CHAPTER III

RESULTS AND DISCUSSION

In reference to the preliminary screening for agrochemicals from the flowers of *D. metel*, the ethanol and ethyl acetate crude extracts of the flowers of *D. metel* exhibited impressive activity against brine shrimp *Artemia salina* Linn. and plant growth inhibition on *Lactuca sativa* Linn. Furthermore, the alkaloid portion revealed satisfied molluscicidal activity test against *Pomacea canaliculata* (golden apple snail). Even though, the leaves of *D. metel* have previously been examined and found that the ethanolic part exhibited high molluscicidal activity against *Pomacea canaliculata*[7], the agricultural-based biological activity of the constituents from flowers of *D. metel* has not been reported. Therefore, it was rationalized to select the flowers of *D. metel* for investigation the chemical constituents and searching for lead agrochemicals.

3.1 The results of extraction

The results of extraction of the flowers of *D. metel* by various solvents according to the procedure described in Chapter II are shown in Table 3.1.

Table 3.1 The results of extraction

Fraction	Crude extract	Weight(g), Percentage% (wt by wt of dried plant materials)
I	EtOH	643.15, 6.43%
II	EtOAc	220.00, 2.20%
III	Alkaloid	31.08, 0.31%

From the result of extraction, it was observed that the alkaloid content in the flowers of *D. metel* was quite high. The flowers of *D. metel* may be considered as a good source for bioactive compound if the alkaloid present exhibited good activity.

3.2 The results of biological activity screening test

3.2.1 Brine shrimp cytotoxicity test

Various crude extracts of the flowers of *D. metel* were preliminarily screened for cytotoxic activity against brine shrimp (*Artemia salina* Linn.) according to the procedures described in Chapter II. The results are displayed in Table 3.2.

Table 3.2 The results of brine shrimp cytotoxicity test

Crude	LC_{50} (ppm) at 24 hours	Activity
Fraction I (EtOH)	34.9	Moderate
Fraction II (EtOAc)	17.2	Moderate
Fraction III (Alkaloid)	728.2	Low

Note: 0-10 μg/mL; High toxicity, 10-100 μg/mL; Moderate toxicity
100-1000 μg/mL; Low toxicity, >1000 μg/mL; No toxicity

According to the above result, Fraction I (ethanol extract) and Fraction II (ethyl acetate extract) displayed interesting cytotoxic activity against brine shrimp. It was interesting that Fraction III (alkaloid fraction) did not reveal significant cytotoxicity against brine shrimp. This presumably implied that the alkaloids present in this fraction possessed low cytotoxic activity. The low cytotoxicity against brine shrimp also suggested that this fraction might not affect on normal cells of human beings or mammal. In the other words, an alkaloid might show cytotoxicity against abnormal cells of human beings or other organisms. Thus, the alkaloid fraction can possibly be used for other works, which were not harmful to human beings or mammal, as insecticide, molluscicide, *etc.* From this reason, an alkaloid fraction perhaps is useful in agrochemical aspects.

3.2.2 Plant growth inhibition on *Lactuca sativa* Linn.

The same three crude extracts were tested for plant growth inhibition on *Lactuca satiya* L. The procedure was described in Chapter II. The results are shown in Table 3.3 and Figure 3.1.

Table 3.3 The effect of crude extracts of *D.metel* on the growth of *Lactuca sativa* L.

Crude	Part	% Inhibition		
		0.1 g	0.5 g	1.0 g
Fraction I (EtOH)	Root	22	100	100
	Shoot	-20	100	100
Fraction II (EtOAc)	Root	-32	100	100
	Shoot	26	100	100
Fraction III (Alkaloid)	Root	-8	3	34
	Shoot	-6	13	21

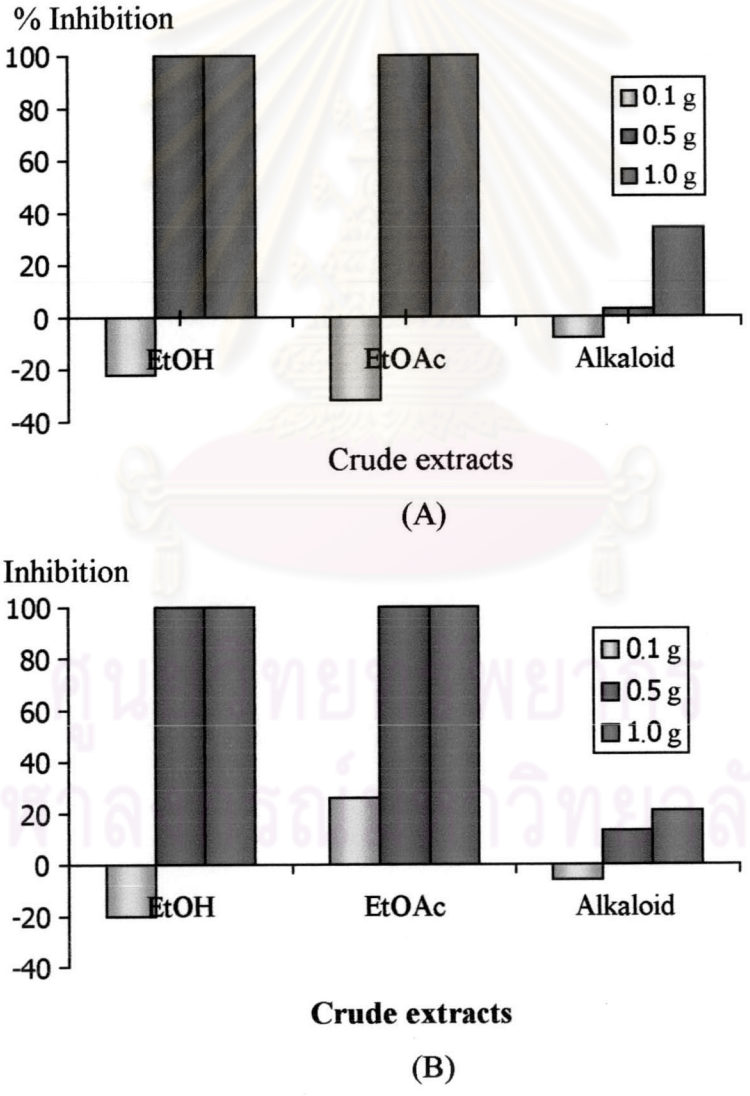


Figure 3.1 The effect of crude extracts of *D. metel* on A) the root growth of *Lactuca sativa* Linn. B) the shoot growth of *Lactuca sativa* Linn.

From the above data, the ethanol and ethyl acetate crude extracts exhibited high plant growth inhibition against both the root and the shoot of *Lactuca sativa* L. at concentrations of 1.0 g and 0.5 g of weight corresponding to dried plant materials. However at low concentration of ethanol crude extract, it showed shoot promotion activity while ethyl acetate extract displayed root promotion. The ethyl acetate crude extract was therefore selected for further investigation of the chemical constituents. In addition, the alkaloid portion exhibited interesting results to reveal low plant growth inhibition at every concentration.

3.2.3 Molluscicidal activity

The crude extracts of the flowers of *D. metel* were also preliminarily screened for molluscicidal activity against *Pomacea canaliculata* Lamarck. according to the procedures described in Chapter II. The results are reported in Table 3.4 and Figure 3.2.

Table 3.4 The results of molluscicidal activity test

Crude	% mortality of snail at 72 hours		
	0.1 g	1.0 g	10 g
Fraction I (EtOH)	56	67	89
Fraction II (EtOAc)	0	22	44
Fraction III (Alkaloid)	22	33	78

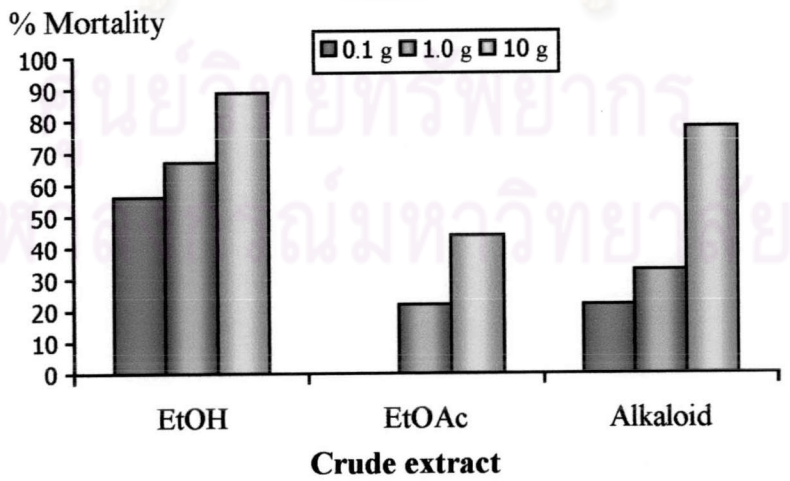


Figure 3.2 The results of molluscicidal activity test

According to the results of molluscicidal activity test, the ethanol and alkaloid crude extracts gave distinctive bioassay result. Thus, chemical investigation of alkaloid crude extract was proceeded.

As a summary, the agrochemical screening results revealed that the ethyl acetate and alkaloid crude extracts had potential for further investigation towards biologically active constituents. The ethyl acetate crude extract displayed medium cytotoxicity against brine shrimp and showed high plant growth inhibition. In addition the alkaloid portion gave high activity against *P. canaliculata.* Based upon the information from biological guide, these crude extracts were selected for further examination in regard to search for new lead agrochemicals.

3.3 The results of the separation of crude extracts

3.3.1 Separation of ethyl acetate crude extract

The ethyl acetate extract as a dark yellow-brown gum, 220.00 g was separated by column chromatography using Marck's silica gel 60 Art 7734 as an adsorbent. The column was initially eluted with dichloromethane, then with dichloromethane-ethyl acetate, ethyl acetate, and ethyl acetate-methanol, respectively. Each fraction approximately 250 mL was collected and concentrated to small volume and then checked by TLC. Fractions containing similar components were combined. The results of separation and combination are shown in Table 3.5.

Table 3.5 The results of the separation of ethyl acetate crude extract (Fraction II)

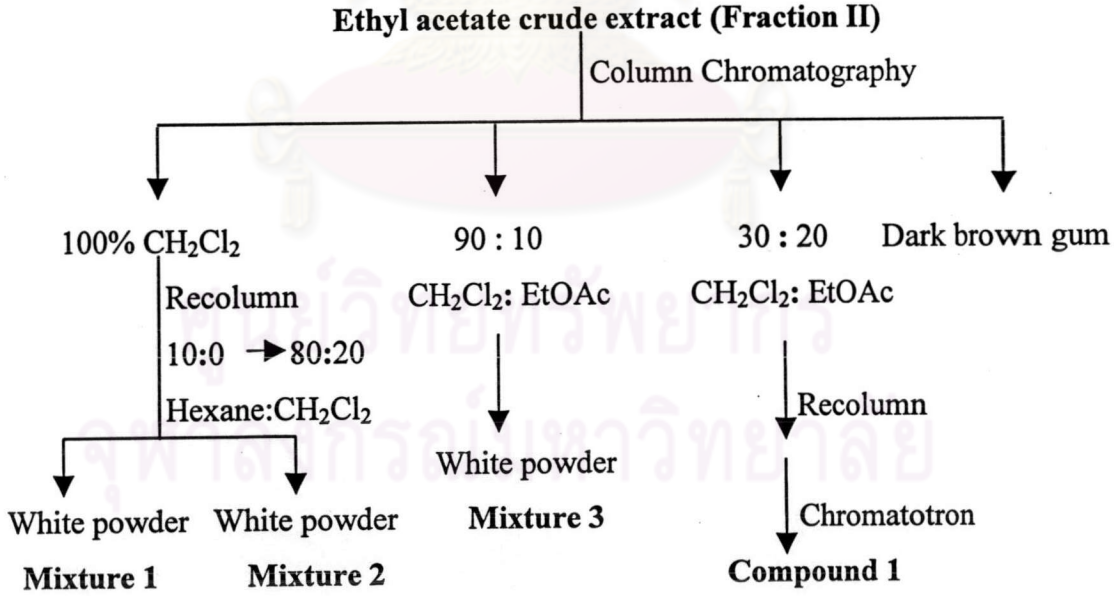
Eluent (% vol/vol)	Fraction No. (250 mL)	Remarks	Weight (g)
100% CH_2Cl_2	1-17 (**EC1**)	White amorphous in pale yellow oil	13.05
10% EtOAc-CH_2Cl_2	18-21 (**EC2**)	White solid in yellow oil **(Mixture 3)**	6.59
20% EtOAc-CH_2Cl_2	22-50 (**EC3**)	Yellow oil	8.35
30% EtOAc-CH_2Cl_2	51-65 (**EC4**)	Yellow oil	7.75
40% EtOAc-CH_2Cl_2	66-80 (**EC5**)	Yellow solid in yellow oil	6.68
50% EtOAc-CH_2Cl_2	81-99 (**EC6**)	Yellow brown oil	9.12
60% EtOAc-CH_2Cl_2	100-115 (**EC7**)	Brown oil	4.43
70% EtOAc-CH_2Cl_2	116-125 (**EC8**)	Brown oil	7.06
80% EtOAc-CH_2Cl_2	126-142 (**EC9**)	Brown oil	8.92
90% EtOAc-CH_2Cl_2	143-157 (**EC10**)	Dark brown viscous liquid	6.58
100%EtOAc	158-190 (**EC11**)	Dark brown viscous liquid	10.13
5% MeOH-EtOAc	191-205 (**EC12**)	Dark brown gum	4.55
10% MeOH-EtOAc	206-250 (**EC13**)	Dark brown gum	8.06

Separation of Fraction EC1

Fraction **EC1** as white amorphous in pale yellow oil (13.05 g) was reseparated by silica gel column chromatography. Gradient solvent using hexane, dichloromethane and methanol was used to elute the column. Each fraction about 50 mL was collected and concentrated to small volume and then checked by TLC using 30% hexane in dichloromethane as a mobile phase. Fractions containing similar components were combined. The results of separation and combination are shown in Table 3.6 and an overview procedure is summarized as presented in Scheme 3.1.

