (1H, d, J = 2.4 Hz, H-8) and 6.35 (1H, d, J = 2.4 Hz, H-6) were also observed. Two signals of five aromatic protons were observed at δ 7.48 (3H, m, H-3', H-4' and H-5') and δ 7.85 (2H, dd, J = 6.4 Hz, and 1.1 Hz, H-2' and H-6') on B-ring. Regarding to these spectral data and the previous report, compound KP1-012P was assigned to be 5,7-dimethoxyflavone which has also been found in this plant (4,7).

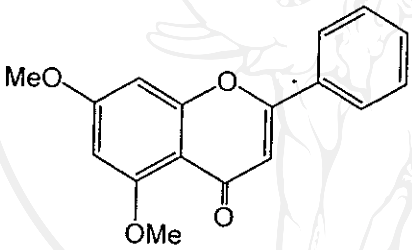


Figure 4.6 The chemical structure of compound KP2-005P and KP2-006-2P

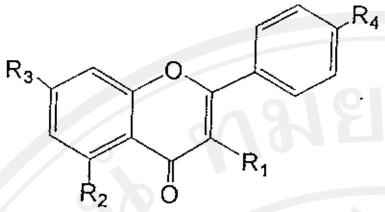


Figure 4.7 Isolated compounds from *n*-hexane and ethyl acetate fractions (5 compounds)

Code	Compounds	R_1	R_2	R_3	R_4
KP1-002P **KP1-004-5P** **KP1-010P**	5-hydroxy-3,7-dimethoxyflavone	OMe	OH	OMe	H
KP1-005-2P **KP1-007-6P**	5-hydroxy-7,4'-dimethoxyflavone	H	OH	OMe	OMe
KP1-006-5P **KP2-007-2-02P**	5-hydroxy-7-methoxyflavone	H	H	OMe	H
KP1-012P **KP2-007-1P**	3,5,7-trimethoxyflavone	OMe	OMe	OMe	H
KP2-005P **KP2-006-2P**	5,7-dimethoxyflavone	H	OMe	OMe	H

Table 4.3 NMR data of compound 5-hydroxy-3,7-dimethoxyflavone, 5-hydroxy-7,4′-dimethoxyflavone and 5-hydroxy-7-methoxyflavone

$C^{\#}$	**5-hydroxy-3,7-dimethoxyflavone** (KP1-002P, KP1-004-5P, KP1-010P)		**5-hydroxy-7,4′-dimethoxyflavone** (KP1-005-2P, KP1-007-6P)		**5-hydroxy-7-methoxyflavone** (KP1-006-5P, KP2-007-2-02P)	
2	-	156.1 [C]	-	164.2 [C]	-	157.8 [C]
3	-	139.9 [C]	6.55 (1H,S)	104.5 [CH]	6.60 (1H,S)	105.9 [CH]
4	-	179.2 [C=O]	-	182.7 [C=O]	-	182.5 [C=O]
5	12.58 (1H,S)	162.3 [C]	12.8 (1H,S)	162.4 [C]	12.65 (1H,S)	162.2 [C]
6	6.36 (1H,d,J=1.6 Hz)	98.2 [CH]	6.35 (1H,d,J=2.0 Hz)	92.8 [CH]	6.30 (1H,d,J=2.0 Hz)	98.2 [CH]
7	-	165.8 [C]	-	162.8 [C]	-	165.6 [C]
8	6.46 (1H,d,J=1.6 Hz)	92.4 [CH]	6.47 (1H,d,J=2.5 Hz)	98.2 [CH]	6.43 (1H,d,J=2.4 Hz)	92.7 [CH]
9	-	130.7 [C]	-	165.7 [C]	-	157.8 [C]
10	-	106.4 [C]	-	105.8 [C]	-	105.7 [C]
1′	-	157.1 [C]	-	123.8 [C]	-	164.0 [C]
2′	8.07 (1H,dd,J=4.0 and 2.0 Hz)	128.8 [CH]	7.01 (1H,d,J=9.0 Hz)	114.7 [CH]	7.81 (1H,dd,J=6.4 and 1.1 Hz)	126.3 [CH]
3′	7.52 (1H,S)	128.6 [CH]	7.35 (1H,d,J=9.0 Hz)	128.2 [CH]	7.47 (1H,S)	129.1 [CH]
4′	7.52 (1H,S)	131.2 [CH]	-	157.9 [C]	7.47 (1H,S)	131.9 [CH]
5′	7.52 (1H,S)	128.6 [CH]	7.35 (1H,d,J=9.0 Hz)	128.2 [CH]	7.47 (1H,S)	129.1 [CH]
6′	8.07 (1H,dd,J=4.0 and 2.0 Hz)	128.8 [CH]	7.01 (1H,d,J=9.0 Hz)	114.7 [CH]	7.81 (1H,dd,J=6.4 and 1.1 Hz)	126.3 [CH]
3-OMe	3.88 (3H,S)	60.6 [OCH_3]	-	-	-	-
7-OMe	3.87 (3H,S)	56.0 [OCH_3]	3.88 (3H,S)	56.0 [OCH_3]	3.81 (3H,S)	55.8 [OCH_3]
4′-OMe	-	-	3.87 (3H,S)	55.7 [OCH_3]	-	-

