CHAPTER 4
RESULTS AND DISCUSSION

4.1 Collection of plant materials

The leaves of *D. glaucum* were collected at Ratchaburi Provinces, Thailand, in October 2010. A voucher specimen (number 01-719) was deposited at the Herbarium of the Department of Biology, Chiang Mai University.

4.2 Extraction and isolation

The dried leaves of *D. glaucum* were extracted with 95% ethanol for 4 days and the combined extracts were evaporated using a rotary evaporator to obtain the crude ethanol extract. The extract resembled a dark green gum. The ethanolic crude extract was subsequently partitioned between water and methanol and extracted with dichloromethane. Successive purifications of this crude residue by column chromatography and preparative TLC gave six alkaloids, assign as DG1, DG2, DG3, DG4, DG5 and DG6. The summary of alkaloids isolation and the possible structure of these compounds as shown in Figure 4.1. All compounds gave positive test to Dragendorff's reagent. Data from spectroscopic technique exhibited DG1 as the same compound with DG6 and DG3 as the same compound with DG4. Alkaloids from the ethanolic crude extract of the leaves of *D. glaucum* are aporphine alkaloids. In previous studies, Karntanakrit *et al* (2011) [25] found four known alkaloids in the methanol extract from leaves and twigs of *D.glaucum.* These compounds are aporphine and quinolizidine alkaloids. The reason of the difference type of

alkaloids in same part and same species of plant may be effect of different place of *D.glaucum*

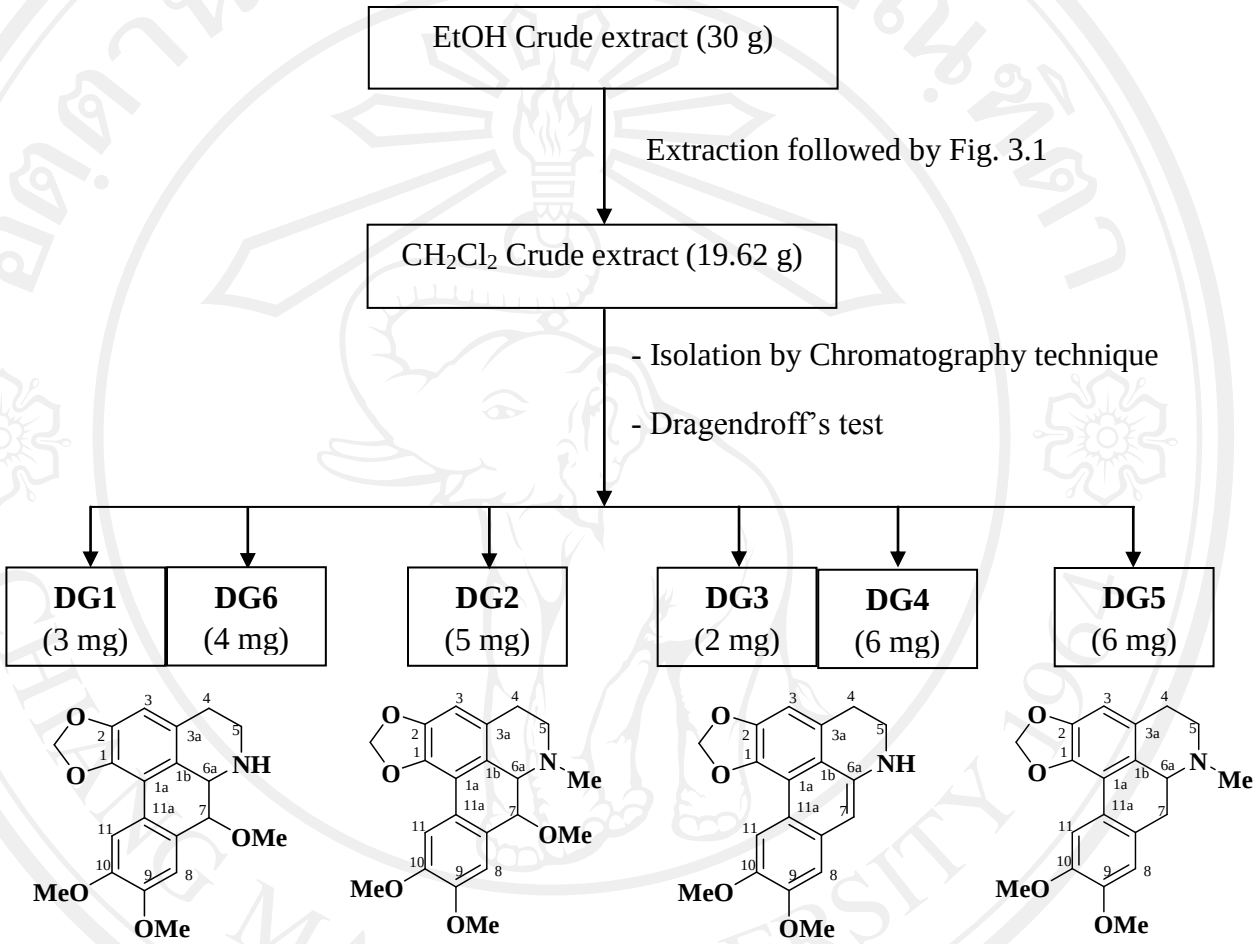

Figure 4.1 The summary of isolation and the possible structure of alkaloids from the leaves of *D. glaucum*

4.3 Structure elucidation

The structural elucidations of the isolated compounds were established by analysis of their spectroscopic data and comparison of the spectral data with those published previously in literatures. The details of the structural elucidation of each compound are as follows.

