CHAPTER 4

RESULTS AND DISCUSSION

Part I Isolation and Cytotoxic Activity Test

- Crude Extraction of Rubiaceous Plants

To date, the cytotoxic activity study of a group of plants in Rubiaceae family against MCF-7 and KB-3-1 cells has not been reported. Therefore, this research had focused on the discovery of Rubiaceous plants in Northern, Thailand, that possessed cytotoxic activity against the two woman-origin cancerous cell lines.

Twenty different Rubiaceae species were collected from Doi Sutep-Poi, Doi Kuhn Dtan, Thailand. Dried powder of plants (approximately 4 gm) were macerated with 95% ethanol, and the extracts were tested for their cytotoxic activity against MCF-7 and KB-3-1 cells. The results of the cytotoxic activity test were shown in Table 3. These plants could be classified into three level according to the criteria given on page 50. *Gardenia obtusifolia* and *Gardenia sootepensis* were the active species with the IC_{50} less than 20 μg/ml. *Ixora cibdela, Mussaenda parva,* and *Psychotria ophioxybides* were found to have the IC_{50} between 20-100 μg/ml, which were the moderately active species. Other fifteen plants were the species their IC_{50}s were either more than 100 μg/ml or unable to find and classified as the inactive species (Table 4). In order to control the precision of the cytotoxic activity test, positive control substances were included in every test plate. In this study, 5-fluorouracil (5-FU) was the positive substance for the test against MCF-7, while vinblastine was used for the test against KB-3-1. The IC_{50} of 47.8 $\pm$ 7.6 μg/ml with the coefficient of variation (%CV) of 15.8% was obtained from 5-FU test (n=6). For

the test against KB-3-1, the IC_{50} of 7.4 $\pm$ 0.8 ng/ml with the coefficient of variation (%CV) of 10.8% was obtained from vinblastine test (n=6).

Some Rubiaceous plants in screening process showed cytotoxic activity, which were corresponding to previously reported literature such as *Hymenodictyon oriexense*, *Canthium glabrum*, *Gardenia jasminoides*, *Uncaria macrophylla*, *Gardenia sootepensis*. In this research, ethanolic extract of *Hymenodictyon oriexense* leaves was inactive against MCF-7 and KB-3-1 cells. From the previous report, ethanolic extract of *H. oriexense* stembark was also inactive when tested in frog and mice[54]. Aswal, B.S. *et al.* found that ethanolic extract of *U. macrophylla* dried aerial parts was inactive against CA-9KB cells[50]. In this research, ethanolic extract of its leaves was inactive against both cell lines. Nair, S.C. *et al.* reported that ethanolic extract of *C. glabrum* dried aerial parts was inactive against CA-nasopharyngeal cells[24] as well as the ethanolic extract of its leaves used in this research was inactive against both cell lines. There was a report about active chemical substances of *G. sootepensis*, that were evaluated for their cytotoxic activity against a panel of human cancer cell lines[22]. Active compounds were coronalolide methyl ester and coronalolide. The potential activity of this plant had been interested. Accordingly, the other plants in the same genus were included for the cytotoxic activity test such as *G. jasminoides, G. obtusifolia,* and *G. erythoclada*. However, only *G. sootepensis* and *G.obtusifolia* were active against cell lines used in this study. From previously reported articles,[28, 29] ethanolic extracts of *G. jasminoides* commercial fruit was also inactive against HELA-S3, CA-JTC-26 cell lines.

While the extract from *G. sootepensis* showed activity against both cell lines (21.5 and 1.0 µg/ml for MCF-7 and KB-3-1 cells, respectively), *G. obtusifolia* Roxb. ex Kurz extract was against both cell lines (10.0 and 1.4 µg/ml for MCF-7 and KB-3-1 cells, respectively). However, *G. sootepensis* and its active constituents had been previously reported for its activity against a panel of cancer cell lines[22,77].

Therefore, *Gardenia obtusifolia* was further studies in order to isolate its active components by using bioassay-guided fractionation technique.

Table 3. Cytotoxic Activity Test of Rubiaceous Plants

Botanical Name	Parts used	IC_{50} (μg/ml)	
		MCF-7	KB-3-1
5-Fluorouracil (μg/ml)	-	47.8± 7.6	
Vinblastine (ng/ml)	-		7.4 ± 0.8
Borreria alata	roots, stem, leaves	-*	-*
Borrevia lavevis	roots, stem, leaves	-*	-*
Canthium glabrum	leaves	-*	-*
Catunaregam spathulifolia	leaves	-*	-*
Gardenia erythoclada	leaves	>100	>100
Gardenia jasminoides	leaves	>100	>100
Gardenia obtusifolia	leaves	10.0	1.4
Gardenia sootepensis	leaves	21.5	1.0
Haldina cordiflora	leaves	>100	>100
Hymenodictyon oriexense	leaves	-*	>100
Ixora cibdela var. *puberula*	leaves	>100	70
Ixora stricta	leaves	-*	100
Lasianthus kurzii	leaves	-*	>100
Mitragyna hirsuta	leaves	-*	>100
Mussaenda parva	leaves	-*	62.5
Paederia pilifera	roots, stem, leaves	-*	>100
Pavetta tomentosa var. *tomentosa*	leaves	>100	>100
Psychotria ophioxybides	leaves	>100	69.5
Tarennoidea wallichii	leaves	-*	-*
Uncaria macrophylla	leaves	-*	-*

*unable to compute IC_{50} from dose-response curve

Table 4. Cytotoxic Activity Categories of Rubiaceae

Categories	Botanical name
active ($IC_{50} \leq 20$ µg/ml)	*Gardenia obtusifolia*
	Gardenia sootepensis
moderately active ($20 < IC_{50} < 100$ µg/ml)	Ixora cibdela
	Mussaenda parva
	Psychotria ophioxybides
inactive ($IC_{50} \geq 100$ µg/ml)	*Haldina cordiflora*
	Hymenodictyon oriexense
	Ixora stricta
	Gardenia erythoclada
	Lasianthus kerri
	Mitragyna hirsuta
	Paederia pilifera
	Pavetta tomentosa
	Gardenia jasminoides
	Borreria alata
	Borrevia lavevis
	Canthium glabrum
	Catunaregam spathulifolia
	Tarennoidea wallichii
	Uncaria macrophylla

Remark : categorization of activity was based on IC_{50}s obtained from the inhibition of either one or both cell lines.

- Extraction of Active Components

After three times maceration of the dried powder of *Gardenia oftusifolia* leaves (1.2 kg) (Figure 10) with 95% ethanol, the wieght dried extract obtained was 159.0 gm, corresponding to 13.3% yield of the dried powder. This extract (79.8 gm) was further isolated by liquid–liquid partition following the scheme on page 52. Resulting extracts were extracts were tested for their cytotoxic activity against MCF-7 and KB-3-1 cells and found that the relatively nonpolar phases and moderately nonpolar contained active components (Table 5). The chloroform extract was found to be the most active fraction against both cell lines. The IC_{50}s obtained from this extract were 4.5 µg/ml for MCF-7 and 1.2 µg/ml for KB-3-1 cells. From chemical test, the chloroform extract gave the positive results to the test for phenolic compounds, deoxy sugar, flavonoids, and triterpenes (Table 6).

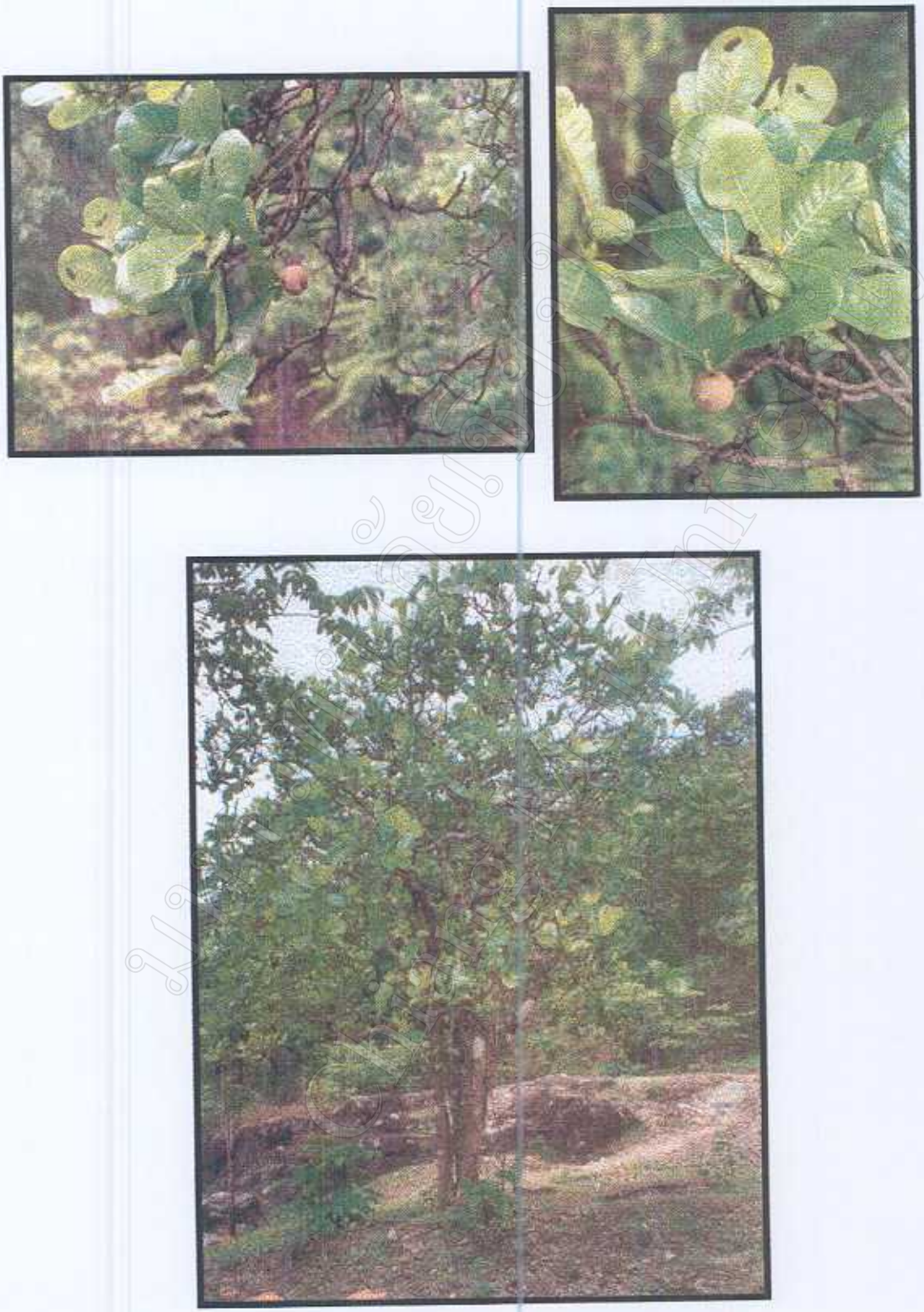


Figure 11. *Gardenia obtusifolia* Roxb. ex Kurz

Table 5. Cytotoxic Activity of *Gardenia obtusifolia* Extracts

Solvents (used for extraction)	% Yield	IC_{50} (µg/ml)	
		MCF-7	KB-3-1
5-fluorouracil (µg/ml)	-	47.8 ±7.6	
vinblastine (ng/ml)	-		7.4 ±0.8
alcohol	13.3	10.0	1.4
hexane	1.3	12.5	1.6
chloroform	8.5	4.5	1.2
ethyl acetate	1.1	-*	-*
aqueous	0.9	-*	-*