A ^1H-NMR (400 MHz in CDCl3) are reported downfield from internal TMS at 0.00 ppm and coupling constants are quoted in Hz. ^{13}C-NMR assignments are related to internal $CDCl_3$ at 77.00 ppm and the chemical shifts are denoted in a square bracket. ^1H and ^{13}C-NMR assignments in $CDCl_3$ are based on DEPT, HMQC and HMBC experiments.

Table 4.4 NMR data of 3,5,7-trimethoxyflavone and 5,7-dimethoxyflavone

C#	**3,5,7-trimethoxyflavone** (KP1-012P, KP2-007-1P)		**5,7-dimethoxyflavone** (KP2-005P, KP2-006-2P)	
2	-	130.8 [C]	-	160.9 [C]
3	-	130.2 [C]	6.65 (1H,S)	109.1 [CH]
4	-	174.1 [C=O]	-	177.0 [C=O]
5	-	141.8 [C]	-	160.7 [C]
6	6.35 (1H,d,J=2.5 Hz).	92.4 [CH]	6.35 (1H,d,J=2.0 Hz)	96.2 [CH]
7	-	109.6 [C]	-	164.1 [C]
8	6.51 (1H,d,J=2.5 Hz)	95.8 [CH]	6.55 (1H,d,J=2.5 Hz)	92.8 [CH]
9	-	158.9 [C]	-	158.9 [C]
10	-	163.9 [C]	-	109.3 [C]
1′	-	152.6 [C]	-	131.5 [C]
2′	8.06 (1H,dd,J=6.5 and 1.3 Hz)	128.1 [CH]	7.85 (1H,dd,J=3.6 and 3.2 Hz)	126.0 [CH]
3′	7.48 (1H,S)	128.4 [CH]	7.48 (1H,S)	129.0 [CH]
4′	7.48 (1H,S)	128.4 [CH]	7.48 (1H,S)	131.2 [CH]
5′	7.48 (1H,S)	128.4 [CH]	7.48 (1H,S)	129.0 [CH]
6′	8.06 (1H,dd,J=6.5 and 1.3 Hz)	128.1 [CH]	7.85 (1H,dd,J=3.6 and 3.2 Hz)	126.0 [CH].
3-OMe	3.89 (3H,S)	55.7 [OCH$_3$]	-	-
5-OMe	3.89 (3H,S)	55.7 [OCH$_3$]	3.93 (3H,S)	56.5 [OCH$_3$]
7-OMe	3.96 (3H,S)	56.4 [OCH$_3$]	3.88 (3H,S)	55.8 [OCH$_3$]

A ^{1}H-NMR (400 MHz in CDCl3) are reported downfield from internal TMS at 0.00 ppm and coupling constants are quoted in Hz. ^{13}C-NMR assignments are related to internal CDCl$_3$ at 77.00 ppm and the chemical shifts are denoted in a square bracket. ^{1}H and ^{13}C-NMR assignments in CDCl$_3$ are based on DEPT, HMQC and HMBC experiments

Then, the methanol fraction was isolated by chromatography techniques as described in chapter III. Test of each fraction for antioxidant showed potent activity in fraction KP3-005. After rechromatography of fraction KP3-005, seven fractions were obtained and evaluated for their antioxidant activity. The active fraction (KP3-005-2P) was separated with RP-CC 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. The LC chromatogram and mass spectrum of KP3-005-2P are shown below.

