

CHAPTER III

RESULTS AND DISCUSSION

3.1 Structural elucidation of the essential oil

The essential oil was obtained as a green-blue oil 7.88 g (0.53 % wt. by wt.) by team distillation. Its IR spectrum as shown in Fig.4 and in Table10, and indicated the presence of alcohol an O-H stretching at 3400-3360 cm^{-1} . The aliphatic C-H stretching at 2929 and 2867 cm^{-1} , =C-H, C=C stretching of unsaturated peak at 3080, 1642 cm^{-1} , and the peak at 1712 cm^{-1} was due to C=O stretching vibration.

Table 10 The IR absorption band assignment of essential oil

| Frequency (cm^{-1}) | Peak intensity | Tentative assignments |
|--------------------------------|----------------|---|
| 3400,3360 | m | O-H stretching vibration of alcohol |
| 3080 | w | =C-H stretching vibration of alkene |
| 2929, 2867 | s | C-H stretching vibration of -CH ₃ , -CH ₂ |
| 1712 | w | C=O stretching vibration of carbonyl |
| 1642 | m | C=C stretching vibration of alkene |
| 1450 | m | C-H bending vibration of -CH ₃ , -CH ₂ |
| 1223, 1131 | w | C-O stretching vibration |
| 888 | m | C-H out of plane bending vibration |

The chemical constituents of the essential oil were analyzed by GC-MS spectrometer and the total ion chromatogram was shown in Fig.5. The chromatogram displayed peak at retention time (R_t) 14.00, 16.64, 25.70, 26.64, 30.87, 31.61, 35.49, 35.68, 38.02, 38.24, 46.20, 49.48, 49.90, 50.97, 52.43, 52.76, 53.02, 54.14, 54.45, 55.13, 58.59, 59.12 mins respectively.

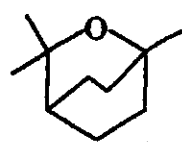
The mass spectrum of each peak was compared with those in the library (NIST database). The identity of several components [23] were obtained as shown in Fig.3 and in Table 11. However, it should be realized that the number of compounds in the database is limited and there may be more than one compound that give the same or a similar fragmentation pattern, thus the identity of these compounds should be regarded as a possibility but not definitive, and comparison of R_t with authentic samples should be done.

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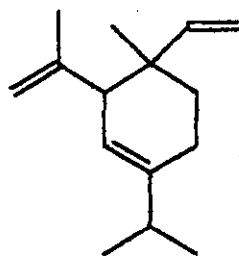
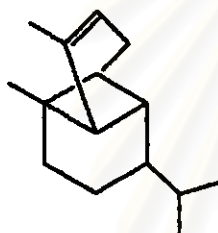
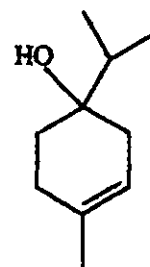
Table 11 The composition of compounds contained in essential oil.

| R_t (min)* | Formular | Name | % areas |
|--------------|----------------------|---------------------|---------|
| 14.00 | 154, $C_{10}H_{18}O$ | cinole | 5.08 |
| 16.64 | - | unidentified | 3.45 |
| 25.70 | 204, $C_{15}H_{24}$ | δ -elemene | 1.88 |
| 26.64 | 204, $C_{15}H_{24}$ | α -copaene | 1.17 |
| 30.87 | - | unidentified | 2.44 |
| 31.61 | 154, $C_{10}H_{18}O$ | 4-terpineol | 11.59 |
| 35.49 | 154, $C_{10}H_{18}O$ | α -terpineol | 2.22 |
| 35.68 | 154, $C_{10}H_{18}O$ | borneol | 1.42 |
| 38.02 | - | unidentified | 1.22 |
| 38.24 | 202, $C_{15}H_{22}$ | α -curcumene | 2.43 |
| 46.20 | 220, $C_{15}H_{24}O$ | caryophyllene oxide | 1.02 |
| 49.48 | 222, $C_{15}H_{26}O$ | elemol | 23.37 |
| 49.90 | - | unidentified | 1.42 |
| 50.97 | 220, $C_{15}H_{24}O$ | spathulenol | 1.46 |
| 52.43 | 222, $C_{15}H_{26}O$ | γ -eudesmol | 6.23 |
| 52.76 | - | unidentified | 1.06 |
| 53.02 | - | unidentified | 2.14 |
| 54.14 | 222, $C_{15}H_{26}O$ | α -eudesmol | 0.44 |
| 54.45 | 222, $C_{15}H_{26}O$ | β -eudesmol | 18.68 |
| 55.13 | - | unidentified | 6.20 |
| 58.59 | - | unidentified | 3.91 |
| 59.12 | - | unidentified | 1.05 |

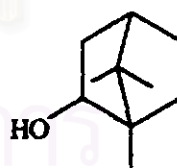
* GC conditions was reported in chapter II



cineole

 δ -elemene α -copaene

4-terpineol

 α -terpineol

borneol

Figure 3 Structure of components from the essential oil of *Z. rubens* Roxb.

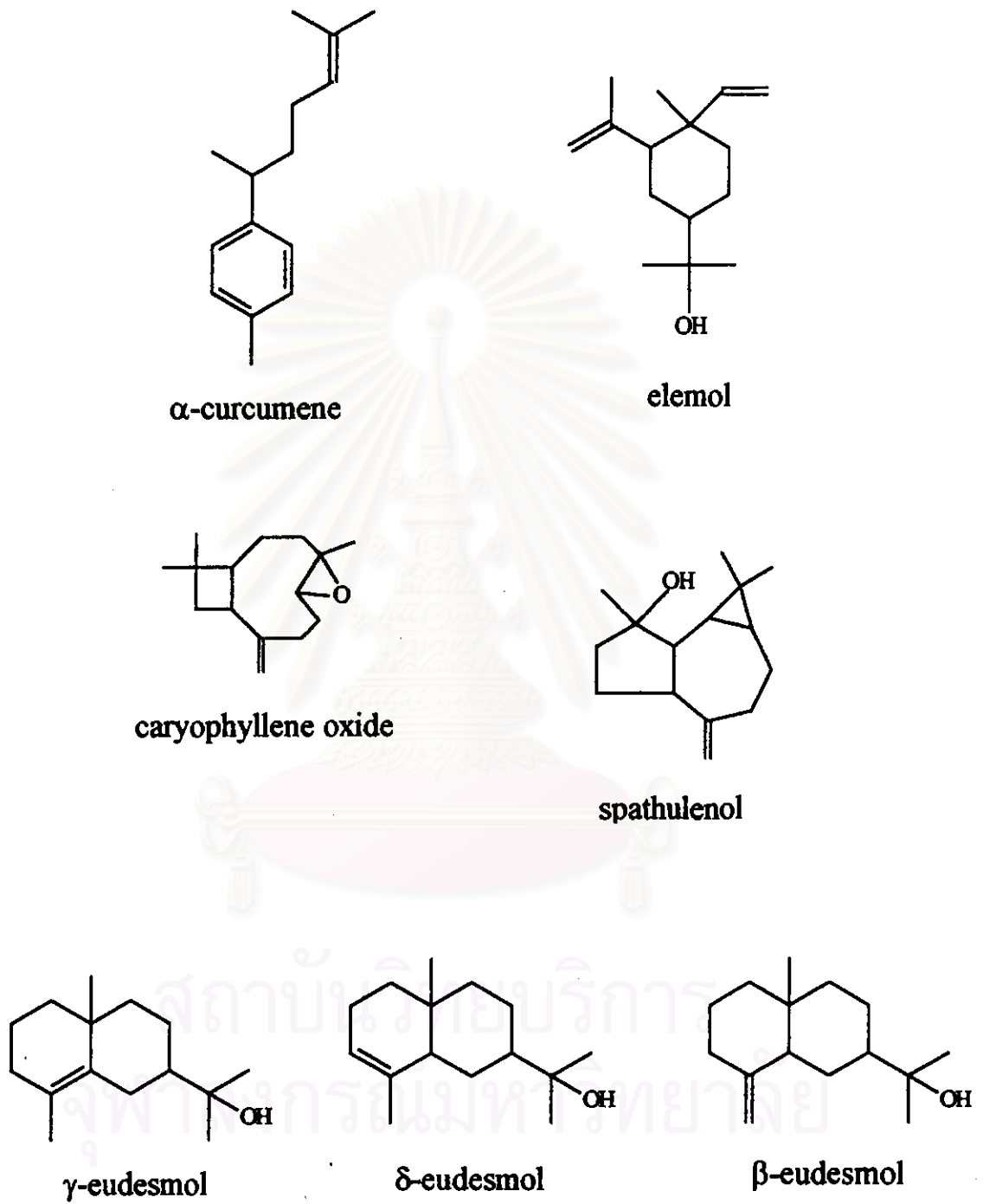


Figure 3 (cont.) Structure of components from the essential oil of *Z. rubens* Roxb.

