CHAPTER IV

RESULT AND DISCUSSION

Part I : Phytochemical Study

The fresh stem bark of *Derris reticulata* Craib was macerated with ethanol. The ethanol extract was then separated by chromatographic technique to afford four pure compounds. The structure elucidations of the isolated compounds were based on data from UV, IR, NMR and Mass spectra, and further comfirmed by comparison with the data reported literatures, as discussed below.

Structure Elucidation of DR-1

DR-1 was obtained as yellow needle crystals from Fraction C (Table 21) by column chromatographic technique using a gradient system of chloroform-ethyl acetate-methanol as eluents.

The IR spectrum of DR-1 (Figure 28) show strong OH absorption at 3250 cm^{-1}. The presence of one or more phenolic OH groups was indicated by the strong colouration with ethanolic ferric chloride. The band at 1620 cm^{-1} was assigned to the chelated flavanone CO group in the molecule. The feature of UV spectrum (Figure 29) showed the characteristic of a flavanone chromophore.

The nature of the groups present in DR-1 structure was indicated by its ^1H-NMR spectrum (Table 22, Figure 32-32.2). The doublets at δ 6.59 (J = 10.0 Hz) and 5.60 ppm (J = 10.0 Hz), each equivalent to one proton, and the singlets at δ 1.43 and 1.44 ppm, together integrating for six protons, are characteristic of the *cis* double bond and *gem*-dimethyl group of a 2,2-dimethylchromene moiety. The presence of a C-γ,γ-dimethylallyl group was inferred from the singlets at δ 1.64 and 1.61 ppm, together integrating for six protons, the doublet at δ 3.19 (2H, J = 7.82 Hz) and the triplet at δ 5.15 ppm (1H, J = 7.33 Hz) of three protons in the allyl group.

Signals due to four aromatic protons (B-ring) were discernible at δ 6.90 (2H, J = 8.3 Hz) and δ 7.39 ppm (2H, J = 8.3 Hz) and these could be readily analysed in

terms of a *p*-disubstituted benzene ring. The salient feature of the NMR spectrum of DR-1 is the ABX system, diagnostic for the C_2 and C_3 protons of a flavanone. The C_2 proton, the X part, appears as a double doublet at δ 5.44 (J_{AX} = 12.7, J_{BX} = 3.0) while the C_3 protons, the AB part, appear at δ 3.14 (J_{AX} = 12.7, J_{AB} = 15.0 Hz) and δ 2.87 ppm (J_{BX} = 3.0, J_{AB} = 15.0 Hz). The two signals of C_3 protons were assigned, according to the *J*-values, for the *trans*- (axial) and *cis*-(equatorial) protons respectively.

The presence of a chelated C_5-OH was evident from the signal of a phenolic proton which appeared as a singlet at low field position (δ 12.43 ppm), whereas the singlet at δ 8.43 was attributed to the other phenolic proton at C-4' position. Chemical evidence for the presence of two phenolic OH groups in DR-1 was provided by acetylation to give dimethyl ether. The NMR spectrum was shown in Figure 30.3.

The substitution pattern of DR-1 was determined from NMR and mass spectral data. The mass spectrum (Figure 27) showed that the base peak at *m/z* 391 (M-15) which resulted by the loss of methyl group of a dimethylchromene moiety. The fragment at *m/z* 351 (M-55) confirmed the presence of a dimethylallyl group ; it might have resulted by the rupture of the bond at the benzylic carbon adjacent to the A-ring. the fragments at *m/z* 271 and *m/z* 120 could be rationalized only if the non-chelate OH group and the γ,γ-dimethylallyl side-chain were assigned to the B- and A-ring, respectively. The non-chelated OH group is therefore located at C-4' while the γ,γ-dimethylallyl side-chain could be located at either C-6 or C-8.

