# Chapter 3

## LABORATORY STUDIES ON THE EFFICIENCY OF CRUDE EXTRACT TO INHIBIT THE GROWTH OF *COLLETOTRICHUM GLOEOSPORIOIDES* (Penz.) Sacc.

### 3.1 Introduction

The works in this Chapter were set up to confirm the effect of crude extract from galanga, sweet flag, and *Rhinacanthus nasutus* Kurz. to inhibit the growth of *Colletotrichum gloeosporioides* (Penz.) Sacc. Bioassay was the main tool using in the Chapter, for which type and species of the fungus used was really the most important point. Infected part of mango fruit peel was cut and cultivated on PDA and maintains the colony for the other experiments.

Study on appropriated solvent were also carried out to select the best and cheapest solvent for extraction. On the separation and purification of active ingredient thin layer chromatography together with column chromatography were running and active ingredient were used for effective dosage study ( $ED_{50}$) and MIC-study.

Expectation from the research result in this chapter was the confirmation of effectiveness of active ingredient that previously reported by some researchers. Moreover plant containing no active substance will also be screened out.

### 3.2 Material and Methods

#### 3.2.1 Isolation and confirmation of *Colletotrichum gloeosporioides* (Penz.) Sacc.

To confirm the true to type of *Colletotrichum gloeosporioides* (Penz.) Sacc. which will be used in the bioassay through out the experiment, fungal parts were isolated from the surface of disease infected mango fruit (variety Nam-Dok-Mai) using Koch's Postulation method (Dhingra and Sinclair, 1986 and Sutton, 1984).

Surface tissue of mango fruit showing disease symptom was firstly cut into tiny quadratic plate at the size approximately 1 sq.mm.. The plates were then sterilised by dipping in the solution of 10% clorox (0.25% $NaHClO_3$) for around 5 min then cleaned up by dipping in sterile distilled water 3 times before incubated on the surface of PDA (Potato Dextrose Agar).

After 8 days of incubation fungal hypha and colonial part were transferred to the malt and yeast extract agar (MYA) for inducing spore formation. After 15 days incubation under room conditions, the specimens were then studied on the colony formed, sporemass colour and fruiting body (acervulus).

### 3.2.2 Preparation of *Cladosporium cladosporioides*

This fungus was also used as bioassay organism due to its black colour of spore, which served for easy observation of clear zone. This fungus can be easily found epiphytic live on plant organs.

In this studies longan leaves were selected to be the fungus donor. The single spore isolation method (Dhingra and Sinclair, 1986) was practiced to isolate the *Colletotrichum cladosporioides*. Longan leaves were firstly washed by sterile distilled water to achieve sporemass. The spore suspension was then diluted 10 times by dilution series method (Dhingra and Sinclair, 1986). The final spore suspension was cultured on PDA media and incubated at room temperature for 48 hr. After confirmation of the black colony and hyaline colour of the colony rim, the fungal specimen was used in all the bioassays.

### 3.2.3 Studies on appropriate solvent for extraction of galanga, sweet flag and *Rhinacanthus nasutus* Kurz.

Fresh rhizome of galanga and sweet flag together with stem and leaf of *Rhinacanthus nasutus* Kurz. were washed in clean water and dried at room temperature for 6 hr before chopped into small pieces. After drying in open-air, at room temperature for 5 days, plant tissue were grounded into tiny pieces (0.5-1 mm in diameter) and further dried for another 4 days. Plant tissues were then macerated in various solvents: dichloromethane, ethylacetate, ethanol, n-pentane, petroleum ether, hexane chloroform, diethyl ether or methanol for 5 days. Plant material was then filtered out by Whatman paper No. 93. Extracted solvent was treated by $Na_2SO_4$ anhydrous for

dehydration before decreased the volume by rotary evaporator and calculateed the percentage of crude extracts as:

$$\text{Percentage yield} = \frac{\text{crude weight}}{\text{Sample weight}} \times 100$$

(Sapyen, 2001 – Personal information)

Dark brown sticky crude extracts were kept at -4°C in deep freezer until used.

Effect of crude extracts on growth and development of *Cladosporium cladosporioides* was used as criterion for selection of the effective plant species. Sticky crude extracts from different solvents were firstly dissolved in adjuvant (25 % acetone: tween-80 = 2:1) in the rate of 1:1 V/V and used as stock solution for preparation of the poison food PDA. Five ml of stock solution was mixed into five ml PDA with special higher concentration of agar (30g, normally 15 g). After autoclaved and kept cool, *Cladosporium cladosporioides* culturing on PDA at 18 days of age was dug out (diameter 1 mm) and transferred on the poison food PDA. The incubation took place under room temperature.

When the colony of the control treatment completely covered the PDA in pettridish, colony diameter on each poison food PDA were measured and the percentage of inhibition was calculated according to Abbott's formula.

$$\text{Percentage of inhibit ion} = \frac{A - B}{A} \times 100$$

A = diameter of colony in control treatment

B = diameter of colony in poison food PDA.

### 3.2.4 Effect of crude extract on growth and development of *Colletotrichum gloeosporioides* (Penz.) Sacc. (Poison food study)

The results from the previous studies to select the appropriate solvent for extraction of active ingredient from galanga, sweet flag and *Rhinacanthus nasutus* Kurz. (3.2.3) revealed that the best solvent for extraction is dichloromethane. Therefore dichloromethane was used in all the left experiments.

According to the standard method recommended by Zehr *et al.* (1978) crude extract of galanga, sweet flag and *Rhinacanthus nasutus* Kurz. were dissolved in adjuvant derived from 25% of acetone : tween 80 = 2:1 . The solvent were mixed into PDA media with the dosage 0, 500, 1000, 5000 and 10,000 ppm, respectively (poison food PDA). The experiment was conducted in completely randomized design (CRD) with 6 replications, *colletotrichum gloeosporioides* (Penz.) Sacc. at 7 days of age culturing on normal PDA was dug out by cork borer (diameter 1 mm) and transferred onto poison food PDA and incubated at room temperature.

Percentage inhibition was calculated according to Abbott's formula as already mentioned in 3.2.3.

### 3.2.5 Purification and identification of antifungal compound from crude extract

#### 1) Screening of the antifungal compound in crude extract by thin layer chromatography

**A. Preparation of TLC-Plate**

Silica gel 60 G was selected for stationary phase. According to the recommendation of Harborne (1998), silica gel 60 G was firstly kept in hot air oven at 120°C for 48 hr to eliminate the moisture content, and kept cool in dessicator. As coating material for the TLC glass plate, dehydrated silica gel was mixed with distilled water at the rate 1:2 weight/volume. The glass plates were coated at the thickness of 0.50 mm and air dried under room temperature on TLC-rack before further dried in hot air oven (120°C) for 4 hr.

**B. Separation of active substances by thin layer chromatograph**

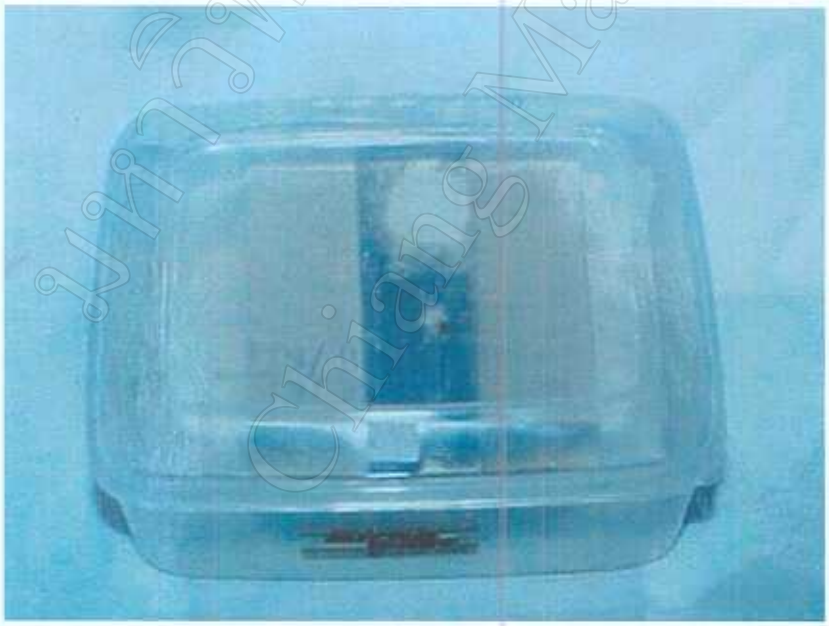
Dichloromethane crude extracts from galanga, sweetflag, and *Rhinacanthus nasutus* Kurz. were firstly dissolved in distilled dichloromethane at the rate 1 g crude extract to distilled dichloromethane 9ml. The solution were spotted on TLC-plate(size 5x2 $cm^2$) with solvent front 15 cm. Eluvents for developing TLC were the mixture of hexane: ethylacetate: methanol in the ratio 80:20:1 V/V/V for galanga, 80:20:5 for sweet flag and 60:40:1 for *Rhinacanthus nasutus* Kurz.. After 2 hr mobile phase running, developed TLC-plates were taken from the tank and evaporated under room temperature. Separation of the chemical compound on stationary phase were detected in Iodine tank ($I_2$–tank , Figure 3.1).

**Figure 3.1** Iodine tank with developed TLC-plate inside (Brown colour is chromatograms of the crude extracts).

**C. TLC-bioassay**

Spore suspension form *Cladosporium cladosporioides* (spore solution mixed with PDB to become $10^6$ spores/ml) were sprayed on developed TLC-plate and incubation in moist chamber at room temperature for 48 hr. Clear zones or the inhibition zone of the active chemical compound were detected and measured for *Rf*

(1)



(2)

**Figure 3.2** Spraying of *Cladosporium cladosporioides* spore suspension on TLC-plate(1) and incubation in moist chamber for 48 hr (2) (Black colour is the sporemass and white colour is clear zone of non- growing area affected by active substance).

**2) Structural elucidation of active ingredients**

The result in 3.2.4 revealed a relatively low inhibition percentage of *Rhinacanthus nasutus* Kurz. crude extract on growth and development of *Colletotrichum gloeosporioides* (Penz.) Sacc.. *Rhinacanthus nasutus* Kurz. was therefore left out from the next experiments to decrease the budget for the chemicals, only galanga and sweet flag remained.

**A. Collection of the active ingredient from TLC - plates**

Structural elucidation of active compounds from galanga and sweetflag were studied by using Mass Spectroscopy. To operate this equipment, large quantity of active compound is required. To collect enough sample, the TLC-plates were developed with the same technique as used in 3.2.5 (B). clear fraction with the same *Rf* value (*Rf* = 037-0.96 ) for sweet flag and 0.50-0.83 for galanga were scraped from TLC-plate including silica gel (Figure 3.3). Total number of 120 TLC-plates were made for each plant species.

**B. Structural elucidation**

Collected the fractions were sent for structural elucidation in the Laboratory of Natural Product Chemistry, in Department of Chemistry, Faculty of Science, Chiang Mai University (Dr. Damrus Sapyen's Laboratory).

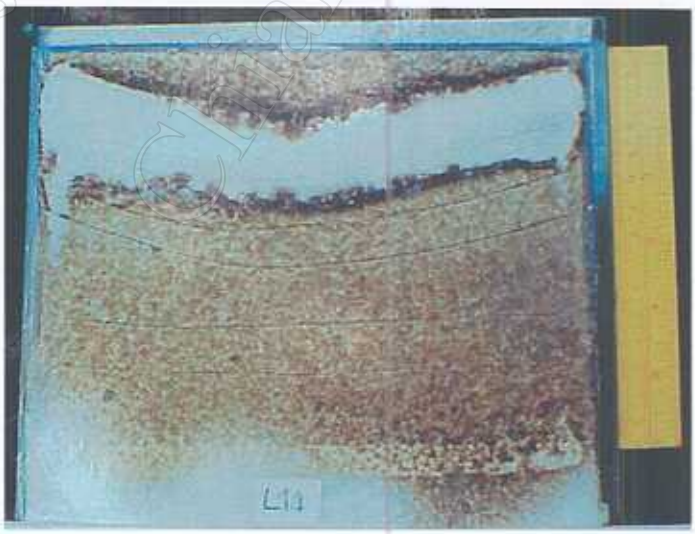


**Figure 3.3** Active fraction on TLC-plate (clear zone) after sprayed *with Cladosporium cladosporioides* spores (black colour and kept moist for 8 days).