3.2 Structural elucidation of Mixture 1

Mixture 1 was obtained as a white needle-like crystals, m.p.129-131 °C from fraction No. 96-103 eluted with 70% CHCl₃-hexane. The IR-spectrum of mixture 1 was shown in Fig.28 and the absorption peaks can be assigned as shown in Table12. Its IR-spectrum showed important absorption bands at 3600-3200 and 1057 cm⁻¹ (O-H and C-O stretching vibration of alcohol), 1638 cm⁻¹ (C=C stretching vibration of olefin), 959 cm⁻¹ (disubstituted vinyl, R₁CH=CHR₂), 801 cm⁻¹ (trisubstituted vinyl, R₁R₂C=CHR₃)

Table 12 The IR-absorption band assignments of Mixture 1

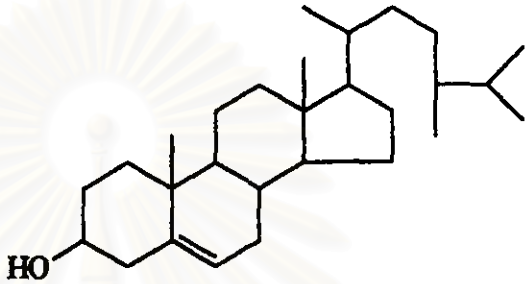
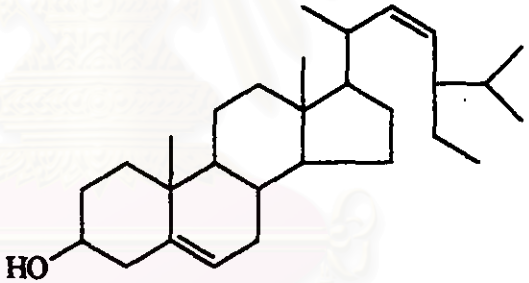
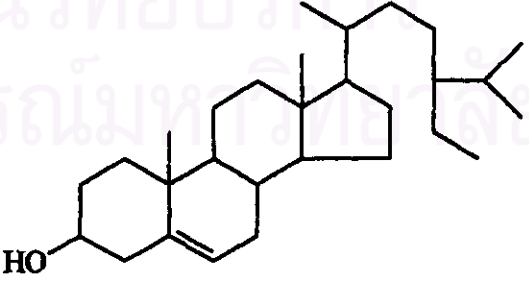
| Frequency (cm ⁻¹) | Peak intensity | Tentative assignments |
|-------------------------------|----------------|---|
| 3600-3200 | m | O-H stretching vibration of alcohol |
| 2961, 2936, 2868 | w | C-H stretching vibration of -CH ₃ , -CH ₂ |
| 1638 | w | C=C stretching vibration of alkene |
| 1464 | m | C-H bending vibration of -CH ₃ , -CH ₂ |
| 1381 | m | C-H bending vibration of -CH ₃ |
| 1057 | m | C-O stretching vibration of alcohol |
| 959 | w | C-H out of plane bending vibration of disubstituted vinyl |
| 801 | w | C-H out of plane bending vibration of trisubstituted vinyl |

The $^1\text{H-NMR}$ spectrum (CDCl_3) of mixture 1 (Fig.30) showed signals at 0.68-2.26 ppm, which were the signals of methyl, methylene, and methine groups of steroids ($-\text{CH}_3$, $-\text{CH}_2$, $-\text{CH}$ respectively). The proton adjacent to the hydroxy group ($-\text{CH-OH}$) was shown as the multiplet signal at 3.50 ppm. While the multiplet signal at 5.09 ppm was due to the disubstituted vinylic protons ($-\text{CH}=\text{CH}-$). The most downfield signal at 5.35 ppm was the signal of trisubstituted vinylic proton ($-\text{CH}=\text{C}-$).

The $^{13}\text{C-NMR}$, DEPT-90 and DEPT-135 spectrum in CDCl_3 (Fig.31) showed the carbon signals at 11.8-56.8 ppm which are CH_3 , CH_2 , and CH of steroid. The olefinic carbon signals were observed at 121.7, 129.3, 138.3 and 140.7 ppm, while the signal at 71.8 ppm are characteristic for the carbon adjacent to the hydroxy group. The mass spectrum (Fig.29) showed a molecular ion peak corresponding to campesterol, stigmasterol, and β -sitosterol at m/e 400 ($\text{C}_{28}\text{H}_{48}\text{O}$), 412 ($\text{C}_{29}\text{H}_{48}\text{O}$), and 414 ($\text{C}_{29}\text{H}_{50}\text{O}$) respectively. The ions fragmentation pattern in the mass spectrum in this mixture indicated that it was a mixture of steroids.

GLC analysis of standard steroids; cholestrol, campesterol, stigmasterol and β -sitosterol was shown in Fig.31. Under these conditions, only 2 peaks of stigmasterol and β -sitosterol were observed at retention time 18.84 and 21.35 min respectively, but the peaks of campesterol did not show up because of column inefficiency. However, EI-MS spectrum showed m/e ions at 414.0, 412.0 and 400.0 which m/e ions peaks at 400.0 could not result from fragmentation from m/e 414.0 and 412.0. The results indicated that mixture 1 was a mixture of campesterol, stigmasterol and β -sitosterol. The structures are shown [24] in table 13.

Table 13 Structure of steroids in Mixture 1

| steroid compound | formular | structural | molecular weight |
|---------------------|-----------------|--|------------------|
| campesterol | $C_{28}H_{48}O$ |  | 400 |
| stigmasterol | $C_{29}H_{48}O$ |  | 412 |
| β -sitosterol | $C_{29}H_{50}O$ |  | 414 |

3.3 Structural elucidation of Mixture 2

Mixture 2 was isolated from fraction No. 194-237 after elution with 100 % CHCl_3 and 2% MeOH-CHCl_3 and was re-chromatographed to afford a white amorphous solid, m.p.273-275 °C.

The IR-spectrum of mixture 2 (Fig.33) could be assigned as shown in Table 14. Its IR-spectrum showed important absorptions at $3600\text{-}3200\text{ cm}^{-1}$ (O-H stretching vibration of alcohol), 1653 cm^{-1} (C=C stretching vibration of olefin), $1072\text{-}1026\text{ cm}^{-1}$ (C-O stretching vibration of OH group of sugar) and 887 cm^{-1} (C-H bending vibration of anomeric axial proton of β -sugar).

Table 14 The IR-absorption band assignments of Mixture 2

| Frequency (cm^{-1}) | Peak intensity | Tentative assignments |
|--------------------------------|----------------|--|
| 3600-3200 | m | O-H stretching vibration of alcohol |
| 2934, 2867 | s | C-H stretching vibration of $-\text{CH}_3$, $-\text{CH}_2$ |
| 1653 | w | C=C stretching vibration of alkene |
| 1463 | m | C-H bending vibration of $-\text{CH}_3$, $-\text{CH}_2$ |
| 1370 | m | C-H bending vibration of $-\text{CH}_3$ |
| 1072-1026 | s | C-O stretching vibration of alcohol of sugar |
| 887 | w | C-H bending vibration of anomeric axial proton of β -sugar |
| 797, 776 | w | C-H out of plane bending vibration of trisubstituted vinyl |

The ^1H -NMR spectrum of mixture 2 (Fig.35) showed the signals at 0.64-2.42 ppm, which were the signals of methyl, methylene, and methine groups of steroids ($-\text{CH}_3$, $-\text{CH}_2$, $-\text{CH}$ respectively). The multiplet signals at 2.72-3.30 ppm were assigned to the protons of a sugar. The proton on carbon attached to sugar ($-\text{CH}-\text{O}-\text{sugar}$) appeared as the multiplet signal at 3.63 ppm. and the signals at 4.20 ppm belonged to the anomeric proton. The multiplet signal at 5.09 ppm was assigned as disubstituted vinyl protons ($-\text{CH}=\text{CH}-$). The last signal at 5.35 ppm was the signal of trisubstituted vinyl proton ($-\text{CH}=\text{C}-$).

The ^{13}C -NMR and DEPT-135 spectrum (Fig.36) showed carbon signals at 11.6-56.1 ppm which were the signals of CH_3 , CH_2 , CH of steroid. The olefinic carbon signals were observed at 121.2, 128.5, 138.1 and 140.4 ppm, while the signal at 61.0, 70.0, 73.4, 76.7, 76.9 and 100.7 ppm were characteristic for a glycoside. The ^{13}C -NMR spectrum of the aglycone corresponded to those of a mixture of campesterol, stigmasterol and β -sitosterol. ^{13}C -NMR spectrum of mixture 2 in the region of 60-100 ppm suggested that it is probably glucose by comparison with those in the literature [24] and the data shown in Table 15.

