CHAPTER III

RESULTS AND DISCUSSION

The antifungal activity of selected essential oils was examined. The biological activity studied included spore germination, mycelial growth and sporulation. The possibility of the utilization of oils as postharvest treatment agents was also investigated. Evaluation of antifungal activity was performed by the agar medium assay. The effects of individual components in effective essential oils were observed by the bioautographic assay. Banana (*Musa* spp.) was used for the application of the essential oils for postharvest disease control.

3.1 Hydrodistillation results

Selected plants were hydrodistillated according to the procedure described in Chapter II for preliminarily antifungal activity screening test. The results of hydrodistillation are shown in Table 3.1.

Table 3.1 The results of hydrodistillation of some selected plants

No	Plant	Plant parts	Weight (g), (% w/w)
1	*Cymbopogon citratus* Stapf.	Aerial part (F: 500 g)	0.49 (0.10)
2	*Ocimum gratissimum* Linn.	Leaves (F: 700 g)	0.59 (0.08)
3	*Ocimum sanctum* Linn.	Leaves(F: 100 g)	0.16 (0.16)
4	*Hyptis suaveolen* Poit.	Leaves (F: 1400 g)	0.08 (0.006)
5	*Piper chaba* Hunt.	Fruits (D: 1000g)	0.58 (0.06)
6	*Piper sarmentosum* Roxb	Leaves (F: 200g)	0.28 (0.14)
7	*Citrus hystrix* DC.	Leaves (F: 200 g)	1.13 (0.57)
8	*Limnophila aromatica* Merr.	Aerial part (F: 400 g)	0.46 (0.12)
9	*Alpinia galanga* Sw.	Rhizomes (F: 400 g)	0.32 (0.08)
10	*Curcuma aromatica* Salisb.	Rhizomes(F: 200 g)	0.33 (0.17)
11	*Curcuma domestica* Valeton.	Rhizomes (F: 200 g)	0.75 (0.38)
12	*Kaempferia galanga* Linn.	Rhizomes (F: 200 g)	0.23 (0.12)
13	*Boesenbergia pandurata* Holtt.	Rhizomes (F: 400 g)	0.18 (0.05)
14	*Zingiber cassumunar* Roxb.	Rhizomes (F: 2800 g)	4.25 (0.15)
15	*Zingiber officinale* Roseoe.	Rhizomes (F: 1700 g)	0.53 (0.03)

D: Dry sample, F: Fresh sample

The hydrodistillation of selected Thai spices yielded variable amounts of essential oils ranging from 0.006 to 0.57 % (w/w) of fresh (or dry) weight. It was found that *Citrus hystrix* DC. (Rutaceae) and *Hyptis suaveolen* Poit. (Lamiaceae) provided the highest and the lowest amount of essential oil, respectively.

3.2 The preliminary screening for antifungal activity

Fifteen essential oils extracted as aforementioned, together with ten commercial grade pure essential oils were preliminarily screened for antifungal activity against *Fusarium oxysporum* 43-68, *Alternaria* sp.43-89 and *Phytophthora* sp. 572. at 1000 ppm. Fungi were treated with tested chemicals in various modes. The antifungal activity commonly used included the suppression of mycelial growth , respiration and spore germination (Heinrich, 1990). The antifungal activity assayed in this experiment included mycelial growth inhibition and spore germination. The results are shown in Tables 3.2 and 3.3.

Table 3.2 The effect of essential oils on mycelial growth inhibition

No	Plant	*Fusarium*	*Alternaria*	*Phytophthora*
1	*Pelargonium graveolens*	+++	++++	++++
2	*Cymbopogon citratus* Stapf.	++	++	++
3	*Cymbopogon nardus* Rendle.	+++	-	++++
4	*Mentha cordifolia* Opiz.	++++	++++	++++
5	*Ocimum gratissimum* Linn.	+++	++++	++++
6	*Ocimum sanctum* Linn.	+++	++++	++++
7	*Rosemarinus officinalis*	+	+	++
8	*Hyptis suaveolens* Poit.	-	ND	ND
9	*Cinnamomum bejolghota* Sweet.	++++	++++	++++
10	*Litsea cubeba* Pers.	++++	++++	++++
11	*Eucalyptus citriodora*	+	-	++
12	*Eugenia caryophyllus* Bullock & Harrison.	++++	++++	++++
13	*Jasminum officinalis*	-	-	-
14	*Piper chaba* Hunt.	+	-	++
15	*Piper sarmentosum* Roxb.	-	ND	ND
16	*Citrus hystrix* DC.	++	+++	++++
17	*Citrus reticulata* Blanco.	-	-	-
18	*Limnophila aromatica* Merr.	+++	++++	++++
19	*Alpinia galanga* Sw.	+++	-	++++
20	*Curcuma aromatica* Salisb.	++	++	-
21	*Curcuma domestica* Valeton.	++	++	++
22	*Kaempferia galanga* Linn.	++	++++	++++
23	*Boesenbergia pandurata* Holtt.	+	-	+++
24	*Zingiber cassumunar* Roxb.	+++	++++	++++
25	*Zingiber officinale* Roseoe.	++++	+	++++

Note: (+) = 1-30 % inhibition, (++) = 31-60 % inhibition, (+++) = 61-85 % inhibition, (++++) = 86-100% inhibition, (-) = not significantly different from control, (ND) = not determined

Tested essential oils were incorporated into the PDA medium, which was then inoculated with fungal mycelium and incubated at room temperature. The reduction in mycelial growth was observed compared to that of the control treatment. The preliminarily screening revealed that the oils from *Mentha cordifolia* Opiz., *Cinnamomum bejolghota* Sweet, *Litsea cubeba* and *Eugenia caryophyllus* Bullock & Harrison. showed complete mycelial growth inhibition in all tested fungi.

The results obtained with the agar medium assay should be assessed critically such as the nature of dissolved test substance in agar medium. The dissolution of active compound in agar medium has an effect upon fungus (Victor, 1991).

In every infectious disease, a series of distinct events occurs in succession to the development and perpetuation of the disease pathogen. The disease cycle had related on disease development. The spore infest in plant debris or contaminate in farm equipment or contaminate in environment can stay alive as resting spore when the condition optimized, resting spore will germinate germ tubes, attack host and cause plant disease. Therefore, each essential oil was further evaluated for spore germination of *Fusarium oxysporum* 43-68 and *Alternaria* sp.43-89. The results are shown in Table 3.3 and Figs. 3.1-3.2.

Table 3.3 The effect of essential oils on conidial germination

No.	Plant	% Germination	
		Fusarium	*Alternaria*
1	*Pelargonium graveolens*	67	63
2	*Cymbopogon citratus* (DC.) Stapf.	89	96
3	*Cymbopogon nardus Rendle.*	100	100
4	*Mentha cordifolia* Opiz .	86	74
5	*Ocimum gratissimum* Linn.	62	79
6	*Ocimum sanctum* Linn.	100	78
7	*Rosemarinus officinalis*	79	100
8	*Hyptis suaveolens Poit.*	ND	ND
9	*Cinnamomum bejolghota* Sweet.	51	46
10	*Litsea cubeba*	98	76
11	*Eucalyptus* citriodora	81	ND
12	*Eugenia caryophyllus* Bullock & Harrison.	54	52
13	*Jasminum officinalis*	88	100
14	*Piper chaba* Hunt.	ND	ND
15	*Piper sarmentosum* Roxb.	ND	ND
16	*Citrus hystrix* DC.	86	100
17	*Citrus reticulata* Blanco.	84	100
18	*Limnophila aromatica* Merr.	67	66
19	*Alpinia galanga* Sw.	79	72
20	*Curcuma aromatica* Salisb.	83	87
21	*Curcuma domestica* Valeton	ND	94
22	*Kaempferia galanga* Linn.	ND	ND
23	*Boesenbergia pandurata* Holtt.	100	ND
24	*Zingiber cassumunar* Roxb.	67	61
25	*Zingiber officinale* Roseoe.	79	ND

