CHAPTER III

RESULT AND DISCUSSION

3.1 Structure of Compound I

Compound I was obtained as colorless crystals from methanol (504 mg). Elemental analysis suggested the molecular formula to be $C_{24}H_{34}O_6$ which was agreed well with m/z 418 molecular ion obtained from FAB and EIMS. The molecular formula of compound I suggested eight degrees of unsaturation. The presence of 2,3-disubstituted furan ring structure was evidenced by the ^{1}H NMR (Table 3.1, Figure 6-7) and ^{13}C NMR (Table 3.1, Figure 8) signals for a pair of doublets of aromatic protons (δ 7.22 and 6.18) and four sp^2 carbons (δ 148.6 s, 140.6 d, 122.6 s and 109.5 d). Infrared and ^{13}C NMR spectra indicated the presence of two carboxyl groups. Thus, three remaining degree of unsaturation equivalents indicated the tricarbocyclic nature.

The ^{13}C , DEPT 135 (Figure 9-10) revealed that Compound I had 18 sp^3 carbons; 6 methyl carbons, 4 methylene carbons, 5 methine carbons, 3 quaternary carbons and also consisted of 2 methine and 2 quaternary sp^2 carbons and two carbonyl groups. The IR spectrum showed ester C=O absorption band at 1738 cm^{-1}, and this was further supported by the presence of two carbonyl (δ 170.4 s and 169.1 s) and two methyl carbons (δ 21.1 q and 20.9 q) in its ^{13}C NMR spectrum. There were two protons in the alpha-position of the acetate group (δ 5.30, dd, J = 4.6 Hz, H-2 and δ 5.24,d,J = 2.4 Hz, H-1). The presence of a hydroxyl group was clear from the IR spectrum (3590 cm^{-1}), the ^{1}H NMR spectrum (δ 3.05) and a mass fragment at m/z 400 $[M-H_2O]^+$. The only oxygenated sp^3 carbons observed in the spectrum was assigned to C-5 (δ 77.0 s). Thus the position C-14 was non-oxygenated, since the 17-methyl signal appeared as doublet (δ 1.04, d, J = 7 Hz) in the ^{1}H NMR spectrum.

Spin decoupling experiments (Figure 22-23) allowed the assignment of H-2, H-3, H-7, H-8, H-9, H-14 and OH. The H-9 proton (δ 2.57) coupled to H-8 and two H-11 protons signals were distinguishable. The H-14 proton was observed at δ 2.64 and was coupled to both the 17-methyl and the H-8.

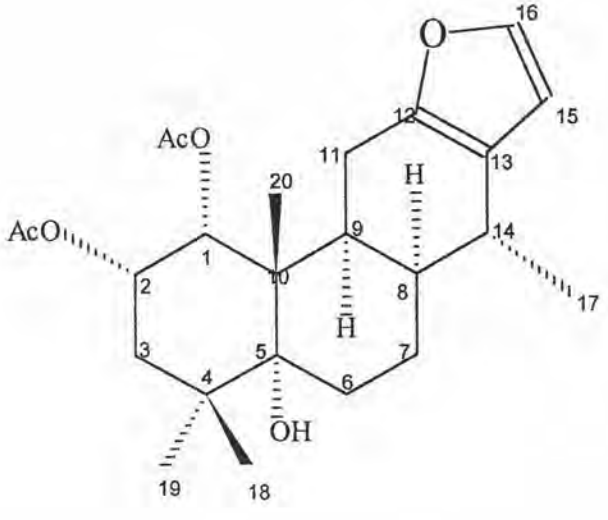
The combination of 2D-NMR techniques, ^{1}H-^{1}H COSY (Figure 19-21), HMBC (Figure 14-18, Table 3.1), HMQC (Figure 12-13) and patterned fragmentation from MS (Scheme 4.1) helped to combine those carbon units to construct the structure of Compound I. Also, it is called ϕ caesalpin. The stereochemistry of Compound I was not fully determined because measuring the coupling constants was not straightforward and no definite NOESY correlations could be obtained.

Table 3.1 ^{1}H NMR , ^{13}C NMR spectrum data and multiple bond C-H correlation of Compound I

