## CHAPTER III

## RESULTS AND DISCUSSION

Nine species of Thai plants were selected for preliminary screening test against *Galleria mellonella* Linn. antifeedant activity. They are eight species of Anonaceae, Euphorbiaceae, Gramineae, Leguminosae, Rutaceae, Sterculiaceae and Zingiberaceae.

### 3.1 The Results of Extraction

The air-dried samples were milled to coarse powder and extracted with 95% ethanol, according to the procedure described in Chapter II. The results of extraction are summarized as shown in Table 3.1.

**Table 3.1** The results of extraction

| Family | Plant | Plant part | Weight of plant (g) | Ethanolic crude extract (g) (% wt by wt) |
|---|---|---|---|---|
| Anonaceae | *Anona squamosa* Linn. | leaves | 346 | 39.49 (11.41) |
| Euphorbiaceae | *Trigonostemon reidioides* (Kurz)Craib | root | 858 | 42.41 (4.94) |
| Gramineae | *Cymbopogon nardus* Rendle | whole plant | 803 | 39.14 (4.87) |
| Leguminosae | *Derris scandens* Benth | whole plant | 998 | 45.53 (4.56) |

**Table 3.1** (cont.)

| Family | Plant | Plant part | Weight of plant (g) | Ethanolic crude extract (g) (% wt by wt) |
|---|---|---|---|---|
| Meliaceae | *Aglaia odorata* Lour | leaves | 1,000 | 14.01 (1.40) |
| | *Azadirachta indica* | branch | 855 | 57.22 (6.69) |
| | var.*siamensis* Valeton | stem | 918 | 25.68 (2.79) |
| Rutaceae | *Murraya paniculata* Jack | leaves | 265 | 55.16 (20.81) |
| Sterculiaceae | *Mansonia gagei* Drumm. | heartwood | 1,004 | 72.26 (7.19) |
| Zingiberaceae | *Zingiber cassumunar* Roxb. | rhizome | 268 | 15.95 (5.95) |

### 3.2 Preliminary Antifeedant Bioassay Results

Each crude extract was preliminarily screened for antifeedant activity against *Galleria mellonella* Linn. (3 rd instar) according to the procedure described in Chapter II. The bioassay results are presented in Table 3.2.

**Table 3.2** The results of the preliminary screening of the crude extract at 0.25% wt by wt against the greater wax moth *G. mellonella* Linn. larvae (3 rd instar)

| Family | Plant | Plant part | Solvent | Antifeedant activity |
|---|---|---|---|---|
| Acanthaceae | *Rhinacanthus communis* Nees* | whole plant | ethanol | ++ |
| Anonaceae | *Anona squamosa* Linn. | leaves | ethanol | ++ |
| Compositae | *Eupatorium odoratum* Linn.* | whole plant | ethanol | ++ |
| | *Sphaeranthus africanus* Linn.* | whole plant | ethanol | +++ |
| Euphorbiaceae | *Euphorbia hirta* Linn.* | whole plant | ethanol | ++ |
| | *Euphorbia hypericifolia* Linn.* | whole plant | ethanol | ++ |
| Gramineae | *Trigonostemon reidioides* (Kurz) Craib | root | ethanol | +++ |
| | *Cymbopogon nardus* Rendle | whole plant | ethanol | ++ |
| | *Imperata cylindrica* Beauv* | root | ethanol | + |
| | | stem | ethanol | +++ |
| Leguminosae | *Derris scandens* Benth | whole plant | ethanol | ++ |
| Meliaceae | *Aglaia odorata* Lour | whole plant | ethanol | ++ |
| | *Azadirachta indica* | branch | ethanol | + |
| | var.*siamensis* Valeton | stem | ethanol | ++ |
| Myrtaceae | *Eugenia caryophyllus* Bullock & Harrison* | flower | ethanol | +++ |
| Rubiaceae | *Litosanthes biflora* Blume* | whole plant | ethanol | - |
| Rutaceae | *Murraya paniculata* Jack | leaves | ethanol | + |
| Sterculiaceae | *Mansonia gagei* Drumm. | heartwood | ethanol | - |
| Zingiberaceae | *Zingiber cassumunar* Roxb. | rhizome | ethanol | +++ |

Note : +++ high antifeedant activity (71 - 100%)
++ medium antifeedant activity (41 - 70%)
+ low antifeedant activity (11 - 40%)
- no antifeedant activity (0 - 10%)

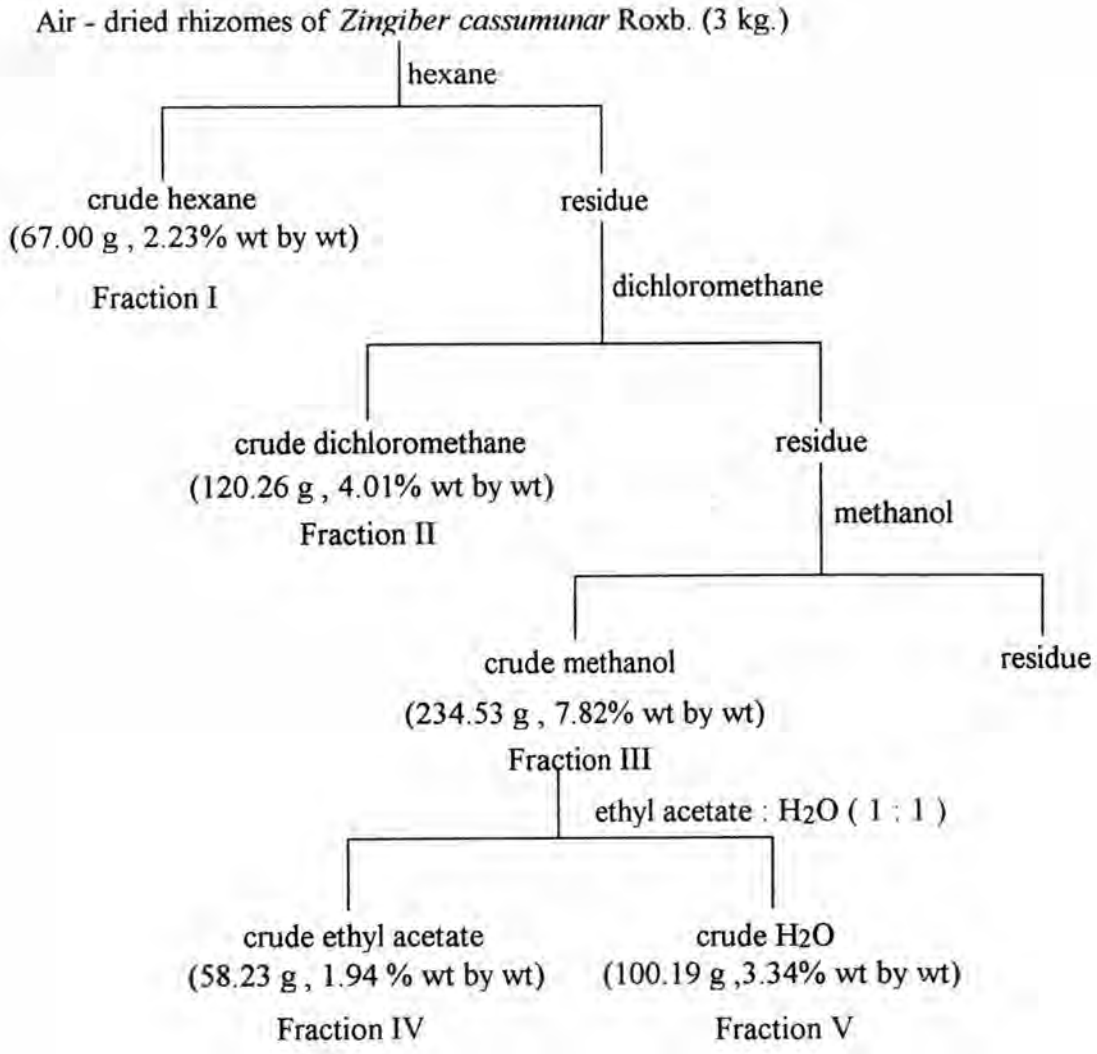
*These crude extracts were obtained from Natural Products Research Unit, Department of Chemistry, Chulalongkorn University.

The ethanolic crude extracts of *E. caryophyllus* , *I. cylindrica* , *S. africanus*, *T. reidiodes* and *Z. cassumunar* showed high antifeedant activity against *G. mellonella* larvae. Among those plants which gave attractive preliminary results, the rhizomes of *Z. cassumunar* were selected for further investigation with the aim to search for insect antifeedant compounds.

## Searching for Insect Antifeedant from *Zingiber cassumunar* Roxb.

### 3.3 The Results of Extraction and Initial Fractionation of the Rhizomes of *Z. cassumunar* Roxb.

The rhizomes of *Z. cassumunar* Roxb. were extracted following the procedure described in Chapter II. The results of extraction and initial fractionation can be summarized as shown in Scheme 3.1.




**Scheme 3.1** The procedure and results of extraction of the rhizomes of *Z. cassumunar* Roxb.

### 3.4 The Results of *Galleria mellonella* Linn. Antifeedant Activity Test

Each crude extract of the rhizomes of *Z. cassumunar* was preliminarily bioassayed for antifeedant activity against the third star stage of *G. mellonella* according to the procedure described in Chapter II. The results are tabulated in Table 3.3 and Fig. 3.1.

**Table 3.3** The results of antifeedant activity against *G. mellonella* Linn.

| Fraction (Solvent extract) | % Antifeedant | Activity* |
|---|---|---|
| I (hexane) | 53.49 | medium |
| II (dichloromethane) | 20.05 | low |
| III (methanol) | 23.23 | low |
| IV (ethyl acetate) | 81.40 | high |
| V (water) | 0.00 | no |

*see note page 37

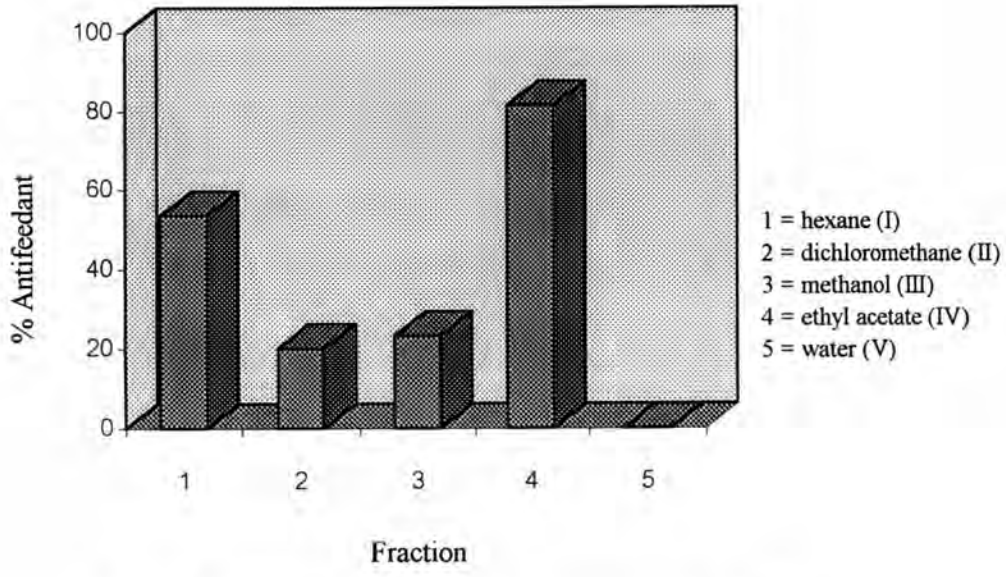



**Fig. 3.1** Antifeedant activity of *Z. cassumunar* Roxb.

### 3.5 Separation

#### 3.5.1 Separation of Fraction I

The hexane crude extract (Fraction I), 65 g as yellow oil was subjected to silica gel column using silica gel 650 g as an adsorbent. The column was initially eluted with *n*-hexane and changed to dichloromethane by gradual introduction of the latter. Finally, the column was stripped with methanol. The eluted solution was collected approximately 250 mL for each fraction. Each portion was concentrated to a small volume and monitored by TLC. The fractions that showed similar components were combined. The results of separation of Fraction I are shown in Table 3.4.

**Table 3.4** The results of the separation of Fraction I

| Eluents | Fraction No. | Remarks | Weight (g) |
|---|---|---|---|
| hexane | 1-17 (IA) | yellow oil | 0.37 |
| 5-40% $CH_2Cl_2$ in hexane | 18-39 (IB) | white oil and white solid | 0.64 |
| 40% $CH_2Cl_2$ in hexane | 40-43 (IC) | pale yellow oil | 2.90 |
| 40-60% $CH_2Cl_2$ in hexane | 44-68 (ID) | pale yellow oil | 7.71 |
| 80% $CH_2Cl_2$ in hexane | 69-74 (IE) | yellow oil | 0.26 |
| 80% $CH_2Cl_2$ in hexane | 75-92 (IF) | pale yellow semisolid | 14.50 |
| 100% $CH_2Cl_2$ | 93-97 (IG) | viscous yellow liquid | 8.41 |
| 100% $CH_2Cl_2$ - 2% MeOH in $CH_2Cl_2$ | 98-108 (IH) | viscous yellow liquid | 9.68 |
| 5%MeOH in $CH_2Cl_2$ | 109-110 (II) | viscous yellow liquid | 6.19 |
| 20% MeOH in $CH_2Cl_2$ | 111-116 (IJ) | viscous yellow liquid | 14.82 |

Each small fraction derived from the separation of Fraction I was further subjected to antifeedant bioassay experiments at dose level 0.25% wt by wt. The antifeedant activity results are reported as shown in Table 3.5 and Fig. 3.2.