**3.2.6 Studies on effective dosage ($ED_{50}$) of active fraction to control *Colletotrichum gloeosporioides* (Penz.) Sacc.**

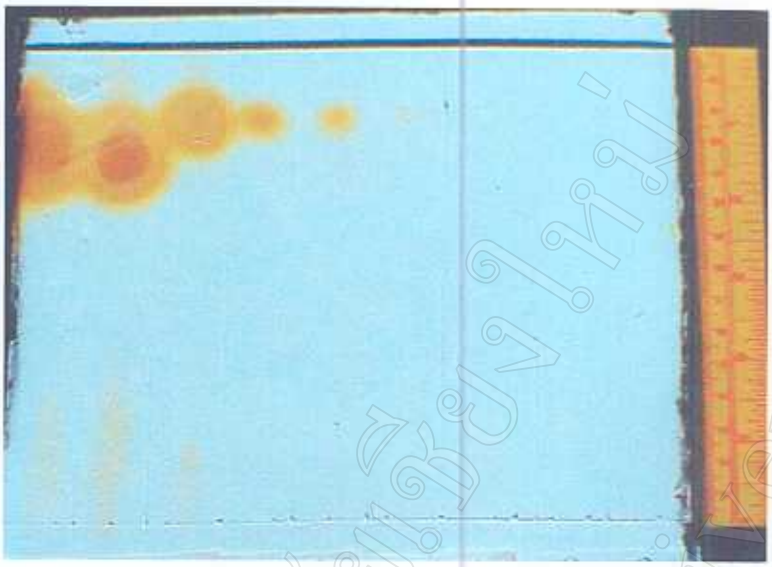
In these studies large quantity of active ingredient is necessary. Separation of active ingredient from crude extract by TLC method is somehow complicate and time consumes. Column chromatography was selected to separate and produce large quantity of the active substance.

Glass column (3.5 cm in diameter x 30 cm length) was packed with dehydrated silica gel 60 G, (stationary phase). Crude extract solution (crude : distilled dichloromethame 1:4 v/v) was loaded on the top of packed column. Elutions were made respectively with the following distilled eluents.

| Eluents for sweetflag | Formulation | Quantity (ml) |
|---|---|---|
| 1. hexane | 100 | 100 |
| 2. hexane : ethylacetate | 90:10 | 100 |
| 3. hexane : ethylacetate : methanol | 85:15:1 | 101 |
| 4. hexane : ethylacetate : methanol | 85:20:2 | 107 |

| Eluents for galanga | Formulation | Quantity (ml) |
|---|---|---|
| 1. hexane | 100 | 100 |
| 2. hexane : ethylacetate | 90:15 | 100 |
| 3. hexane : ethylacetate | 85:20 | 100 |
| 4. hexane : ethylacetate : methanol | 85:20:1 | 101 |

Down washed solutions were gradually collected for each 50 ml. Not all the fractions contained the active substance. To screen out the fraction with active ingredient, those collected fractions were separately spotted on preparative TLC-plate (PTLC). Only fractions showed mobile phase with the same *Rf* value as in the previous TLC-plate studies (3.2.5,2), A) were selected out for the future experiments. (Figure 3.4, 3.5 ).



L14 $R_f$ 0.50-0.83

**Figure 3.4** Fractional group arrangement by PTLC from a crude extract of galanga (Brown spots).

L14 $R_f$ 0.50-0.83

**Figure 3.5** Confirmation PTLC-bioassay on the fractional group arrangement by *Cladosporium cladosporioides* (Confirming clear zone according to active spots in Figure 3.4).

**1) Efficiency to inhibit spore germination**

Selected fractions containing active ingredient from galanga and sweet flag were mixed into PDA. The dosages for mixing were similar at 0, 50, 100, 150 and 200 ppm. After incubation under room temperature for 6 hr, germination of spore from *Colletotrichum gloeosporioides* (Penz.) Sacc. on the surface of poison food PDA was checked by light microscope, and randomly counted the germination of 500 spores. Percentage inhibition on spore germination was calculated by Abbott's formula :

$$\text{Percentage of spore inhibition} = \frac{A - B}{A} \times 100$$

A = amount of germinating spore in control treatment

B = amount of germinating spore in other treatments.

**2) Effective dosage studies ($ED_{50}$)**

The same methodologies were used but with different concentration of the fractions to mix into the poison food PDA. Dosage was varied from 0 to 50, 100, 200 and 500 ppm. By the time when colony of control treatment completely spread over PDA, diameter of the *Colletotrichum gloeosporioides* (Penz.) Sacc. colony in the other treatments were measured and calculated the percentage inhibitory by Abbott's formula

$$\text{Percentage of inhibition} = \frac{A - B}{A} \times 100$$

A = diameter of colony in control treatment

B = diameter of colony in other treatments.

The $ED_{50}$ values were then calculated by first transfer the percentage of inhibition value into probit value by using probit table. Dosage respond curve (DR- Curve) was plot to demonstrate the 50% inhibition level (Sommart, 1985).

**3) Minimum inhibitory concentration studies (MIC)**

*Cladosporium cladosporioides* was used in these studies. Spore suspension was smeared on normal PDA. Paper disc (made from Whatman filter paper No.1 with 1 mm diameter) dipped in various concentration of active fractions were then placed on the surface of PDA. The solution of the fractions were 0,10,20,30,40,50,60,70,80,90,100,200,300 ppm. The experiment was conducted on completely randomized design (CRD) with 3 replications. On each plate 1 paper disc were placed.

Data collections on minimum concentration were made when the colony of *Colletotrichum cladosporioides* in the control treatment completely covered the PDA. Clear zone surrounding paper disc represented the inhibitory efficiency of the active fraction.

## 3.3 Results and Discussion

### 3.3.1 Basic characteristics of *Colletotrichum gloeosporioides* (Penz.) Sacc. and *Cladosporium cladosporioides*

As shown in Figure 3.6 hyphae of *Colletotrichum gloeosporioides* (Penz.) Sacc. spreaded out on PDA and showed many layers of ring pattern, which compiled of conidiomata. Colony consisted of aerial mycelium, of which the colour varied from grey to dark grey. Sporemass was orange in colour. Mycelium compiled of septate hypha (Figure 3.7). Conidia were formed on conidiophore, which developed in acervulus (fruiting body). Conidia was rod shape, oblong to elliptic, with the average size 3.23 x 13.45 μm (mean from 60 conidia, 1 μm = 0.001 mm.).

Sukmark (1977) also reported the similar size of conidia (3.2 x 13.4 µm). Furthermore the average size of acervulus was 39.5 x 41.2 µm. Sutton (1980) reported the diversification of conidia shape from straight, cylindrical apex, obtuse, base truncate but the similar in size of 3.5-6 x 12-17 µm.

*Cladosporium cladosporioides* showed firstly pale brown colony on PDA. During the time of development central part of the colony form the bulking spore with dark brown to black colour, where as the outer ridge of the colony still remained in pale brown in colour (Figure 3.6).

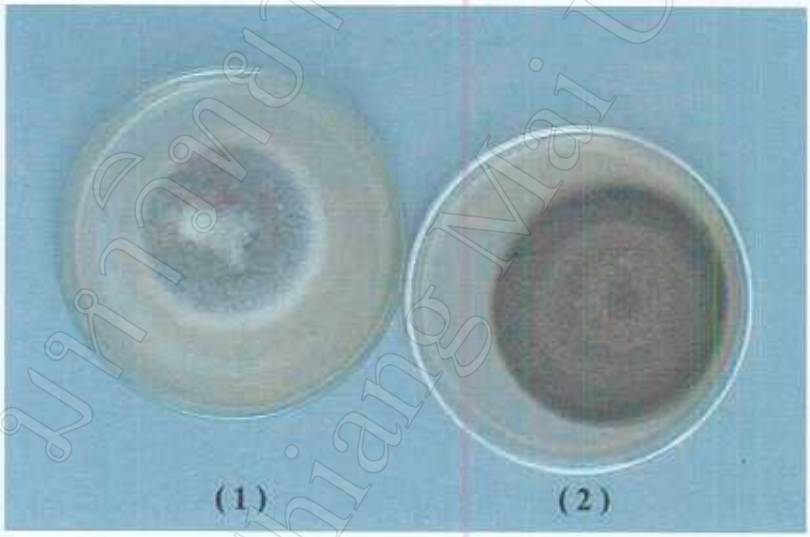
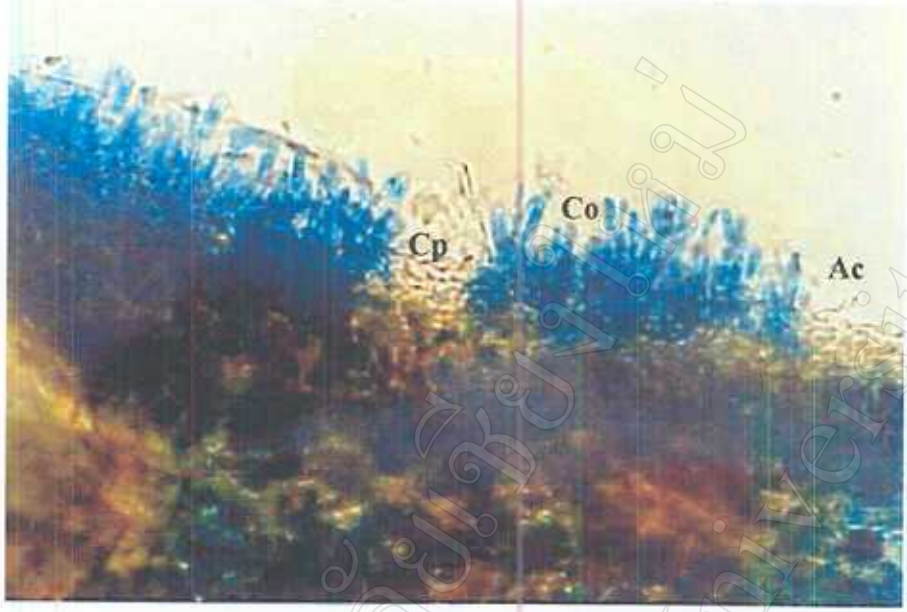



**Figure 3.6** Ring layers of *Colletotrichum gloeosporioide* (Penz.) Sacc. (1) and black colour sporemass of *Cladosporium cladosporioides* (2) at 15 days on PDA.

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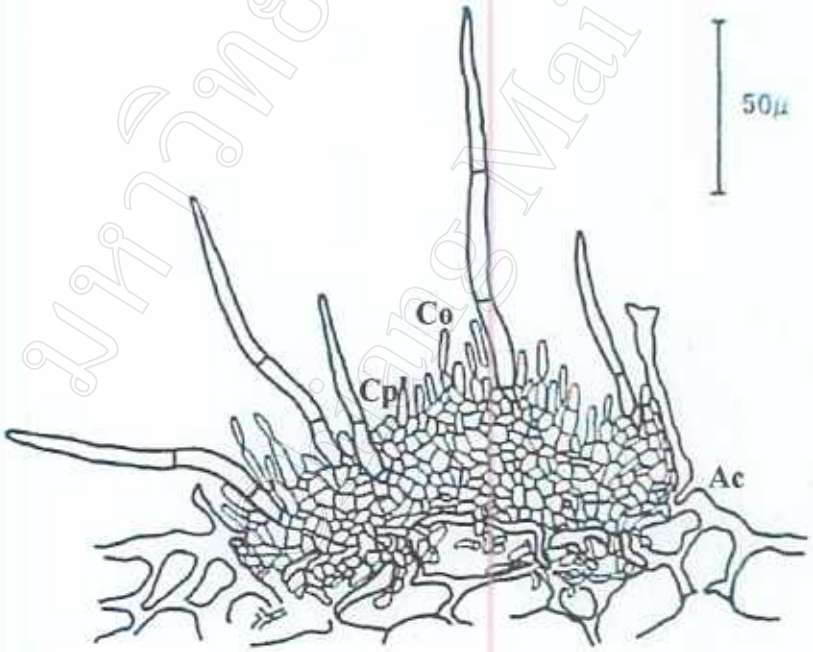



(2)

**Figure 3.7** Acervulus (Ac), conidiophore (Cp), and conidia (Co), of *Colletotrichum gloeosporioides* (Penz.) Sacc. from mango peel (1) and drawing (2).