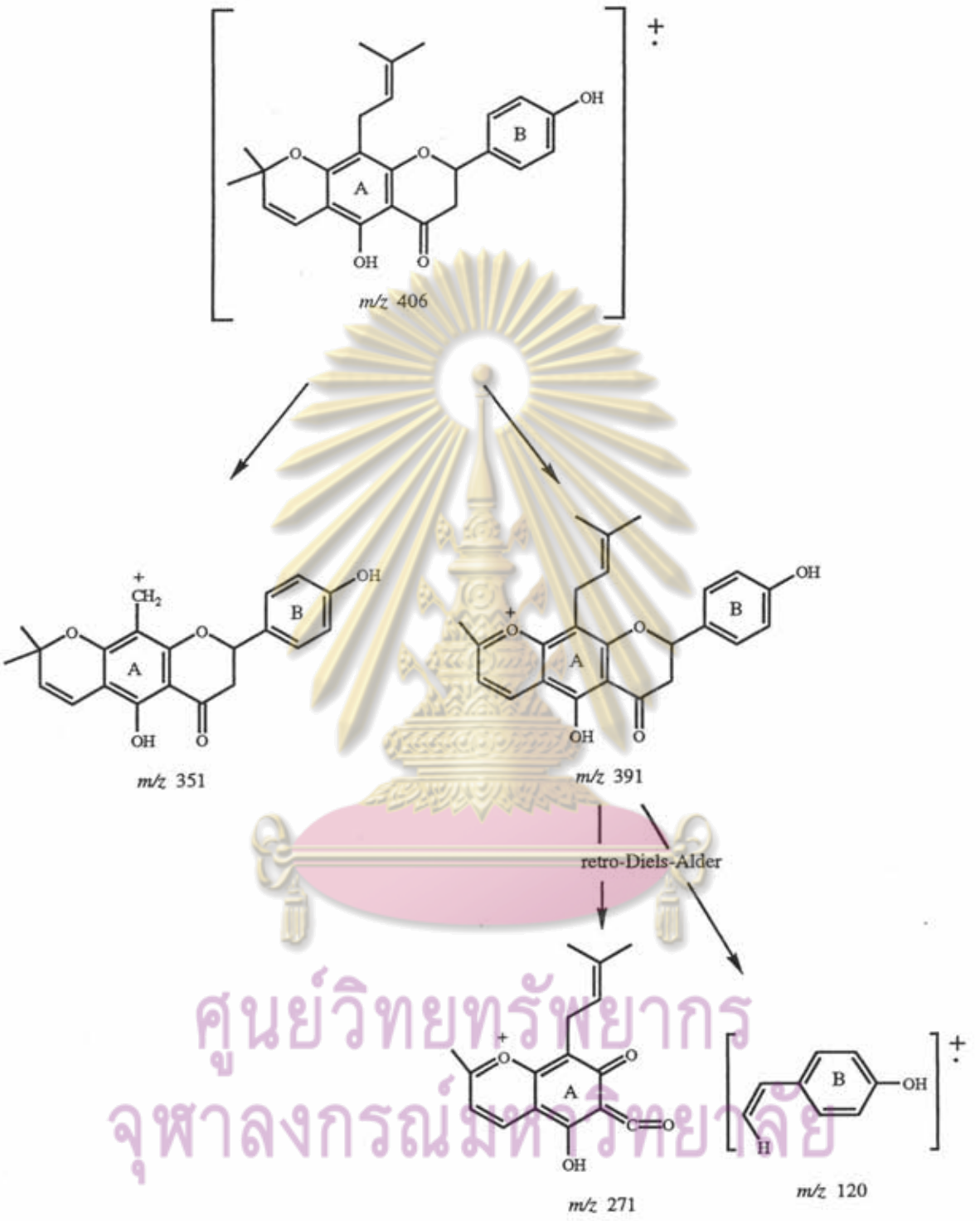
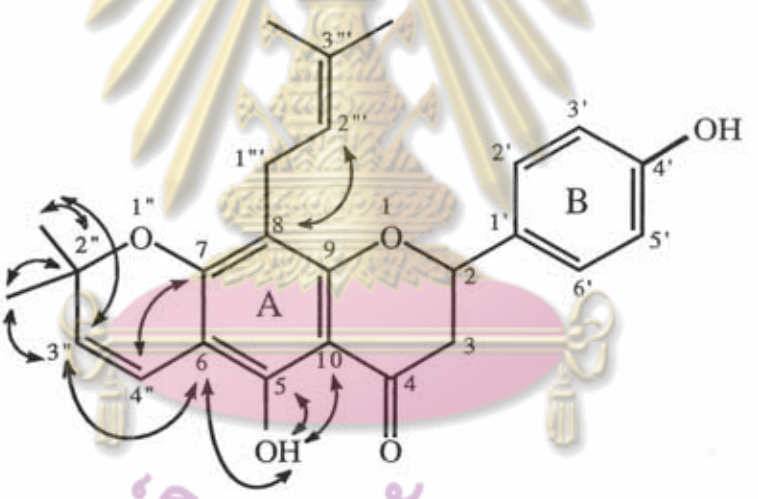


Figure 24 Mass spectral fragmentation of DR-1

The heteronuclear multiple bond connectivity (HMBC) and the correlation spectroscopy via long range coupling experiments (COLOC) were confirmed that the dimethylallyl group is located at C-8. The HMBC exhibited the C-H long range (2-3 bonds) correlation of DR-1 that C-8 carbon (δ 109.26) showed the correlation to C-2''' proton.

