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Herbal Plant Crude Extracts and Essential Oils to Control Anthracnose Disease in *Dendrobium* 'Earsakul'

W. Kaewduangta[1]*, W. Sutthisa[2], M. Nakornriab[3] and P. Rermkratog[1]

[1] Department of Agricultural Technology, Faculty of Technology, Mahasarakham University, Mahasarakham 44150, Thailand.
[2] Department of Biology, Faculty of Science, Mahasarakham University, Mahasarakham 44150, Thailand.
[3]Department of Chemistry, Faculty of Science, Mahasarakham University, Mahasarakham 44150, Thailand.

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The effect of crude extracted and essential oils was compared for controlling anthracnose disease in *Dendrobium* 'Earsakul'. *Dendrobium* 'Earsakul' was tested to determine the efficiency of five Thai medicinal herbs: galangal, garlic, lemongrass, turmeric and ginger on the growth inhibition of *Colletotrichum* sp. as the pathogens of anthracnose or leaf blight disease. Each medicinal plant was blended and macerated in 95% ethyl alcohol and sterile distilled water at ratio 200 g: 400 mL for seven days. Crude extracts and essential oil were tested for inhibition of their antifungal potentials against *Colletotrichum* sp. by food poison technique. Results in the laboratory and under greenhouse condition showed that 8,000 ppm essential oil of ginger inhibited the growth of *Colletotrichum* sp. both before and after disease infection at 100% similar with the chemical compound Mancozeb.

Keywords: *Dendrobium* 'Earsakul', plant extract, essential oil, Thai medicinal herb, *Colletotrichum* sp.

Anthracnose or leaf blight disease is caused by fungi in the genus *Colletotrichum*, a common group of pathogens that are responsible for diseases on many plant species. Anthracnose infects the aerial portion of the orchid and leaves are most often attacked. Leaf tips turn brown, beginning at the apex and proceeding toward the base. Dark brown or light gray patches develop, sometimes as concentric rings or as numerous dark bands across the leaf. The affected area is usually sharply defined and somewhat sunken, while the remainder of the leaf appears normal. Sporing bodies develop in the infected area. Flowers develop watery, black or brown pustules which are usually raised and occur on the underside of older sepals and petals. The spots may merge and cover the entire flower[1,2]. The control of this pathogen remains a challenge and is still based upon multiple applications of fungicides. Chemical control is effective and efficient but can lead to the development of pathogen resistance, chemical residues in fruit, phytotoxicity to other organisms or environmental and public health problems, as well as the occurrence of fungicide resistant pathogen strains. This has stimulated research on alternative methods to control diseases which are needed because of the negative effects of synthetic chemicals which increase the risk of high levels of toxic residues[3,4,5,6]. Natural plant extracts are important sources of new agrochemicals with large antimicrobial spectrum properties for the control of plant diseases[7,8]. However, the potential toxic effect of applying pesticides to plants on soil beneficial organisms needs to be addressed. Another method is to use plant pathogen control substances which are environmentally friendly. Both these methods

* To whom all correspondence should be addressed.
E-mail: waranyoo.k@msu.ac.th

leave toxic residues in the environment but do not harm the ecosystem. Disease management using plant essential oils has been applied as an eco-friendly control method[9,10]. Many plant essential oils showed different levels of antimicrobial efficacies to various ranges of plant fungal and bacterial pathogens, and efficiently reduced the major diseases in crops. *Colletotrichum* sp. which infect diverse economically important crops have been successfully managed by plant essential oils and their individual components[11]. The present work therefore aimed to evaluate plant crude extracts and essential oil for their antifungal activities against *Colletotrichum* sp. that could possibly lead to their use to control anthracnose disease *in vitro* and in the greenhouse

MATERIALS AND METHODS

Disease protection activity of the crude plant extracts and essential oils were tested on detached pseudobulbs or leaves of orchid. Experiments were conducted in the science laboratory at the Department of Agricultural Technology, Faculty of Technology, Mahasarakham University, Thailand.

