CHAPTER III

RESULTS AND DISCUSSION

3.1 Yield of crude extracts from four cultivars pomelo peels.

In this study, Pomelo albedo peels of the cultivars Khao Taeng-gwa (KT), Khao Yai (KY), Khao Nam Pheung (KN), Tong Dee (TD) and flavedo peel of KT were separately macerated in methanol (3x200 mL) for 9 days with stirring. The slurry was filtered and the methanol extract was concentrated with a rotary evaporator under reduced pressure at 35 °C. The methanolic crude extract of KN gave the highest % yield (w/w) of the albedo dry peels, followed by KT, KY and TD cultivar. Methanolic extract of KT flavedo peel gave the lowest % yield (w/w) (Table 3.1).

3.2 Screening of free-radical scavenging, tyrosinase inhibition and UV absorption activities from crude pomelo peels.

Screening of free-radical scavenging, tyrosinase inhibition and UV absorption activities in KT, KY, KN and TD methanolic crude extracts (Table 3.1) indicated strongest free-radical scavenging and tyrosinase inhibition activities in the KT cultivar (Figure 3.1). Moreover, extracts of KT and KY cultivars showed both UV-A and UV-B absorption property while those of KN and TD cultivar showed only UV-B absorption activity (Figure 3.2).

As a result, KT albedo peel was selected for further study. It should also be mentioned here that KT cultivar is one of a popular pomelo in Thailand.

Table 3.1 Preliminary screening tests of free-radical scavenging, tyrosinase-inhibition and UV absorption activities in methanolic crude extracts (10,000 mg/L) from pomelo peels of various cultivars.

Cultivars of crude extracts (in MeOH)	**% Yield (w/w)**	**Free-radical scavenging activity**	**Tyrosinase-inhibition activity**	**UV absorption activity, λ_{max} (nm)**
KT (albedo)	40.23	++	*	281, 327
KY (albedo)	35.36	+	NA	284, 332
KN (albedo)	41.7	+	NA	278
TD (albedo)	33.7	+	NA	282
KT (flavedo)	32.93	++	*	284, 322
Standard	-	BHT	Kojic acid	-