Table 3.6 The results of the separation of Fraction **EC1**

Eluent (%vol/vol)	Fraction No. (50 mL)	Remarks	Weight (g)
100% hexane	1-6	Yellow wax	trace
10% CH_2Cl_2-hexane	7-15	White solid in yellow oil **(Mixture 1)**	0.92
20% CH_2Cl_2-hexane	16-33	Yellow oil	0.16
40% CH_2Cl_2-hexane	34-50	White solid in yellow	0.60
50% CH_2Cl_2-hexane	51-70	oil **(Mixture 2)**	0.85
60% CH_2Cl_2-hexane	71-109	Yellow oil	0.27
80% CH_2Cl_2-hexane	110-117	Yellow oil	Trace
100% CH_2Cl_2	118-147	Yellow oil	1.94
5% MeOH-CH_2Cl_2	148-158	Yellow oil	1.13
10% MeOH-CH_2Cl_2	159-183	Yellow viscous liquid	2.35
20% MeOH-CH_2Cl_2	184-190	Yellow viscous liquid	0.19



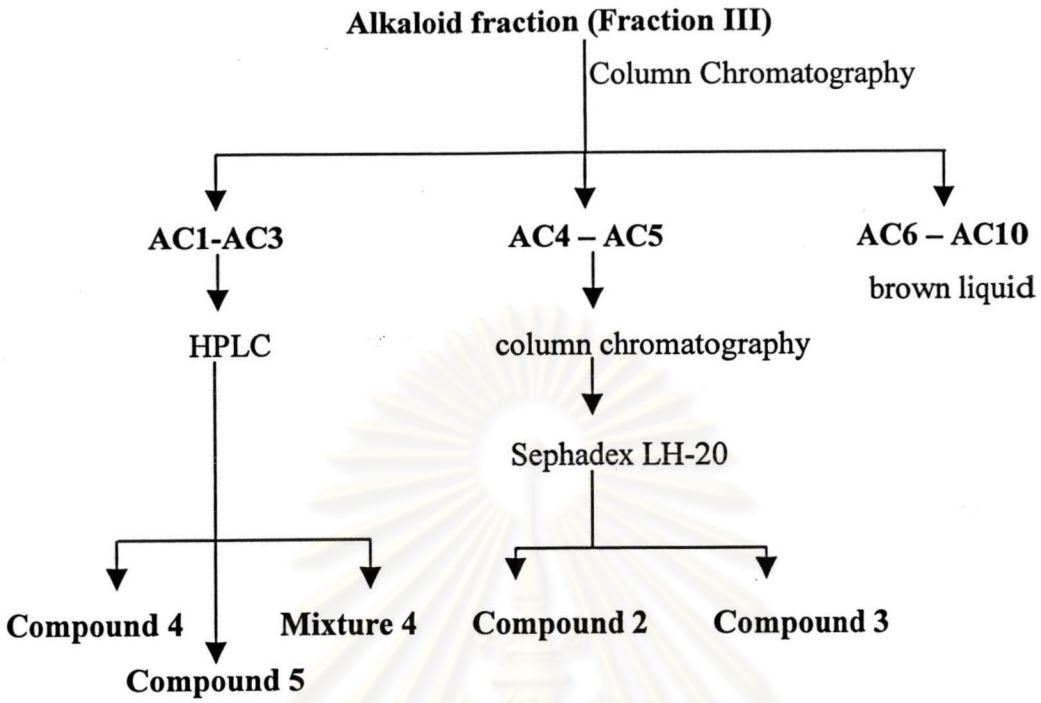
Scheme 3.1 The summarized separation of ethyl acetate crude extract

3.3.2 Separation of alkaloid fraction

The alkaloid fraction as deep yellow viscous liquid (28.6 g) was separated by aluminum oxide column chromatography. This column was eluted with gradient chloroform: methanol in a stepwise fashion. All fractions were checked by TLC and fractions displaying similar components were combined. Silica gel and sephadex column chromatographies were utilized for further purification of each fraction. The results of the separation of alkaloid fraction are shown in Table 3.7 and an overview procedure is summarized as presented in Scheme 3.2.

Table 3.7 The results of the separation of the alkaloid fraction (Fraction III)

Eluent (% vol/vol)	Fraction No. (50 mL)	Remarks	Weight(g)
100% $CHCl_3$	1-4 (**AC1**)	Pale yellow liquid	Trace
5% MeOH-$CHCl_3$	5-9 (**AC2**)	Pale yellow liquid	1.03
10% MeOH-$CHCl_3$	10-20 (**AC3**)	Yellow liquid	0.37
20% MeOH-$CHCl_3$	21-42 (**AC4**)	Yellow liquid	8.89
30% MeOH-$CHCl_3$	43-60 (**AC5**)	Deep yellow liquid	6.71
50% MeOH-$CHCl_3$	61-76 (**AC6**)	Deep yellow liquid	3.07
60% MeOH-$CHCl_3$	77-87 (**AC7**)	Yellow brown liquid	1.08
70% MeOH-$CHCl_3$	88-94 (**AC8**)	Yellow brown liquid	Trace
80% MeOH-$CHCl_3$	95-106 (**AC9**)	Yellow brown liquid	Trace
100% MeOH	107-140 (**AC10**)	Brown liquid	1.00



Scheme 3.2 The separation of alkaloid fraction

3.4 Purification, properties and structural elucidation of isolated substances

3.4.1 Purification, properties and structural elucidation of Mixture 1

Mixture **1** as white solid, 0.49 g (0.22% w/w of ethyl acetate extract) was obtained from ethyl acetate crude extract by eluting with 100% CH_2Cl_2 and repeated column chromatography by using hexane in dichloromethane as a mobile phase. Its spot on TLC could not be detected under UV light but be visualized when dipping in 10% H_2SO_4 in ethanol. This mixture showed blue-green solution with Liebermann-Burchad's reagent, which indicated the presence of steroid compound. The melting point of this mixture was 66-68 °C. The TLC showed R_f value of 0.47 in hexane: dichloromethane (3:7) as a solvent system.

The IR spectrum (Figure 1) showed the C-H stretching vibration of CH_2, CH_3 at 2919 (s) and 2852 (s) cm^{-1} and C=O stretching vibration of an ester at 1738(s) cm^{-1}. The additional absorption peak at 1175 (m) was the characteristic of C-O stretching vibration. From the IR spectroscopic data, Mixture **1** was roughly identified as a mixture of steroid ester. In order to verify its structure, the basic hydrolysis of this

mixture was set up. Two parts, designated as Mixture **1a** (an alcoholic part) and Mixture **1b** (a carboxylic part) were gained.

Study on Mixture 1a

Mixture **1a** (0.06 g) as white needle crystal had melting point of 117-118 °C. The TLC exhibited only one brown spot after dipping with 10% H_2SO_4 in ethanol with R_f value of 0.58 in hexane: ethyl acetate (4:1) as a solvent system. Reacting with Liebermann-Burchard's reagent, it showed blue-green color solution, which is the characteristic of steroid compound.

The IR spectrum (Figure 2) revealed the characteristic absorption peak of O-H stretching vibration of hydroxy group at 3424 cm^{-1} and C-O stretching vibration at 1050 cm^{-1}. The C-H stretching and bending vibration of CH_2 and CH_3 were observed at 2926, 2844, 1466 and 1373 cm^{-1}. From the chemical reaction and spectroscopic data, Mixture **1a** could be identified as a mixture of steroids. Thus, the GC technique was used for further analyzing the components of this mixture. The GC analysis of Mixture **1a** was compared with standard steroids, namely compesterol, stigmasterol, and β-sitosterol. The result of analysis (Figure 3.3) showed the two major peaks at retention time of 24.45 and 27.80 min, corresponding to stigmasterol and β-sitosterol, respectively. The percent composition of this mixture is presented as shown in Table 3.8.

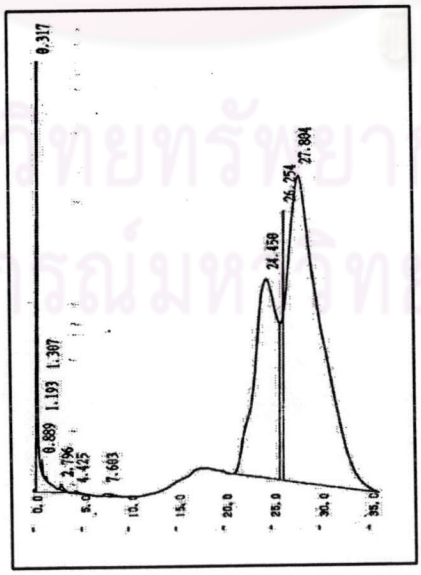
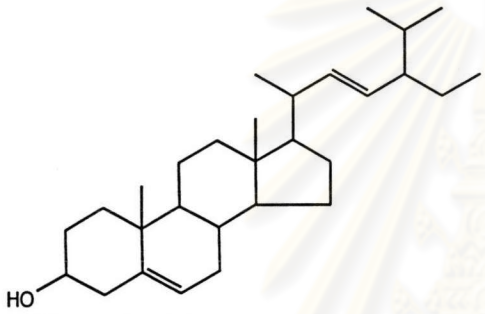


Figure 3.3 Chromatogram of Mixture **1a**

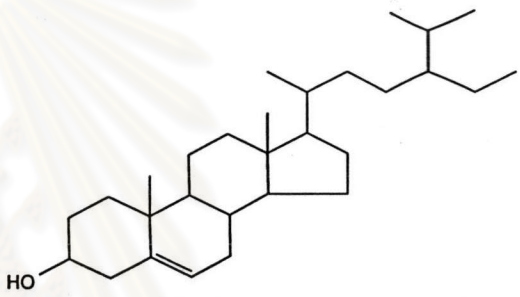
Table 3.8 The composition of steroids in Mixture **1a**

Name	Retention time (min)	% composition
Stigmasterol	22.29	31
β-sitosterol	27.52	69

From the color test, spectroscopic data and GC analysis, Mixture **1a** could be deduced the structure as a mixture of stigmasterol and β-sitosterol in ratio approximately 1:2.



Stigmasterol



β-Sitosterol

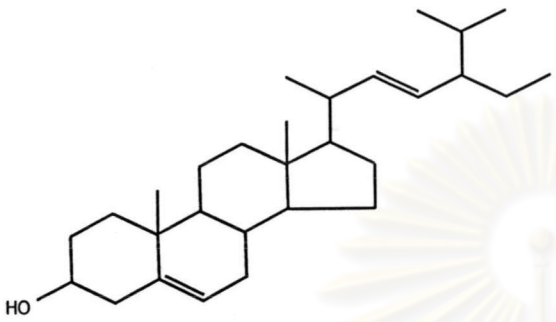
Study on Mixture 1b

Mixture **1b** as white amorphous solid (0.05 g), melting point 41-42°C was designated for the acidic part derived from basic hydrolysis of Mixture **1**.

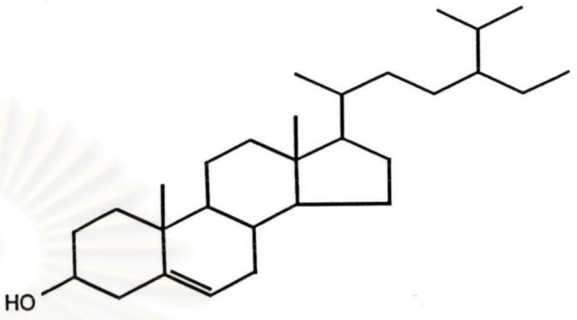
The IR spectrum (Figure 3) showed the broad absorption peak of O-H stretching vibration of carboxylic acid at 3000-3500 cm^{-1}, C=O stretching vibration of carboxylic acid at 1696 (s) cm^{-1} and C-O stretching vibration at 1291 cm^{-1}. The additional absorption peaks of C-H stretching and bending vibration of CH_2 and CH_3 were observed at 2926, 2844, 1466 and 1373 cm^{-1}.

This acidic part was transformed into its methyl ester with trimethylsilyl diazomethane by methylation method. The methyl ester derivative was further analyzed by GC-MS (DB-35 column). The GC-MS spectrum (Figure 4) gave two major peaks with retention time of 8.40 and 9.26 minutes. The corresponding molecular ion peaks were observed at m/z 270 and 298, respectively. The mass spectra (Figures 5-6) compared with NIST databases were well corresponded to methyl palmitate and methyl stearate.

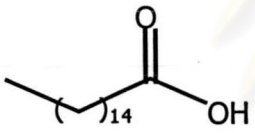
According to the chemical reaction, spectroscopic data and GC-MS analysis, it could be concluded that Mixture **1** was steroidal ester. The steroidal part of Mixture **1** was a mixture of stigmasterol and β-sitosterol and the other part (acidic part) was a mixture of palmitic acid and stearic acid.