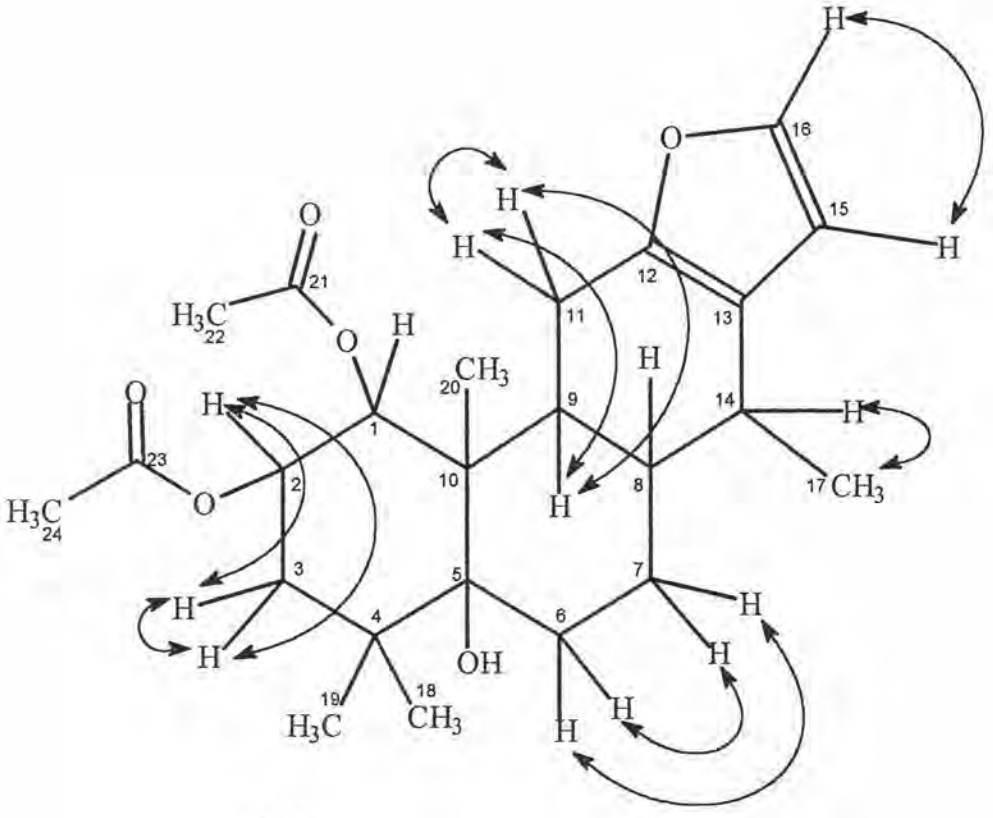
Position	δ_H (ppm)	δ_C (ppm)	multiple bond correlation with proton at δ_H
1	5.24 (d , 2.4)	75.0 d	1.38, 2.02, 2.14, 2.57, 5.30
2	5.30 (ddd,2.9,4.6,12.9)	67.5 d	1.38, 2.02, 5.24
3	2.02 (d , 13)	35.7 t	5.23, 5.30
	1.38 (dd , 4.4,13.0)		
4	—	40.3 s	1.38, 1.68, 2.02
5	—	77.0 s	3.05, 1.38, 1.68, 5.23
6	1.68 (m)	25.6 t	1.42, 1.95
7	1.95 (m)	23.9 t	1.02, 1.68
	1.42 (m)		
8	1.84 (m)	34.2 d	1.42, 2.57, 2.64
9	2.57 (m)	32.7 d	1.84, 2.28, 2.39, 2.64, 5.23
10	—	44.9 s	1.68, 2.39, 2.55, 3.05, 5.23
11	2.39 (dd , 10.5,16.6)	22.1 t	1.84, 2.57
	2.28 (dd , 6.6,16.6)		
12	—	148.6 s	2.28, 2.39, 2.64, 6.20, 7.20
13	—	122.6 s	1.04, 2.28, 22.39, 6.20
14	2.64 (m)	31.3 d	1.84, 1.95, 2.55
15	6.18 (d , 1.7)	109.5 d	2.64, 7.22
16	7.22 (d , 0.98)	140.6 d	6.18
17	1.04 (d ,7)	17.4 q	1.84, 2.64
18	1.19 (s)	26.0 q	1.10, 1.38
19	1.10 (s)	28.2 q	1.19, 2.02

Table 3.1 (continued)

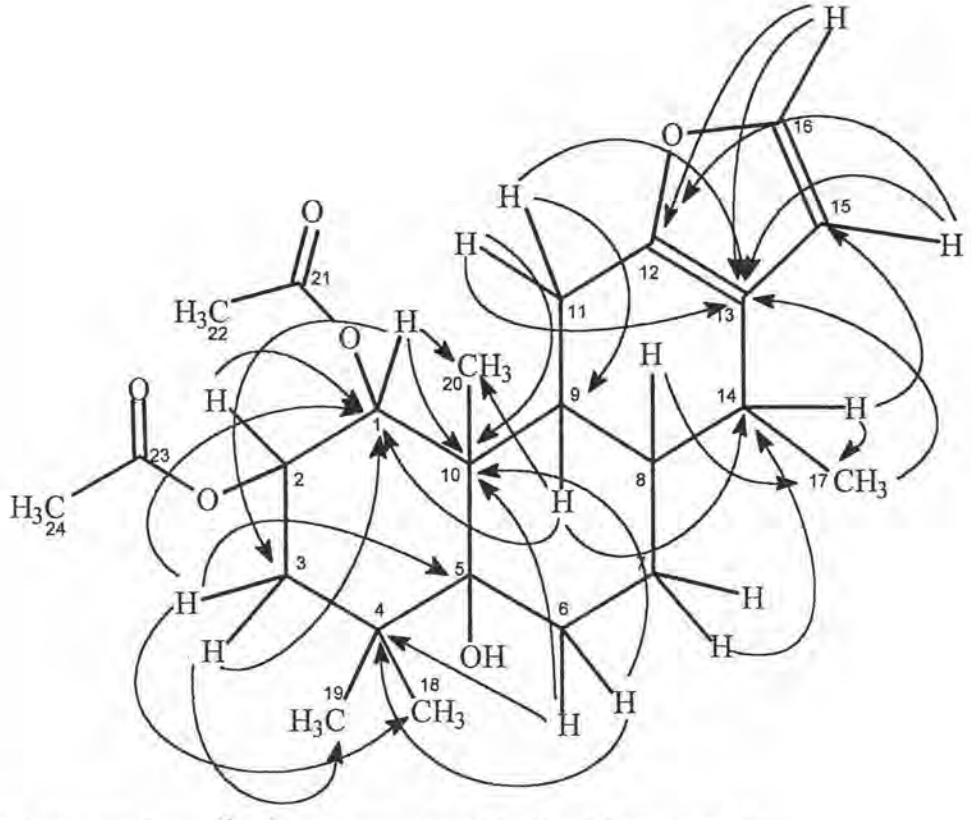
Position	δ_H (ppm)	δ_C (ppm)	multiple bond correlation with proton at δ_H
20	1.22 (s)	17.4 q	2.55, 5.23
21	—	169.1 s	2.14
22	2.14 (s)	21.1 q	—
23	—	170.4 s	1.98
24	1.98 (s)	21.0 q	—