4.3.1 DG1 (Fraction 5.2.2.5)

DG1 was isolated as a pale yellow gum; IR (film) ν_{max} 3434 (N-H stretching), 2922 (CH stretch), 1689, 1648 (C=C stretching), 1519 (CH stretching of aromatic), 1463 (CH-CH_2 bending), 1221, 1246 (C-O stretching) cm^{-1}. The characteristic absorption bands at 1648, 1519, 1463 and 1404 cm^{-1} indicated the presence of the skeleton of an aporphine alkaloid [34]. The IR spectrum as shown in Figure 4.2. The ^{1}H-NMR of DG1 showed that two aromatic methoxy groups at δ_H 3.96 and 3.97 ppm. The methoxy group attached with C-7 was observed at δ_H 3.62 ppm. The signal at δ_H 2.75 and 3.55 ppm belong to two sets of methylene protons. Methylenedioxy protons showed two doublet at δ_H 5.97 and 6.17 ppm. Three singlets of aromatic protons were presented at δ_H 7.74, 6.99 and 6.64 ppm. The ^{1}H-NMR spectrum of DG1 as shown in Figure 4.3 and summarized in Table 4.1. The ^{13}C-NMR showed that signal of methylenedioxy carbon at δ_C 101 ppm. Two aromatic carbons attached with methoxy group were presented at δ_C 148.4 and 156.9 ppm. Three methoxy carbon were presented at δ_C 59.1, 56.1 and 55.9 ppm. These assignment were supported by the observed HMBC correlation between C-9 to H-8, H-11 and H(9,10-OCH_3) and C-1b to H-8, H-11, H-3, H-6a and H-7. The possible structure is shown in Figure 4.4.

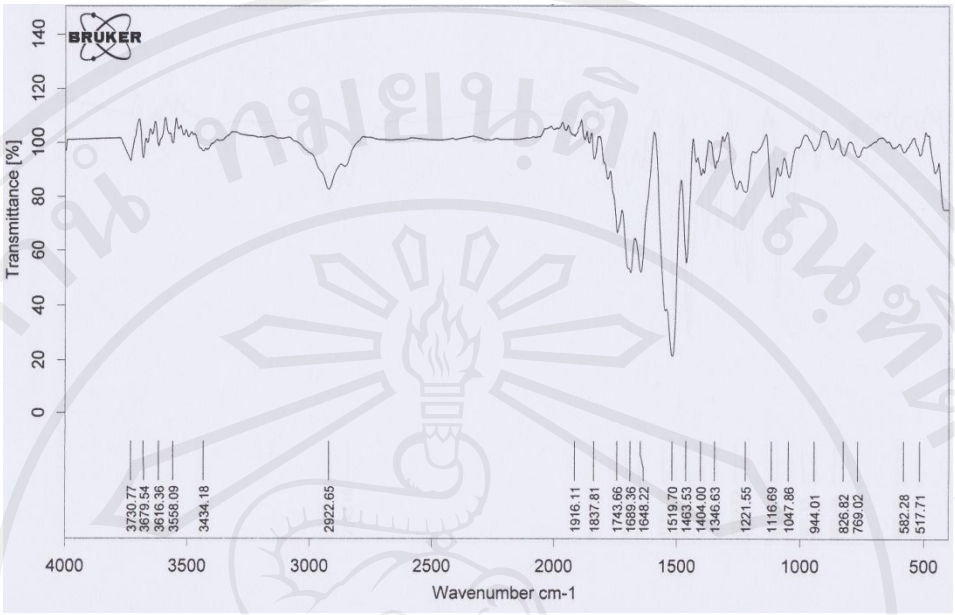

Figure 4.2 IR spectrum of DG1

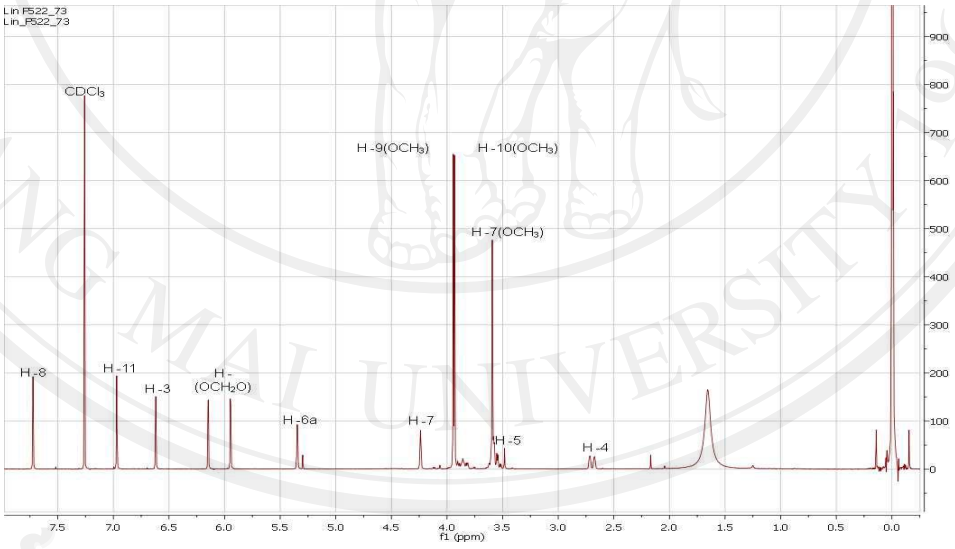

Figure 4.3 ^{1}H-NMR spectrum of DG1

Table 4.1 ^{13}C- NMR (*100* MHz) and ^{1}H-NMR (*400* MHz) spectroscopic data of **DG1** in $CDCl_3$

Position	**δ_C**	**δ_H**	**HMBC**
1	142.7	-	H-3,H(OCH_2O)
1a	122.8	-	H-11, H-6a
1b	117.7	-	H-11(w), H-3, H-6a,H-7
2	148.0	-	H-3,H(OCH_2O)
3	106.0	6.64 (1H, *s*)	
3a	116.7	-	H-11(w), H-3, H-6a
4	24.8	2.75 (2H,*m*)	H-3
5	65.4	3.55 (2H,*m*)	
6a	68.0	5.37 (1H, *d*, *J= 2.4* Hz)	H-11,H(7-OCH_3)
7	70.0	4.26 (1H, *m*)	H(7-OCH_3)
7a	126.4	-	H-8, H-6a
8	111.1	7.74(1H, *s*)	H-11(w), H-6a
9	148.1	-	H-8, H-11, H(9,10-OCH_3)
10	156.9	-	-
11	111.8	6.99(1H, *s*)	
11a	124.7	-	H-8, H-11, H-6a
OCH_2O	101.0	5.97 (1H, *d*, *J=1.*2 Hz) 6.17 (1H,*d*, *J=1.2* Hz)	H(OCH_2O)
7-OCH_3	59.1	3.62 (3H, *br s*)	
9-OCH_3	56.1	3.97 (3H, *br s*)	
10-OCH_3	55.9	3.96 (3H, *br s*)	