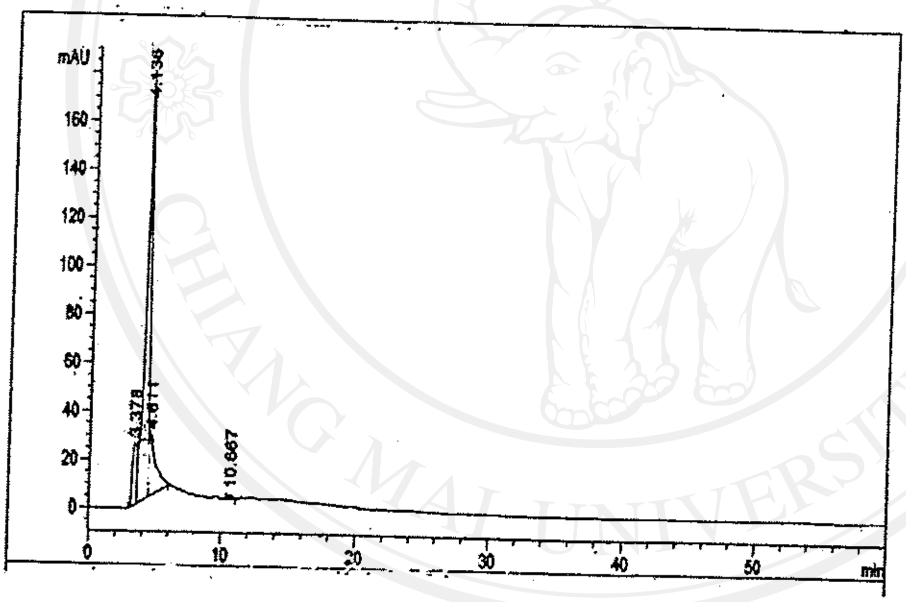


Figure 4.8 The LC chromatogram of KP3-005-2P from methanol extract of *K. parviflora*

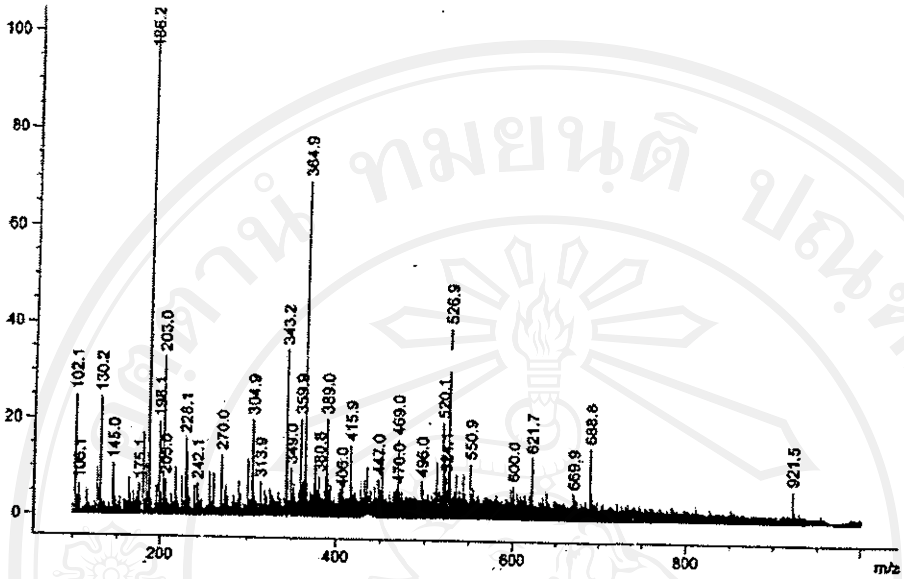


Figure 4.9 The mass spectrum of KP3-005-2P from methanol extract of *K. parviflora*

All of the isolated compounds from the *n*-hexane and ethyl acetate extract of rhizomes of *K. parviflora* are flavones derivatives: **1)** 5-hydroxy-7,3'-dimethoxyflavone (KP1-005-2P), **2)** 5-hydroxy-3,7-dimethoxyflavone (KP1-004-5P), **3)** 5-hydroxy-7-methoxyflavone(KP1-006-5P), **4)** 3,5,7-trimethoxyflavone (KP1-012P) and **5)** 5,7-dimethoxyflavone (KP2-005P), respectively. The physical properties of crude extracts and isolated compounds are shown in Table 4.3.

Table 4.5 The physical properties of crude extracts and isolated compounds from *n*-hexane, ethyl acetate and methanol fractions from rhizomes of *K. parviflora*

Solvent of partition	**Code**	**Character**	**Melting point**
***n*-hexane**	Crude extract	Gold-yellow, paste	-
	KP1-002P KP1-004-5P KP1-010P	Yellow, needle	145.4-145.8
	KP1-005-2P KP1-007-6P	Yellow, needle	167.6-168.9
	KP1-006-5P KP2-007-2-02P	Yellow, plates	136.3-137.2
	KP1-012P KP2-007-1P	Colorless, plates	166.0-168.9
Ethyl acetate	Crude extract	Light-yellow	-
	KP2-005P KP2-006-2P	White, needle	147.9-150.5
Methanol	Crude extract	Dark-purple, sticky mass	-
	KP3-005-2-02	Purple, sticky mass	-

According to Table 4.8, we selected one compound from repeated compounds to assess antioxidant activity. A list of selected compounds is shown as following;

1. **KP1-004-5P** is a representative of 5-hydroxy-3,7-dimethoxyflavone.
2. **KP1-005-2P** is a representative of 5-hydroxy-7,4′-dimethoxyflavone.
3. **KP1-006-5P** is a representative of 5-hydroxy-7-methoxyflavone.
4. **KP1-012P** is a representative of 3,5,7-dimethoxyflavone.
5. **KP2-005P** is a representative of 5,7-dimethoxyflavone.

