CHAPTER III

RESULTS AND DISCUSSIONS

I. *Clausena harmandiana* Pierre

Dried, powdered root bark of *Clausena harmandiana* Pierre was extracted by refluxing with hexane. The hexane extract was concentrated on rotary evaporator to yield a gummy residue (Scheme 1). A total 5 gm of gummy residue was separated by means of adsorption column chromatography on 60 gm of silica gel G 60 (230-400 mesh ASTM) and eluted with chloroform. A 25 ml of each fraction was collected as F_1, F_2, F_3... . A total of 90 fractions of 25 ml each were obtained. Each fraction was monitored by tlc in solvent system E for F_1 - F_{23} and solvent system B for F_{23} - F_{90}.

Fractions 5-10, which showed some interesting compounds on tlc plate, were combined and dried on rotary evaporator to yield a 2.2033 g residue. Fractions 12-14 were also combined due to the similar pattern on the plate. Only these two combined fractions, containing the interesting compounds, were attempted to obtain the pure compounds.

The combined fractions 5-10 were rechromatographed on a column of 60 gm silica gel G 60 (230-400 mesh ASTM) with benzene as eluent. Fractions (10 ml each) were collected. Fractions 15-20, which showed a single spot on tlc plate with solvent system E

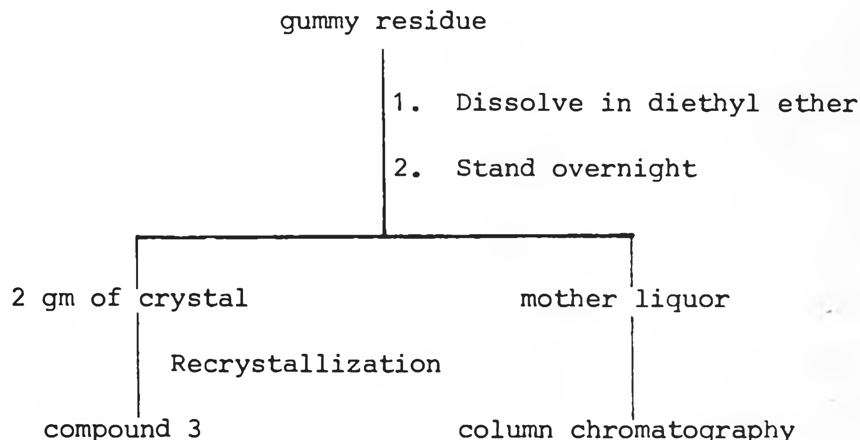
(table 6), were combined and the solvent was removed on rotary evaporator. The residue on crystallization from acetone, gave needle-like yellow crystals designated as compound 1.

The combined fractions 12-14 were rechromatographed over a column of 60 gm silica gel G 60 (230-400 mesh ASTM) using benzene as eluent. Fractions (20 ml each) were collected. Fractions 20-25 were combined, concentrated and crystallized from acetone to give compound 2.

II. *Micromelum minutum* Wight and Arn.

Dried, powdered stem bark of *Micromelum minutum* was extracted the methods shown in scheme 2. The gummy residue (12 gm) was dissolved in diethyl ether and standed overnight to give 2 gm of crystal (scheme 3). This crystal was recrystallized by dissolving in a small amount of chloroform; and acetone was added in proper amount to form a saturated solution. The mixture was left to recrystallize at room temperature. The crystal was separated and recrystallized with acetone. The process of recrystallization with acetone was repeated at least three times or until the pure crystal was obtained. The pure crystal then was assigned as compound 3 (680 mg).

The combined mother liquor, obtained from the obove process, was concentrated to give a 10 gm of gummy residue. The residue was then chromatographed on silica gel G 60 (230-400 mesh ASTM) and eluted with petroleum ether, 100 ml; petroleum ether : diethyl ether (98 : 2), 100 ml; petroleum ether : diethyl ether (96 : 4), 100 ml and so on. A 25 ml of each fraction was collected as F_1, F_2... .



Scheme 3 Isolation of compounds from *Micromelum minutum*

Fractions 14-23 were combined and concentrated, so were fractions 28-29. These two combined fractions showed interesting compounds on the tlc plate; and the attempt to obtain these compounds was performed.