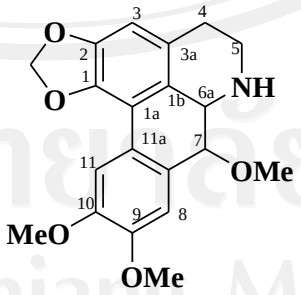

Figure 4.4 The possible structure of DG1 and DG6

4.3.2 DG6 (Fraction 6.7.1, the same compound as DG1)

DG6 was isolated as a pale yellow gum; IR (film) ν_{max} 3615 (N-H stretching), 2921 (CH stretch), 1647, 1518 (CH stretching of aromatic), 1463 (CH-CH_2 bending), 1222 (C-O stretching), 1116 cm^{-1} and 1647, 1518, 1463 cm^{-1} (skeleton of a aporphine alkaloid). The IR spectrum as shown in Figure 4.5. The ^{1}H-NMR of DG6 showed that two aromatic methoxy groups at same position δ_H 3.94 ppm. The methoxy group attached with C-7 was observed at δ_H 3.62 ppm. The signal at δ_H 2.71 and 3.55 ppm belong to two sets of methylene protons. Methylenedioxy protons showed two doublet at δ_H 5.95 and 6.15 ppm. Three singlets of aromatic protons were presented at δ_H 7.72, 6.98 and 6.62 ppm. The ^{1}H-NMR spectrum of DG6 exhibited DG6 as the same compound as DG1 but the NMR spectrum of DG6 has more impurity. The ^{1}H-NMR spectrum of DG6 as shown in Figure 4.6 and summarized in Table 4.2. The possible structure is shown in Figure 4.4.

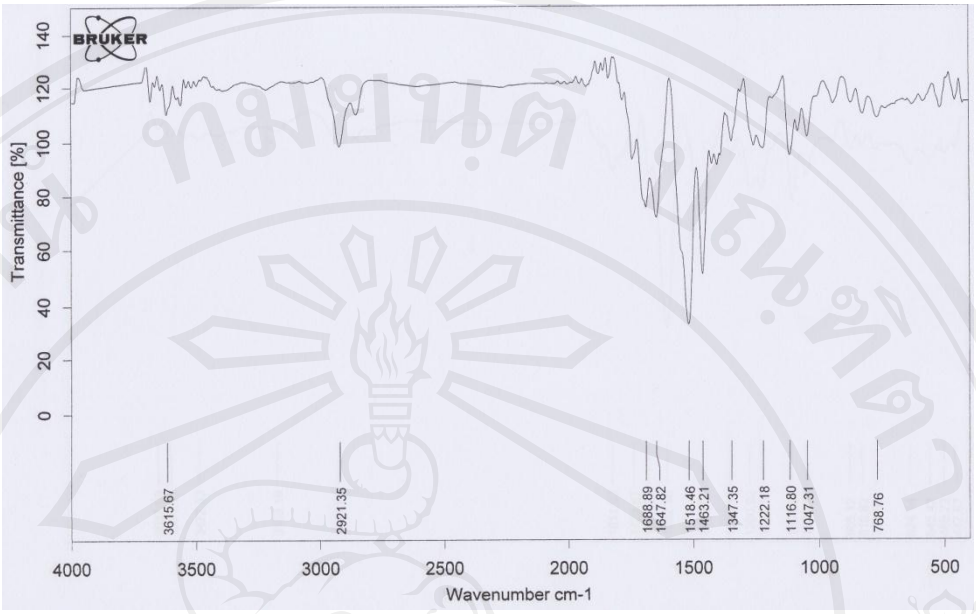

Figure 4.5 IR spectrum of DG6

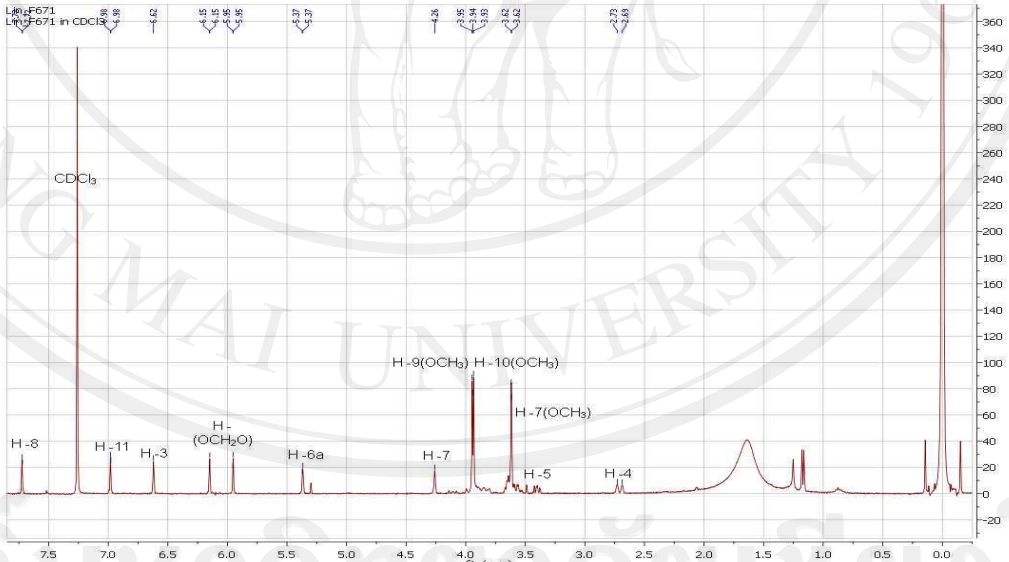

Figure 4.6 ^{1}H-NMR spectrum of DG6

Table 4.2 ^{1}H-NMR (*400* MHz) spectroscopic data of DG6 in $CDCl_3$

Position	**δ_H**
1	-
1a	-
1b	-
2	-
3	6.62 (1H, *s*)
3a	-
4	2.71 (2H,*m*)
5	3.55 (2H,*m*)
6a	5.37 (1H, *d, J= 2.4* Hz)
7	4.26 (1H, *m*)
7a	-
8	7.72(1H, *s*)
9	-
10	-
11	6.98(1H, *s*)
11a	-
OCH_2O	5.95 (1H, *d, J=1.*2 Hz) 6.15 (1H,*d, J=1.2* Hz)
7-OCH_3	3.62 (3H, *br s*)
9-OCH_3	3.94 (3H, *br s*)
10-OCH_3	3.94 (3H, *br s*)