Antioxidant activity of isolated compounds

Four fractions of crude extracts and five isolated compounds were investigated their antioxidant by free radical decolourization assay (ABTS^{0+} Assay) and ferric reducing ability power assay (FRAP Assay).

Total antioxidant activity:

1. Free radical decolourization assay (ABTS^{0+} Assay)

Calibration curves

A plot of the calibration curves of the three standard antioxidant, which included ascorbic acid, quercetin and pyrogallol, were shown in Figure 4.1. Data are shown in Table 4.4, 4.5, 4.6.

Table 4.6 The data used to construct calibration curve for ascorbic acid standard

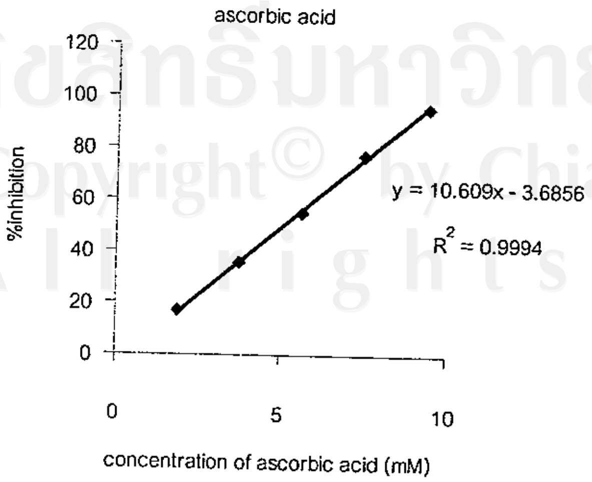
Concentration of ascorbic acid (mM)	ABTS scavenging activity, %
0.50	16.757
1.00	35.683
1.50	55.149
2.00	77.145
2.50	95.845

Table 4.7 The data used to construct calibration curve for quercetin standard

Concentration of quecetin (mM)	ABTS scavenging activity, %
0.05	3.839
0.10	10.289
0.25	31.752
0.50	60.253

Table 4.8 The data used to construct calibration curve for pyrogallol standard

Concentration of pyrogallol (mM)	ABTS scavenging activity, %
0.10	7.995
0.25	25.655
0.50	53.839
1.00	95.799



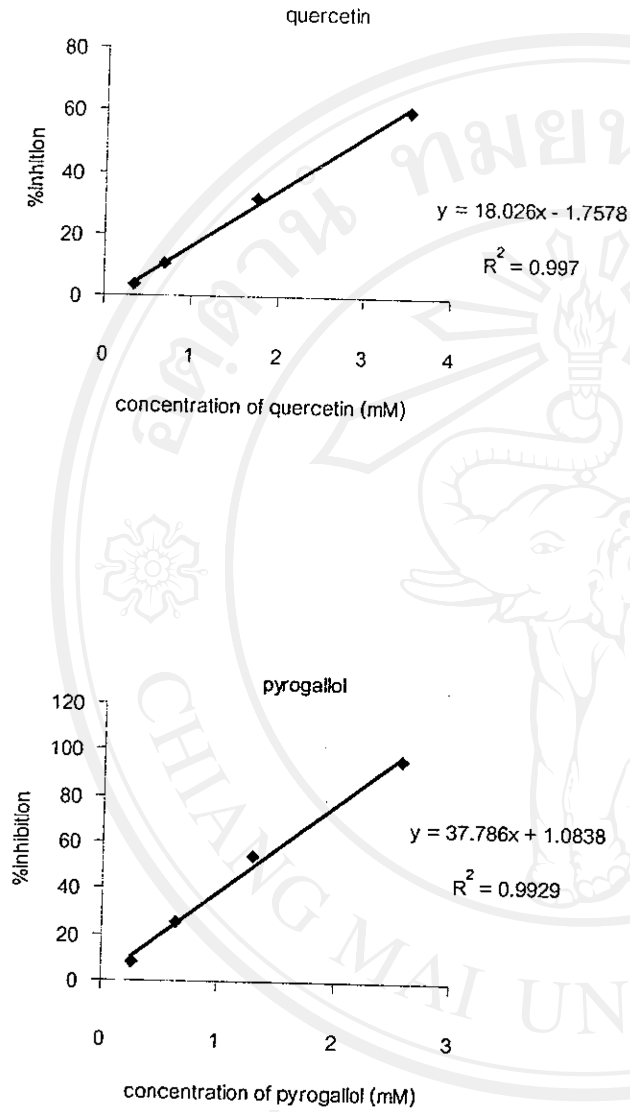


Figure 4.10 Concentration-response curves for the absorbance at 743 nm for $ABTS^{0+}$ as a function of concentration of standard ascorbic acid (a), quercetin (b) and pyrogallol (c) solutions.