* unable to compute IC_{50} from dose-response curve

Table 6. Chemical Tests of *Gardenia obtusifolia* Extracts

Extracts	Phenolic Moiety	Flavonoids	Sugar	Unsaturated lactone ring	Deoxysugar	Triterpenes / Steroids	Saponins	Alkaloids	Anthraquinones
crude extract	+[a]	+	+	+	+	purple	-	-	-
hexane extract	-[b]	-	-	-	-	purple	-	-	-
chloroform extract	+	+	+	-	+	purple	-	-	-
ethyl acetate	+	+	+	+	+	-	-	-	-
aqueous extract	+	+	+	-	+	-	-	-	-

+[a] representing positive result

-[b] representing negative result

- Fractionation of the Chloroform Extract

In order to separate active components from the chloroform extract, chromatographic fractionation was carried out on a silica gel 60 column (7.5 cm i.d.). Silica gel packing material is suitable for the adsorption of polar components from nonpolar solvent system. The fractionation of the chloroform extract on silica gel 60 column provided 12 fractions. The cytotoxic activity test was carried out and revealed that the highest activity was obtained from chloroform:ethyl acetate (1:1) fraction (Fraction F) with the IC_{50} of 1.2 µg/ml against KB-3-1 and IC_{50} of 4 µg/ml against MCF-7. From the chemical test, the extract from this fraction was positive to flavonoids and triterpenes test (Table 6). Unlike other fractions, the chloroform:ethyl acetate (1:1) fraction gave highest percent yield. Therefore, the chloroform:ethyl acetate (1:1) fraction was chosen for the further studies.

Isolation of Active Compounds

- Isolation of Active Compounds From Chloroform:ethyl acetate (1:1) Fraction

The chloroform:ethyl acetate (1:1) fraction was further purified by using a silica gel 60 column as a stationary phase and chloroform/ethyl acetate gradient (Table 8) as a mobile phase. Eight fractions were obtained from the fractionation. Fraction 2 possessed an IC_{50} of 1 µg/ml against KB-3-1 and IC_{50} of 4 µg/ml against MCF-7. The fraction 3 exhibited cytotoxic activity with IC_{50} of 12 µg/ml against MCF-7 and IC_{50} of 1.9 µg/ml against KB-3-1. Both fraction 2 and fraction 3 were positive to the flavonoids and triterpenes test (Table 11).

In the last step, two active fractions (fr. 2 and 3) were rechromatographed on silica gel 60 column. Fraction 2 was isolated and gave eight subfractions (Table 9). A yellow compound crystallized from subfraction iii, iv, and v after these subfractions were concentrated. The yellow compound was washed with petroleum ether and filtered. The filtrate was concentrated and washed again. This yellow compound

designated as GO.1. The GO.1 exhibited cytotoxic activity against MCF-7 with the IC_{50} of 7 µg/ml, but was inactive against KB-3-1 cells.

Fraction 3 was isolated and gave six subfractions (Table 10). Fraction 3 was isolated and gave eight subfractions. A yellow compound was crystallized from subfraction d after concentration. The yellow compound was washed with petroleum ether and filtered. The filtrate was concentrated and washed again. Similarly, the yellow compound was designated as GO.2. Contrasting to the GO.1, the GO.2 exhibited cytotoxic activity against KB-3-1 with the IC_{50} of 2.5 µg/ml, but it was inactive against MCF-7. According to the above results, the GO.1 and the GO.2 exhibited selectivity differently against both cell lines. Purification to obtain the GO.1 and GO.2 generated pure compounds with lower cytotoxic activity compound to the activity of fractions 2 and 3. The rationale of this finding should be addressed in the future.

The chemical class of these two crystals revealed to be flavonoids (Table 11). From thin layer chromatography (TLC), high-performance liquid chromatography (HPLC), and narrow range of melting point these GO.1 and GO.2 were confirmed to be relatively pure substances (Figures 12-15). The GO.1 was found to be more nonpolar than the GO.2. When the criteria of cytotoxic activity were considered, the GO.2 has the IC_{50} less than 4 µg/ml and was considered as the cytotoxic substances, whereas the GO.1 was inactive.

Table 7. Cytotoxic Activity of Fractions from Chloroform Extract

Fraction	Eluent	%Yield	Remarks	IC_{50} (µg/ml)	
				MCF-7	KB-3-1
A	n-hexane, mixture of n-hexane and n-hexane :chloroform (7:3)	0.01	colorless solution	>100	>100
B	n-hexane : chloroform (7:3)	0.51	pale brown sticky mass	87	-*
C	mixture of n-hexane : chloroform (7:3) and chloroform	<0.01	pale yellow sticky mass	-*	-*
D	chloroform	0.55	brown - yellow sticky mass	>100	82
E	mixture of chloroform and chloroform : ethyl acetate (1:1)	<0.01	black sticky mass	51	20
F	chloroform : ethyl acetate (1:1)	0.71	black - brown sticky mass	4	1.2

Table 7. (cont.)

Fraction	Eluent	%Yield	Remarks	IC_{50} (µg/ml)	
				MCF-7	KB-3-1
G	mixture of chloroform : ethyl acetate (1:1) and ethyl acetate	0.01	yellow sticky mass	48	22
H	ethyl acetate	0.14	brown sticky mass	26	22
I	mixture of ethyl acetate and ethyl acetate : methanol (1:1)	0.01	yellow sticky mass	38	22
J	ethyl acetate : methanol (1:1)	1.02	black brown sticky mass	42	21
K	mixture of ethyl acetate : methanol (1:1) and methanol	<0.01	black brown sticky mass	35	3.1
L	methanol	0.26	pale brown sticky mass	84	26

* unable to compute IC_{50} from dose-response curve

Table 8. Cytotoxic Activity of Fractions from Chloroform:Ethyl acetate (1:1) Extract

Fraction	Eluent	%Yield	Remarks	IC_{50} (µg/ml)	
				MCF-7	KB-3-1
1	chloroform	0.02	black brown sticky mass	100	27
2	chloroform	0.13	black brown sticky mass	4	1
3	gradient of ethyl acetate in chloroform (0%, 5%)	0.08	yellow brown sticky mass	12	1.9
4	gradient of ethyl acetate in chloroform (10%, 20%, 30%, 50%)	0.05	yellow brown sticky mass	22	12
5	gradient of ethyl acetate in chloroform (50%, 70%); 50% methanol in ethyl acetate	0.06	brown sticky mass	28	23
6	30%,50% ethyl acetate in methanol	0.08	brown sticky mass	50	24
7	30% ethyl acetate in methanol	0.04	brown sticky mass	53	26
8	methanol	0.01	brown sticky mass	28	12

Table 9. Cytotoxic Activity of Subfractions from Fraction 2 Extract

Subfraction	Eluent	%Yield	Remarks	IC_{50} (µg/ml)	
				MCF-7	KB-3-1
i	2% n-hexane in chloroform	0.01	black mass	62	10
ii	2% n-hexane in chloroform	0.03	black brown mass	7	2
iii	2% n-hexane in chloroform	<0.01	yellow mass	14	2
iv	2% n-hexane in chloroform; chloroform	<0.01	yellow mass	6	1.6
v	chloroform	<0.01	yellow mass	11	2.1
vi	chloroform : diethyl ether (1:1)	<0.01	yellow mass	20	2.1
vii	chloroform : diethyl ether (1:1)	<0.01	yellow mass	9	1.9
viii	chloroform : diethyl ether (3:7); diethyl ether; methanol	<0.01	yellow mass	20	1.9
GO.1 compound	-	0.03	yellow crystals	7	-*

* unable to compute IC_{50} from dose-response curve

Table 10. Cytotoxic Activity of Subfractions from Fraction 3 Extract

Subfraction of fraction 3	Eluent	%Yield	Remarks	IC_{50} (µg/ml)	
				MCF-7	KB-3-1
a	10% n-hexane in chloroform	0.01	black mass	93	>100
b	10% n-hexane in chloroform	<0.01	yellow mass	19	2.5
c	10% n-hexane in chloroform	<0.01	yellow mass	60	8
d	10% n-hexane in chloroform; chloroform	0.01	yellow mass	16	18
e	chloroform	<0.01	yellow mass	42	14
f	chloroform : diethyl ether (1:1), (3:7); diethyl ether; methanol	0.01		41	30
GO.2 compound	-	<0.01	yellow crystals	-*	2.5

* unable to compute IC_{50} from dose-response curve

Table 11. Chemical Tests of Active Fractions and Compounds

Fractionation	Phenolic Moiety	Flavonoids	Sugar	Unsaturated lactone ring	Deoxysugar	Triterpenes / Steroids	Saponins	Alkaloids	Anthraquinones
$CHCl_3$:EtOAc (1:1)	+[a]	+	-[b]	+	-	purple	-	-	-
Fraction 2	+	+	-	-	-	purple	-	-	-
Fraction 3	+	+	-	-	-	purple	-	-	-
GO. 1 compound	+	+	-	-	-	-	-	-	-
GO. 2 compound	+	+	-	-	-	-	-	-	-

+[a] representing positive result

-[b] representing negative result

Figure 12. TLC chromatograms of fraction 2 and 3 of chloroform:ethyl acetate (1:1) fraction, and GO.1, GO.2 on silica gel 60 developed by chloroform:ethyl acetate (7:3), detected with UV spectrophometer at λ 254, 365 nm. ($Rf_{GO.1}$= 0.42, $Rf_{GO.2}$= 0.26)

Figure 13. TLC chromatograms of fraction 2 and 3 of chloroform:ethyl acetate (1:1) fraction, and GO.1, GO.2 on silica gel 60 developed by chloroform:diethyl ether (7:3), detected with UV spectrophometer at λ 254, 365 nm. ($Rf_{GO.1}$= 0.81, $Rf_{GO.2}$= 0.44)