Note: % germination calculated compare with control

ND= not determined

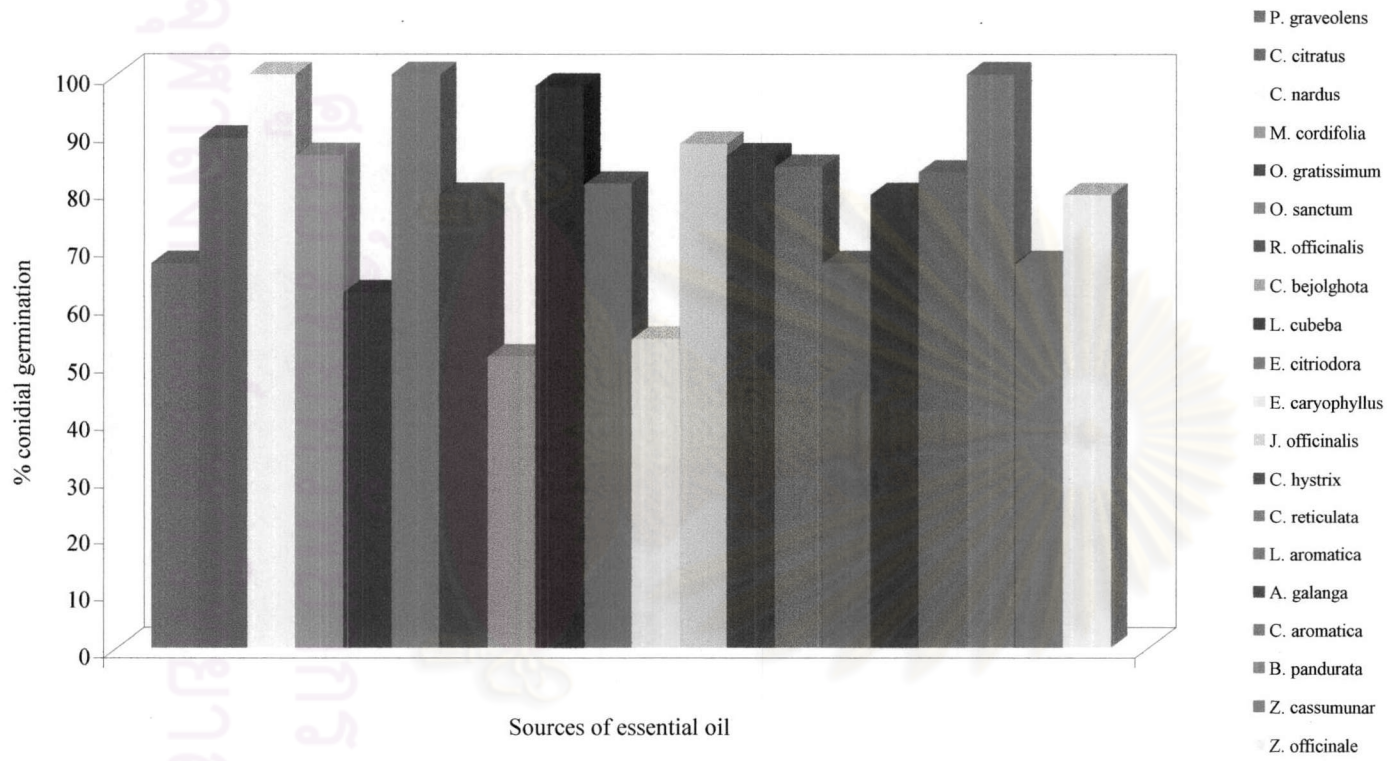


Fig. 3.1 The effect of essential oils on spore germination of *F. oxysporum* 43-68

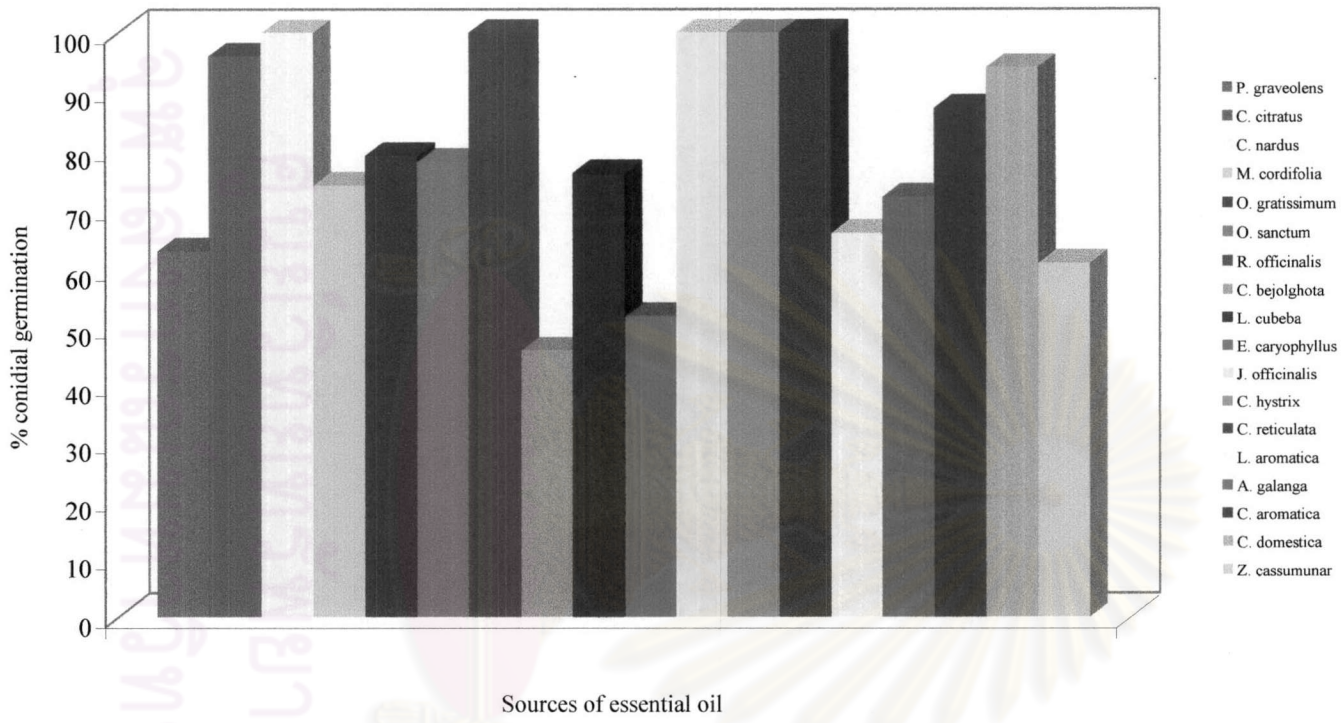


Fig. 3.2 The effect of essential oils on spore germination of *Alternaria* sp. 43-89

The preliminary screening for mycelial growth inhibition activity against *Photophthora* sp. 572. revealed that fourteen oils (sample no. 1, 3-6, 9-10, 12, 16, 18-19, 22 and 24-25) exhibited complete inhibition. Five (sample no. 4, 9-10, 12 and 25) and ten (sample no. 1, 4-6, 9-10, 12, 18, 22 and 24) oils were detected for the effect on *F. oxysporum* 43-68 and *Alternaria* sp. 43-89, respectively. Considering the difference in chemical resistance between fungal spores and vegetative cells, it was suggested that the fungal spores be more tolerant than the vegetative cells. *Phytophthora* sp. which are extremely sensitive to heat and desiccation (Alexopoulos, 1996), may be more susceptible to chemicals or other components in medium than *F. oxysporum* and *Alternaria* sp.

The results of conidial germination after 36-hour incubation were varied depending on type of the essential oil. The highest germination percentage was observed in the control plates. The antifungal activity against *F. oxysporum* 43-68 revealed that fifteen essential oils (sample no. 1-2, 4-5, 7, 10-11, 13, 16-20 and 24-25) exhibited slight germination suppression (more than 60% germination). Three essential oils (sample no. 3, 6 and 23) did not show any inhibition activity (100% germination). Only two essential oils from *Cinnamomum bejolghota* Sweet. and *Eugenia caryophyllus* Bullock & Harrison. (sample no. 9 and 12) exhibited moderate inhibition activity at the concentration of 1000 ppm.

The antifungal activity against conidial germination of *Alternaria* sp. 43-89 revealed that eleven essential oils (sample no. 1-2, 4-6, 10, 18-21 and 24) exhibited slight germination suppression (more than 60% germination). Five essential oils (sample no. 3, 7, 13 and 16-17) did not show significant inhibition activity (100% germination). The essential oils from *Cinnamomum bejolghota* Sweet. and *Eugenia caryophyllus* Bullock & Harrison. (sample no. 9 and 12) also showed moderate activity.

Although, *Fusarium oxysporum* 43-68 and *Alternaria* sp. 43-89 are two representatives of higher fungi but they are susceptible to distinct compound. *Fusarium oxysporum* has been previously reported that it was more resistant to antifungal agents than *Alternaria* sp. (Oku, 1993).