4.3.3 DG2 (Fraction 5.3.2)

DG2 was isolated as a red-brown gum; IR (film) ν_{max} IR (film) ν_{max} 2927 (CH stretching), 1689 (C=C stretching), 1518 (CH stretching of aromatic), 1462 (CH-CH_2 bending), 1259 (C-O stretching), 1116 cm^{-1} and 1647, 1518, 1462 cm^{-1} (skeleton of a aporphine alkaloid). The IR spectrum as shown in Figure 4.7. The ^{1}H-NMR of DG2 shown that methyl proton attached with N atom at δ_H 2.19 ppm and two aromatic methoxy groups at δ_H 3.93 and 3.94 ppm. The methoxy group attached with C-7 was observed at δ_H 3.60 ppm. The signal at δ_H 2.69 and 3.50 ppm revealed the presence of two methylene protons. Methylenedioxy protons were observed at δ_H 5.95 and 6.15 ppm. Three singlets of aromatic protons were presented at δ_H 6.62, 7.72 and 6.99 ppm. From the data of ^{1}H-NMR of DG2 indicated that structure of DG2 similarly to DG1 but their structures only differed by presence N-methyl substituent. The ^{1}H-NMR spectrum of DG2 as shown in Figure 4.8 and summarized in Table 4.3. The possible structure is shown in Figure 4.9.

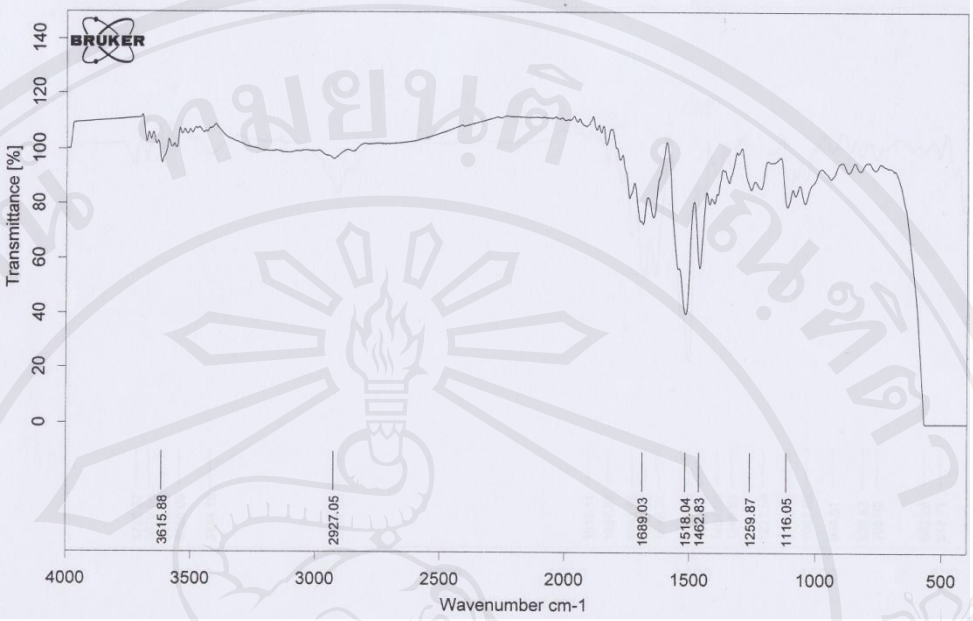

Figure 4.7 IR spectrum of DG2

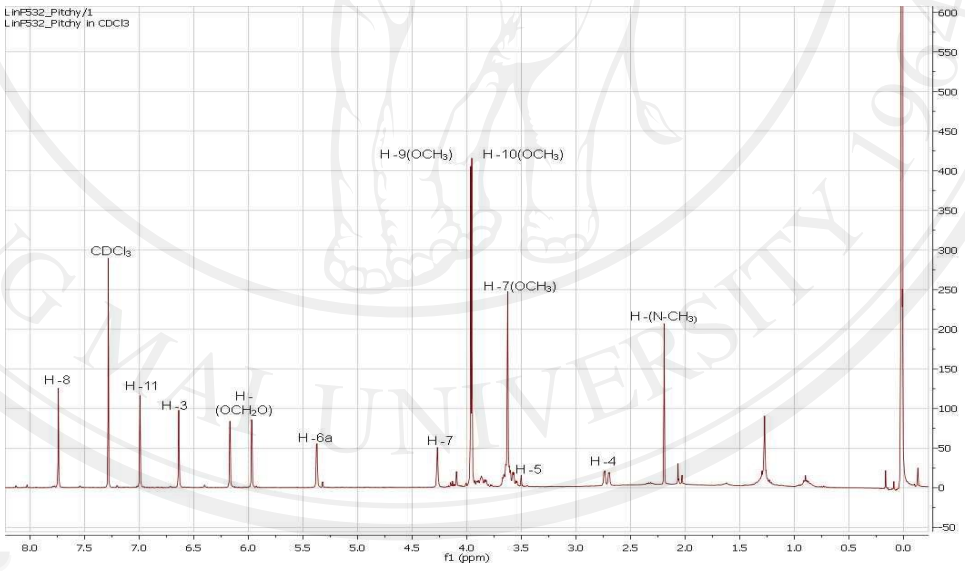

Figure 4.8 ^{1}H-NMR spectrum of DG2

Table 4.3 ^{1}H-NMR (*400* MHz) spectroscopic data of DG2 in $CDCl_3$

Position	δH
1	-
1a	-
1b	-
2	-
3	6.62 (1H,*s*)
3a	-
4	2.69 (2H,*m*)
5	3.50 (2H,*m*)
6a	5.35 (1H, *d*, *J= 2.4* Hz)
7	4.24 (1H,s)
7a	-
8	7.72 (1H, *s*)
9	-
10	-
11	6.99(1H, *s*)
11a	-
OCH_2O	5.95 6.15
7-OCH_3	3.60 (3H, *br s*)
9-OCH_3	3.93 (3H, *br s*)
10-OCH_3	3.94 (3H, *br s*)
N-CH_3	2.19 (3H, *s*)