The expansion of its spectrum exhibited the correlation of C-2" carbon (δ 79.01 ppm) showed the correlation to 2"-CH_3 proton signals, and the 2"-CH_3 carbons (δ 28.50 ppm) correlated to C-3" proton. The C-10 carbon (δ 103.52 ppm), C-5 carbon (δ 157.52 ppm) and C-6 carbon (δ 103.46 ppm) showed the correlation to 5-OH proton and C-6 carbon also correlated to C-3" proton. At δ 160.30 ppm, C-7 carbon showed the correlation to C-4" proton. It indicated that the dimethylallyl group is located at C-8.



m/z 406

The proton assignments of DR-1 were confirmed by comparison of the recorded data of lupinifolin, a known flavanone isolated from *Tephrosia lupinifolia* (Smalberger, Vleggaar and Weber, 1974) and *Lonchocarpus miniflorus* (Mahmoud and Waterman, 1985). The proposed proton assignments of DR-1 are shown in Table 34.

Table 34 Proposed ^{1}H-NMR Assignment of DR-1

H-Position	δ H (multiplicity, *J* (H_3))	Category
2	5.44 (dd, 12.7, 3.0)	H
3	2.87 (dd, 15.0, 3.0)	H-*cis*
	3.14 (dd, 15.0, 12.7)	H-*trans*
5-OH	12.43 (s)	OH
2',6'	7.39 (br d, 8.3)	2H
3',5'	6.90 (d, 8.3)	2H
4'-OH	8.43 (s)	OH
2"-CH_3	1.43 (s)	CH_3
	1.44 (s)	CH_3
3"	5.60 (d, 10.0)	H
4"	6.59 (d, 10.0)	H
1'"	3.19 (br d, 7.82)	2H
2'"	5.15 (t, 7.33)	H
3'"-CH_3	1.61 (br s)	CH_3
	1.64 (br s)	CH_3

The ^{13}C-NMR spectrum (Figure 31-31.1) supported this structure. This reported data was based on the interpretation of spectra obtained from various ^{13}C-NMR techniques which included the proton decoupling, the ^{13}C-^{1}H HETCOR, the HMBC and the distortionless enhancement by polarization transfer (DEPT) experiment. They indicated twenty-five carbon signals which were the signals of 11 quaternary carbons, 9 methine carbons, 1 methylene carbons and 4 methyl carbons. The proposed carbon assignments of DR-1 are shown in Table 35.

Table 35 Proposed ^{13}C-NMR Assignments of DR-1

C-Position	δ (ppm)	Category
2	80.00	CH
3	43.64	CH_2
4	198.09	C
5	157.52	C
6	103.46*	C
7	160.30	C
8	109.26	C
9	160.69	C
10	103.52*	C
1'	131.04	C
2'	128.98	CH
3'	116.39	CH
4'	158.79	C
5'	116.39	CH
6'	128.98	CH
2"	79.01	C
2"-CH_3	28.50	CH_3
2"-CH_3	28.50	CH_3
3"	127.13	CH
4"	116.52	CH
1'"	22.25	CH_2
2'"	123.75	CH
3'"	131.37	C
3'"-CH_3	26.09	CH_3
3'"-CH_3	18.18	CH_3

* interchangeable

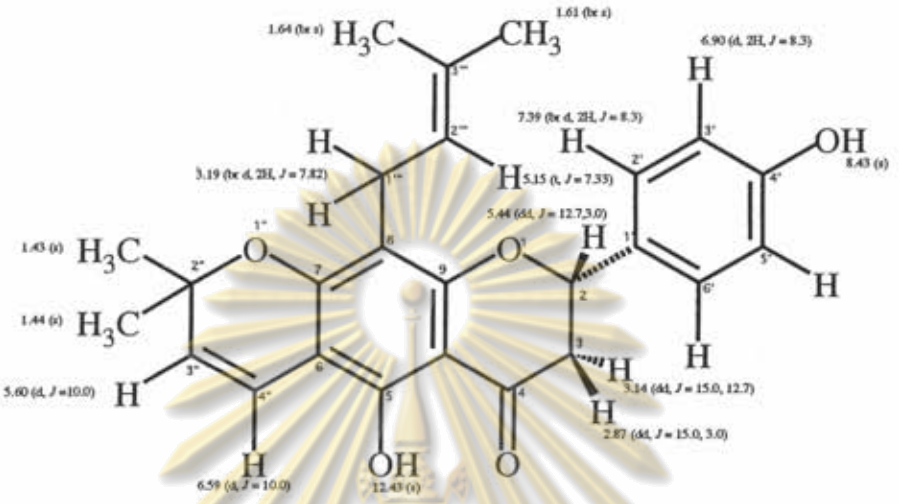
In this investigation, an attempt to completely assign all the ^{13}C-NMR signals was made the results from analysis of the HMBC and COLOC spectrum of DR-1 and suggested that the previously published (van Zyl, Rall and Roux, 1979) assignment should be revised. The three signals (C-6, C-8 and C-10) were in the range of non-oxygenated carbon with *ortho/para* oxygenation (90-125 ppm) (Harborne and Mabry,