The antifungal activity on mycelial growth and spore germination greatly affects the costs and benefits of fungal disease control. If the substances were active for inhibition of both mycelial growth and spore germination, it would then be able to control all the subsequent steps in the disease cycle. However, if the active substances

had pronounced affected on either mycelial growth or spore germination inhibition, then the efficiency of the substances on disease control may be improved when applied with other treatments. This study showed that the essential oils from *Cinnamomum bejolghota* Sweet. and *Eugenia caryophyllus* exhibited active inhibition of both mycelial growth and spore germination.

3.3 Further study on antifungal activity of selected essential oil

Further evaluation of antifungal activity of selected essential oils was determined by agar medium assay and their IC_{50}'s were calculated. Each selected oil was diluted to the final concentration of 1, 10, 100 and 1000 ppm. A mycelial disc inoculated each plate, and the fungi were allowed to grow. The antifungal activity was observed 7 days (*F. oxysporum*) and 14 days (*Alternaria* sp.) after inoculation.

3.3.1 Fusarium oxysporum 43-68 mycelial growth inhibition

According to the results of the preliminary screening, five essential oils showing complete inhibition against *F. oxysporum* 43-68 were further investigated for mycelial growth inhibition activity. The results are shown in Table 3.4 and Fig. 3.3.

Table 3.4 Mycelial growth inhibition percentage and IC_{50} of selected essential oils against *F. oxysporum* 43-89

Plant	Inhibition percentage				IC_{50}[a] (ppm)
	1 ppm	10 ppm	100 ppm	1000 ppm	
Mentha cordifolia Opiz.	0.58	7.49	15.93	100	195
Cinnamomum bejolghota Sweet.	0.95	3.07	15.71	100	177
Litsea cubeba Pers.	3.27	4.92	34.01	100	125
Eugenia caryophyllus Bullock &Harrison.	1.72	5.18	38.77	100	115
Zingiber officinale Roseoe.	2.50	28.83	61.54	100	73

[a] Inhibition percentage of each concentration was calculated as IC_{50} using Probit Analysis Program

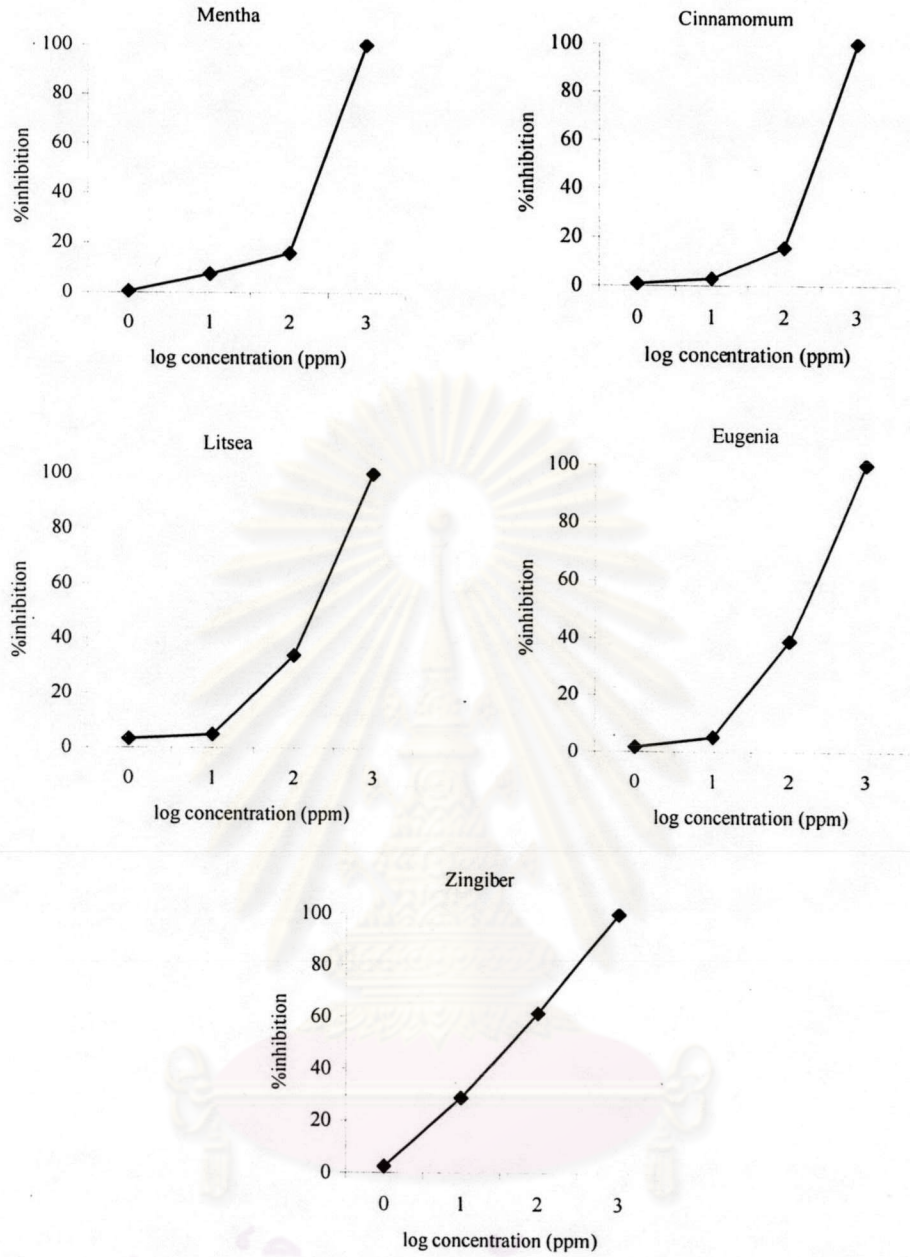


Fig. 3.3 Concentration effect of essential oils on mycelial growth of *Fusarium oxysporum* 43-68

Table 3.5 Sporulation inhibition percentage of selected essential oils

Plant	control plate (spore/cm^2)	Treated plate[a] (spore/cm^2)	% Sporulation
Mentha cordifolia Opiz.	2.8x10^5	185	0.07
Cinnamomum bejolghota Sweet.	3.0 x10^5	136	0.05
Litsea cubeba Pers.	2.6 x10^5	818	0.31
Eugenia caryophyllus Bullock &Harrison.	2.8 x10^5	248	0.09
Zingiber officinale Roseoe.	4.0 x10^5	1175	0.29

Note : [a] concentration : 100 ppm of tested essential oil

: sporulation percentage of each essential oil was calculated compare with sporulation in control plate

: sporulation was determined by collected spore by scrapped surface with glass rod and estimated by haemocytometer and converted to spores per square centimeter of plate culture.

All selected essential oils showed complete mycelial growth inhibition against *Fusarium oxysporum* 43-68 at 1000 ppm. Essential oil from *Zingiber officinale* Roseoe displayed the lowest IC_{50} at 73 ppm. Sporulation of the fungi was significantly suppressed by all essential oils tested.

3.3.2 *Alternaria* sp. 43-89 mycelial growth inhibition

Ten essential oils showing high activity from the preliminary screening test against *Alternaria* sp. 43-89 were evaluated for the mycelial growth inhibition and IC_{50} at various concentrations. The results are shown in Table 3.5 and Fig. 3.4.