++ sample's activity $\approx$ 1/50 of BHT's activity

\+ sample's activity $\approx$ 1/100 of BHT's activity

* sample's activity $\approx$ 1/100 of kojic acid activity

NA no activity at 10,000 mg/L

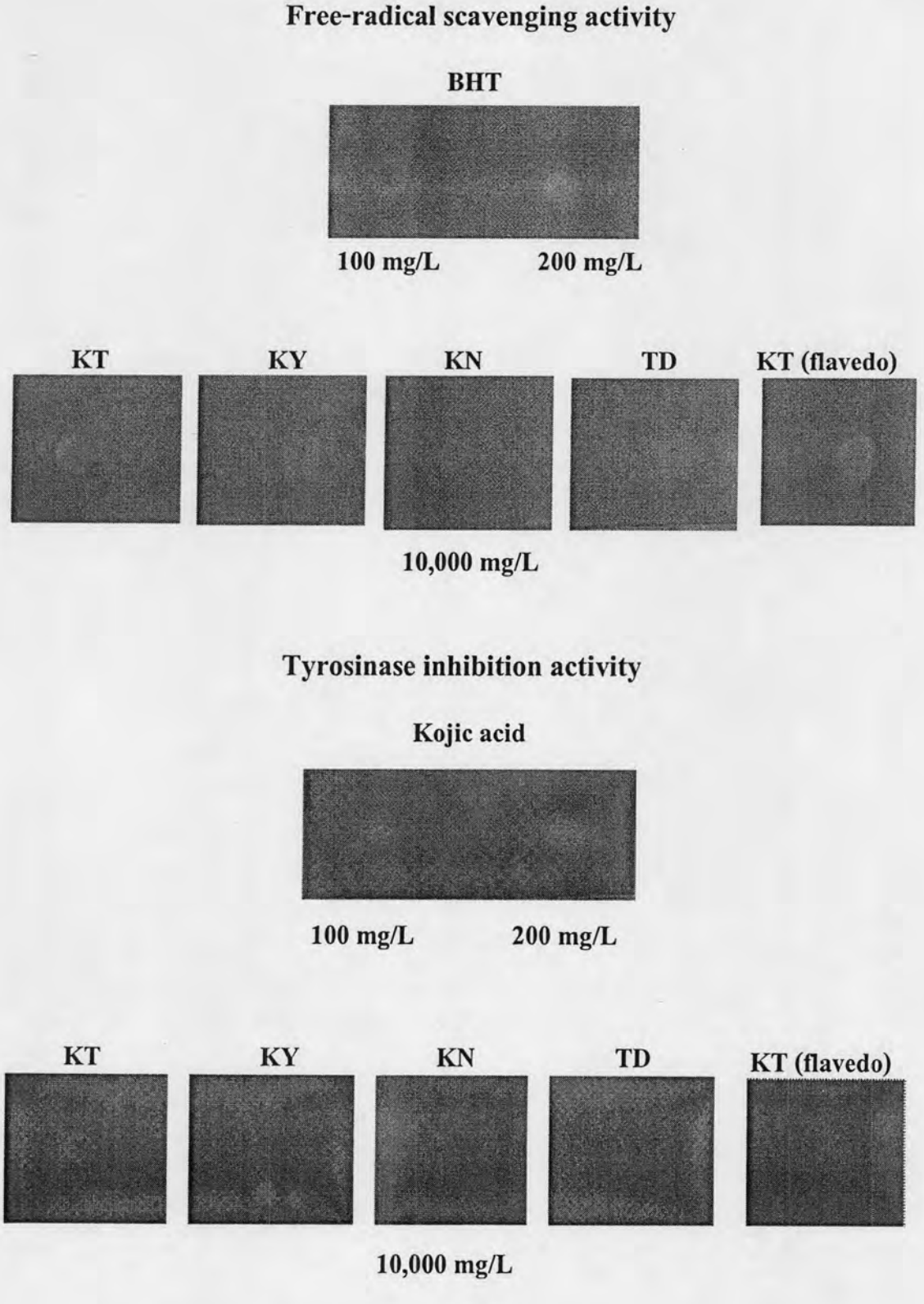


Figure 3.1 Free-radical scavenging and tyrosinase inhibition activities of KT, KY, KN, TD albedo peels and KT flavedo peel were tested at concentration 10,000 mg/L compared with standard (Kojic acid and BHT) by TLC autographic assay.