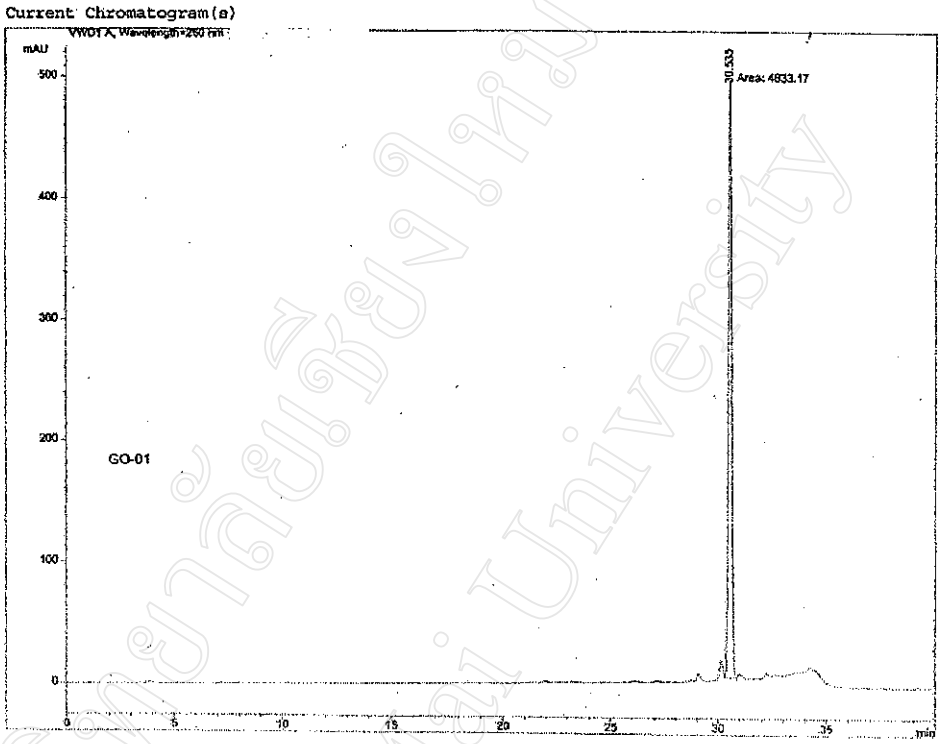


Figure 14. HPLC Chromatogram of GO.1, retention time 30 min.

Chromatographic conditions: Column Inertsil® ODS-3, 5 µm, i.d. 4.6 mm x 250 mm length; mobile phase-gradient elution with methanol in 0.1% trifluoroacetic acid, linear gradient of 15% methanol to 95% methanol with in 30 minutes; flow rate 1.00 ml/min; ultraviolet detection at wavelength 260 nm

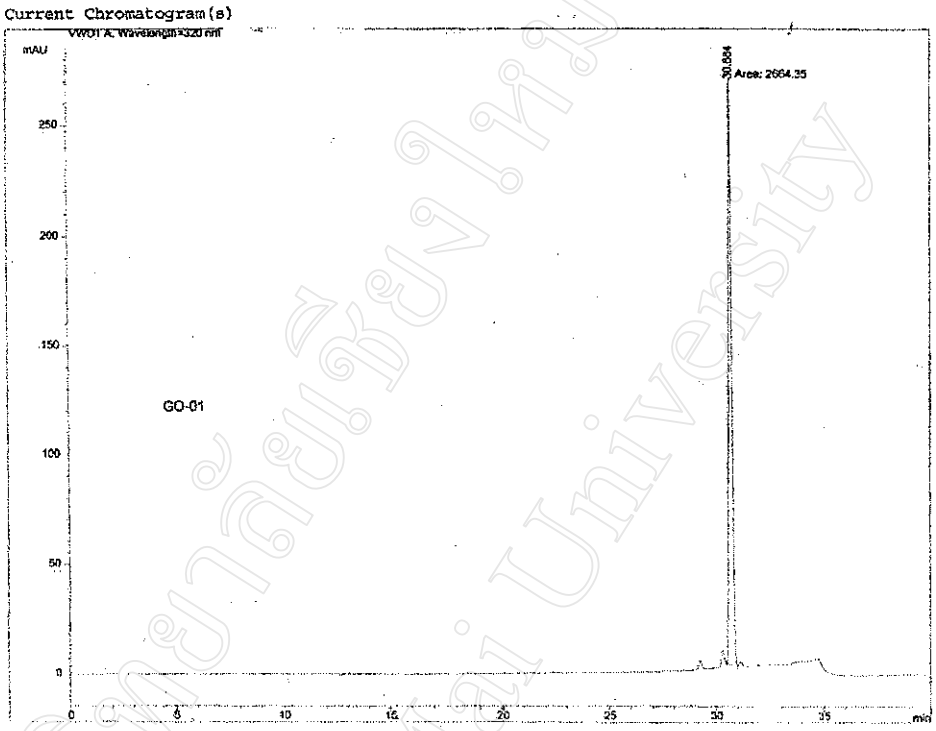


Figure 14. HPLC Chromatogram of GO.1, retention time 30 min, cont.

Chromatographic conditions: Column Inertsil® ODS-3, 5 µm, i.d. 4.6 mm x 250 mm length; mobile phase-gradient elution with methanol in 0.1% trifluoroacetic acid, linear gradient of 15% methanol to 95% methanol with in 30 minutes; flow rate 1.00 ml/min; ultraviolet detection at wavelength 320 nm

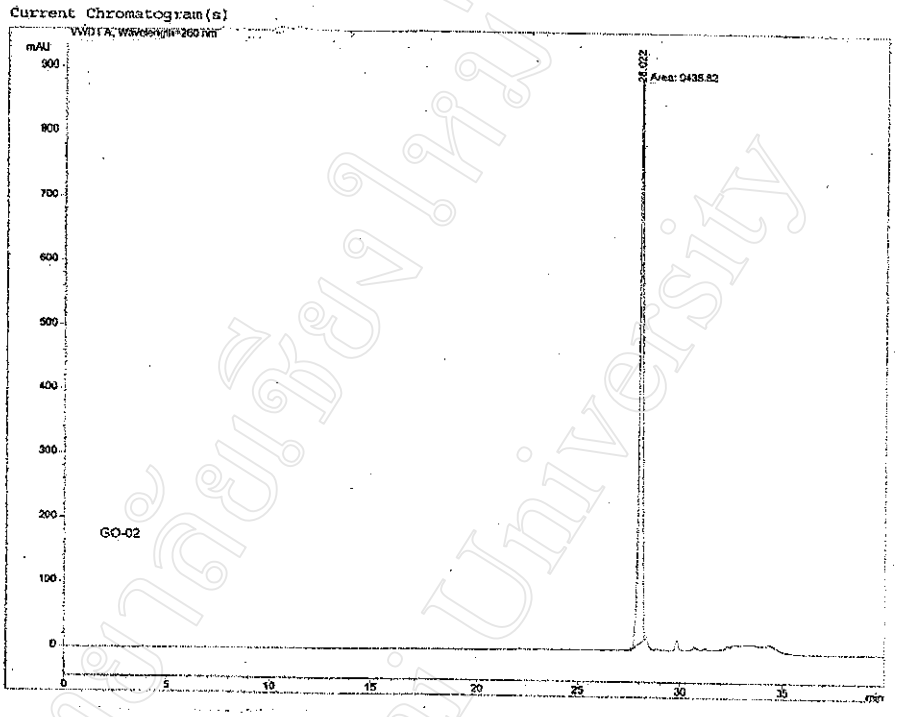


Figure 15. HPLC Chromatogram of GO.2, retention time 28 min.

Chromatographic conditions: Column Inertsil® ODS-3, 5 μm, i.d. 4.6 mm x 250 mm length; mobile phase-gradient elution with methanol in 0.1% trifluoroacetic acid, linear gradient of 15% methanol to 95% methanol with in 30 minutes; flow rate 1.00 ml/min; ultraviolet detection at wavelength 260 nm

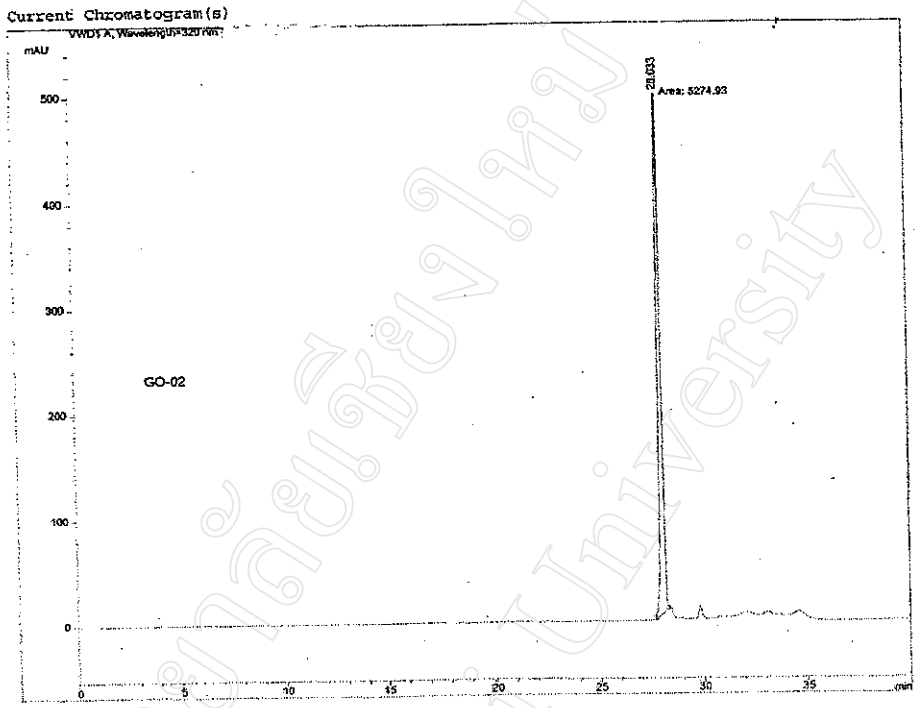


Figure 15. HPLC Chromatogram of GO.2, retention time 28 min, cont.