Table 3.6 Mycelial growth inhibition percentage and IC_{50} of selected essential oils against Alternaria sp. 43-89

Plant	Inhibition percentage				IC_{50} [a]
	1 ppm	10 ppm	100 ppm	1000 ppm	(ppm)
Pelargonium graveolens	13.14	9.56	15.17	100	303
Mentha cordifolia Opiz.	7.43	8.42	3.22	100	-
Ocimum gratissimum Linn.	15.85	17.25	22.86	100	233
Ocimum sanctum Linn.	15.48	11.37	8.22	100	-
Cinnamomum bejolghota Sweet.	3.80	4.79	28.73	100	139
Litsea cubeba Pers.	-2.82	-3.24	11.69	100	-
Eugenia caryophyllus Bullock & Harrison.	6.10	4.69	23.94	100	162
Limnophila aromatica Merr.	15.49	16.48	28.17	100	177
Kaempferia galanga Linn.	12.68	11.27	26.76	100	158
Zingiber cassumunar Roxb.	2.70	5.40	33.11	100	127

Note: [a] Inhibition percentage of each concentration were calculated IC_{50} using Probit Analysis Program

(-) : can not analyzed by Probit Analysis Program

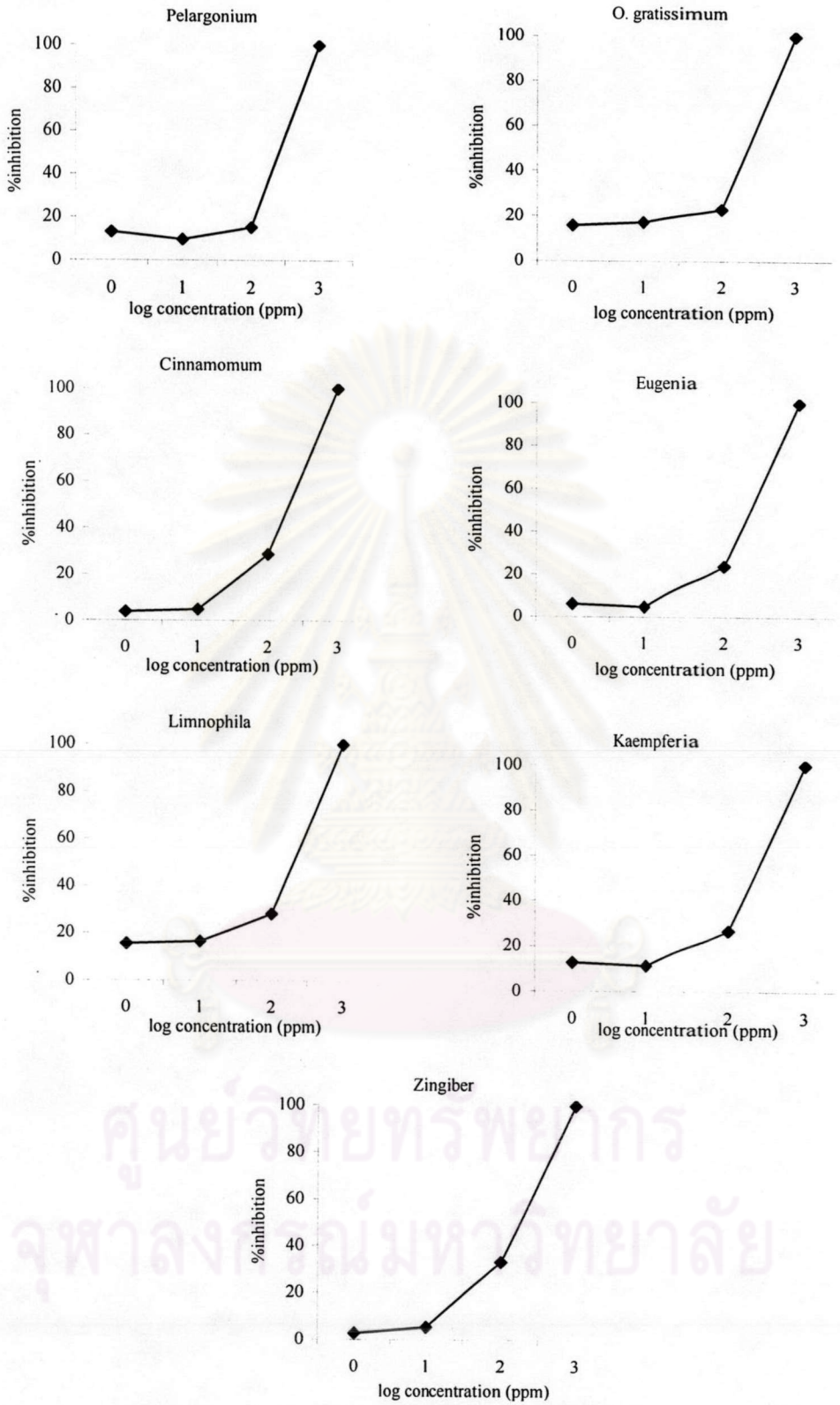


Fig. 3.4 Concentration effect of essential oil on mycelial growth inhibition of *Alternaria* sp. 43-89

Table 3.7 Sporulation inhibition percentage of selected essential oils

Plant	control plate (spore/cm^2)	Treated plate[a] (spore/cm^2)	% Sporulation
Pelargonium graveolens	$5.5x10^5$	0	0
Mentha cordifolia Opiz.	$3.0 x10^5$	114	0.04
Ocimum gratissimum Linn.	$3.0 x10^5$	134	0.04
Ocimum sanctum Linn.	$3.2 x10^5$	$1.5 x10^4$	4.69
Cinnamomum bejolghota Sweet.	$4.2 x10^5$	248	0.06
Litsea cubeba Pers.	$3.3 x10^5$	$5.9 x10^5$	179
Eugenia caryophyllus Bullock & Harrison.	$3.3 x10^5$	$1.1 x10^4$	3.31
Limnophila aromatica Merr.	$3.3 x10^5$	159	0.05
Kaempferia galanga Linn.	$4.2 x10^5$	170	0.04
Zingiber cassumunar Roxb.	$5.2 x10^5$	562	0.12

Note : [a] concentration : 100 ppm of tested essential oil

sporulation percentage of each essential oil was calculated compare with sporulation in control plate

: sporulation was determined by collected spore by scrapped surface with glass rod and estimated by haemocytometer and converted to spores per square centimeter of plate culture.

The high level of mycelial growth inhibition was detected from the essential oils from *Zingiber cassumunar* Roxb., *Cinnamomum bejolghota* Sweet., *Kaempferia galanga* Linn., *Eugenia caryophyllus* Bullock & Harrison. and *Limnophila aromatica* Merr. with IC_{50} of 127, 139, 158, 162 and 177, respectively. In addition, the essential oil from *Pelargonium graveolens* exhibited very high antifungal activity. It completely suppressed *Alternaria* sporulation at 100 ppm. Other essential oils exhibited significantly suppression except the essential oil from *Litsea cubeba* Pers. was not effective.

Clove oil and cinnamon oil have been previously reported to have antifungal property against several phytopathogenic fungi, such as *Aspergillus* spp., *Penicillium* spp. The initiation of the fungal growth on PDA was delayed for 6 days compared to 2 days of the control (Muftah and Lloyd, 1982). Moreover, cinnamon oil at the concentration as low as 0.02% (v/v) inhibited mycelial growth and aflatoxin production of *Aspergilus niger* in yeast extract sucrose broth (Bullerman, Lieu and

Seier, 1977). The clove bud oil also showed complete inhibition of *Botrytis cinerea* spore germination at 0.78% (v/v) up to 24 h whereas the cinnamon leaf oil inhibition the spore germination at 1.56 % (v/v) up to 40 h (Wilson *et al.* 1997). In addition, clove oil has been investigated for antifungal activity towards sensitive fungus species such as *Alternaria alternata, Fusarium chlamydosporum, Helminthosporum oryzae* and *Rhizoctonia bataticola*, conidial cell lysis and mycelial growth inhibition were detected at 0.05%-20% (v/v) concentration (Beg and Ahmad, 2002). The results obtained in this study supported the information from those experiments. The strong antifungal activity against *Fusarium oxysporum* 43-68 and *Alternaria* sp 43-89. was observed in clove oil and cinnamon oil.

Ginger oil also showed strong antifungal activity towards *Fusarium oxysporum* 43-68. This is the first report of the antifungal activity of essential oil from ginger.

Essential oil from *Ocimum gratissismum* and *Pelargonium graveolens* had been tested for the antifungal activity against dermatophyte fungi (Lima *et al.* 1993) and food spoilage bacteria (Doemam and Deans, 2000), respectively. However, there was no other report on activity of the extracts from these plants against *Fusarium oxysporum* and *Alternaria* sp.

3.4 Evaluation for antifungal activity of essential oil component by bioautographic assay

Addition to the investigation of IC_{50} for all essential oils displayed in Table 3.3, active components were attempted to seek for in each essential oil using bioautographic assay.

3.4.1 Bioautographic assay for antifungal activity against *Fusarium oxysporum* 43-68

Component in five-selected crude essential oils from *Mentha cordifolia* Opiz, *Cinnamomum bejolghota* Sweet., *Eugenia caryophyllus* Bullock & Harrison., *Litsea cubeba* and *Zingiber officinale* Roseoe. were separated on TLC plate using Hex: EtOAc (85 : 15) as the developing solvent. The TLC plate was sprayed with spore suspension and was observed inhibition zone after incubating in a water agar for 3 days.