**Table 3.5** The results of antifeedant activity of *G. mellonella* of Fraction IA-IJ

| Fraction | %Antifeedant |
|---|---|
| IA | 47.91 |
| IB | 5.75 |
| IC | 38.35 |
| ID | 40.15 |
| IE | 49.02 |
| IF | 66.59 |
| IG | 54.11 |
| IH | 32.67 |
| II | 7.30 |
| IJ | 25.00 |

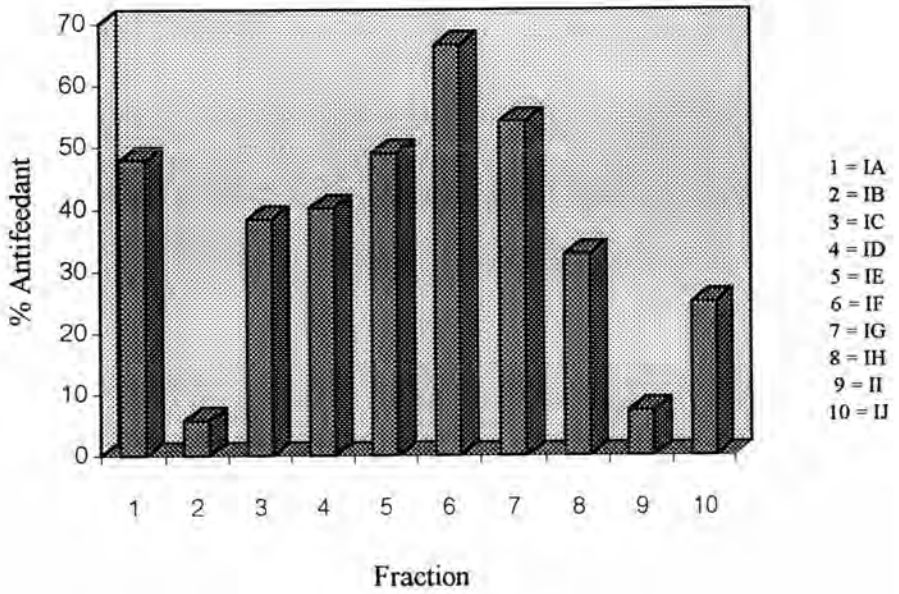



**Fig 3.2** Antifeedant activity of Fraction I

### 3.5.2 Separation of Fraction IF

According to the antifeedant results (Table 3.5, Fig. 3.2), Fraction IF, 11.46 g was reseparated by using silica gel column chromatography. A mixture of dichloromethane and hexane, dichloromethane and a mixture of dichloromethane and methanol were used as eluents. About 100 mL of eluent was collected for each fraction and then concentrated to about 30 mL. Each fraction was monitored by TLC. The results of the separation of Fraction IF are revealed in Table 3.6.

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

| Eluents | Fraction No. | Remarks | Weight (g) |
|---|---|---|---|
| 50-75% $CH_2Cl_2$ in hexane | 1-5 (IFA) | pale yellow wax in yellow oil | 0.87 |
| 75% $CH_2Cl_2$ in hexane | 6 (IFB) | yellow liquid | 1.46 |
| 75% $CH_2Cl_2$ in hexane | 7-11 (IFC) | pale yellow semisolid | 4.09 |
| 75-80% $CH_2Cl_2$ in hexane | 12-21 (IFD) | yellow liquid | 3.27 |
| 80-100% $CH_2Cl_2$ in hexane | 22-29 (IFE) | viscous yellow liquid | 0.95 |
| 20% MeOH in $CH_2Cl_2$ | 30 (IFF) | viscous dark brown liquid | 2.20 |

In order to follow the antifeedant activity, each fractionated portion was resubjected to the bioassay experiments. The results of the antifeedant bioassay of each fraction derived from the separation of Fraction IF are recorded in Table 3.7 and Fig. 3.3.

**Table 3.7** The results of antifeedant activity against *G. mellonella* of each fraction derived from the separation of Fraction IF

| Fraction | %Antifeedant |
|---|---|
| IFA | 55.15 |
| IFB | 0.00 |
| IFC | 92.00 |
| IFD | 36.41 |
| IFE | 39.12 |
| IFF | 43.66 |

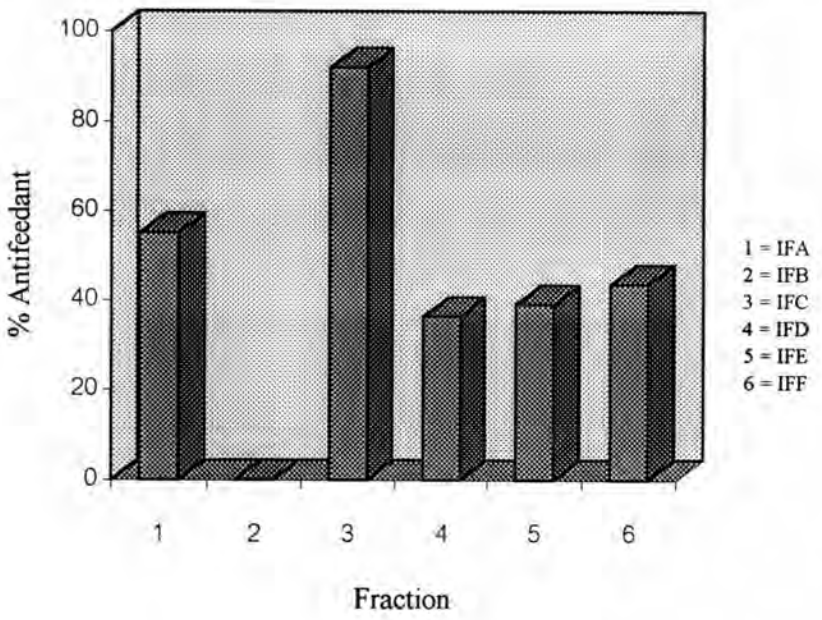



**Fig. 3.3** Antifeedant activity of Fraction IF

It was very clear that Fraction IFC revealed attractive antifeedant results. The TLC of this fraction showed a long tail spot using $CH_2Cl_2$ as a solvent system. Thus, this fraction (4.09 g) was reseparated by silica gel column chromatography using 75% dichloromethane in hexane as an eluent. Other general procedure was carried out as aforementioned. The results of the separation of Fraction IFC are tabulated in Table 3.8.

**Table 3.8** The results of separation of Fraction IFC

| Eluents | Fraction No. | Remarks | Weight (g) |
|---|---|---|---|
| 75% $CH_2Cl_2$ in hexane | 1-2 (IFCA) | pale yellow liquid | 0.45 |
| | 3-7 (IFCB) | pale yellow liquid | 1.07 |
| | 8-20 (IFCC) | pale yellow semisolid and white needle | 1.66 |
| | 21-24 (IFCD) | pale yellow semisolid | 0.26 |
| | 25-28 (IFCE) | pale yellow liquid | 0.34 |
| | 29-60 (IFCF) | pale yellow liquid | 0.12 |

In order to follow the antifeedant activity, each fractionated portion was resubjected to the bioassay experiments. The results of the antifeedant bioassay of each fraction derived from the separation of fraction IFC are recorded in Table 3.9 and Fig. 3.4.

**Table 3.9** The results of antifeedant activity of *G. mellonella* of each fraction derived from the separation of Fraction IFC

| Fraction | %Antifeedant |
|---|---|
| IFCA | 39.40 |
| IFCB | 42.90 |
| IFCC | 92.05 |
| IFCD | 33.43 |
| IFCE | 43.73 |
| IFCF | 50.21 |

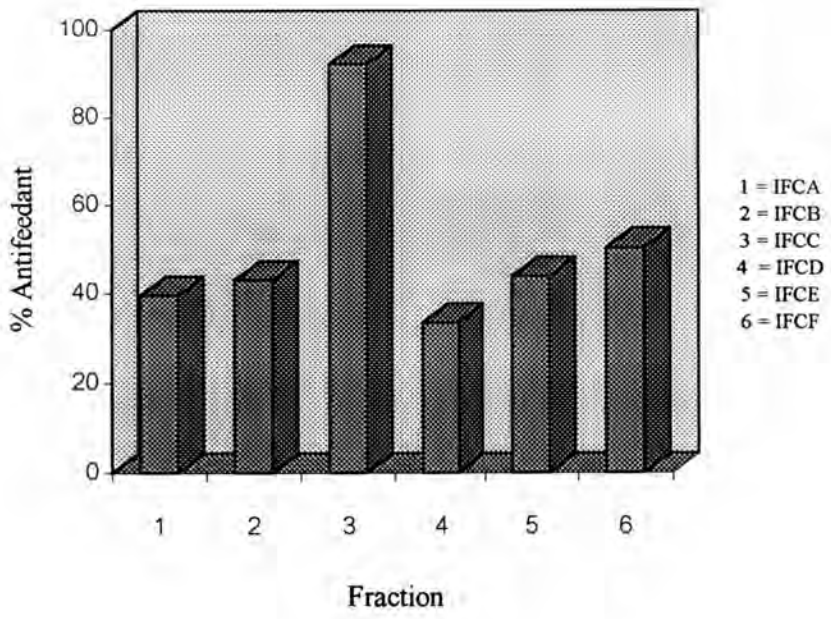



**Fig. 3.4** Antifeedant activity of Fraction IFC

It was obvious that Fraction IFCC showed high antifeedant activity. TLC of this fraction also showed a spot with long tail using $CH_2Cl_2$ as a solvent system. This fraction was further analyzed by the aids of GC-MS. The results of GC-MS analysis are shown in Fig. 3.5.-3.11. A capillary column DB5 was used and the analytical conditions employed were as follows: programmed temperature 60 °C, 1 min then 60-200 °C (10 °C/min), injector temperature 200 °C, ion source temperature 200 °C, ionization voltage 70 eV.

