CHAPTER III

RESULTS AND DISCUSSION

3.1 Effect of dichloromethane crude extract for antifungal activity

3.1.1 Mycelial growth inhibition

The dichloromethane crude extract was prepared at final concentrations of 150, 170, 190, 200, 230 250 500 and 1000 ppm. After a mycelial disc was inoculated on each plate, the fungi were allowed to grow. The antifungal activity was observed after the fungi reached the edge of control plates. The results are shown in Table 3.1.

Table 3.1 The mycelial growth inhibition of dichloromethane crude extract

Concentration of dichloromethane crude extract (ppm)	Inhibition (%)	SD	IC_{50} (ppm)
0	0.0	±0.00	
150	3.3	±0.23	
170	20.7	±0.32	
190	51.3	±0.23	190.0
200	74.3	±0.51	
230	83.3	±0.29	
250	98.3	±0.29	
500	100.0	±0.00	
1000	100.0	±0.00	

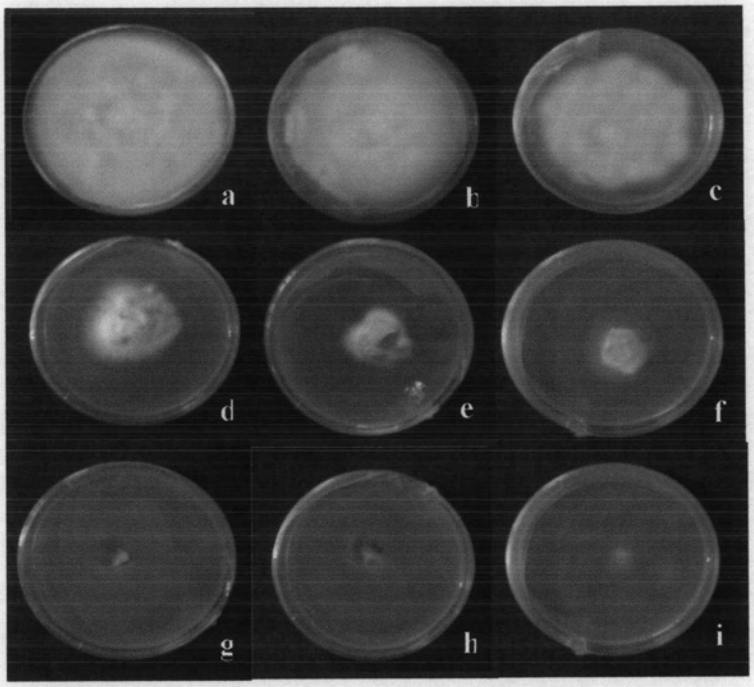
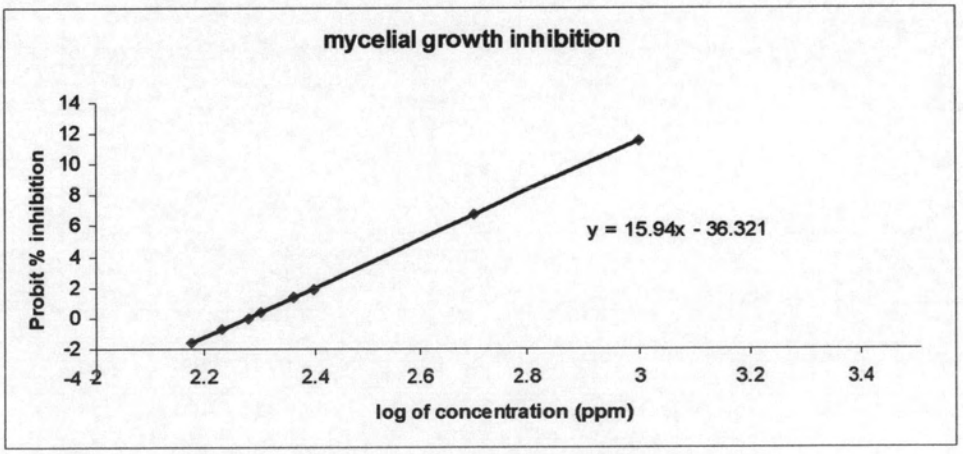