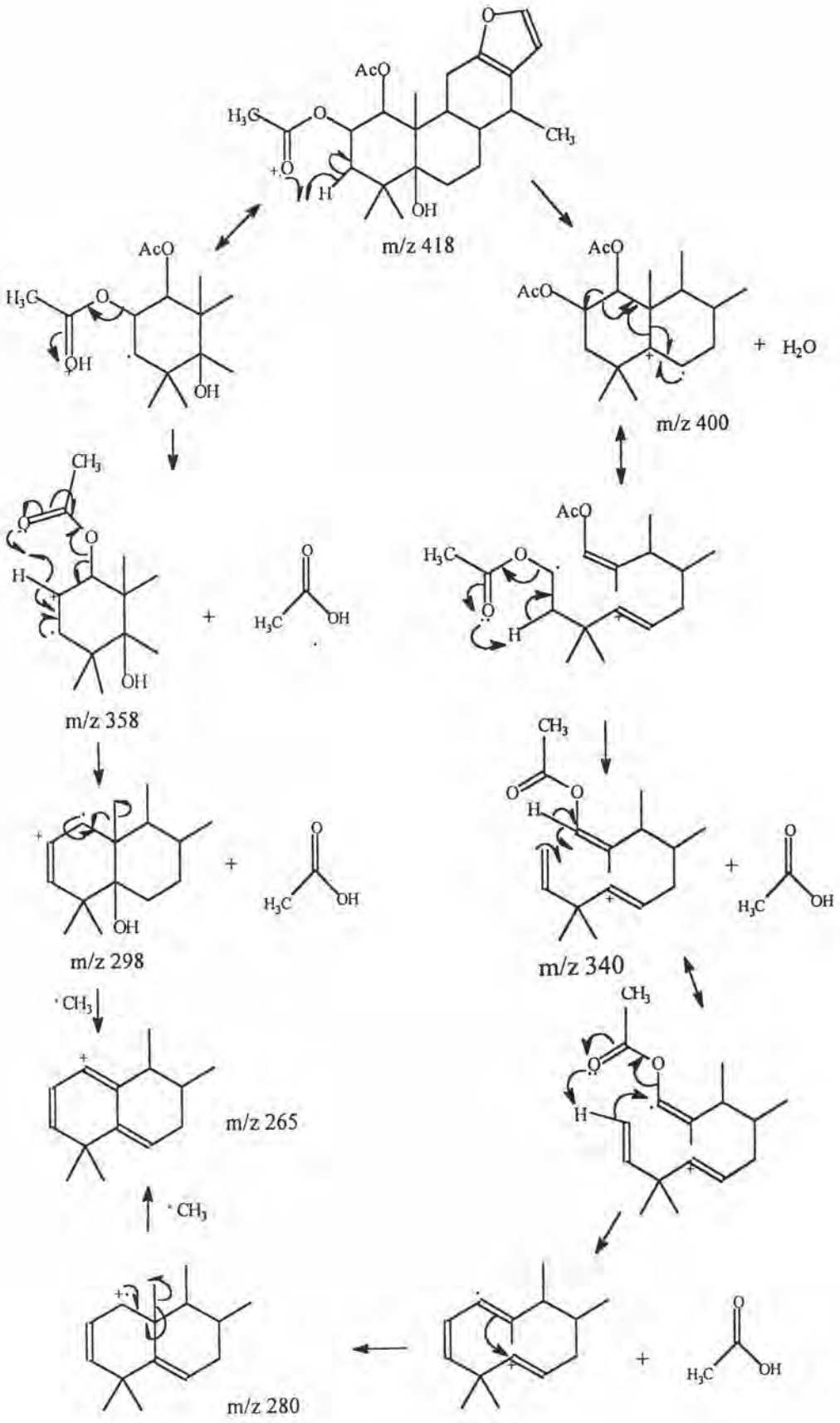
(I) ϕ caesalpin



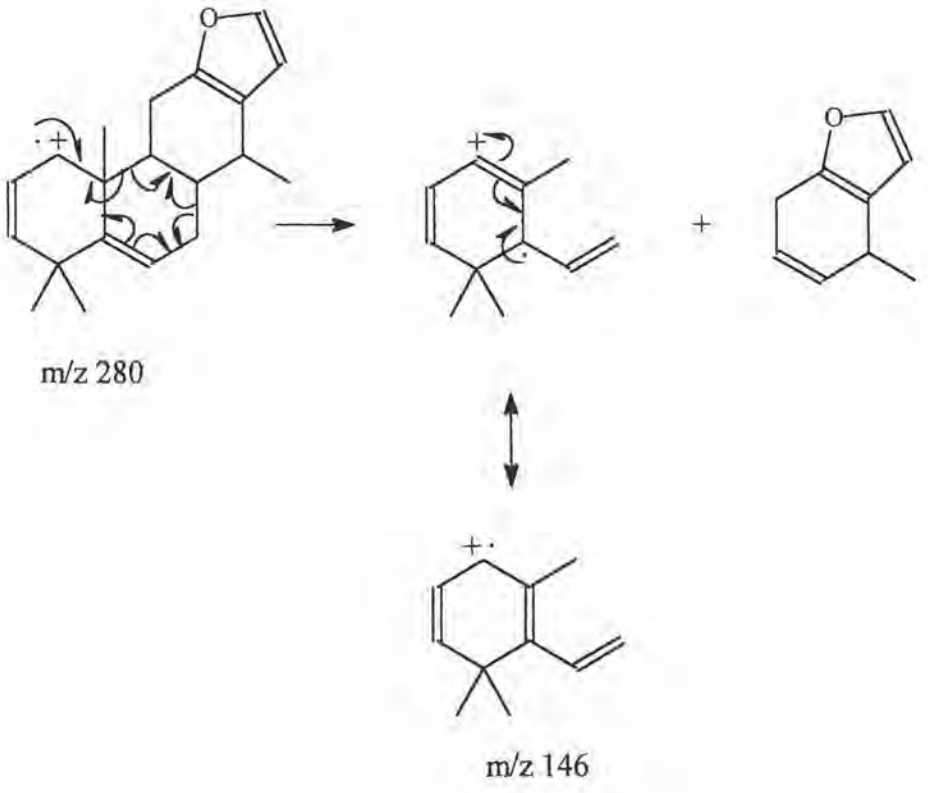
Most significant ^{1}H-^{1}H coupling observed in COSY spectra of Compound I



Most significant ^{13}C-^{1}H observed in HMBC of Compound I



Scheme 3.1 The MS fragmentation pattern of Compound I



Scheme 3.1 (continued)

3.2 Structure of Compound II

Compound II was obtained as colorless crystals from chloroform (10 mg). Its FAB and EI mass spectra showed a molecular ion at m/z 450 for a $C_{24}H_{34}O_8$ with a melting point of 149-151 ^{o}C. Its IR spectrum (Figure 25) revealed the absorption band of hydroxyl group at 3573-3500 cm^{-1} and infrared bands at 1715 and 1654 cm^{-1}, suggesting the presence of an α ,β -butenolide moiety. A C=O stretching band of ester was also observed at 1739cm^{-1}.