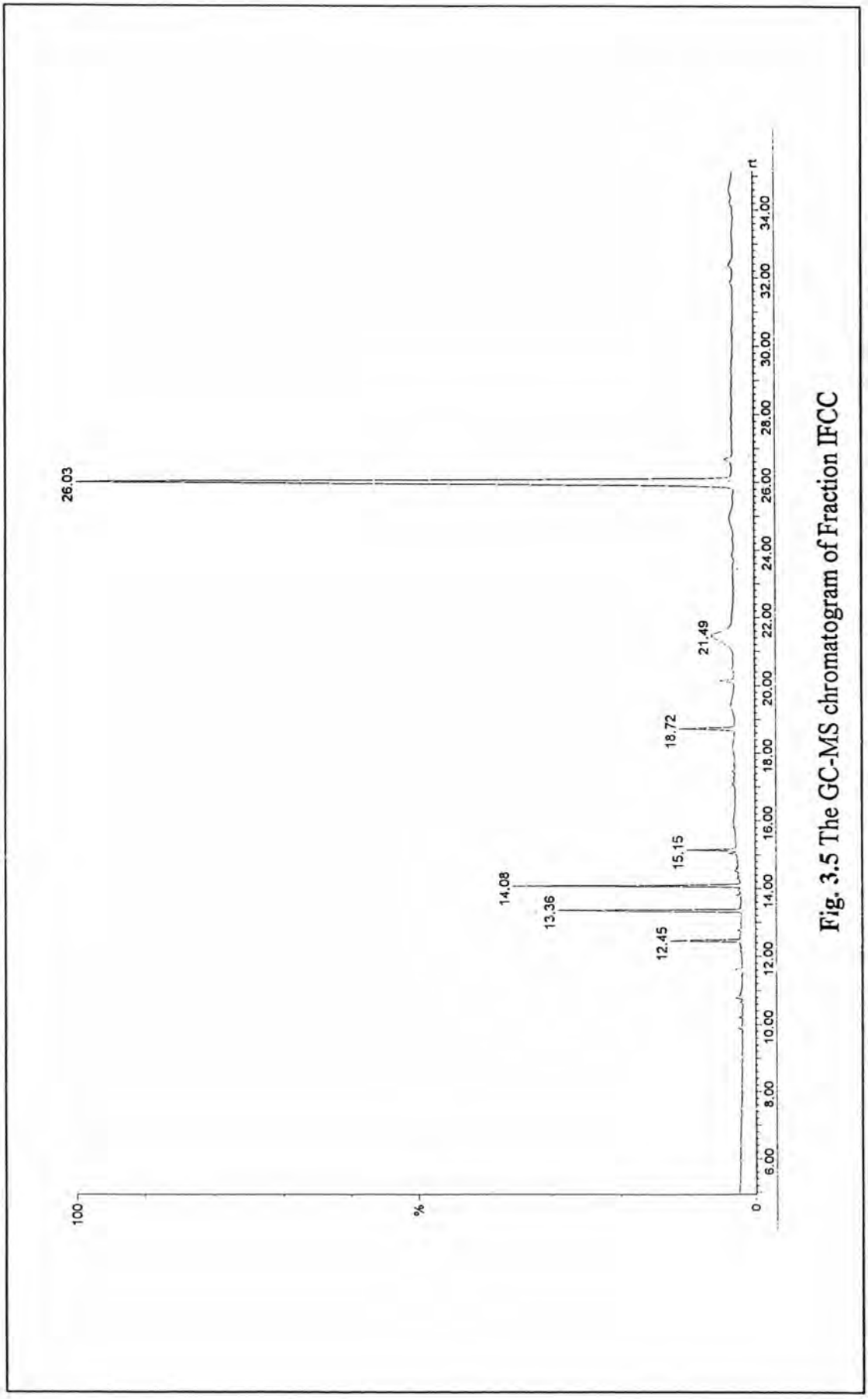



**Fig. 3.5** The GC-MS chromatogram of Fraction IFCC

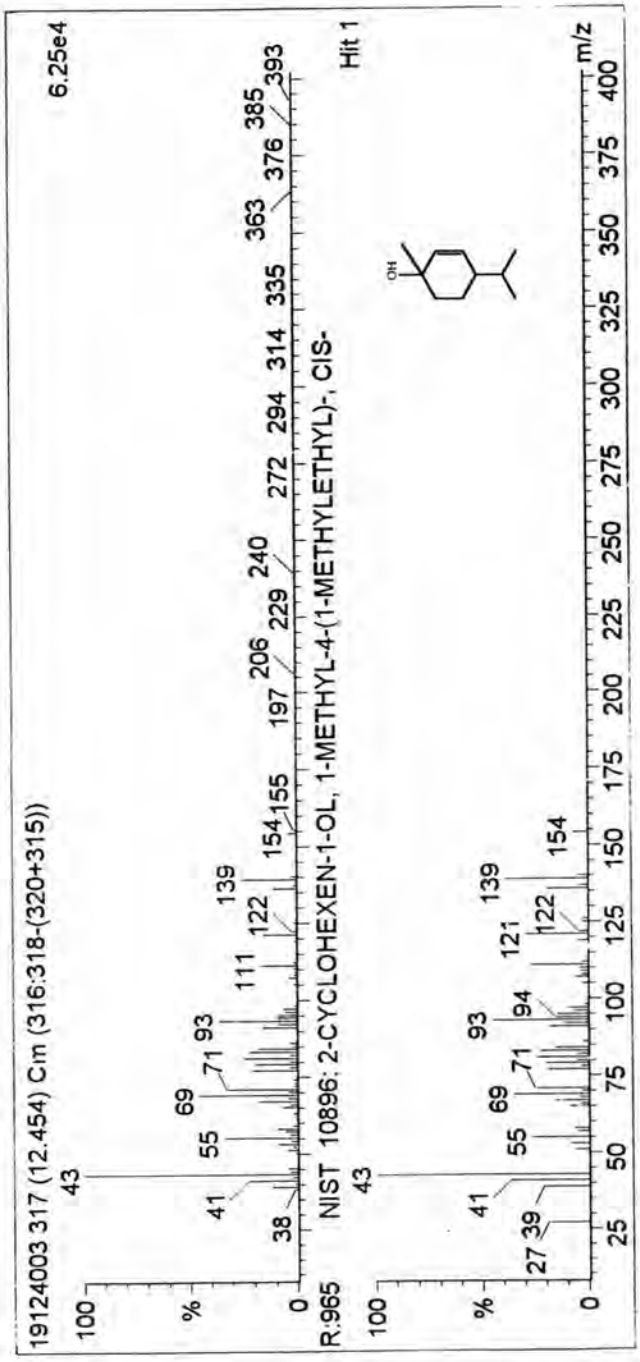



**Fig. 3.6** The mass spectrum of Fraction IFCC at retention time 12.45 min

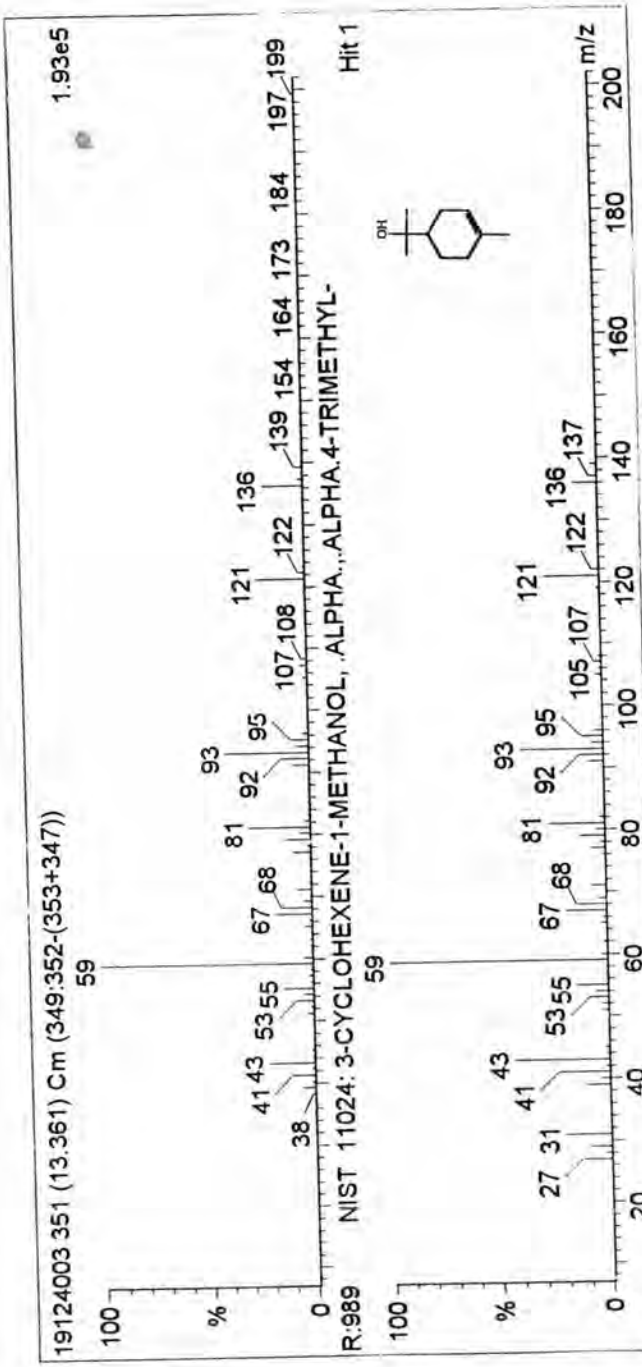



**Fig. 3.7** The mass spectrum of Fraction IFCC at retention time 13.36 min

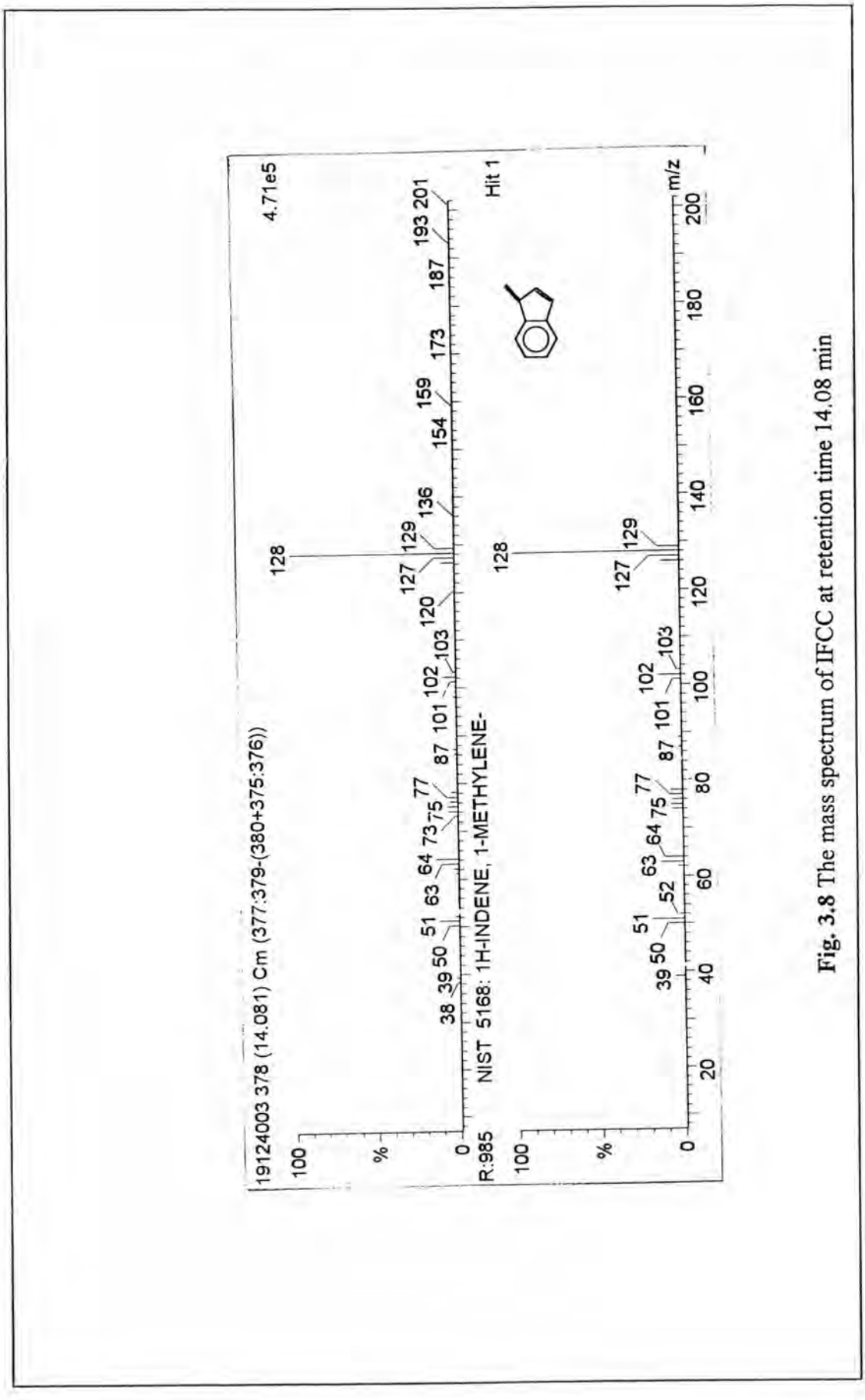



**Fig. 3.8** The mass spectrum of IFCC at retention time 14.08 min

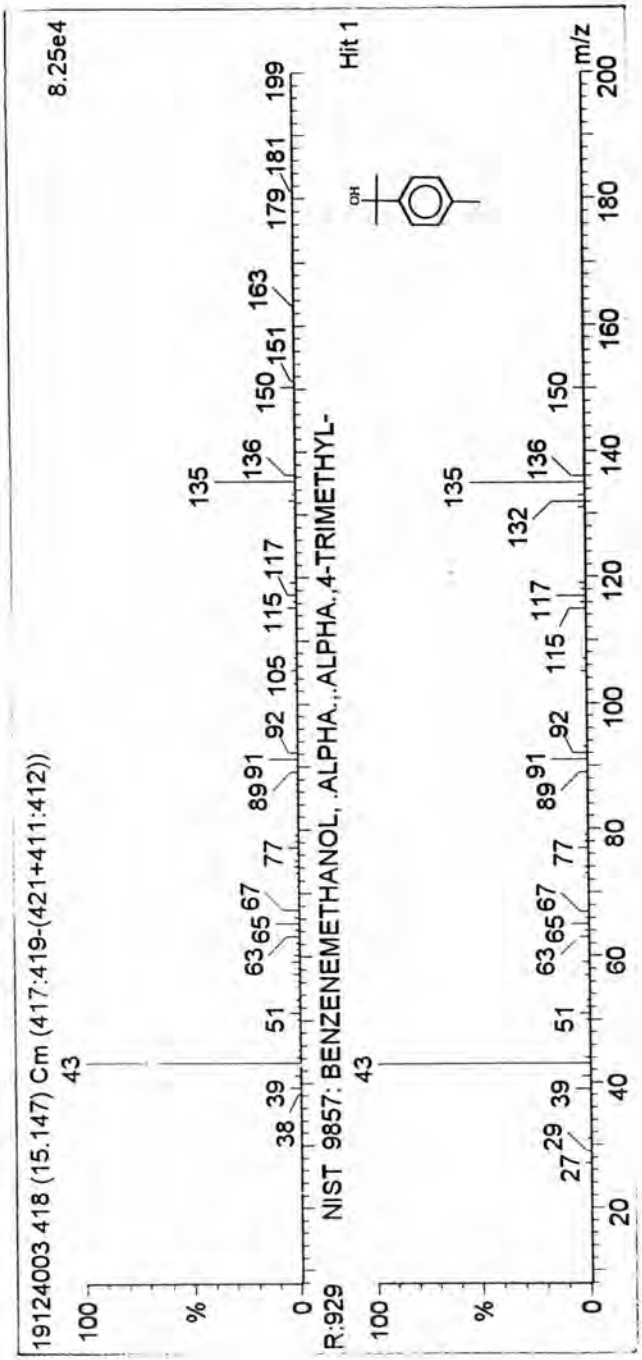



**Fig. 3.9** The mass spectrum of Fraction IFCC at retention time 15.15 min

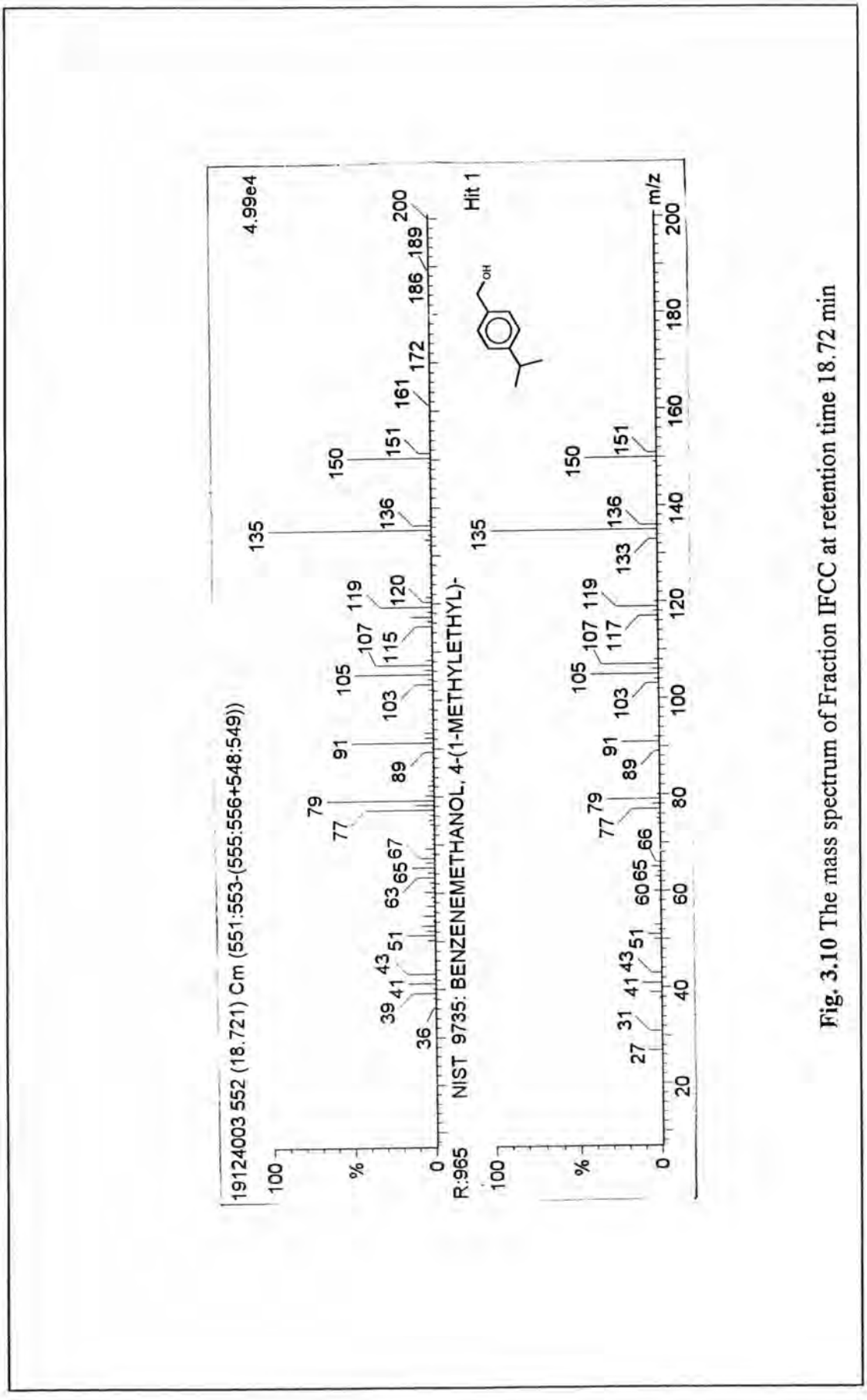



**Fig. 3.10** The mass spectrum of Fraction IFCC at retention time 18.72 min

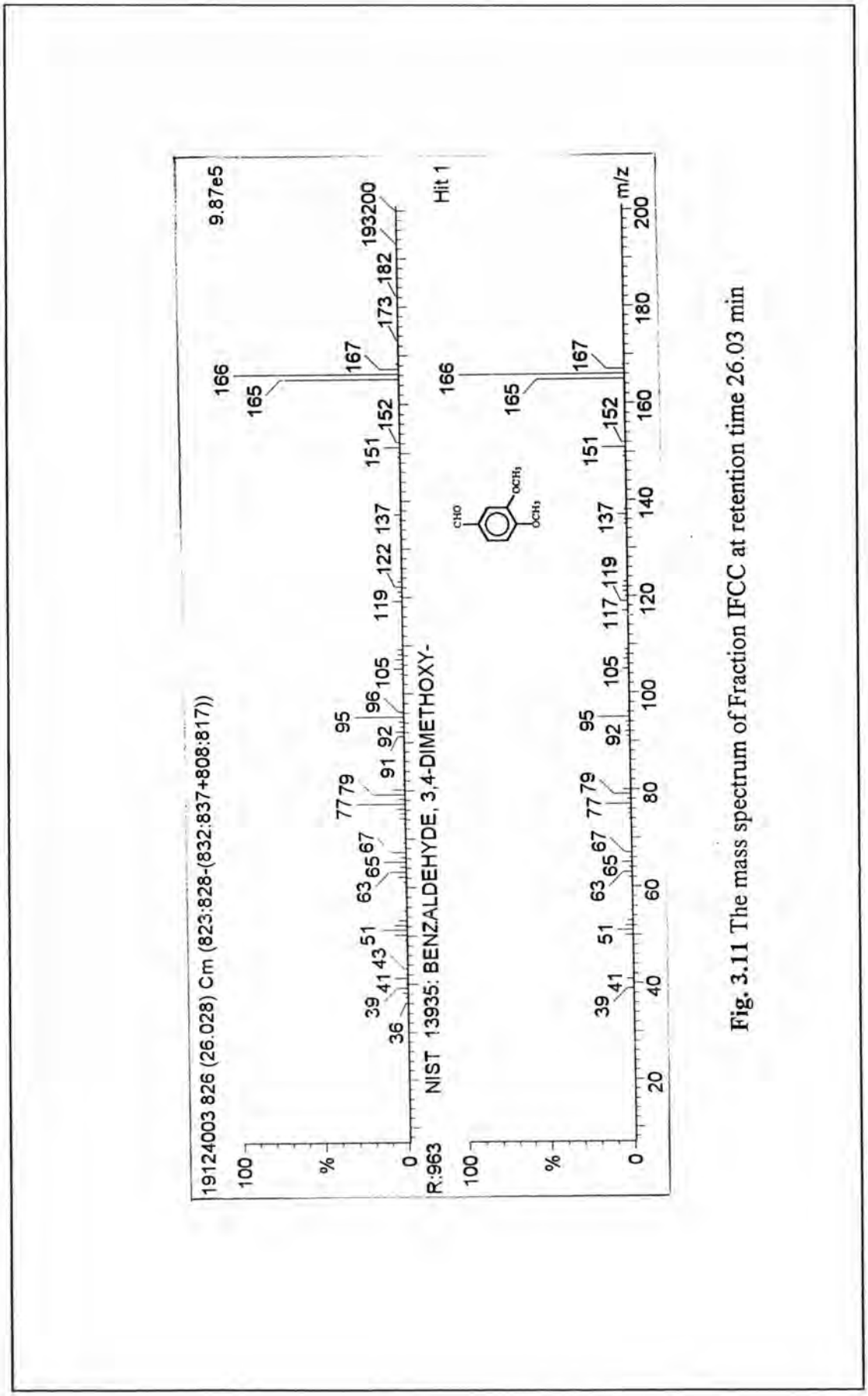
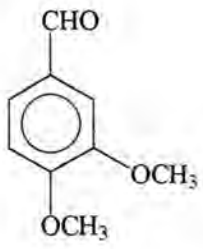
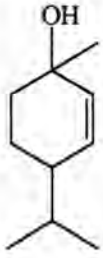



**Fig. 3.11** The mass spectrum of Fraction IFCC at retention time 26.03 min

The gas chromatogram (Fig. 3.5) clearly showed that there were six major components in this mixture. The main component appeared at 26.03 minute was suggested to be 3,4-dimethoxybenzaldehyde (veratraldehyde) **(I)** according to the matching of mass fragmentation pattern obtained from the GC-MS library NIST. Others occurred at 12.45, 13.36, 14.08, 15.15 and 18.72 minutes might be 1-methyl-4-(1-methylethyl)-2-cyclohexen-1-ol **(II)**, α,α-4-trimetryl-3-cyclohexane-1-methanol **(III)**, 1-methlene-1 H- indene **(IV)**, α,α-trimethylbenzenemethanol **(V)** and 4-(1-methylethyl)benzenemethanol **(VI)**, respectively. The composition and component present in this fraction are tabulated in Table 3.10.

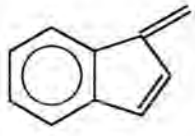



**(I)**



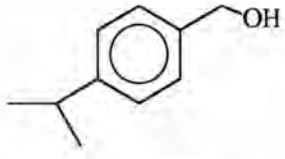

**(II)**


**(III)**



**(IV)**


**(V)**




**(VI)**

**Table 3.10** The possible composition in Fraction IFCC analyzed by GC-MS

| Possible compound | Structure formula | Retention time (min) | % Composition |
|---|---|---|---|
| 3,4-dimethoxybenzaldehyde **(I)** | $C_9H_{10}O_3$ | 26.03 | 77.60 |
| 1-methyl-4-(1-methylethyl-2-cyclohexen-1-ol **(II)** | $C_{10}H_{14}O$ | 12.45 | 2.77 |
| α,α-4-trimethyl-3-cyclohexane-1-methanol **(III)** | $C_{10}H_{14}O$ | 13.36 | 6.93 |
| 1-methylene-1H-indene **(IV)** | $C_{10}H_8$ | 14.08 | 8.77 |
| α,α- trimethylenzenemethanol **(V)** | $C_{10}H_{18}O$ | 15.15 | 1.85 |