Fig. 3.1 The mycelial inhibition of crude dichloromethane extract against *Ascosphaera apis* fungi at several concentration.
(a, 000 ppm; b, 150 ppm; c, 170 ppm; d,190 ppm; e, 200 ppm; f, 230 ppm; g, 250 ppm; h, 500 ppm and I, 1000 ppm)

Fig. 3.2 Linear regression representing the probit of percent inhibition in



relation to the logarithm of concentrations (ppm).

As summarized in Table 3.1, the results revealed a dose dependence for the maximal observed inhibition recorded at the highest concentration tested as 250, 500 and 1000 ppm. Fig. 3.1 showed the mycelial inhibition of crude dichloromethane extract against *Ascosphaera apis* fungi at several concentrations. The result indicated that the higher concentration of crude extract, the more inhibition of fungus. Determining the IC_{50} of propolis concentration by measuring inhibition of fungus was performed. The results revealed that IC_{50} of dichloromethane crude extract is 190 ppm. The percent inhibition was then transformed into Probit, and the graph was traced for this Probit in relation to the logarithm of dichloromethane crude extract concentration. Fig. 3.2 presents graph of mycelial growth inhibition of crude extract to *Ascosphaera apis* incubated at 30°C on a PDA after 7 days.

3.1.2 Inhibition of *Ascosphaera apis* spore germination

The effect of dichloromethane crude extract as the inhibitor on spore germination of *Ascosphaera apis* is shown in Fig. 3.3 and Table 3.2.

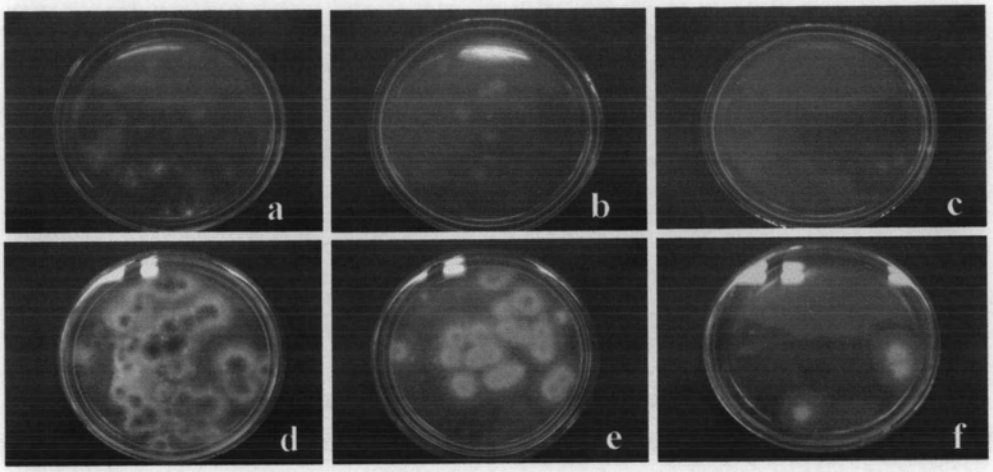


Fig. 3.3 The spore germinate inhibition of crude dichloromethane extract to *Ascosphaera apis* (Suspensions of *Ascosphaera apis* conidia dissolved in sterile distilled water, a: 1 hr and d: 24 hr, conidia dissolved in DMSO, b: 1 hr and e: 24 hr, conidia dissolve in 1,000 ppm dichloromethane crude extract, c: 1 hr and f: 24 hr)

Table 3.2 Inhibitory effects of dichloromethane crude extract against the spore germination of *Ascosphaera apis* at different times