1982). The two closely located signals at δ 103.46 and 103.52 ppm were assigned to C-6 and C-10 respectively (or might be interchanged) owing to the similar environment of the two carbons. Thus the last signal of the group at 109.26 ppm was arbitrary assigned to C-8.

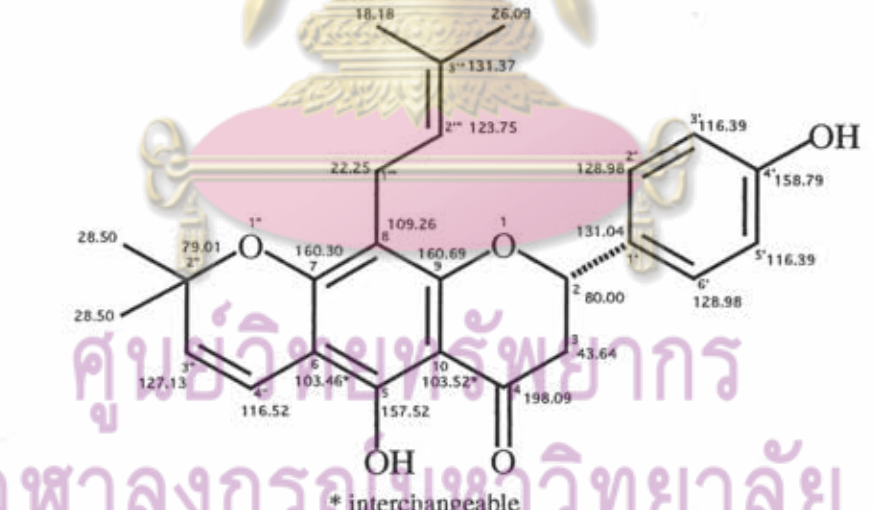
The HMBC and COLOC experiments showed that C-3" proton correlated to C-6 carbon at δ 103.46 ppm and C-8 carbon (δ 109.26 ppm) correlated to C-2"' proton.

In addition, the assignments of C-6 carbon (δ 108.1 ppm) and C-8 carbon (δ 102.2 ppm) (both quaternary carbons) in the previous report (van Zyl, Rall and Roux, 1979) should be revised.

From ^{1}H-NMR and ^{13}C-NMR assignments, DR-1 was determined as lupinifolin. The structure of which with ^{1}H-NMR and ^{13}C-NMR assignments are shown below.



^{1}H-NMR assignment of DR-1



^{13}C-NMR assignment of DR-1

Structure Elucidation of DR-2

DR-2 was obtained as white amorphous powders from Fraction E (Table 21) by column chromatographic technique with petroleum ether : ethyl acetate : methanol (7:3:1) as eluents.

The ^{1}H-NMR spectrum of DR-2 showed a heterocyclic olefinic proton of isoflavone (H-C-2) as a sharp singlet in a low magnetic field at δ 8.24 ppm. A ring of this isoflavone must have been hydroxylated at both C-5 and C-7. Together with a hydrogen-boned C-5-OH resonating at δ 12.88 ppm and a singlet at δ 6.36 ppm assignable to an A-ring proton not at C-8 but at C-6. The ^{1}H-NMR at δ 6.92 and 7.64 ppm (both 2H, two d, *J*=8.0 Hz) were characteristics of 4'-hydroxylated B-ring protons.

The three sets of proton signals attributable to a prenyl group were assigned at δ 1.64 and 1.76 ppm (each 3H, two br s), 3.43 (2H, br d, *J*=6.0 Hz) and 5.17 ppm (1H, t). The proposed proton assigments of DR-2 are shown in Table 36.

Table 36 Proposed ^{1}H-NMR Assignment of DR-2.