Plant material

To prepare the experimental *Dendrobium* 'Earsakul' orchid plants, the first step involved inducing protocorm-like bodies of orchid to seedlings on MS media[12] with 0.5 mg/L benzylaminopurine (BAP) and 0.5 mg/L dichlorophenoxy acetic acid (2,4-D) for 8 weeks. Seedling growth was then induced on VW media[13] with 15 mg/L chitosan for 8 weeks with subculture once a month. Finally, the seedlings were transplanted in the greenhouse after one year.

Isolation of target pathogen

Colletotrichum sp. was isolated from pseudobulbs or leaves of orchid showing anthracnose lesions. An isolate of the pathogen grown as a pure culture was maintained in PDA (potato dextrose agar) medium as a stock culture.

Inoculum disc: Seven days old culture of the test fungus was used for the preparation of inoculum discs 5 mm in diameter.

Preparation of plant crude extract and essential oils

Sample collection: Potential plant crude extracts and essential oils were selected by screening the efficiency of five Thai medicinal herbs (galangal, garlic, lemongrass, turmeric and ginger) on the growth inhibition of *Colletotrichum* sp.

The method for the preparation of plant crude extracts and essential oils from five Thai medicinal herbs (galangal, garlic, lemongrass, turmeric and ginger) was as follows. For plant crude extracts, firstly, fresh plant bulbs or rhizomes were selected and washed thoroughly 2-3 times with running tap water followed by distilled water. They were then artificially heated by air drying in a hot air oven at 50°C for 72 hrs or until stable dry weight. Secondly, each plant sample was mixed in a blender and the powder was soaked in ethanol. An aqueous extract was prepared by blending 200 g of each plant bulb or rhizome in 400 mL 95% ethanol for seven days. The macerate was filtered through double-layered muslin cloth and centrifuged at 8,000 rpm at 10°C for 30 minutes. The supernatant was filtered through Whatman No. 1 filter paper followed by evaporation using an R-205 Buchi rotary evaporator to remove the ethanol to obtain concentrates. The crude extracts were kept at 4°C in sterile universal bottles until required for use. Essential oils were separated from the crude extracts by the water distillation process.

The inhibitory effects of plant extracts and their antifungal potentials against *Colletotrichum* sp. which cause anthracnose disease in *Dendrobium* 'Earsakul' orchid were tested by food poison technique[14]. Each of the plant crude extracts were dissolved in 1%DMSO and a volume of 5.5 mL of each concentrate was aseptically poured into a Petri dish followed by the addition of 9.5 mL of melted PDA and then agitated gently to achieve a thorough mixing of the contents. For the control set, no extract was used. After solidification of the media, one inoculum disc of the test fungus was aseptically inoculated upside down at the center of the Petri dish and incubated at 25°C. Average radial growths of the fungal colonies were measured on the seventh day of incubation. The treatments were as follows:

Experiment 1

To screen the efficiency of plant crude extracts and essential oils for the control of *Colletotrichum* sp. *in vitro* by PDA standard medium with plant crude extracts and essential oils for seven days. This experiment was conducted

in a CRD (completely randomized block design) with four replications. Four discs were prepared for repeated experiments per each replication. Four individual experiments were performed *in vitro*.

T1 Control (distilled water)
T2 1,500 ppm Mancozeb
T3 10,000 ppm DMSO (1% DMSO)
T4 10,000 ppm Galangal extract
T5 80,000 ppm Garlic extract
T6 7,500 ppm Lemongrass extract
T7 20,000 ppm Turmeric extract
T8 8,530 ppm Ginger extract
T9 3,000 ppm Galangal essential oil
T10 100 ppm Garlic essential oil
T11 500 ppm Lemongrass essential oil
T12 2,500 ppm Turmeric essential oil
T13 8,000 ppm Ginger essential oil

Following observations, the percentage inhibition of diameter growth (PIDG) values was determined according to the equation below:

$$\text{Inhibition percentage} = 100 - \left[\frac{\text{Diameter of sample}}{\text{Diameter of control}} \times 100\right]$$

Experiment 2

To study the efficiency of plant crude extracts and essential oils for the control of *Colletotrichum* sp. by the inoculation modified detached leaf technique *in vitro*. This experiment was conducted at 7x2 factorials in CRD for 14 treatments. Seven factor A substances were selected