Concentration (ppm)	Percent inhibition (%)		
	0 hr	1 hr	24 hr
1000	95.8	95.3	93.4
DMSO	19.1	64.5	67.6

The results revealed that the inhibitory effect of the dichloromethane crude extract of propolis was higher than DMSO (Table 3.2). The highest inhibition of dichloromethane crude extract at 1000 ppm was recorded at 0 hr. The effects of inhibited spore by propolis 1000 ppm at each time was not significantly different ($p \leq 0.05$) while those by DMSO at each time was significantly different ($p \leq 0.05$). However, the

result indicated that there was significantly different between dichloromethane crude extract of propolis and DMSO ($p \leq 0.05$).

3.2 The fractionation of dichloromethane crude extract

A part of the dichloromethane crude extract (31 g) was mixed with silica gel. It was then fractionated by quick column chromatography, eluted with hexane-EtOAc and EtOAc-MeOH as solvent systems (approximately 3,000 ml per system). Each fraction was monitored by TLC using EtOAc in hexane (3:7, 4:6, 6:4, 8:2, EtOAc and 0.5:9.5 MeOH/EtOAc, respectively) as the developing solvent. Fractions with similar chromatographic patterns were combined to yield seven fractions of the bioactive compounds as summarized in Table 3.3 and Fig. 3.4.

Table 3.3 The fractionation of dichloromethane crude extract by quick column chromatograph.

Fraction number	Eluent	Remarks	Weight (g)
1	Hexane – 1:9 EtOAc/Hexane	yellow	0.14
2	2:8 EtOAc/ Hexane	pale yellow	5.43
3	3:7EtOAc/Hexane	yellow viscous	2.72
4	4:6 EtOAc/Hexane	orange	2.68
5	6:4 EtOAc/Hexane	light brown	1.92
6	8:2 EtOAc/Hexane	brown	0.55
7	EtOAc – 1:9 MeOH/EtOAc	dark brown	0.98

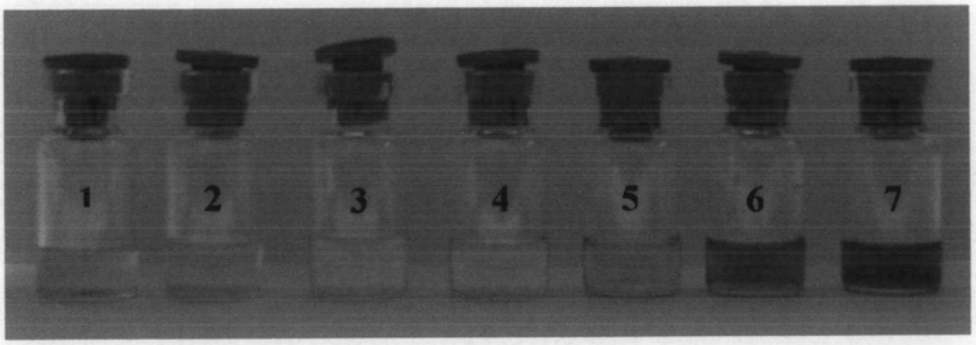


Fig. 3.4 Colourations of seven fractions of dichloromethane crude extract.

As the result, the dichloromethane extract could be subfractionated to seven fractions. Fraction 2 gave the highest yield. The colour of each fraction was shown in Fig. 3.4.

3.2.1 TLC bioautographic assay of fractionation of dichloromethane crude extract

Seven fractions of dichloromethane crude extract were subjected to bioactivity testing on TLC. The seven fractions were loaded on TLC and resolved with EtOAc/hexane 8:2. The TLC plate was dipped into 10% (v/v) H_2SO_4 in ethanol followed by heating to visualize the spots. For the plate used in the bioautographic assay system, it was dried, then sprayed with conidial suspension of *Ascosphaera apis* in liquid media and incubated in a moisture chamber for 3 days. The result is presented in Fig. 3.5.