| 4-(1-methylethyl)benzenemethanol **(VI)** | $C_{10}H_{18}O$ | 18.72 | 2.08 |

### 3.5.3 Separation of Fraction IFCC

To gain an idea from GC-MS analysis that the major component (more than 77%) present in this active fraction was veratraldehyde, an attempt to isolate this compound was carried out. Thus, Fraction IFCC 0.65 g was reseparated by using chromatotron and the results are present in Table 3.11.

**Table 3.11** The results of the separation of Fraction IFCC

| Eluents | Fraction No. | Remarks | Weight (g) |
|---|---|---|---|
| hexane | 1 | white liquid | 0.01 |
| 10-20 % EtOAc in hexane | 2 | yellow liquid | 0.08 |
| 30-40 % EtOAc in hexane | 3 | pale yellow solid | 0.03 |
| 50 % EtOAc in hexane | 4 | pale yellow solid (Compound **1**) | 0.35 |
| 60-80 % EtOAc in hexane | 5 | yellow liquid | 0.07 |
| 90% EtOAc in hexane | 6 | yellow liquid | 0.05 |

### 3.5.4 Structural Elucidation of Compound 1

Compound **1** as a pale yellow solid was separated from Fraction IFCC by chromatotron. This compound 0.35 g ($6.6 \times 10^{-4}$ % wt by wt of dried rhizomes) had a melting range of 42-44 °C, $R_f$ value 0.30 (dichloromethane). This compound gave the same $R_f$ value as authentic veratraldehyde. In addition, the Co-TLC of both compounds was also found to give the same $R_f$ values.

The IR spectrum of this compound as shown in Fig 3.12 revealed characteristic absorption peaks of an aldehyde moiety at 2840 (C-H stretching of aldehyde) and 1680 (C=O stretching), respectively. Other signals were tentatively assigned as shown in Table 3.12.

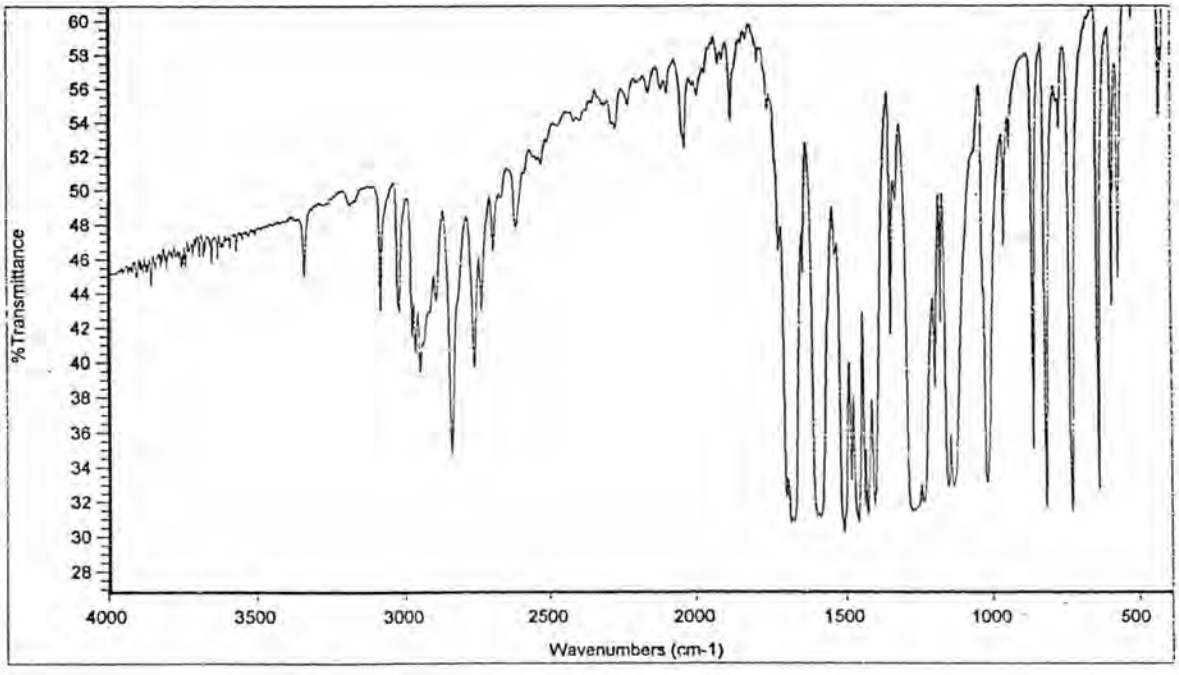



**Fig. 3.12** The IR spectrum of Compound **1**

**Table 3.12** The IR absorption band assignments of Compound 1

| wave number ($cm^{-1}$) | intensity | tentative assignment |
|---|---|---|
| 3080-3020 | weak | C-H stretching of aromatic |
| 2980-2890 | weak | C-H stretching; of $CH_3$- |
| 2840 | medium | C-H stretching of aldehyde |
| 1680 | strong | C=O stretching of aldehyde |
| 1590-1510 | strong | C=C stretching of aromatic |

The $^1H$ NMR spectrum of Compound **1** (Fig. 3.13) clearly showed 2 sets of methoxy protons (3H each) at 3.89 and 3.92 ppm. A signal around 6.93-7.41 ppm with 3H integration could be assigned for aromatic protons. The singlet signal at 9.80 ppm was no doubt to be an aldehyde proton.