The crude essential oil from *Mentha cordifolia* Opiz. was chromatographed on TLC plate. Five different spots could be detected by UV_{254} and only two spots were clearly seen with orange and pink coloration. When the TLC plate was sprayed with spore suspension, only one inhibition zone was detected. The representative TLC plates are shown in Fig. 3.5

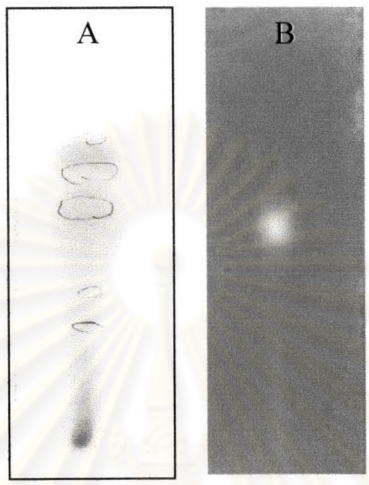


Fig. 3.5 Bioautographic pattern of *Mentha cordifolia* Opiz. against *F. oxysporum* 43-68 (A) and TLC profile (B)

The TLC pattern of *Cinnamomum bejolghota* Sweet. revealed solely one spot under UV_{254}. An additional gray-brown zone of cinnamaldehyde was observed. This spot also exhibited inhibition zone after conidial suspension of *Fusarium oxysporum* was sprayed. The representative TLC plates are shown in Fig. 3.6.

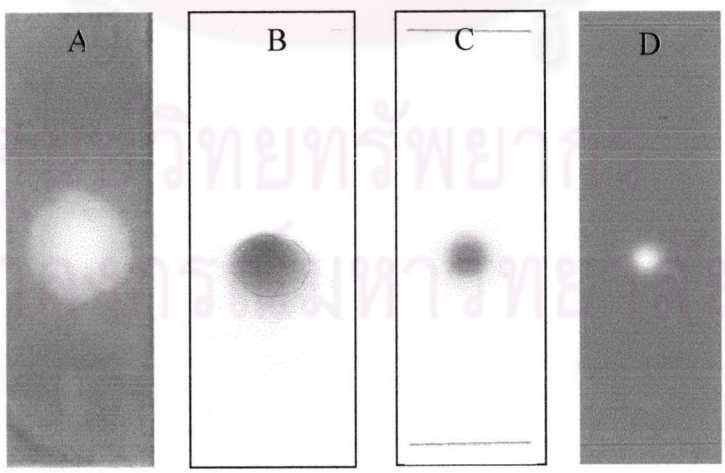


Fig. 3.6 Bioautographic pattern of *Cinnamomum bejolghota* Sweet against *F. oxysporum* 43-68 (A), cinnamon oil profile (B), authentic cinnamaldehyde (C) and bioautographic pattern of authentic cinnamaldehyde (D)

Essential oil from *Eugenia caryophyllus* Bullock & Harrison. clearly showed one spot under UV_{254} with a pink zone of eugenol. This spot also exhibited inhibition zone. In addition, imperceptible clear zone was observed from the spot present at the lower spot of eugenol. The TLC profiles are shown in Fig. 3.7.

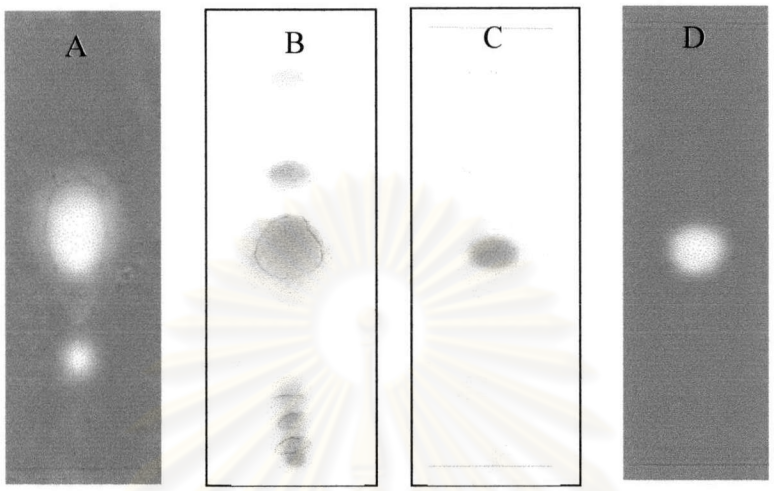


Fig. 3.7 Bioautographic pattern of *Eugenia caryophyllus* Bullock & Harrison. against *F. oxysporum* 43-68 (A), clove oil profile (B), authentic eugenol (C) and bioautographic pattern of authentic eugenol (D)

The essential oil from *Litsea cubeba,* exhibited gray-purple spot as a major component. This spot exhibited as well an inhibition zone. The illustration of TLC plates are shown in Fig. 3.8.

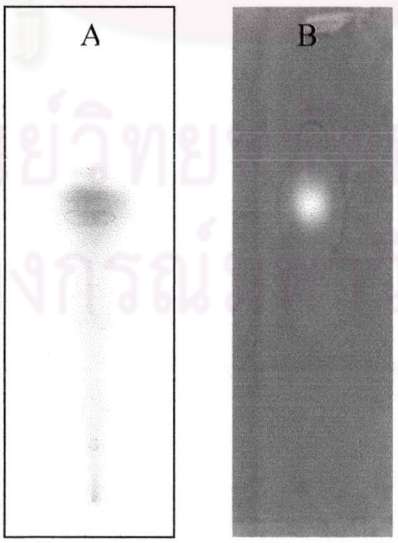


Fig. 3.8 Bioautographic pattern of *Litsea cubeba* against *F. oxysporum* 43-68 (A) and TLC profile (B)

For the essential oil from *Zingiber officinale* Roseoe., five spots were detected under UV_{254}. These spots had a clear yellowish zone but did not show inhibition zone. The diagram of TLC plates is shown in Fig. 3.9.

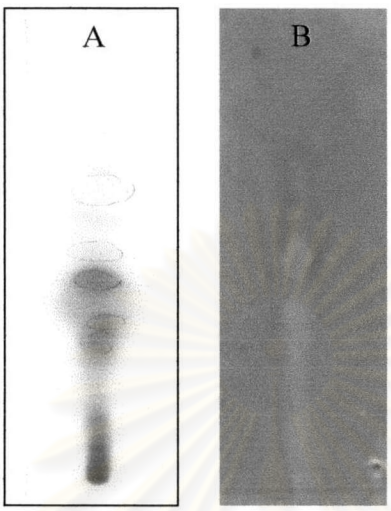


Fig. 3.9 Bioautographic pattern of *Zingiber officinale* Roseoe. against *F. oxysporum* 43-68 (A) and TLC profile (B)

3.4.1 Bioautographic assay against *Alternaria* sp. 43-89

Ten selected essential oils previously determined for IC_{50} in Table 3.6 were investigated for the antifungal activity by bioautographic assay against *Alternaria* sp. Each crude essential oil was developed on TLC plate using Hex: EtOAc = 85 : 15 as a developing solvent, sprayed with spore suspension and observed inhibition zone. The results displayed below.

The essential oil from *Cinnamomum bejolghota* Sweet. displayed one spot that absorbed UV_{254} and showed gray-brown zone of cinnamaldehyde. This spot also exhibited an inhibition zone. The illustration of TLC pattern is presented as shown in Fig. 3.10.

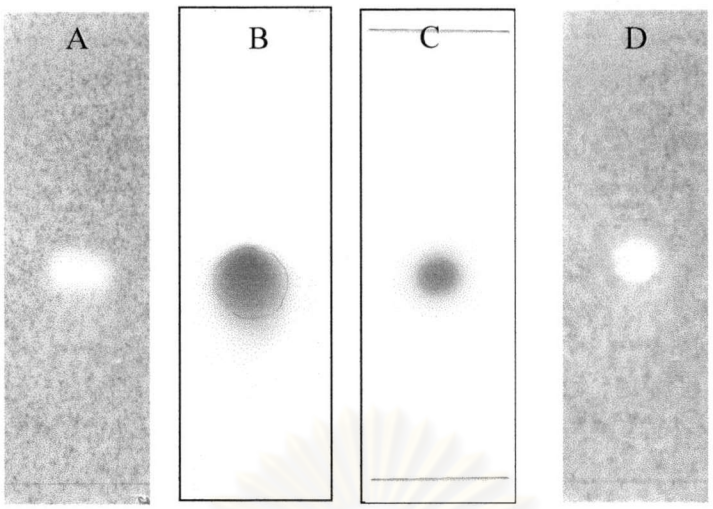


Fig. 3.10 Bioautographic pattern of *Cinnamomum bejolghota* Sweet. against *Alternaria* sp. 43-89 (A), cinnamon oil profile (B), authentic cinnamaldehyde (C) and bioautographic pattern of authentic cinnamaldehyde (D)

The inhibition zone of the essential oil from *Eugenia caryophyllus* Bullock & Harrison. against *Alternaria* sp. also evidently presented the same spot as eugenol. The other spots did not show inhibition activity. The pattern of bioautographic plate is shown in Fig. 3.11.