### 3.3.2 Appropriate solvent studies

As shown in Table 3.1 ethanol, methanol, and dichloromethane were the best three solvents, which gave the highest percentage yield by all the studied plant species. However dichloromethane gave a higher crude extract yield from galanga and sweetflag, but lower when extract the *Rhinacanthus nasutus* Kurz. From the observation crude extract from alcoholic solvent, there was still more water left back in the crude extract than those from dichloromethane extracted.

Percentage inhibition of the crude extract on *Cladosporium cladosporioides* were compared between the extracts from galanga, sweetflag and *Rhinacanthus nasutus* Kurz. In Table 3.2, again dichloromethane crude extract from all the studied plant species showed the highest percentage inhibition on growth of *Cladosporium cladosporioides* followed by methanol extracted.

**Table 3.1** Percentage yield (w/w) of crude extract from galanga, sweetflag and *Rhinacanthus nasutus* Kurz. when using different solvent extraction.

| Solvent | Galanga | Sweetflag | *Rhinacanthus nasutus* Kurz. |
|---|---|---|---|
| Hexane | 0.92 | 0.97 | 0.77 |
| ethylacetate | 0.84 | 0.89 | 0.83 |
| n-pentane | 0.86 | 0.81 | 0.54 |
| chloroform | 1.24 | 0.93 | 0.59 |
| petroleum ether | 0.95 | 0.84 | 0.84 |
| diethyl ether | 0.84 | 0.86 | 0.91 |
| ethanol | 1.35 | 1.50 | 1.59 |
| methanol | 1.37 | 1.55 | 1.55 |
| dichloromethane | 1.47 | 1.61 | 1.37 |

**Table 3.2** Percentage inhibition of crude extracts from galanga, sweetflag, and *Rhinacanthus nasutus* Kurz. when studied with *Cladosporium cladosporioides* and poison food PDA.

| Solvent | Percentage inhibition | | |
|---|---|---|---|
| | Galanga | Sweetflag | *Rhinacanthus nasutus* Kurz. |
| h-pentane | 47.50 $^{d}$ | 68.33 $^{b}$ | 42.05 $^{cd}$ |
| petroleum ether | 48.30 $^{d}$ | 63.33 $^{bc}$ | 59.17 $^{c}$ |
| diethylether | 52.50 $^{cd}$ | 63.33 $^{bc}$ | 60.00 $^{c}$ |
| chloroform | 54.17 $^{cd}$ | 59.69 $^{c}$ | 60.00 $^{c}$ |
| ethyl acetate | 65.00 $^{c}$ | 63.33 $^{bc}$ | 36.67 $^{d}$ |
| ethanol | 67.50 $^{c}$ | 70.83 $^{b}$ | 70.83 $^{b}$ |
| hexane | 69.17 $^{c}$ | 65.83 $^{bc}$ | 35.83 $^{d}$ |
| methanol | 79.17 $^{b}$ | 68.33 $^{b}$ | 70.83 $^{b}$ |
| dichloromethane | 86.67 $^{a}$ | 86.67 $^{a}$ | 75.00 $^{a}$ |
| % CV. | 16.96 | 15.82 | 17.14 |

Means in the same column followed by different superscript differs significantly at $p<0.05$

Sornsrikampol (1997) and Lertvirasawat (1998) also studied the effect of crude extract from galanga derived from dichloromethane on growth of *Cladosporium cladosporioides*, and found the percentage crude extract yield at 1.75%, which similar to the result achieved from this experiment. Both authors also confirmed the best effectiveness of dichloromethane crude extract to control *Cladosporium cladosporioides.*

### 3.3.3 Effect of crude extract on growth and development of *Colletotrichum gloeosporioides* (Penz.) Sacc.

Variations of dichloromethane crude extract from galanga, sweetflag and *Rhinacanthus nasutus* Kurz. were made from 0, 500, 1000, and 10,000 ppm by mixing in PDA (poison food PDA). Percentage of inhibition on growth and development of *Colletotrichum gloeosporioides* (Penz.) Sacc. were calculated according to Abbott's formula.

Crude extract of galanga and sweet flag gave the better result than crude extract from *Rhinacanthus nasutus* Kurz. (Table 3.3 ). In the case of concentration, crude extract from sweetflag at 10,000 ppm could completely inhibit growth whereas crude extract of galanga gave around 84.26% inhibition.

**Table 3.3** The inhibition percentage of crude extracts from galanga, sweetflag and *Rhinacanthus nasutus* Kurz. on the colonial growth of *Cladosporium gloeosporioides* (Penz.) Sacc.

| Plant | Concentration (ppm) | | | |
|---|---|---|---|---|
| | 500 | 1,000 | 5,000 | 10,000 |
| Sweetflag | 46.85 [d 1/] | 80.55 [a] | 89.38 [a] | 100 [a] |
| Galanga | 52.47 [a] | 57.04 [b] | 57.04 [b] | 84.26 [b] |
| *Rhinacanthus nasutus* Kurz. | 0.25 [b] | 9.57 [c] | 9.57 [c] | 50.47 [c] |
| CV. | 31.33% | 16.56 | 19.35 | 14.45 |

Means in the same column followed by different superscript differs significantly at $p<0.05$

Crude extract from galanga revealed a relative lower percentage inhibition of only 57.04% when applied at 1,000 ppm (Table 3.3 and Figure 3.8, 3.9). So far, there is no study on dichloromethane crude extract from sweetflag, galanga and *Rhinacanthus nasutus* Kurz. to control *Colletotrichum gloeosporioides* (Penz.) Sacc.. Bhassabutra (1997) studied on ethylacetate crude extract and found the best effective from Sweetflag, *Rhinacanthus nasutus* Kurz. and galanga, respectively. Korpraditsakul (1990, and 1991) studied on ethanol crude extract found slightly

different results that *Rhinacanthus nasutus* Kurz. could show the best controlling effect than sweetflag and galanga, respectively.

These finding reminded the effect of solvent on the efficiency of crude extract to control the growth of *Colletotrichum gloeosporioides* (Penz.) Sacc. According to Harborne (1998) active ingredient in plant material are differed in chemical structure. Dissolved in any solvent to be related to its polarity and non-polarity of the chemical compound and solvent. High polarity could be extracted by alcoholic solvent; and moderately dissolved in dichloromethane, ethylacetate can also easily extract the high polarity substances. The non-polar ingredient can be easily extracted by water.

Comparison between the solvents; ethylacetate, ethanol and dichloromethane, which already studied in this experiment, dichloromethane crude extract had another advantage of less water residue in the product. So that the dehydration process requires less amount of $Na_2SO_4$ anhydrous. Therefore in the next experiment dichloromethane was chosen to be the extracting solvent for all the studied plant species.

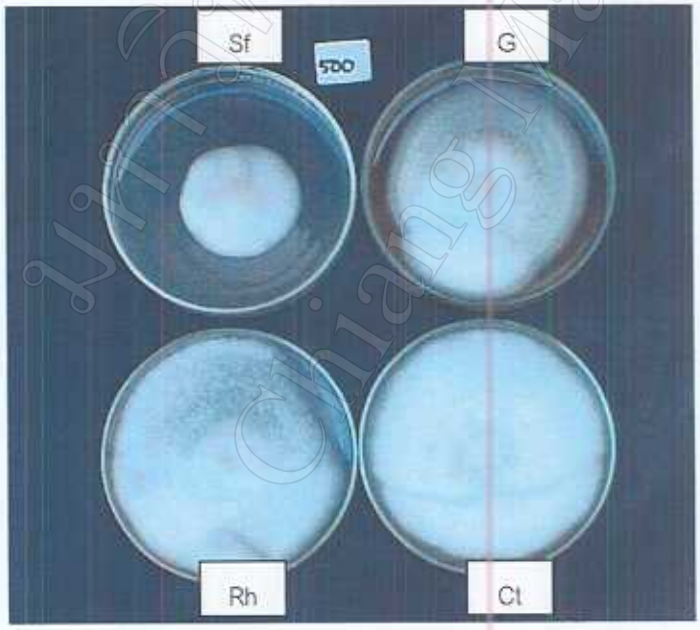



**Figure 3.8** Colony of *Colletotrichum gloeosporioides* (Penz.) Sacc. on poison food PDA mixed with crude extract from galanga (G), sweetflag (Sf), and *Rhinacanthus nasutus* Kurz. (Rh) at 500 ppm and control (Ct).


**Figure 3.9** Colony of *Colletotrichum gloeosporioides* (Penz.) Sacc. on poison food PDA mixed with crude extract from galanga (G) sweetflag (Sw) and *Rhinacanthus nasutus* Kurz. (Rh) at 1,000 ppm and control (Ct).

### 3.3.4 Screening of the antifungal compound in crude extracts from galanga, sweet flag and *Rhinacanthus nasutus* Kurz.

The studies were made by thin layer chromatograph – bioassay (TLC-bioassay) using *Cladosporium cladosporioides* as indicating fungi after spraying of spore suspension on developed TLC-plate and incubated in moist chamber kept at room temperature for 48 hr.

In the case of crude extract from the sweetflag that showed in Figure 3.11 and the Table 3.4. Four clear zones existed with *Rf* value at 0, 0.20 – 0.27, 0.42 – 0.53 and 0.67 – 0.96. These clear zones were name as A10, A11, A12 and A13, respectively.

From this experiment crude extract from *Rhinacanthus nasutus* Kurz. showed no inhibitory effect on growth of *Colletotrichum gloeosporioides* (Penz.) Sacc. This plant species was therefore

eliminated from the whole experiments in order to save time and chemical cost. Kritsanapan (1993) and Youngviset (1993) confirmed the positive effect of *Rhinacanthus nasutus* Kurz. to control fungal development. These studies were however different from what was done in this experiment in especially the type of solvent used. Ethanol was used by Krisanapan and Youngviset whereas dichloromethane was used in this experiment. This reminded a better dissolve of antifungal compound in *Rhinacanthus nasutus* Kurz. in ethanol but not so well in dichloromethane.

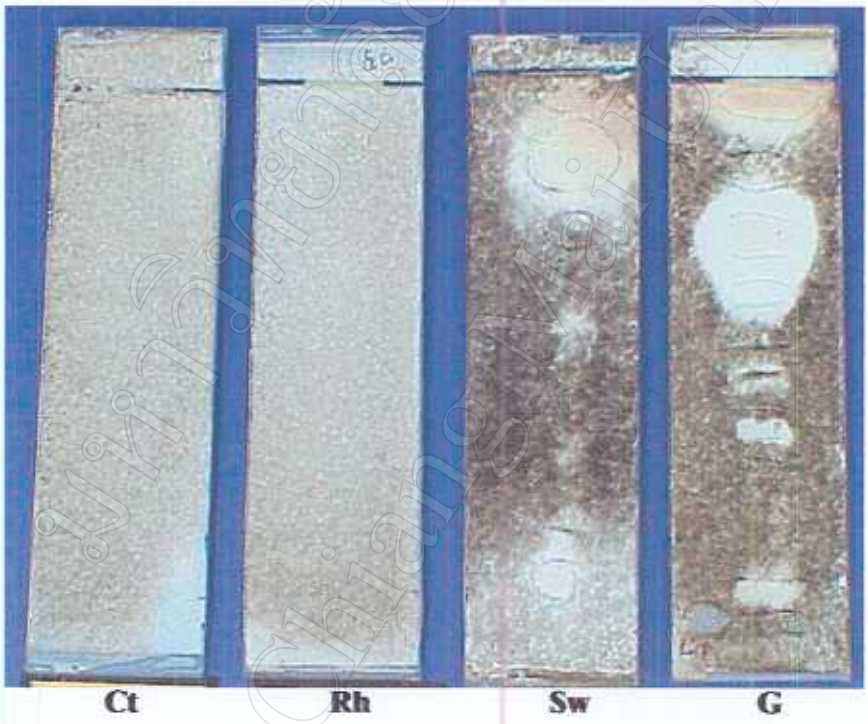



**Figure 3.10** Clear zones of control treatment (CT), *Rhinacanthus nasutus* Kurz. (Rh), sweetflag (Sw), and galanga (G) on TLC-bioassay plate using *Cladosporium cladosporioides* as indicator.