Antioxidant activity of isolated compounds and crude extract of *K. parviflora* as determined by scavenging ABTS radical expressed as mg ascorbic acid /g sample (a), mg quercetin/g sample (b) and mg pyrogallol/g sample (c) solutions are shown in Table 4.7.

Table 4.7 The antioxidant activity by ABTS^{0+} Assay of isolated compounds from *n*-hexane, ethyl acetate and methanol extracts of *K. parviflora* as a function of concentration of standard expressed as mg of ascorbic acid/g sample (a), mg quercetin/g sample (b) and mg pyrogallol/g sample (c) solutions.

Fraction	**Code**	**ABTS Scavenging activity in mg standard/g sample**		
		Ascorbic acid	**Quercetin**	**Pyrogallol**
***n*-hexane**	Crude extract	0.735	0.413	0.183
	KP1-004-5P	0.162	0.074	0.021
	KP1-005-2P	0.311	0.162	0.063
	KP1-006-5P	0.516	0.562	0.046
	KP1-012P	0.216	0.232	0.019
Ethyl acetate	Crude extract	1.384	0.795	0.365
	KP2-005P	0.238	0.245	0.020
Methanol	Crude extract	1.705	0.984	0.455
	KP3-005	2.143	1.287	0.555

<u>**Antioxidant activity by the ABTS^{0+} Assay**</u>

As shown in Table 4.7, the antioxidant activity of crude extracts and their isolated compounds of *K. parviflora* was determined by scavenging ABTS radical ranged from 2.143 to 0.162 mg ascorbic acid/ g dry wt. material. The highest activity revealed in methanol extract followed by ethyl acetate and *n*-hexane extract, 1.705, 1.384, 0.725 mg ascorbic acid/ g dry wt. material, respectively. Among isolated compounds, KP3-005 showed the highest free radical scavenging activity whereas compound KP1-006-5P (5-hydroxy-7-methoxyflavone) exhibited comparatively high antioxidant activity, compounds KP1-004-5P (5-hydroxy-3,7-dimethoxyflavone), compound KP2-005P (5,7-dimethoxyflavone), compound KP1-005-2P (5-hydroxy-7, 4'-dimethoxyflavone) and compound KP1-012P (3,5,7-trimethoxyflavone) showed comparatively low antioxidant activity. Comparison antioxidant activity between crude extracts and isolated compounds revealed that all of crude extracts showed higher activity than their isolated compounds.

2. Ferric reducing ability power assay (FRAP Assay)

Calibration curve

A plot of the calibration curves of the Fe_2SO_4 standard antioxidant is shown in Figure 4.2. Data are shown in Table 4.8.

Table 4.10 The data used to construct calibration curve for Fe_2SO_4 standard

Concentration of Fe_2SO_4 (μM)	Ferric reducing ability, %
1000	0.624
500	0.335
250	0.166
100	0.084
50	0.038

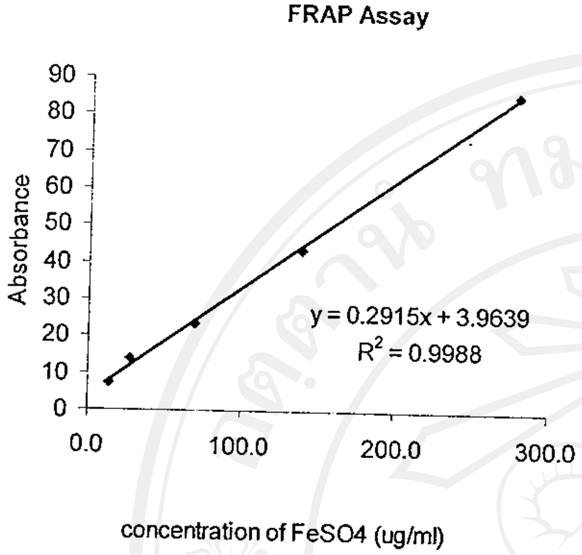


Figure 4.11 Calibration curve for the absorbance at 593 nm for FRAP Assay as a function of concentration of standard Fe_2SO_4

Antioxidant activity by the FRAP Assay

As displayed in Table 4.9, the FRAP values for investigated extracts of *K. parviflora* varied in a widely range between 662.806 and 212.095 mg Fe_2SO_4/ g dry wt. material. Among these, methanolic extract showed the highest FRAP value, 289.059 mg Fe_2SO_4/ g dry wt. material, whereas ethyl acetate and *n*-hexane extracts show comparatively low FRAP values, 333.571 and 380.501 mg Fe_2SO_4/ g dry wt. material, respectively. Comparison of FRAP values between isolated compounds found that compounds KP3-005 from methanolic extract showed the highest reducing power, while compound KP2-005P (5,7-dimethoxyflavone), compound KP1-012 (3,5,7-trimethoxyflavone), compound KP1-005-2P (5-hydroxy-7,3'-dimethoxyflavone) and compound KP1-004-5P (5-hydroxy-3,7-dimethoxyflavone) exhibited comparatively high reducing power as well as compound KP1-006-5P (5-hydroxy-7-methoxyflavone) showed comparatively low reducing power. Comparison of reducing power of the crude extracts and isolated compounds obtained from *K. parviflora* found that there was no significant statistic difference between them.