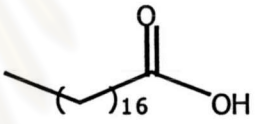
Stigmasterol



β-Sitosterol



Hexadecanoic acid (palmitic acid)



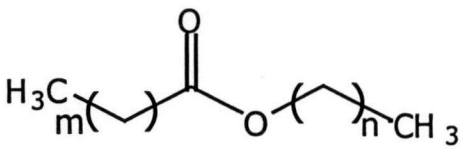
Octadecanoic acid (stearic acid)

3.4.2 Purification, properties and structural elucidation of Mixture 2

Mixture **2** (1.19 g, 0.54% w/w of ethyl acetate extract) was eluted with 100% dichloromethane and repeated column chromatography with dichloromethane in hexane (10:0 to 4:1) solvent system (see Tables 3.4-3.5). This mixture was inactive under UV light and in 10% H_2SO_4 ethanolic solution. The white amorphous solid obtained after recrystallization with a mixture of hexane-ethyl acetate and its melting point was 61-62°C. It showed negative tests to Liebermann-Burchard's and Br_2 in chloroform reagents which signified the characteristic of saturated hydrocarbon and no steroidal moiety present in this mixture.

The IR spectrum (Figure 7) showed an absorption peak belonging to C=O stretching vibration of ester at 1704 cm^{-1} (s) and C-H stretching vibration at 2914 and 2844 cm^{-1}. The C-H bending vibration of CH_2 was exhibited at 1459 cm^{-1}. In addition, the absorption peak at 719 cm^{-1} was due to CH_2 (for chain, 4 carbons). From this

spectroscopic data, Mixture **2** was identified as a mixture of saturated long chain aliphatic ester.



Mixture 2

3.4.3 Purification, properties and structural elucidation of Mixture 3

This mixture (1.50 g, 0.68% w/w of ethyl acetate extract) was isolated from ethyl acetate crude extract by silica gel column chromatography eluting with 90% dichloromethane in ethyl acetate and was recrystallized in chloroform and methanol to obtain white powder (m.p. 100-106 °C). This mixture could not be detected on TLC under UV light, but a brown spot could be visulized with 10% H_2SO_4 in ethanol. Reacting with Liebermann-Burchard's reagent, it showed blue-green color solution, which indicated the characteristic of steroid compound. The GC-MS technique was used to analyze for the structure of the composition in this mixture.

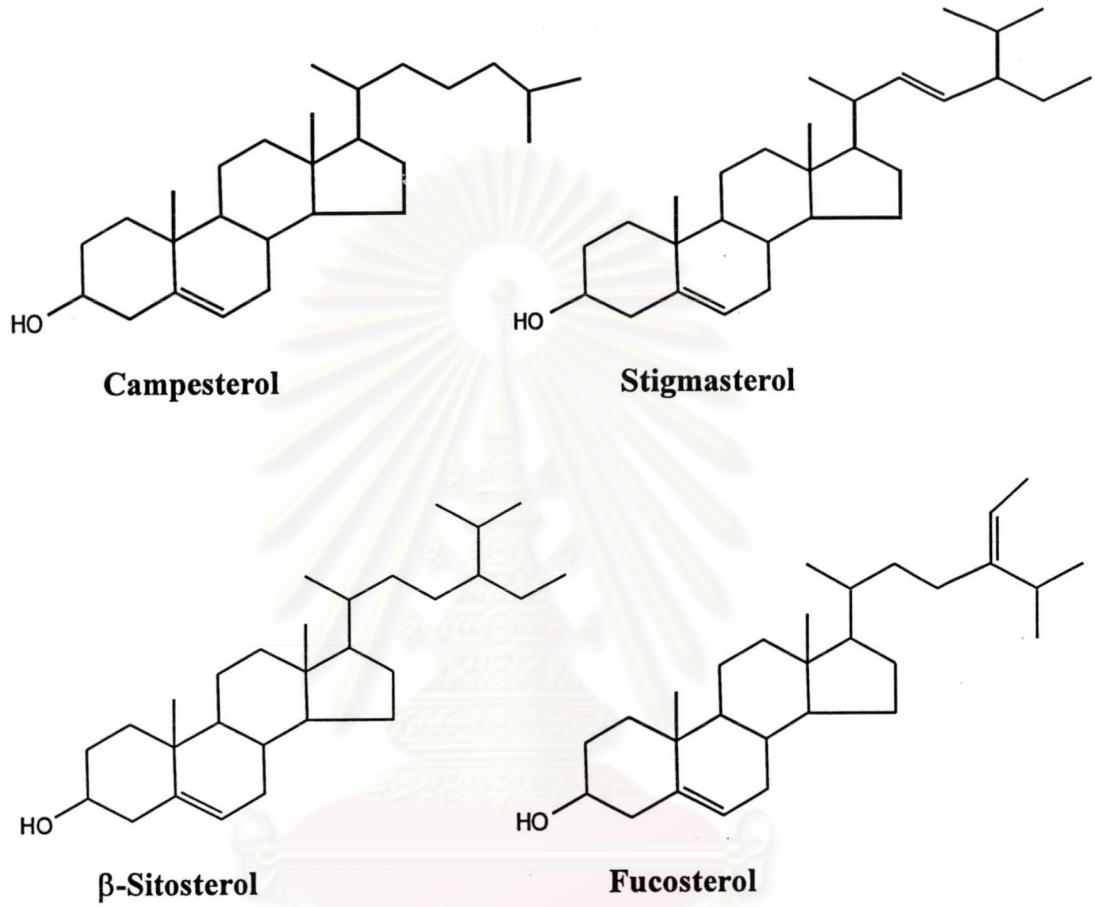
The GC-MS spectrum (Figures 8-12) gave 4 peaks at retention times of 7.33, 7.60, 8.26 and 8.49 minutes, with the molecular ion peak at m/z 400, 412, 414 and 412, respectively. The mass fragmentation patterns of these four components were well matched and corresponded to campesterol, stigmasterol, β-sitosterol and fucosterol (NIST database). The %composition of each steroid analyzed by GC-MS is shown in Table 3.9.

Table 3.9 The composition of steroids in Mixture **3**

Name	Retention time (min)	% composition
Campesterol	7.33	29
Stigmasterol	7.60	27
β-sitosterol	8.26	25
Fucosterol	8.49	19

From Table 3.9, it could be concluded that Mixture **3** was a mixture of campesterol, stigmasterol, β-sitosterol, and fucosterol. It was also observed that the

composition of campesterol, stigmasterol and β-sitosterol were in comparable amount in this mixture while fucosterol being a minor component. The structures of each component in Mixture **3** are shown below.



Campesterol **Stigmasterol**

β-Sitosterol **Fucosterol**

Mixture 3

3.4.4 Purification, properties and structural elucidation of Compound 1

After elution with 60% dichloromethane in ethyl acetate of ethyl acetate extract, Compound **1** was obtained as yellow solid in yellow oil. To purify this compound, silica gel column chromatography using 30% hexane in ethyl acetate as mobile phase, and chromatotron were employed to ultimately gain as yellow amorphous solid. The yellow small needle m.p. 274-275°C, 30 mg, 0.014% w/w of ethyl acetate extract, was obtained after recrystallization with a mixture of acetone-methanol. It showed a yellow single spot with R_f value of 0.49 (solvent system: 20% ethyl acetate in dichloromethane).

Its IR spectrum (Figure 13) showed a major absorption band of hydroxy group at 3000-3500 cm^{-1}. The strong absorption peaks at 1661 and 1610 cm^{-1} revealed the presence of α,β-unsaturated carbonyl functional group and the characteristic absorption peak of aromatic moiety was observed at 1501 cm^{-1}. In addition the strong absorption peak of C-O stretching vibration was detected at 1171 cm^{-1}.

The EI mass spectrum (Figure 14) of this compound gave a molecular ion peak at *m/z* 286 in accordance with a molecular formula $C_{15}H_{10}O_6$ (MW. 286.24). The base peak and the pattern of fragmentation were similar to the characteristic mass spectra of flavonoid compounds.[55]

The ^{1}H-NMR spectrum of Compound **1** (DMSO-d_6, Figure 15) exhibited two doublet aromatic proton signals with 2H integration at δ 6.93 (1H each, d, J = 6.90 Hz) and 8.11 (1H, d, J = 6.89 Hz). In addition, two singlet signals with 1H integration appeared at δ 6.41 and 6.20 for *meta*-positioned aromatic ring proton. The four signals of hydroxy groups were exhibited at δ 7.68, 9.42, 10.15 and 12.50 ppm.

The ^{13}C-NMR and DEPT-135 spectra (DMSO-d_6, Figures 16-17) signified that Compound **1** possessed a flavone skeleton based on total 15 carbons. The carbon signal, however displayed only 13 signals because of two methine carbons signals being co-incidence at the same chemical shift: δ 115.5 and 129.9 ppm. The ketone carbonyl carbon clearly showed the signal at δ 176.5 ppm, which revealed the characteristic feature of a flavonol compound (tetrahydroxyflavone). The quaternary carbon signals of aromatic ring were detected at δ 147.2 and 157.4 ppm, respectively. In addition, five quaternary carbon signals in flavonol skeleton were observed at δ 164.7, 161.7, 159.7, 122.9 and 103.8 ppm and two methine carbons of aromatic ring appeared at δ 98.5 and 93.7 ppm.

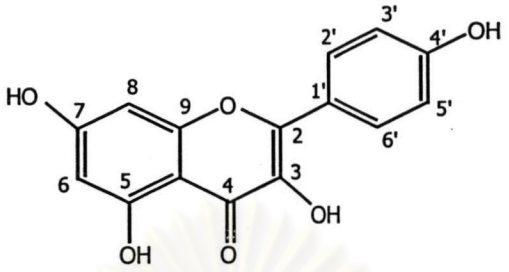
Moreover, the ^{1}H-^{1}H COSY spectrum (Figure 18) supplied informative data to prove the actual structure of this compound. The proton of aromatic rings at δ 6.93 and 8.11 ppm were correlated with the protons of benzene ring. From the spectral data, it could be concluded that Compound **1** was a flavonol compound containing four hydroxy groups. The ^{1}H and ^{13}C NMR chemical shift assignments of this compound compared with those of kaempferol reported in literature[55-56] are illustrated in Table 3.10.

Table 3.10 The comparison of ^{1}H and ^{13}C chemical shift assignments of Compound **1** with kaempferol[55-56]

Position	Chemical shift (ppm)			
	Compound **1**		Kaempferol	
	^{1}H-NMR	^{13}C-NMR	^{1}H-NMR	^{13}C-NMR
2	-	136.6	-	133.5
3	9.42	122.9	-*	122.6
4	-	176.5	-	187.0
5	12.50	159.7	12.52	160.6
6	6.20 (1H, d, J = 2.07 Hz)	98.5	6.20 (1H, d, J = 2.0 Hz)	97.6
7	7.68	164.7	-*	165.2
8	6.41 (1H, d, J = 2.07 Hz)	93.7	6.45 (1H, d, J = 2.0 Hz)	97.3
9	-	161.9	-	160.7
10	-	103.7		105.0
1′	-	147.2	-	147.0
2′	8.11 (2H, d, J = 6.89 Hz)	129.9	8.06 (2H, d, J = 9.0)	127.6
3′	6.93 (2H, d, J = 6.90 Hz)	115.5	6.95 (2H, d, J = 9 Hz)	115.6
4′	10.15	157.4	-*	156.5
5′	6.93 (2H, d, J = 6.90 Hz)	115.5	6.95 (2H, d, J = 9 Hz)	115.6
6′	8.11 (2H, d, J = 6.89 Hz)	129.9	8.06 (2H, d, J = 9 Hz)	127.6