The ^{1}H NMR spectrum of Compound II (Table 3.2 , Figure 27-28) showed signals in down field region at δ 5.69 which was assigned to H-15. There were two protons in the alpha-position of the acetate group at δ 5.28 , m , H-2 and 5.23 , d , H-1. The presence of two hydroxyl groups at δ 3.52 and 3.07 were assigned to 12-OH and 5-OH, respectively. There were six methyl groups at δ 2.20 (OAc), 1.99 (OAc), 1.22 (17-Me), 1.17 (18-Me), 1.11 (20-Me), 1.09 (19-Me).

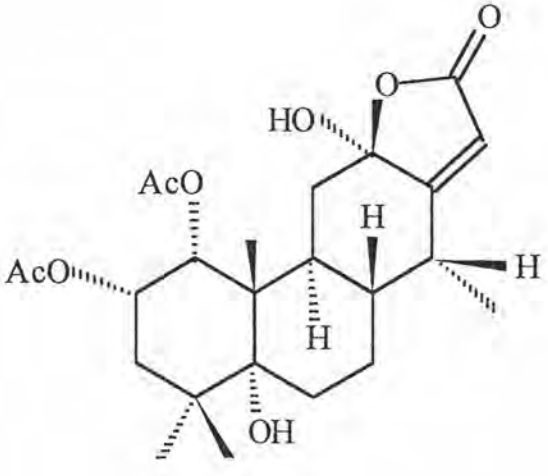
The ^{13}C NMR (Table 3.2, Figure 29-31), DEPT 135 (Figure 32) revealed that Compound II had 19 sp^3 carbons; 6 methyl carbons, 4 methylene carbons, 5 methine carbons, 4 quaternary carbons and also consisted of 1 quaternary and 1 methine sp^2 carbons and three carbonyl carbons. The carbon signals (δ 76.57) in the downfield region of the ^{13}C NMR spectrum was typical of the oxygenated sp^3 quaternary carbon and was assigned to C-5. A carbon signal at δ 105.3 in the further downfield region was assigned to a characteristic sp^3 carbon of hemiketals. Its spectroscopic properties were very similar to those reported for neocaesalpin B as shown in Table 4.2 [14] . In comparison of the spectroscopic data of neocaesalpin B with those of compound II and patterned fragmentation from EI-MS suggested that Compound II was indeed neocaesalpin B (II).

Table 3.2 ^{1}H NMR and ^{13}C NMR Spectrum Data for Compound II and Neocaesalpin B

Protons	δ_H (ppm)		Carbons	δ_C (ppm)	
	Compound II $CDCl_3$,500 MHz	Neocaesalpin B pyridine-d_5, 500 MHz		Compound II $CDCL_3$,500 MHz	Neocaesalpin B pyridine-d_5, 500 MHz
H-1	5.23 (d,2.0)	5.54 (d,2.4)	C-1	74.8 d	74.3 d
H-2	5.28 (m)	5.52 (ddd,2.4, 4.1,13.1)	C-2	67.2 d	67.8 d
H-3α	2.1 (m)	2.30(dd,13.1, 13.1)	C-3	35.8 t	36.0 t
H-3β	1.32 (m)	1.40(dd,4.1, 13.1)			
H-4	—	—	C-4	40.2 s	40.3 s
H-5	—	—	C-5	76.6 s	76.4 s
H-6α	1.71 (m)	1.69 (dd,2.7, 13.3)	C-6	25.4 t	26.0 t
H-6β		1.58			
H-7α	1.97 (m)	2.15-2.20	C-7	23.3 t	23.8 t
H-7β	1.24 (dd)	1.24			
H-8	1.66-1.55 (m)	1.58	C-8	39.4 d	39.5 d
H-9	2.59 (m)	3.10 (ddd,2.7, 12.6,12.6)	C-9	32.3 d	33.1 d
H-10	—	—	C-10	44.9 s	45.2 s
H-11α	2.14(dd,2.5, 12.7)	2.42 (dd,2.7, 12.6)	C-11	38.0 t	38.9 t
H-11β	1.43 (dd,13.7 4.9)	1.33 (dd,12.6, 12.6)			
H-12	—	—	C-12	105.3 s	106.5s

Table 3.2 (continued)

Protons	δ_H (ppm)		Carbons	δ_C (ppm)	
	Compound II	Neocaesalpin B		Compound II	Neocaesalpin B
H-13	—	—	C-13	170.5s	171.0s
H-14	2.96 (m)	2.88(dq,4.6 7.2)	C-14	36.6d	36.9d
H-15	5.69 (s)	5.82 (s)	C-15	133.5d	113.2d
H-16	—	—	C-16	172.7s	174.4s
17-Me	1.22(d,7.1)	1.35 (d,7.2)	C-17	12.6s	13.2q
18-Me	1.17 (s)	1.17 (s)	C-18	28.2q	28.4q
19-Me	1.09 (s)	1.10 (s)	C-19	25.7q	25.4q
20-Me	1.11 (s)	1.05 (s)	C-20	16.9 s	17.0 q
OAc	1.99 (s)	2.01 (s)	$CH_3\underline{C}O$	169.7 s	170.2 s
	2.20 (s)	2.15 (s)		170.3 s	170.3 s
			$\underline{C}H_3$ CO	21.0 q	20.8 q
				20.9 q	20.9 q



(II) Neocaesalpin B

3.3 Structure of MixtureIII

Mixture III was colorless needle-like crystals and melting point was 135 -136 °C. The ^{1}H NMR spectrum (Figure 35) showed the signal at δ 0.65-1.3 ppm which were the signals of methyl proton that substituted at C-18, C-19 and at side chain of the steroidal compounds. The signals at δ 1.3-2.3 ppm were the signal of methylene and methine proton of the steroids. The chemical shift at 3.53 ppm indicated the proton attached to a carbon bearing a hydroxyl group (CH-OH). The olefinic signals at δ 5.14 ppm and 4.95 ppm are *trans*-disubstituted vinyl protons (H-22 and H-23). And the olefinic signal at δ 5.33 could be assigned as H-6 which was trisubstituted vinyl proton. The ^{13}C NMR spectrum (Table 3.3, Figure 36) showed 25 signals in region of 11.9-57.0 ppm which indicated sp^3 CH_3 , CH_2, CH and C. The olefinic carbon signals were evidenced at 121.8 ppm (C̲H=C) and 140.8 ppm (CH=C̲).