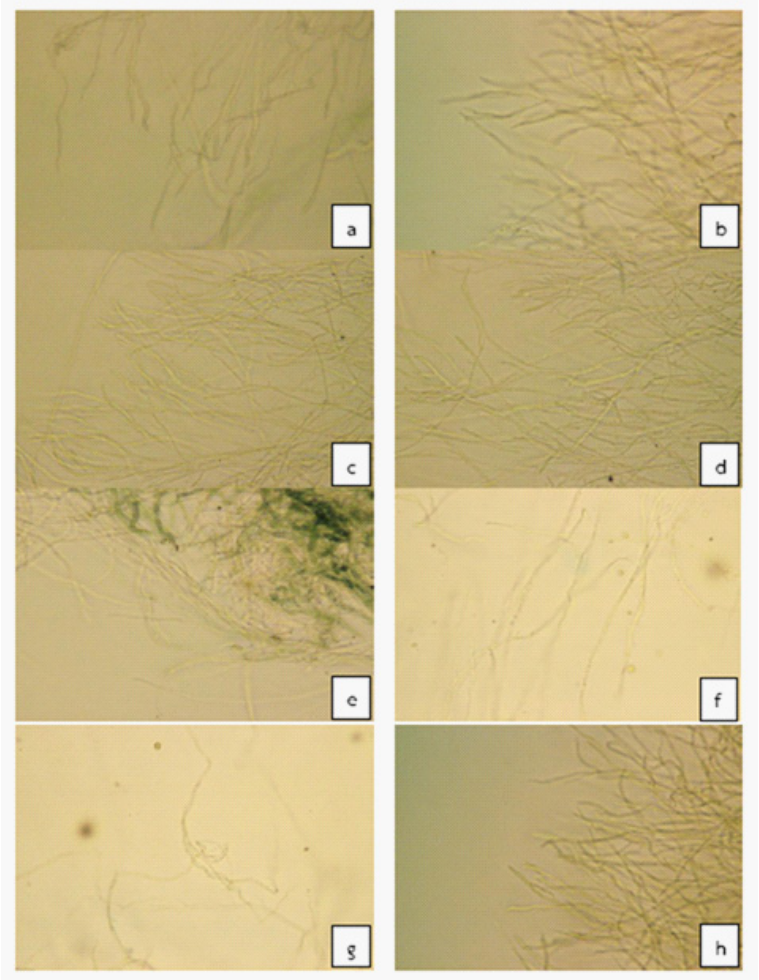

Fig. 1. Characteristics of *Colletotrichum* sp. mycelium causing anthracnose disease tested with different treatment for seven days. (a) Control (PDA), (b) DMSO, (c) 7,500 ppm Lemongrass extract, (d) 20,000 ppm Turmeric extract, (e) 8,530 ppm Ginger extract, (f) 100 ppm Garlic essential oil, (g) 3,000 ppm Galangal essential oil, (h) 2,500 ppm Turmeric essential oil

from Experiment 1 (Control, Mancozeb, DMSO, 10,000 ppm Galangal extract, 80,000 ppm Garlic extract, 500 ppm Lemongrass essential oil and 8,000 ppm Ginger essential oil), and the two factor B time periods were the time of usage (before and after pathogen infection) with four replications and 10 leaves per replication. Following observations, the PIDG values were determined according to the equation below:

Experiment 3

To study the efficiency of plant crude extracts and essential oils for the control of *Colletotrichum* sp. by the inoculation modified detached leaf technique *in vivo* (greenhouse). This experiment was conducted at 6x2 factorials in CRD for 14 treatments. Six factor A substances were selected from Experiment 2 (Control, Mancozeb, DMSO, 10,000 ppm Galangal extract, 80,000 ppm Garlic extract, and 8,000 ppm Ginger essential oil), and the two factor B time periods were the time of usage (before and after pathogen infection) with four replications and 10 plants per replication.

Statistical analyses

ANOVA (analysis of variance) was used to determine the effects of anthracnose treatments at both the laboratory and greenhouse. Means were compared using Duncan's multiple range tests. Statistical analyses were performed using SPSS version 22 (IBM SPSS Statistics 22.Ink).