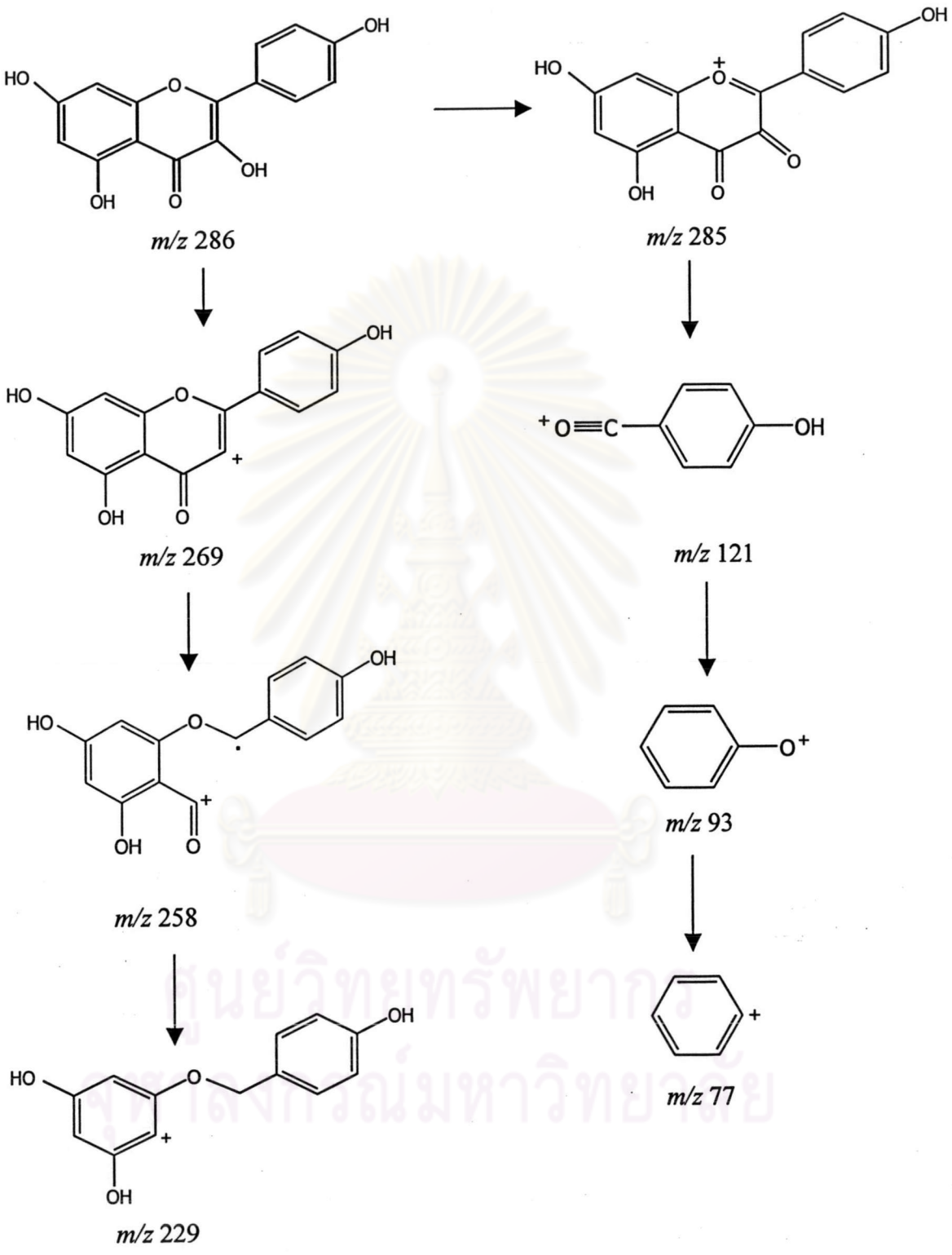
* not reported

From the above spectroscopic data, Compound **1** can be deduced as 3,4',5,7-tetrahydroxyflavone or kaempferol. Its structure is shown below.



Compound 1

The EI mass spectrum (Figure 14), supported the proposed structure of Compound **1**. It revealed the molecular ion at m/z 286 $[M]^+$. The possible mass fragmentation pattern was proposed as shown in Scheme 3.3.



Scheme 3.3 The proposed mass fragmentation pattern of Compound **1**

3.4.5 Purification, properties and structural elucidation of Compound 2

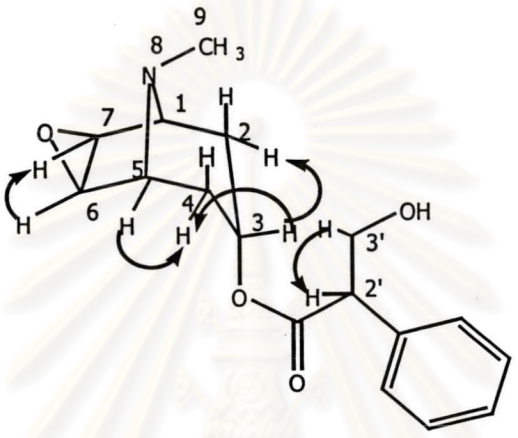
The yellow liquid of Compound **2** (11.64 g, 37.45% w/w of alkaloid fraction) was isolated from Fraction AC2 of alkaloid portion by eluting with 10% methanol in chloroform and repeated column chromatography by eluting with 15% methanol in chloroform. It was further purified with sephadex by using 50% methanol in chloroform as a mobile phase. Its spot on TLC can be detected by dipping with Dragendorff's reagent with R_f 0.63 (20% methanol in chloroform).

The IR spectrum of Compound **2** (Figure 19) showed the O-H stretching band at 3200-3400 cm^{-1}. The absoption peak of C-H stretching of epoxide and aromatic was detected in the same range at 3027 cm^{-1}. In addition, the C-H stretching of CH_2 was observed at 2938 cm^{-1}. The strong absorption peak at 1727 cm^{-1} revealed the presence of α,β unsaturated esters and the characteristic absorption peak of C-O stretching was detected at 1050 cm^{-1}. Furthermore, the medium absorption peak of C-H stretching of N-CH_3 appeared at 1175 cm^{-1} and the absorption peak of epoxide was found in the finger print at 855 cm^{-1}.

The ^{1}H-NMR spectrum ($CDCl_3$, Figure 20) exhibited a multiplet signal of protons in aromatic ring at δ 7.25 ppm (5H). The multiplet signal with integration of two protons detected at δ 3.74 ppm could be assigned for protons on carbon bearing an electronegative atom such as OH group (CH_2-OH). The chemical shift of α-ester proton (2′ position) appeared at 4.10 ppm. Moreover, the proton signal of alicyclic ring displayed a singlet signal of methine proton at δ 3.10 and 2.96 (1H each, s), two singlet signals of methine protons of epoxide at δ 2.65 and 3.38 ppm. The multiplet signal of equatorial proton of methylene group appeared at δ 2.10 ppm while a pair of axial proton displayed as doublet signals at δ 1.51 (1H, d, J = 15.19 Hz) and 1.28 (1H, d, J = 15.31 Hz). The multiplet signal of methine proton linked with oxygen ester at δ 4.97 ppm and the additional singlet signal with integration of three protons appeared at δ 2.37 ppm could be assigned for methyl group connected to N atom (CH_3-N). The OH broad signal appeared at 2.27 ppm.

The ^{13}C-NMR spectrum ($CDCl_3$, Figure 21) displayed twelve carbon signals which could be assigned as follows: the carbonyl ester carbon at 171.8 ppm, five methine aromatic carbons at δ 128.0, 128.9 (2C each) and 127.9 (1C) and one additional quaternary carbon at δ 135.7 ppm. The signals at δ 54.4 and 63.8 ppm could be assigned for C-2′ and C-3′ which linked with OH group, respectively. In

addition, the methylene and methine signals of alicyclic ring were detected at δ 30.6 and 57.5 ppm, respectively. The chemical shift at δ 56.3 ppm could be assigned for epoxide carbon in alicyclic ring. The signal at δ 66.7 ppm consistant with a carbon directly connected to -OCOR and additional alkyl amine carbon was observed at 41.8 ppm. Gathering from all spectroscopic data obtained, it was found that this compound should be a tropane alkaloid, scopolamine. The structure is shown below.



In addition, the ^{1}H-^{1}H COSY spectrum (Figure 22) showed that the H-2 and H-4 protons in axial position were correlated to the H-2 and H-4 protons in an equatorial position. The equatorial H-4 and H-2 protons were also correlated to H-3 and H-5 protons. Moreover, H-2′ proton was correlated to H-3′ proton. On the other hand, the H-1 and H-5 protons had no coupling interaction with H-6 and H-7 protons. The ^{1}H and ^{13}C NMR chemical shift assignments of Compound **2** compared with scopolamine are reported in Table 3.11.

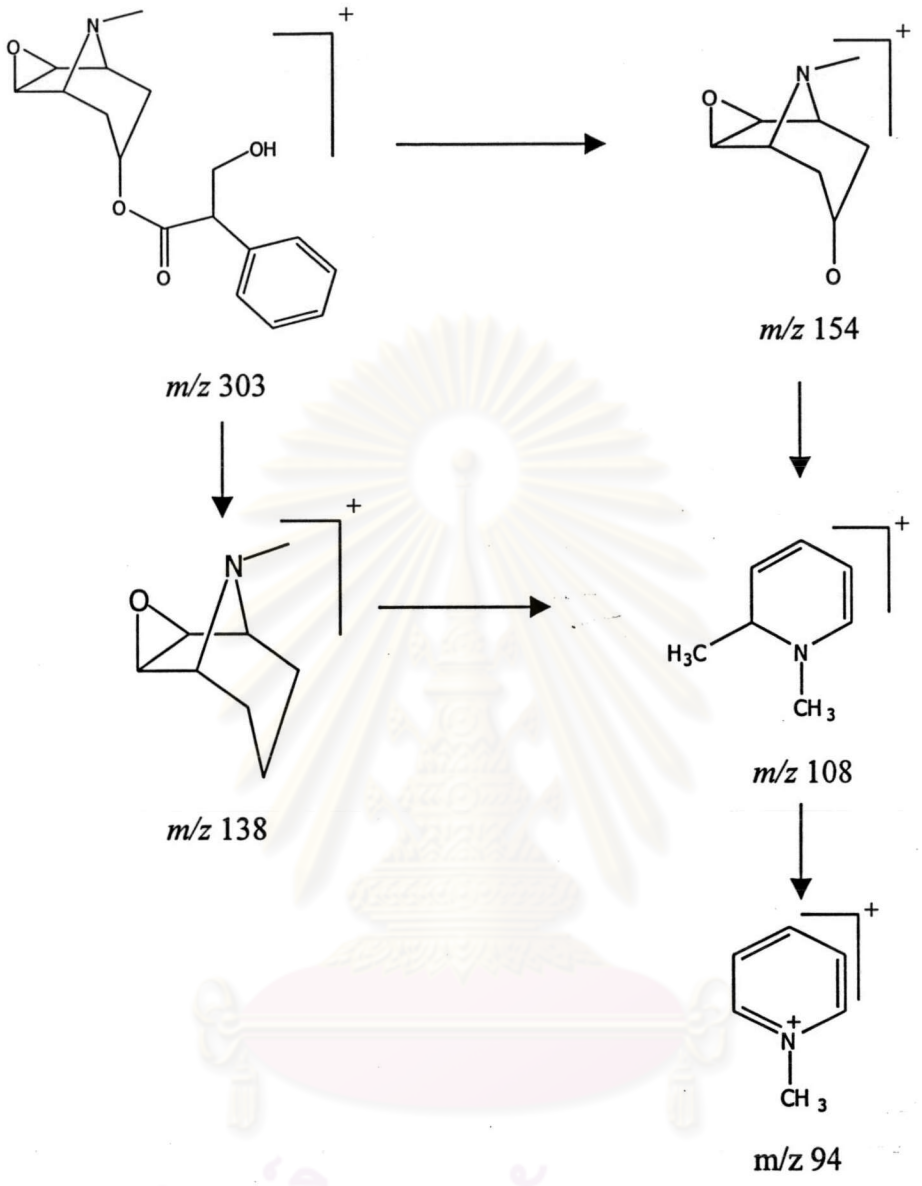
Table 3.11 The ^{1}H and ^{13}C NMR chemical shift assignments of Compound **2** and reported scopolamine[57-60]

Position	Chemical shift (ppm)			
	Compound **2**		Scopolamine	
	^{1}H-NMR	^{13}C-NMR	^{1}H-NMR	^{13}C-NMR
1	3.03 (1H, s)	57.6	2.95 (1H, s)	57.8
2β*	1.99 (1H, m)	30.4	2.05 (2H, m)	30.3
2α**	1.51 (1H, d, J = 15.19)		1.50 (1H, d, J = 10.15)	
3	4.93 (2H, m)	66.7	4.99 (1H, m)	66.4
4β*	1.99 (1H, m)	30.6	2.05 (2H, m)	30.4
4α**	1.28 (1H, d, J = 15.31)		1.25 (1H, d, J = 10.00)	
5	2.90 (1H, s)	57.6	3.07 (1H, s)	57.8
6	2.67 (1H, s)	56.3	2.65 (1H, s)	55.5
7	3.35 (1H, s)	55.9	3.34 (1H, s)	56.4
9	2.38 (3H, s)	41.8	2.42 (3H, s)	42.1
1′		171.8		171.9
2′	4.04 (1H, m)	54.4	4.13 (1H, m)	54.3
3′	3.73 (2H, m)	63.8	3.73 (2H, m)	63.9
4′		135.7		135.7
5′	7.18 (1H, m)	128.9	7.28 (1H, m)	128.5
6′	7.18 (1H, m)	128.0	7.27 (1H, m)	128.0
7′	7.18 (1H, s)	128.0	7.27 (1H, m)	128.0

* β is equatorial proton

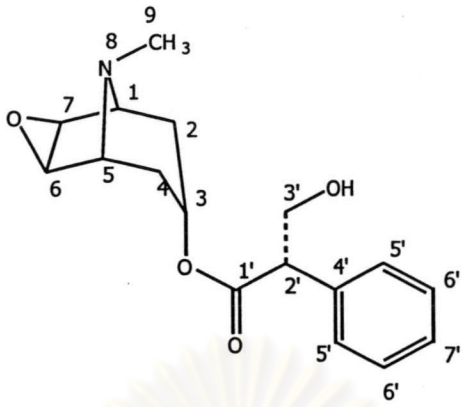
** α is axial proton

The mass spectrum (Figure 23) of this compound showed the molecular ion peak at m/z 303, which indicated that this compound should contain nitrogen. The molecular formula was proposed as $C_{17}H_{21}O_4N$. The mass fragmentation pattern of this compound is presented as shown in Scheme 3.4.[48,58-59]



Scheme 3.4 The possible mass fragmentation pattern of Compound **2**

According to the mass fragmentation pattern, ^{1}H and ^{13}C NMR data, this compound could be deduced to be scopolamine.