The NMR spectrum suggested that Mixture III was a mixture of steroidal compounds possessing a hydroxyl group. GC technique was then used to identify Mixture III by comparison the chromatogram of Mixture III with that of the standard mixture of steroids : campesterol, stigmasterol, β - sitosterol (Figure37). The retention times of the standard steroid were 17.42, 18.38 and 20.51 min respectively. The retention times of Mixture III was 17.86 and 20.80 min which revealed that Mixture III was mixtures of stigmasterol and β-sitosterol. EI mass spectra (Figure 34) revealed a molecular ion peak at M^+ = 414 which also corresponded to the molecular formula $C_{29}H_{50}O$ of β-sitosterol. The peak at m/z = 412 corresponded to the molecular ion peak of stigmasterol was also observed. Comparison of the ^{13}C NMR spectrum of Mixture III and β-sitosterol [33] was also carried out to further confirm the structure as presented in Table 3.3.

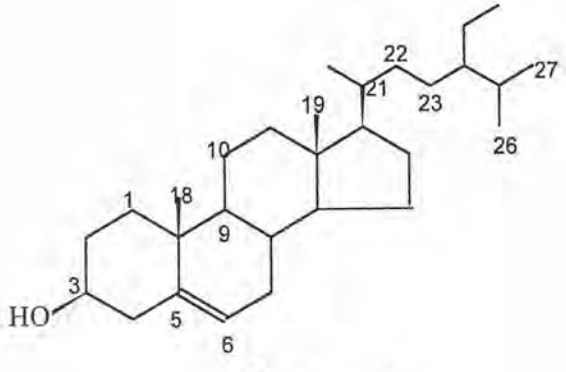
Table 3.3 ^{13}C NMR Spectrum of Mixture III compared with β-Sitosterol

Position	Chemical shift (ppm)	
	β-sitosterol	Mixture III
1	37.4	37.4
2	31.8	31.7
3	71.9	72.0
4	42.4	42.5
5	140.9	140.8
6	121.8	121.8
7	32.0	32.1
8	32.0	32.1
9	50.3	50.1
10	36.6	36.5
11	21.1	21.1
12	39.4	40.0
13	42.2	42.5
14	56.8	57.0
15	24.3	24.5
16	28.2	28.5
17	56.2	56.1
18	11.9	12.0
19	19.4	19.6
20	36.2	36.3
21	19.1	19.2
22	34.0	34.1
23	29.3	29.5
24	50.3	50.1

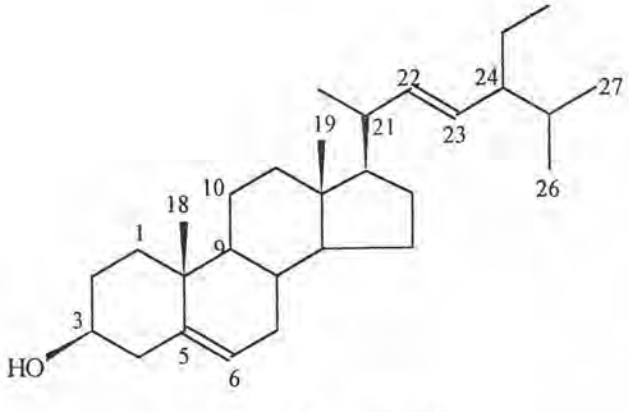
Table 3.3 (continued)

Position	Chemical shift (ppm)	
	β-sitosterol	Mixture III
25	26.2	26.2
26	18.8	18.8
27	19.8	20.1
28	23.1	23.2
29	11.9	11.9

These results suggested that Compound III was mixture of stigmasterol and β-sitosterol.