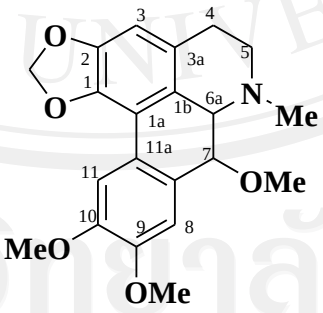

Figure 4.9 The possible structure of DG2

4.3.4 DG3 (Fraction 4.4.1)

DG3 was isolated as a colorless gum; IR (film) ν_{max} IR (film) ν_{max} 3620, 2919 (CH stretching), 1699, 1516 (CH stretching of aromatic), 1212 cm^{-1} (C-O stretching) and 1650, 1513, 1456 cm^{-1} (skeleton of a aporphine alkaloid). The IR spectrum as shown in Figure 4.10. The ^{1}H-NMR of DG3 exhibited the absence of protons at C-6a and 7-OCH_3 and showed a broad singlet of two methoxy protons at δ_H 3.93 ppm. From the data of ^{1}H-NMR indicated that structure of DG3 similarly to DG1 but their structure only differed by absence methoxy group at C-7. The ^{1}H-NMR spectrum of DG3 as shown in Figure 4.11 and summarized in Table 4.4. The possible structure is shown in Figure 4.12.

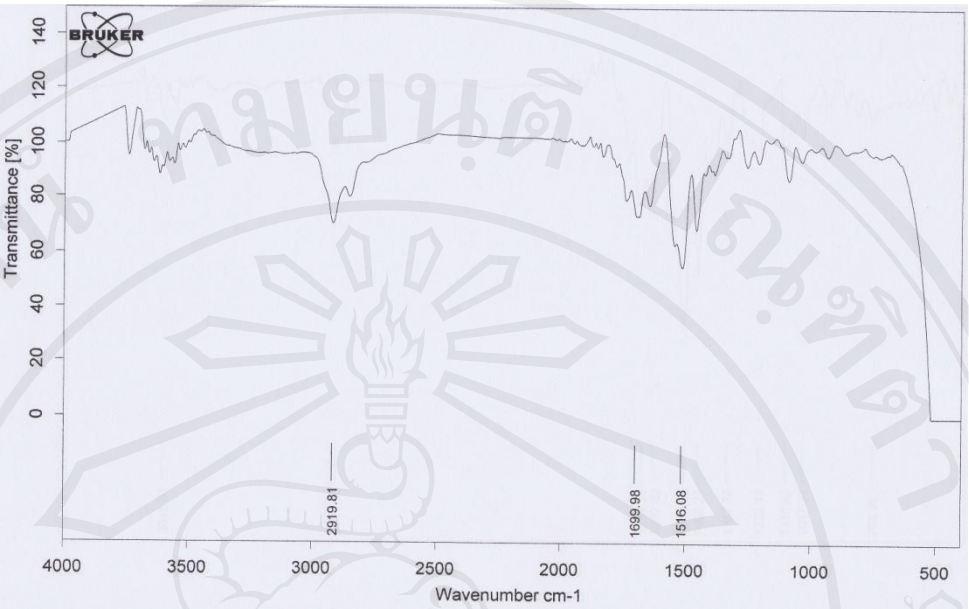

Figure 4.10 IR spectrum of DG3

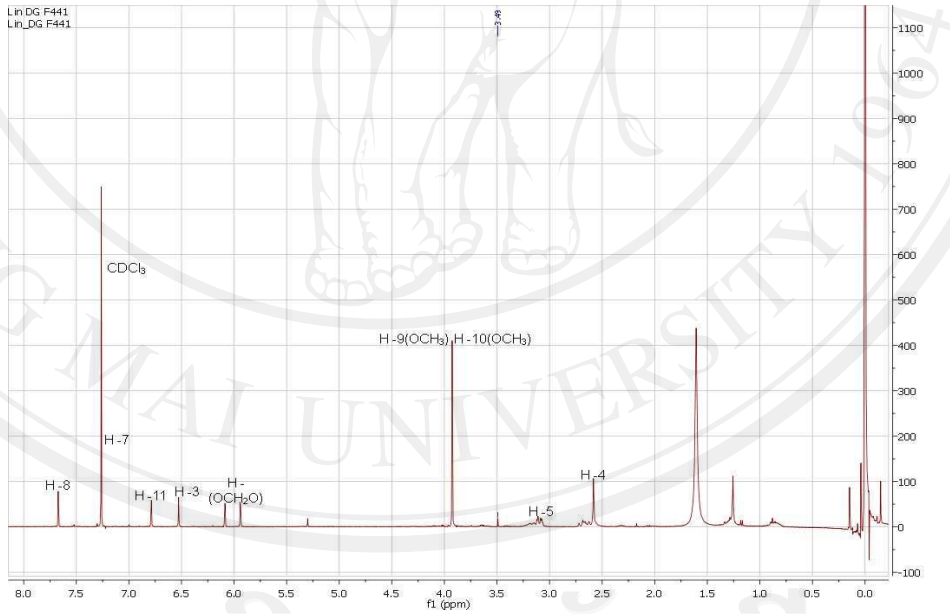

Figure 4.11 ^{1}H-NMR spectrum of DG3

Table 4.4 ^{1}H-NMR (*400* MHz) spectroscopic data of DG3 in $CDCl_3$

Position	δ_H
1	-
1a	-
1b	-
2	-
3	6.53 (1H,*s*)
3a	-
4	2.66 (2H,*m*)
5	3.10 (2H,*m*)
6a	-
7	7.26 (1H, *s*)
7a	-
8	7.67 (1H,*s*)
9	
10	
11	6.79 (1H, *s*)
11a	
OCH_2O	5.94 (1H,*d*,*J*=1.4 Hz) 6.09 (1H,*d*,*J*=1.5 Hz)
7-OCH_3	-
9-OCH_3	3.93(3H, *br s*)
10-OCH_3	3.93(3H, *br s*)

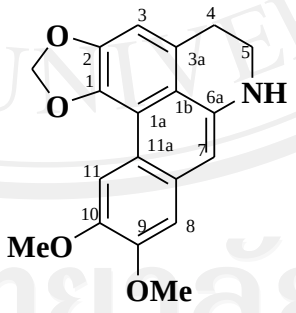

Figure 4.12 The possible structure of DG3 and DG4

4.3.5 DG4 (Fraction 4.3.6.1, the same compound as DG3)

DG4 was isolated as a colorless gum; IR (film) ν_{max} 3436 (N-H stretching), 2914, 2843 (CH stretch), 1513 (CH stretching of aromatic), 1456 (CH-CH_3 bending), 1259, 1210 (C-O stretching), 1091 (C-N stretching) cm^{-1} and 1650, 1513, 1456 cm^{-1} (skeleton of a aporphine alkaloid). The IR spectrum as shown in Figure 4.13. From the result of TLC co-spot found that DG3 and DG4 was the same compound. The data from ^{1}H-NMR of DG4 exhibited a broad singlet of two methoxy protons at δ_H 3.93 ppm and absence of protons at C-6a and 7-OCH_3 which was the same as DG3. The ^{1}H-NMR spectrum of DG4 as shown in Figure 4.14 and summarized in Table 4.5. The possible structure is shown in Figure 4.12.