Compound 2

3.4.6 Purification, properties and structural elucidation of Compound 3

Compound **3** was obtained as yellow liquid (6.30 mg, 0.02% w/w of alkaloid fraction) from Fraction AC4-5. After performing repeat column chromatography eluting by 10% methanol in chloroform, it was purified by sephadex using 50% methanol in chloroform as a mobile phase. This compound manifested a single spot with R_f value of 0.82 in 20% methanol in chloroform as a solvent system. It gave an orange spot on TLC after dipping in Dragendroff's reagent, which is the characteristic of alkaloid.

The IR spectrum (Figure 24) showed the OH stretching vibration at 3200-3400 cm^{-1}. The C-H stretching and bending vibration of aliphatic was detected at 2918 cm^{-1} and 1634 cm^{-1}, respectively. Moreover, the C-O stretching vibration of alcohol exhibited at 1042 cm^{-1}.

The mass spectrum (Figure 25) displayed the molecular ion peak at *m/z* 141, corresponding to molecular formula $C_8H_{15}ON$. Other significant fragmentation ions were detected at 124, 113, 96 and 83. The pattern was found to be similar to that of a tropane alkaloid.

The ^{1}H-NMR spectrum ($CDCl_3$, Figure 26) demonstrated the broad with 1H integration signal of hydroxy proton at 5.08 ppm. The spectrum also showed the presence of an alicyclic ring, which could be assigned as follows: the methine protons showed singlet signal at δ 3.27 of H-1 and H-5 and triplet signal at δ 4.10 (1H, t, J = 5.04 Hz) of H-3. Moreover the doublet signal of a methylene proton at δ 2.40 (1H each, d, J = 14.60 Hz), could be assigned for an equatorial proton of H-2 and H-4. While an axial of H-2 and H-4 proton showed a doublet signal at δ 1.77 (1H each, d, J = 14.64 Hz). In addition, other methylene protons as two multiplet signals appeared

at 2.08 and 2.19 ppm, could be designated as equatorial and axial protons of H-6 and H-7, respectively.

The ^{13}C-NMR and DEPT 135 spectra ($CDCl_3$, Figures 27-28) exhibited five alicyclic ring carbon signals which could be assigned as follows: two methylene carbon signals at δ 26.1(2C) and 39.5 (2C), two methine carbon signals at δ 61.3 (2C) and 64.6 (1C). The methyl of amine displayed a distinctive signal at 40.6 ppm. The ^{1}H and ^{13}C NMR chemical shift assignments of Compound **3** compared with tropine[58-60] are illustrated in Table 3.12.

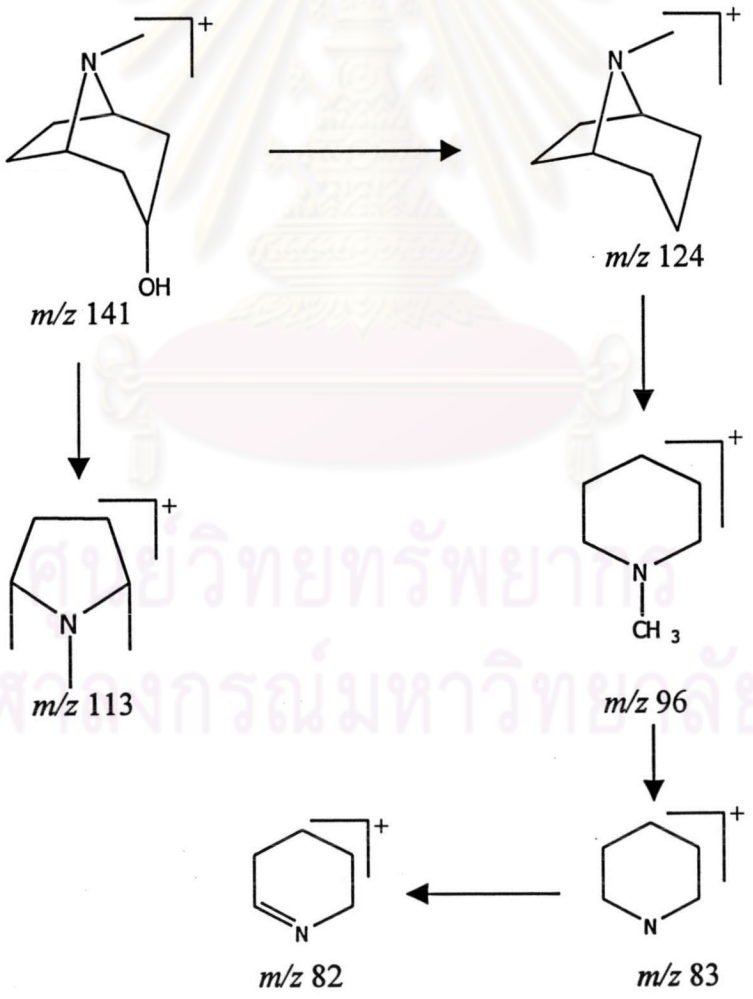
Table 3.12 The ^{1}H and ^{13}C NMR chemical shift assignments of Compound **3** and reported tropine.[58-60]

Position	Chemical shift (ppm)			
	Compound **3**		Tropine	
	^{1}H	^{13}C	^{1}H	^{13}C
1	3.27 (1H, s)	61.3	3.06 (1H, s)	60.4
2α	1.77 (1H, d, J = 14.64 Hz)	39.5	1.79 (1H, d, J = 16.80 Hz)	39.1
2β	2.32 (1H, d, J = 14.60 Hz)		2.09 (1H, d, J = 16.00 Hz)	
3	4.10 (1H, t, J = 5.04 Hz)	64.6	4.10 (1H, t, J = 4.00 Hz)	63.6
4α	1.77 (1H, d, J = 14.64 Hz)	39.5	1.79 (1H, d, J = 16.80 Hz)	39.1
4β	2.32 (1H, d, J = 14.60 Hz)		2.09 (1H, d, J = 16.00 Hz)	
5	3.27 (1H, s)	61.3	3.06 (1H, s)	60.0
6α	2.08 (1H, m)	26.1	1.98 (1H, s)	25.8
6β	2.19 (1H, m)		2.08 (1H, s)	
7α	2.08 (1H, m)	26.1	1.98 (1H, s)	25.8
7β	2.19 (1H, m)		2.08 (1H, s)	
N-CH_3	2.40 (3H, s)	40.6	2.25 (3H, s)	40.2
OH	5.08 (1H, b)		-*	

* not reported

From the above spectroscopic data, it could be deduced that this compound should be a tropane alkaloid bearing one hydroxy group. The possible structure of Compound **3** is illustrated below

The information obtained from mass spectrum gave an expected molecular ion peak at m/z 141 and a series of fragmentation ions at m/z 124, 113, 96, 83, and 82. The possible mass fragmentation pattern is displayed in Scheme 3.5.[49,61]



Scheme 3.5 The mass fragmentation pattern of Compound **3**

According to spectroscopic data and fragmentation pattern, it could be concluded that Compound **3** was tropine. The structure is demonstrated below.

Compound 3

Isolation and purification of Fraction AC 1-3

Fractions **AC1-3,** 1.40 g was obtained from the separation of an alkaloid fraction by using chloroform in methanol as a gradient mobile phase and purified by reversed-phase HPLC with Econospher RC18 column (250 mm length) using methanol in water as an eluent. The flow rate was 1.0 mL/min, the injection volume was 250 µL. UV at 254 nm was used as a detector. After fractionation by HPLC using 50% methanol in water as an eluent, four compounds could be obtained as presented in the chromatogram in Figure 3.4.

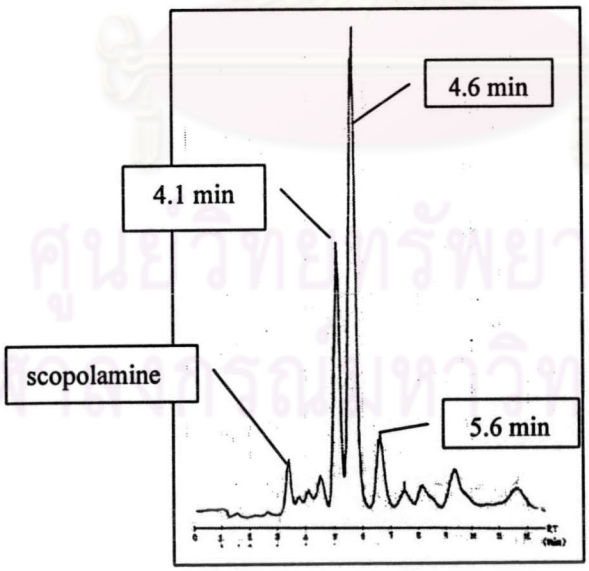
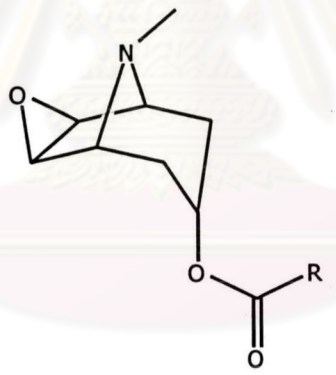


Figure 3.4 HPLC chromatogram of Fraction **AC1-3**

3.4.7 Properties and structural elucidation of Compound 4

The yellow liquid, (2.0 mg, 0.006% w/w of alkaloid fraction) was isolated from Fraction **AC1-3** of alkaloid fraction with retention time of 4.1 min by HPLC. It showed only yellow spot on TLC after dipping in Dragendorff's reagent with R_f value of 0.80 (20% methanol in chloroform). The IR spectrum (Figure 29) of this compound showed OH stretching of alcohol at 3200-3500 cm^{-1} and C-H stretching of aliphatic at 2926 cm^{-1}. The strong absorption peak of carbonyl of ester was detected at 1723 cm^{-1} and C=C stretching showed weak absorption at 1653 and 1447 cm^{-1}. Moreover two strong bands ascribed for C-O stretching were detected at low frequency at 1194 and 1066 cm^{-1}.

Since this compound was obtained in such a small amount, the complete structural elucidation could not be possible. However, this compound should be a derivative of scopolamine. The chemical structure of this compound was different from scopolamine only in the part of the substituent in the acidic part. The proposed stucture is shown below.



Compound **4**

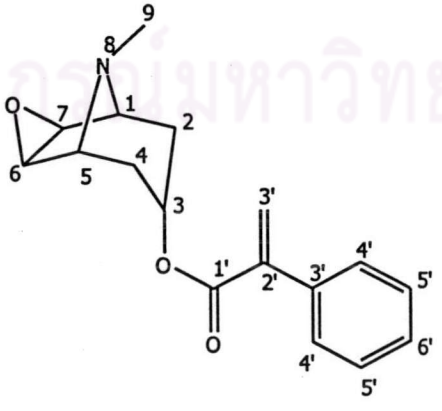
3.4.8 Properties and structural elucidation of Compound 5

Compound **5** as yellow liquid (4.0 mg, 0.013% w/w of alkaloid fraction) was obtained from Fraction **AC1-3** by HPLC technique. This compound showed a sharp peak on chromatogram at retention time of 4.6 min. It showed one spot on TLC after dipping with Dragendorff's reagent with R_f value of 0.84 (20% methanol in chloroform). Its IR spectrum (Figure 30) showed strong peak of carbonyl of ester at 1719 cm^{-1} and OH stretching at 3200-3500 cm^{-1}. Moreover the C-O stretching of ester was detected at 1198 and 1050 cm^{-1} and C-H stretching at 2938 cm^{-1}. The C=C stretching of aromatic ring absorption was detected at 1642 cm^{-1} and C=C stretching of alkene showed absorption peak at 1439 cm^{-1}. The GC chromatogram (Figure 31)

displayed the major peak at retention time of 24.79 min, which was in good agreement with molecular ion peak at m/z 285, calculated for $C_{17}H_{19}O_3N$ (Figure 32).

The ^{13}C NMR spectrum ($CDCl_3$, Figure 33) displayed low intensity signals of C=O at δ 166.0 ppm, olefinic carbons at δ 142.5 and 122.5 ppm, and aromatic carbons at δ 128.5 and 137.0 ppm. The signals of tropane ring could be observed as five characteristic signals and could be assigned as follows: methylene and methine signals of tropane ring at δ 31.6 and 58.7 ppm, respectively and an epoxide carbon at δ 56.8 ppm. The signal at δ 67.4 ppm consistent with a carbonyl ester in alicyclic ring and additional alkyl amine carbon was observed at δ 42.8 ppm. From the spectral data accessed, it was found that the possible structure of this compound should be another derivative of scopolamine.

The ^1H and ^1H-^1H COSY spectra ($CDCl_3$, Figures 34 and 35) exhibited the signal of aromatic protons at δ 7.40 ppm and signals of olefinic protons at δ 5.98 and 6.10 ppm (1H each, d, J = 1.2 Hz). The olefinic proton displayed the correlation in COSY spectrum. The proton signals of tropane ring revealed the signals similar to those in a tropane ring of scopolamine, which could be assigned as follows: the signals of methylene protons of H-2 and H-4 displayed multiplet signals at δ 1.31 and 1.67 ppm (m, 2H each) for the axial protons and a multiplet signal at δ 2.3 ppm (m, 2H each) for equatorial protons. Methine protons of H-6 and H-7 revealed the signal at δ 3.07 and 3.74 ppm (1H each, s). Two methine protons of H-1 and H-5 could be detected at δ 3.38 and 3.48 ppm (1H each, s) and the signal at δ 5.20 ppm (1H, m) was assigned for the proton of H-3. Methyl protons attached to N- displayed the signal at δ 2.62 ppm (s, 3H).