β-sitosterol



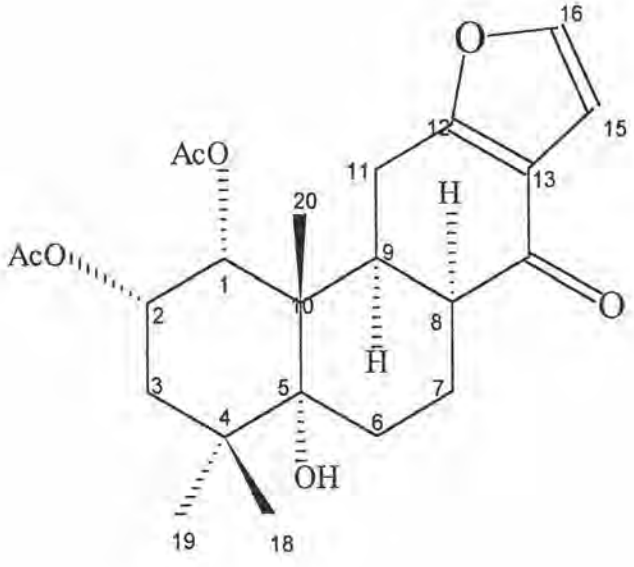
stigmasterol

3.4 Structure of Compound IV

Compound IV was obtained as yellow oil (7.2 mg). Its MS showed a molecular at m/z 418. The ^{13}C NMR (Table 3.4 , Figure 40-48), DEPT 135 (Figure 44), DEPT 90 (Figure 45) revealed that Compound IV had 5 methyl carbons, 4 methylene carbons, 6 methine carbons, 5 quaternary carbons and also consisted of three carbonyl groups Its NMR spectrum provided the molecular formula C_{23} H_{30} O_7 , which indicated eight degree of unsaturation. Three remaining double-bond equivalents indicated the tricarbocyclic nature. The presence of a 2,3-disubstituted furan ring structure was evidenced from the existence of an oxygen atom and the ^{1}H NMR signals for a pair of doublets at δ 7.26 and 6.60 (Table 4.4 , Figure 40) and ^{13}C NMR signals of sp^2 carbons at δ 165.48 s , 142.93 d , 119.89 s and 106.47 d. The presence of two acetate groups was evidenced from two carbonyls (δ 170.35 s and 169.36 s) and two methyl carbons (δ 21.11 s and 20.89 s) in its ^{13}C NMR spectrum. There were two protons in the alpha-position of the acetate groups (δ 5.34, H-2 and δ 5.26, H-1). The ^{13}C NMR spectrum (δ 76.44 , s , C-5) and a mass fragment at m/z 400 $[M-H_2O]^+$ exhibited a hydroxy group. The NMR spectra were similar to those of Compound I. In comparison of the ^{1}H NMR resonance of Compound IV with those of compound I, the signals in downfield at δ 7.26 and δ 6.60 were doublets of aromatic proton and were assigned to H-15 and H-16. They were shifted downfield from those signals of Compound I (δ 7.22 and δ 6.18). Compound IV has one carbonyl carbon (δ 195.30 s) in the downfield region of ^{13}C NMR spectrum and was assigned to C-14. This was further supported by two methine carbons (δ 40.0 , C-8, δ 43.7, C-9) and quaternary sp^2 carbon (δ 165.5, C-12) that conjugated to carbonyl carbon (C-14) that their signals were shifted downfield from those signals of Compound I. ^{13}C NMR spectrum of Compound I indicated three methine carbons at δ 34.19 C-8 , δ 32.69 C-9 and δ 31.28 C-14 and the 17-methyl signal was a doublet at δ 1.04. On the other hand, the Compound IV has only two methine carbons at those region in ^{13}C NMR spectrum and has no doublet of methyl protons in ^{1}H NMR spectrum. Furthermore, the ^{13}C NMR showed only signal of

methyl carbon at chemical shift between 16.50 to 18.00 and was shifted downfield due to carbonyl group at C-14. Morever the position C-11 was methylene carbon (δ 20.54).

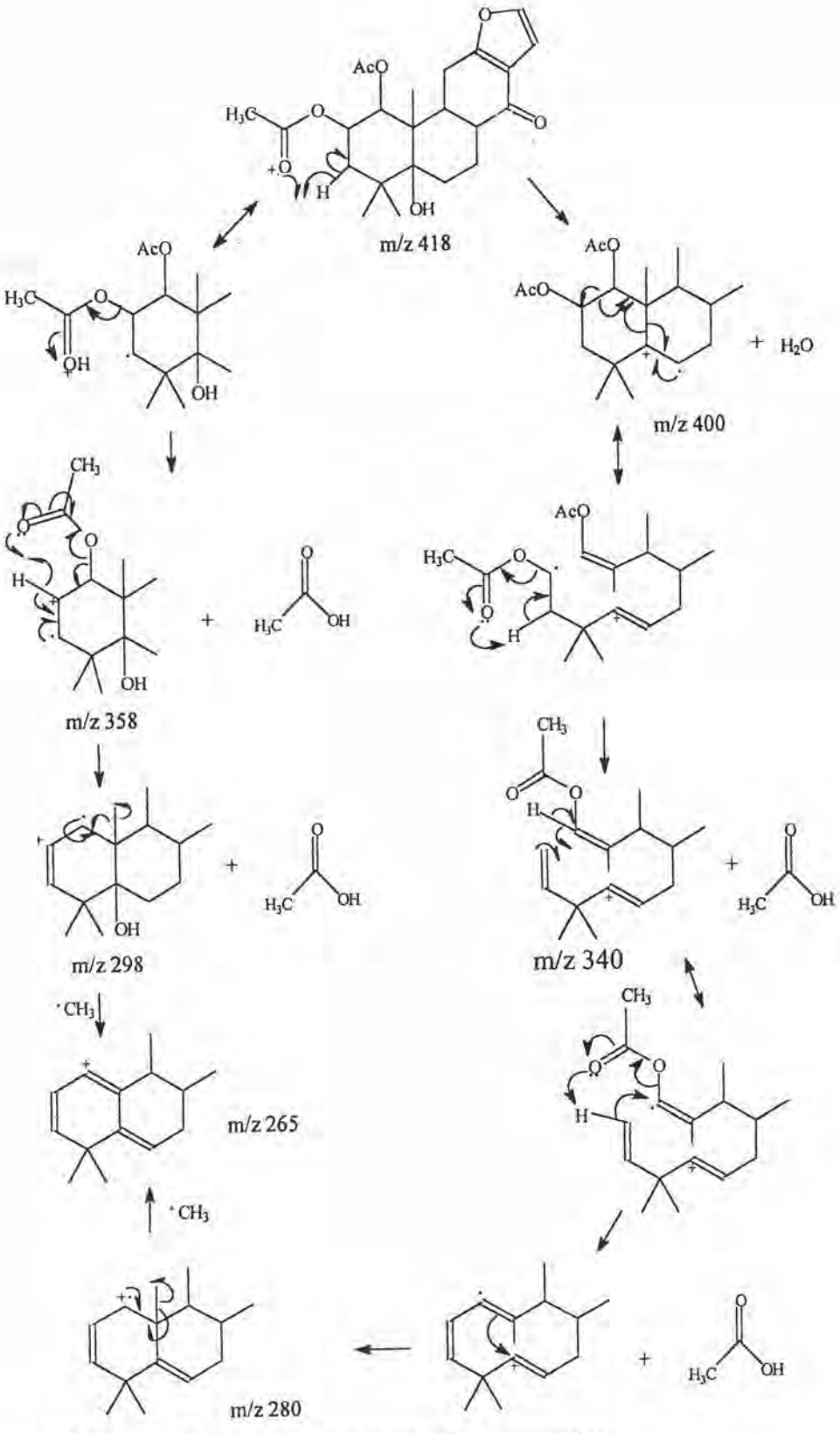
Due to limit quatity of Compound IV, no information on 2D NMR was available. However, a combination of ^{1}H NMR, ^{13}C NMR spectrum, fragmentation patterned from MS (Scheme 3.2) and the comparison data of Compound IV with those of Compound I and also other furanoditerpene which have been studies, the structure of compound IV was proposed as shown below and it is called η caesalpin. If this structure corrects, it would be a new compound from *Caesalpinia major* (Medik.) Dandy &Exell.