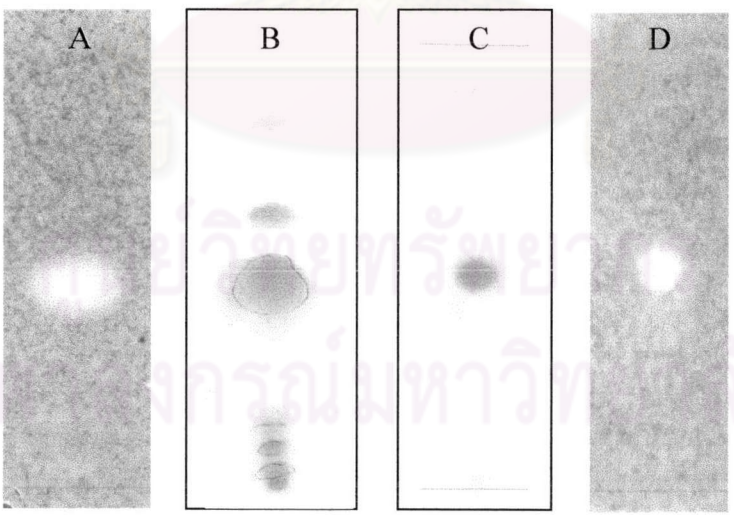


Fig. 3.11 Bioautographic pattern of *Eugenia caryophyllus* Bullock & Harrison. against *Alternaria* sp. 43-89 (A), clove oil profile (B), authentic eugenol (C) and bioautugraphic pattern of authentic eugenol (D)

From the results presented in Table 3.5, essential oils from *Cinnamomum bejolghota* Sweet. and *Eugenia caryophyllus* Bullock & Harrison. clearly exhibited inhibition zone while the rest of selected essential oils did not show inhibition zone on TLC plates.

Essential oils normally consist of many different volatile compounds. It has been suggested that the antifungal activity is the result of many compounds acting synergistically (Jenny, 2000). This means that the individual component itself may not be effective. In this research it was found that the crude essential oils with antifungal activity might possibly compose with only one major active substance.

3.5 Evaluation of antifungal activity of fractionated clove oil

Eugenol was separated from other components in clove oil by CH_2Cl_2 extraction as described in Chapter II. The isolated fractions were further examined for antifungal activity. The results are shown in Table 3.6 and Fig. 3.12.

Table 3.8 Mycelial growth inhibition percentage of the fractionated clove oil against *Fusarium oxysporum* 43-68

Fungal strain	Fractions	Percentage inhibition				IC_{50} (ppm)
		1 ppm	10 ppm	100 ppm	1000 ppm	
Fusarium oxysporium 43-68	Eugenol	-1.18	14.12	63.53	100	81
	Other components	9.41	14.12	27.06	63.53	759
Alternaria sp. 43-89	Eugenol	2.63	14.47	61.84	100	81
	Other components	2.63	2.63	2.63	53.95	954

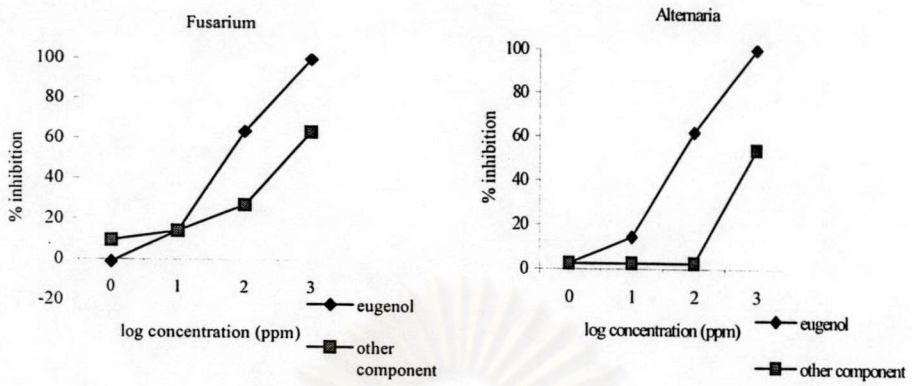


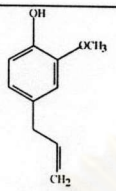
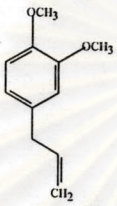
Fig. 3.12 The effect of fractionated clove oil on mycelial growth inhibition of *F. oxysporum* 43-68(A) and *Alternaria* sp. 43-89 (B)

The results revealed that the eugenol fraction showed higher antifungal activity than the others. Therefore, it is possible that the most active compound in the clove oil is eugenol.

3.6 The antifungal activity of eugenol, cinnamaldehyde and their derivatives

The essential oils from *Eugenia caryophyllus* Bullock & Harrison. and *Cinnamomum bejolghota* Sweet. exhibited potential antifungal activity. The major components: eugenol, cinnamaldehyde and related compounds were evaluated for antifungal activity against *Fusarium oxysporum* 43-68. The results are displayed in Tables 3.9 and 3.10.

Table 3.9 Mycelial growth inhibition percentage of eugenol and eugenol methyl ether against *F. oxysporum* 43-68

Compound	Structure	Inhibition percentage				IC_{50} (ppm)
		1 ppm	10 ppm	100 ppm	1000 ppm	
Eugenol		22.50	30.00	72.50	100	62
Eugenol methyl ether		7.50	8.75	25.00	100	117

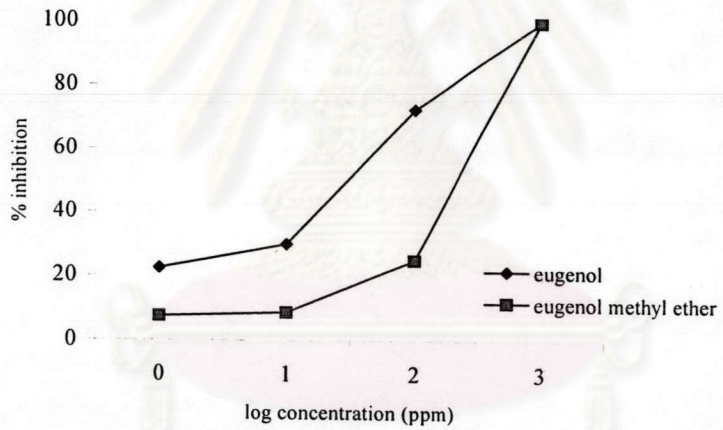
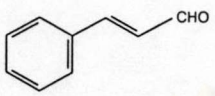
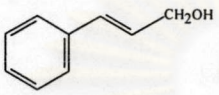
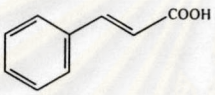
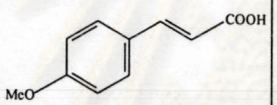


Fig. 3.13 Concentration effect of eugenol and eugenol methyl ether on *Fusarium oxysporum* 43-68 mycelial growth inhibition

Table 3.10 Mycelial growth inhibition percentage of cinnamaldehyde and its derivatives against *F. oxysporum* 43-68

Compound	Structure	Inhibition percentage				IC_{50} (ppm)
		1 ppm	10 ppm	100 ppm	1000 ppm	
Cinnamalde-hyde		11.25	20.00	75.00	100	66
Cinnamyl alcohol		12.50	20.00	26.25	91.25	183
Cinnamic acid		-1.45	0	5.80	100	216
4-Methoxy cinnamic acid		-4.35	0	5.80	47.83	>1000