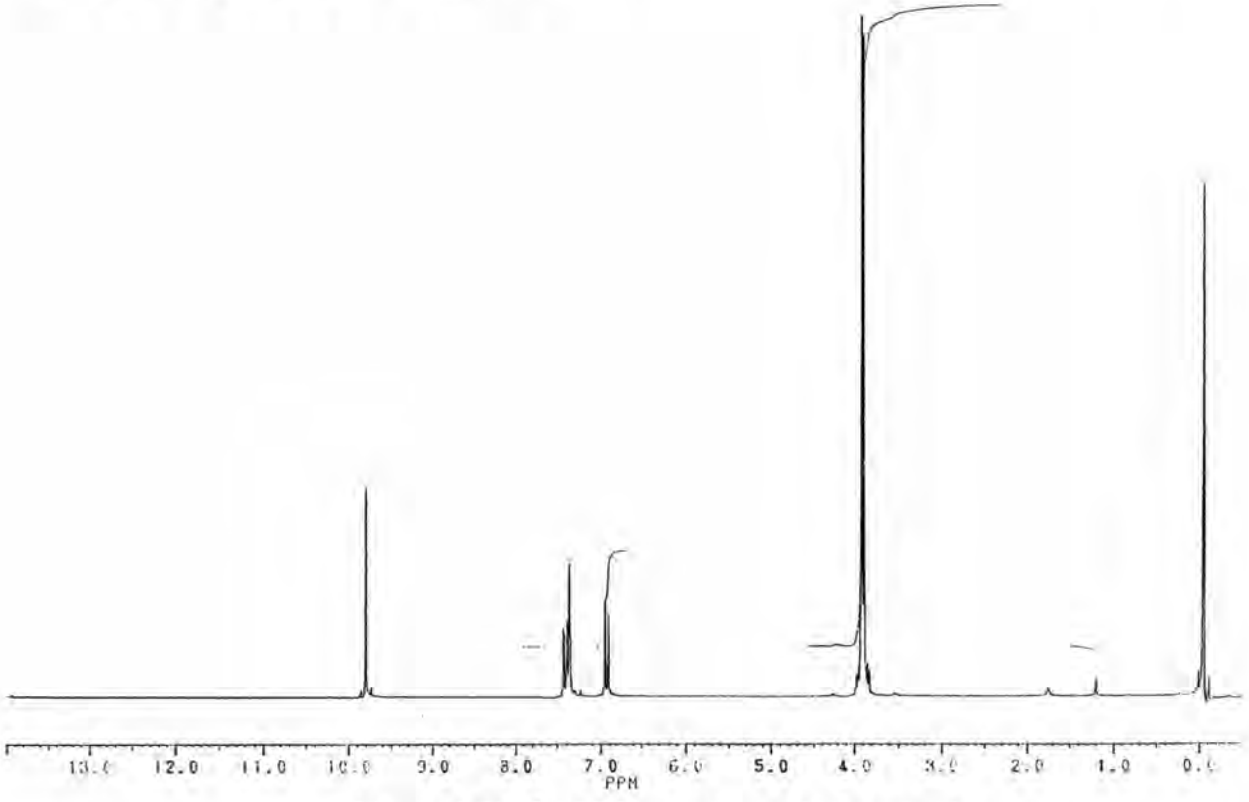



**Fig. 3.13** The $^1H$ NMR spectrum of Compound **1**

The $^{13}$C-NMR spectrum of Compound 1 (Fig. 3.14) exhibited 9 carbon signals. Two methoxy carbons were detected at 56.0 and 56.2 ppm. The aromatic carbons were found in the range of 109.0-154.5 ppm, while the aldehyde carbon was present at 190.9 ppm.

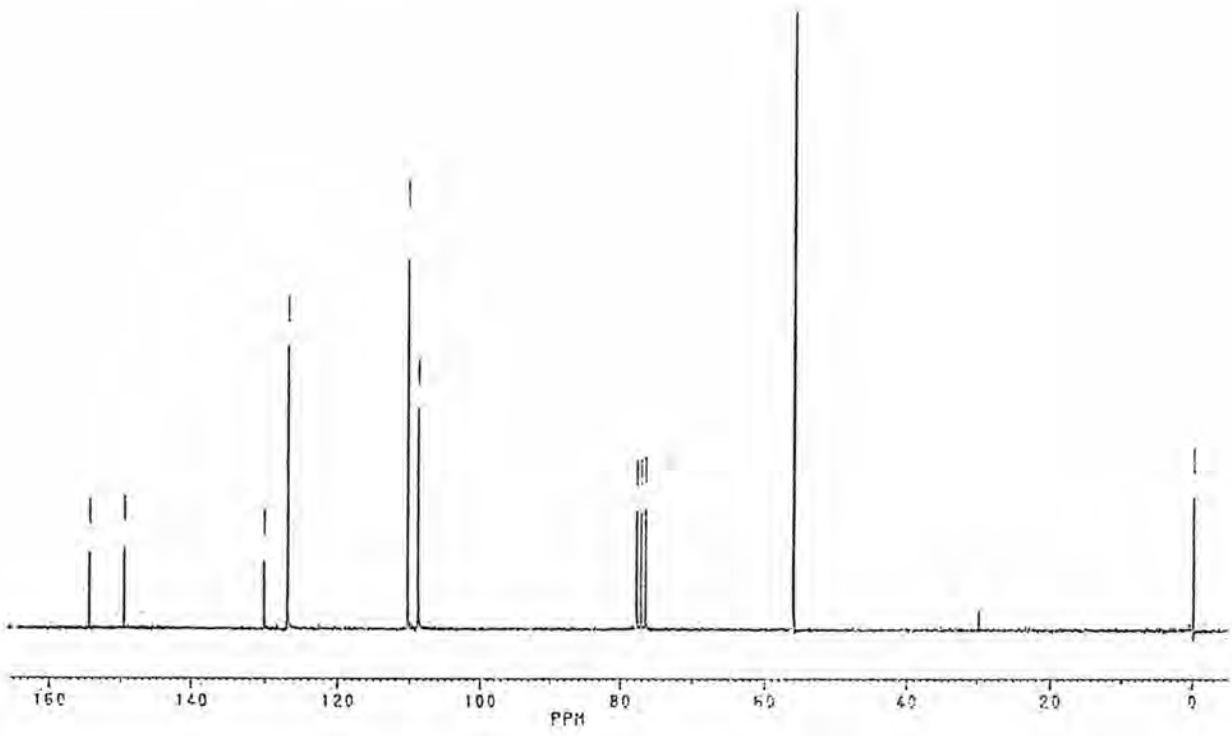


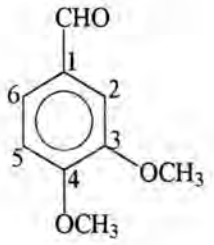

**Fig. 3.14** The $^{13}$C NMR spectrum of Compound 1

The tentative assignment of signals from the $^{1}$H- and $^{13}$C-NMR spectra could be summarized in Table 3.13.

**Table 3.13** The $^{1}H$- and $^{13}C$-NMR signal assignments of Compound **1**

| Position | Chemical Shift (ppm) | |
|---|---|---|
| | $^{1}H$ | $^{13}C$ |
| 1 | - | 130.2 |
| 2 | 7.36 | 110.5 |
| 3 | - | 149.7 |
| 4 | - | 154.5 |
| 5 | 6.93 | 109.0 |
| 6 | 7.41 | 126.8 |
| $-OCH_3$ | 3.89, 3.92 | 56.0, 56.2 |
| -CHO | 9.80 | 190.9 |

All spectroscopic evidence and by the comparison with an authentic specimen, Compound **1** had no doubt to be 3,4-dimethoxybenzaldehyde or veratraldehyde having the structure :




Compound **1** was then subjected to the antifeedant activity test. It was found that this compound showed an antifeedant activity around 53%. Another set of experiment was then performed to observe the effects of the amount of veratraldehyde on antifeedant activity. The results are recorded in Table 3.14 and Fig 3.15.

**Table 3.14** The results of antifeedant activity of veratraldehyde

| Concentration (% wt by wt) | % Antifeedant |
|---|---|
| 5.00 | 71.60 |
| 1.00 | 60.45 |
| 0.50 | 55.45 |
| 0.25 | 53.36 |

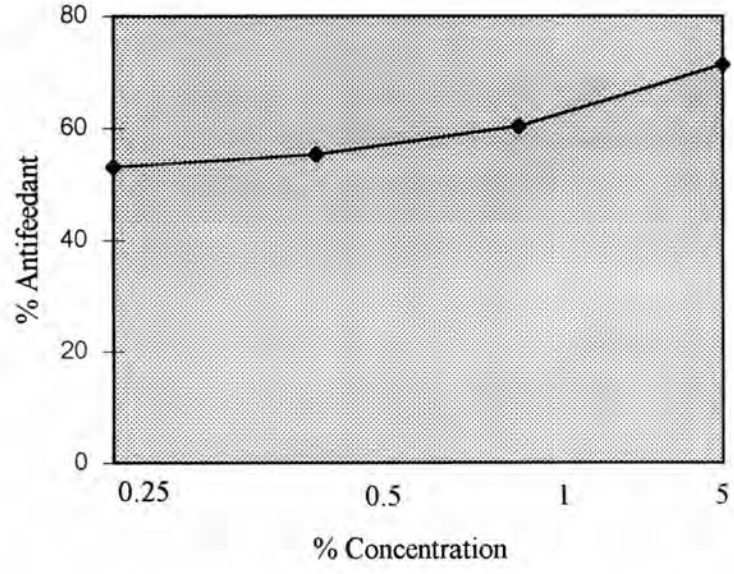



**Fig. 3.15** Antifeedant activity of veratraldehyde

**Statistical Analysis**

The statistical analysis of veratraldehyde is presented in Table 3.15.

**Table 3.15** Consumption of veratraldehyde in choice tests by third-instar larvae of greater wax moth

| Dosage (% wt by wt) | $\bar{X}$ consumption (SEM) of treated (g compounds / g larvae food)[a] | $\bar{X}$ consumption (SEM) of control (g compounds / g larvae food)[a] | $\bar{X}$ % antifeedant (SEM)[b] | *t* value | df |
|---|---|---|---|---|---|
| 5.00 | 0.0078 a | 0.0273 a | 53.36 a | 37.75 | 5 |
| 1.00 | 0.0101 b | 0.0256 a | 55.45 b | 15.82 | 5 |
| 0.50 | 0.0116 c | 0.0262 a | 59.94 c | 13.58 | 5 |
| 0.25 | 0.0127 d | 0.0273 a | 71.59 d | 13.14 | 5 |

[a] Mean in each column followed significantly different by the same letter are not significantly different ($P$= 0.05 ; Fisher's least significant difference (LSD)

[b] Mean in each column followed significantly different by the same letter are not significantly ($P$= 0.05 ; as determined by *t* test)

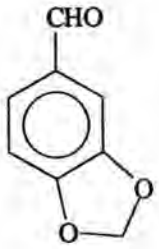
From Table 3.15, it was found that the weight loss average in a treated bowl at dose level 5, 1, 0.5 and 0.25 % wt by wt within 48 hr was of significantly statistical difference at 95 %, whereas the average of the weight loss in a control bowl at similar dose levels and time was not significantly statistical difference. The mean of % antifeedant and the difference between treatment and controlled bowl were also found to be significant difference in statistics.

From the above experiment, it could be concluded at this point that veratraldehyde was an effective antifeedant against greater wax moth larvae.

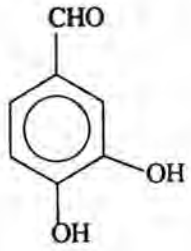
The two bioassay methods that are widely accepted are no-choice and choice tests. Choice tests are useful in detecting small differences in food acceptability when it was reduced. The results of antifeedant activity mainly depend on the emphasis of the necessity of using both types of bioassays in any comprehensive feeding preference study. Thus, a closer examination of the obtained data and description of some incidental behavioral observations made during the assays may help to explain the differences in the two tests methods.

**Structure Activity Relationship Study (SAR)**

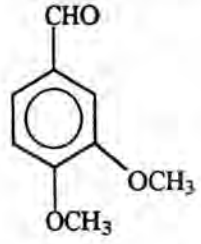
Learning from previous results, several known commercially available compounds which are of related structures to veratraldehyde, namely piperonal **(I)**, 3,4-dihydroxybenzaldehyde **(II)**, veratraldehyde **(III)**, anisaldehyde **(IV)**, veratric acid **(V)**, salicylaldehyde **(VI)**, syringaldehyde **(VII)**, vanillic acid **(VIII)**, 2,4-dihydroxybenzaldehyde **(IX)**, 2,3,4-trihydroxybenzaldehyde **(X)** and a synthetic compound, 3′,4′-dimethoxyphenyl-(1*E*)-butene-3-one **(XI)** were selected for structure and antifeedant activity relationship study. The results are presented in Table 3.16.




**(I)**

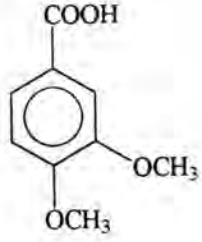



**(II)**

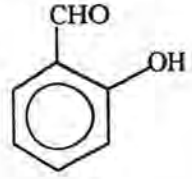



**(III)**

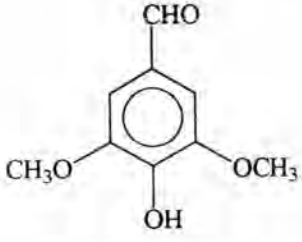

**(IV)**



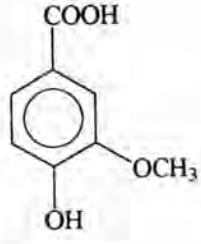

**(V)**



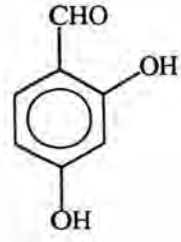

**(VI)**



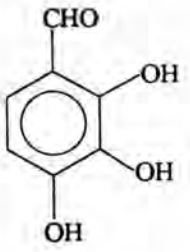

**(VII)**



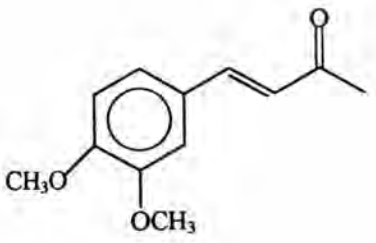

**(VIII)**




**(IX)**




**(X)**




**(XI)**

**Table 3.16** The results of antifeedant activity of Compound **(I)** - **(XI)**

| Entry | Compound | % Antifeedant |
|---|---|---|
| 1 | piperonal **(I)** | 96.87 |
| 2 | 3,4-dihydroxybenzaldehyde **(II)** | 73.60 |
| 3 | veratraldehyde **(III)** | 53.13 |
| 4 | anisaldehyde **(IV)** | 45.21 |
| 5 | veratric acid **(V)** | 18.89 |
| 6 | salicylaldehyde **(VI)** | 16.49 |
| 7 | syringaldehyde **(VII)** | 15.32 |
| 8 | vanillic acid **(VIII)** | 5.92 |
| 9 | 2,4-dihydroxybenzaldehyde **(IX)** | 0.00 |
| 10 | 2,3,4-trihydroxybenzaldehyde **(X)** | 0.00 |
| 11 | 3′,4′-Dimethoxyphenyl-(1*E*)-butene-3-one **(XI)** | 0.00 |

The results obtained from the SAR study were meaningful and strongly supported the necessity of structure-activity relationship study. To illustrate this, it was found that the aldehyde functional group seemed important for this antifeedant category. The change of -CHO to -COOH in Compound III to Compound V (entries 3 and 5) significantly reduce an antifeedant activity from 53 to 19 %. In addition, Compound XI was synthesized to alter the aldehyde functional group to enone moiety (-CH=CH-C(O)$CH_3$). The activity of the latter was also found to drop significantly.