The combined fractions 14-23 were chromatographed on a column of 120 gm silica gel G 60 (230-400 mesh ASTM) using benzene as eluent. A 15 ml of each fraction was collected. Fractions 45-50 were combined and rechromatographed on a column of 60 gm silica gel G 60 (230-400 mesh ASTM) with benzene as eluent. Fractions (10 ml each) were collected. The combined fractions 16-18 were concentrated on rotary evaporator and dissolved in acetone. After leaving for a few days, a crystal was formed. Recrystallization in acetone obtained a pure compound which was assigned as compound 5 (25 mg).

The combined fractions 28-29 were rechromatographed on a column of 60 gm silica gel G 60 (230-400 mesh ASTM) using chloroform as eluent. Fractions (15 ml each) were collected and only fraction 8 was taken for crystallization. A pure crystal was obtained from

acetone and assigned as compound 4.

The identification of these isolated compounds was demonstrated on the basis of the following data :

1. <u>Compound 1</u> This compound (18.9 mg, 0.02% yield) was obtained from the root bark of *Clausena harmandiana*. It was crystallized from acetone, and gave a bright yellow crystal.

The identification and structure elucidation of this compound were based on these following data :

molecular weight : 309 (EIMS) Indicate that the number of N in formular is odd.

melting point : 150-151.5℃ (uncorrected)

tlc : This compound was spotted on tlc plates, developed in solvent system A, B, C, D, E, and F, dried in open air; the detection was performed with uv light and spray reagents. This compound gave one spot on tlc in these solvent systems. It gave a quenching spot on the fluorescent background of tlc under uv light (254 nm). It gave brown color with benzidine reagent, indicated that there is a phenolic group in this compound.

R_f values : 0.89 in solvent system A

0.94 in solvent system B

0.92 in solvent system C

0.72 in solvent system D

0.89 in solvent system E

0.68 in solvent system F

uv spectrum : λ_{max}^{MeOH} = 207 nm (log ε = 4.22)
(Fig. 11) 233 nm (log ε = 4.23)
255 nm (log ε = 4.26)
303 nm (log ε = 4.54)
340 nm (log ε = 3.87)

ir spectrum : ν_{max}^{KBr} = 3350 cm^{-1} (O-H or N-H stretching)
(Fig. 12) 1619 cm^{-1} (C=O stretching)
900-675 cm^{-1} (out of plane ⁝ C-H bending)

mass spectrum (EIMS) : = m/e (% relative intensity) = 309 (100.0),
(Fig. 13) 294 (15.08), 266 (14.34), 255 (13.09), 254 (74.81), 253 (24.02), 238 (10.69), 225 (12.83).

^{1}H-nmr spectrum (Fig. 14,15)

Proton-nmr spectrum was obtained by using deuterochloroform ($CDCl_3$) as solvent and TMS as reference standard. Six protons of two methyl groups showed a singlet signal at 1.77 and 1.88 ppm. The two protons of methylene showed doublet signal at 3.59 ppm due to their coupling with olefinic proton (H_{11}). The olefinic proton gave a triplet signal at 5.30 ppm. The methoxy proton certainly showed a singlet signal at 3.88 ppm. H_7 was assigned to be a signal at 6.85 ppm due to an ortho coupling with H_8 and meta coupling with H_5 (dd, J = 8.1 and 2 Hz). The H_8 showed a doublet signal at 7.80 ppm (J = 8.1 Hz). The chemical shift of H_4 and H_5 was assigned to be at 7.86 and 6.89 ppm, and both of them showed high intensity signals

which overlapped to the signals of H_7 and H_8, respectively. To confirm these assignments, the ^{1}H-nmr spectrum in hexadeuterodimethyl sulfoxide (DMSO-d_6) was performed (Fig. 15). The signals of H_4 and H_5 were moved to downfield and showed at 8.16 and 6.99 ppm. The signal of H_4 at 8.16 ppm showed a singlet and that of H_5 at 6.99 ppm was clearly splitted into double due to its coupling with H_7. The chemical shift of proton attached to nitrogen (-NH-) was at 8.15 ppm in $CDCl_3$, but the signal was moved to downfield at 11.26 ppm in DMSO-d_6. This effect might be caused by the hydrogen bond forming between NH and solvent.