The antioxidant activity by ABTS^{0+} Assay and FRAP Assay of isolated compounds from *n*-hexane, ethyl acetate and methanol extracts of *K. parviflora* expressed as mg standard/g sample are shown in table 4.9.

Table. 4.11 The antioxidant activity by ABTS^{0+} Assay and FRAP Assay of isolated compounds from *n*-hexane, ethyl acetate and methanol extracts of *K. parviflora.*

Fraction	Code	ABTS scavenging activity in mg ascorbic acid/ g sample	FRAP Value in mg Fe_2SO_4/ g sample
***n*-hexane extract**	Crude extract	0.735	252.140
	KP1-004-5P	0.162	333.975
	KP1-005-2P	0.311	271.199
	KP1-006-5P	0.516	577.738
	KP1-012P	0.216	239.088
Ethyl acetate extract	Crude extract	1.384	291.634
	KP2-005P	0.238	239.088
Methanol	Crude extract	1.705 .	333.520
	KP3-005	2.143	212.095

In this study, the antioxidant activity of crude extracts and their isolated compounds of *K. parviflora* were examined by the two model systems, the $ABTS^{\circ+}$ and FRAP assay. The details of both methods can be explained as following.

1. $ABTS^{\circ+}$ free radical decolorization assay

ABTS, normally, is a colorless molecule, reduced ABTS, which has been oxidized by various agents (in this study is $K_2S_2O_8$) and the characteristic blue-green color develop. When $ABTS^{\circ+}$ is mixed with any substance that can be reduced to its original colorless ABTS form again; in contrast, the reacted substance is oxidized. The total antioxidant activity of investigated samples determined by $ABTS^{\circ+}$ free radical scavenging assay is based on their ability to donate electron to free radical (41, 71-72, 74). Therefore, flavonoid compounds which contain several electron donating groups enhance free-radical scavenging activity. For example, quercetin satifies advantage structures for effective radical-scavenging including 5-OH group in ring A, 3-OH group in ring B, 2,3 double bond and 4-oxo function in molecule (as described in chapter II), thus distinguish better antioxidant activity (21, 23, 43-44, 56, 70-71).

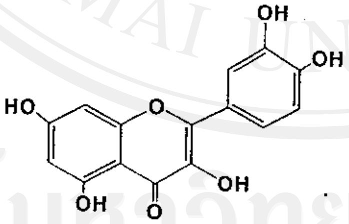
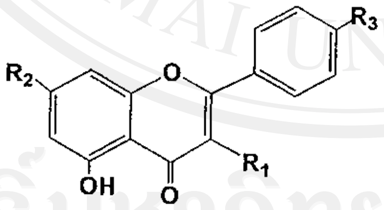
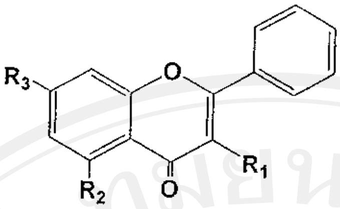


Figure 4.12 Quercetin

In this study, vitamin C is used as standard. Total antioxidant activity can be expressed as scavenging activity in mg vitamin C/g sample measuring the concentration of vitamin C solution with an equivalent antioxidant potential to a standard concentration of the compound under investigation. This activity reflects the ability of electron-donating antioxidants to scavenge the ABTS$^{\circ+}$ radical cation absorbing in the near-IR region at 734 nm compared with vitamin C. Our findings revealed that the methanol fraction showed the highest scavenging activity. This fraction could be contained more electron donors in flavonoid molecules than *n*-hexane and ethyl acetate extracts. The order of scavenging activity of respective rhizome extracts and their isolated compounds is as follow: methanol extract > ethyl acetate extract > *n*-hexane extract > KP1-006-5P > KP1-005-4P > KP2-005P > K1-012P > KP1-005-2P. Among isolated compounds, 5-hydroxy-7-methoxyflavone (KP1-006-5P) gave the highest free radical-scavenging activity. Compounds KP1-005-4P (5-hydroxy-3,7-dimethoxyflavone) exhibited lower activity whereas other compounds including 5,7-dimethoxyflavone, 3,5,7-trimethoxyflavone and 5-hydroxy-7,4'-dimethoxyflavone showed comparatively low antioxidant activity. The figures of all isolated compounds are shown as following.