Chromatographic conditions: Column Inertsil® ODS-3, 5 µm, i.d. 4.6 mm x 250 mm length; mobile phase-gradient elution with methanol in 0.1% trifluoroacetic acid, linear gradient of 15% methanol to 95% methanol with in 30 minutes; flow rate 1.00 ml/min; ultraviolet detection at wavelength 320 nm

Part II Physicochemical Properties

- GO.1

Compound GO.1 was obtained as yellow amorphous powder in larger amounts (0.02 % yield from dried leaves powder) than the GO.2. Its melting point (mp.) was 166.7-168.8 °C. The IR spectrum[73] revealed strong absorption bands of OH (3100-3700 cm^{-1}, broad), C-H (2800-3000 cm^{-1}, weak), C=O (1650-1705 cm^{-1}, medium), C=C (1600-1500 cm^{-1}, strong), C-O (1200-1400 cm^{-1}, strong) (Figures 17, 18). However, medium intensity of C=O absorption was observed, which could be due to hydrogen bonding between C=O and OH on C-5 and C-4. The IR spectra showed the presence of hydroxyl and aromatic groups.

The presence of a flavonoid skeleton (Figure 19) in its molecule was suggested by UV spectrum which displayed characteristic absorption maxima (λ_{max}) of flavonoid at 260, 278, and 348 nm (Figure 16)[78]. The UV spectra of most flavonoids are consisted of two major absorption maxima, one of which occurs in the range of 240-285 nm (band II, ring A) and the other in the range of 300-400 nm (band I, ring B) [79]. Types of flavonoids such as flavones, flavonols, isoflavones, flavanones, dihydroflavonol could be distinguished by using the UV spectra. Except flavones and flavonols, the other flavonoids mentioned above do not have the conjugation between the A- and B- rings. The UV charecteristics of these compounds likely exhibited low intensity of band I absorption and a shoulder on band II[79]. Therefore, the structure of GO.1 could be either flavones or flavonols which showed two dominant UV absorption bands of band I (B-ring) and band II (A-ring). The UV spectrum of GO.1 (Figure. 16) showed absorption maxima of band I and band II at 348 nm and 260-278 nm, respectively. The absorption of band I is useful for distinguishing flavones from flavonols, since band I UV absorption of flavones is between 340-350 nm, while band I UV absorption of flavonols is between 352-385 nm. From this information, the GO.1 was likely to be a flavone.

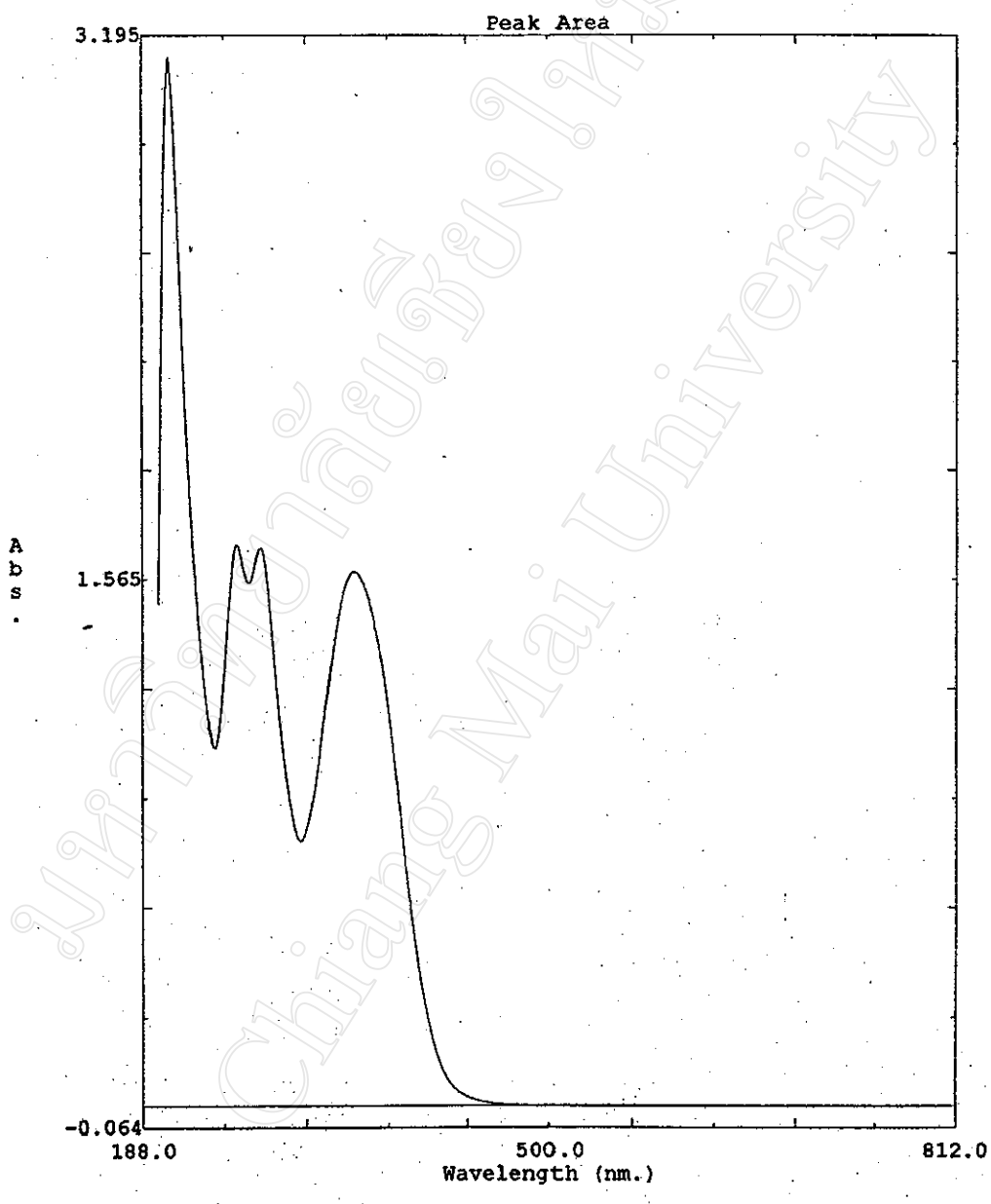


Figure 16. UV spectrum of GO.1 (in methanol)

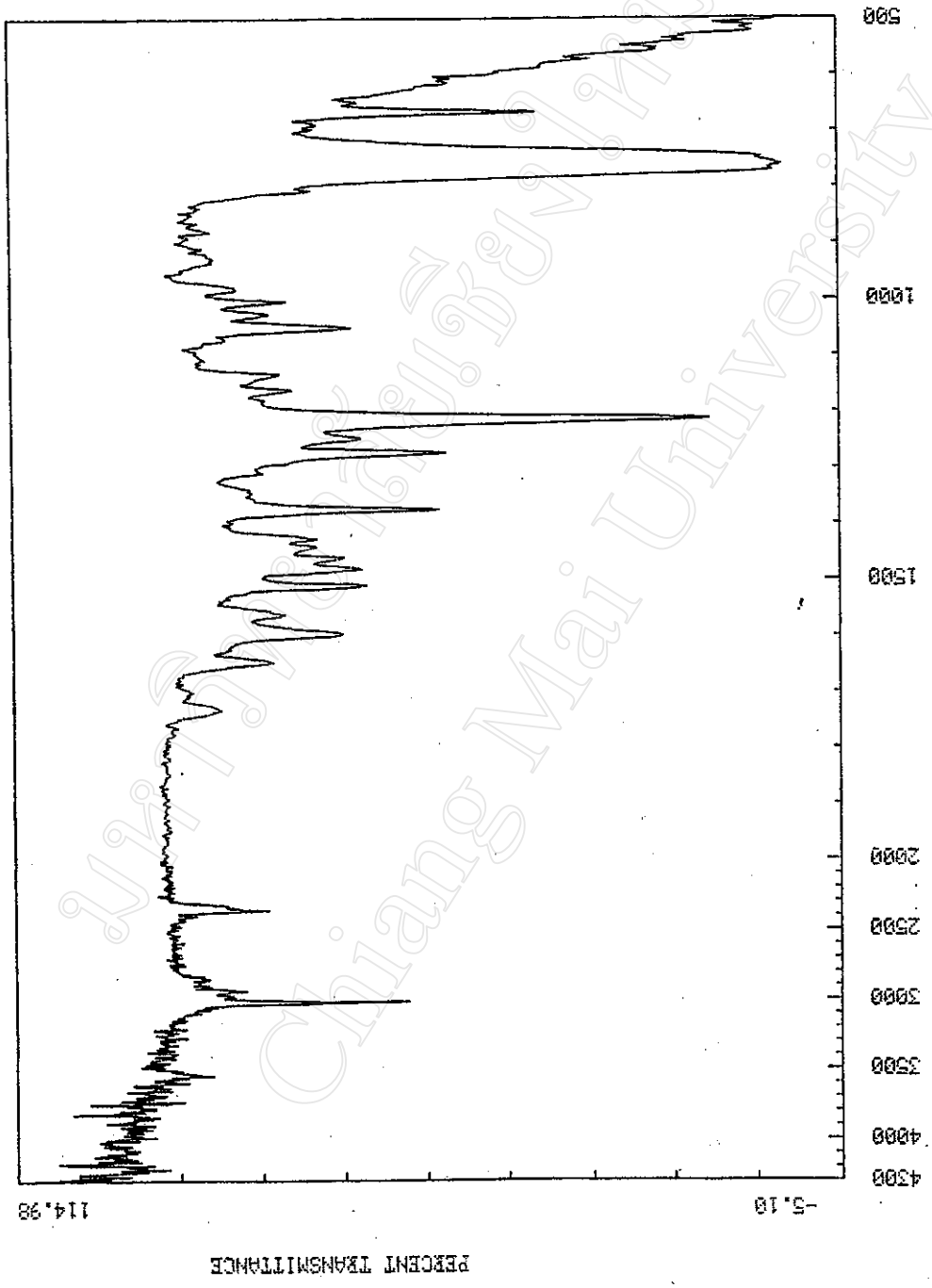


Figure 17. IR spectrum of GO.1 (dry film on sodium chloride)