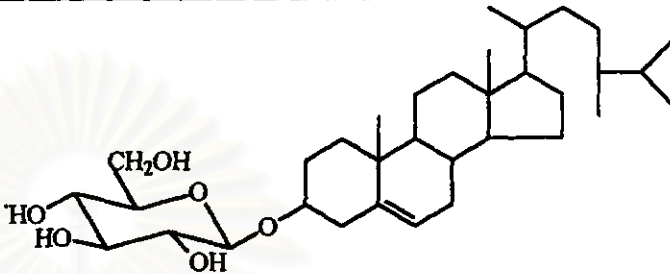
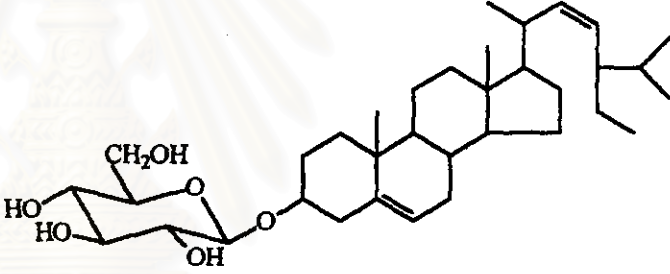
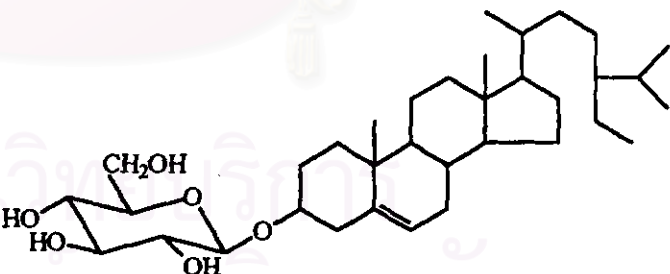
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Table 15 Partial ^{13}C - NMR spectrum of sugar in mixture 2 with steroid-3-O- β -D-glucopyranoside

| carbon position | mixture 2 | steroid-3-O- β -D-glucopyranoside |
|-----------------|-----------|---|
| C-1 | 100.69 | 100.74 |
| C-2 | 73.41 | 73.43 |
| C-3 | 76.92 | 76.93 |
| C-4 | 70.04 | 70.06 |
| C-5 | 76.65 | 76.69 |
| C-6 | 61.03 | 61.05 |

The mass spectrum (Fig.34) showed the molecular ion peaks of campesterol, stigmasterol, and β -sitosterol at m/e 400($\text{C}_{28}\text{H}_{48}\text{O}$), 412 ($\text{C}_{29}\text{H}_{48}\text{O}$), and 414 ($\text{C}_{29}\text{H}_{50}\text{O}$) respectively. The fragmentation ion mass spectrum pattern of this mixture indicated that it was a mixture of steroids. The above results of ^1H -NMR, ^{13}C -NMR, EI-MS spectra and a literature search indicated that mixture 2 was a mixture of steroid glucoside; campesteryl-3-O- β -D-glucopyranoside, stigmasteryl-3-O- β -D-glucopyranoside, and β -sitosteryl-3-O- β -D-glucopyranoside.

Table 16 Structure of steroid glycoside in Mixture 2

| steroid glycoside compound | structure |
|---|--|
| <p>campesterol-3-O-β-D-glucopyranoside</p> |  |
| <p>stigmasterol-3-O-β-D-glucopyranoside</p> |  |
| <p>β-sitosterol-3-O-β-D-glucopyranoside</p> |  |

3.4 Structure elucidation of Compound 3

Compound 3 was converted into compound 3A after an attempt to purify by preparative GLC. Compound 3A was obtained as a colorless oil. The IR spectrum of compound 3A (Fig.39) could be assigned as shown in Table 17. The spectrum showed important absorption bands at 3082, 1642, 890 cm^{-1} ($-\text{CH}=\text{CH}_2$ stretching vibration), 2929,2860 cm^{-1} (C-H stretching vibration of CH_2 , CH_3).

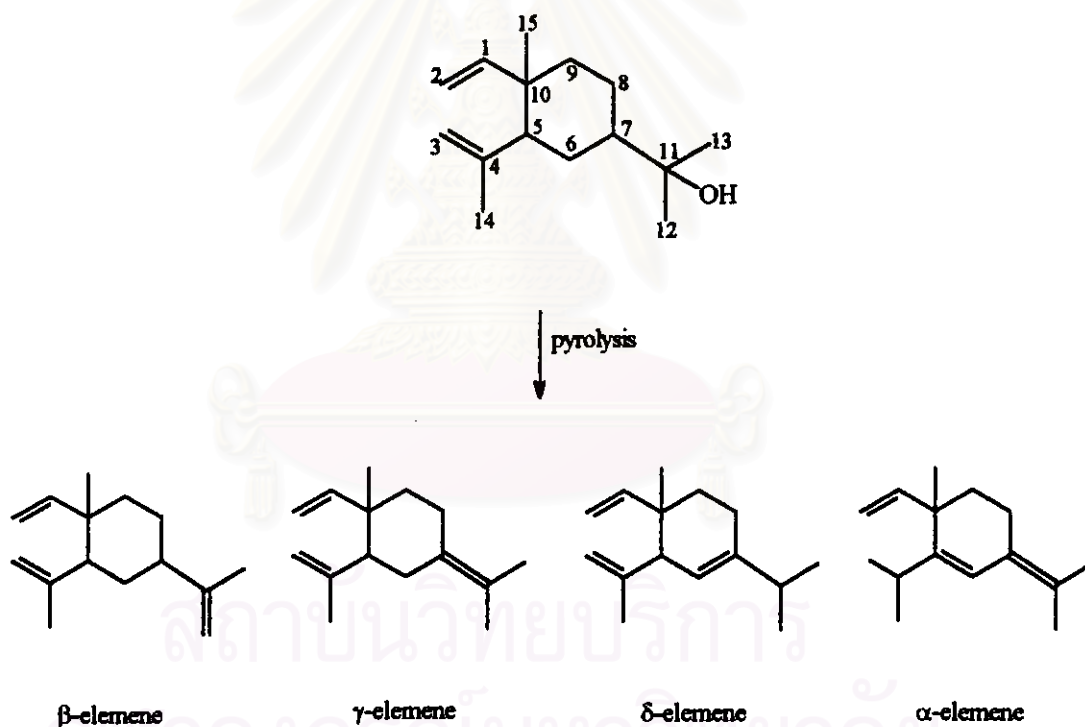
Table 17 The IR-spectrum band assignments of compound 3A

| Frequency (cm^{-1}) | Peak intensity | Tentative assignment |
|--------------------------------|----------------|---|
| 3082 | m | C-H stretching vibration of alkene |
| 2929,2860 | s | C-H stretching vibration of $-\text{CH}_2$, $-\text{CH}_3$ |
| 1642 | m | C-H stretching vibration of alkene |
| 1440,1375 | m | C-H bending vibration of $-\text{CH}_2$, $-\text{CH}_3$ |
| 890 | s | $-\text{C}=\text{CH}$ out of plane bending of alkene |

The ion fragmentation patterns in the mass spectrum of each component in the GC chromatogram of compound 3A was compared to the data in the library (NIST database). Analysis of the starting oil fraction No.19-22 before preparative GLC suggested that it mainly consisted of elemol (Fig.40), a sesquiterpene which possesses a hydroxy group (M.W.. 222, formula $\text{C}_{15}\text{H}_{26}\text{O}$). After preparative GLC, however, GC-MS analysis of each fractions revealed that the peak corresponding to elemol disappeared and a new peak with a shorter retention time 18.5 min (Fig.41) was apparent in fraction I (compound 3A).

^1H and ^{13}C -NMR spectrum were different from the starting oil, especially the signal of hydroxy group in the ^1H -NMR spectrum of fraction I which had disappeared.

A literature survey suggested that pyrolysis of elemol [25,26] could result in dehydration which could give many elemene type products. There are several isomers of elemene including β -elemene, γ -elemene, δ -elemene and α -elemene as shown in Scheme 2.



Scheme 2 Products from pyrolysis of elemol

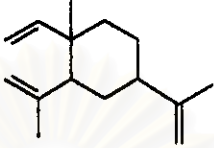
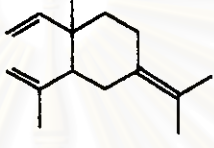
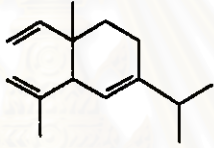
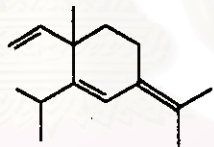
The data above suggested that fraction I consisted of an elemene which could have resulted from pyrolysis of elemol during preparative GLC at high temperature and this reaction may be catalyzed by the silica surface of chromosorb-P. Furthermore, ^1H and ^{13}C -NMR spectra suggested that only one isomer of elemene was obtained and that this could be β -elemene, γ -elemene, δ -elemene or α -elemene.