The chemical shifts and multiplicities of ^{1}H-nmr of compound 1 in $CDCl_3$ and DMSO-d_6 were summarized in table 8.

Most of the data obtained corresponded with the data from literature (21) so this compound was identified to be 6-methoxyheptaphylline (53). The only significant difference of data was the assignment of ^{1}H-nmr. The assignment of H_5 and H_8 was at 6.89 and 7.80 ppm, while that in the literature (21) was at 7.45 and 7.30 ppm. In the literature the chemical shift of H_8 was found upper-field than that of H_5, but in this experiment the signal of H_8 was found lower-field than that of H_5. The ^{1}H-nmr spectrum in DMSO-d_6 was clearly proved that this assignment of H_5 and H_8 was corrected and it was agreed with the assignment of other known alkaloids in this type (42,43).

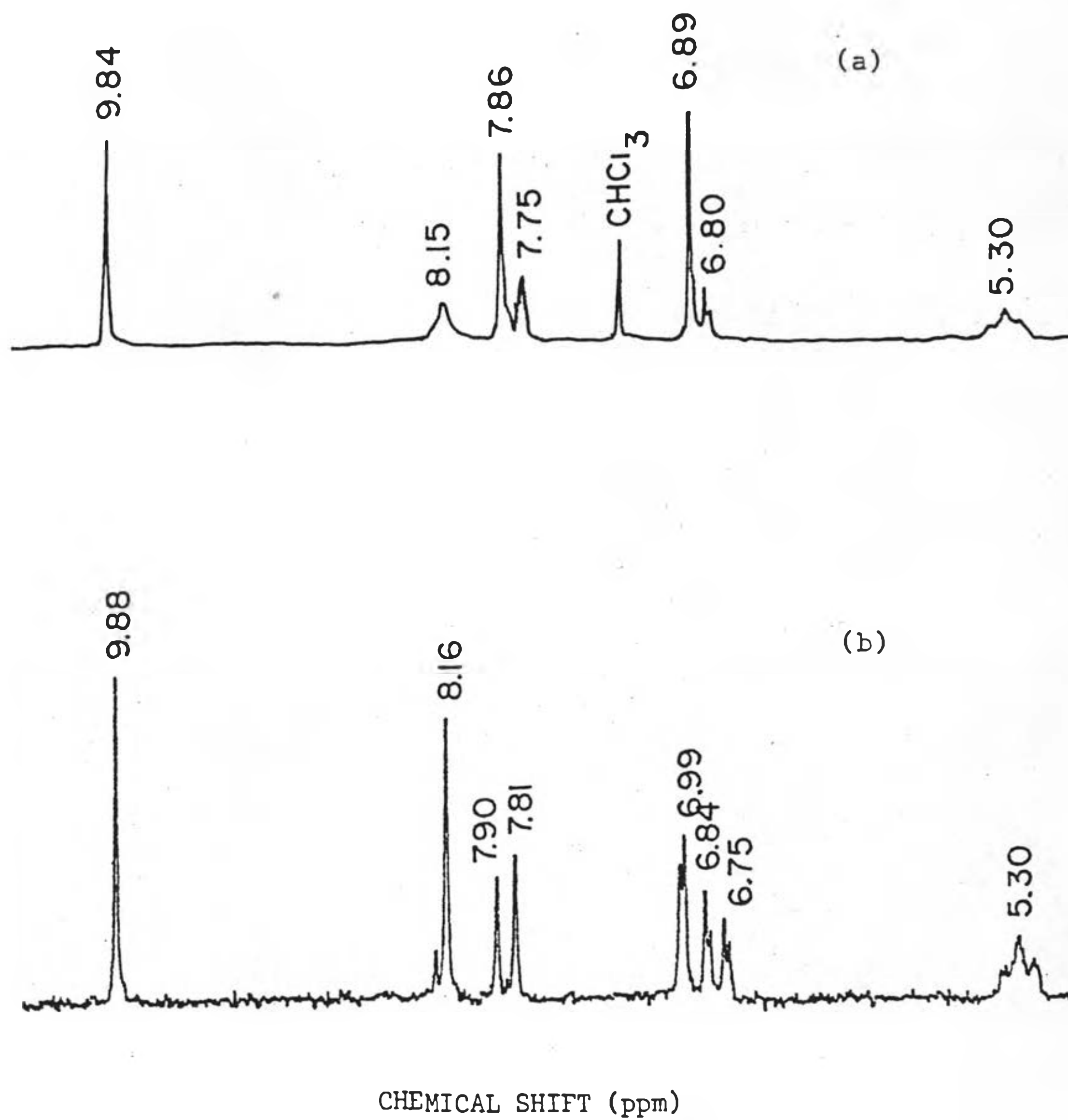
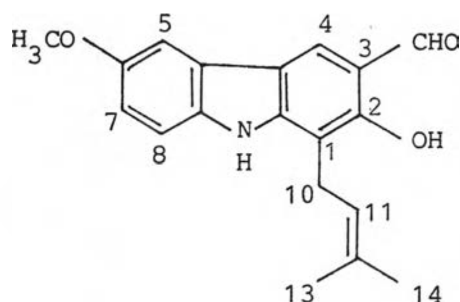