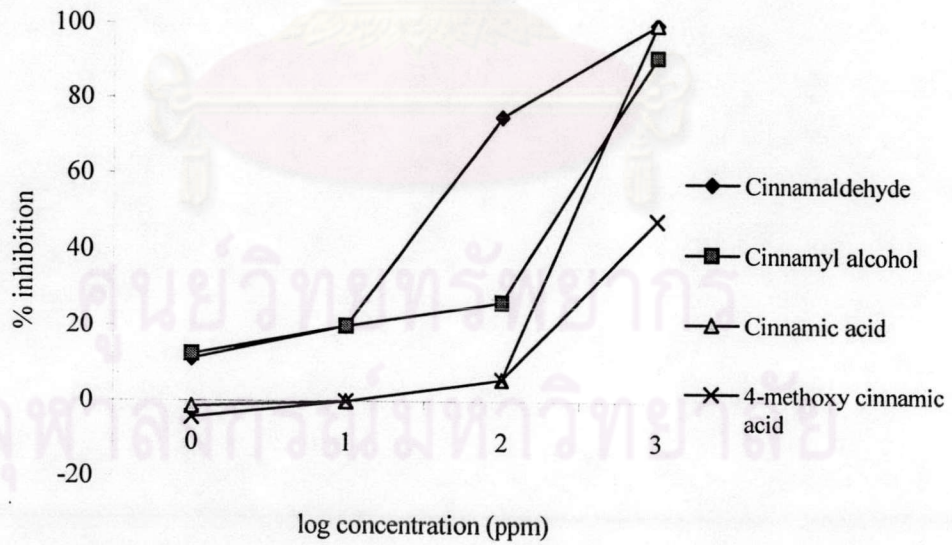
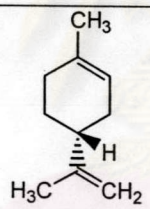
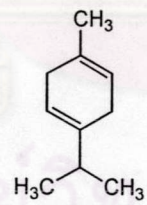
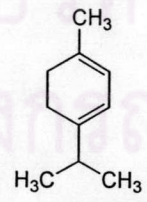


Fig. 3.14 Concentration effect of cinnamaldehyde and its derivative on *Fusarium oxysporum* 43-68 mycelial growth inhibition

Eugenol and eugenol methyl ether, two monoterpenes, showed strong antifungal activity. Cinnamaldehyde, cinnamyl alcohol and cinnamic acid are phenylpropane aldehyde, alcohol and acid, respectively. As shown in Tables 3.9 and 3.10, it was found that the aldehyde functional group exhibited stronger antifungal activity than those of the alcohol and carboxylic acid.

The pure commercial cymene and β-myrcene has been tested to have antifungal effect on *Penicillium digitatum* and found that they were not effective (Dimitra *et al.* 2000). The cymene and β-myrcene are the monoterpene hydrocarbons. Therefore, the antifungal activity of other monoterpene hydrocarbons was studied. Some monoterpenes, limonene, γ-terpinene and α-terpinene were selected for tested antifungal activity. The results are showed in Table 3.11.

Table 3.11 Mycelial growth inhibition percentage of limonene, γ-terpinene and α-terpinene against *F. oxysporum* 43-68

Compound	Structure	Inhibition percentage				IC_{50} (ppm)
		1 ppm	10 ppm	100 ppm	1000 ppm	
Limonene		1.43	0	1.43	4.29	>1000
γ-terpinene		1.43	1.43	4.29	4.29	>1000
α-terpinene		1.43	0	0	7.14	>1000

It was found that none of them showed antifungal activity at any concentration. It appears that the structure of the most abundant compounds in

essential oil might indicate level of the antifungal activity. The results obtained in this study supported those reported previously that the antifungal activity ranges from higher to lower level in compounds: phenol, aldehyde and hydrocarbon (Dimitra *et al.* 2000).

The IC_{50} of these compounds were calculated and it was found that the most effective component was eugenol following by cinnamaldehyde, eugenol methyl ether, cinnamyl alcohol and cinnamic acid, respectively. Eugenol has been reported to have the antifungal effects on eight strains of *Aspergillus* spp. and *Penicillium* spp. Furthermore, eugenol showed growth inhibition activity in dose-related manner against foodborne pathogens and *E. coli* 0157:H7 (Jeongmok, Maurice and Cheng-I, 1995). Many reports classified eugenol as a phenol derivative and assumed that their effects might be the same as those of other phenol derivatives. In bacteria, the active sites of phenol are considered to be the cell wall, the cytoplasmic membrane and the cytoplasm, with the effect at these three sites depending on the concentrations used (Chuenchit and Flege, 1982). Moreover, it is well known that OH-group is much more reactive than other functional group and perhaps can easily form hydrogen bonds with active site of target enzymes (Farag *et al.* 1989).

Cinnamaldehyde completely inhibitied the growth thiabendazole-resistant *Fusarium sambucinum* strains *in vitro* at 0.1 % (v/v) level. *In vivo,* cinnamaldehyde also reduced the decay of potato tuber with dry rot disease when treated as emulsified compounds (Steven and Gayland, 1994). In addition, cinnamaldehyde has been reported to completely suppress the growth and aflatoxin synthesis of *Aspergillus niger* at 200 ppm (Mahmoud, 1994). However, cinnamaldehyde exhibited only mild antifungal activity against several *Rhizopus* species (Thompsom, 1989). The effect of phytotoxicity of cinnamaldehyde was also tested and it was found that the compound completely inhibited the sprouting of potato tubers when dipped in 1 % (v/v) emulsion for 10 seconds (Vaughn and Spencer, 1993).

3.7 The effects of exposure time on conidial germination of *Fusarium oxysporum* 43-68

From the preliminary screening test, crude essential oil from *Cinnamomum bejolghota* Sweet. and *Eugenia caryophyllus* Bullock & Harrison. exhibited moderate conidial germination suppression. Thus, it was interesting to test whether the antifungal activity could be improved if the exposure time was extended. Therefore,

the effect of exposure time on conidial germination was studied and the results are shown in Table 3.12.

Table 3.12 Conidial germination percentage of *Fusarium oxysporum* 43-68 after exposing to the crude essential oils at various length of time

Plant	Exposure time (min)	% Germination
Cinnamomum bejolghota Sweet.	5	74.45
	10	65.69
	15	62.04
Eugenia caryophyllus Bullock & Harrison.	5	79.20
	10	62.41
	15	52.55
Control		100

Note : control condition : spread spore suspension on PDA after 36 h. germinated spore were count as Colony Forming Unit (CFU) and calculated % germination

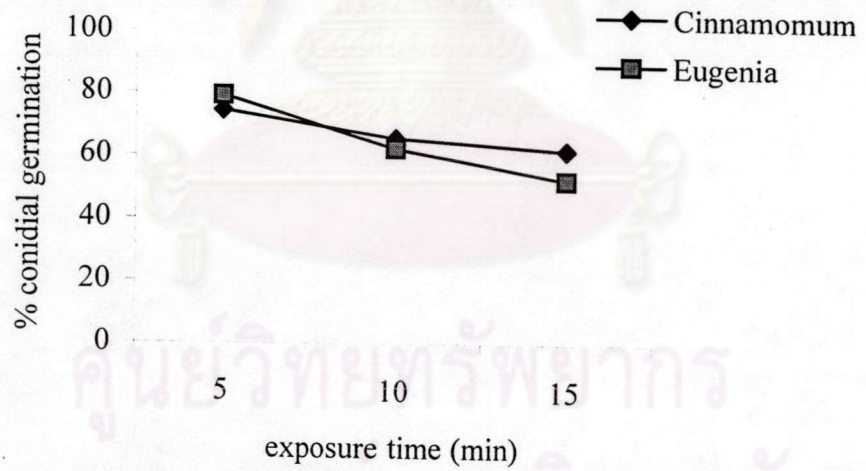


Fig. 3.15 Conidial germination percentage of *Fusarium oxysporum* 43-68 after exposing to the crude essential oils at various length of time

The effect of exposure time on *F. oxysporum* conidial germination was investigated. Clove oil and cinnamon oil showed conidial germination at 52.55% and 62.04%, respectively when exposure with those extracts for 15 minute.

3.8 Application of essential oils as the postharvest disease control agents

Essential oils from *Cinnamomum bejolghota* Sweet. and *Eugenia caryophyllus* Bullock & Harrison. showed strong antifungal activity on agar medium assay and bioautograpgic assay. Those essential oils were used as agents for controlling postharvest disease on banana and the results are shown in Table 3.13 and Fig. 3.16.