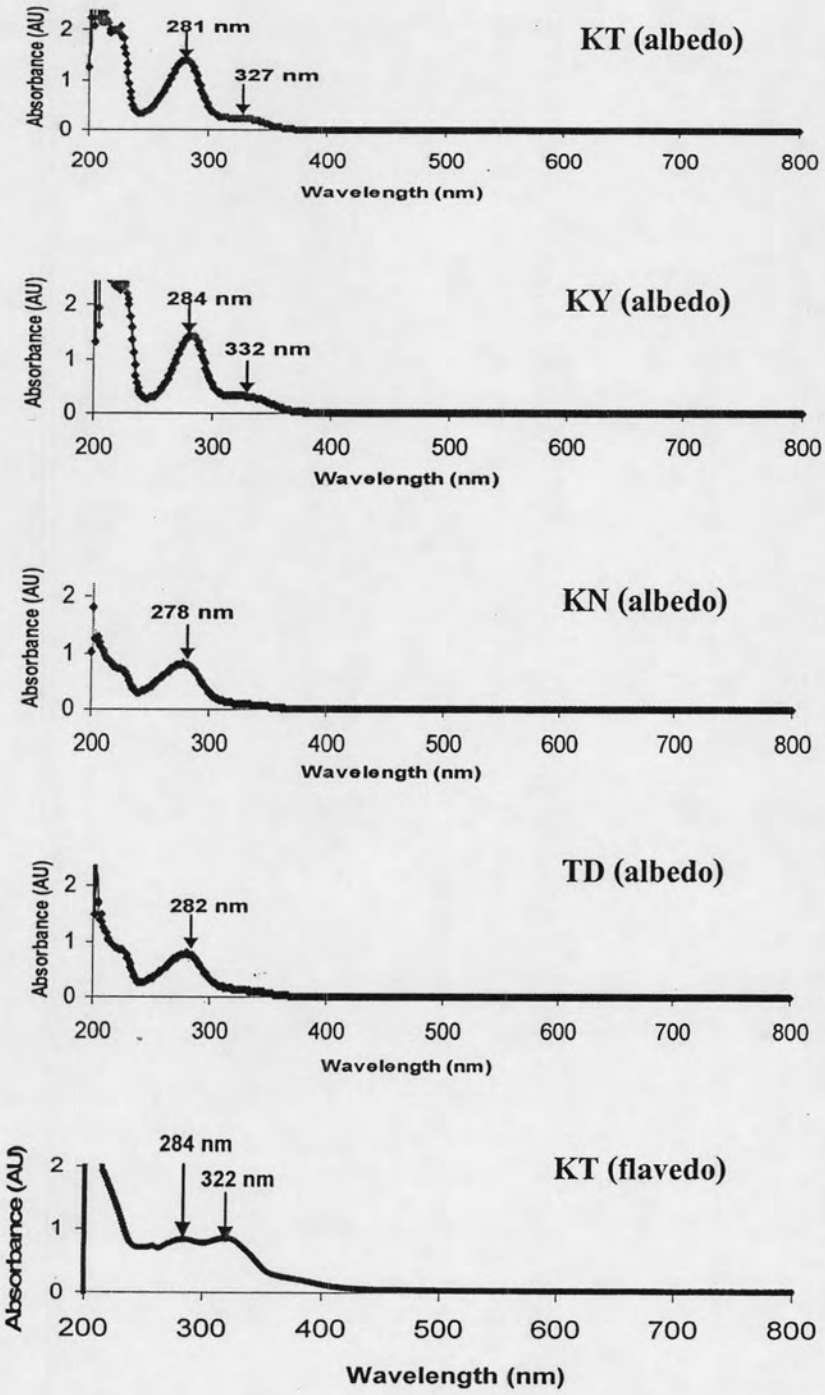


Figure 3.2 UV spectra of 50 mg/L methanolic crude extracts of pomelo peels from various cultivars

3.3 Constituents of the crude MeOH extract.

TLC analysis of crude MeOH extract implied that naringin was the major component (Figure 3.3). As a result, a process to isolate pure naringin from this crude methanol was pursued.

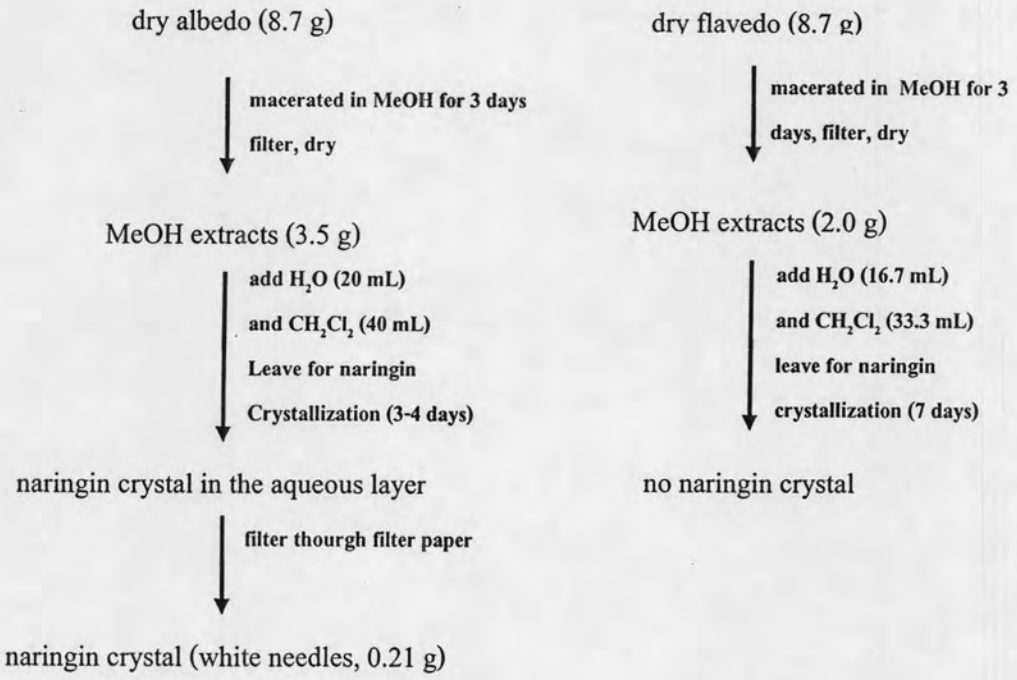
Lane 1 = crude MeOH extract

Lane 2 = naringin standard

Figure 3.3 TLC plate using solvents system MeOH: CH_2Cl_2 (2:3)