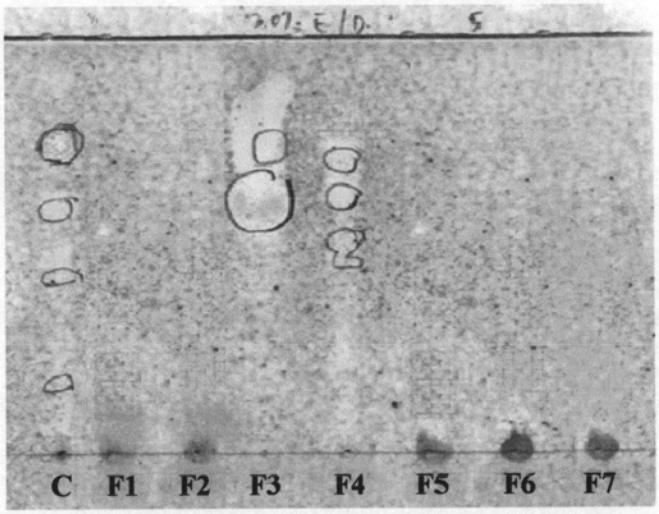


Fig. 3.5 TLC bioautographic pattern of the fractions of dichloromethane crude extract, sprayed with *Ascosphaera apis* compared with control (DMSO) treatment.

After staining and destaining, a typical bioautographic pattern is shown in Fig. 3.5. Fractions 3 and 4 showed a clear inhibition zone to *Ascosphaera apis*. Fractionation of dichloromethane crude extract was not clearly separated. TLC pattern of each fraction displayed a mixture of some constituents similar to another fraction. Thus, TLC profile of fractions 3 and 4 had similar pattern. Fraction 3 which gave the highest yield was selected for further investigation of active constituents.

Table 3.4 The spores germination inhibition of the seven fractions of dichloromethane crude extract.

Fraction number	Inhibition (%)					IC_{50} (ppm)
	1	10	100	500	1000	
1	9.67	9	13.33	9	9	-
2	10.67	9.33	10.33	11	10	-
3	9	11	33.67	72	93	110
4	8.67	10.67	8.33	42.33	73	675
5	9.33	8	10	14	38.33	137,870
6	7.67	8.67	11.33	24.67	42	12,346
7	8.33	9.67	8	9.67	8.67	-

The results in table 3.4 revealed that fraction 3 of dichloromethane crude extract showed the most inhibition at the lowest concentration of IC_{50} at 110 ppm, followed by fraction 4 at 675 ppm, respectively.

The concentration of IC_{50} for fractions 1 and 2 were not found because of they did not inhibit spore of fungal on TLC plate. The percent inhibition was transformed into Probits, and the graph was traced for this Probit in relation to the logarithm of dichloromethane crude extract concentration. Linear regression representing the Probit of percent inhibition is shown in Fig. 3.6 presenting the graph for *Ascosphaera apis* after 3 days on TLC plate in a moisture chamber.