H-position	DR-2 δ H (multiplicity, *J*(Hz)	Lupiwighteone δ H (multiplicity, *J*(Hz)	Category
2	8.24 (s)	8.26 (s)	H
5-OH	12.88 (s)	12.98 (s)	OH
6	6.36 (s)	6.37 (s)	H
2', 6'	7.64 (d, *J*=8.0 Hz)	7.46 (d, *J*=8.7)	2H
3', 5'	6.92 (d, *J*=8.0 Hz)	6.90 (br d, *J*=8.7)	2H
1"	3.43 (br d, *J*=6.0 Hz)	3.45 (br d, *J*=7.3)	2H
2"	5.17 (t)	5.25 (t)	H
4"	1.76 (br s)	1.81 (br s)	CH_3
5"	1.64 (br s)	1.66 (br s)	CH_3

The assignments of protons was confirmed by comparison of the data of lupiwighteone (in acetone -d_6) previously reported by Hashidoko, Tahara and Mizutani, 1986.

The IR spectrum of DR-2 (Figure 36) suggested the presence of a hydroxyl group and a ketone group. The IR spectrum assignments of DR-2 were shown in Table 37.

Table 37 IR Spectrum Assignment of DR-2

Range of Absorption (cm^{-1})	Intensity	Assignment
3400, 3200	medium	O-H stretching of R-OH
1660	high	C=C stretching of alkene
1580	weak	C=O stretching
1260	high	C-O stretching
1415	high	O-H inplane bending of phenol
820, 840, 880	medium	out-of-plane C-H bending of aromatic hydrocarbons

In the MS spectrum, the molecular ion was detected at *m/z* 338 ($C_{20}H_{18}O_5$) ; fragments *m/z* 323 (M^+-15), 283 (M^+-55) and 270 (M^+-68, arising from fission at a prenyl side-chain). Significantly, the mass fragmentation loss parts of the prenyl side-chain followed by retro-Diels-Alder (RDA) fission to give fragments at *m/z* 165 and 118 as shown in Figure 35.

From the retro-Diels-Alder fragments at *m/z* 118 and *m/z* 165 could be rationalized only that the non-chelated OH-groups are located at C-4' and C-7 to the B and A ring respectively (Figure 25). These are in agreement with ^{13}C-NMR data.