3.4 Isolation of naringin from KT peel.

After many experiments, it was found that the best process to isolate naringin from the peels involved a simple liquid-liquid extaction (dichloromethane/water) coupled with crystallization. Methanolic extract of albedo peel (3.5 g) was dissolved in water (20 mL) and transferred into a separating funnel. Dichloromethane (40 mL) was added and the mixture was swirled. The mixture was left for 3-4 days at room temperature to allow complete crystallization of naringin (0.21 g, 2.4 % (w/w)) in the aqueous layer (scheme 3.1). Similar process was carried out with the methanolic extract of flavedo peel, but no naringin crystal could be obtained (scheme 3.1). The result indicates that only the albedo part not the flavedo, is the reservoir of naringin.



Scheme 3.1 Isolation of naringin from pomelo peels

3.5 Naringin purity.

Through methanol extraction followed by liquid-liquid (CH_2Cl_2/H_2O) extraction and crystallization, naringin could be isolated from only albedo peel effectively. Purity of the obtained naringin crystal was determined by reversed-phase HPLC comparing with naringin standard. The chromatogram for naringin from pomelo peel (Figure 3.4 A) shows that the obtained naringin is of higher purity than the standard 97 % naringin (Figure 3.4).

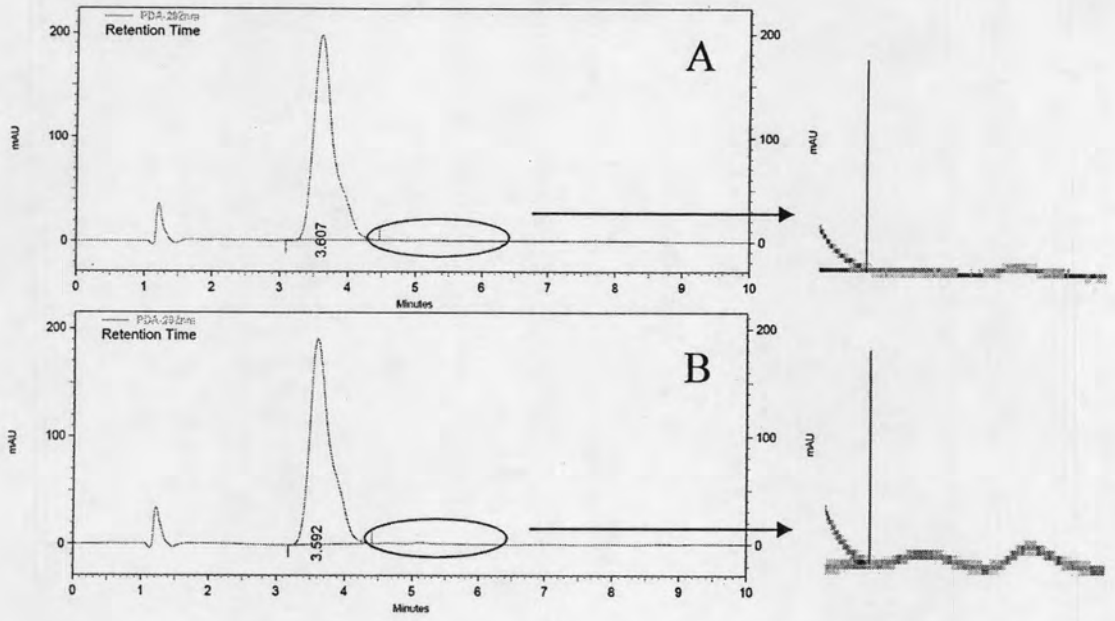


Figure 3.4 HPLC chromatogram of naringin from pomelo peel A) the obtained naringin B) standard naringin of 97 % purity

3.6 Total phenolic contents.

Total phenolic content was measured by Folin–Ciocalteu method, using the standard curve of gallic acid (R^2 = 0.99). The content of total phenolic compounds in crude methanol extract of pomelo peel was 5.94 mg GAE for 100 g of dry crude (Figure3.5). The high content of phenolic compounds agreed well with the fact that ≈ 12.18 mg of naringin (polyphenolic compound) could be isolated from 17.88 mg of this crude.