The ^1H -NMR spectrum (Fig.42) of compound 3A showed the presence of three methyl protons at 0.99 (3H,s), 1.69 (3H,m) and 1.71 (3H,m), six olefinic protons ($\text{CH}_2=\text{C}-$) at 4.57, 4.80 (2H,m), 4.69 (2H,m), 4.89,4.91 (2H,m). and another olefinic proton at 5.80 (1H, dd, $J = 10, 18$ Hz) ppm.

The ^{13}C -NMR spectrum (Fig.43) exhibited totally 15 signals of carbon, DEPT 90 and DEPT 135 experiments (Fig.44) further revealed the presence of three methyl carbons at 16.6, 21.1 and 24.7 ppm, six methylene carbons at 26.8, 32.9, 39.9, 108.2, 109.8, 112.1 ppm, three methine carbons at 45.7, 52.8, 150.3 ppm and three quarternary carbons at 39.8, 147.7 and 150.4 ppm. These data suggested that the compound 3A should be β -elemene according to the number of different types of carbons as shown in Table 18.

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Table 18 The structure of elemene isomer

| Elemene compound | Structure | Type of carbon |
|-------------------|---|---|
| β -elemene |  | C-3, CH-3, CH ₂ -6, CH ₃ -3 |
| γ -elemene |  | C-4, CH-2, CH ₂ -5, CH ₃ -4 |
| δ -elemene |  | C-3, CH-4, CH ₂ -4, CH ₃ -4 |
| α -elemene |  | C-3, CH-1, CH ₂ -6, CH ₃ -5 |

Two dimension NMR techniques were used to assist the assignment of ^1H and ^{13}C -NMR spectra of the compound. The one bond C-H correlation (HMQC) data is shown in Fig.45 and Table 19.

Table 19 ^1H and ^{13}C -NMR spectral data of Compound 3A by HMQC

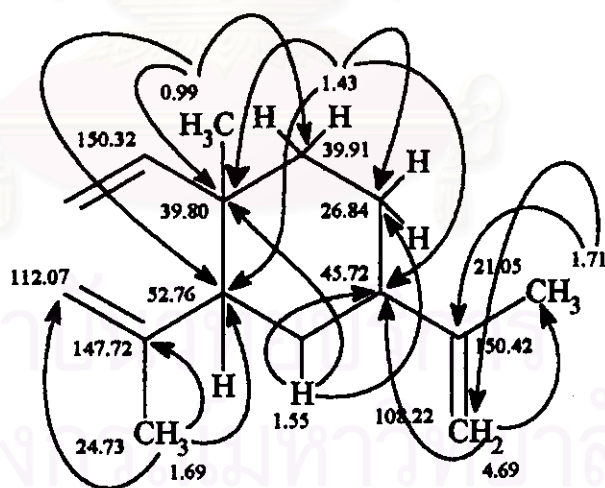
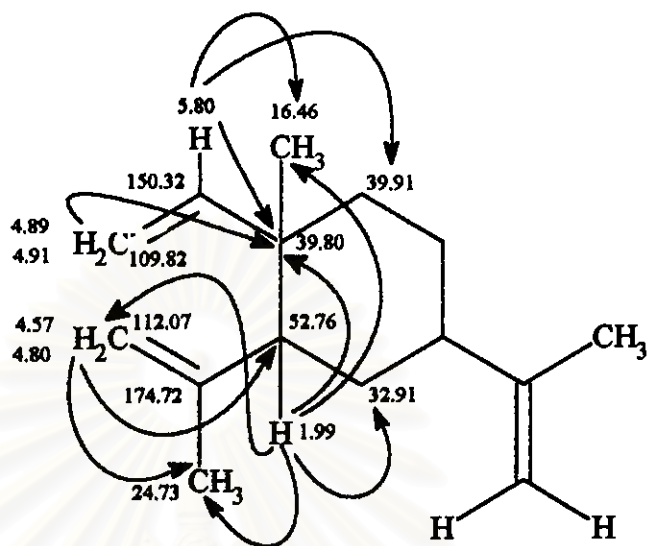
| ^{13}C -NMR (ppm) | ^1H -NMR (ppm) |
|----------------------------|-------------------------|
| 150.2 (d) | 5.80 |
| 109.8 (t) | 4.89, 4.91 |
| 112.1 (t) | 4.57, 4.80 |
| 147.7 (s) | - |
| 52.8 (d) | 1.99 |
| 32.9 (t) | 1.55 |
| 45.7 (d) | 1.93 |
| 26.8 (t) | 1.60 |
| 39.9 (t) | 1.43 |
| 39.8 (s) | - |
| 150.4 (s) | - |
| 108.2 (t) | 4.69 |
| 21.1 (q) | 1.71 |
| 24.7 (q) | 1.69 |
| 16.5 (q) | 0.99 |

A COSY experiment (Fig.46) revealed that the proton at 1.69 ppm was coupled to the proton at 4.57 and 4.80 ppm. The proton at 1.71 ppm was coupled to the proton at 4.69 ppm and the proton at 5.80 ppm was coupled to the proton at 4.89 and 4.91 ppm. Furthermore, information from HMBC experiments was consistent with β -elemene as shown in Fig.42 and Table in 20.

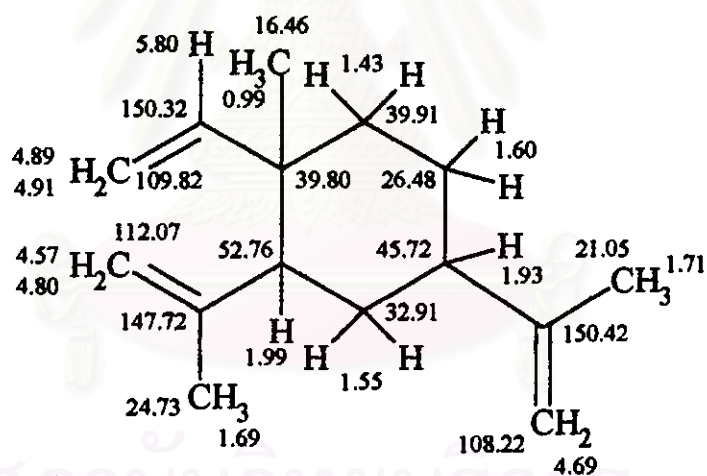
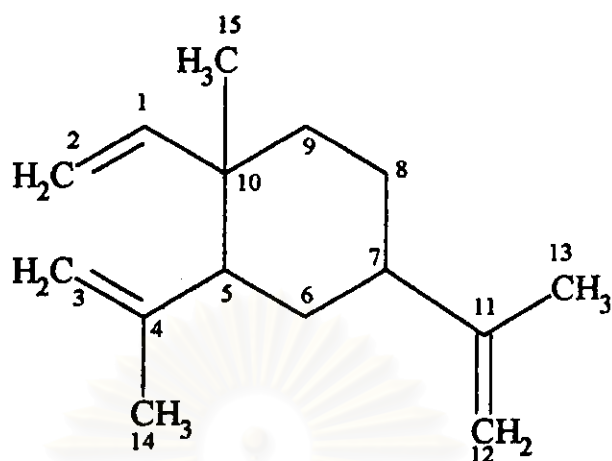
Table 20 The One bond and Multiple bond correlation of compound 3A

| Carbon position | ¹ H-NMR (ppm) | One bond correlation Attached carbon (ppm) | Multiple bond correlation Attached carbon (ppm) |
|-----------------|--------------------------|--|---|
| C-1 | 5.80 | 150.3 | 16.5, 39.8, 39.9 |
| C-2 | 4.89, 4.91 | 109.8 | 39.8 |
| C-3 | 4.57, 4.80 | 112.1 | 24.7, 52.8 |
| C-4 | - | 147.7 | - |
| C-5 | 1.99 | 52.8 | 16.5, 24.7, 32.9, 39.8, 112.1 |
| C-6 | 1.55 | 32.9 | 26.8, 39.8, 45.7 |
| C-7 | 1.93 | 45.7 | - |
| C-8 | 1.60 | 26.5 | 21.1 |
| C-9 | 1.43 | 39.9 | 26.8, 39.8, 45.7, 52.8 |
| C-10 | - | 39.8 | - |
| C-11 | - | 150.4 | - |
| C-12 | 4.69 | 108.2 | 21.1, 45.7 |
| C-13 | 1.71 | 21.1 | 45.7, 108.2, 150.4 |
| C-14 | 1.69 | 24.7 | 52.8, 112.1, 147.7 |
| C-15 | 0.99 | 16.5 | 39.8, 39.9, 52.8, 150.3 |

The HMBC data confirmed the following structure below:



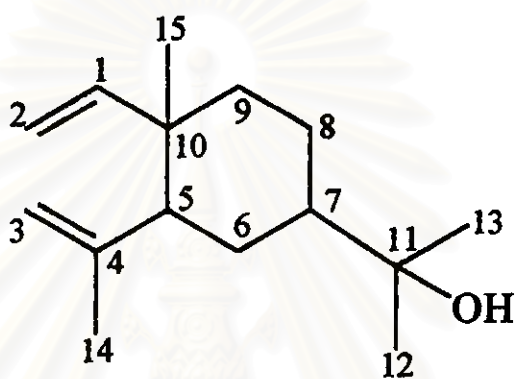
Most significant correlations observed in HMBC of compound 3A



The spectral data is consistent with the identification of compound 3A as β -elemene [23]. Therefore, it could be deduced that compound 3 was elemol which obtained from hexane extract.