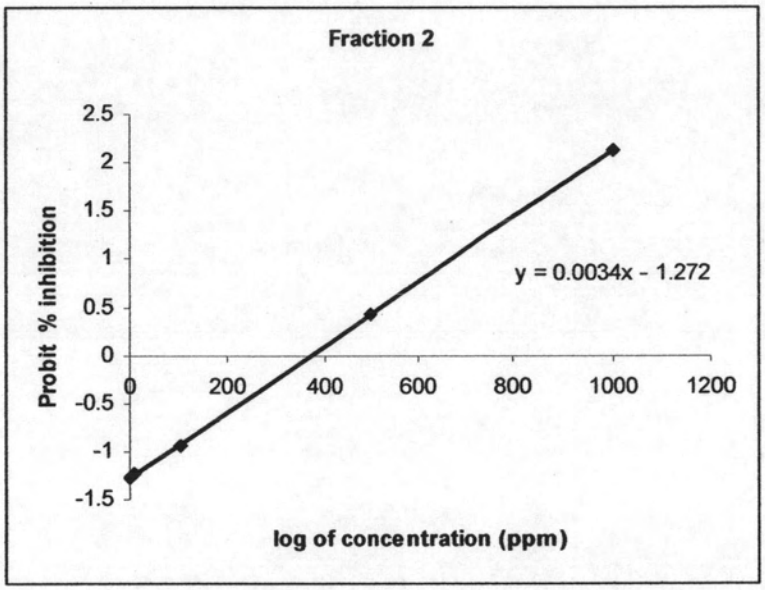


Fig. 3.6 Linear regression representing the Probit of percent inhibition in relation to the logarithm of concentrations of fraction 2 (ppm)

IC_{50} of fraction 3 was determined by measuring spore germinate inhibition. The result showed the concentration of IC_{50} at 110 ppm, it was the lowest IC_{50} to inhibit spores germination of fungi (Fig 3.5).The percent inhibition was transformed into Probits, and the graph was traced for this Probit in relation to the logarithm of concentration as shown in Fig. 3.7.

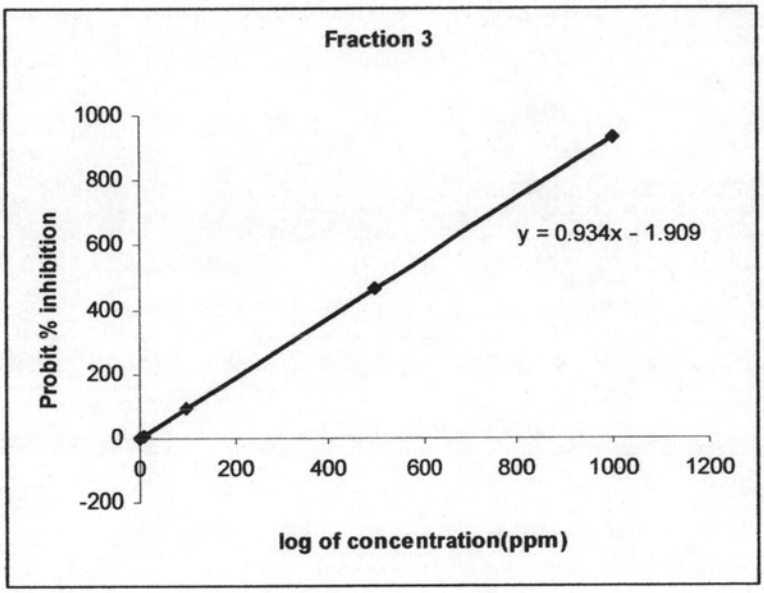


Fig. 3.7 Linear regression representing the Probit of percent inhibition in relation to the logarithm of concentrations of fraction 3 (ppm)

IC_{50} of fraction 4 was determined by measuring spore germinate inhibition. The result showed the concentration of IC_{50} at 675 ppm, it was the second lower IC_{50} to inhibit spores germination of fungi (Fig. 3.5). The percent inhibition was transformed into Probits, and the graph was traced for this probit in relation to the logarithm of concentration as shown in Fig. 3.8.