Fig. 10 ^{1}H-nmr Spectrum (90 MHz) of Compound 1. (6-Methoxyheptaphylline) : (a) $CDCl_3$; (b) DMSO-d_6.

Table 8 ^{1}H-nmr Chemical Shifts and Multiplicities of Compound 1 in $CDCl_3$ and DMSO-d_6

Proton	Chemical Shift (ppm) $CDCl_3$	Chemical Shift (ppm) DMSO-d_6	Multiplicity
$-CH_3$ $-CH_3$	1.77,1.88	1.68,1.84	singlet
$-CH_2-$	3.59	3.54	doublet
$-OCH_3$	3.88	3.86	singlet
H_{11}	5.30	5.30	triplet, broad
H_7	6.85	6.80	doublet doublet, J = 8.1, 2 Hz
H_5	6.89	6.99	singlet (in $CDCl_3$) doublet (in DMSO-d_6), J = 2 Hz
H_4	7.86	8.16	singlet
H_8	7.80	7.86	doublet, J = 8.1 Hz
-NH-	8.15	11.26	singlet
$-C(=O)-H$	9.84	9.88	singlet
-OH	11.61	11.54	singlet



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2. Compound 2 This compound (30.8 mg, 0.03% yield) was obtained from the root bark of *Clausena harmandiana*. It was crystallized form acetone, and it gave bright yellow crystals. The identification and structure elucidation of this compound were based on these following data :

molecular weight : 241 (EIMS)

melting point : 218-219°C (uncorrected)

tlc : This compound was spotted on tlc plates, developed in solvent system A, B, C, D, E, and F, dried, and then visualized under uv light and with spray reagents. This compound gave only one spot on tlc in these solvent systems. It gave a violet spot on the fluorescent background of tlc under uv light (254 nm). It gave brown color with benzidine reagent which indicated that there is a phenolic group in the molecule.

R_f values : 0.76 in solvent system A

0.77 in solvent system B

0.69 in solvent system C

0.39 in solvent system D

0.56 in solvent system E

0.35 in solvent system F

uv spectrum : λ_{max}^{MeOH} = 340 nm (log ε = 3.89)
(Fig. 16)
300 nm (log ε = 4.58)
244 nm (log ε = 4.32)
225 nm (log ε = 4.30)

ir spectrum : ν_{max}^{MeOH} = 3280 cm^{-1} (O-H or N-H stretching)
(Fig. 17)
1640 cm^{-1} (C=O stretching)
900-675 cm^{-1} (out of plane $\stackrel{\cdots}{—}$ C-H bending)

mass spectrum (EIMS) : m/e (% relative intensity) = 241 (100.0),
(Fig. 18)
240 (11.10), 227 (11.13), 226 (71.08),
198 (28.00), 120 (11.75)

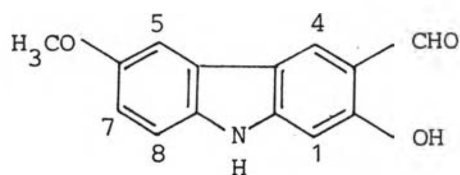
^{1}H-nmr spectrum (Fig. 19)

The ^{1}H-nmr chemical shifts and multiplicities of this compound were shown in table 9.