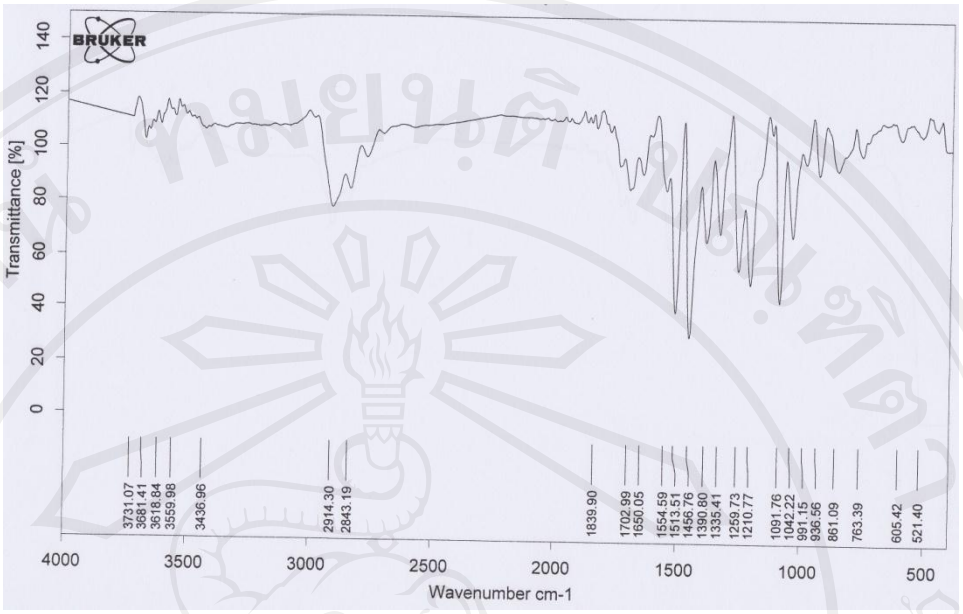

Figure 4.13 IR spectrum of DG4

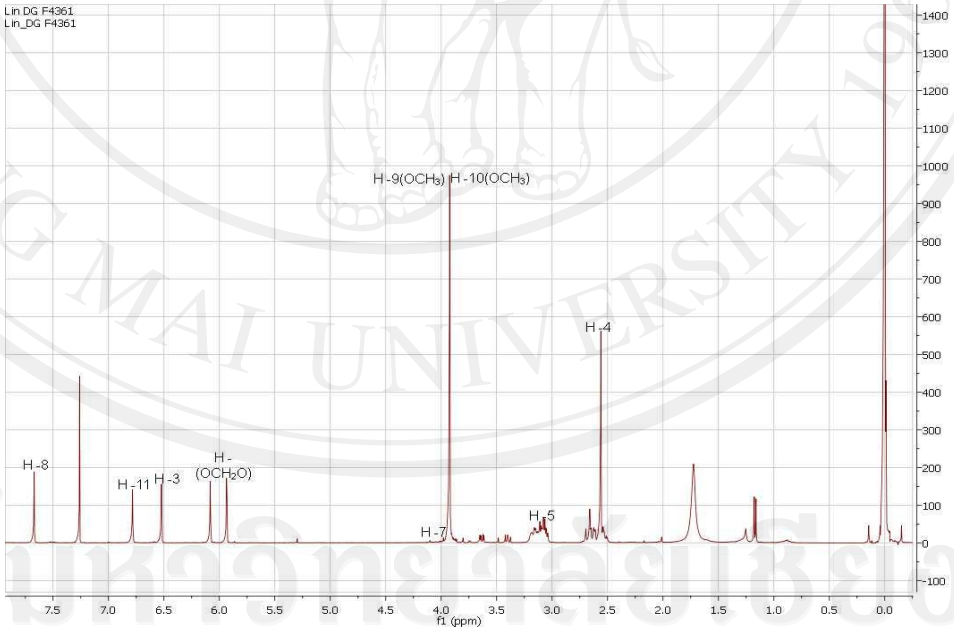

Figure 4.14 ^{1}H-NMR spectrum of DG4

Table 4.5 ^{1}H-NMR (*400* MHz) spectroscopic data of DG4 in $CDCl_3$

Position	**δ_H**
1	-
1a	-
1b	-
2	-
3	6.52 (1H,*s*)
3a	-
4	2.65 (2H,*m*)
5	3.09 (2H,*m*)
6a	-
7	4.10 (1H, *s*)
7a	-
8	7.67 (1H,*s*)
9	
10	
11	6.78 (1H, *s*)
11a	
OCH_2O	5.94 (1H,*d*,*J*=1.4 Hz)
	6.08 (1H,*d*, *J*=1.4Hz)
7-OCH_3	-
9-OCH_3	3.92(3H, *br s*)
10-OCH_3	3.92(3H, *br s*)

4.3.6 DG5 (Fraction 6.3.1)

DG5 was isolated as a yellow-brown gum; IR (film) ν_{max} 3590, 2911 (CH stretching), 1811, 1592 (CH stretching of aromatic), 1449 (CH-CH_2 bending), 1260 (C-O stretching), 1092 (C-N stretching) cm^{-1}. The IR spectrum as shown in Figure 4.15. The ^{1}H-NMR of DG5 showed that methyl proton attached with N atom at δ_H 2.07 ppm and two aromatic methoxy groups at the same position at δ_H 3.94 ppm. The Methylenedioxy protons were observed at δ_H 5.94 and 6.09 ppm. Three singlets of aromatic protons were presented at δ_H 6.53, 7.74 and 6.96 ppm. From the data of ^{1}H-NMR of DG5 indicated that the structure of DG2 similarly to DG2 but in the NMR spectrum of DG5 has more impurity and the proton positions at δ_H 4.84 ppm. could not be identified. However, the signal of H-7 (OCH_3) at 3.60 ppm.could not be observed in the ^{1}H-NMR spectrum of DG5. The ^{1}H-NMR spectrum of DG5 as shown in Figure 4.16 and summarized in Table 4.6. The possible structure is shown in Figure 4.17.