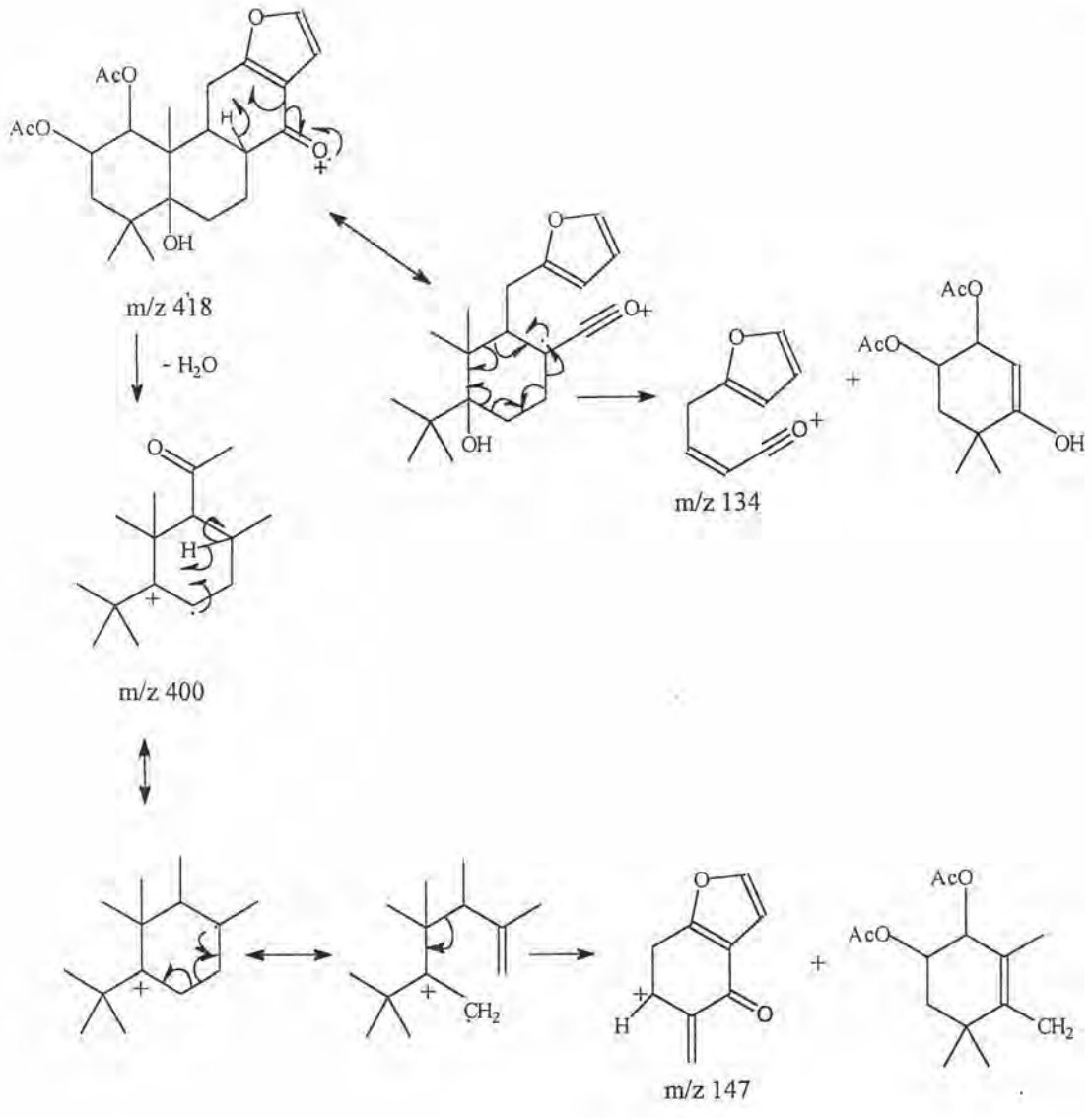
(IV) η- caesalpin

Table 3.4 ^{1}H NMR and ^{13}C NMR Spectrum Data for Compound IV

Position	δ_H (ppm)	δ_C (ppm)
1	5.26 d	74.5 d
2	5.34 m	67.3 d
3	1.28-2.11 m	35.7 t
4		40.1 s
5		76.4 s
6		25.4 t
7		22..9 t
8		40.0 d
9		43.7 d
10		45.0 s
11		195.3 s
12		165.5 s
13		119.9 s
14	2.13-3.10 m	20.5 t
15	6.60 d	106.5 d
16	7.26 d	142.9 d
17	1.16 s	25.7 q
18	1.06 s	28.1 q
19	1.27 s	17.8 q
$C\underline{H}_3CO$	2.12 s	21.1 q
	1.93 s	20.9 q
$CH_3\underline{C}O$		170.4 s
		169.4 s