The ^1H and ^{13}C spectra of Compound **5** revealed the similar pattern to those of Compound **2**. The different peak that differed from Compound **2** were the peak

belonging to a substituted group at C-2′. The CH_2OH group at C-2′ in Compound **2** was replaced by the olefinic group in Compound **5.** The comparison of ^{1}H and ^{13}C-NMR chemical shift assignments of Compounds **2** and **5** are reported in Table 3.13.

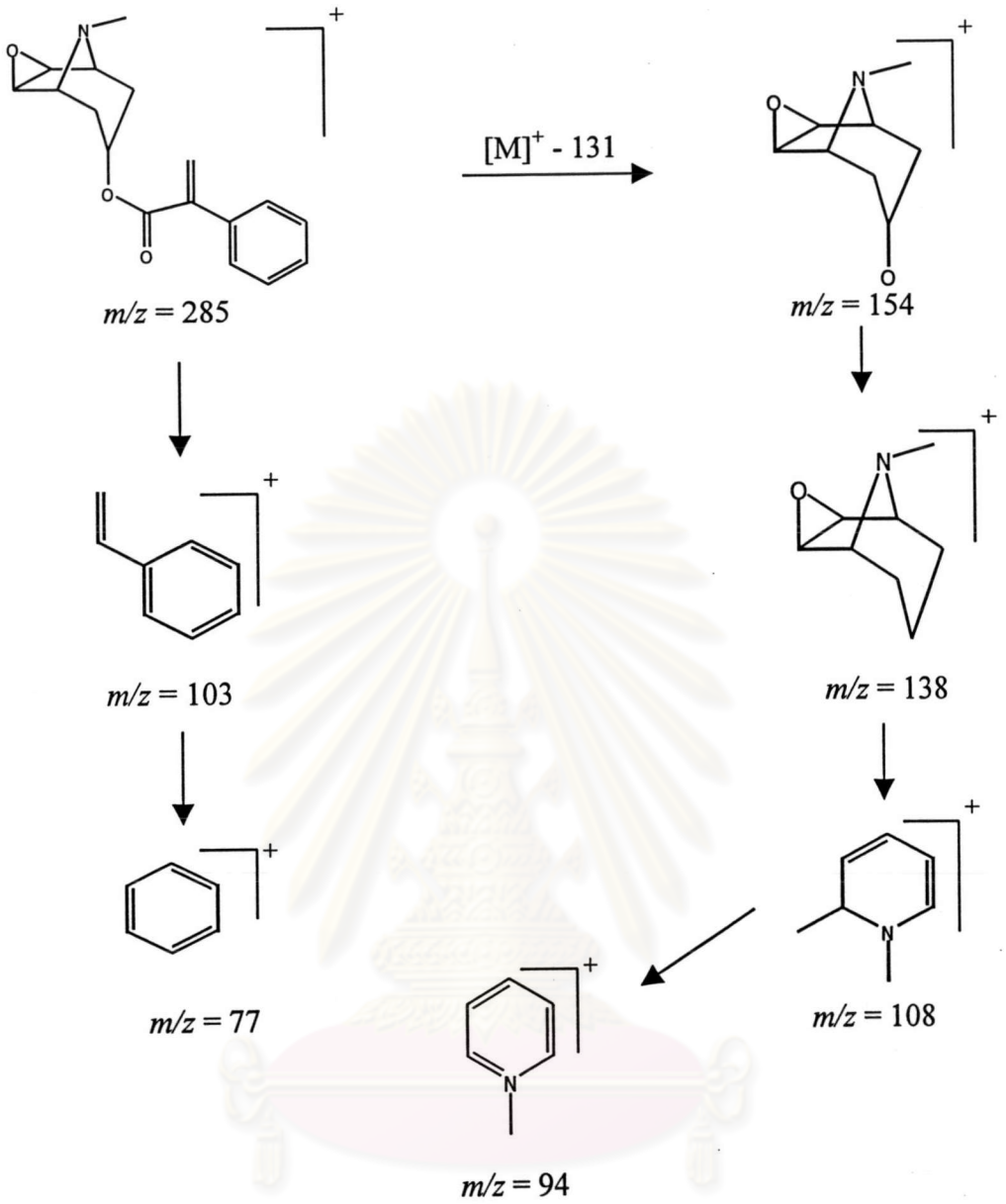
According to the spectroscopic data, it could be concluded that the possible structure was aposcopolamine with the molecular weight of 285.34. The EI mass spectrum (Figure 32) also supported the proposed structure of Compound **5**. It revealed a significant molecular fragmentation ion at 285 (M^+). The ester function was cleaved to generate RCOO and RCO ions from the molecular ion, giving rise to the prominent ions at *m/z* 138 and *m/z* 154, respectively. Cleavage of the 1,7 and 5,6 bonds of the symmetrical substituted tropane nucleus, followed by the loss of hydrogen radical and the ester function at C-3, led to the stable *N*-methylpyridinium cation (*m/z* 94). The fragment for C_8H_7 appeared at *m/z* 103. Other fragmentation ions were proposed as shown in Scheme 3.6.[62]

Table 3.13 The ^{1}H and ^{13}C NMR chemical shift assignments of Compounds **2** and **5**.

Position	Chemical shift (ppm)			
	Compound **2**		Compound **5**	
	^{1}H-NMR	^{13}C-NMR	^{1}H-NMR	^{13}C-NMR
1	3.03 (1H, s)	57.6	3.48 (1H, s)	58.7
2β*	1.99 (1H, m)	30.4	2.30 (2H, m)	31.6
2α**	1.51 (1H, d, J = 15.19 Hz)		1.67 (1H, m)	
3	4.93 (2H, m)	66.7	5.20 (1H, m)	67.4
4β*	1.99 (1H, m)	30.6	2.30 (2H, m)	31.6
4α**	1.28 (1H, d, J = 15.31 Hz)		1.31 (1H, m)	
5	2.90 (1H, s)	57.6	3.38 (1H, s)	58.7
6	2.67 (1H, s)	56.3	3.07 (1H, s)	56.8
7	3.35 (1H, s)	55.9	3.74 (1H, s)	56.8
9	2.38 (3H, s)	41.8	2.62 (3H, s)	42.8
1′		171.8		166.0
2′	4.04 (1H, m)	54.4		142.5
3′/3′a	3.73 (2H, m)	63.8	5.98 (1H, d, J = 1.2 Hz)	63.9
3′b	-	-	6.10 (1H, d, J = 1.2 Hz)	
4′		135.7		137.0
5′	7.18 (1H, m)	128.9	7.40 (1H, m)	128.5
6′	7.18 (1H, m)	128.0	7.40 (1H, m)	128.5
7′	7.18 (1H, s)	128.0	7.40 (1H, m)	128.5

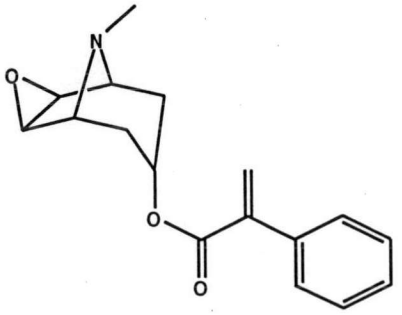
* β is an equatorial proton

** α is an axial proton



Scheme 3.6 The possible mass fragmentation pattern of Compound **5**

According to spectroscopic data and fragmentation pattern, it could be concluded that Compound **5** was aposcopolamine. The structure is demonstrated below.



Compound 5

3.4.9 Properties and structural elucidation of Mixture 4

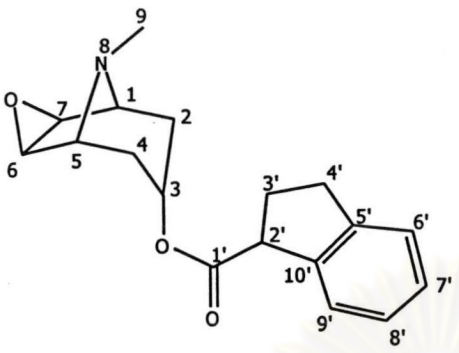
Mixture **4** as white amorphous solid (9.7 mg, 0.031% w/w of alkaloid fraction) was obtained from Fraction **AC1-3** and purified by HPLC. This mixture showed a yellow spot on TLC with R_f value of 0.85 (20% methanol in chloroform) visualized when dipping in Dragendorff's reagent. Its melting point was 120-125 °C.

The IR spectrum (Figure 36) showed the weak absorption of C-H stretching at 2949 and 2918 cm^{-1}. The strong absorption of C=O stretching was observed at frequency 1716 cm^{-1} and C-O stretching showed weak absorption at 1042 cm^{-1}. Moreover the C-N stretching and C-H stretching of epoxy were observed at 1198 and 859 cm^{-1}, respectively.

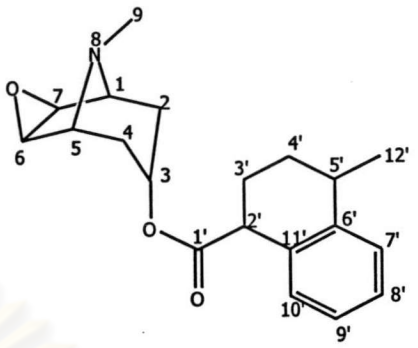
The ^{13}C NMR, DEPT135, and gHSQC spectra ($CDCl_3$, Figures 37-39) of Mixture **4** showed two series of signals, which could be assigned as follows: methyl carbon signals of amine (N-CH3) at δ 43.0 ppm, methylene carbon signals of C-1 and C-5 at δ 58.5 and 58.4 ppm, methylene carbon signals of C-6 and C-7 at δ 56.9 and 56.8 ppm, methylene carbon signals of C-2 and C-4 at δ 31.8 and 31.7 ppm and the carbon signal of C-3 at δ 67.4 and 67.9 ppm. The carbonyl ester signal was observed at δ 173.6 ppm. In addition the carbons of aromatic signals were detected at δ 127.4, 127.6, 128.1, 128.5, 128.9, 130.1 and 132.4 ppm and quaternary carbon signals of aromatic was appeared at δ 134.6, 137.4 and 145.4 ppm. Moreover the methine and methylene carbon signals of cyclopentyl and cyclohexyl was observed at δ 45.5 (C-2′), 57.0 (C-5′) 23.5 (C-3′), and 34.5 ppm (C-4′) and methyl signal was displayed at 29.5 ppm.

The gHSQC and ^1H NMR spectra ($CDCl_3$, Figures 39-40) also revealed two series of signals as follows: aromatic protons were detected at δ 7.00-7.22 ppm, methylene and methine protons of tropane ring displayed at δ 1.45 ppm (2H each, m) for equatorial protons of H- 2 and H-4, δ 2.15 (2H each, m) for axial protons of H-2 and H-4, δ 2.78 ppm (1H, d, J = 2.8 Hz) or for H-6, δ 2.90 ppm (d, 1H, J = 2.8 Hz) for H-7, 3.05 (1H, m) for H-1, 3.10 (1H, m) for H-5, δ 2.15 ppm (d, 1H, J = 2.8 Hz) for equatorial protons of H-2 and H-4, δ 3.22 ppm (1H, d, J = 2.8 Hz), δ 4.98 ppm (1H, m), and δ 5.08 ppm (1H, m). The methyl protons of amine showed two signals at δ 2.45 and 2.50 ppm. The proton of cyclopentyl and cyclohexyl was observed at δ 2.00 (2H, m), 1.18 (3H, s), 1.45 (2H, m), 2.15 (2H, m), 2.55 (2H, m), 3.78 (1H, t,

J = 5.6 Hz), 2.78 (1H, d, J = 2.8 Hz). The possible chemical structure of Mixture **4** is shown below.



Component 1



Component 2

Mixture 4

The complete assignments of the protons of Component **1** were established from the analysis of ^{1}H-^{1}H COSY, ^{1}H-^{1}H NOESY and CIGAR spectral data. In the CIGAR spectrum (Figures 41-43), the H-2′ showed the correlation with carbonyl ester at δ 173.4 ppm. Moreover, the H-2′ signal displayed the correlation with C-10′, C-3′, C-4′ and C-9′. The COSY spectrum (Figure 41), in addition, exhibited the correlation between H-2′ and H-3′. The H-3′ signal displayed the correlation with H-4′ and H-2′ in the COSY spectrum. In the CIGAR spectrum, H-3′ also showed the correlation with the carbonyl ester, C-2′ and C-4′. The H-4′ was manifestly correlated with the carbonyl ester, C-3′, C-5′, and C-6′. Moreover, the aromatic protons between δ 7.00-7.22 ppm exhibited the correlation with C-6′, C-2′, C-5′, and C-11′. In the NOESY spectrum of Mixture **4**, the H-2′ showed the correlation with H-3′ (δ_H 1.90 ppm), and H-4′ (δ_H 2.55 ppm). The aromatic protons at δ_H 7.20 ppm of C-9′ coupled with the other aromatic proton (δ_H 7.0 ppm) and the methine proton (δ_H 3.78 ppm) of H-2′. The protons of tropine moiety displayed the correlation as those observed in Compounds **2** and **5**. Other correlation of Mixture **4** is reported in Tables 3.14 and 3.15.