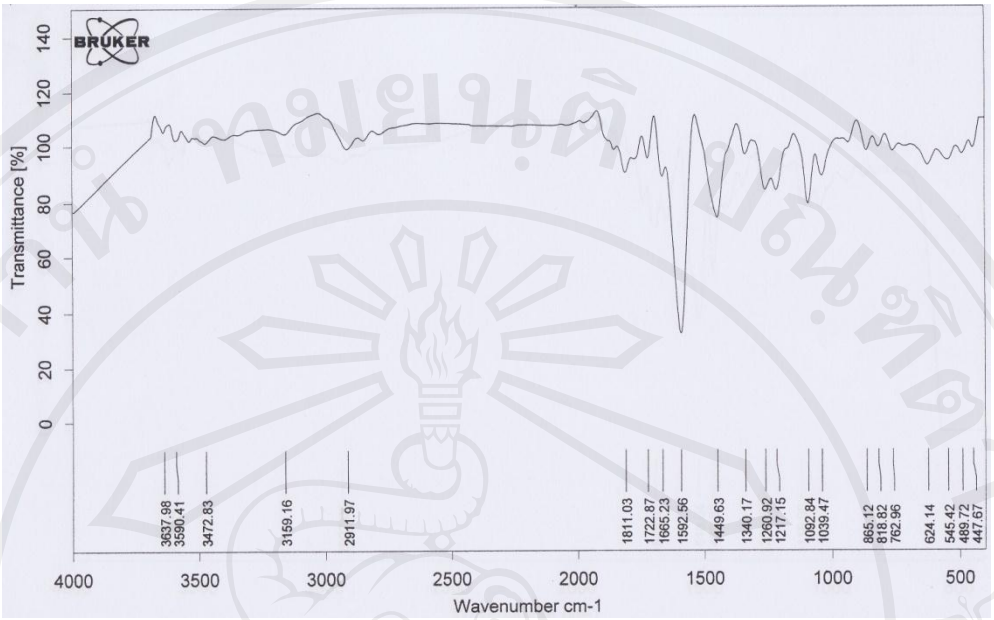

Figure 4.15 IR spectrum of DG5

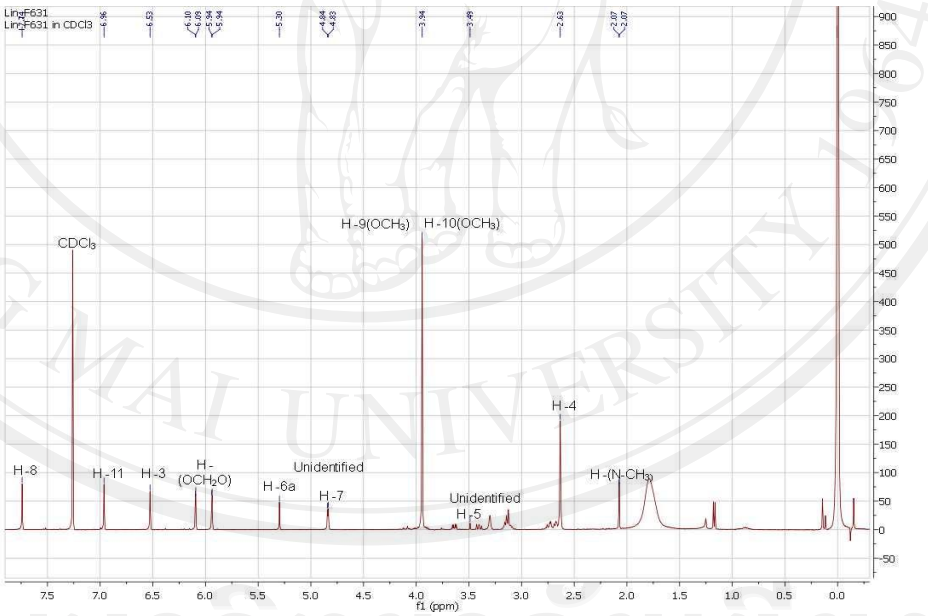

Figure 4.16 ^{1}H-NMR spectrum of DG5

Table 4.6 ^{1}H-NMR (*400* MHz) spectroscopic data of DG5 in $CDCl_3$

Position	δH
1	-
1a	-
1b	-
2	-
3	6.53 (1H,*s*)
3a	-
4	2.63 (2H,*s*)
5	3.49 (2H,*m*)
6a	5.30 (1H, *d*, *J= 2.4* Hz)
7	4.84 *
7a	-
8	7.74 (1H, *s*)
9	-
10	-
11	6.96(1H, *s*)
11a	-
OCH_2O	5.94 6.09
7-OCH_3	-
9-OCH_3	3.94 (3H, *br s*)
10-OCH_3	3.94 (3H, *br s*)
N-CH_3	2.07 (3H, *s*)

* Not confirmed

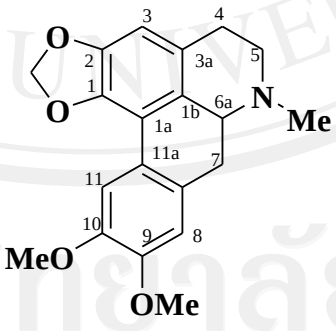

Figure 4.17 The possible structure of DG5

4.4 Determination of efficiency on acetylcholinesterase inhibitory activity by TLC bioautography

A study of the acetycholinesterase inhibitory activity of the isolated compounds from *D. glaucum* was performed by TLC bioautography assay in which AChE inhibition appeared as white spots on the purple background of the chromatogram (Figure 4.18). The result showed that DG1, DG2, DG3, DG4, DG5 and DG6 were able to inhibit the acetylcholinesterase activity with a minimum inhibit requirement (MIR) of 100-250 ng as shown in table 4.7. DG2 and DG5 were the most active compounds with a MIR 100 ng. On the other hand, DG1, DG3 and DG6 found to be the active compounds with a MIR 200 ng and DG4 found to be the active compounds with a MIR 250 ng. Nevertheless, ^{1}H NMR exhibited that DG1 and DG6, DG3 and DG4 were the same compound. The acetycholinesterase inhibitory activities of DG3 and DG4 were difference may be because of more impurity in DG4 compared with DG3. Moreover, all of these compounds exhibited weak inhibitory activities against AChE compared with galanthamine but when compared with *Stemona* alkaloids, [15] these compounds exhibited moderate inhibitory activities against AChE. The MIR of some *Stemona* alkaloids are shown in Table 4.8.