Compound 3 was elemol which occurs in several natural product and was reported previously in *Manila elemi*, *Java citronella* oils and other essential oil.

The structure of elemol is shown below:



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3.5 Structural elucidation of Compound 4

Compound 4 was isolated from fraction No. 100-155 and was eluted with 60%-80% CHCl_3 -hexane as a yellow solid m.p. 229-231 °C

The IR-spectrum of compound 4 (Fig.48) could be assigned as shown in Table 21. The spectrum showed important absorption bands at 3500-3100 cm^{-1} (O-H stretching vibration), 1659 cm^{-1} (C=O stretching vibration of conjugated ketone), 1587, 1508, cm^{-1} (C=C stretching vibration of aromatic), 1336 cm^{-1} (C-H stretching and C-H bending vibration of $-\text{CH}_3$, $-\text{CH}_2$).

Table 21 The IR-spectrum band assignments of compound 4

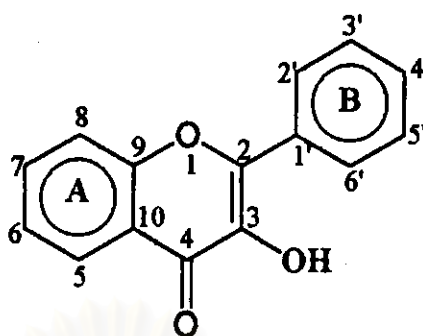
| Frequency (cm^{-1}) | Peak intensity | Tentative assignment |
|--------------------------------|----------------|---|
| 3500-3100 | s | O-H stretching vibration |
| 2975, 2839 | w | C-H stretching vibration of $-\text{CH}_3$, $-\text{CH}_2$ |
| 1659 | s | C=O stretching vibration of conjugated ketone |
| 1587, 1508 | s | C=C stretching vibration of aromatic |
| 1336 | m | C-H bending vibration of $-\text{CH}_3$, $-\text{CH}_2$ |
| 1157, 1114 | m | C-O stretching symmetric vibration of C-O-C |
| 1092, 1046 | m | C-O stretching symmetric vibration of C-O-C |
| 838, 804 | w | =C-H out of plane bending of aromatic |

The $^1\text{H-NMR}$ spectrum (Fig.50) of compound 4 showed the presence of two methoxy groups at 3.84 (6H,s), three hydroxy groups at 9.28(1H,s), 9.51(1H, br s) and 12.41(1H,s) and two aromatic protons with a meta-relationship at 6.32(1H,d, $J = 2.4$ Hz) and 6.67(1H, d, $J = 2.4$ Hz), another aromatic proton at 7.07 (1H,d, $J = 8.8$ Hz), 7.76(1H,dd, $J = 8.5, 10.6$ Hz) and 7.71 (1H,d, $J = 2.1$ Hz) ppm.

The $^{13}\text{C-NMR}$ spectrum exhibited 17 signals (Fig.51) DEPT 90 and DEPT 135 experiments (Fig.52) showed two methoxy carbons at 55.6 and 56.0 ppm, five tertiary carbons at 91.9, 97.5, 111.8, 114.8 and 119.8 ppm, nine quaternary carbons at 401.0, 123.4, 136.4, 146.2, 146.8, 149.5, 156.1, 160.4, and 164.9 ppm respectively, and no methylene carbons. One carbonyl carbon of a conjugated ketone was present at 176.1 ppm.

The mass spectrum (Fig.49) showed a molecular ion peak at M^+ 330 and other fragments at m/e 315, 287, 259, 231, 167, 151 and 123.

The above data indicated that compound 4 has 17 carbons,14 protons, two methoxy groups and three hydroxy groups. This compound suggested that there were at least five oxygen atoms in the molecule. The molecular ion at m/e 330 indicated that the molecular formula was $\text{C}_{17}\text{H}_{14}\text{O}_7$ corresponding to a degree of unsaturation of 11. However, the molecular formula suggested that this compound has another two oxygens in the molecule. These requirements could be fulfilled by a flavonol skeleton as shown below.



The basic skeleton of flavonol

The $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ data showed the presence of five substituents, which were two methoxy groups at 3.84 (6H,s), three hydroxy groups at 9.28 (1H,s), 9.51 (1H,br s) and 12.41 (1H,s) ppm. One of the hydroxy group, showing a signal at 12.41 ppm, was unusually deshielded, which could result from H-bonding with the carbonyl carbon at the position C-4, hence, this hydroxy group should be located at the position C-5.

For further information, two dimensional NMR-HMQC which showed one bond H-C correlation, was performed and the correlation of ^1H and $^{13}\text{C-NMR}$ chemical shifts was shown in Fig.53 and Table 22.

The spectrum showed that the protons of the two methoxy group at 3.84 ppm were attached to the carbons at 55.6 and 56.0 ppm, the proton at 6.32 ppm was attached to the carbon at 97.5 ppm, the proton at 6.67 ppm was attached to the carbon at 97.9 ppm, the proton at 7.07 ppm was attached to the carbon at 111.8 ppm, the proton at 7.66 ppm was attached to the carbon at 119.8 ppm, the proton at 7.71 ppm was attached to the carbon at 114.7 ppm.

Table 22 ^1H and ^{13}C -NMR spectral data of compound 4 by HMQC

| ^{13}C -NMR (ppm.) | Attached proton (ppm) |
|-----------------------------|-----------------------|
| 55.6 | 3.84 |
| 56.0 | 3.84 |
| 91.9 | 6.67 |
| 97.5 | 6.32 |
| 111.8 | 7.07 |
| 114.8 | 7.71 |
| 119.8 | 7.66 |

To confirm the data above a H-C long range coupling experiment HMBC (Heteronuclear multiple bond correlation) was performed and the results are shown in Fig.54 and in Table 23. This indicated that the hydroxy proton at 12.41 ppm was near to the carbon at 156.1 (C-5) ppm, and two meta protons at 6.32 and 6.67 ppm were attached to the carbon at 97.5 (C-6) and 91.9 (C-8) ppm respectively, the hydroxy proton at 9.28 ppm was attached to the carbon at 146.2 (C-3') ppm because this proton showed a cross peak with the carbon at 114.8 (C-2') and 149.5 (C-4') ppm which beared the proton at 7.71 ppm and the methoxy protons at 3.84 ppm respectively. Another hydroxy proton at 9.51 ppm was attached to the carbon at 136.4 (C-3) ppm as this proton showed a cross peak with the carbon at 146.8 (C-2') ppm which was a quarternary carbon. The proton at 7.07 ppm was attached to the

carbon at 111.8 (C-5') ppm because this proton showed a cross peak with the carbon at 123.4 (C-1') and 146.24 (C-3') ppm, and the proton at 7.66 ppm was attached to the carbon at 119.8 (C-6') ppm because it showed a cross peak with the carbon at 114.7 ppm.

Table 23 The one bond and multiple bond correlation of compound 4

| Carbon position | proton (ppm) | One bond correlations Attached carbon (ppm) | Multiple bond correlations Attached carbon (ppm) |
|-----------------|-----------------|---|--|
| C-2 | - | 146.8 | - |
| C-3 | - | 136.4 | - |
| C-4 | - | 176.1 | - |
| C-5 | - | 156.1 | - |
| C-6 | 6.32 | 97.5 | 91.9, 104.0, 160.4, 164.9 |
| C-7 | - | 164.9 | - |
| C-8 | 6.67 | 91.9 | 97.5, 104.0, 156.1, 164.9 |
| C-9 | - | 160.4 | - |
| C-10 | - | 104.0 | - |
| C-1' | - | 123.4 | - |
| C-2' | 7.71 | 114.8 | 119.8, 146.2, 146.8 149.5 |

Table 23 (cont.)