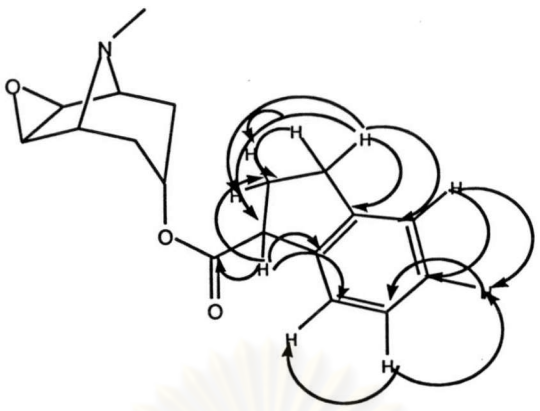
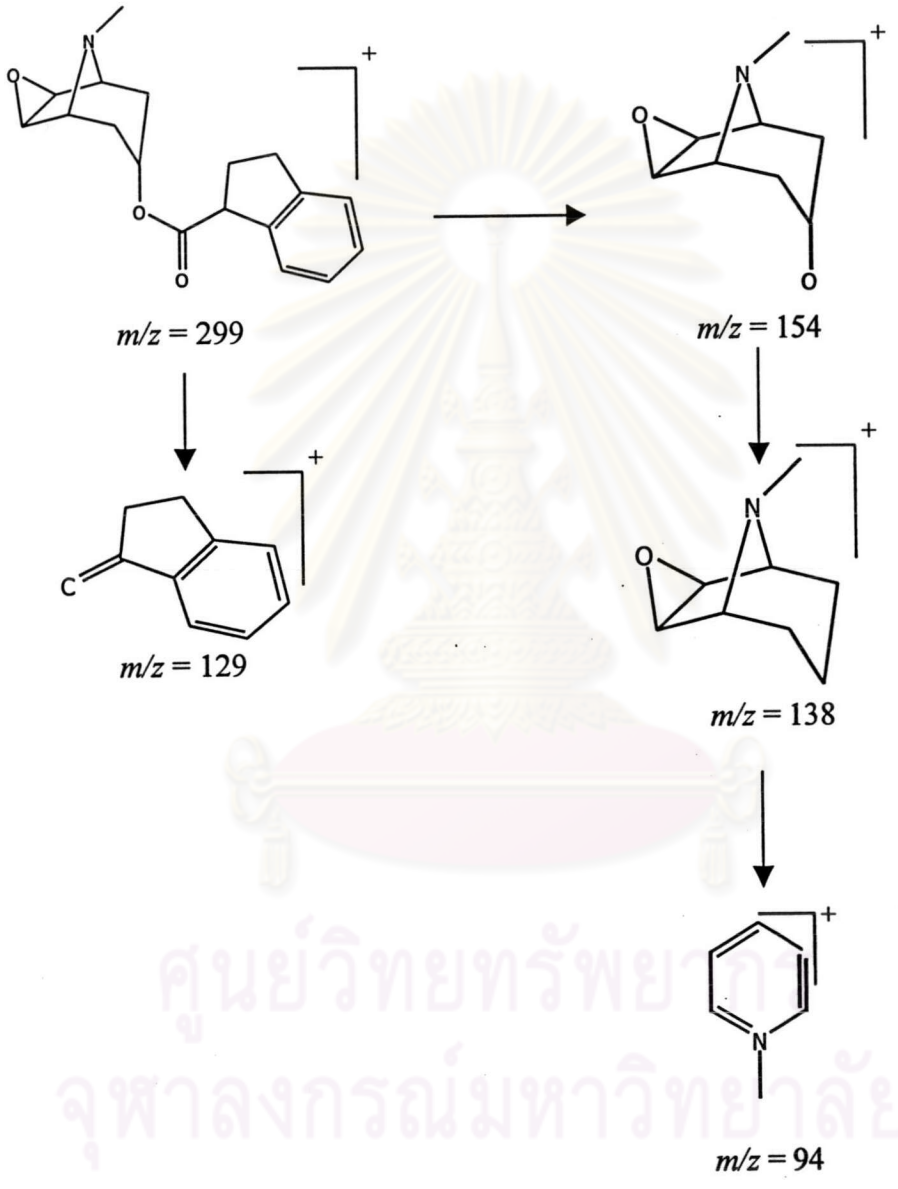


Table 3.14 The ^{1}H and ^{13}C NMR data of Component **1** of Mixture **4**

Position	^{13}C	^{1}H	gCOSY	CIGAR
1	58.3	3.05, m, 1H	H-2α, H-2β	C-3, C-2, C-7, N-CH_3
2α	31.0	1.45, m, 1H	H-2β	C-3, C-1, C-1′
2β		2.15, m, 1H	H-2α, H-3, H-1	C-3, C-1, C-1′
3	67.0	5.08, m, 1H	H-2α, H-2β, H-4α, H-4β	C-2, C-4, C-1, C-5 C-1′
4α	31.0	1.45, m,1H	H-4β	C-3, C-5, C-1′
4β		2.15, m, 1H	H-4α, H-3, H-5	C-3, C-5, C-1′
5	58.5	3.10, m, 1H	H-4α, H-4β	C-3, C-4, C-6, N-CH_3
6	56.8	2.78, d, 1H J = 2.8Hz	H-6	C-5
7	56.9	2.90, d, 1H J = 2.8Hz	H-7	C-1
N-CH_3	43.0	2.50, s, 3H		C-1, C-5
1′	173.5			
2′	45.5	3.78, t, 1H J = 5.6 Hz	H-3′	C-1′, C-3′, C-4′, C-10′, C-9′
3′	23.5	1.90-2.05, m, 2H	H-2′, H-4′α, H-4′β	C-1′, C-10′
4′α	34.5	2.15, m ,1H	H-3′, H-4′β	C-1′, C-2′, C-3′, C-10′,C-5′
4′β		2.55, m, 1H	H-3′, H-4′α	
5′	145.4*			
6′	132.4	7.20, m, 1H	H-7′	C-4′, C-8′, C-5′
7′	128.5	7.20, m, 1H	H-6′, H-8′	C-5′, C-8′, C-6′, C-9′
8′	128.1	7.00, m, 1H	H-7′, H-9′	C-7′, C-9′
9′	130.1	7.20, m, 1H	H-8′	C-10′, C-8′, C-7′
10′	134.6*			

* Quaternary carbon

The mass spectrum (Figure 44) displayed the molecular ion peak at m/z 299 ($[M]^+$), and other fragmentation ions at m/z 154 (M^+-COR), 138 (M^+-OCOR), and 129 (R^+). The proposed fragmentation pattern of Component **1** is shown in Scheme 3.7.



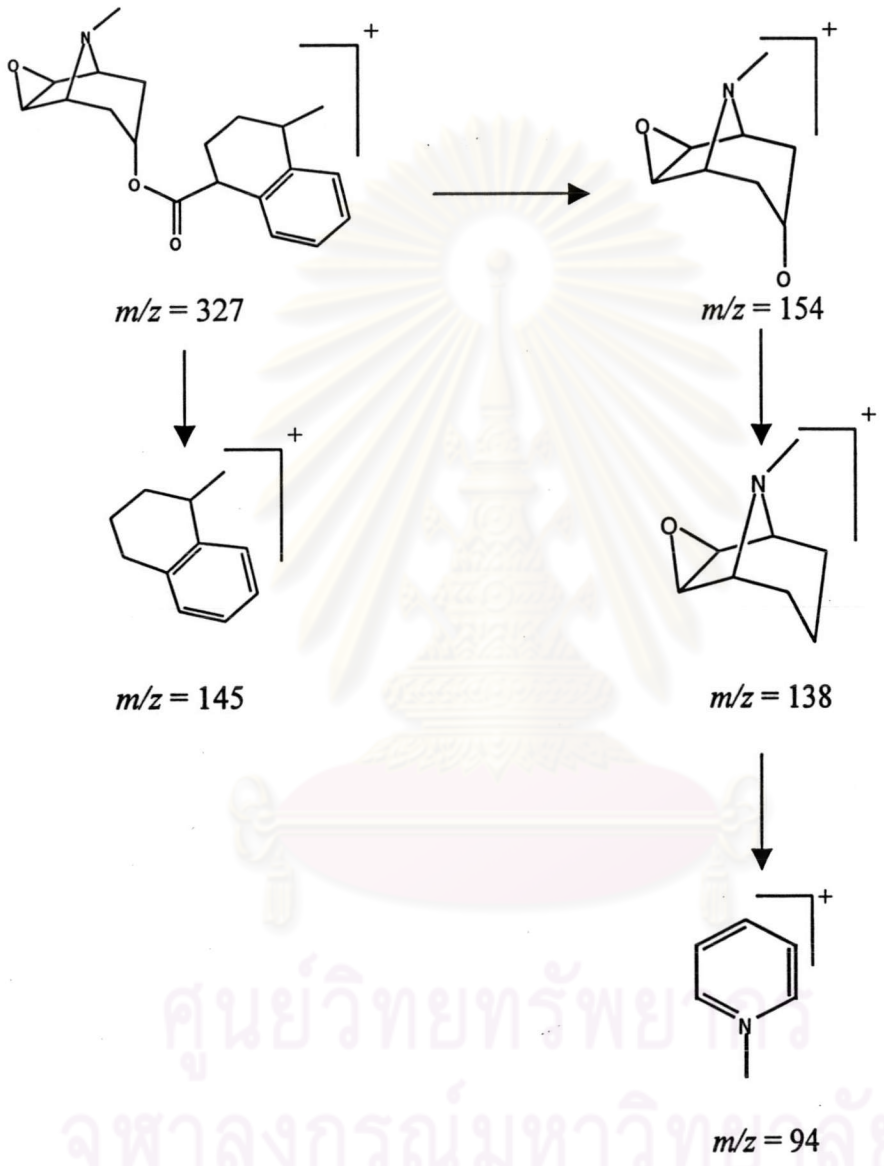
Scheme 3.7 The proposed mass fragmentation pattern of Componenet **1**

Table 3.15 The ^{1}H and ^{13}C NMR data of Component **2** of Mixture **4**

Position	^{13}C	^{1}H	gCOSY	CIGAR
1	58.3	3.05, m, 1H	H-2α, H-2β	C-3, C-2, C-7, N-CH_3
2α	31.0	1.45, m, 1H	H-2β	C-3, C-1, C-1′
2β		2.15, m, 1H	H-2α, H-3, H-1	C-3, C-1, C-1′
3	67.0	5.08, m, 1H	H-2α, H-2β, H-4α, H-4β	C-2, C-4, C-1, C-5 C-1′
4α	31.0	1.45, m,1H	H-4β	C-3, C-5, C-1′
4β		2.15, m, 1H	H-4α, H-3, H-5	C-3, C-5, C-1′
5	58.5	3.10, m, 1H	H-4α, H-4β	C-3, C-4, C-6, N-CH_3
6	56.8	2.78 1H,d, J = 2.8 Hz	H-6	C-5
7	56.9	2.90 1H, d, J = 2.8Hz	H-7	C-1
N-CH_3	43.0	2.50, s, 3H		C-1, C-5
1′	173.5*			
2′	45.5	3.78 1H, t, J = 5.6 Hz	H-3′	C-1′, C-3′, C-4′, C-10′, C-9′
3′	23.5	1.90-2.05, m, 2H	H-2′, H-4′α, H-4′β	C-1′, C-10′
4′α	34.5	2.15, m ,1H	H-3′, H-4′β	C-1′, C-2′, C-3′, C-10′, C-5′
4′β		2.55, m, 1H	H-3′, H-4′α	
5′	57.0	2.78 1H, d, J = 2.8 Hz	H-4′β	
6′	145.4*			
7′	127.4	7.20, m, 1H	H-7′	C-5′, C-8′, C-6′, C-9′
8′	128.5	7.22, m, 1H	H-7′, H-9′	C-6′, C-9′, C-7′, C-10′
9′	128.1	7.00, m, 1H	H-8′, H-10′	C-8′, C-10′
10′	127.6	7.20, m, 1H	H-9′	C-11′, C-9′
11′	137.4*			
12′	29.5	1.18, s, 3H	-	C-5′, C-12′

* Quaternary carbon

The mass spectrum (Figure 44) of Component **2** is similar to that of Component **1**, which displayed the molecular ion peak at m/z 327 ($[M]^+$), and other fragmentation ions at m/z 154 (M^+-COR), 138 (M^+-OCOR) and 145 (R^+). The proposed mass fragmentation pattern of Component **2** is shown in Scheme 3.8.



Scheme 3.8 The proposed mass fragmentation pattern of Component **2**

3.5 The results of biological activities test of isolated substances

All isolated substances from the flowers of *D. metel* were assayed for biological activity using brine shrimp cytotoxicity test, plant growth inhibition test, and molluscicidal activity. In addition, the insecticidal activity against *Spodoptera litura* was also conducted using vial contact method. The results of biological activity study of these substances are reported below.

3.5.1 The results of brine shrimp cytotoxicity test of isolated substances

Six isolated substances and atropine were tested for cytotoxicity with brine shrimp microwell method. The results are reported in Table 3.16.