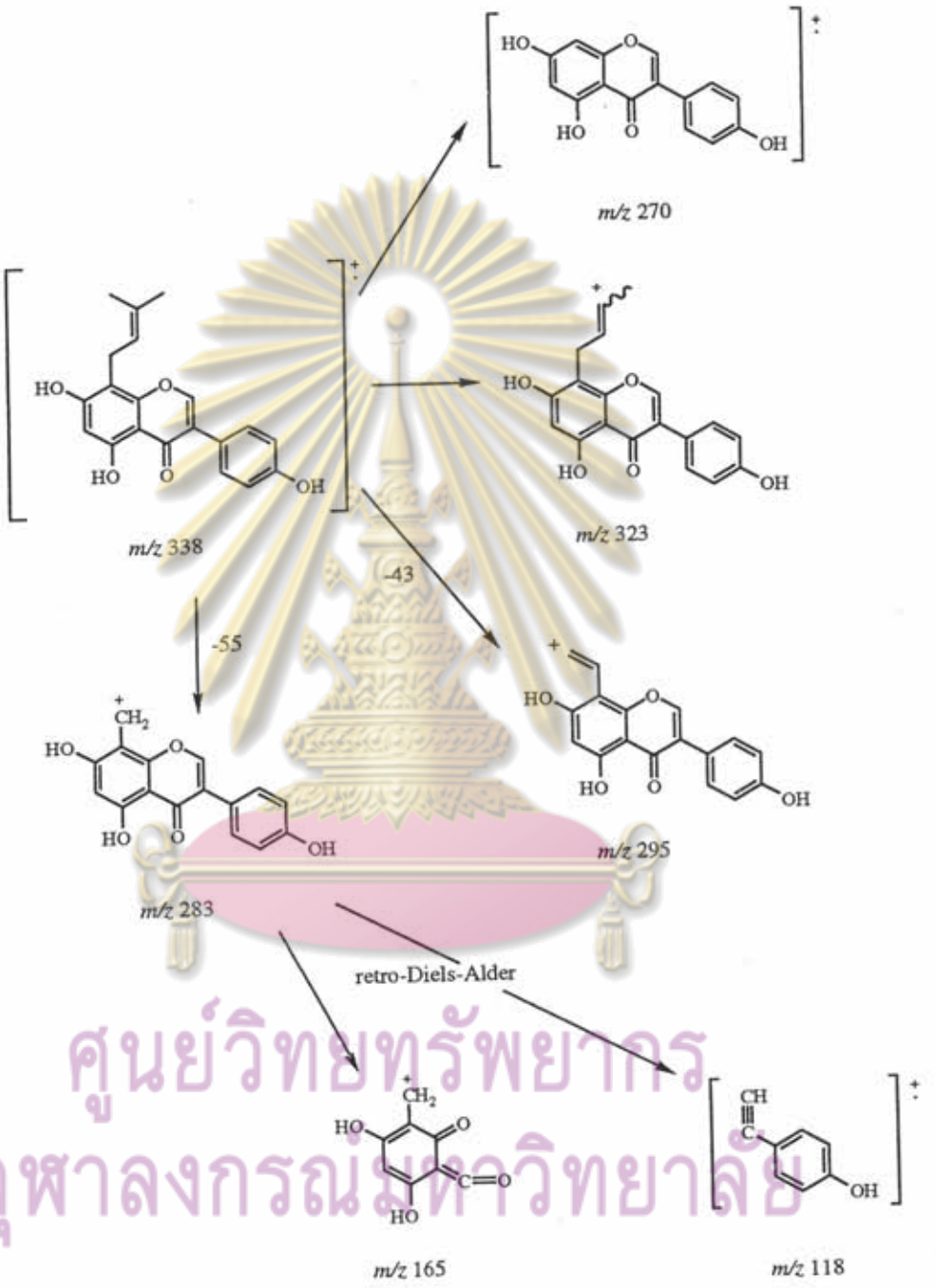


Figure 25 Mass spectral fragmentation of DR-2

The ^{13}C-NMR spectrum (Figure 38-38.1) supported this structure and was shown in Table 38.

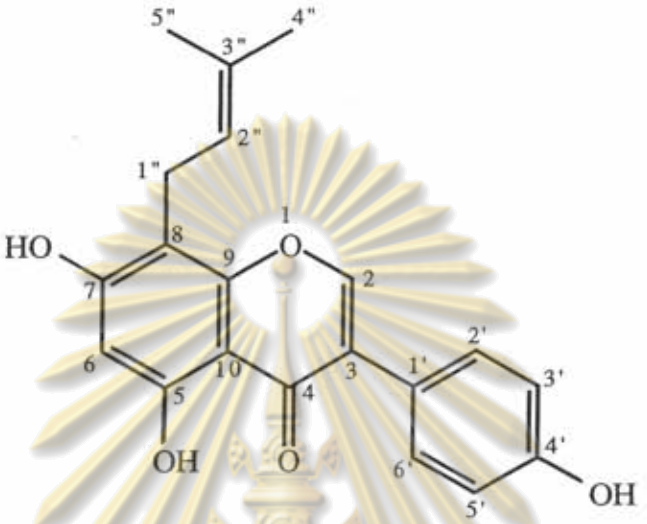
Table 38 Proposed ^{13}C-NMR Assignment of DR-2.

C-Position	DR-2 δ (ppm)	Lupiwighteone δ (ppm)	Category
C-2	154.3	153.78	CH
C-3	123.3*	122.10	C
C-4	182.0	180.55	C
C-5	161.6	159.61	C
C-6	99.5	98.58	CH
C-7	162.2	161.66	C
C-8	107.2	105.97	C
C-9	156.3	154.89	C
C-10	106.3	104.57	C
C-1'	123.1*	121.40	C
C-2'	131.2	130.04	CH
C-3'	116.0	115.09	CH
C-4'	158.4	157.38	C
C-5'	116.0	115.09	CH
C-6'	131.2	130.04	CH
C-1"	22.0	21.02	CH_2
C-2"	123.7*	122.21	CH
C-3"	132.0	130.88	C
C-4"	25.8	25.30	CH_3
C-5"	17.9	17.30	CH_3

* interchangeable

The assignments of carbons was confirmed by comparison with the recorded data of lupiwighteone (in DMSO -d_6) previously reported by Fukai, Wang and Nomura, 1989.

From ^{1}H-NMR and ^{13}C-NMR assignments of DR-2 (in acetone d_6), the structure of which was determined as lupiwighteone the structure is shown below.



Part II : Pharmacognostic Specification

The genus *Derris* belongs to the family Leguminosae. Many species are used as medicinal , fish-poisonings and insecticides in various asian countries.

Owing to the similarity of *Derris reticulata* Craib and *Derris scandens* Benth. in both external and internal characters. The pharmacognostic specification was used to distinguish these two species. The results from this investigations were summarized as follows.