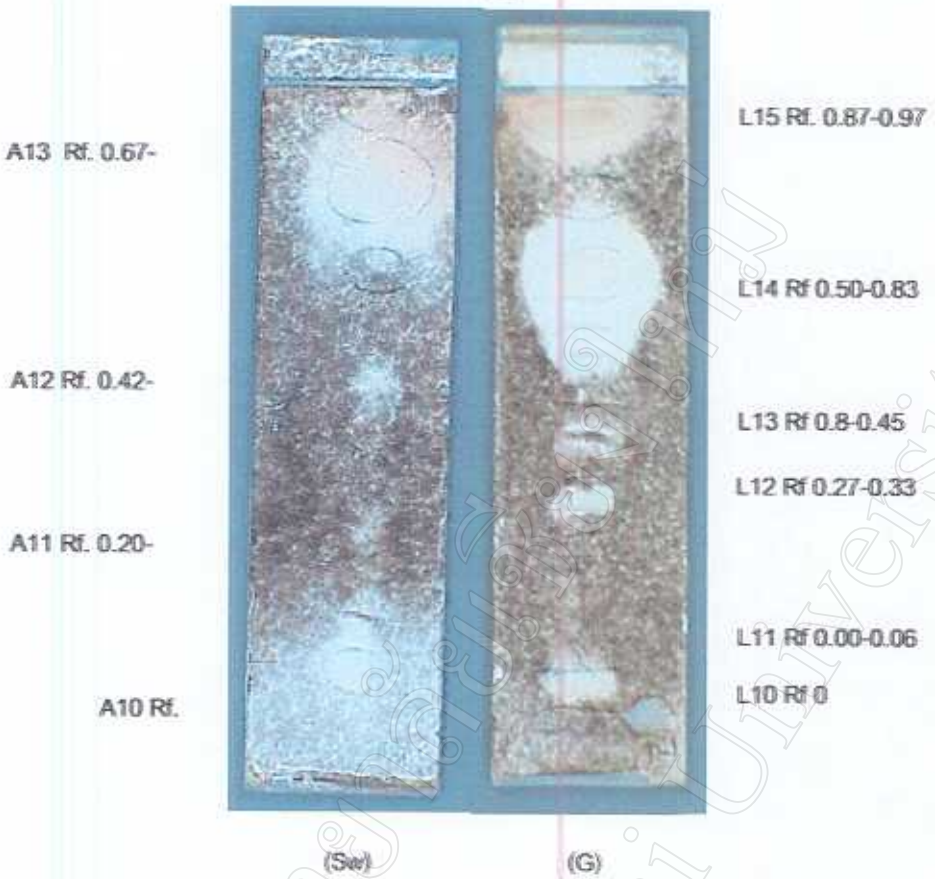



**Figure 3.11** *Rf* of each fraction on TLC-plate of sweet flag (Sw) and galanga (G).

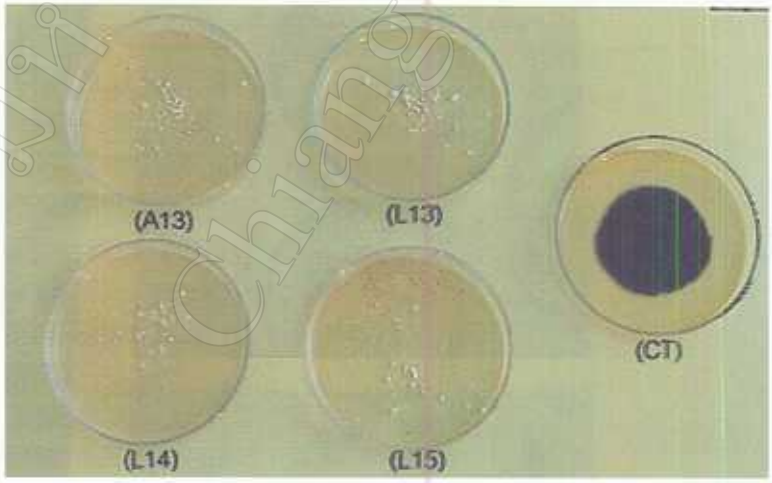



**Figure 3.12** No spore germination and mycelium development was found on the plate treated with A13, L13, L14 and L15 but well grew in control treatment (Ct).

Crude extract from galanga showed 5 clear zones (Figure 3.11 (G)) with *Rf* value at 0.00-0.06, 0.27 – 0.33, 0.38 – 0.45, 0.50 – 0.83 and 0.87 – 0.97 which named L10, L11,L12, L13, L14 and L15, respectively. One mobile fraction on TLC-plate found in $I_2$-tank with *Rf* value. (L11) showed no clear zone.

In the case of *Rhinacanthus nasutus* Kurz. Which showed in $I_2$ - tank studied (Figure 3.10) 3 mobiles fractions, could not show any clear zone (Figure 3.12). This means a non–inhibitory efficiency of crude extract from *Rhinacanthus nasutus* Kurz. to control growth and development of *Colletotrichum gloeosporioides* (Penz.) Sacc. when extracted with dichloromethane.

**Table 3.4** *Rf* value of clear zone from TLC-bioassay of crude extract from sweetflag and galanga.

| Crude extract | *Rf* value | Code | Note |
|---|---|---|---|
| Sweet flag | 0 | A10 | + |
| | 0.20-0.27 | A11 | + |
| | 0.42-0.53 | A12 | + |
| | 0.67-0.96 | A13 | + |
| Galanga | 0 | L10 | + |
| | 0.00-0.06 | L11 | - |
| | 0.27-0.33 | L12 | + |
| | 0.38-0.45 | L13 | + |
| | 0.50-0.83 | L14 | + |
| | 0.87-0.97 | L15 | + |

Note: + = with clear zone

\- = without clear zone

In order to confirm the persistence of effectiveness in controlling fungi growth, the clear zone on silica gel were scraped. The powders were spreaded over the normal PDA to allow the living spore or mycelium of *Cladosporium cladosporioides* to germinate. It was found that even after 14 days of incubation, no spore germination or mycelium development was found in the plate treated with A13, L13, L14 and L15 (Figure 3.13, 3.14)

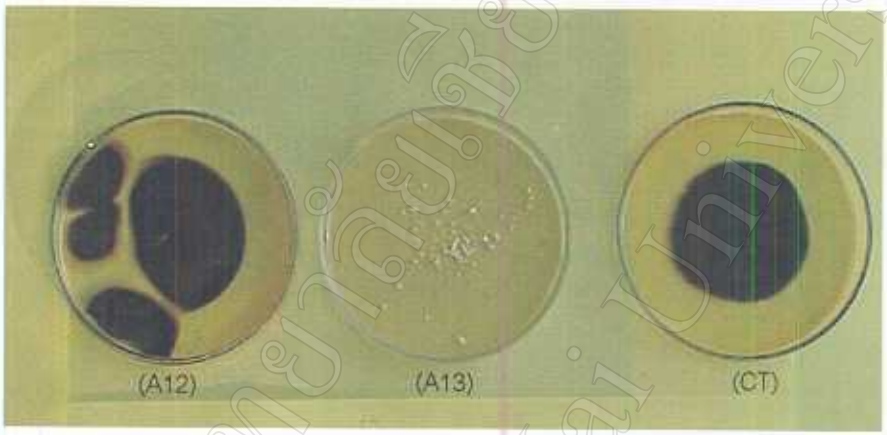



**Figure 3.13** Germination of spore and growth of mycelium of *Cladosporium cladosporioides* on PDA after 14 days of treatment with A12 and A13 compared to control (Ct).

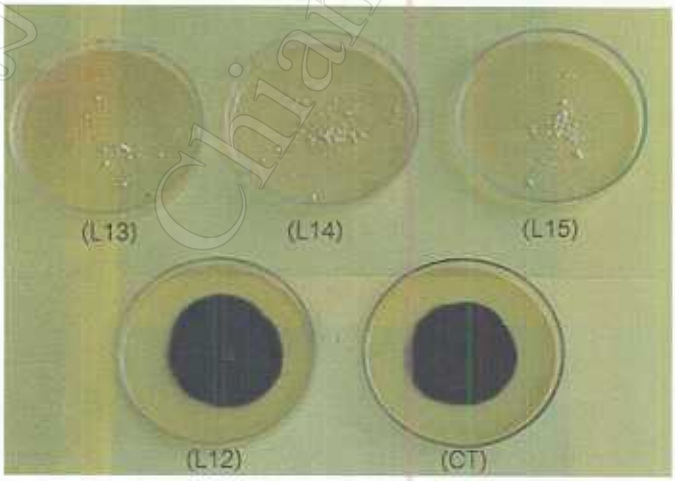



**Figure 3.14** Germination of spore and growth of mycelium of *Cladosporium cladosporioides* on PDA after 14 days of treatment with L12, L13, L14 and L15 compared to control (Ct).

### 3.3.5 Chemical structure elucidation of active ingredient from sweet flag and galanga and sweet flag.

The scraped silica gel saturated with active substances were repeatedly extracted with dichloromethane and dehydrated with $Na_2SO_4$ anhydrous. After 3 times cleaned up of impurity through PTLC-technique, the purified final liquid was sent for GC-MS studies at Faculty of Science, Chiang Mai University.

**1) Sweet flag**

In Table 3.5, Figure 3.15 and Figure 3.16 are data file and resolutions from GC-MS study. The highest peak (*Rt* 7.91 – 8.73) revealed cis ß– asarone, trans – asarone and α -asarone as the major component in the purified active compound.

Study on chemical structure through IR – resonane (Figure 3.16) revealed the proposed fragmentation of cis β – asarone as shown in Figure 3.17 (Sapyen, 2001 – Personal information).

**Table 3.5** Information from data file of beta-asarone.

```
Information from Data File:
     File:           C:\HPCHEM\1\DATA\DR20305.D
     Operator:       Pisan
     Date Acquired:  21 Mar 2001  10:55
     Method File:    112RT
     Sample Name:    beta-Asarone
     Misc Info:
     Vial Number:    5

Search Libraries:    C:\DATABASE\WILEY275.L          Minimum Quality:   0

Unknown Spectrum:    Apex
Integration Events:  Chemstation Integrator - events.e
```