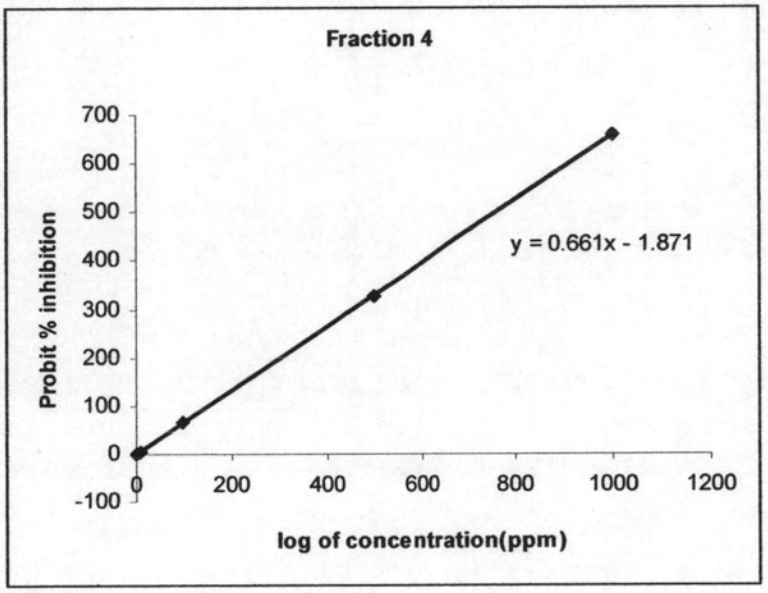


Fig. 3.8 Linear regression representing the Probit of percent inhibition in relation to the logarithm of concentrations of fraction 4 (ppm)

IC_{50} of fraction 5 was determined by measuring spore germination inhibition. The result showed very high concentration of IC_{50} at 137,870 ppm. It was too high IC_{50} to inhibit spores germination of fungi. Fig. 3.5 showed that this fraction did not inhibit spore germination. The percent inhibition was transformed into Probits, and the graph was traced for this Probit in relation to the logarithm of concentration as shown in Fig. 3.9.

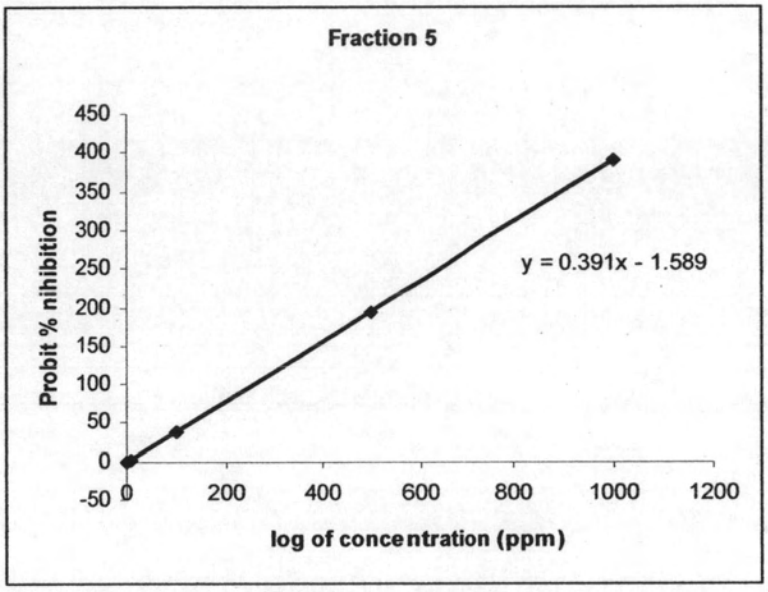


Fig. 3.9 Linear regression representing the Probit of percent inhibition in relation to the logarithm of concentrations of fraction 5 (ppm)

IC_{50} of fraction 6 was determined by measuring spore germinate inhibition. The result showed very high concentration of IC_{50} at 12,346 ppm. Moreover the result in Fig. 3.5 indicated that this fraction did not show inhibition of spore germination. The percent inhibition was transformed into Probits, and the graph was traced for this Probit in relation to the logarithm of concentration as shown in Fig. 3.10.