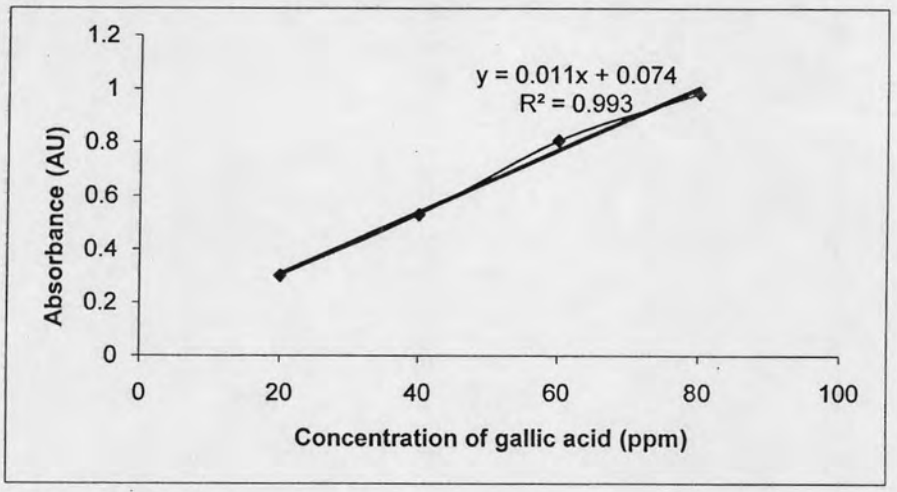


Figure 3.5 Calibration curve of gallic acid of concentration at 20, 40, 60 and 80 mg/L

3.7 Tyrosinase inhibition activity.

In this work, for tyrosinase inhibition activity of the obtained naringin was assayed. The result indicated the IC_{50} values of 4914.77 and 27.39 mg/L for naringin and kojic acid, respectively (Figure 3.6). This is the first report of mild tyrosinase inhibition activity of the compound.

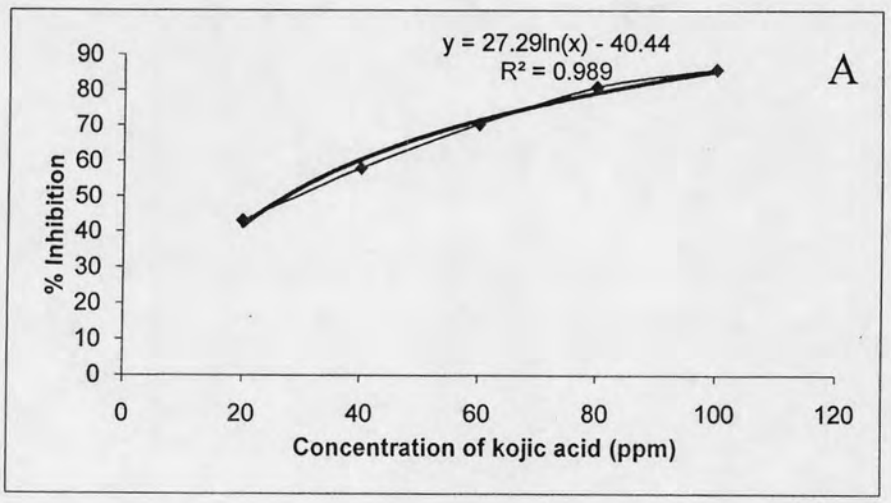


Figure 3.6 % Tyrosinase inhibition capacity of A) Kojic acid and B) naringin. Results are means of three replicates