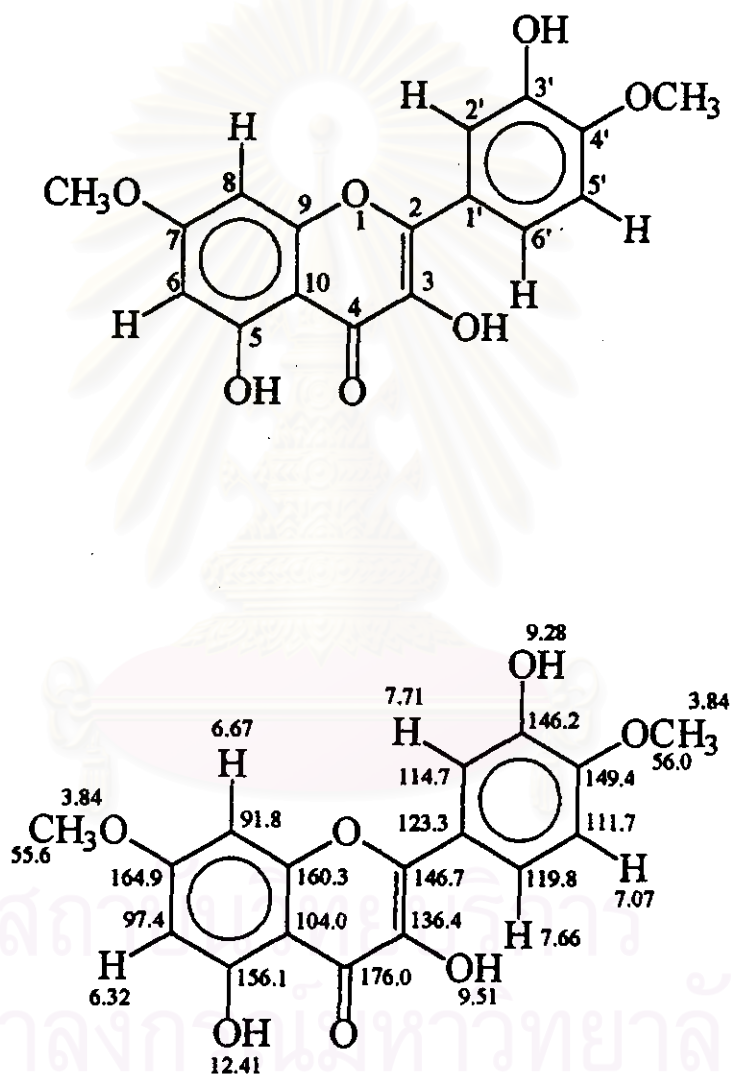
| Carbon position | proton (ppm) | One bond correlations | Multiple bond correlations |
|-----------------|-----------------|--------------------------|----------------------------|
| | | Attached carbon (ppm) | Attached carbon (ppm) |
| C-3' | - | 146.2 | - |
| C-4' | - | 149.5 | - |
| C-5' | 7.07 | 111.8 | 123.4, 146.2 |
| C-6' | 7.66 | 119.8 | 114.7 |
| methoxy in C-7 | 3.84 | 55.6 | 164.9 |
| methoxy in C-4' | 3.84 | 56.0 | 149.5 |
| hydroxy in C-3 | 9.51 | - | 146.8, 176.1 |
| hydroxy in C-5 | 12.41 | - | 97.45, 104.03, 160.38 |
| hydroxy in C-3' | 9.28 | - | 114.78, 149.46 |

The above results indicated that compound 4 was ombuin which is a known compound, by comparison of its ^1H and ^{13}C -NMR with those reported [27] which was closely matched as shown in Table 24.

Table 24 ^1H and ^{13}C -NMR spectral data of compound 4 compared with the literature

| Position | ^{13}C -NMR (lit.) (ppm) ombuin | ^{13}C -NMR (ppm) compound 4 | ^1H -NMR (ppm) compound 4 |
|-----------------|--|---|--|
| C-2 | 146.7 | 146.7 | - |
| C-3 | 136.4 | 136.4 | - |
| C-4 | 176.0 | 176.0 | - |
| C-5 | 156.0 | 156.1 | - |
| C-6 | 97.4 | 97.4 | 6.32 |
| C-7 | 164.9 | 164.9 | - |
| C-8 | 91.8 | 91.8 | 6.67 |
| C-9 | 160.4 | 160.3 | - |
| C-10 | 104.0 | 104.0 | - |
| C-1' | 123.4 | 123.3 | - |
| C-2' | 114.8 | 114.7 | 7.71 |
| C-3' | 146.2 | 146.2 | - |
| C-4' | 149.4 | 149.4 | - |
| C-5' | 111.7 | 111.7 | 7.07 |
| C-6' | 119.8 | 119.8 | 7.66 |
| methoxy in C-7 | 55.6 | 55.6 | 3.84 |
| methoxy in C-4' | 55.9 | 56.0 | 3.84 |
| hydroxy in C-3 | - | - | 9.51 |
| hydroxy in C-5 | - | - | 12.41 |
| hydroxy in C-3' | - | - | 9.28 |

Thus the complete assignment of the ^1H and ^{13}C -NMR chemical shift of compound 4 can be shown as follow:



The spectral data is consistent with the identification of compound 3 as 3',5-Dihydroxy-4',7-dimethoxyflavonol or ombuin [23,27]

This compound has been reported previously in leaves of *Polygonum hydropiper*, *Prosopis juliflora* bark and has been found in numerous other natural sources. [23,28-29] It has various interesting activities; antioxidative activity for protection against the rancidity of fats and oils in food.[29] and also has mutagenic activity in *Salmonella typhimurium* TA 98.[30]



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3.6 Structural elucidation of Compound 5

Compound 5 was isolated from fraction No. 100-155 by elution with 60%-80% CHCl_3 -hexane and was re-chromatographed to afford yellow needle-like crystals, m.p. 178-180 $^\circ\text{C}$.

The IR-spectrum of compound 5 (Fig.55) could be assigned as shown in Table 25. The spectrum showed important absorption bands at 3400-3100 cm^{-1} (O-H stretching vibration), 1649 cm^{-1} (C=O stretching vibration of conjugated ketone), 1595, 1503, and 1466 cm^{-1} (C=C stretching vibration of aromatic).

Table 25 The IR-spectrum band assignments of Compound 5

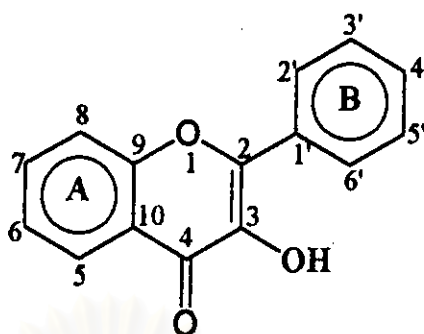
| Frequency (cm^{-1}) | Peak intensity | Tentative assignment |
|-----------------------------------|----------------|---|
| 3400-3100 | s | O-H stretching vibration |
| 2966, 2946 | w | C-H stretching vibration of $-\text{CH}_3$, $-\text{CH}_2$ |
| 1649 | s | C=O stretching vibration of conjugated ketone |
| 1595, 1503, 1466 | s | C=C stretching vibration of aromatic |
| 1353, 1310 | w | C-H bending vibration of $-\text{CH}_3$, $-\text{CH}_2$ |
| 1257, 1194, 1158 | s | C-O stretching vibration asymmetric of C-O-C |
| 1089, 1031 | m | C-O stretching vibration symmetric of C-O-C |
| 879, 833 | m | =C-H out of plane bending of aromatic |

The ^1H -NMR spectrum (Fig.57) of compound 5 showed two methoxy groups at 3.88 (3H,s) and 3.89 (3H,s) ppm, two hydroxy groups at 6.61 (1H,s) and 11.73 (1H,s) ppm and two aromatic protons with a *meta*-relationship at 6.37 (1H,d, $J = 2.1$ Hz) and 6.48 (1H, d, $J = 2.1$ Hz) ppm and another pair of aromatic protons at 7.01 (2H, d, $J = 9$) and 8.16 (2H, $J = 9$ A₂B₂ system) ppm.

The ^{13}C -NMR spectrum (Fig.58) exhibited 17 signals, DEPT 90 and DEPT 135 experiments (Fig.59) showed two methoxy carbons at 55.4 and 55.8 ppm, six tertiary carbons at 92.2, 97.9, 114.1 (2C) and 129.4 (2C) ppm, nine quaternary carbons at 103.9, 123.2, 135.7, 145.7, 156.8, 160.8, 161.6, 165.7, and 175.2 ppm respectively, but no methylene carbon was found, and one carbonyl carbon of a conjugated ketone was present at 175.2 ppm.

The mass spectrum (Fig.56) showed a molecular ion peak at M^+ 314 and other fragments at m/e 299, 285, 271, 243, 167 and 135.