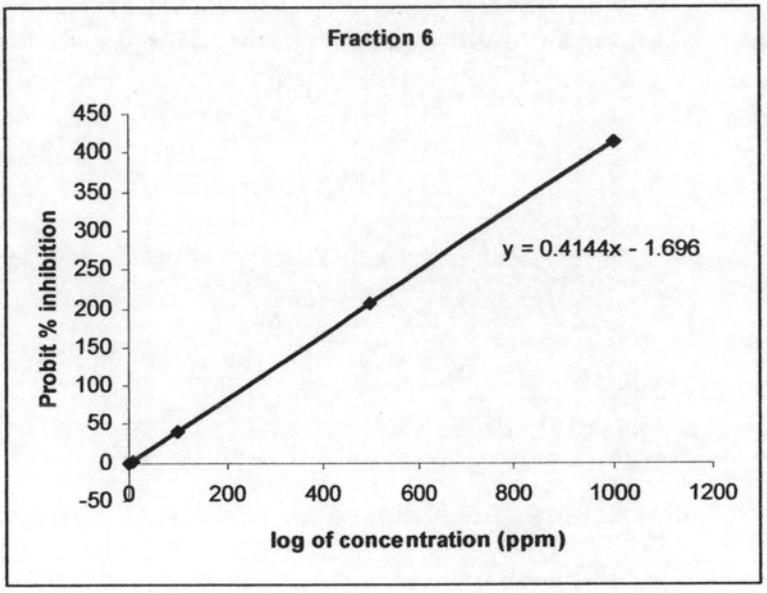


Fig. 3.10 Linear regression representing the Probit of percent inhibition in relation to the logarithm of concentrations of fraction 6 (ppm)

IC_{50} of fraction 7 was determined by measuring spore germinate inhibition. The result did not show concentration of IC_{50}, as it did not show inhibition of spore germination in Fig 3.5. The percent inhibition was transformed into probit, and the graph was traced for this probit in relation to the logarithm of concentration as shown in Fig. 3.11.

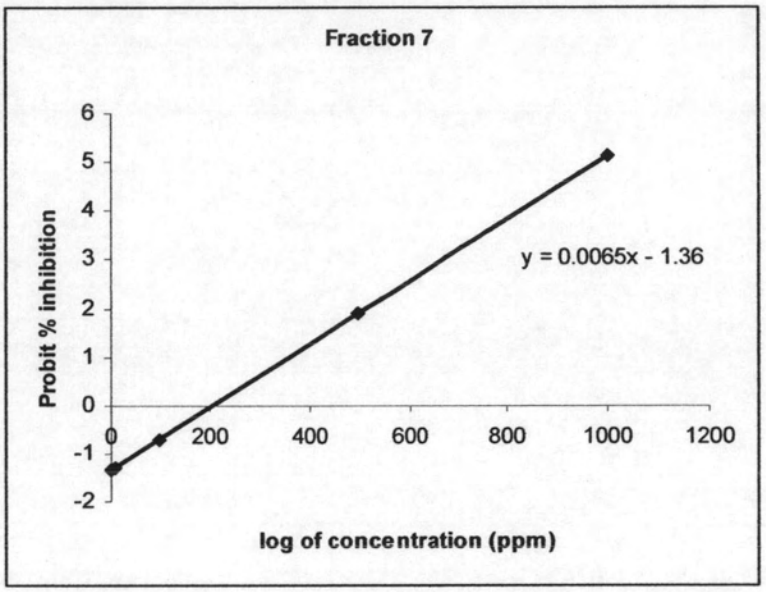
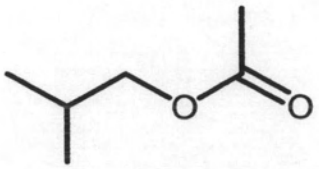


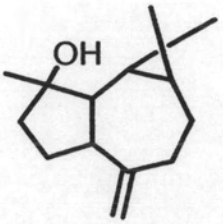
Fig. 3.11 Linear regression representing the Probit of percent inhibition in relation to the logarithm of concentrations of fraction 7 (ppm)