| Pk# | RT | Area% | Library/ID | Ref# | CAS# | Qual |
|---|---|---|---|---|---|---|
| 1 | 6.15 | 3.17 | C:\DATABASE\WILEY275.L | | | |
| | | | TRANS-METHYL ISOEUGENOL | 62469 | 006379-72-2 | 98 |
| | | | CIS-METHYL ISOEUGENOL | 62468 | 006380-24-1 | 98 |
| | | | ANISYL ACETONE $$ PARA METHOXY PHE | 62472 | 000104-20-1 | 97 |
| 2 | 6.52 | 0.37 | C:\DATABASE\WILEY275.L | | | |
| | | | CIS-METHYL ISOEUGENOL | 62468 | 006380-24-1 | 98 |
| | | | TRANS-METHYL ISOEUGENOL | 62469 | 006379-72-2 | 98 |
| | | | ANISYL ACETONE $$ PARA METHOXY PHE | 62472 | 000104-20-1 | 97 |
| 3 | 6.79 | 1.03 | C:\DATABASE\WILEY275.L | | | |
| | | | Cyclohexanone, 3-ethenyl-3-methyl- | 105814 | 058437-65-3 | 90 |
| | | | Cyclohexanone, 3-ethenyl-3-methyl- | 105815 | 058437-65-3 | 90 |
| | | | Cyclohexanone, 3-ethenyl-3-methyl- | 105817 | 058437-66-4 | 46 |
| 4 | 7.12 | 1.01 | C:\DATABASE\WILEY275.L | | | |
| | | | Naphthalene, 1,2-dihydro-1,1,6-tri | 57820 | 030364-38-6 | 80 |
| | | | 1,4,5,8-Tetramethyl-1,4-dihydronap | 85446 | 068185-75-1 | 72 |
| | | | CALACORENE $$ 1,1,6-trimethyl-1,2- | 57844 | 000000-00-0 | 72 |
| 5 | 7.29 | 1.64 | C:\DATABASE\WILEY275.L | | | |
| | | | EUASARONE | 93383 | 005353-15-1 | 96 |
| | | | cis-Asarone $$ Benzene, 1,2,4-trim | 93311 | 005273-86-9 | 91 |
| | | | CIS-ISOELEMICIN | 93382 | 005273-84-7 | 91 |
| 6 | 7.91 | 70.43 | C:\DATABASE\WILEY275.L | | | |
| | | | cis-Asarone $$ Benzene, 1,2,4-trim | 93314 | 005273-86-9 | 99 |
| | | | cis-Asarone $$ Benzene, 1,2,4-trim | 93311 | 005273-86-9 | 99 |
| | | | trans-Asarone $$ .alpha.-Asarone $ | 93317 | 002883-98-9 | 98 |
| 7 | 8.73 | 17.49 | C:\DATABASE\WILEY275.L | | | |
| | | | cis-Asarone $$ Benzene, 1,2,4-trim | 93314 | 005273-86-9 | 99 |
| | | | TRANS(.ALPHA.)-ASARONE | 93480 | 000000-00-0 | 98 |
| | | | trans-Asarone $$ .alpha.-Asarone $ | 93316 | 002883-98-9 | 98 |
| 8 | 8.87 | 0.78 | C:\DATABASE\WILEY275.L | | | |
| | | | 1,2,2-TRIMETHYL-1-(P-TOLYL)-CYCLOP | 87265 | 016982-00-6 | 83 |
| | | | Benzene, 1-methyl-4-(1,2,2-trimeth | 87269 | 016982-00-6 | 66 |
| | | | Benzene, 1-methyl-4-(1,2,2-trimeth | 87267 | 016982-00-6 | 66 |
| 9 | 8.94 | 0.96 | C:\DATABASE\WILEY275.L | | | |
| | | | (+)-3,8-Dimethyl-5-(1-methylethyli | 103911 | 000000-00-0 | 95 |
| | | | Aristolone $$ 2B-Cyclopropa[a]naph | 103769 | 006831-17-0 | 89 |
| | | | 5(1H)-Azulenone, 2,4,6,7,8,8a-hexa | 103914 | 006754-66-1 | 60 |
| 10 | 9.35 | 2.47 | C:\DATABASE\WILEY275.L | | | |
| | | | Benzaldehyde, 2,4,5-trimethoxy- (C | 80178 | 004460-86-0 | 98 |
| | | | Benzaldehyde, 2,4,5-trimethoxy- (C | 80176 | 004460-86-0 | 98 |
| | | | Benzaldehyde, 2,4,5-trimethoxy- (C | 80177 | 004460-86-0 | 98 |
| 11 | 10.82 | 0.65 | C:\DATABASE\WILEY275.L | | | |
| | | | N-Phenyl-2,4,6-trimethylaniline | 96851 | 023592-67-8 | 72 |
| | | | (E)-2-hydroxy-4'-aminostilbene $$ | 96820 | 110983-41-0 | 59 |
| | | | 3-hydroxy-1,2-dimethyl-9H-carbazol | 96819 | 118538-97-9 | 53 |

```
Wed Mar 21 11:20:26 2001
```

```
File       : C:\HPCHEM\1\DATA\DR20305.D
Operator   : Pisan
Acquired   : 21 Mar 2001  10:55     using AcqMethod 112HT
Instrument :   GC/MS Ins
Sample Name: beta-Asarone
Misc Info  :
Vial Number: 5
```

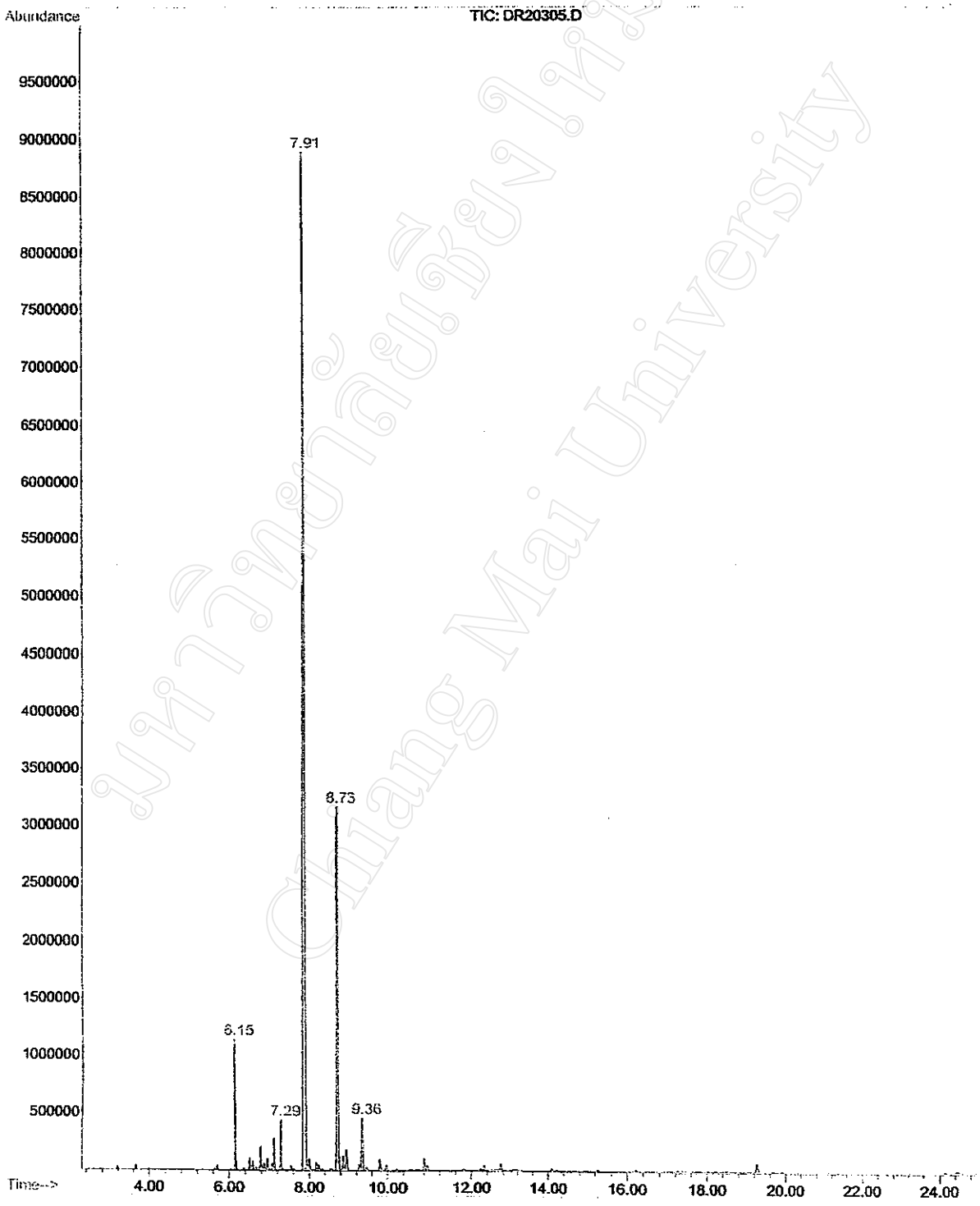
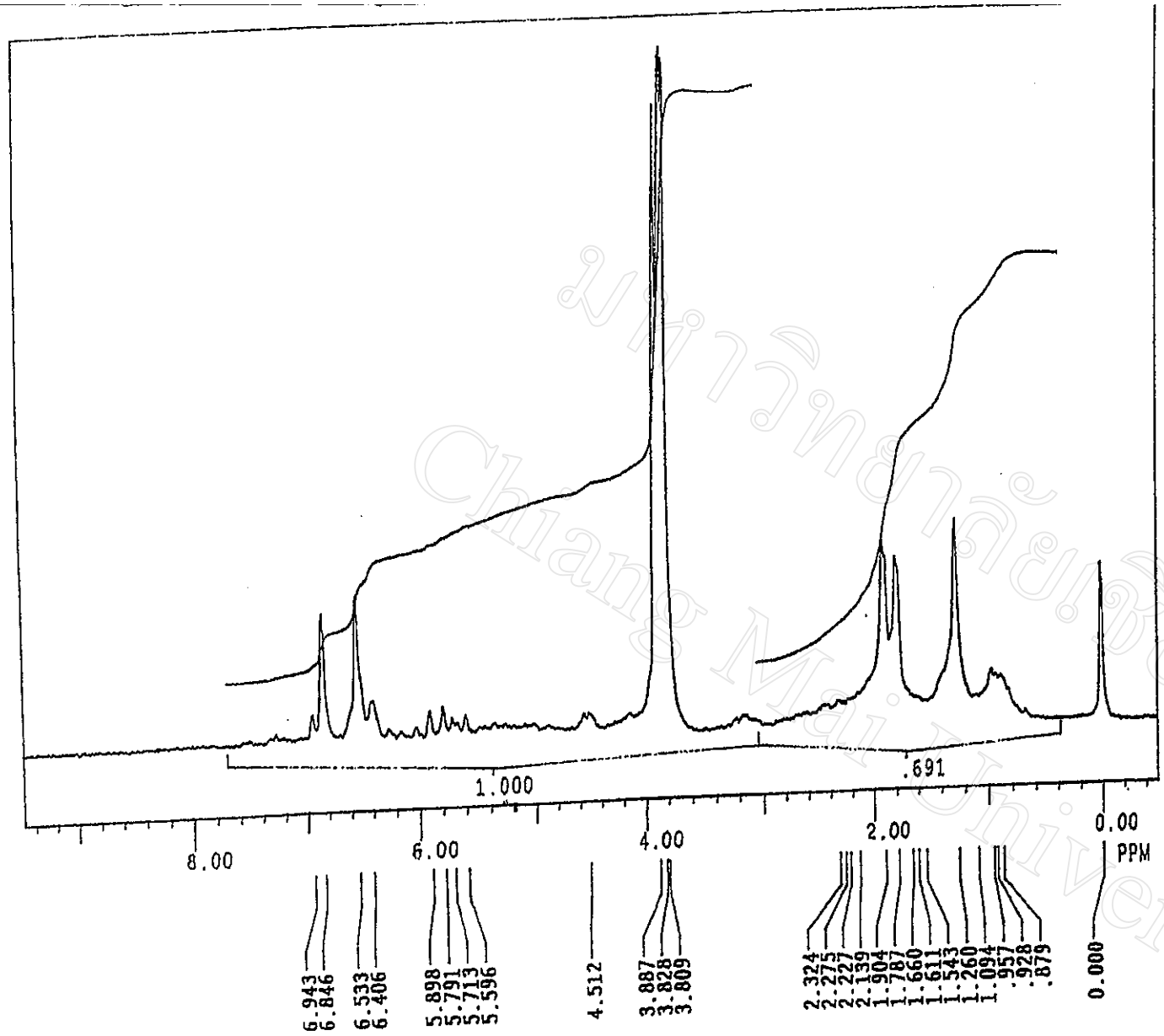



**Figure 3.15** Data file of GC-MS of beta –asarone fraction.




```
** ACQUISITION COMMENT **

Sample  : 8 - ASDROUE

Solvent : CDCL3
Conc.   : -
Refer.  : TMS
Tube D. : 5 mm
Operator: S.ARAMRUENG
Date    : 16/3/2001
Memo    : DAMRUS

**  ACQUISITION PARAMETER  **

Acquis. mode    : Normal
Spectrum width  :        20 pp
No. of acquis.  :         8
Pulse interval  :       5.0 se
Data point      :         4 k


Pulse width 90  :        20 mi

**  PROCESSING PARAMETER **

Proce.data point:         4 k
Display range   :     10.00 pp
Display gain    :      1.00
Gain mode       : Normal
```