Compounds	R_1	R_2	R_3
KP1-005-2	H	OMe	OMe
KP1-005-4	OMe	OMe	H
KP1-006-5	H	OMe	H



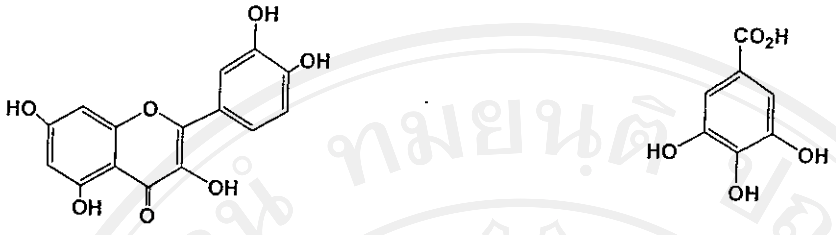
Compounds	R_1	R_2	R_3
KP1-012	OMe	OMe	OMe
KP2-005	H	OMe	OMe

Figure 4.13 Summary of isolated compounds obtained from *n*-hexane and ethyl acetate fractions

These results indicated that crude extracts and their isolated compounds from non-polar fractions displayed comparatively low antioxidant activity due to they less contain electron donors in their molecules. These also support our accumulative data that among structurally homologous flavones, scavenging activity increases according to the total number of –OH groups (21, 23, 78). Beside that, the presence of methoxy groups in flavonoid structure does not enhance the effectiveness against free-radical scavenging activity due to methoxy group may reflect steric effects and/or perturb planarity thus reflect the ability of flavonoid molecule to delocalize electrons, lending to the lower stability of flavonoid radicals and resulting in less antioxidant activity (43, 71, 76).

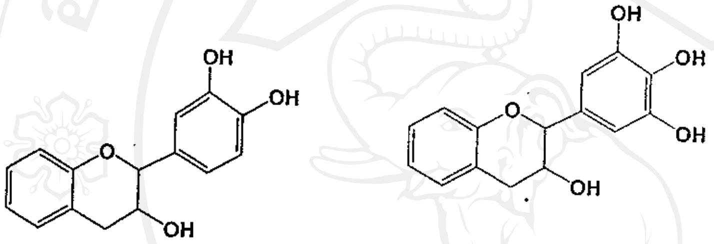
2.2 Ferric Reducing Ability Power Assay (FRAP assay)

As mentioned before, $ABTS^{\circ+}$ free radical scavenging assay of phenolic compounds was based on their ability to donate electron to free radical. Among homologous flavonoid, scavenging activity is in agreement with total number of OH-groups whereas among subclass of flavonoid, both configuration and total hydrogen-donating groups are also effect scavenging activity. On the other hand, FRAP assay measures the antioxidant effect of any substance in the reaction medium as reducing ability. The mechanism of this method is different from free radical-scavenging assay. This method involves neither a pro-oxidant nor an oxidizable substrate. The antioxidant activity of investigated samples determined by FRAP assay base on their ability to reduce oxidizing species or oxidants (37). It depends upon the reduction of ferrictripyridyltriazine (Fe^{3+}-TPTZ) complex to the ferroustripyridyltriazine (Fe^{2+}-TPTZ) by reductant at low pH. Fe^{2+}-TPTZ has an intensive blue color and can be monitored at 593 nm. Therefore, its really measures is the ability of compound to reduce Fe^{3+} to produce Fe^{2+}, thus Fe^{2+} is a well known pro-oxidant and can react with H_2O_2 to produce OH° the most harmful radical found in vivo, some antioxidants such as ascorbic acid and uric acid can reduce both reactive species and Fe^{3+} and their ability in reducing Fe^{3+}may reflect their ability in reducing reactive species (37, 48, 58). Several studies suggested that phenolic compounds, iron bind to polyphenol *via* *ortho*-dihydroxy (catechol), e.g. catechin, quercetin or trihydroxy-benzene (galloyl) group e.g. epicatechin, gallic acid. Beside that, 4-keto and 5-OH moiety may also contribute to the chelation of iron (43, 70-71). Based on these data, flavonoid isolated compounds which contained vicinal OH group or 4-keto and/or 3- or 5-OH in their molecules should be exhibited high reducing power.