Comparing a set of Compounds I,II and III, it gave informative results. The presence of aldehyde functional group together with a small change in structure of I and II dramatically increased the antifeedant activity. Among hydroxyl substituents present in the structure, it was obviously revealed that positions of hydroxy groups were necessary. For instance, Compound II which has hydroxy groups at 3 and 4 positions displayed attractive results, while Compound IX and X which have hydroxyl groups at 2,4- and 2,3,4- positions did not show any activity. Thus, the ideal structural pattern of the antifeedant in this class should be of an aldehyde functional group and substituents at 3 and 4 positions, preferably alkoxy or hydroxy groups.

### 3.5.5 Separation of Fraction II

Fraction II (dichloromethane fraction), 60 g was separated by silica gel column chromatography. The eluting system was intially commenced with 50% dichloromethane-hexane and changed to dichloromethane by gradual introduction of dichloromethane, dichloromethane, a mixture of dichloromethane-ethyl acetate, ethyl acetate, a mixture of ethyl acetate-methanol and finally methanol. Other separation procedures were carried out by the same way as those conducted for Fraction I. The combined fractions from the separation of crude dichloromethane are shown in Table 3.17.

**Table 3.17** The results of the separation of Fraction II by silica gel column

| Eluents | Fraction No. | Remarks | Weight (g) |
|---|---|---|---|
| 50% $CH_2Cl_2$ in hexane | 1-4 (IIA) | pale yellow liquid | 0.65 |
| 60-80% $CH_2Cl_2$ in hexane | 5-24 (IIB) | yellow liquid | 4.33 |
| 80% $CH_2Cl_2$ in hexane | 25-32 (IIC) | yellow liquid | 0.45 |
| 80% $CH_2Cl_2$ in hexane | 33- 42 (IID) | yellow viscous liquid | 0.63 |
| 80% $CH_2Cl_2$ in hexane-100% $CH_2Cl_2$ | 43-71 (IIE) | dark red liquid | 11.48 |
| 2% EtOAc in $CH_2Cl_2$ | 72-73 (IIF) | dark red liquid | 0.18 |
| 2% EtOAc in $CH_2Cl_2$ | 74 (IIG) | red-orange crystal | 0.12 |
| 2-20% EtOAc in $CH_2Cl_2$ | 75-102 (IIH) | red-orange needle in dark red viscous liquid | 11.39 |
| 20% EtOAc in $CH_2Cl_2$ -100% EtOAc | 103-147 (III) | dark red viscous liquid | 13.45 |
| 2% MeOH in EtOAc-100% MeOH | 148-180 (IIJ) | dark red viscous liquid | 2.94 |

Each small fraction derived from the separation of Fraction II was subjected to antifeedant activity study at dose level 0.25% wt by wt. The results are shown in Table 3.18 and Fig 3.16.

**Table 3.18** The results of antifeedant activity of *G. mellonella* of Fraction IIA- IIJ

| Fraction | % Antifeedant |
|---|---|
| IIA | 25.02 |
| IIB | 52.51 |
| IIC | 47.56 |
| IID | 30.60 |
| IIE | 19.90 |
| IIF | 6.22 |
| IIG | 31.09 |
| IIH | 48.47 |
| III | 67.87 |
| IIJ | 2.94 |

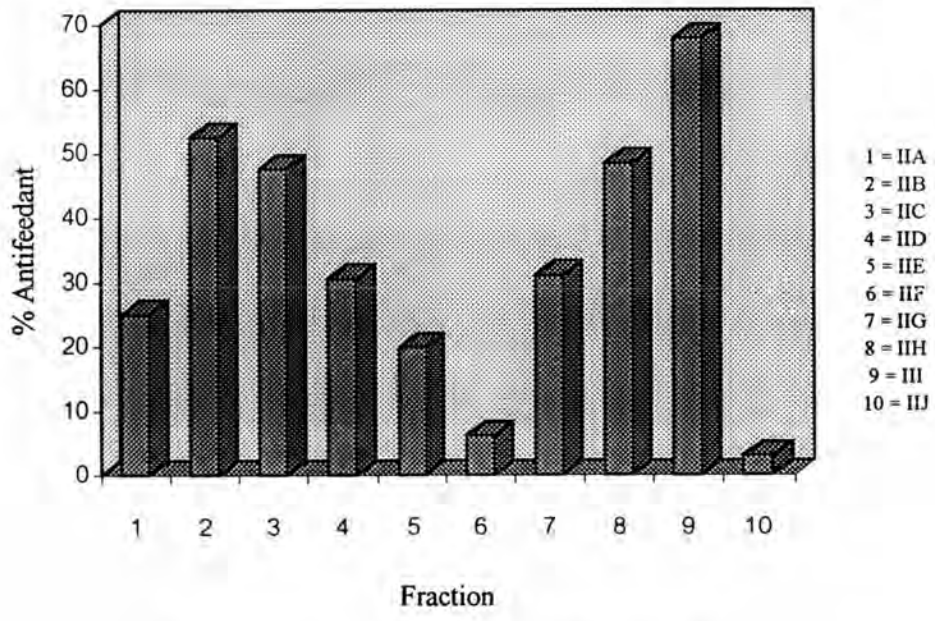



**Fig. 3.16** Antifeedant activity of Fraction II

### 3.5.6 Reseparation of Fractions IIH and III

According to attractive antifeedant results of Fractions IIH and III, these two fractions were combined (14 g) and reseparated by silica gel column chromatography. Each fraction was collected approximately 100 mL and concentrated to 25 mL. Each portion was monitored by TLC and the fractions which had similar components were combined. The results of the separation of combined Fractions IIH and III are shown in Table 3.19.

**Table 3.19** The results of the separation of combined Fractions IIH and III.

| Eluents | Fraction No. | Remarks | Weight (g) |
|---|---|---|---|
| 5% EtOAc in $CH_2Cl_2$ | 1-2 (IIHIA) | pale yellow liquid | 0.04 |
| 10-20% EtOAC in $CH_2Cl_2$ | 3-4 (IIHIB) | yellow solid in yellow liquid | 0.42 |
| 20-50% EtOAc in $CH_2Cl_2$ | 5-10 (IIHIC) | dark red viscous liquid | 1.78 |
| 60% EtOAc in $CH_2Cl_2$-100% EtOAc | 11-18 (IIHID) | dark red viscous liquid | 7.74 |
| 20% MeOH in EtOAc-100% MeOH | 19-26 (IIHIE) | dark red viscous liquid | 3.03 |

### 3.5.7 Purification and Identification of Compound 2

Fraction IIHIB contained yellow solid in yellow liquid (see Table 3.19). After the yellow liquid was washed with dichloromethane, remained solid was recrystallized with a mixture of hexane and dichloromethane to furnish a bright yellow needle 42 mg ($0.33 \times 10^{-3}$ % wt by wt by dried rhizome). This solid was designated as Compound **2**, m.p. 183-184 °C.

The IR spectrum of Compound **2** is shown in Fig 3.17 and the tentative assignment for this compound is tabulated in Table 3.20.

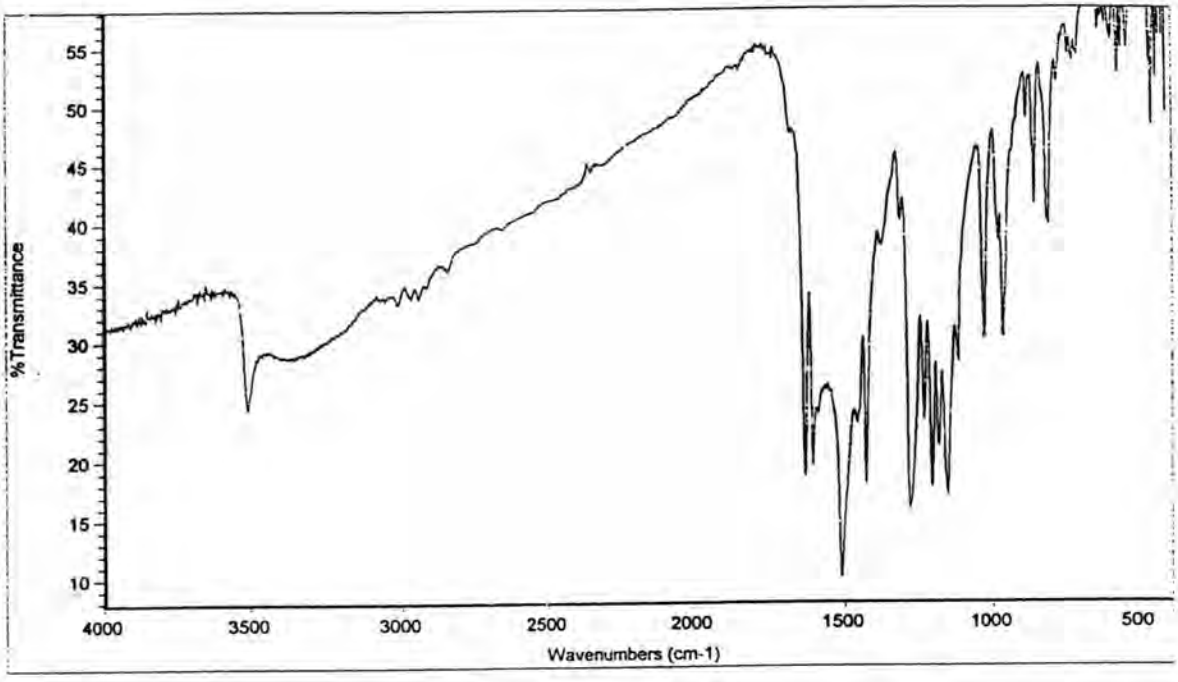



**Fig. 3.17** The IR spectrum of Compound **2**

**Table 3.20** The IR absorption band assignments of Compound **2**

| wave number ($cm^{-1}$) | intensity | tentative assignment |
|---|---|---|
| 3020-3080 | weak | O-H stretching |
| 3100-3550 | weak, broad | C-H stretching of aromatic moiety |
| 2960-2980 | weak | C-H stretching of $-CH_2-$ |
| 1640 | strong | C=O stretching of ketone |
| 1500 | strong | C=C stretching of aromatic ring |
| 1030-1290 | strong | C-O stretching |

The $^1$H-NMR spectrum of Compound **2** (Fig. 3.18) displayed signals at δ (ppm) : 3.80 (6H, s, $CH_3O$-), 6.04 (2H, s, -$CH_2$-), 6.67 (2H, d, J= 14.01 Hz, H-C=CH-Ar), 6.81 (2H, d, J = 8.60 Hz, Ar-H), 7.14 (2H, d, J = 8.22 Hz, Ar-H), 7.31 (2H, s, Ar-H), 7.55 (2H, d, J = 15.48 Hz, HC=CH-CO) and 9.57 (2H, br, s, HO-).

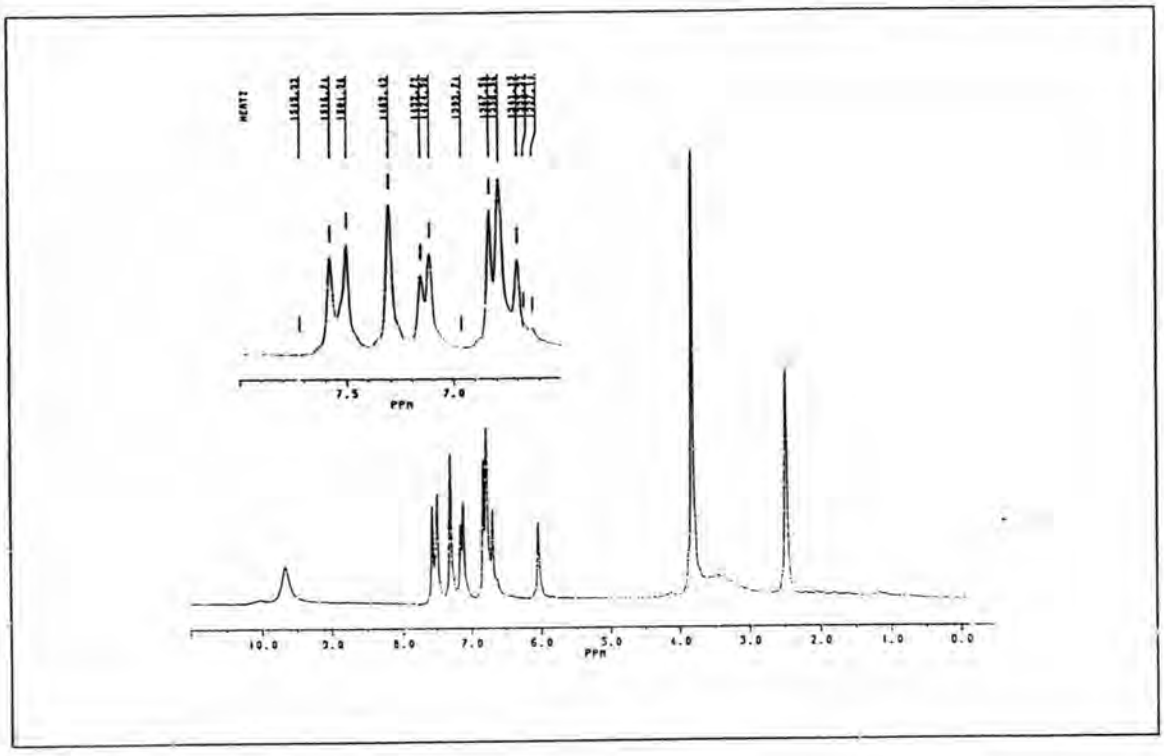



**Fig**. 3.18 The $^1$H NMR spectrum of Compound **2**

The $^{13}C$ NMR spectrum of Compound **2** (Fig. 3.19) showed signals at δ (ppm) 56.2 (2C, $\underline{C}H_3O$), 101.1(-C(O)-$\underline{C}H_2$-C(O)-),111.5, 116.2, 121.3, 126.3, 141.1, 149.5 (1C each, aromatic carbons), 123.4 ($\underline{C}$=C Ar), 148.3 (C=C-C(O) and 183.4 (-C(O)-).