HITACHI R-1500

**Figure 3.16** Acquisition comment of beta-asarone from IR-resonance (HITACHI R-1500)

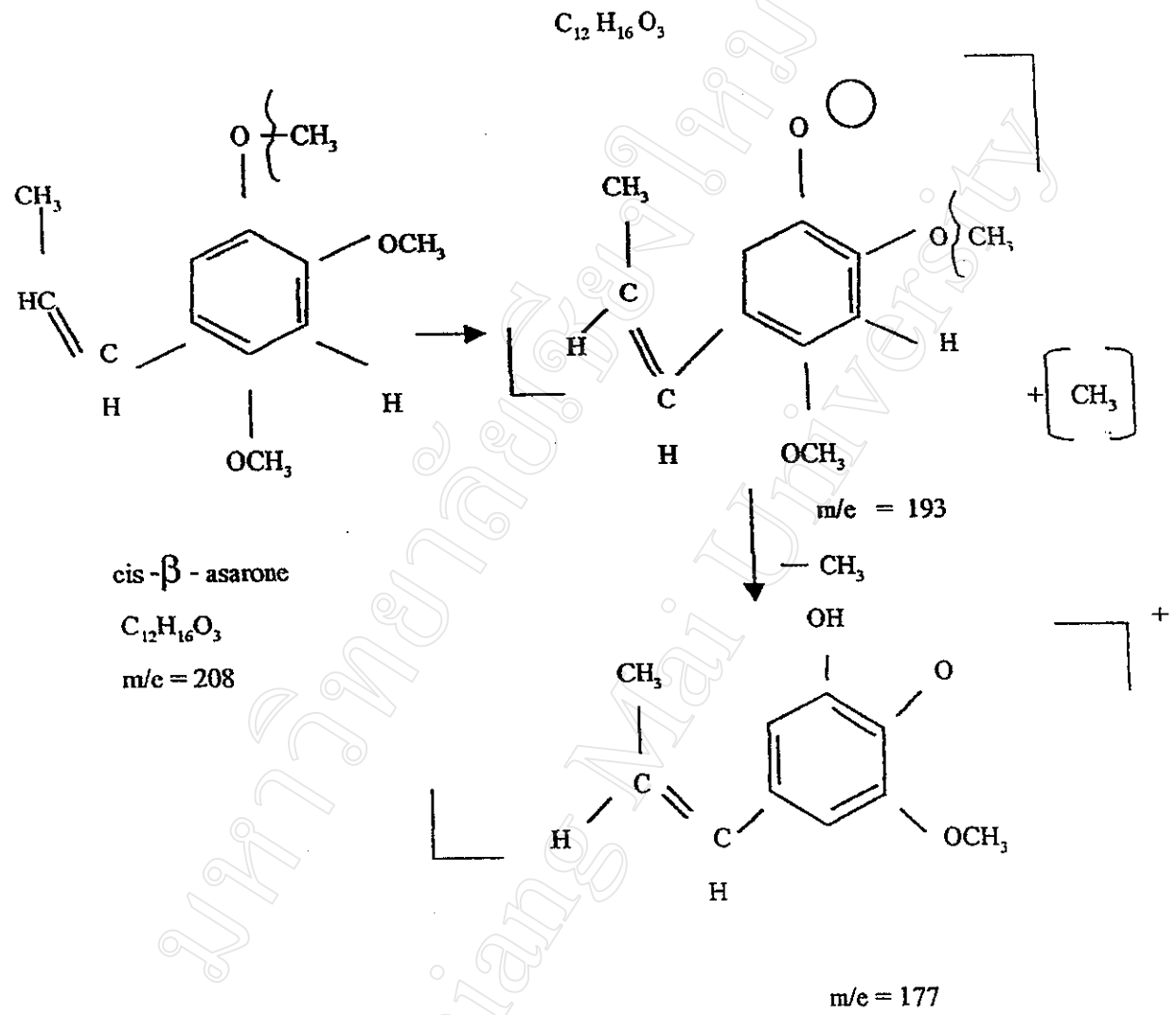



**Figure 3.17** Proposed fragmentation of cis-beta- asarone.

**2) Galanga**

Data file from GC-MS analysis showed the active substance at the time 8.258 min as the major component in the fraction (Figure 3.18 , 3.19 ). IR – study recommended the chemical structure as $1'$ – acetoxychavicol acetate (Figure 3.20) which the proposed fragmentation showed in Figure 3.21.

Structural elucidation of active substance from sweet flag was the first time conducted ever. No report has been done on this active substance before, in especially as the antifungal substance to control *Colletotrichum gloeosporioides* (Penz.) Sacc..

For $1'$ – acetoxychavicol acetate, which was confirmed to be the active ingredient in galanga crude extract and showed efficiency substance to control *Colletotrichum gloeosporioides* (Penz.) Sacc. The same results both from GC – MS studied and antifungal efficiency also reported by Srisornkampol (1996) and Lertvirasawat (1997).

```
File       : C:\HPCHEM\1\DATA\DR20304.D
Operator   : Pisan
Acquired   : 21 Mar 2001  10:25     using AcqMethod 112HT
Instrument :   GC/MS Ins
Sample Name: acetoxy chavicol
Misc Info  :
Vial Number: 4
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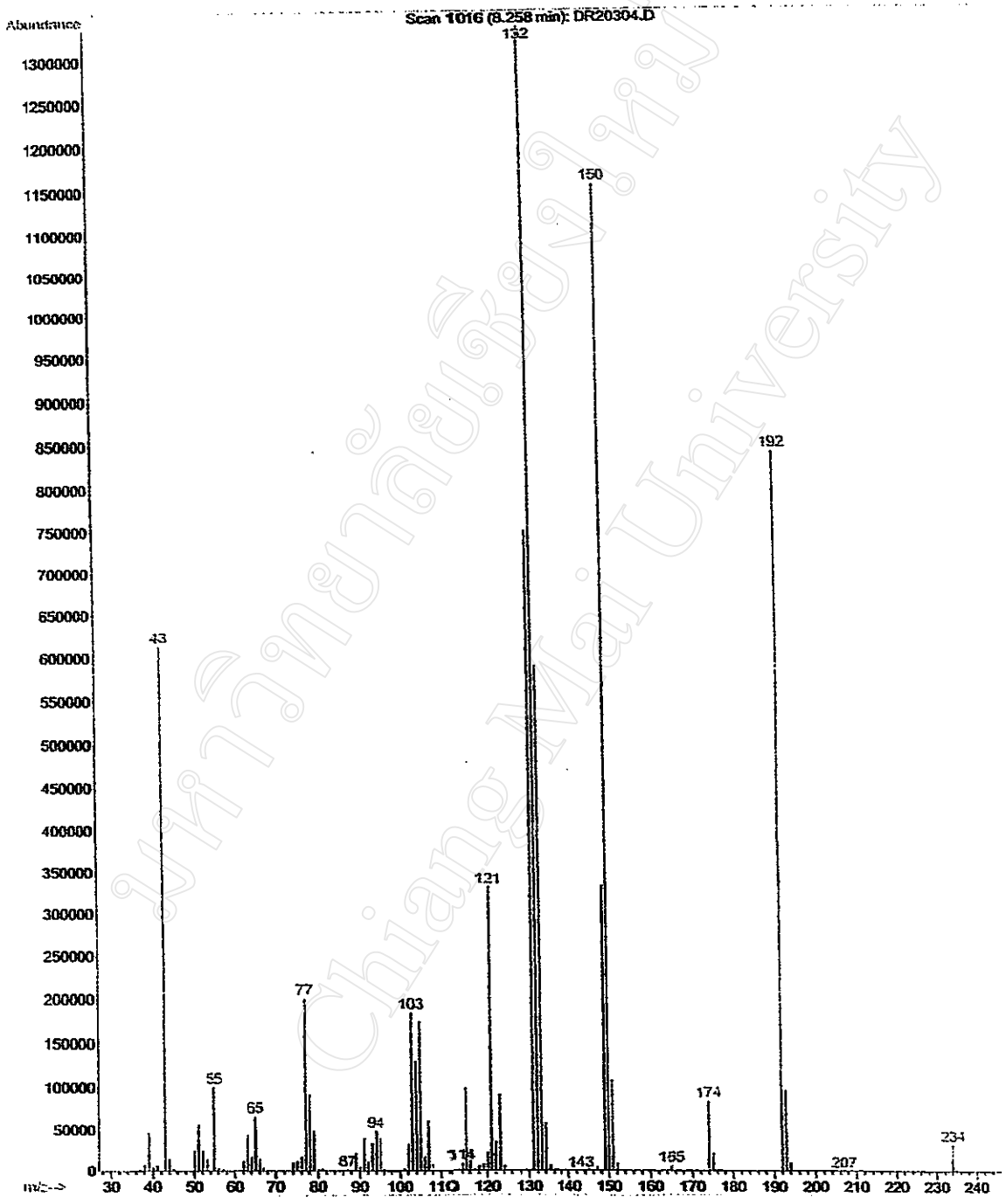



**Figure 3.18** The data file of 1′– acetoxychavicol acetate from GC-MS.