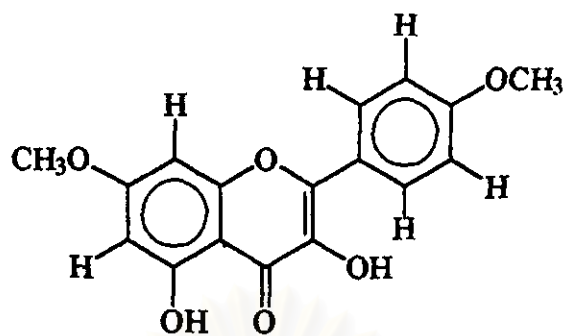
The above result showed that compound 5 has a molecular weight of 314 and contained 17 carbons, 14 protons, two oxygens in two methoxy groups and two oxygens in two hydroxy groups. Assuming the molecule contains only C,H and O, therefore, the molecular formula should be C₁₇H₁₄O₆. The molecular formula obtained suggested a degree of unsaturation of 11 which indicated that compound 4 probably possesses a flavonol skeleton according to the general structure shown below:



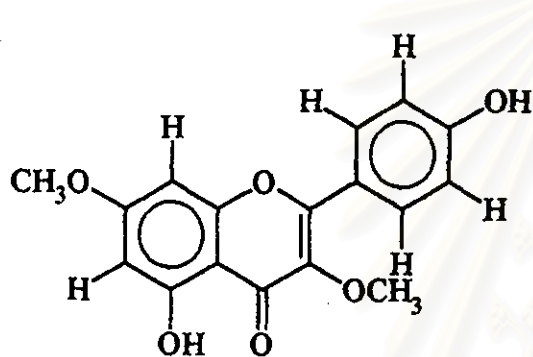
The basic skeleton of flavonol

From the ^1H -NMR spectrum, it can be deduced that compound 5 possesses four substituents, which are two methoxy groups and two hydroxy groups. These could be located on any of the carbon at positions C- 3, 5, 6, 7, 8, 2', 3', 4', 5' and 6'. One of the hydroxy groups showed a signal at 6.61 ppm should be at the 3-carbon position according to the basic skeleton of flavonol compound. Another hydroxy group showing a signal at 11.74 ppm was unusually deshielded and suggested the presence of H-bonding with the carbonyl carbon of the conjugated ketone at the position C-4. Therefore this hydroxy group should be located at the 5-carbon position. There is an aromatic A_2B_2 system which is characteristic for *para*-substituted aromatic rings. This is possible only for ring B because ring A already possess *ortho*-substituents. Another two aromatic protons with a *meta* relationship as suggested by $J = 2.1$ Hz could then be assigned to ring A, and the two methoxy groups should be located on ring A and B each.

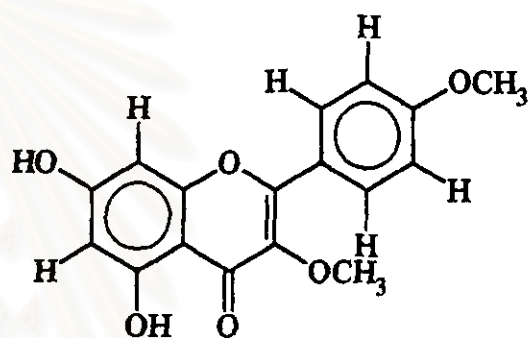
The possible structures are:



1



2



3

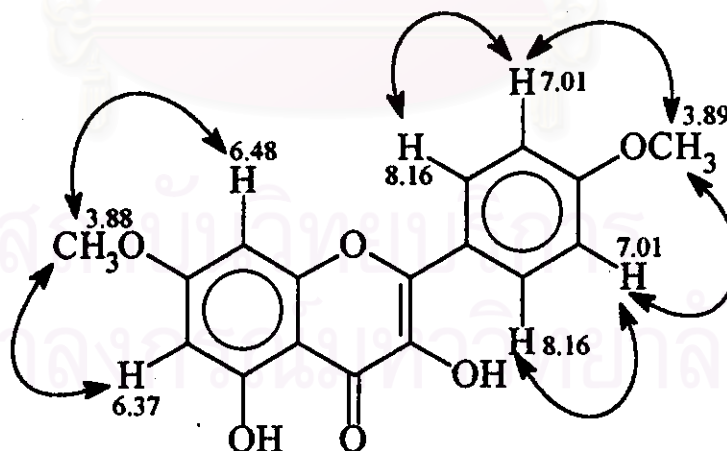
Two dimensional NMR were used to determine the structure. One bond C-H correlation (HMQC) (Fig.60) data showed that the protons of the methoxy groups at 3.88 and 3.89 ppm were attached to the carbon at 55.4 and 55.8 ppm respectively, and the protons at δ 6.38, 6.48, 7.03, and 8.16 ppm were attached to the carbons at 97.9, 92.2, 114.1, and 129.4 ppm respectively, in Table 26.

Table 26 ^1H and ^{13}C -NMR spectral data of compound 5 by HMQC

| Carbon (ppm) | Attached proton (ppm) |
|--------------|-----------------------|
| 145.7 | - |
| 135.6 | - |
| 175.2 | - |
| 156.8 | - |
| 97.8 | 6.37 |
| 165.7 | - |
| 92.2 | 6.48 |
| 160.8 | - |
| 103.9 | - |
| 123.2 | - |
| 129.3 | 8.16 |
| 114.0 | 7.01 |
| 161.1 | - |
| 114.0 | 7.01 |
| 129.3 | 8.16 |
| 55.4 | 3.88 |
| 55.8 | 3.89 |
| - | 6.61 |
| - | 11.73 |

NOE difference spectroscopy experiments (Fig.61-63) on compound 5 also provided additional information. Irradiation of the proton at 7.01 ppm caused enhancement of the signal at 8.16 and 3.89 ppm. This data indicated that proton at 7.01 ppm was near to the proton at 8.16 ppm and near to one of the methoxy groups. While irradiation of the proton at 8.16 ppm caused enhancement of the signal at 7.01 ppm only. This indicated that the proton at 7.01 ppm was near to the proton at 8.16 ppm. In contrast, irradiation of the methoxy proton at 3.89 ppm only caused enhancement of the signal at 7.01 ppm. These data confirmed that the proton at 7.01 ppm was located between the protons at 8.16 ppm and the protons of the methoxy group at 3.89 ppm.

The only structure that agrees with all the data above is shown below.



Most significant interaction observed in NOE of compound 5

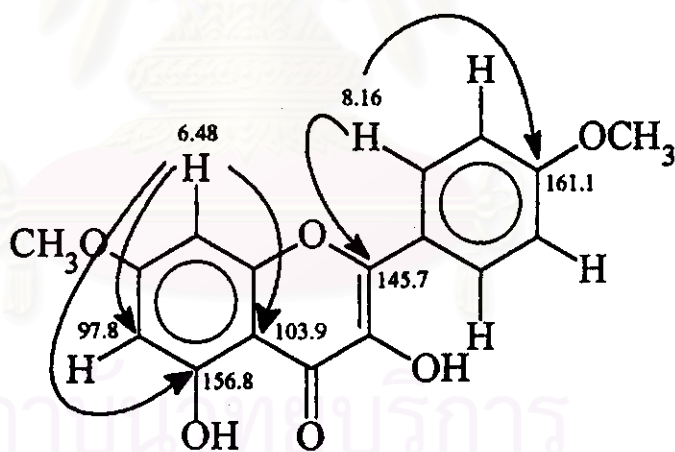
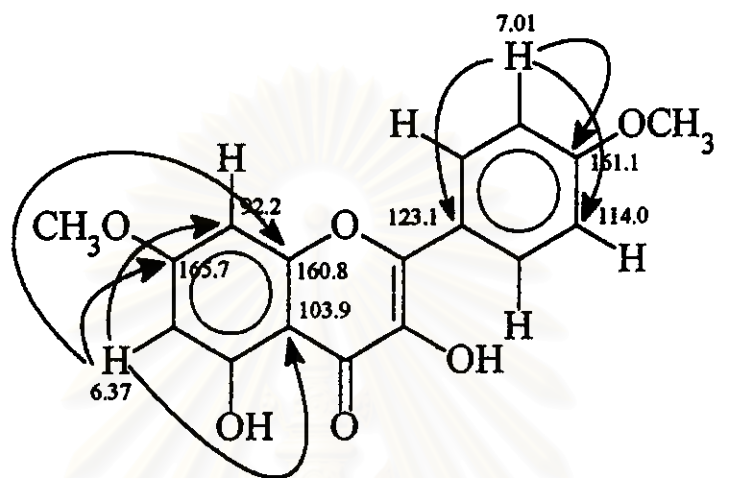
Similarly, irradiation of the proton at 6.48 ppm caused enhancement of the signal at 3.88 ppm in methoxy group. Irradiation of the proton at 6.37 ppm caused enhancement of the signal at 3.88 ppm of the same methoxy group. This data indicated that protons of the methoxy group at 3.88 ppm was near to the protons at 6.48 and 6.37 ppm. Irradiation of the methoxy protons at 3.88 ppm caused enhancement of both signals at 6.37 and 6.48 ppm. As a result these data confirmed that the protons in the methoxy group at 3.88 ppm located between the protons at 6.48 and 6.37 ppm.