Table 3.16 The brine shrimp cytotoxicity test of isolated substances

Substance	LC_{50} at 24 hours (ppm)	Activity
Mixture **1**	>1000	Not toxic
Mixture **2**	>1000	Not toxic
Mixture **3**	888.59	Low
Compound **1**	134.81	Low
Compound **2**	313.70	Low
Compound **3**	not test	Not test
Atropine	>1000	Not toxic

Note: 0-10 μg/mL; High toxicity, 10-100 μg/mL; Moderate toxicity
100-1000 μg/mL; Low toxicity, >1000 μg/mL; No toxicity

From the above data, a mixture of steroidal ester (Mixture **1**) and a mixture of long chain ester did not exhibit cytotoxic activity against brine shrimp. On the other hand, a mixture of steroids (Mixture **3**) showed low cytotoxicity (LC_{50} 888.59 ppm) and kaempferol (Compound **1**) displayed medium cytotoxic activity against brine shrimp. The medium cytotoxic activity of ethyl acetate extract revealed the corresponding correlation with the result derived from isolated substances. Thus, Compound **1** was the best candidate possessing cytotoxic activity in ethyl acetate extract. In addition, Compound **1** was preliminarily tested for anti-oxidant activity with DPPH solution, and revealed the positive test for this regard. Scopolamine (Compound **2**) exhibited low cytotoxic activity (LC_{50} 313.70 ppm). The activity result

of Compound **2** was also in good agreement correlated with that obtained earlier from the alkaloid fraction. Atropine, isolated from the leaves of *D. metel*, was another substance tested. It did not show cytotoxic activity against brine shrimp.

3.5.2 The results of plant growth inhibition on *Lactuca sativa* L. of isolated substances

From the purification of various crude extracts, six substances were isolated. Five substances including a mixture steroid ester, a long chain ester, a mixture of steroid, kaempferol and scopolamine were tested for plant growth inhibition on *L. sativa.* The results are reported in Tables 3.17 and Figure 3.5.

Table 3.17 Plant growth inhibition test of isolated substances

Substance	Part	% inhibition of various concentration (ppm)		
		10	100	1000
Mixture **1**	Root	25	39	76
	Shoot	9	-26	34
Mixture **2**	Root	57	59	20
	Shoot	8	27	20
Mixture **3**	Root	13	19	7
	Shoot	-18	-20	-41
Compound **1**	Root	4	6	-3
	Shoot	33	11	13
Compound **2**	Root	5	17	91
	Shoot	5	8	30

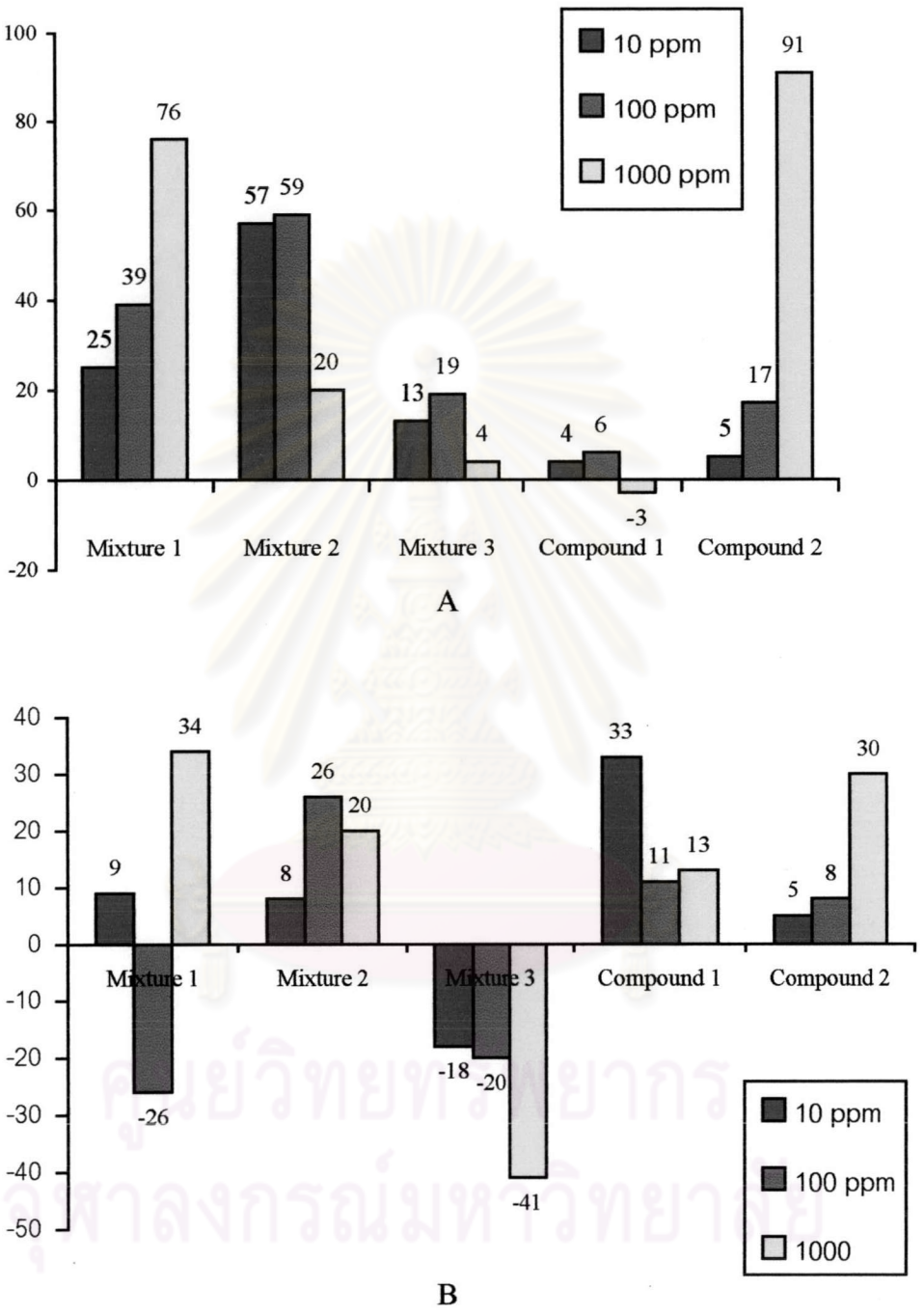


Figure 3.5 The result of isolated substances on A) root growth inhibition and B) shoot growth inhibition against *L. sativa.*

From the above data, Mixture **1** (steroid ester) and Compound **2** (scopolamine) displayed increasingly inhibition activity on the root growth when the concentration was increased. Other isolated substances showed different trend of activity on the root growth. For instance, a mixture of steroid (Mixture **3**) showed increasing promotion activity when the concentration was increased. Thus, Compound **2** and Mixture **1** revealed the best inhibition activity on root growth inhibition. Besides, Compound **2** revealed a good trend of activity on shoot growth inhibition. The comparison of activity between alkaloid fraction and Compound **2** revealed that Compound **2** exhibited higher inhibition activity than alkaloid fraction. In the case of ethyl acetate extract and isolated substances from this extract, it was found to display the same trend of activity.

3.5.3 The results of molluscicidal activity against *P. canaliculata* of isolated substances

Seven isolated substances from flowers and leaves of *D. metel* including steroid ester, long chain ester, a mixture of steroids, kaempferol, scopolamine and atropine were tested for molluscicidal activity against *P. canaliculata* (gloden apple snail). The results are exhibited in Table 3.18 and Figure 3.6.

Table 3.18 Mortality of *P. canaliculata* at 72 hours

Substance	% mortality of snail at 72 hours			
	10 ppm	100 ppm	1000 ppm	control
Mixture **1**	0	0	0	0
Mixture **2**	0	0	0	0
Mixture **3**	0	0	0	0
Compound **1**	0	0	22	0
Compound **2**	22	56	78	0
Atropine	0	11	22	0

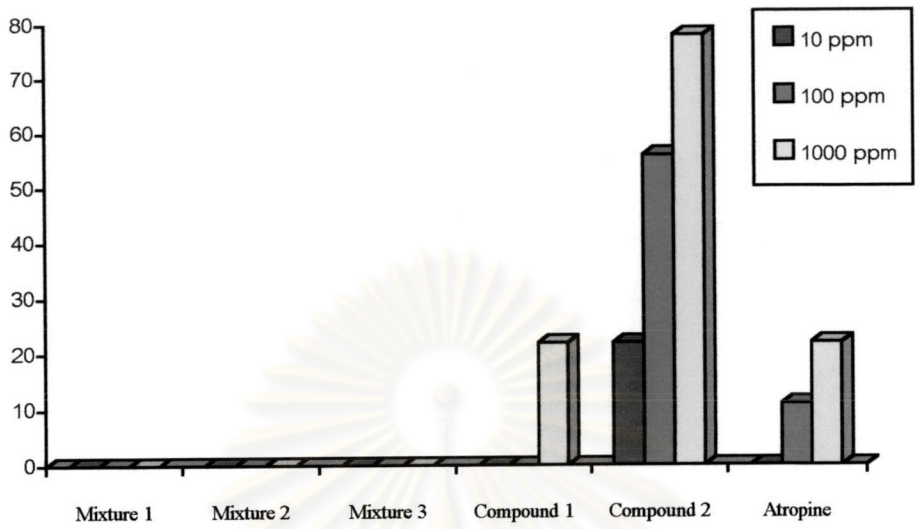


Figure 3.6 Mortality of *P. canaliculata* at 72 hours

The toxicity of isolated substances was evaluated against golden apple snail. The results of percent mortality of Compound **1**, at concentration of 1000 ppm, 100 ppm and 10 ppm, were 22%, 11% and 0%, respectively. On the other hand, Compound **2** displayed more intriguing results. It expressed 78%, 50% and 22% mortality at concentration of 1000, 100 and 10 ppm, respectively. The last compound, found in the leaves of *D. metel*, was atropine. Its results displayed the percent mortality of 22%, 11% and 0% at concentration of 1000, 100, and 10 ppm, respectively. From this result, it could be concluded that scopolamine and atropine exhibited molluscicidal activity against *P. canaliculata* more than other substances tested. While kaempferol only revealed the toxicity against *P. canaliculata* at 1000 ppm.

3.5.4 The results of insecticidal activity on *Spodoptera litura* of some isolated compounds

The selected compounds from the alkaloid fraction namely scopolamine, atropine and tropine were assayed for insecticidal activity with vial contact toxicity method. The results are displayed in Table 3.19 and Figure 3.7.

Table 3.19 Mortality of neonate larvae of *S. litura.*

mg/vial	Atropine		Scopolamine		Tropine	
	Died	Percentage	Died	Percentage	Died	Percentage
0.01	19	42	14	31	24	53
0.05	18	40	10	26	25	56
0.1	19	42	19	42	28	60
0.5	18	40	27	60	35	78
1.0	22	49	30	67	43	96

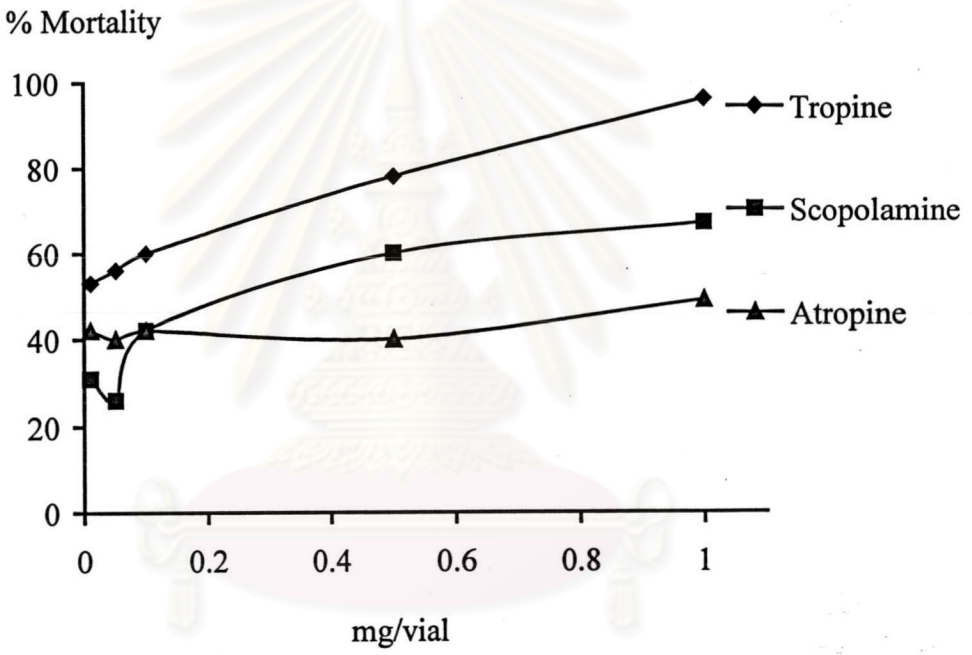


Figure 3.7 The mortality of neonate larvae of *S. litura.*

From the above data, the pronounced contact toxicity of scopolamine, atropine, and tropine towards neonate larvae of *S. litura* was observed. Tropine was highly active causing significantly increasing mortality of larvae when the concentration of sample increased. Other compounds displayed unsatisfied results regarding the toxicity against *S. litura*. Calculating by Probit analysis program, the LD_{50} of tested compounds are reported in Table 3.15.

Table 3.20 The LD_{50} of selected compounds against *S. litura* by contact toxicity vial test.

Compounds	LD_{50} (mg/vial)
Atropine	1.44
Scopolamine	0.47
Tropine	-0.05

In this experiment the doses applied were ranged from 0.01-1.0 mg/vial. Tropine was the most active compounds in this bioassay with LD_{50} of –0.05 that meant the dose range performing in this experiment was not proper for tropine. Thus, the dose ranged should be 0.001-0.1 mg/vial. Scopolamine and atropine were other active compounds with LD_{50} 0.47 and LD_{50} 1.44 mg/vial.