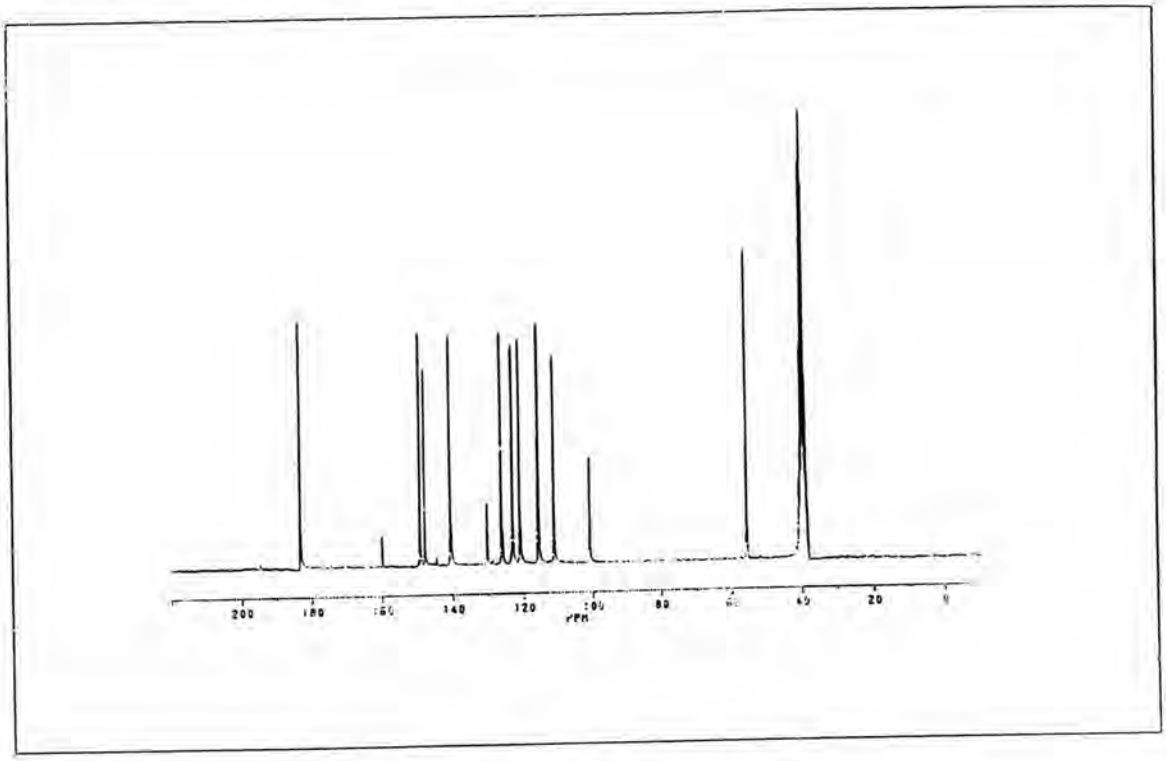
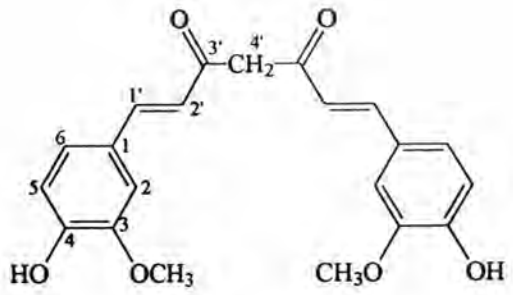


**Fig. 3.19** The $^{13}$ C NMR spectrum of Compound **2**

All physical property and spectroscopic evidence implied that the possible structure for this compound was curcumin (the structure is shown below), a common constituent widely distributed in *Z. cassumunar*. The comparative $^{1}H$ and $^{13}C$-NMR data are tabulated in Table 3.21.




Curcumin ($C_{21}H_{20}O_6$)

**Table 3.21** The $^{1}H$- and $^{13}C$-NMR signal assignments of Compound **2**

| Position | Chemical Shift (ppm) | |
|---|---|---|
| | $^{1}H$ | $^{13}C$ |
| 1 | - | 127.3 |
| 2 | 7.31 | 111.5 |
| 3 | - | 148.3 |
| 4 | - | 149.5 |
| 5 | 6.82 | 116.2 |
| 6 | 7.14 | 121.3 |
| 1′ | 7.55 | 141.1 |
| 2′ | 6.71 | 123.4 |
| 3′ | - | 183.4 |
| 4′ | 6.04 | 101.1 |
| - $OCH_3$ | 3.80 | 56.2 |
| - OH | 6.04 | - |

Compound **2**, curcumin was tested for antifeedant activity. It was found that at dose level 0.25% this compound exhibited around 23 % antifeedant activity. In addition, the variation of the amount of curcumin towards the antifeedant activity was investigated. The results are displayed in Table 3.22 and Fig. 3.20.

**Table 3.22** The results of antifeedant activity of curcumin

| % Concentration | % Antifeedant |
|---|---|
| 5.00 | 74.20 |
| 1.00 | 50.67 |
| 0.5 | 40.23 |
| 0.25 | 23.25 |

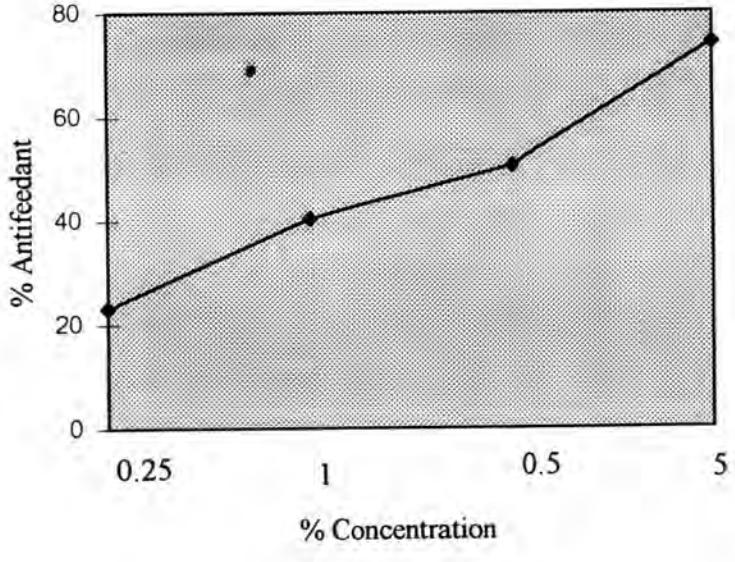



**Fig. 3.20** Antifeedant activity of curcumin

**Statistical Analysis**

The statistical analysis of curcumin is presented in Table 3.23.

**Table 3.23** Consumption of curcumin in choice tests by third-instar larvae of greater wax moth

| Dosage (% wt by wt) | $\bar{X}$ consumption (SEM) of treated (g compounds / g larvae food) | $\bar{X}$ consumption (SEM) of control (g compounds / g larvae food) | $\bar{X}$ % antifeedant (SEM) | *t* value | df |
|---|---|---|---|---|---|
| 5.00 | 0.0084 a | 0.0324 a | 74.20 a | 32.03 | 5 |
| 1.00 | 0.0166 b | 0.0337 a | 50.67 b | 20.61 | 5 |
| 0.50 | 0.0211 c | 0.0348 a | 39.56 c | 15.57 | 5 |
| 0.25 | 0.0267 d | 0.0352 a | 23.24 d | 9.35 | 5 |

[a] Mean in each column followed significantly different by the same letter are not significantly different ($P$= 0.05 ; Fisher's least significant difference (LSD)

[b] Mean in each column followed significantly different by the same letter are not significantly ($P$= 0.05 ; as determined by *t* test)

The results of statistical analysis shown in Table 3.23 revealed that mean of loss weight in treated bowl and % antifeedant activity are significantly different at 95% at dose level 5%, 1%, 0.5% and 0.25% wt by wt. On the other hand, the mean of loss weight in control bowl is not significantly different at 95 % of all dose levels.

### 3.5.8 Separation of Fraction IV

Based upon the preliminary antifeedant activity (see Table 3.23), Fraction IV (ethyl acetate fraction) gave the most promising results. Thus, 45 g of crude extract was further separated into small fractions by quick column chromatography using silica gel 60G Art. 7731 as an adsorbent. The column was initially eluted with 50% hexane-dichloromethane and gradually changed to dichloromethane, ethyl acetate-dichloromethane, ethyl acetate, methanol - ethyl acetate and methanol, respectively. Approximately 1 L of solvent was collected for each fraction and then concentrated to about 20 mL. Each fraction was monitored by TLC and similar fractions were combined. The results of the separation of ethyl acetate crude extract are shown in Table 3.24.

**Table 3.24** The results of the separation of Fraction IV

| Eluents | Fraction No. | Remarks | Weight (g) |
|---|---|---|---|
| 50% $CH_2Cl_2$ in hexane | 1 ( IVA ) | yellow oil | 6.24 |
| 50% $CH_2Cl_2$ in hexane | 2 ( IVB) | yellow viscous liquid | 2.95 |
| 50-75% $CH_2Cl_2$ in hexane | 3-5 ( IVC) | dark red viscous liquid | 6.79 |
| 75-100% $CH_2Cl_2$ in hexane | 6 ( IVD ) | white needle crystal mixed with dark red viscous liquid | 2.25 |
| 100% $CH_2Cl_2$ - 10% EtOAc in $CH_2Cl_2$ | 7-11 ( IVE ) | dark red viscous liquid | 7.45 |
| 10-80% EtOAc in $CH_2Cl_2$ | 12-20 ( IVF ) | dark red viscous liquid | 15.26 |
| 100% EtOAc - 10% MeOH in EtOAc | 21-25 ( IVG ) | dark red liquid | 4.46 |
| 10-40% MeOH in EtOAc | 26-30 ( IVH ) | dark red liquid | 4.69 |

Each small fraction derived from the separation of Fraction IV was further subjected to antifeedant activity test at dose level 0.25% wt by wt. The results of antifeedant activity test are presented in Table 3.25 and Fig. 3.21.

**Table 3.25** The results of antifeedant activity of *G. mellonella*, of each small fraction derived from the separation of Fraction IV

| Fraction | % Antifeedant |
|---|---|
| IVA | 59.34 |
| IVB | 92.75 |
| IVC | 88.68 |
| IVD | 0.00 |
| IVE | 2.51 |
| IVF | 86.80 |
| IVG | 50.62 |
| IVH | 46.86 |

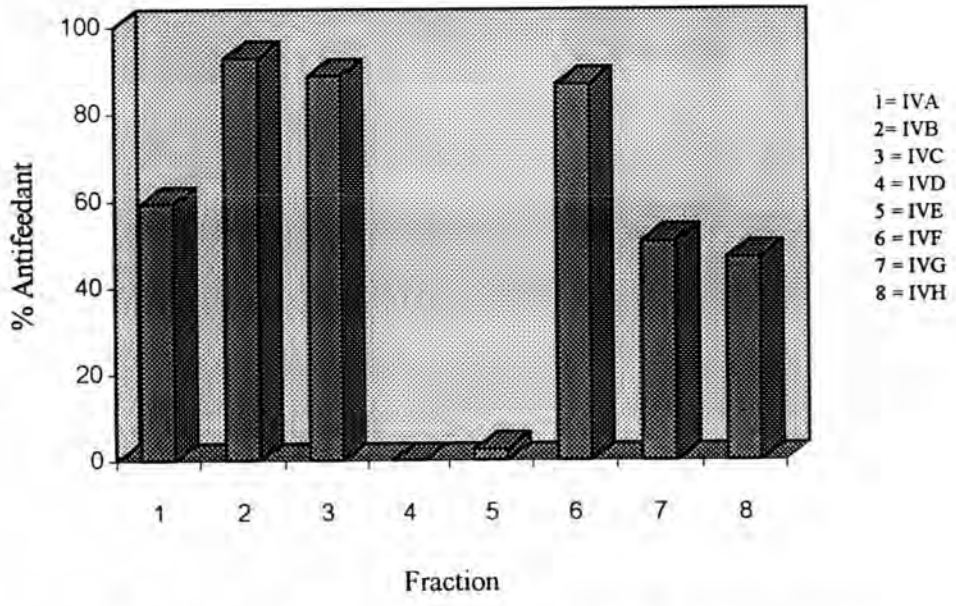



**Fig. 3.21** Antifeedant activity of each fraction derived from the separation of Fraction IV

As it was clearly revealed in Fig. 3.21, Fractions IVB, IVC and IVF exhibited very high antifeedant activity. Fractions IVB and IVC were then combined and reseparated by column chromatography to follow this bioassay activity.