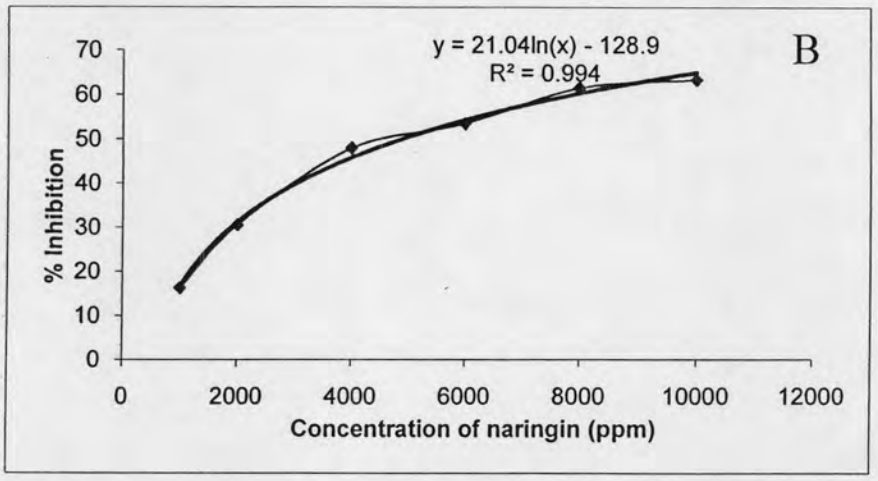


Figure 3.6 % Tyrosinase inhibition capacity of A) Kojic acid and B) naringin. Results are means of three replicates

3.8 Comparision of naringin content in pomelo peels.

Isolation of naringin from methanolic extracts of KY, KN and TD was carried out. The results showed that all cultivars gave significant amount of naringin (Table 3.2) with the highest yield from KT albedo peel. The naringin content of albedo peels showed the highest % yield (w/w) in KY (2.78 %) followed by KT (2.36 %), KN (2.13 %) and TD (1.61 %) cultivar.

Table 3.2 % Yield of naringin from pomelo peels of various cultivars.

Cultivars albedo peels	% Yield (w/w) of naringin
KT	2.36
KY	2.78
KN	2.13
TD	1.61

As results we gave higher naringin content than previously process reported about 5 times from pomelo peels [65].

3.9 Characterization of naringin.

The isolated naringin was characterized by ^{1}H and ^{13}C NMR, FT-IR, MS and UV–Vis spectroscopy techniques. Naringin (naringenin-7-*O*-neohesperidoside): white needles (0.21 mg); mp 171 °C (acetone, ethanol); high-resolution positive ESI MS m/z 603.17 $[M+Na]^{+}$: Calcd. 580.18 (M^{+} of $C_{27}O_{14}H_{32}$); UV λ_{max} (MeOH) 281, 327 nm; FT-IR (KBr), IR spectrum showed absorption band of O—H stretching (very board) at 3426 cm^{-1}, C—H stretching at 2923 cm^{-1}, C=O stretching at 1641 cm^{-1}, C=C stretching at 1577 and 1514 cm^{-1} of aromatic ring and absorption band of C—O stretching at 1207, 1179, 1128 and 1068 cm^{-1}.

Naringin ^{1}H-NMR (acetone-d_6); Naringenin δ 2.73 (1H, d, *J*=16.8 Hz, 3-eq), 3.20, 3.24 (1H, dd, *J*=13.2, 17.6 Hz, 3-ax), 5.43, 5.46 (1H, d, *J*=12.8 Hz, H-2), 6.13 (2H, s, H-6), 6.11 (2H, s, H-8), 6.86 (2H, d, *J*=8.4 Hz, H-3', 5'), 7.36 (2H, d, *J*=8.4 Hz, H-2', 6'), 8.66 (1H, s, H-4'), 12.0 (1H, s, H-5); Glucose δ 3.45 (m, 1H, H-4''), 3.57 (m, 1H, H-3''), 3.65 (m, 3H, H-2'', 5'', 6''), 5.13, 5.15 (1H, d, *J*=7.2 Hz, H-1" (7GlcH1)); Rhamnose δ 1.23 (3H, d, *J*=6.4 Hz, H-6'''), 3.37 (m, 1H, H-4'''), 3.57 (m, 1H, H-2'''), 3.88 (m, 2H, H-3''', 5'''), 5.3 (2H, s, H-1'''); Naringin ^{13}C-NMR (acetone-d_6); Naringenin δ 44.4, 44.5, (C-3), 81.0, 81.1 (C-2), 97.2, 97.2 (C-8), 98.4, 98.5 (C-6), 105.5 (C-10), 117.2 (C-3', 5'), 130.1,130.1 (C-2',6'), 131.4, 131.5 (C-1'), 158.9 (C-4'), 165.0, 165.1 (C-9),165.6 (C-5), 167.1, 167.2 (C-7), 199.0 (C-4); Glucose δ 63.3 (C-6''), 72.2 (C-4'), 78.4 (C-5''), 78.6 (C-3''), 79.8 (C-2''), 100.0, 100.0 (C-1''); Rhamnose δ 19.3 (C-6'''), 70.2 (C-5'''), 72.8 (C-3'''), 73.2 (C-2'''), 74.7 (C-4'''), 102.5, 102.5 (C-1''') (Table 3.2).