Most of the data, compared with those of the known compound in the literature (16), indicated that this compound was lansine (54). The only small difference was the assignment of the chemical shifts of protons at position 1, 5, 7, and 8. This assignment agreed with those reported of alkaloids in this type (42,43).

Table 9 ^{1}H-nmr Chemical Shifts (ppm) and Multiplicities of Compound 2 in DMSO-d_6

Proton	Chemical Shift (ppm)	Multiplicity
$-OCH_3$	3.78	singlet
H_7	6.74	double doublet, J = 9, 2 Hz
H_1	6.81	singlet
H_5	6.91	doublet, J = 2 Hz
H_8	7.88	doublet, J = 9 Hz
H_4	8.26	singlet
-C(=O)-H	10.06	singlet
-NH-	10.91	broad
-OH	11.36	singlet



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3. <u>Compound 3</u> This compound (680.90 mg, 0.14% yield) was obtained from *Micromelum minutum*. It was recrystallized from acetone, and it afforded white crystals. The identification and structure elucidation of this compound were based on these following data :

molecular weight : 258 (EIMS)

melting point : 127-128℃ (uncorrected)

tlc : This compound was spotted on tlc plates, developed in solvent system A, B, C, D, E, and F, dried in open air; the detection was performed with uv light and spray reagents. This compound gave one spot on tlc in these solvent systems. It fluoresced under uv light (366 nm). It gave a brown spot on tlc when exposed with I_2 vapor and gave a positive reaction with Dragendorff's reagent.

R_f values : 0.76 in solvent system A

0.80 in solvent system B

0.69 in solvent system C

0.40 in solvent system D

0.56 in solvent system E

0.35 in solvent system F

uv spectrum : λ_{max}^{MeOH} = 322 nm (log ε = 4.13)

(Fig. 20) 217 nm (log ε = 4.53)

ir spectrum : ν_{max}^{KBr} = 1705, 1715 cm^{-1} (a lactone function of coumarins)(28)

(Fig. 21) 1605 cm^{-1} (C=C stretching)

1100 cm^{-1} (C-O bond)

890 cm^{-1} (=CH_2 wag of methylene)

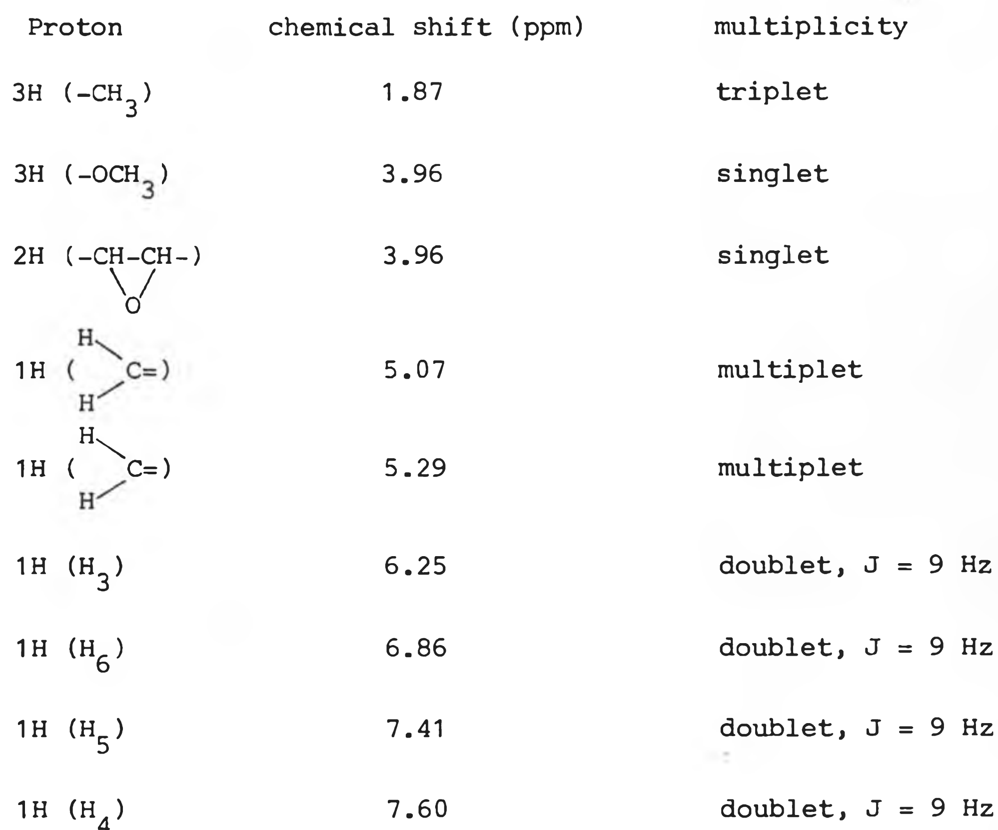
810 cm^{-1} (assymmetrical stretching)