3.3 Structural elucidation of isolated compounds.

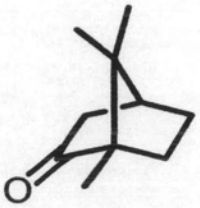
After fractionation and evaluation the antifungal activity, Fractions 3 and 4 were biologically active. GC-MS suggested the possible components including as camphor, 6- oxohuperzine A, 2,6-diphenyl-1,7-dihydrodipyrrolo[2,3-b:3',2'-E] pyridine and 2, 4 – bis (dimethylbenzyl) - 6 - *t*- butylphenol of Fraction 3, 2-methylpropyl ester, 1H-cycloprop[e]azulen-7-ol of Fraction 4. The structures of compounds are in Fig. 3.12.



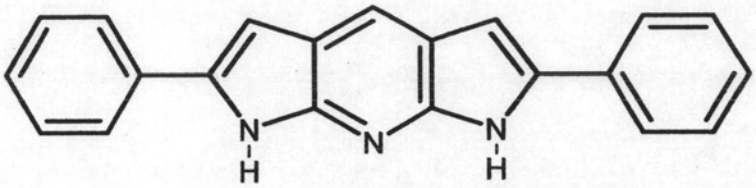
2-methylpropyl ester



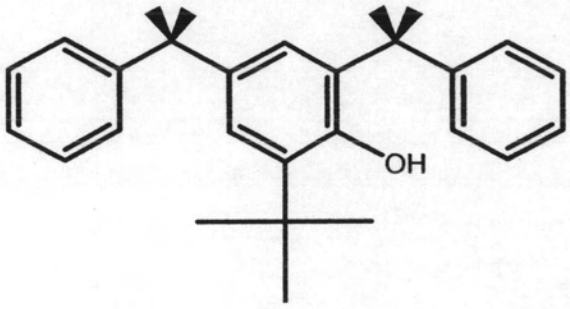
1H-Cycloprop[e]azulen-7-ol



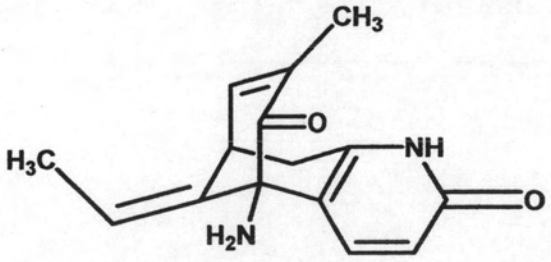
Camphor



2,6-Diphenyl-1,7-dihydrodipyrrolo [2,3-b:3',2'-E] pyridine



2, 4 – Bis (dimethylbenzyl) - 6 - t- butylphenol



6-oxohuperzine

Fig. 3.12 Structures of compounds analyzed from Fractions 3 and 4 propolis of the nest of *Trigona laeviceps* by GC-MS.

The propolis of stingless bee *Trigona laeviceps* has received considerable attention, with several reports of its analysis but how it is carried by workers, what is the fresh composition of the resin, The comparison of resin composition between nest of stingless bee *Trigona laeviceps* and other species in Thailand have not been studied.

Patricio, *et al.* 2002 found that only mature foragers carried resin on their legs and only on the third pair of tibia in each of the three species. Analysis showed it to be a complex mixture of constituents of plant resins. Each species of stingless bee had different groups of constituent. Although each species has a different home range, at the time of collection they were housed at the same place. It is interesting therefore that they were foraging on different plants and were collecting different resins. It had important biologically active chemical constituents of propolis from different geographic locations and the corresponding plant sources (Biesmeijer and Toth, 1998). This thesis chose the nest of *Trigona laeviceps* species to study because, it is the most common species in Thailand. Its nesting site is usually found in a cavity of human architecture and it can build the nest entrance in short time. We can find and collect propolis from the nest of *Trigona laeviceps* species easier than other species.

Moreover, propolis from nest of *Trigona laeviceps* had biologically active chemical constituents, which did not take a risk with human and environments, to inhibit *Ascosphaera apis*.