Table 3.2 1D and 2D NMR spectroscopic data for naringin in acetone-d_6.

Position	δ_H, mult. (*J* in Hz)	δ_C	COSY	HMBC
naringenin				
2	5.43 (1H, d, *J*=12.8)	81.0 81.1	H-3ax	C-2', C-6'
	5.46			
3	3-ax,	44.4 44.5	H-3eq	C-4
	3.20 (1H, d, *J*=12.8)			
	3.24			
	3-eq,		H-3ax	
	2.73 (1H, d, *J*=12.8)			
4	-	199.0	-	-
5	12.0 (1H, s)	165.6	-	C-5, C-6, C-10
6	6.13 (2H, s)	98.5	-	C-5, C-6, C-10
7	-	167.1 167.2	-	-
8	6.11 (2H, s)	97.2 97.2	-	C-7, C-10
9	-	165.0 165.1	-	-
10	-	105.5	-	-
1'	-	131.4 131.5	-	-
2', 6'	7.36 (2H, d, *J*=8.4)	130.1 130.1	H-3', H-5'	C-2, C-4', C-2', 6'
3', 5'	6.86 (1H, d, *J*=8.4)	117.2	H-2', H-6'	C-1', C-4', C-3',5'
4'	8.66 (1H,s)	158.9	-	C-4', C-3',5'
glucose				
1''	5.13 (1H,d, *J*=7.2)	100.0 100.0	H-2''	C-7, C-1'''
	5.15			
2''	3.65 (m, 1H)	79.8	H-1''	C-1''
3''	3.55 (m, 1H)	78.6	-	-
4''	3.45 (m, 1H)	72.2	-	-
5''	3.65 (m, 1H)	78.4	-	C-2''
6''	3.65 (m, 1H)	63.3	-	C-5''
	3.8 (m, 1H)			-

Position	δ_H, mult. (*J* in Hz)	δ_C	COSY	HMBC
rhamnose				
1'''	5.3 (2H,s)	102.5 102.5	-	C-3''', C-5'''
2'''	3.55 (m, 1H)	73.2	-	-
3'''	3.88 (m, 1H)	72.8	-	C-1''', C-2'''
4'''	3.37 (m, 1H)	74.7	-	-
5'''	3.88 (m, 1H)	70.2	-	C-2''', C-4'''
6'''	1.24 (3H, d, *J*=6.4)	19.3	H-5'''	-

The ^{1}H-^{1}H COSY spectrum of naringin revealed the presence of the following connectivities of; naringenin, CH (2) to CH (3-ax), CH (3-ax) to CH (3-eq), CH (2',6') to CH (3',5'); glucose, CH (1'') to CH (2''); rhamnose, CH (5''') to CH (6''') (Figure 3.7).