Quercetin

Gallic acid



Catechin

Epicatechin

Figure 4.14 Structure of flavonoids which reduce metal ion

The antioxidant potential of the crude extracts and isolated compounds of *K. parviflora* determined by FRAP Assay are given in Table 4.9. Methanol extract exhibited the highest FRAP values (333.520 mg $FeSO_4$/g sample) as has been recorded in $ABTS^{\circ+}$ methods. The order of FRAP values of respective rhizome extracts and their isolated compounds is as follows: methanol extract > ethyl acetate extract > *n*-hexane extract > KP1-006-5P > KP1-004-5P > KP1-005-2P > K1-012P > KP2-005P. Among isolated compounds, 5-hydroxy-7-methoxyflavone (KP1-006-5) displayed the highest FRAP values. Metal ions reducing may from at the 5-OH group. Compound 5-hydroxy-3,7-dimethoxyflavone (KP1-004-5) exhibited comparatively high reducing power, reducing metal ions may occur at 5-OH or 3-OCH_3.

Nevertheless, reducing metal ion at 3-OCH_3 less found (compare with 3- or 5-OH group) due to steric hindrance. Substitution 3-OH group with -OCH_3 may reduce electron delocalize since unplanarity of flavonoid molecule. However, comparison between polymethoxylated-flavones; increasing the number of methoxy group may enhance the effectiveness of reducing power. For example, KP2-005P (5,7-dimethoxyflavone) gave lower FRAP values than KP1-012P (3,5,7-trimethoxyflavone) due to it contain less methoxy groups in molecule. Increasing methoxy groups in flavonoids should be elevated steric effects and lending to less antioxidant activity (43, 71, 76). But the obtained results revealed that increasing methoxy group gave the higher reducing power. This feature may elevate the opportunity of flavonoid molecule to chelate with metal ion, resulting in high activity (44, 56, 65). Nevertheless, comparison between reference standards and isolated compounds obtained from non-polar fractions revealed that isolated compounds showed low reducing power due to they contain a few of -OH or electron donating groups in their molecules (21, 23, 41). From these results, it may be concluded that aside from the presence and total number of OH groups, the position and structure of –OH groups also play an important role for reduce reactive species (43, 70-71, 75).

3. Correlation between $\text{ABTS}^{\circ\pm}$ and FRAP assay

The obtained results indicated that the total antioxidant activity of rhizome extracts measured by ferric reducing ability power are proportional to $\text{ABTS}^{\circ+}$ radical decolorization assay. The results from both assays showed that compound KP3-005-2 (unidentified) from methanolic extract of *K. parviflora* show the highest antioxidant activity. The high activity of this fraction could possible attributed to a greater of electron-donor in their molecules. However, LC chromatogram and mass spectrum of KP3-005-2 suggested that this fraction is required to investigate the chemical constituents as well as the relationship between antioxidant activity and chemical structures.

Comparison antioxidant activity of the crude extracts and isolated compounds obtained from *K. parviflora* found that all of crude extracts showed higher activity than their isolated compounds, except KP3-005-2 displayed higher activity than its crude extract (methanol fraction). These results correspond with published literatures that antioxidant activity are proportion to the degree of hydroxylation. Several –OH groups enhance antioxidant of flavonoids for radical scavenging and reducing reactive species (37, 41, 42, 48, 56, 58, 71-72, 74).

Comparison between isolated compounds, some compounds with higher free radical scavenging activity did not always have higher reducing power. Interestingly, compound KP1-006-5P (5-hydroxy-7-methoxyflavone) exhibited high free radical scavenging activity, compound KP1-005-4P (5-hydroxy-3,7-dimethoxyflavone) and KP2-005P (5,7-dimethoxyflavone) showed comparatively high activity. All of compounds when evaluated by FRAP assay found that compound KP1-005-4P (5-hydroxy-3,7-dimethoxyflavone) exhibited the highest FRAP value whereas KP1-006-5P (5-hydroxy-7-methoxyflavone) showed comparatively high FRAP value and KP2-005P (5,7-dimethoxyflavone) showed the lowest FRAP value. These mean that the lack of correlation occurs between ABTS^{0+} and FRAP assay in these samples. These results may cause from different mechanisms of flavones in each assay (37, 42, 48, 58, 71-72, 74). As mentioned ealier, the antioxidant activity of investigated samples determined by free radical scavenging activity is based on their ability to donate electron to free radical. So flavonoids or phenolic compounds which have several OH-groups or electron donating groups show strong free-radical scavenging activity (21, 23, 44). Whereas FRAP assay involve both the configuration and the total number of methoxy groups in flavonoid molecules. Several hydroxyl group, particularly, 3',4'-catechol or 3 and 5 -OH are required for reduce metal ion. Beside that, substitution -OH group by $-OCH_3$ group may also contribute to the reducing of reactive species but less activity (48, 73). Therefore, flavonoid compounds which effective in free-radical scavenging may not promote metal chelation. These results indicate that the role of SAR in antioxidant activity of flavonoids largely depend on the assay and the conditions used. From these results, we can conclude that the molecular structure of different flavonoid compounds play the important roles in antioxidant activity (48, 71, 73, 76).