```
File       : C:\HPCHEM\1\DATA\DR20304.D
Operator   : Pisan
Acquired   : 21 Mar 2001  10:25     using AcqMethod 112HT
Instrument :   GC/MS Ins
Sample Name: acetoxy chavicol
Misc Info  :
Vial Number: 4
```

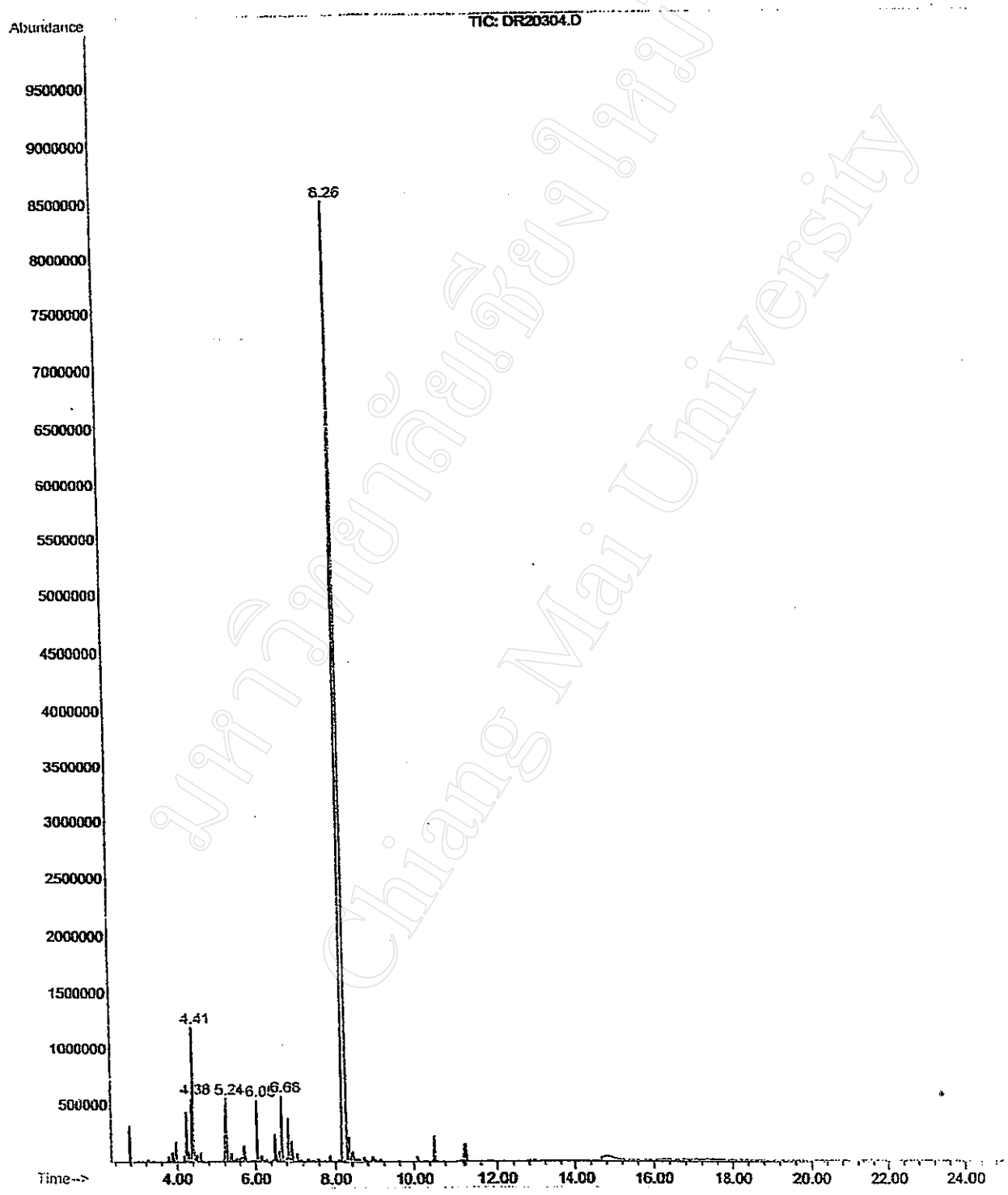



**Figure 3.19** The major component as 1′– acetoxychavicol acetate in the data file of GC-MS.

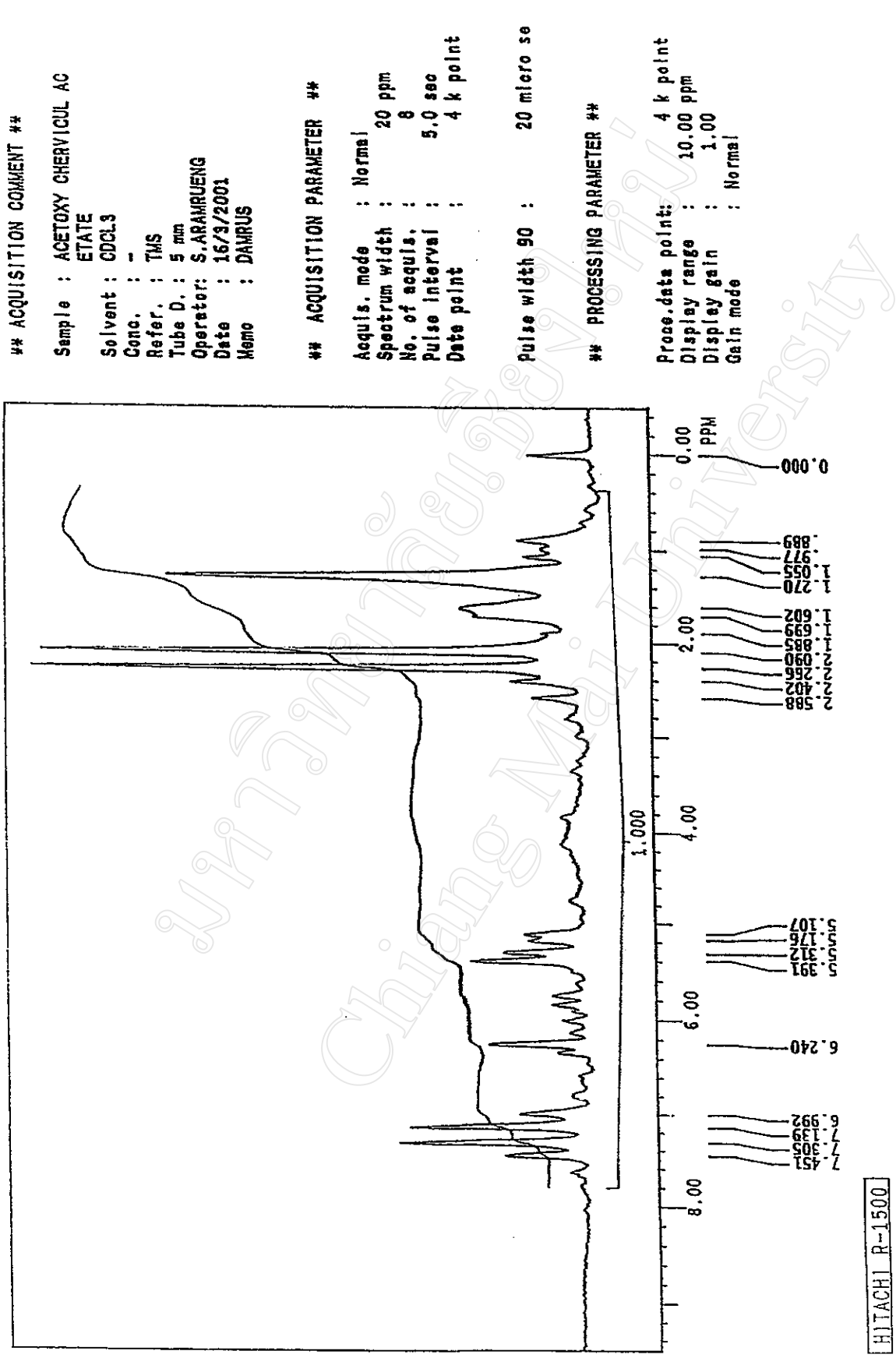



**Figure 3.20** Acquisition comment of 1′– acetoxychavicol acetate from IR –resonace (HITACHI R-1500).

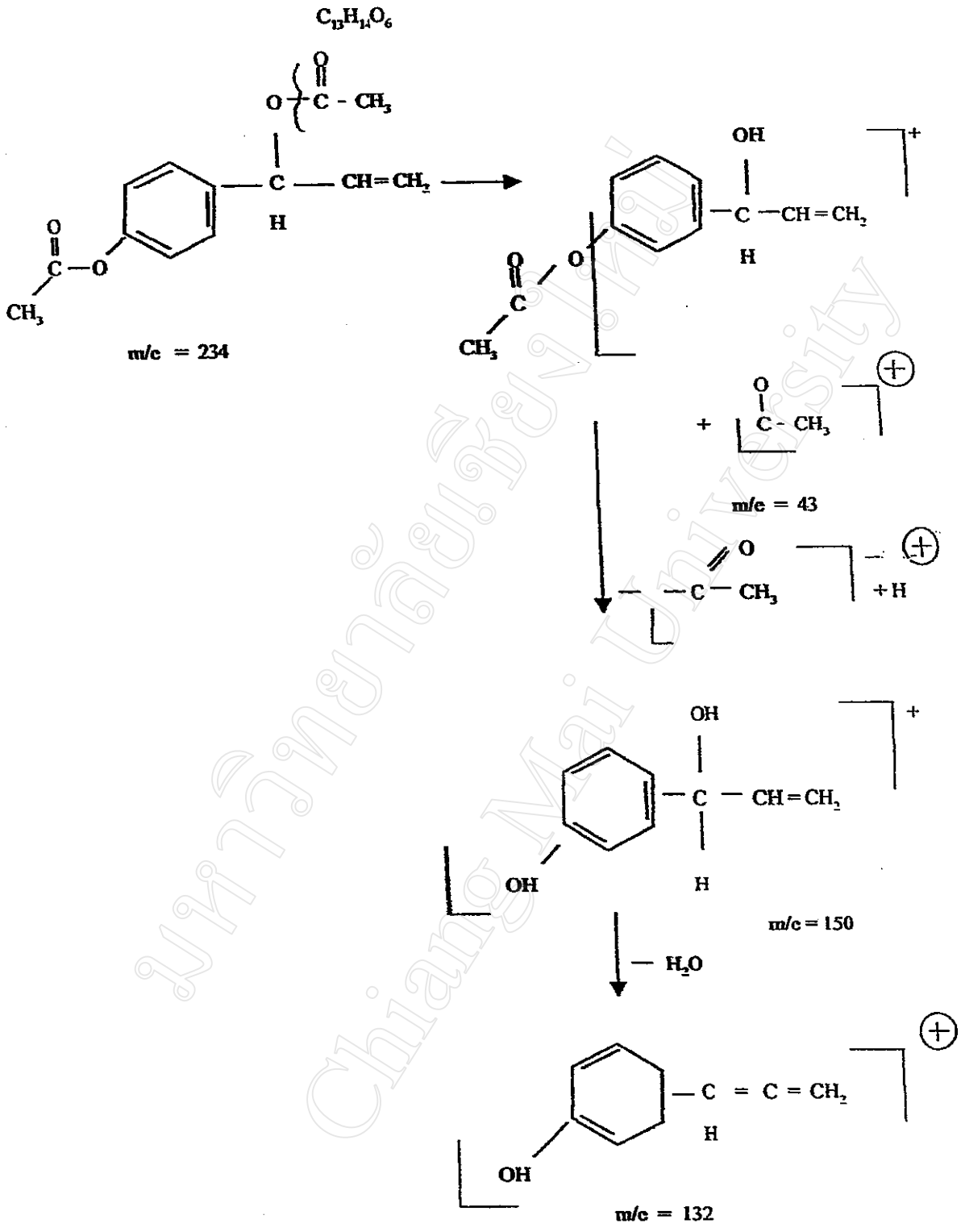


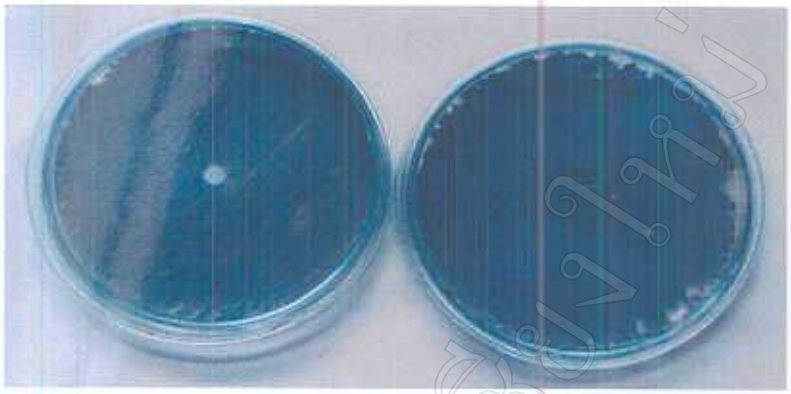

**Figure 3.21** Proposed fragmentation of 1′ acetoxychavicol acetate.

**3.6.6 Selection of the most efficiency antifungal ingredient**

The studies were divided in to three parts; Minimum inhibitory concentration (MIC) study, effective dosage ($ED_{50}$) and spore germination inhibition.

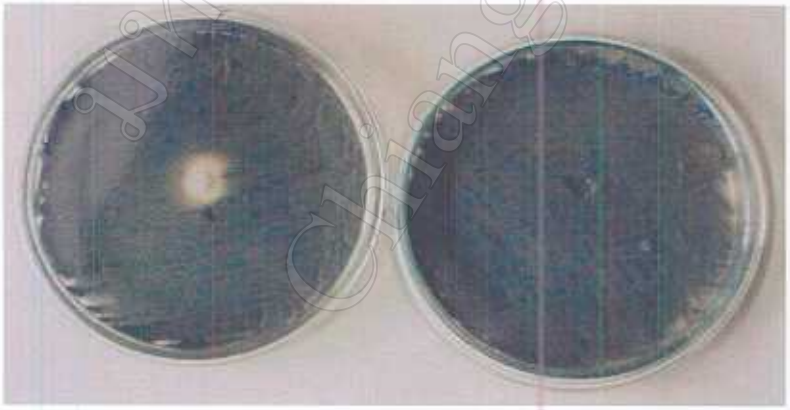
**1) Minimum inhibitory concentration (MIC) study**

As showed in Figure 3.22, clear Zones of Cladosporium cladosporioides grown on PDA were clearly observed firstly in the treatment with paper disc saturated with β-asarone at 70 ppm and with 1′ acetoxychavical acetate at 100 ppm. Sornsrikampol (1996) also showed the similar result of 100 ppm. when study on the purified extraction from galanga. But no report was found doing on sweetflag or β-asarone.



Control $H_2O$

MIC β-asarone 70 ppm.



MIC 1′ acetoxychavical acetate 100 ppm.

**Figure 3.22** Clear zone of *Colletotrichum cladosporioides* surroundy paper disc saturated with β - asarone at the concentration 70 and 1′–acetoxychavicol at 100 ppm. compared to control.

**2) Effective dosage ($ED_{50}$)**

As shown in Table 3.6, β - asarone (active ingredient in sweet flag) trend to have more efficiency to control *Colletotrichum gloeosporioides* (Penz.)Sacc. than 1′–acetoxychavicol acetate from galanga. At the concentration of 500 ppm β - asarone inhibit the growth at 65.74%, whereas 1′–acetoxychavicol acetate inhibited only 55.55%. After calculation with dosage response curve (DR-curve) the 50% inhibition was achieved for β - asarone at 200 ppm and for 1′–acetoxychavicol acetate at 350 ppm (Figure 3.23 ).

$ED_{50}$ (β - asarone) = 200 ppm

$ED_{50}$ (1′– acetoxychavicol acetate) = 350 ppm

There are so far vary lacking of information on $ED_{50}$ of natural substance to control *Colletotrichum gloeosporioides* (Penz.) Sacc. in especially the active substance from galanga. Only Bhasabuttra (1997) reported $ED_{50}$ value for sweet flag to control *Colletotrichum gloeosporioides* (Penz.) Sacc. at 200 ppm.

**Table 3.6** Percent inhibition of active substance on mycelium growth of *Colletotrichum gloeosporioides* (Penz.) Sacc. according to Abbott's formula.

| Plant | Active ingredient | Concentration (ppm) | | | |
|---|---|---|---|---|---|
| | | 50 | 100 | 200 | 500 |
| Sweet flag | β - asarone | 17.22 $^{1/}$ | 31.48 | 42.59 | 65.74 |
| | Probit | 4.0545 | 4.5172 | 4.8132 | 5.405 |
| Galanga | 1′ – acetoxychavicol acetate | 9.26 | 15.56 | 43.52 | 55.55 |
| | Probit | 3.675 | 4.010 | 4.840 | 5.140 |

Means in the same column followed by different superscript differs significantly at $p<0.05$

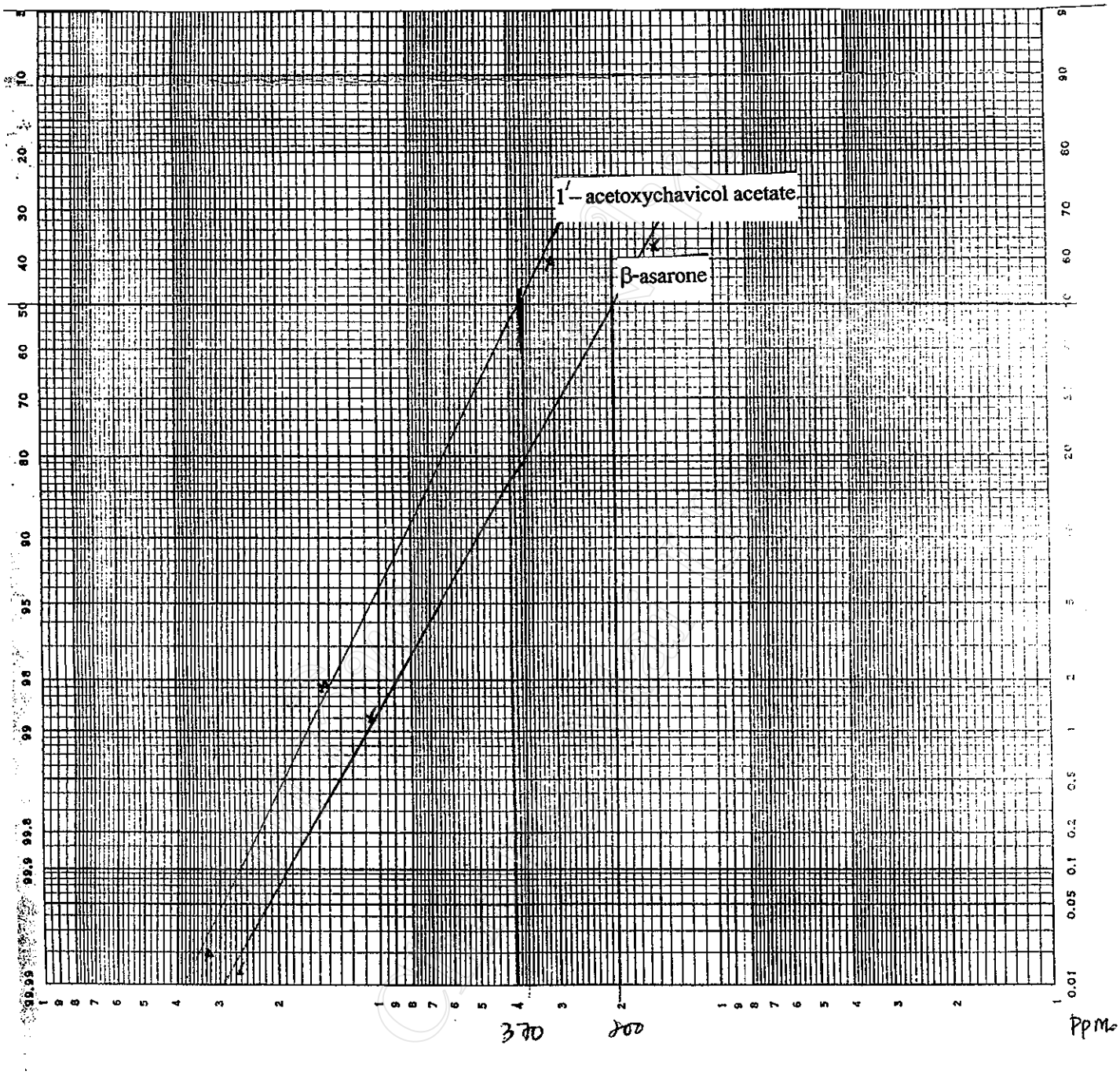