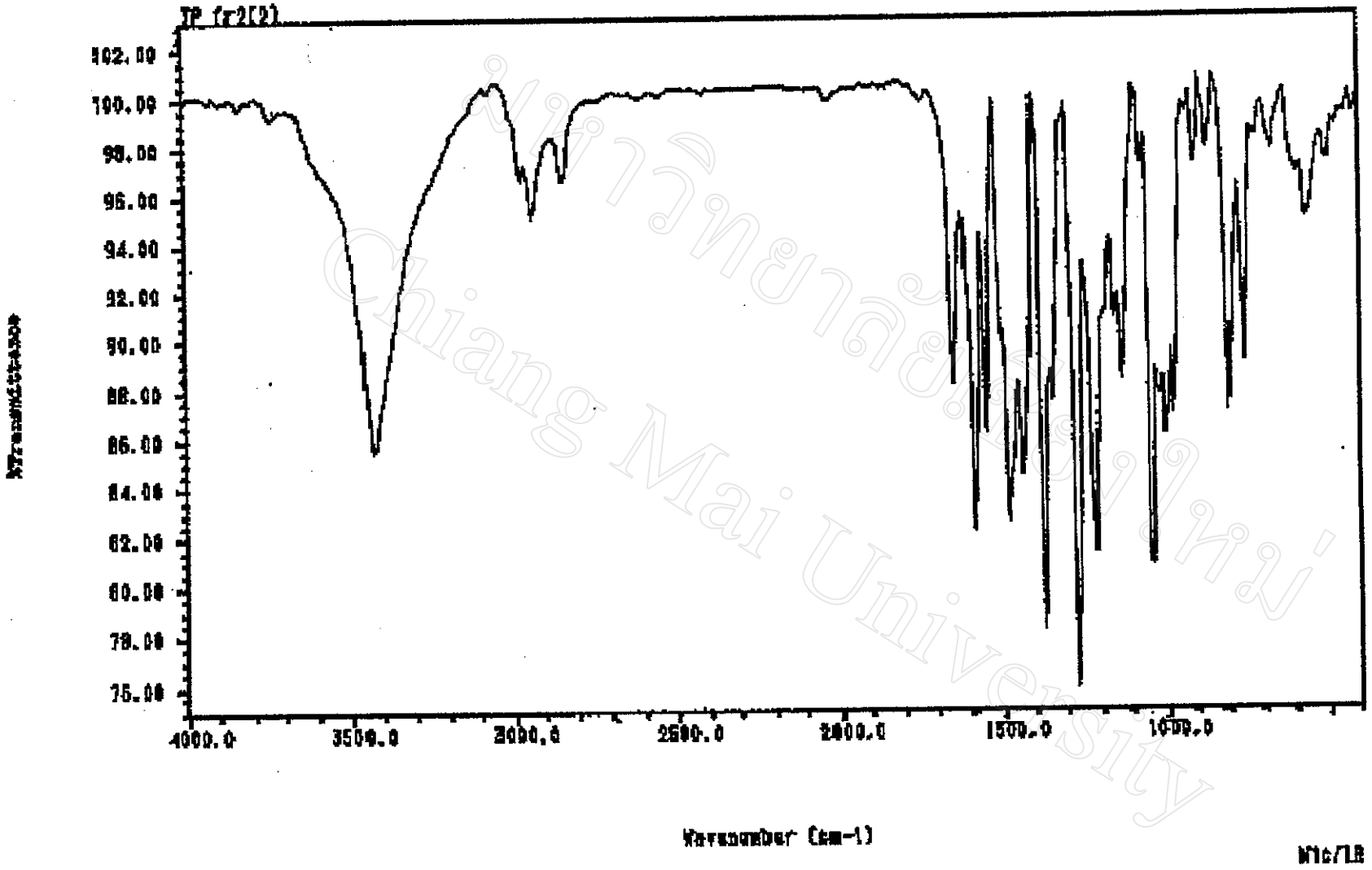


Figure 18. IR spectrum of GO.1 (KBr, pressed disc technique)

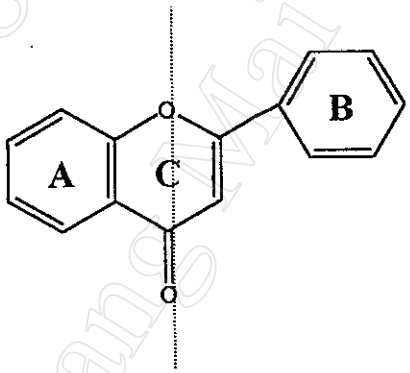
In addition to UV spectrum, spectral data from ^{1}H- and ^{13}C- NMR were more useful to assign the GO.1 structure. The GO.1 can be assigned as a known flavone; 5,3'-dihydroxy–3,6,7,8,4'-pentamethoxyflavone[80-83] (Figure 20). The ^{1}H-NMR spectrum provided the signals of 3 aromatic protons, in addition to 5 methoxyl, 2 hydroxyl groups. The ^{13}C-NMR spectrum showed the signal of 1 carbonyl carbon, 5 methoxyl carbons, and 15 aromatic carbons.

The signals of hydroxyl protons in ^{1}H-NMR spectrum at 12.400 and 5.734 ppm were assigned as 5-OH, 3'-OH, respectively. The aromatic proton signals were the signals of H-2' (d, J = 1.8 Hz), H-5' (d, J = 8.5 Hz), and H-6' (dd, J = 1.8, 8.5 Hz). The remaining signals were the signals of OCH_3-3, OCH_3-6, OCH_3-7, OCH_3-8, and OCH_3-4' (Table 12, Figures 22-23).

The ^{13}C-NMR spectrum supported the ^{1}H-NMR assignment. The spectrum exhibited 20 carbons. The carbonyl carbon signal at 179.361 ppm was assigned as C-4. The signals at 149.120 and 145.633 ppm were assigned as C-5 and C-3', respectively. The signals at 60.139, 62.150, 61.724, 61.170, and 56.069 ppm were assigned as OCH_3-3, OCH_3-6, OCH_3-7, OCH_3-8, and OCH_3-4'. The remaining signals were the signals of aromatic carbons (Table 13, Figure 24).

However, the exact structure of the GO.1 could not be proposed by using only available data, since the positions of problematic hydroxyl and methoxyl groups on B ring could be interchangeable. In order to confirm the structure of the GO.1, the chemical shifts obtained from ^{1}H-NMR spectrum were directly compared with the chemical shifts of reference compounds reported in literature[63] (Table 12, Figure 21). The chemical shifts obtained from ^{1}H-NMR spectrum of the GO.1 were closely matched to the chemical shifts of the reference compound I (5,3'-dihydroxy-3,6,7,8,4'-pentamethoxyflavone), where the hydroxyl and methoxyl groups were on 3' and 4', respectively. Thus, this research was proposed the structure of GO.1 as 5,3'-dihydroxy-3,6,7,8,4'-pentamethoxyflavone. From previous reports, this

compound was isolated from *Gutierrezia microcephala*, *Polanisia trachysperma*, *Calycadenia truncata*, *Calycadenia mollis* and *Polanisia dodecandra* and revealed its cytotoxic activity against various cancer cell lines[80-83]. This flavone and other 78 related structures were studied for their structure cytotoxic activity relationship (SAR)[85]. The substitution on the B ring may be quite stringent. The reversal of hydroxyl and methoxyl groups on 3' and 4' positions changed the mean GI_{50} of the compounds I (from 0.13 μM to 7.6 μM). The substitution of 3-methoxyl group on C ring is essential for cytotoxic activity compared to a 3-hydroxyl or hydrogen substituent.



Band II; Benzoyl Band I; Cinnamoyl

Figure 19. Flavonoid skeleton[79]

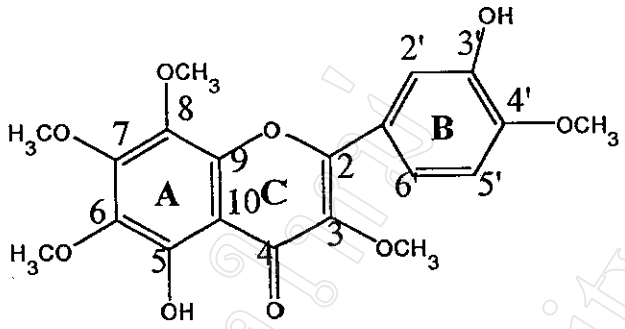
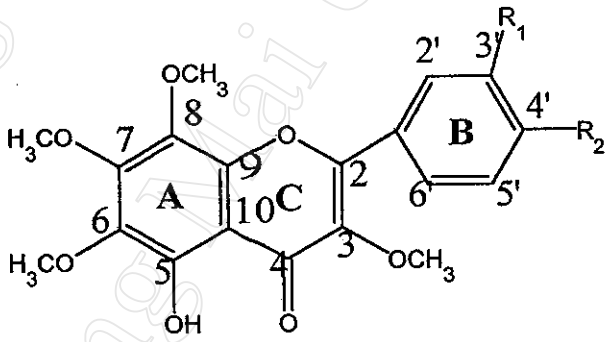


Figure 20. Proposed structure of GO.1, 5,3'-dihydroxy–3,6,7,8,4'-pentamethoxyflavone



Reference Compounds	substituted groups	
	R_1	R_2
I	-OH	$-OCH_3$
II	$-OCH_3$	-OH

Figure 21. Structures of reference compounds[83]:

I = 5,3'-dihydroxy–3,6,7,8,4'-pentamethoxyflavone

II = 5,4'-dihydroxy–3,6,7,8,3'-pentamethoxyflavone

Table 12. Comparison of ^{1}H-NMR data of GO.1 and Reference compounds (I and II)

H-position	δ (ppm) and Category		
	GO.1 in this study* ($CDCl_3$)	Reference compounds ($CDCl_3$)**[83]	
		I	II
OH-5	12.400, s	12.40, s	12.39, s
OCH_3-3	3.880, s	3.88, s	3.88, s
OCH_3-6	3.956, s	3.95, s	3.95, s
OCH_3-7	4.106, s	4.11, s	4.11, s
OCH_3-8	3.948, s	3.96, s	3.96, s
OH-3'	*5.734 s*	*5.73, s*	-
OCH_3-3'	-	-	*3.98, s*
OH-4'	-	-	*6.02, s*
OCH_3-4'	*3.997 s*	*4.00, s*	-
H-2'	7.776 d (J = 1.8 Hz)	7.77, d (J = 2.2 Hz)	7.80, d (J = 1.5 Hz)
H-5'	6.995 d (J = 8.5 Hz)	7.00, d (J = 7.0 Hz)	7.07, d (J = 7.5 Hz)
H-6'	7.792 dd (J = 1.8, 8.5 Hz.)	7.78, dd (J = 2.2, 7.0 Hz)	7.78, dd (J = 1.5, 7.5 Hz)

s = singlet , d = doublet, dd = doublet of doublet, J = coupling constant, * = JOEL JNM-A500; 500 MHz, ** = Bruker AC-300; 300 MHz.

Table 13. Comparison of ^{13}C-NMR data of GO.1 and Reference compounds (I and II)

C-position	δ (ppm)		
	GO.1 in this study* ($CDCl_3$)	Reference compounds** ($CDCl_3$)[83]	
		I	II
C-2	155.868	155.8	155.9
C-3	138.907	138.9	138.6
C-4	179.361	179.4	179.3
C-5	149.120	149.1	149.1
C-6	136.142	136.2	136.2
C-7	152.909	152.9	152.9
C-8	132.903	132.9	132.9
C-9	144.948	144.9	144.9
C-10	107.510	107.5	107.5
C-1'	123.685	123.5	122.5
C-2'	114.551	114.6	110.8
C-3'	145.633	145.6	146.4
C-4'	148.992	149.0	148.6
C-5'	110.510	110.5	114.9
C-6'	121.630	121.6	122.8
OCH_3-3	60.139	60.1	60.1
OCH_3-6	62.150	62.1	62.1
OCH_3-7	61.724	61.7	61.7
OCH_3-8	61.170	61.2	61.2
OCH_3-4'	56.069	56.1	-
OCH_3-3'	-	-	56.0

* = JOEL JNM-A500; 125 MHz, ** = Bruker AC-300; 300 MHz.