of epoxy ring)(44)

mass spectrum (EIMS) : m/e (% relative intensity) = 258 (100.0),
(Fig. 22) 229 (51.18), 199 (49.39), 189 (77.06),
187 (62.14), 131 (45.24)

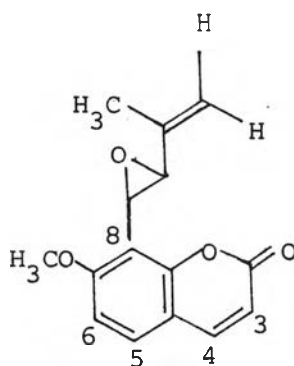
^{1}H-nmr spectrum (Fig. 23)

The ^{1}H-nmr spectrum of this compound in $CDCl_3$ were summarized as follows :



Proton	chemical shift (ppm)	multiplicity
3H (-CH_3)	1.87	triplet
3H (-OCH_3)	3.96	singlet
2H (-CH-CH-) (epoxide, O bridging)	3.96	singlet
1H ($H_2C=$)	5.07	multiplet
1H ($H_2C=$)	5.29	multiplet
1H (H_3)	6.25	doublet, J = 9 Hz
1H (H_6)	6.86	doublet, J = 9 Hz
1H (H_5)	7.41	doublet, J = 9 Hz
1H (H_4)	7.60	doublet, J = 9 Hz

Ir spectrum provided a good evidence for the identification of a lactone functional group of coumarin (28). Melting point and mass spectrum suggested that this compound might be phebalosin (55)(29). In our experience, most of coumarins gave strong molecular ion peaks. This was because the forming of stable molecular ions were available by losing of one electron from oxygen of pyrone ring. Because the reference compound was not available, ^{1}H-nmr was performed. The signals of two protons of epoxide ring, AB coupling, were at the same position of methoxy proton. The integration indicated a presence of five protons at this signal. The multiplet at 5.07 ppm might be assigned to be a terminal olefinic proton which was trans to methyl group due to the long range coupling of *trans* was bigger than that of *cis*. Therefore the multiplet at 5.29 ppm was assigned to be an olefinic proton which was *cis* to methyl group. The doublets (J = 9 Hz) at 6.25 and 7.60 ppm, which arise from the *cis* protons H_3 and H_4 of the pyrone ring, were an indication and confirmation of the presence of a coumarin nucleus and also showed that the pyrone ring was unsubstituted. The H_4 doublet, falled at 7.60 ppm in $CDCl_3$, indicated a lacking of oxygen function at C_5 in coumarin. The doublets at 7.41 and 6.86 ppm, due to H_5 and H_6, pointed out that the H_8 was absent (45). All of the data obtained corresponded to the data in the literature (46,47), therefore the compound was identified to be phebalosin (55)



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4. Compound 4   A total of 25.0 mg, 0.01% yield, of this compound was obtained from the stem bark of *Micromelum minutum*. It was recrystallized from acetone, and it afforded colorless needle crystals. The identification and structure of this compound were based on these following data :

molecular weight : 288 (EIMS)

melting point : 211-212℃ (uncorrected)

tlc : This compound was spotted on tlc plates, developed in solvent system A, B, C, D, E, and F, dried in open air; the detection was performed with uv light and spray reagents. This compound gave only one spot on tlc in every solvent systems. It fluoresced under uv light (366 nm); and gave a brown spot in I_2 vapor.