Scheme 3.2 The MS fragmentation pattern of Compound IV



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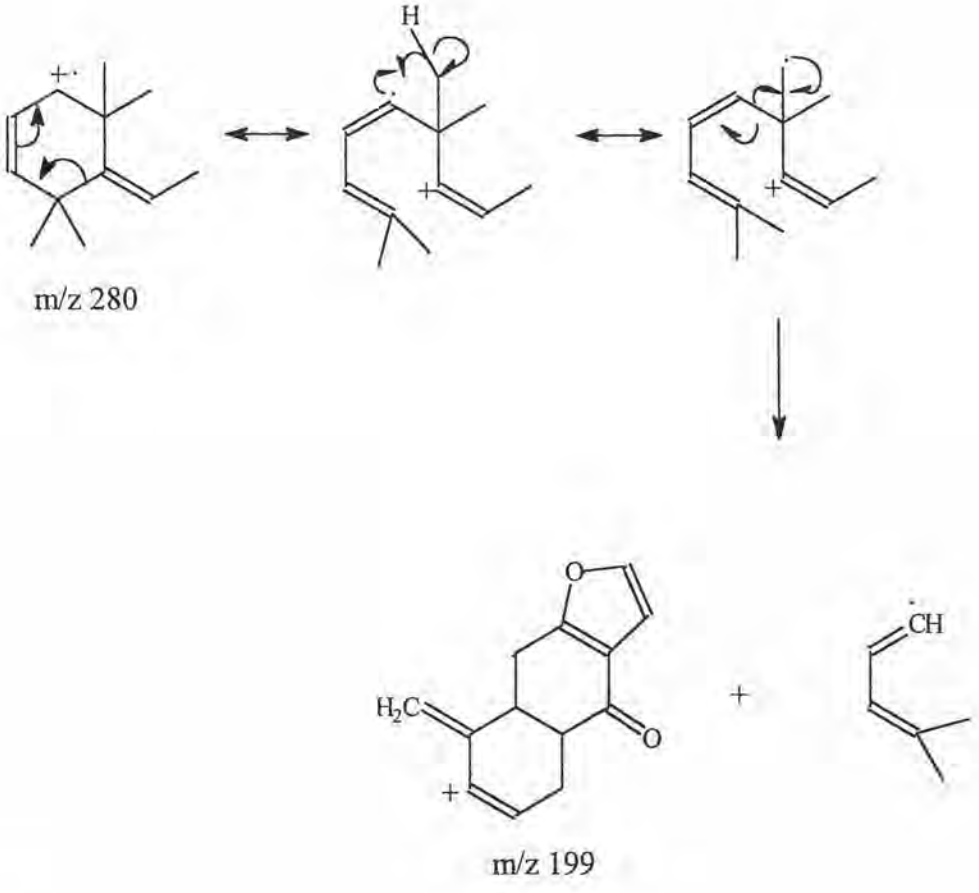

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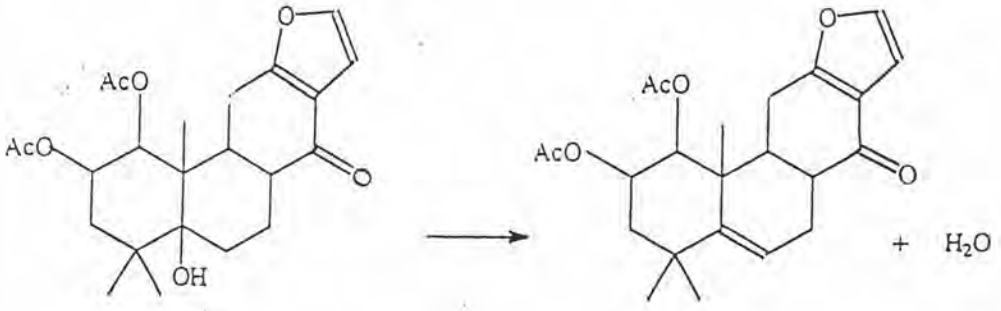

m/z 418

Scheme 3.2 (continued)

3.5 Biological Activity Test of Compound I

Preparation and procedure of Insect Antifeedant Activity

The worms used in the test were called Greater Wax Moth(*Galleria mellonella* Linn.) and the larvae was obtained from the damage combs from the Bee Research Unit Chulalongkorn University, Tambol Bangkhantak, Amphur Muang, Samut Songkhram, Thailand. The adult larvae was fed on artificial diet and they were kept in the temperature room at 27°C at the Bee Research Unit Department of Biology, Chulalongkorn University. The larval food was modified according to Duthy et al, 1962 [34]. The larvae food consists of ceresol : bee pollen (3:1) and honey : distilled water : glycerol (1:1.4 : 1.1). Two grams of food were put in a square folded aluminium foil bowl of size 3x3 cm^2 . The food bowls were weighed before use. The Compound I in dichloromethane at various concentrations (percentage wt. by wt. of food) were dropped into the food bowls, for the test. The control food was prepared by dropping the same solvent without Compound I dissolved. The solvent was allowed to evaporate from each food bowl by air drying for three hours. Then each bowl was reweighed and then placed pair-wise (test + control) in a plastic box. Ten worms of the same size, 0.75 cm length, were chosen and put in the same box. Its was kept in the incubator at temperature around 34-36°C. After two days, the worms were counted. After removing the worms, food bowls were weighed to determine the loss weight from tested food and control food. Antifeedant activity was expressed as % T/C value, where:

%T/C = (1 – loss weight of tested sample/ loss weight of controlled sample) x 100

* T/C value of 100% represents total inhibition of feeding activity.

The result of Antifeedant Activity Tests

The compound I obtained from the seed kernels of *Caesalpinia major* (Medik.) Dandy&Exell. as mentioned above was tested for antifeedant activity against the Greater Wax Moth, *Galleria mellonella* Linn. The result are shown in Table 3.5.

Table 3.5 The antifeedant activity of the compound I from the seed kernels of *Caesalpinia major* (Medik) Dandy&Exell. against Greater Wax Moth, *Galleria mellonella.*

Concentration (%w/w)	%Antifeedant
0.25	46.38
0.10	36.99
0.05	5.66

Note : 71-100% = high antifeedant activity

41-70% = medium antifeedant activity

11-40% = low antifeedant activity

0-10% = no antifeedant activity

w/w = weight of compound I / weight of larvae food

From the results of antifeedant test, compound I was found to have no antifeedant activity against this Greater Wax Moth. Due to limited quantity of other compounds isolated from *Caesalpinia major* (Medik.) Dandy&Exell., no information on the antifeedant activity test was available. However, other biological activities are still interesting and may be carried out in the future.