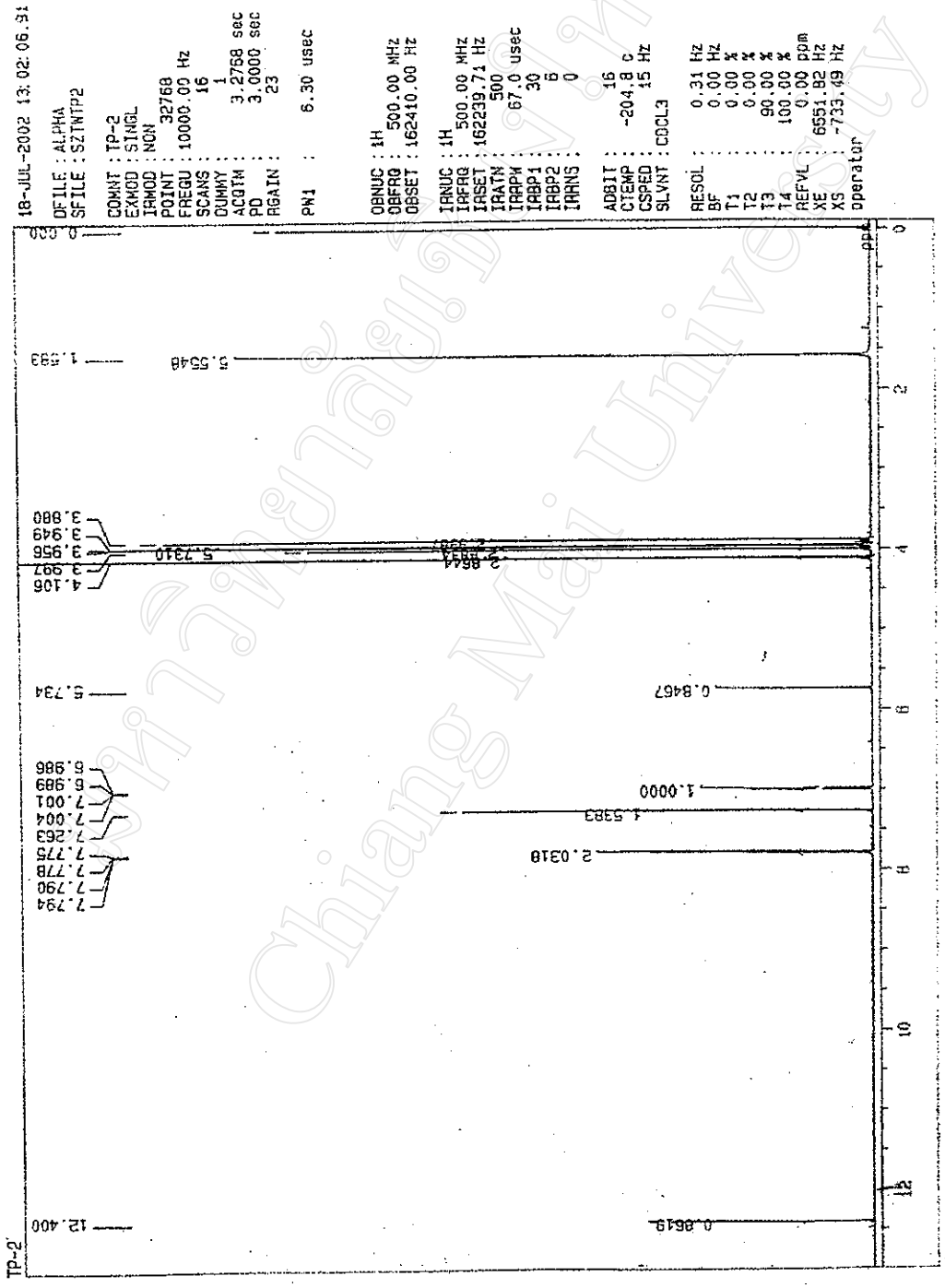


Figure 22. ^{1}H-NMR spectrum of GO.1 (in $CDCl_3$, 500 MHz)

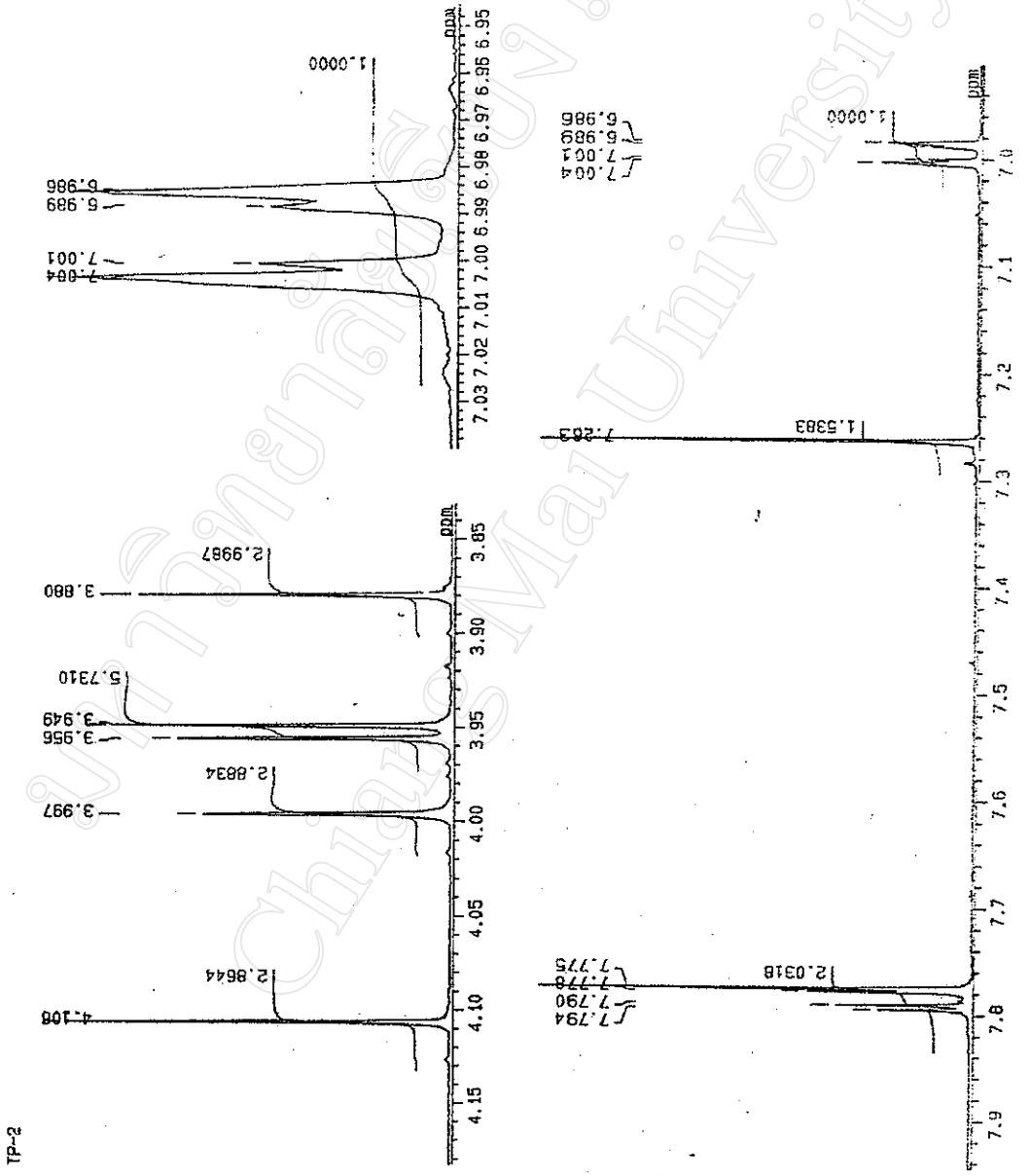


Figure 22. ^{1}H-NMR spectrum of GO.1 (in $CDCl_3$, 500 MHz), cont.