Quantitative Value of Leaf of *Derris reticulata* Craib and *D. scandens* Benth.

Table 39 shows various values of the leaf measurement of *Derris reticulata* craib and *D. scandens* Benth.. It can be seen that the values of the two species were markedly different and thus could be used for distinguish the plants.

Table 39 Data of Leaf Measurement

Data	*D. reticulata* Craib	*D. scandens* Benth.
Mean of Stomatal Number (lower epidermis)	372.81 (342.11 - 421.05)	723.68 (605.21 - 815.79)
Mean of Stomatal Index (lower epidermis)	11.69 (10.40 - 13.27)	23.37 (20.44 - 25.44)
Standard deviation of the Stomatal Index	0.753	1.097
Mean of Palisade Ratio	9.64 (8.75 - 11.25)	5.99 (5.00 - 7.25)
Standard deviation	0.768	0.740
Mean of Vein-Islet Number	20.45 (17.50 - 23.25)	14.95 (13.00 - 17.75)
Standard deviation	1.812	1.295
Mean of Veinlet Termination Number	0.65 (0.25 - 1.25)	1.675 (1.25 - 2.25)
Standard deviation	0.300	0.3237

The Microscopical Study of Powdered Drug Characteristic

1. The Microscopical Character of Powdered Stem of *Derris reticulata* Craib

The powder is pale yellowish-brown in colour and sweet taste. The diagnostic characters are (Figure 14) :

1. the occasional fragments of brownish polygonal cork cells in sectional view.
2. the medullary ray in tangential longitudinal view surrounded by calcium oxalate prism sheath.
3. the abundant fragments of groups of fibres with thick, lignified walls and narrow lumen. Some groups of fibres are found surrounded by calcium oxalate prism sheath.

4. the fragments of xylem parenchyma, composed of rectangular cells with moderately thickened walls and numerous pits.

5. the large, lignified bordered pitted vessels, which are usually found in small fragments.

6. the starch granules which are found scattered ; they are simple and very occasional compound with 2 to 4 components. Each granule is nearly spherical but some of them are different shapes. The diameter is usually 4 to 7 μ (range 4 to 27 μ).

7. the scattered calcium oxalate prisms.

8. the sclereids which are oval to rectangular and usually found in small groups.

9. the occasional brownish substances.

2. The Microscopical Character of Powdered Stem of *Derris scandens* Benth.

The powder is pale brown in colour. The diagnostic characters are (Figure 15) :

1. the occasional fragments of browinsh polygonal cork cells in surface view.

2. the fragments of parenchymatous cells containing starch granules.

3. the abundant fragments of groups of fibres with thick, lignified walls and narrow lumen. Some groups of fibres are found surrounded by calcium oxalate prism sheath.

4. the fragments of xylem parenchyma in longitudinal view, composed of rectangular cells with moderately thickened walls and numerous pits.

5. the fragments of xylem ray in radial longitudinal view, composing of ray parenchyma with underlying fibres or xylem parenchyma.

6. the large, lignified bordered pitted vessels, which are usually found in small fragments.

7. the sclereids are oval or rectangular and usually found in small groups.

8. the starch granules which are found scattered as well as in the parenchymatous cells ; they are simple or compound with 2 to 3 components. Each granule is nearly spherical and 3 to 11 μ in diameter.

9. the scattered calcium oxalate prisms.

10. the occasional brownish substances.

The common characters of both powdered drugs are the presence of calcium oxalate sheath fibres, bordered pitted vessels, brownish cork, brownish substances and starch grains. Their differences could be distinguished and shown in Table 44.

Table 40 Comparison of the Organoleptic and Microscopic Character of Powdered Stem of *Derris reticulata* Craib and *D. scandens* Benth.

D. reticulata Craib	*D. scandens* Benth.
1. pale-yellowish brown powder and good sweet taste.	1. pale brown powder and slightly astringent taste
2. the multiseriate medullary ray in tangential longitudinal view surrounded by fibres and calcium oxalate prism sheath.	2. the medullary rays are usually found in radial longitudinal view whilst the uniseriate medullary rays in tangential longitudinal view are occasionally found in a row of thin-walled cells or frequently cannot be observed and are often seen as spindle space among the group of fibres.
3. the starch granules ; they are simple and very occasional compound with 2 to 4 compoents. Each granules is nearly spherical but some of them are different shapes. The diameter is usually 4 to 7 μ	3. the starch granules ; they are simple or compound with 2 to 3 components. Each granules is nearly spherical and 3 to 11 μ in diameter.