**Figure 3.23** Dosage response curve (DR-curve) of β-asarone and 1′– acetoxychavicol acetate.

$ED_{50}$, beta-asarone = 200 ppm

1′– acetoxychavicol acetate = 370 ppm

**3) Efficiency to inhibit spore germination**

Inhibition of spore germination was also calculated by Abbott's formula and showed in Table 3.7. Sweetflag also showed a higher percentage inhibition of spore germination than galanga in especially at the concentration of 50 and 100 ppm. At 150 ppm both β - asarone and 1′ – acetoxychavicol acetate gave the best result to control spore germination of 94.78 and 91.76%, respectively. (Figure 3.23, 3.24, 3.25) The results confirmed the work of Bhasabuttra (1997) on sweetflag active substance to control spore germination of *Colletotrichum gloeosporioides* (Penz.) Sacc.

According to Harborne *et al.* (1999) crude extract from sweetflag (*Acorus calamus* L.) contains both cis – isomer of β - asarone and trans – isomer of α - asarone. Both substance has antifungal activity, insect chemostericant, and insect attractant. However β - asarone was reported to be carcinogenic in animal study. Essential oil from sweetflag (calamus oil) has been banned in USA, except α - asarone . In this experiment, we also found both cis-and trans – isomer of asarone. For safety use of sweetflag crude extract. β - asarone must be firstly separated, in especially application in food product. Thinking about additional investment cost for this separating procedure of β - asarone, 1′ – acetoxychavicol acetate form galanga may be more interesting. There are many reports on non – carsinogenic activity of 1′ – acetoxychavicol acetate.

**Table 3.7** Percentage inhibition of spore germination of *Colletotrichum gloeosporioides* (Penz.) Sacc. as affected by active substance from sweetflag and galanga.

| Plant | Active ingredient | Concentration (ppm) | | |
|---|---|---|---|---|
| | | 50 | 100 | 150 |
| Sweetflag | β - asarone | 62.77 a | 76.97 a | 94.78 ns |
| Galanga | 1′ – acetoxychavicol acetate | 41.61 b | 67.95 b | 91.76 |
| C.V. % | | 18.31 | 26.35 | 14.01 |

Means in the same column followed by different superscript differs significantly at $p<0.05$

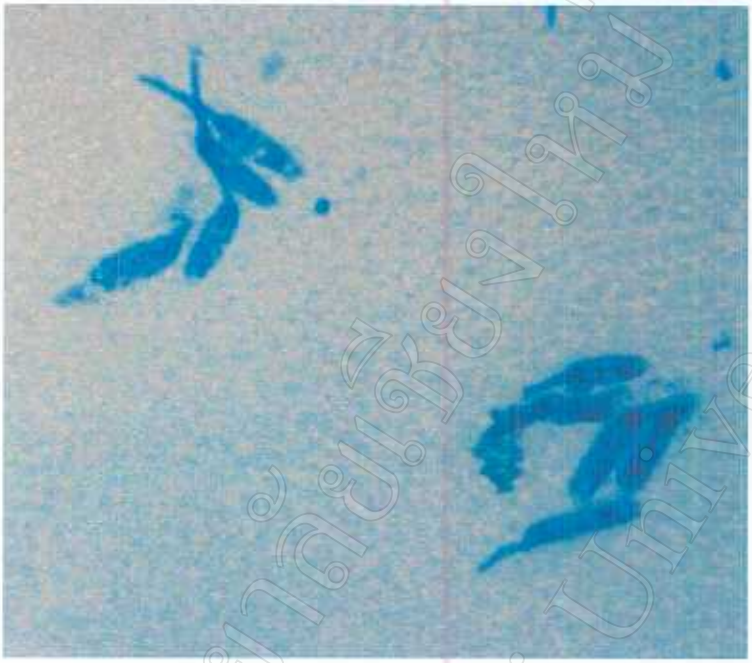


**Figure 3.24** Germination of spore of *Colletotrichum gloeosporioides* (Penz.) Sacc. in control treatment.

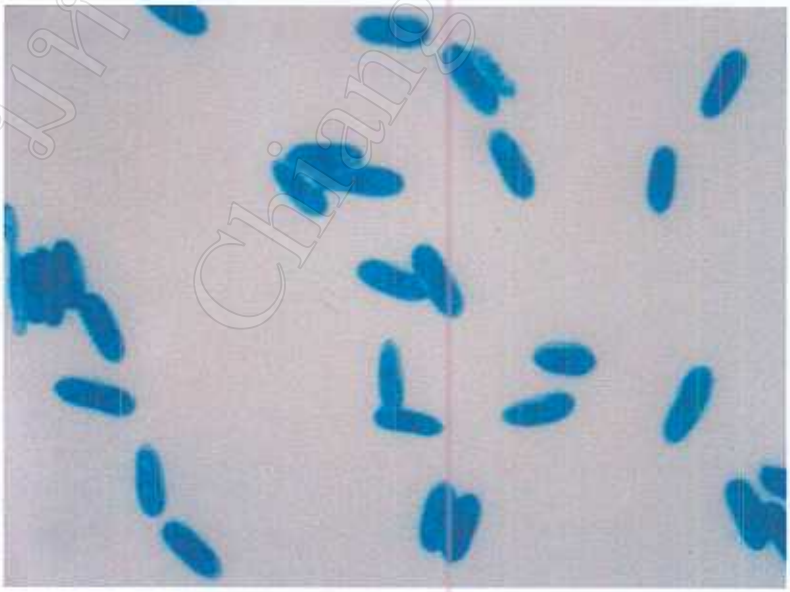


**Figure 3.25** Inhibition of spore germination in *Colletotrichum gloeosporioides* (Penz.)Sacc. when treated with 1′ – acetoxychavicol acetate.

### 3.4 Conclusion

3.4.1 Among the studied plants and solvents, only dichloromethane crude extract from galanga and sweetflag showed a positive efficiency to inhibit spore germination of *Colletotrichum gloeosporioides* (Penz.) Sacc.. Dichloromethane crude extract from *Rhinacanthus nesuhes* Kunz. had no antifungal efficiency to control *Colletotrichum gloeosporioides* (Penz.) Sacc..

3.4.2 Structural elucidation by GC-MS and IR - resonance confirmed the active ingredient in galanga and sweetflag to be $1'$ – acetoxychavicol acetate and CIS - β - asarone, respectively. TLC-bioassay with *C. cladosporioides* still showed more than one clear zone with different *Rf* value. This required more detail study on chemical structure of active ingredient both in galanga and sweetflag.