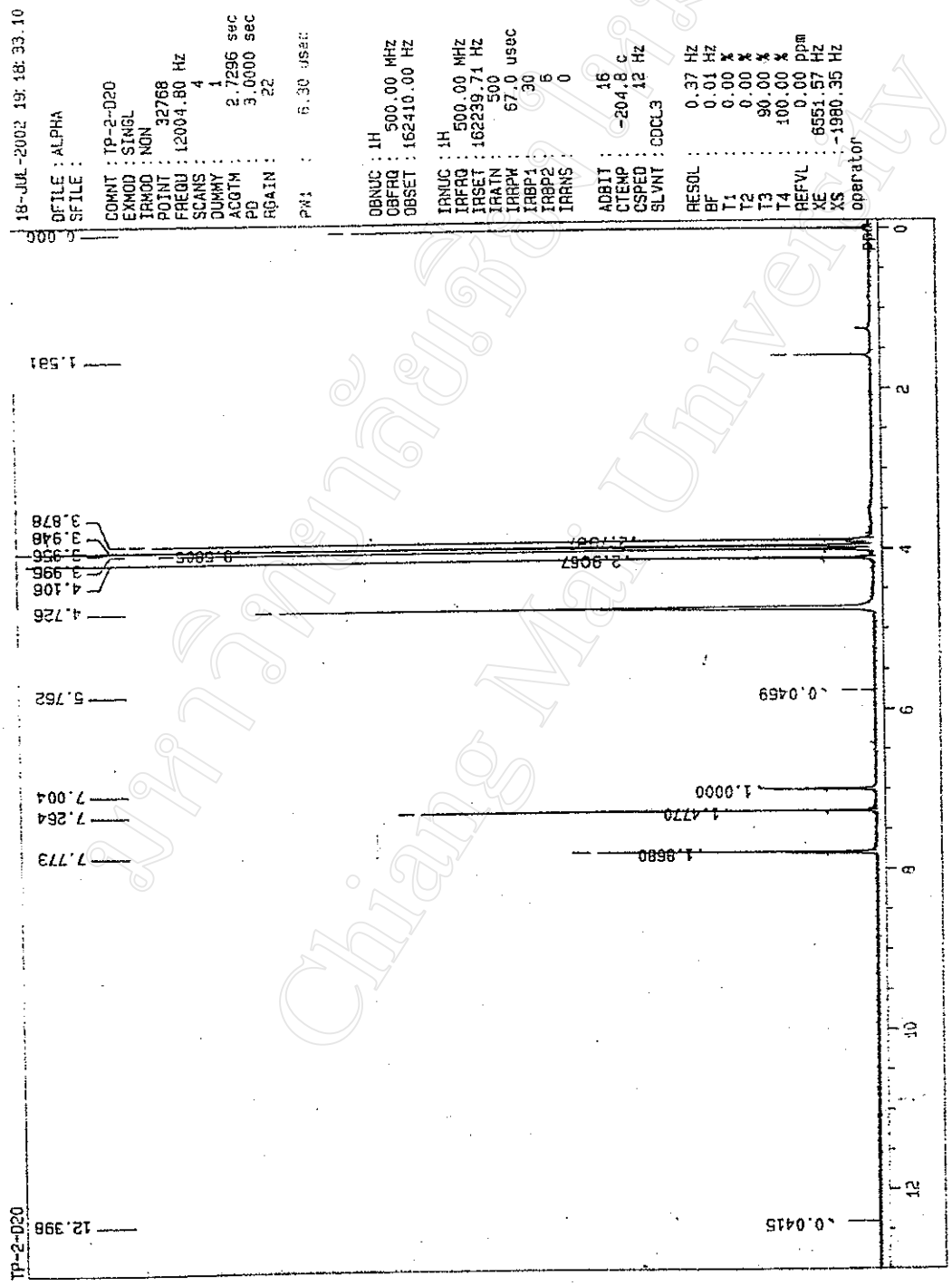


Figure 23. ^{1}H-NMR spectrum of GO.1 (add D_2O , in $CDCl_3$, 500 MHz)

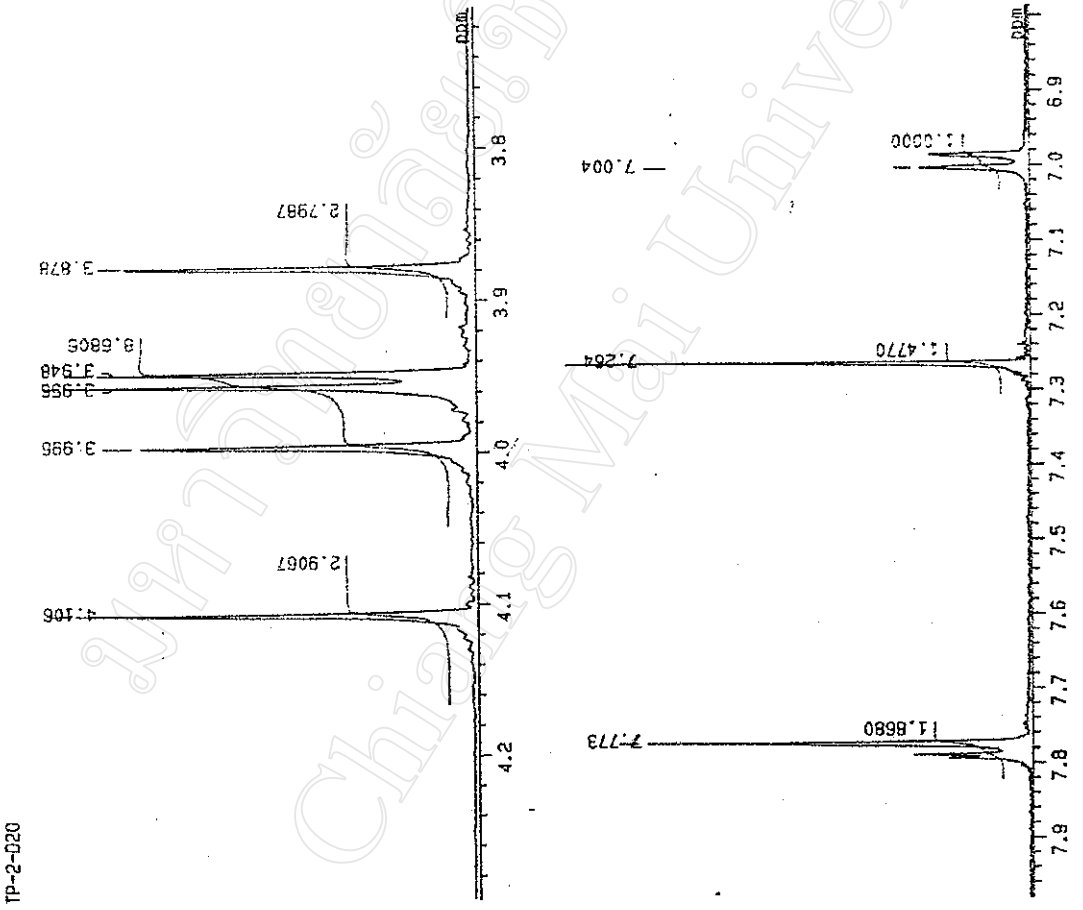


Figure 23. ^{1}H-NMR spectrum of GO.1 (add D_2O, in $CDCl_3$, 500 MHz), cont.

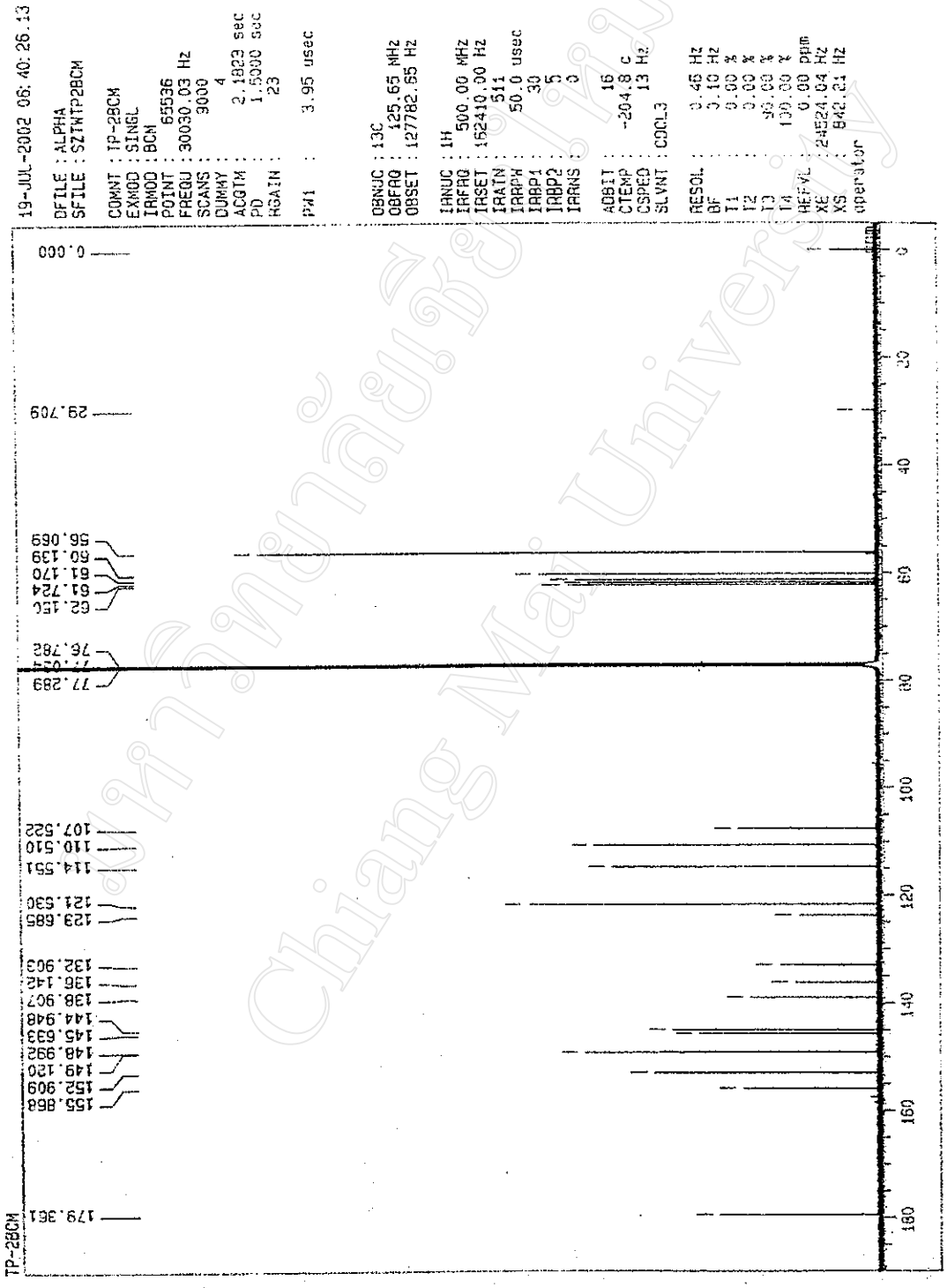


Figure 24. ^{13}C-NMR spectrum of GO.1 (in $CDCl_3$, 125 MHz)