3. The Microscopical Character of Powdered Leaf of *Derris reticulata* Craib

The powder is yellowish-green in colour. The diagnostic characters are (Figure 16).

1. the abundant fragments of the lamina in surface view showing upper epidermis with slightly thick-walled cells, stomata absent and underlying palisade cells. Paracytic stomata are present on the lower epidermis.

2. the fragments of the lamina in sectional view showing the upper epidermis, a single layer of thin-walled palisade cells, the spongy cells, some of them containing calcium oxalate prisms and the lower epidermis.

3. the fragment of parenchyma with/without calcium oxalate prisms.

4. the fragment of fibres attached with spiral vessels, and calcium oxalate prism sheath.

5. the occasional nonglandular uniseriate multicellular (3 cells) trichomes.

4. The Macroscopical Character of Powdered Leaf of *Derris scandens* Benth.

The powder is green in colour. The diagnostic characters are (Figure 17).

1. the abundant fragments of the lamina in surface view showing upper epidermis with slightly thick-walled cells, stomata absent or rarely found. Paracytic stomata and cicatrix of trichome are present on the lower epidermis.

2. the fragments of the lamina in sectional view showing the upper epidermis, a single layer of thin-walled palisade cells, the spongy cells and the lower epidermis.

3. the fragment of the mesophyll with small vessels and fibres.

4. the parenchyma in longitudinal view.

5. the occasional calcium oxalate prisms.

6. fragments of spiral, pitted and bordered pitted vessels.

7. the scattered nonglandular bicellular trichomes.

The common characters of both powdered leaves are :

1. the abundant fragments of the lamina in surface view and sectional view.
2. the calcium oxalate prisms.
3. the nonglandular multicellular trichomes.
4. the parenchyma cells.
5. vessels

Their differences could be distinguished and were shown in Table 45.

Table 41 Comparison of the Organoleptic and Microscopic Character of Powdered Leaf of *Derris reticulata* Craib and *D. scandens* Benth.

D. reticulata Craib	*D. scandens* Benth.
1. yellowish-green powder.	1. green powder.
2. the abundant fragments of epidermis and rarely found the cicatrix on the lower epidermis.	2. the abundant fragments of epidermis and showing the cicatrix on the lower epidermis.
3. the nonglandular multicellular (3 cells) trichomes, 230 to 261 μ long and rarely found.	3. the nonglandular bicellular trichomes, 91 to 162 μ long. They are found scattered, conical with a long apical cell and a faintly striated cuticle.

Two-Dimensional Thin-Layer Chromatographic Pattern of Chemical Constituents

Two-dimensional thin-layer chromatographic patterns of chemical constituents of the stem of *Derris reticulata* Craib and *D. scandens* Benth. have indicated marked differences from species to species and could be used as an important tool as an additional in the identification and differentiation of the plants.

A coding system has been set up for the purpose of describing the individual spot occurring on the chromatogram, involving :

1. R_f values obtained in two different solvent systems.
2. fluorescence and quenching of the substances under ultraviolet light before or after spraying with spray reagents.
3. colours of spots obtained from treatment with various detection reagents.

Determination of the sameness of compounds occurring in different species can be done by direct matching of the code numbers.

Two-dimensional development is particular valuable for mixtures of many components. If the components of a mixture are not completely separated by development in a single direction, it may be possible to resolve them by this method. Reproducible results can be obtained by controlling of the conditions affecting the Rf values such as the layer thickness, the temperature and the time interval for drying, moisture content of the chromatogram, the amount of sample applied, saturation of the chromatography tanks, the compositions of the solvent systems, and the conditions and the time interval of the intermediate drying after each development. In the present work, ambient temperature throughout the development of the chromatogram was about 30-35°C.

Determination of Thin-Layer Chromatographic Pattern of Cha-aem Thai from Various Local Traditional Drug Distributor

The identification for the corrected species of Cha-aem Thai from various local traditonal drug distributors was made by using Thin-layer chromatographic pattern of the extract with those of authentic plant (*Derris reticulata* Craib, Cha-aem nuea). The

result (Figure 18-21) showed that Cha-aem Thai which were secured from various local traditional drug distributors are *D. reticulata* Craib, Cha-aem nuea.

Determination of Thin-Layer Chromatographic Pattern of Sugar

For the aqueous portion (DR-W), the presence of free sugar was determined by using Thin-layer chromatographic pattern in order to compare with authentic mono- and di-saccharides. The result (Figure 22-23) showed that the free sugar in the aqueous portion was sucrose which may be the major sweetener of this plant.