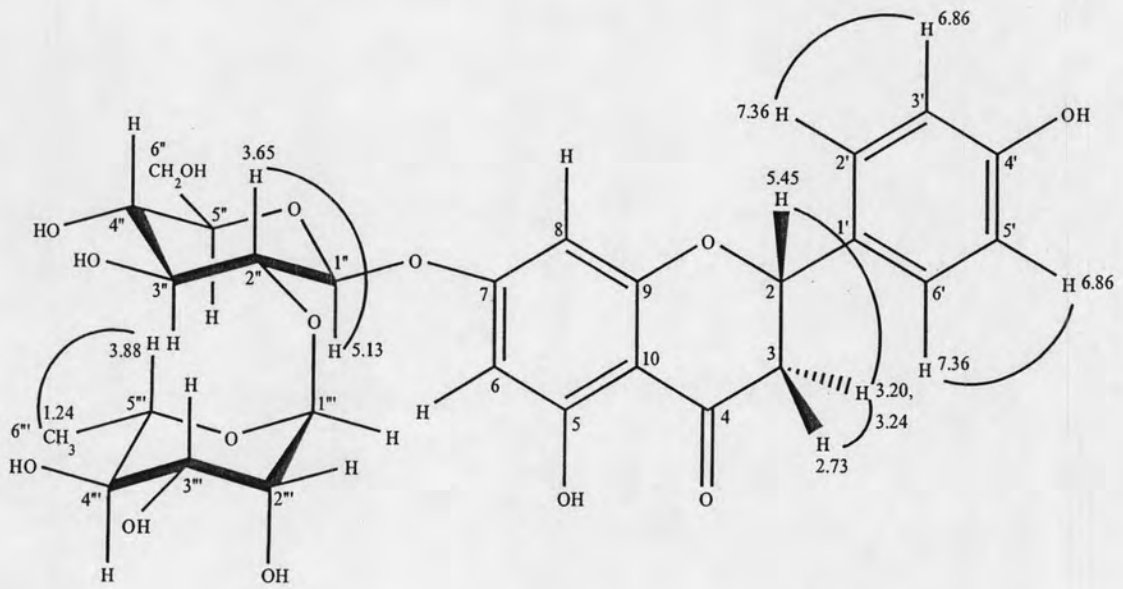


Figure 3.7 The COSY correlation of naringin

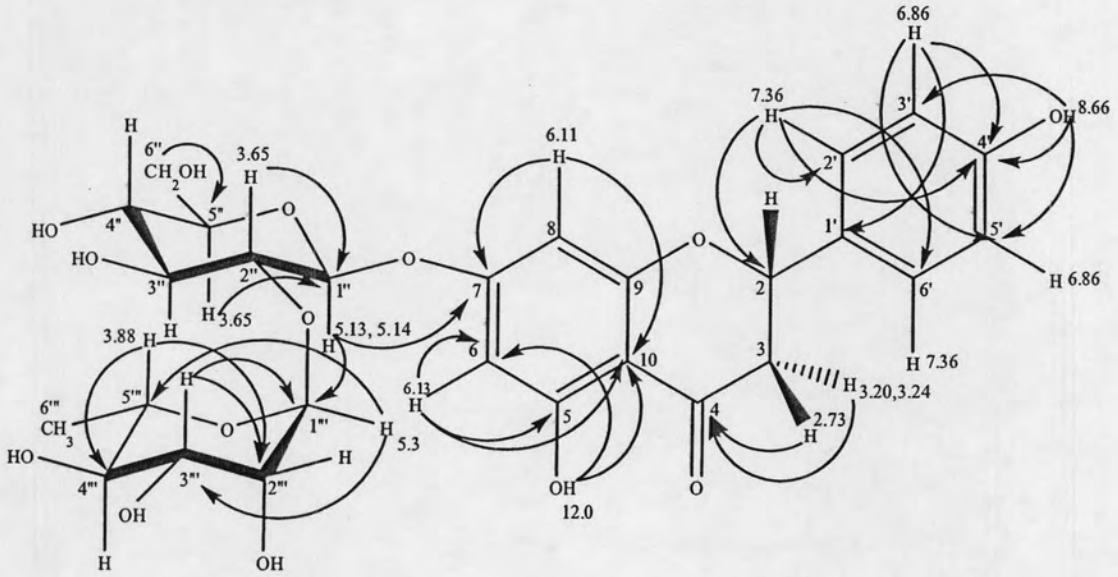


Figure 3.8 The HMBC correlation of naringin

The HMBC correlations of naringin are shown in Figure 3.8. The correlations comfirm naringin structure.