R_f values : 0.64 in solvent system A
0.90 in solvent system B
0.61 in solvent system C
0.16 in solvent system D
0.38 in solvent system E
0.13 in solvent system F

uv spectrum : λ_{max}^{MeOH} = 222 nm (log ε = 3.99)
320 nm (log ε = 3.86)

ir spectrum : ν_{max}^{KBr} = 1770 cm^{-1} (γ-lactone)
1725 cm^{-1} (coumarin lactone)
810 cm^{-1} (asymmetrical stretching of epoxy ring)

mass spectrum (EIMS) : m/e (% relative intensity) = 288 (100.0),
(Fig. 26) 229 (48.15), 214 (18.16), 213 (40.30),
186 (17.34), 43 (15.75)

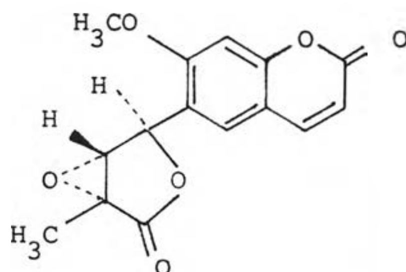
^{1}H-nmr spectrum (Fig. 27)

The ^{1}H-nmr spectrum of this compound in $CDCl_3$ were summarized as follows :

proton	chemical shift (ppm)	multiplicity
3H ($-CH_3$)	1.67	singlet
3H ($-OCH_3$)	3.95	singlet
1H (H_4')	4.04	singlet
1H (H_5')	5.55	singlet
1H (H_3)	6.32	doublet, J = 9 Hz
1H (H_8)	6.87	singlet
1H (H_5)	7.37	singlet
1H (H_4)	7.65	doublet, J = 9 Hz

The doublets at 6.32 and 7.65 ppm indicated and confirmed the presence of a coumarin nucleus. The singlets at 6.87 and 7.37 ppm due to H_8 and H_5, therefore there was a substitution at position 6.

The data obtained indicated that this compound was micromelin (micromelumin)(56), and were agreed with the data from literature (48).



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5. Compound 5 This compound (30.5 mg, 0.01% yield) was obtained from the stem bark of *Micromelum minutum*. It was crystallized from acetone, and gave colorless crystals. The identification and structure elucidation of this compound were shown in the following data :

molecular weight : 412 or 414 (EIMS)

melting point : 137-138°C (uncorrected)

tlc : This compound was spotted on tlc plates, developed in solvent system A, B, C, D, E, and F, dried in open air; the detection was performed with uv light and spray reagents. This compound gave one spot on tlc in these solvent system (normal-phase systems). It did not show a spot under uv light (366 and 254 nm). It gave blue color with Liebermann-Burchard reagent indicated that this compound might be a sterol (36). It also gave a brown spot with I_2 vapor.

R_f values : 0.70 in solvent system A

0.80 in solvent system B

0.69 in solvent system C

0.24 in solvent system D

0.48 in solvent system E

0.48 in solvent system F

ir spectrum : ν_{max}^{KBr} = 3450 cm^{-1} (O-H stretching)

(Fig. 28) 2800-3000 cm^{-1} (C-H stretching)

1650 cm^{-1}, low intensity (C=C stretching)

1080 cm^{-1} (C-O stretching)(49)

mass spectrum (EIMS) : m/e (% relative intensity) = 414 (95.25),

(Fig. 29) 412 (94.09), 400 (100.0), 396 (46.02),
382 (50.62),

(Fig. 30) m/e (% relative intensity = 412 (100.0),

400 (78.36), 392 (39.52), 382 (38.59)

^{1}H-nmr spectrum (Fig. 31)

The ^{1}H-nmr spectrum showed these following signals :

- A broad band at 0.68-2.3 ppm, originating from the unresolved multiplets of the equivalent protons of the steroid skeleton, was a characteristic of steroids.