In The H-C long range coupling information obtained by HMBC experiment (heteronuclear multiple bond correlation) (Fig.64). The aromatic proton at 6.38 ppm showed cross peaks with the carbons at δ 62.2 (C-8), 165.7 (C-7), 160.8 (C-9) and 103.9 (C-10) ppm. The proton at 6.48 ppm showed cross peaks with the carbons at 97.9 (C-6), 103.9 (C-10) and 156.9 (C-5) ppm. The proton at 7.01 ppm showed cross peaks with the carbons at 123.2 (C-1'), 114.1 and 161.2 (C-4') ppm. The proton at 8.16 showed cross peaks with the carbons at 129.4 (c-2'), 145.7 (C-1) and 161.2(C-4') ppm as shown in Table 27.

Table 27 The one bond and multiple bond correlation of compound 5

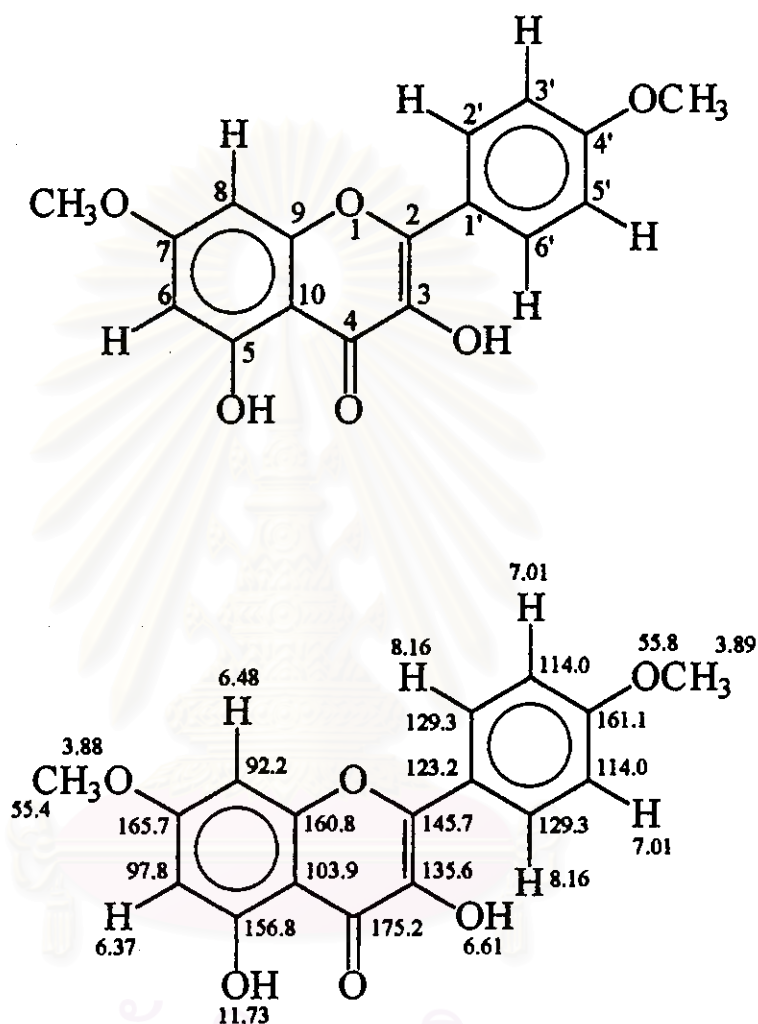
| Carbon position | proton (ppm) | One bond correlations Attached carbon (ppm) | Multiple bond correlations Attached carbon (ppm) |
|-----------------|-----------------|---|--|
| C-2 | - | 145.7 | - |
| C-3 | - | 135.6 | - |
| C-4 | - | 175.2 | - |
| C-5 | - | 156.8 | - |
| C-6 | 6.37 | 97.8 | 92.2, 165.7, 160.8 |
| C-7 | - | 165.7 | - |
| C-8 | 6.48 | 92.2 | 97.8, 103.9, 156.8 |
| C-9 | - | 160.8 | - |
| C-10 | - | 103.9 | - |
| C-1' | - | 123.2 | - |
| C-2' | 8.16 | 129.3 | 129.3, 161.1 |
| C-3' | 7.01 | 114.0 | 123.2, 114.0, 161.1 |
| C-4' | - | 161.1 | - |
| C-5' | 7.01 | 114.0 | 123.2, 114.0, 161.1 |
| C-6' | 8.16 | 129.3 | 129.3, 161.1 |
| methoxy in C-7 | 3.88 | 55.4 | |
| methoxy in C-4' | 3.89 | 55.8 | |
| hydroxy in C-3 | 6.61 | - | |
| hydroxy in C-5 | 11.73 | - | |

The HMBC data also confirmed the possible structure suggested by NOE experiment



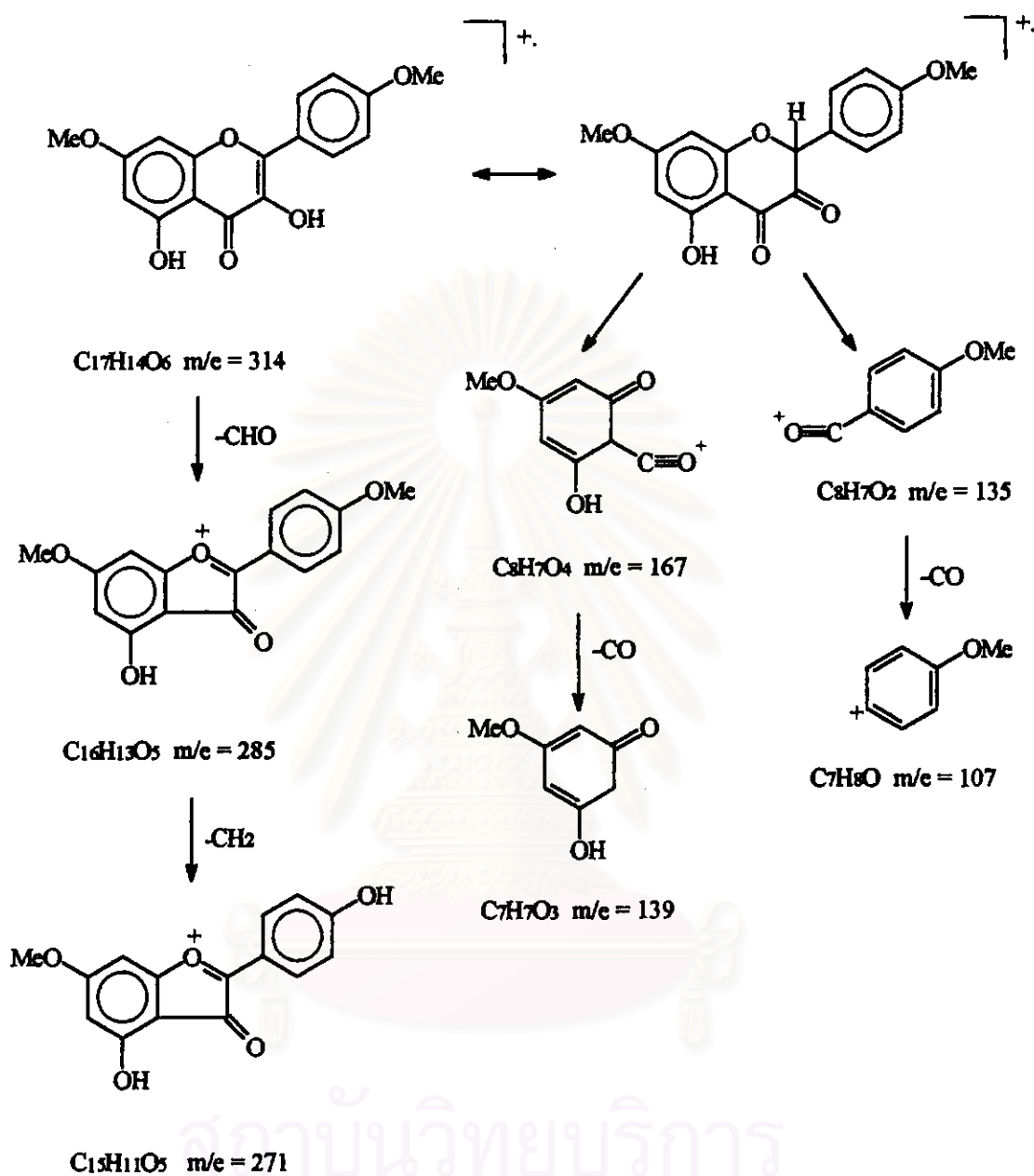
Most significant correlation observed in HMBC of compound 5

Thus the complete assignment of the ^1H and ^{13}C -NMR chemical shifts of compound 5 are as shown:



The spectral data is consistent with the identification of compound 5 as 5-Hydroxy-4',7-dimethoxyflavonol or Kaempferol 4',7-dimethyl ether[23]

This compound has been reported previously in the fern *Cheilanthes farinosa*, also found in *Butula nigra* and other plants [23,31-32] which possessed inhibition of TPA-induced inflammation activity.[33]



Scheme 3 The possible mass fragmentation patterns of compound 5 [34-35]