Table 3.13 *F. oxysporum* infection percentage on banana treated the crude essential oils

	Total area (cm^2)	Infected area [a] (cm^2)	% Infection area	Level of disease severity [b]
Un-inoculated control	136.73	6.27	4.59	2
Inoculated control	119.87	19.33	16.13	4
Cinnamomum bejolghota Sweet.	83.27	1.75	2.10	1
Eugenia caryophyllus Bullock & Harrison.	93.67	0.9	0.96	1

Note : [a] infection area based on tissue necrosis

[b] level 1 = cottony hyphae less than 10 % of total fungal colony

level 2 = cottony hyphae 10 -25 % of total fungal colony

level 3 = cottony hyphae 25 - 50 % of total fungal colony

level 4 = cottony hyphae more than 50 % of total fungal colony

un-inoculated control : banana without inoculated fungal spore and without treated the crude essential oil

inoculated control : banana inoculated with fungal spore but without treated the crude essential oil

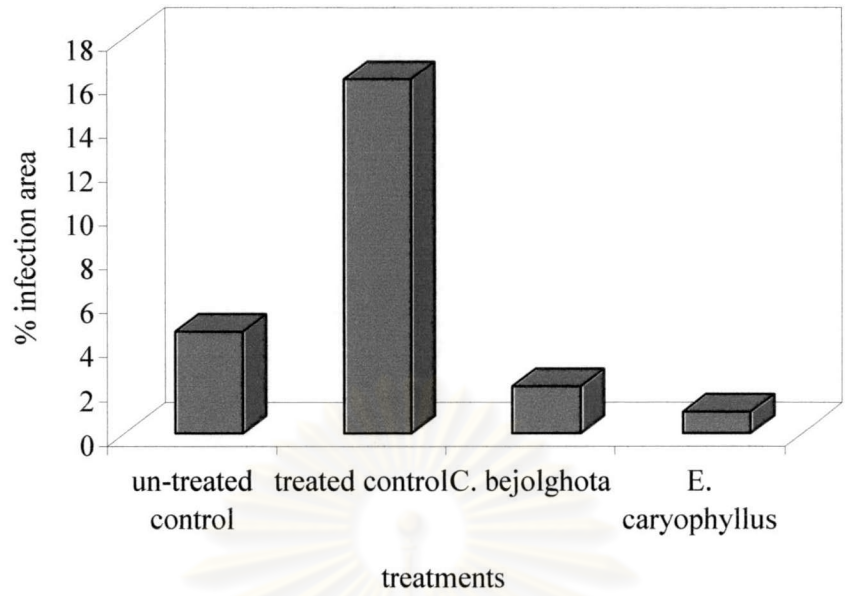


Fig. 3.16 *F. oxysporum* infection percentage on banana treated the crude essential oils

Analyses of inoculated fruit treats with emulsified crude essential oil of *Cinnamomum bejolghota* Sweet. and *Eugenia caryophyllus* Bullock & Harrison. demonstrated significant reduction in area of decayed tissue at 87 and 94%, respectively (Table 3.13 and Fig. 3.16). The level of disease severity exhibited consequential differences between the control and the treatment. However, heterogeneous emulsions of both essential oils caused a physiological damage occurred as the browning on peel (data not show). However, those compounds have no effect on ripening and smell disorder of product.

The method of inoculation and the essential oil exposure had affected on postharvest disease control. If fruits were deeply penetrated by pathogen or the time of the exposure not extremely, the antifungal activity may not be effective. The increase efficiency of activity of those compounds when applied to commercial condition product may be much higher due to increased concentration.

The application of essential oil as an antifungal agent has been reported in strawberries. Thyme oils efficient reduced *Botrytis cinerea* and *Rhizopus stolonifer* infection in inoculated strawberries (Reddy *et al*. 1998). The monoterpenes, such as cuminaldehyde, perillaldehyde, salicylaldehyde and carvone significantly reduced natural *Penicillium* infection in tulip bulbs, but all of compounds tested did not affect

the bacterial population. Furthermore, those compounds had no effect on the total stalk length or the flowering capacity of tulip bulbs (Eddy, Yke and Leon 1995). Carvacrol and *p*-anisaldehyde (oxidation product of anethole) were found to be effectively inhibited fungi causing postharvest disease (Caccioni and Guizzardi, 1994). The effect of temperature on the antifungal activity was also studied, cinnamon extract remained stable after heat treatments up to 100 °C for 20 min (Wilson *et al.* 1997), and its antimicrobial activity was not significantly affected by autoclave (Jeng-Leun, Chiu-Ping and Pau-Chuan, 2001). Therefore, it is possible to use essential oil from cinnamon as an efficient antifungal agent in postharvest processing with heat-treatment due to its thermostable property.

3.9 Analysis of the components of *Limnophila aromatica* Merr. essential oil

Based on the mycelial growth inhibition test the crude essential oil of *Limnophila aromatica* Merr. was found to exhibit antifungal activity (Table 3.6). Nonetheless, its chemical compositions have not been studied. Thus, GC-MS technique was used to elucidate the chemical constituents of this essentail oil. The components of essential oil from *Limnophila aromatica* Merr. were determined by comparing the mass spectra of oil components and mass spectra from NIST data library. The analysis of essential oil by GC-MS showed 16 peaks separated (Fig. 1 in appendix C) identified as 7 monoterpenes, 4 sesquiterpenes. The results are shown in Table 3.14 and Fig. 3.17 (The GC-MS pattern, mass spectra and possible mass fragmentation are displayed in appendix C).

Table 3.14 Possible chemical constituents of the essentail oil from *Limnophila aromatica* Merr. as analyzed by GC-MS

Peak No.	R_t (min)	Compound	Composition (%)
1	8.19	1-methyl-5-(1-methylethenyl)-,(R) Cyclohexene	0.96
2	8.31	Eucalyptol	21.56
3	11.96	Unknown	1.36
4	12.57	1-methyl-4-(1-methylethyl)-cyclohexanol	1.21
5	13.27	long-chain hydrocarbon	5.90
6	13.50	pulegone	27.97
7	15.85	*p*-menth-1(7)-en-9-ol	8.80
8	16.50	long-chain hydrocarbon	0.20
9	18.58	Unknown	2.33
10	19.22	pinene	23.03
11	19.46	Isocaryophyllene	0.34
12	19.78	2-menthyl-5-(1-methylethenyl)-acetate, 2-cyclohexen-1-ol	5.23
13	20.42	∝-caryophyllene	0.38
14	20.98	long-chain hydrocarbon	0.19
15	24.30	Caryophyllene oxide	0.43
16	24.73	3,7-dimethyl-10-(1-methylethyl)-3,7-cyclodecadien-1-one,	0.17

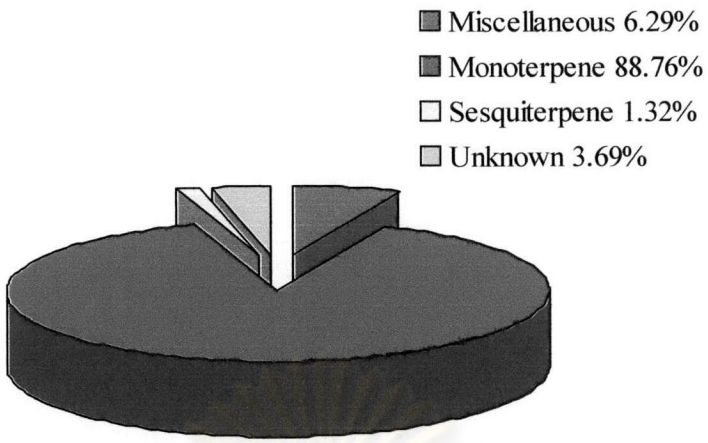


Fig. 3.17 Percentages of terpenoid groups found in the essential oil from *Limnophila aromatica* Merr.

From the component profile, peak number 6 should be major component in this essential oil. According to, comparing mass spectrum of peak no. 6 and NIST mass spectra data library. Pulegone was identified as major component. The antifungal activity of essential oil form *Limnophila aromatica Merr.* exhibited moderate mycelial growth inhibition against *F. oxysporum* 43-68. However, this essential oil displayed strongly antifungal activity towards *Alternaria* sp. 43-89 and *Phytophthora* sp. 572. In addition, *Limnophila* oil slightly suppressed conidial germination of *Fusarium oxysporum* 43-68 and *Alternaria* sp. 43-89. The activity of components of *Limnophila* oil was also evaluated by bioautographic assay. However, this oil did not show any clear zone on the bioautographic plate. Therefore, the antifungal activity of *Limnophila* oil was possibly the result of the synergistic effect between all compounds.