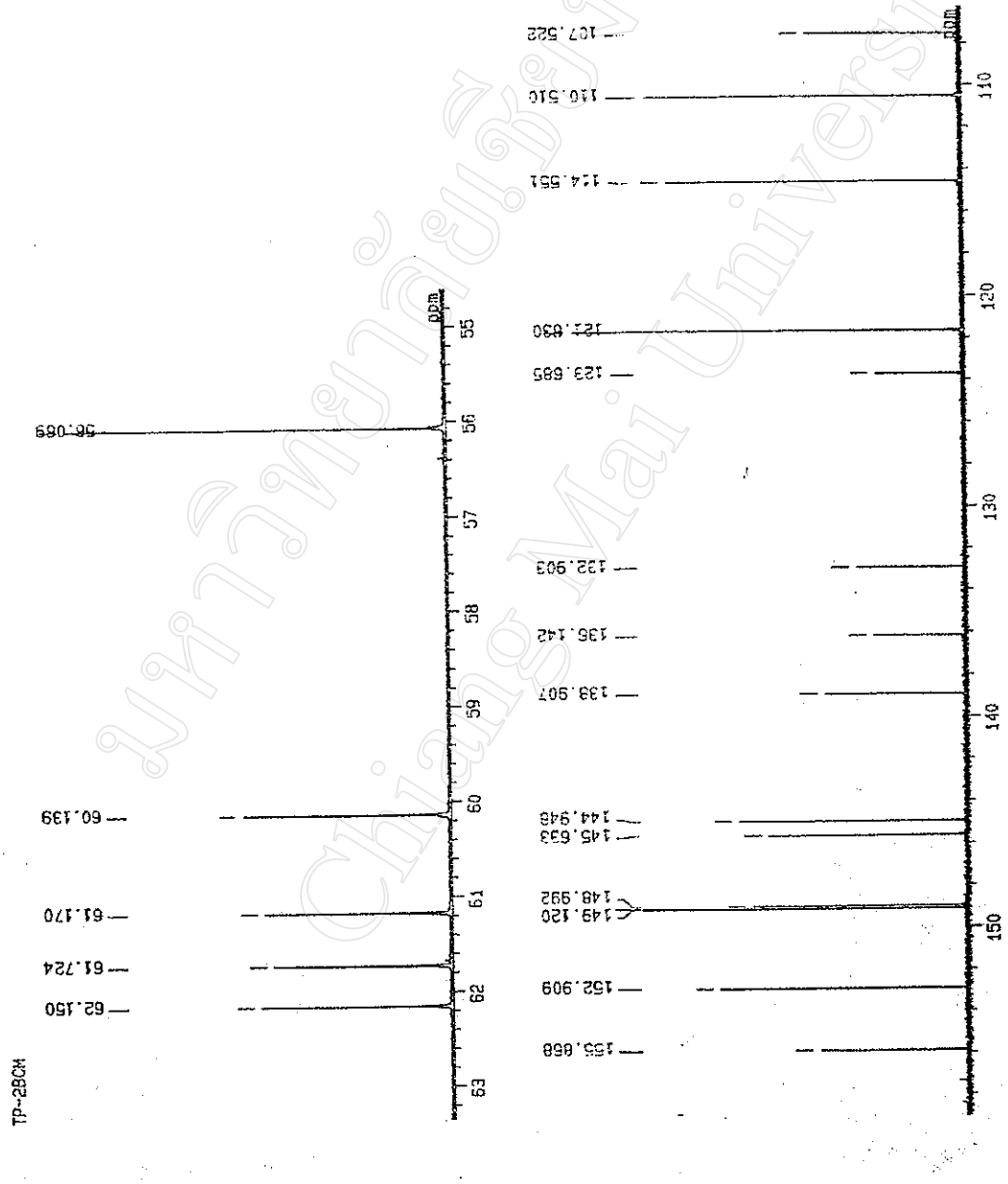


Figure 24. ^{13}C-NMR spectrum of GO.1 (in $CDCl_3$, 125 MHz), cont.

- GO.2

Compound GO.2 was obtained as yellow amorphous powder, mp. 206.6-208.4 °C in relatively small amounts (<0.01 % yield from dried leaves powder) compared to the GO.1. The IR spectrum revealed strong absorption of C-H (2800-3000 cm^{-1}), C=C (1600-1500 cm^{-1}), C-O (1200-1400 cm^{-1}) (Figure 26). The presence of a flavonoid skeleton in its molecule was suggested by UV spectrum which displayed characteristic absorption maxima of flavonoid (λ_{max}) at 259, 275, and 353 nm (Figure 25) and a possible flavone skeleton[78,79]. However, the exact structure of GO.2 could not be proposed due to a limit quantity of sample.

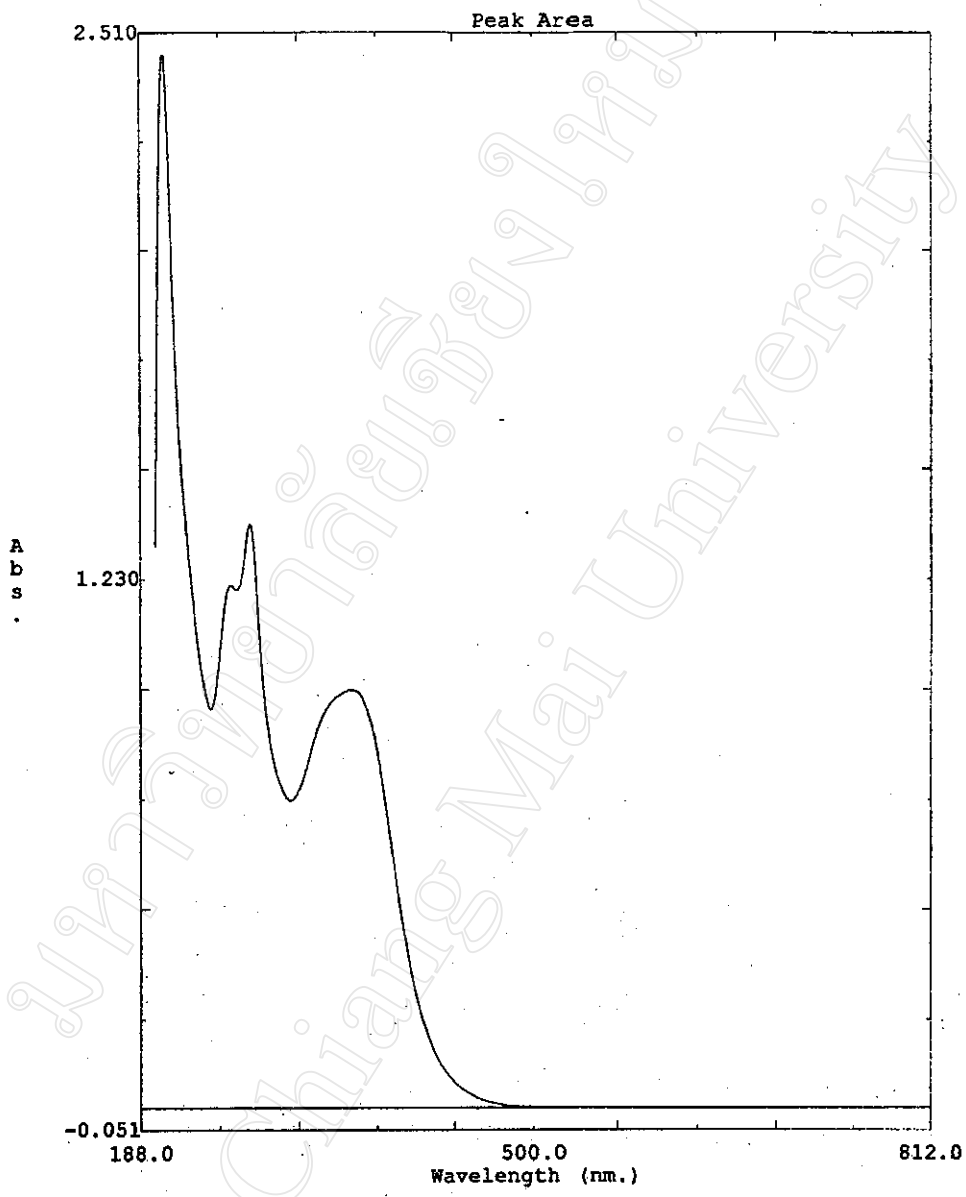


Figure 25. UV spectrum of GO.2 (in methanol)

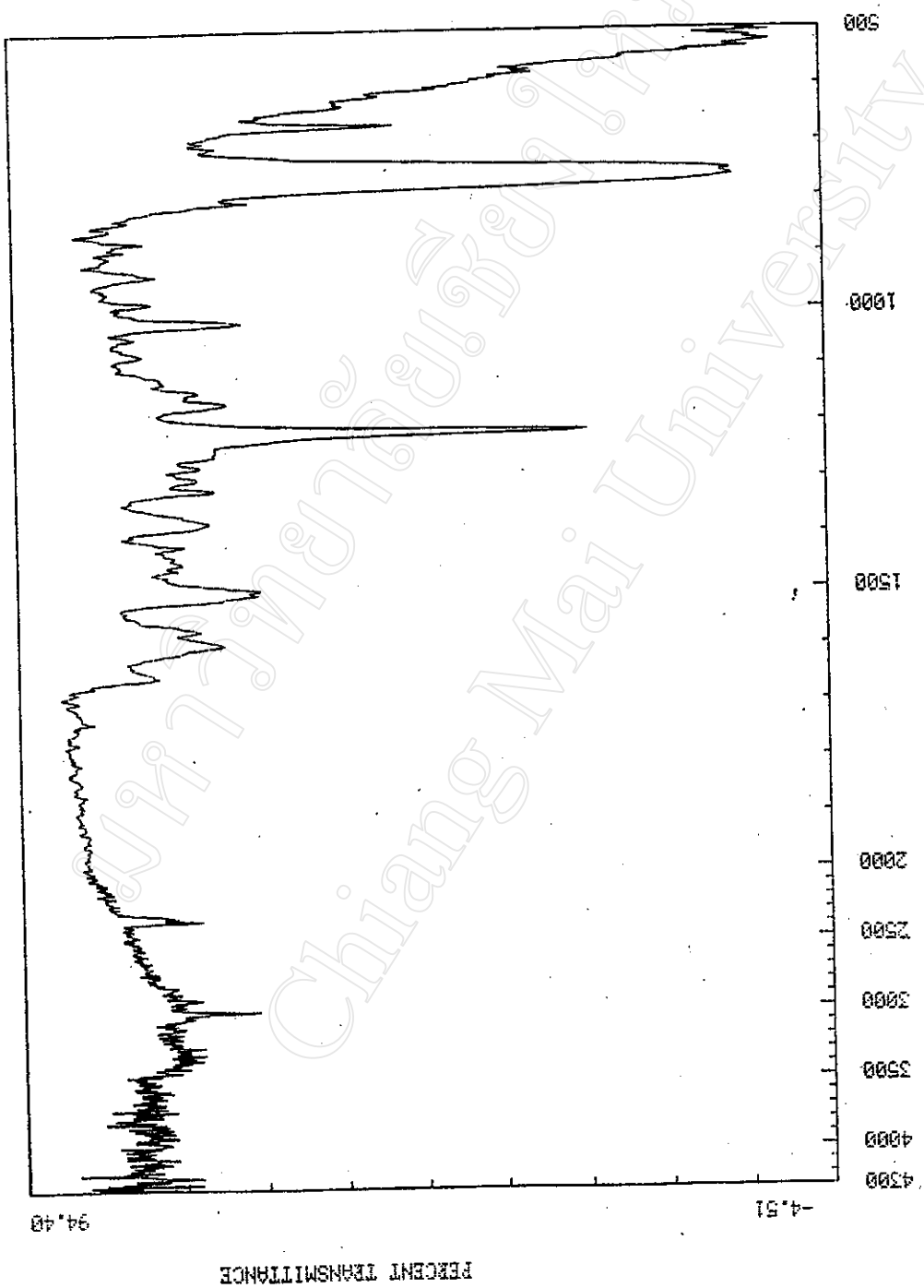


Figure 26. IR spectrum of GO. 2 (dry film on sodium chloride)