- A very broad multiplet at 3.5 ppm originated from the 3α proton.

- A broad multiplet at 5.09 ppm, AB pattern coupling, originated from olefinic protons.

- A broad multiplet at 5.36 ppm originated from ethylenic proton.

- The characteristic peaks of 18- and 19- methyl groups were at 0.68 and 1.01 ppm, respectively (49).

According to the data obtained, this compound was characterized to be a sterol. More experiments are needed for identifying the structure of this compound (50,51,52). Because a sterol was commonly found in many plants and this compound was out of our interest, the more information for identification of this compound was not performed.

Conclusions

Clausena harmandiana, family Rutaceae, was found in the northeast of Thailand. A report of using this plant as folkloric medicine has been known as stomachica. Its root bark gave pungent odor, and tasted bitter and acrid.

The investigation of the root bark of this plant was previously done; the presence of 5 coumarins clausarin, dentatin, osthol, xanthoxyletin, and nordentatin as well as one carbarzole alkaloid heptaphylline was reported (15). The further investigation of the root bark of this plant was performed. We could isolate the two more carbazole aklaloids 6-methoxyheptaphylline and lansine. The structures of these two alkaloids were established on the basis of chemical and spectroscopic methods. Both compounds gave brown color with benzidine reagent indicating the presence of phenolic group. This was confirmed by adding a few drops of sodium hydroxide solution into each solution of alkaloids in methanol. Bathochromic shifts from 303 to 310 nm for 6-methoxyheptaphylline and from 302 to 307 for lansine appeared in uv spectra. The ir spectra of both compounds showed -OH and -NH stretching and aldehyde C=O stretching. EIMS showed an odd number of molecular ion (309 for 6-methoxyheptaphylline and 241 for lansine) indicated that these compounds might contain a nitrogen atom. The ^{1}H-nmr spectra of these compounds were analysed. In 6-methoxyheptaphylline, the solvent effects in DMSO-d_6 was performed for structure analysis (40,41). Hydrogen-bonding between solvent (DMSO-d_6) and the alkaloid provided better signal

seperations relative to those in deuterochloroform. Lansine, which could only be dissolved in DMSO-d_6, also showed good signal seperations which could be analysed. The comparison of spectroscopic data of these compounds with the known compounds from literatures were made and most of them were identical. 6-Methoxyheptaphylline and lansine have been previously found in the root bark of *Clausena indica* Oliv. (21) and the leave of *Clausena lansium* (16), respectively. The result of this inrestigation shows the first report of these two alkaloids in the root bark of *Clausena harmandiana.*

Micromelum minutum (Forst.f.) Wight and Arn. (Synonym *M. pubescens* Blume) belongs to the family Rutaceae. It has a wide range extending from Indo-China to the Pacific (53). In this study, the plant specimen was collected from Nakornsrithammarat province, Thailand.

The chemical constituents of the leave of this plant have been previously reported, containing a pyranoquinoline alkaloid flindersine (54) and four coumarins micropubescin (54), osthol (48), micromelumin (micromelin)(48), and microminutin (55).

In this study, three compounds were isolated from the stem bark of this plant. The first compound is phebalosin, which was previously obtained from the leave of *Phebalium tuberculosum*, *Phebalium drummondii* (46), *Murraya paniculata*, and *Murraya exotica* (29). The second compound is micromelumin (micromelin), which was isolated from the leave of *Micromelum minutum* (48), *Micromelum integerrimum* (56), and *Micromelum zeylanicum* (18) and from the tree

bark of *Casearia gravenolens* (57). And the third compound is a sterol. All of three compounds were analysed on the basis of chemical and spectroscopic methods. The first two compounds, phebalosin and micromelin, the coumarins, showed the spectroscopic data (^{1}H-nmr, ir) corresponded to the known ones in the literatures. The third compound, characterized as a sterol, was out of our interest, therefore more spectroscopic data were not obtained.