Table 4.7 Minimum inhibitory concentration of **DG1-DG6** required to inhibit AChE

Alkaloid compound from the leaves of *D. glaucum*	Minimum inhibitory requirement (ng)
DG1	200
DG2	100
DG3	200
DG4	250
DG5	100
DG6	200
Galantamine	5

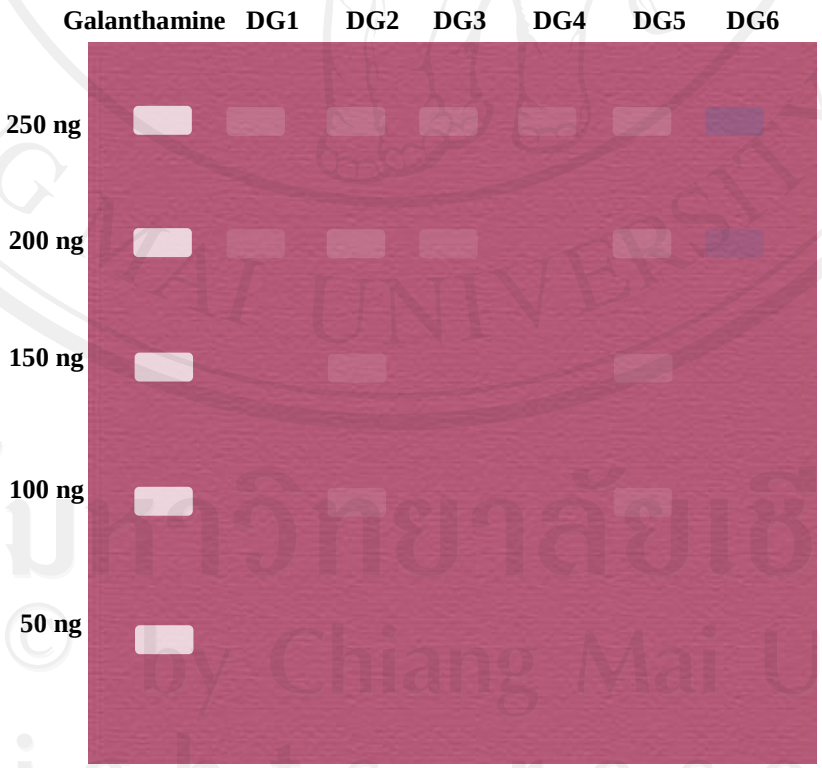

Figure 4.18 Bioautographic thin layer chromatography showing the acetylcholinesterase inhibition of the alkaloid compounds from *D. glaucum* and standard (galanthamine)

Table 4.8 Minimum inhibitory concentration of *Stemona* alkaloids required to inhibit AChE

Stemona alkaloids	Minimum inhibitory requirement (ng)
(1'*R*)-hydroxystemofoline	5
(11*Z*)-1′,2′-didehydrostemofoline	10
Methoxystemofoline	50
Oxystemofoline	50
Stemofolenol	100
Stemocurtisinol *N*-oxide	100
Stemocurtisine *N*-oxide	500
Stemocuticinol	1000
Stemocurticine	>1000

The structure of compounds which have the high acetylcholinesterase inhibitory activity exhibited the methyl group attached with N atom such as physostigmine, sanguinine and galantamine. The structures of these compounds as shown in Figure.4.19. In this research, the results showed that DG2 and DG5 had the high effect to inhibit acetycholinesterase. Their structures showed the methyl group attached with N atom while the structure of DG1 and DG3 had H-atom attached with N atom. Therefore, it could be possible that the methyl group attached with N atom causes the acetycholinesterase inhibition of these compounds.

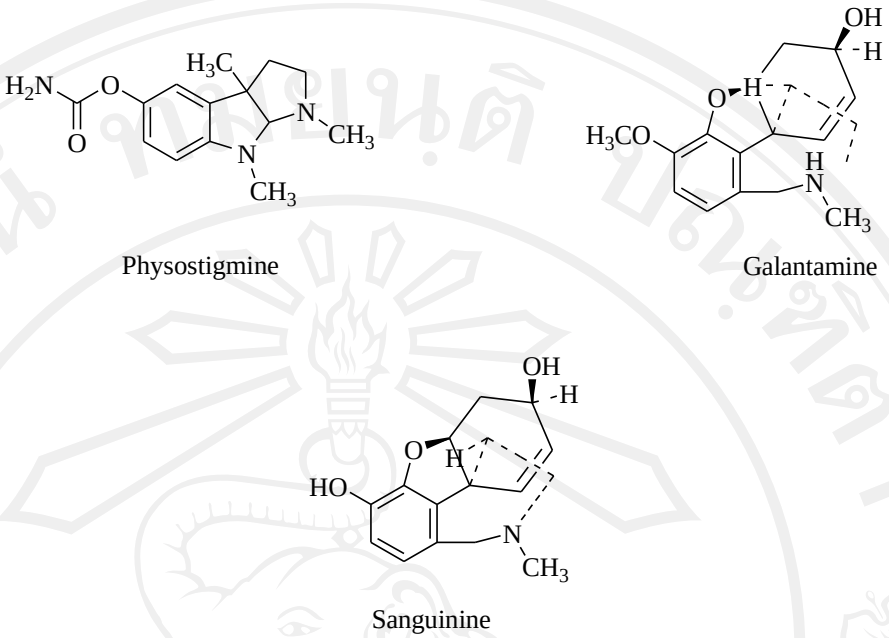

Figure 4.19 The structures of some alkaloids which the high acetycholinesterase inhibitory activity.

However, all of the alkaloids in this work exhibited weak inhibitory activities against AChE compared with galanthamine. Therefore, application of these compounds for using as insecticide should be modified some parts of the structure to give the higher effect to inhibit acetycholinesterase.