### 3.5.9 Reseparation of Fractions IVB and IVC

Combined Fractions IVB and IVC, 9.74 g was separated by column chromatography. Each fraction was collected for 200 mL and was concentrated to 25 mL. By using TLC, fractions which had similar components were combined. The results of the separation of combined Fractions IVB and IVC are tabulated in Table 3.26.

**Table 3.26** The results of the separation of combined Fractions IVB and IVC

| Eluents | Fraction No. | Remarks | Weight (g) |
|---|---|---|---|
| 50-75% $CH_2Cl_2$ in hexane | 1-13 (IVBCA) | pale yellow semisolid | 1.78 |
| 75% $CH_2Cl_2$ in hexane | 14-17 (IVBCB) | yellow viscous liquid | 1.50 |
| 75% $CH_2Cl_2$ in hexane | 18-19 (IVBCC) | yellow viscous liquid mixed with white semisolid | 0.72 |
| 100% $CH_2Cl_2$ | 20-23 (IVBCD) | brown viscous liquid | 1.88 |
| 100% $CH_2Cl_2$ | 24-27 (IVBCE) | brown semisolid | 0.58 |
| 20% MeOH in $CH_2Cl_2$ | 28-35 (IVBCF) | dark brown viscous liquid | 2.26 |
| 20-100% MeOH in $CH_2Cl_2$ | 36-50 ( IVBCG) | brown solid | 0.39 |

Each small fraction attained from the separation of combined Fractions IVB and IVC, was further examined for antifeedant activity at dose level 0.25% wt by wt. The results are displayed in Table 3.27 and Fig. 3.22.

**Table 3.27** The results of antifeedant activity of *G .mellonella* of each fraction derived from the separation of combined Fractions IVB and IVC

| Fraction | % Antifeedant |
|---|---|
| IVBCA | 92.00 |
| IVBCB | 47.21 |
| IVBCC | 51.31 |
| IVBCD | 18.87 |
| IVBCE | 53.11 |
| IVBCF | 68.03 |
| IVBCG | 30.21 |

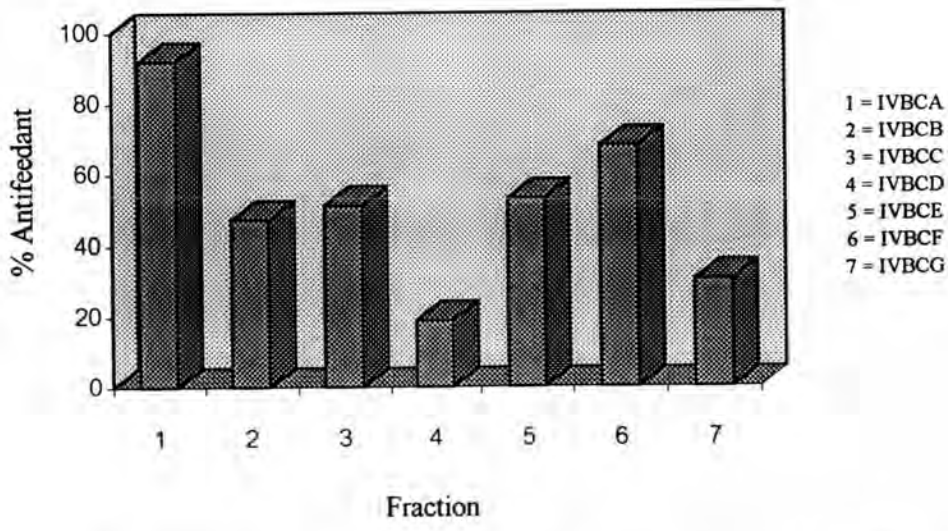



**Fig. 3.22** Antifeedant activity of each fraction obtained from the separation of combined Fractions IVB and IVC

From Fig. 3.22, it was clearly observed that Fraction IVBCA possessed very high antifeedant activity. TLC of this fraction showed a spot with a long tail using $CH_2Cl_2$ as a solvent system. Thus, fraction (1.78 g) was further separated by chromatotron.

### 3.5.10 Separation of Fraction IVBCA

The pale yellow semisolid obtained from Fraction IVBCA (1.78 g) was chromatographed on silica gel $PF_{254}$ Art.7749.1000 (45 g) using a chromatotron. Hexane, a mixture of hexane and ethyl acetate were used as eluents. While the mixture was eluted by solvent, the UV lamp with wavelength 254 nm was set above the plate. Each absorption band was collected into a fraction. Fractions of about 50 mL of solution were collected and checked for similarity by TLC. The results of the separation of Fraction IVBCA are shown in Table 3.28.

**Table 3.28** The results of the separation of Fraction IVBCA by chromatotron

| Eluents | Fraction No. | Remarks | Weight(g) |
|---|---|---|---|
| hexane | 1 | pale yellow liquid | 0.29 |
| 5% EtOAc in hexane | 2 | white needle mixed with pale yellow liquid | 0.38 |
| 10% EtOAc in hexane | 3 | white needle mixed with pale yellow liquid | 0.31 |
| 15% EtOAc in hexane | 4 | white needle mixed with pale yellow liquid | 0.26 |
| 20% EtOAc in hexane | 5 | viscous yellow liquid | 0.32 |
| 30% EtOAc in hexane | 6 | viscous yellow liquid | 0.09 |
| 40% EtOAc in hexane | 7 | viscous yellow liquid | 0.01 |
| 60% EtOAc in hexane | 8 | viscous yellow liquid | 0.009 |

Eight fractions were obtained from the separation of Fraction IVBCA. All small fractions were subjected to bioassay. It was found that only the fraction No. 5 (see Table 3.28) revealed very high antifeedant activity (95 %). This fraction was therefore reseparated by using chromatotron. The results are shown in Table 3.29.

**Table 3.29** The results of the separation of Fraction 5 by chromatotron

| Eluents | Remarks | Weight (g) |
|---|---|---|
| hexane | white liquid | 0.04 |
| 5-10 % EtOAc in hexane | pale yellow liquid | 0.05 |
| 20 % EtOAc in hexane | pale yellow liquid | 0.06 |
| 30 % EtOAc in hexane | pale yellow semisolid (Fraction A) | 0.02 |
| 40-70 % EtOAc in hexane | pale yellow semisolid | 0.05 |

Fraction A, obtained from the reseparation of Fraction 5 (see Table 3.29) contained a major spot with a long tail present on TLC. The $^{1}$H-NMR spectrum of this mixture clearly revealed the similar pattern of this mixture to that of veratraldehyde (Compound **1**). Thus, the co-TLC of this mixture and veratraldehyde was performed and it was found that the major spot of the mixture gave the same $R_f$ value as that of veratraldehyde. Thus, it may conclude that the major component in this active fraction was the same as that obtained in a hexane crude extract (Fraction I).

### 3.5.11 Separation of Fraction IVF

From the results of antifeedant activity (Fig. 3.15), Fraction IVF was found to be one of promising fractions that showed high activity. This fraction (15.26 g) was therefore separated by column chromatography and tried to follow the activity. Each fraction collected approximately 100 mL was concentrated to a small volume (25 mL) and monitored by TLC. The fractions that had the same components were combined. The results of the separation of Fraction IVF are shown in Table 3.30.

**Table 3.30** The results of the separation of Fraction IVF

| Eluents | Fraction No. | Remarks | Weight (g) |
|---|---|---|---|
| 10% EtOAc in $CH_2Cl_2$ | 1-5 ( IVFA) | dark red viscous liquid | 0.35 |
| 10-20% EtOAc in $CH_2Cl_2$ | 6-16 ( IVFB) | dark red viscous liquid | 2.82 |
| 20-40% EtOAc in $CH_2Cl_2$ | 17-24 ( IVFC) | dark red viscous liquid | 3.58 |
| 40-60% EtOAc in $CH_2Cl_2$ | 25-35 ( IVFD) | dark red viscous liquid | 2.40 |
| 60-80% EtOAc in $CH_2Cl_2$ | 36-47 ( IVFE) | dark red viscous liquid | 1.20 |
| 100% EtOAc -20% MeOH in EtOAc | 48-58 ( IVFF) | dark red viscous liquid | 3.91 |

Each small fraction received from the separation of Fraction IVF, was further subjected to antifeedant activity at dose level of each small fraction 0.25% wt by wt. The results are shown in Table 3.31 and Fig. 3.23.

**Table 3.31** The results of antifeedant activity of *G. mellonella* of each small fraction derived from the separation of Fraction IVF

| Fraction | % Antifeedant |
|---|---|
| IVFA | 34.92 |
| IVFB | 33.57 |
| IVFC | 85.76 |
| IVFD | 94.27 |
| IVFE | 50.79 |
| IVFF | 45.32 |

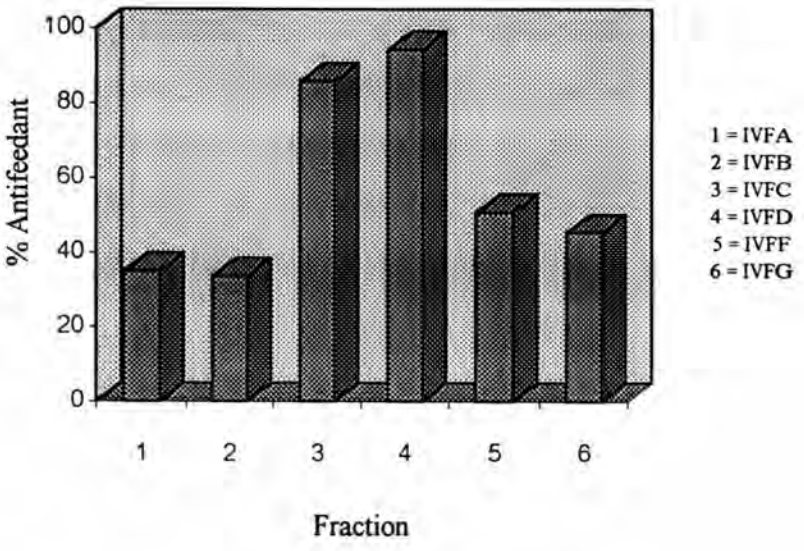



**Fig. 3.23** Antifeedant activity of Fraction IVF

The results of antifeedant activity of Fractions IVFC and IVFD (see Fig. 3.25) were quite attractive. Thus, there two fractions were combined and reseparated by column chromatography.

### 3.5.12 Reseparation of Combined Fractions IVFC and IVFD

Combined Fractions IVFC and IVFD, 5.98 g was separated by column chromatography using the same procedure as previously described. Each fraction was collected approximately 50 mL and concentrated to a small volume and monitored by TLC. The fractions which revealed similar components were combined. The results of the reseparation of combined Fractions IVFC and IVFD are shown in Table 3.32.

**Table 3.32** The results of the separation of combined Fractions IVFC and IVFD

| Eluents | Fraction No. | Remarks | Weight (g) |
|---|---|---|---|
| 10% EtOAc in $CH_2Cl_2$ | 1-20 (IVFCDA) | dark red viscous liquid | 0.50 |
| 20-40% EtOAc in $CH_2Cl_2$ | 21-38 (IVFCDB) | dark red viscous liquid | 1.54 |
| 40-60% EtOAc in $CH_2Cl_2$ | 39-45 (IVFCDC) | dark red viscous liquid | 1.22 |
| 60% EtOAc in $CH_2Cl_2$ | 46-55 (IVFCDD) | dark red viscous liquid | 0.98 |
| 60% EtOAc in $CH_2Cl_2$-100% MeOH | 56-101(IVFCDE) | dark red viscous liquid | 0.75 |

Employing the same fashion as that performed earlier, each small fraction derived from the separation of combined Fractions IVFC and IVFD were then subjected to antifeedant activity at dose level 0.25% wt by wt. The results are shown in Table 3.33 and Fig. 3.24.

**Table 3.33** The results of antifeedant activity of *G. mellonella* of each small fraction derived from the separation of Fractions IVFC and IVFD

| Fraction | % Antifeedant |
|---|---|
| IVFCDA | 48.40 |
| IVFCDB | 23.95 |
| IVFCDC | 34.10 |
| IVFCDD | 85.04 |
| IVFCDE | 35.60 |

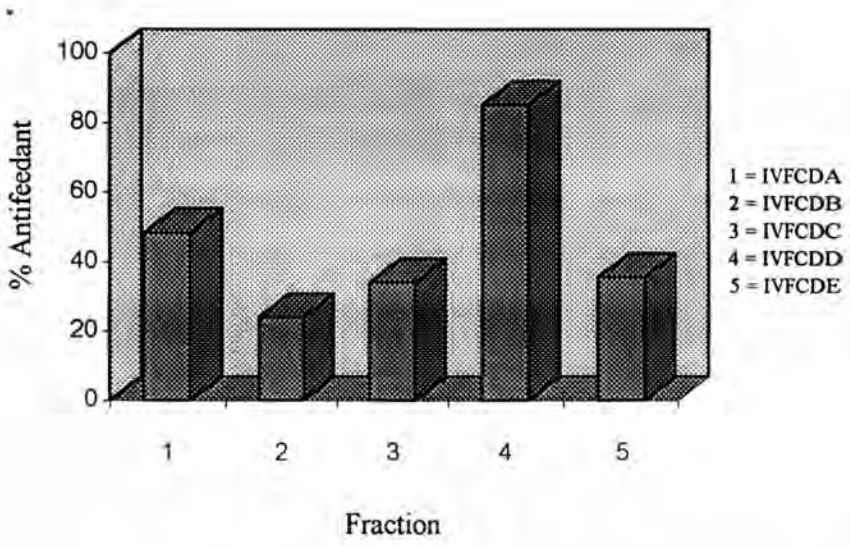



**Fig. 3.24** Antifeedant activity of combined Fractions IVFC and IVFD

It could obviously seen that Fraction IVFDD exhibited high antifeedant activity. The TLC of this fraction showed that there were at least four compounds. All components were found not to give the same $R_f$ value as either veratraldehyde (Compound **1**) or curcumin (Compound **2**). This fraction, in fact should further be purified. Unfortunately this fraction was left only small amount. Therefore, the further separation was not possible. However, it could be noted that another promising antifeedant should be present in this particular fraction.