RESULTS

1. The efficiency of plant crude extracts and essential oils for the control of *Colletotrichum* sp. *in vitro* by PDA standard medium with plant crude extracts and essential oils for seven days. Laboratory results showed that plant crude extracts of galangal (10,000 ppm), garlic (80,000 ppm), and essential oils from lemongrass (500 ppm) and ginger (8,000 ppm) inhibited the growth of *Colletotrichum* sp. at 100% compared with the control and the chemical compound Mancozeb (Table 1). Other treatments were not effective in inhibiting the growth of *Colletotrichum* sp. Under a compound microscope, the mycelium showed growth and abnormalities such as bent, knotted, twisted and kinked hypha (Figure 1).
2. The efficiency of plant crude extracts and essential oils for the control of *Colletotrichum* sp. by the inoculation modified detached leaf technique *in vitro*. Laboratory results for the main study, factor A substances and time factor interaction B were significant at 99%. Ginger essential oil (8,000 ppm) inhibited the growth of *Colletotrichum* sp. at 100% similar with the chemical compound Mancozeb both before and after disease infection (Table 2).

Table 1. Efficiency of plant crude extracts and essential oils for control of *Colletotrichum* sp. *in vitro* by PDA medium for seven days

Treatments	Inhibition percentage (%) for 7 days						
	1	2	3	4	5	6	7
Control (distilled water)	0.00b	0.00b	0.00f	0.00f	0.00d	0.00d	0.00e
Mancozeb	100.00a	100.00a	100.00a	100.00a	100.00a	100.00a	100.00a
DMSO	100.00a	100.00a	65.02d	39.15d	17.06c	0.00d	0.00e
10,000 ppm Galangal extract	100.00a	100.00a	100.00a	100.00a	100.00a	100.00a	100.00a
80,000 ppm Garlic extract	100.00a	100.00a	100.00a	100.00a	100.00a	100.00a	100.00a
7,500 ppm Lemongrass extract	100.00a	100.00a	68.65c	45.33c	18.48c	2.19d	0.00e
20,000 ppm Turmeric extract	100.00a	100.00a	71.21b	50.05b	31.98b	10.77c	0.00e
8,530 ppm Ginger extract	100.00a	100.00a	100.00a	100.00a	100.00a	100.00a	85.67b
3,000 ppm Galangal essential oil	100.00a	100.00a	100.00a	100.00a	100.00a	83.64b	69.12d
100 ppm Garlic essential oil	100.00a	100.00a	62.64e	24.46e	0.00d	0.00d	0.00e
500 ppm Lemongrass essential oil	100.00a	100.00a	100.00a	100.00a	100.00a	100.00a	100.00a
2,500 ppm Turmeric essential oil	100.00a	100.00a	100.00a	100.00a	100.00a	84.72b	71.17c
8,000 ppm Ginger essential oil	100.00a	100.00a	100.00a	100.00a	100.00a	100.00a	100.00a
F-test	**	**	**	**	**	**	**
CV (%)	8.62	28.26	4.06	1.81	1.34	1.86	1.76

** = significant difference at p =0.01. Means within a column followed by the same letter do not differ significantly according to DMRT.

3. The efficiency of plant crude extracts and essential oils for the control of *Colletotrichum* sp. was tested by the inoculation modified detached leaf technique *in vivo* (greenhouse). Results in the greenhouse for the main study, factor A substances and time factor interaction B were significant at 99%. Leaves treated with ginger essential oil (8,000 ppm) did not show symptoms of anthracnose disease compared with the control and the chemical compound Mancozeb when used both before and after disease infection. Essential oil of ginger (8,000 ppm) showed 100% inhibition of *Colletotrichum* sp. compared with the control and the chemical compound Mancozeb. (Table 3).

DISCUSSION

This experiment used the DMSO solvent to compare treatments. Results indicated that DMSO had no effect on anthracnose inhibition compared with the control. This finding was consistent with Pothikhawet et al. (2013)[15] who tested alfalfa and radish seeds inoculated with *Aspergillus parasiticus* or *A. niger* and then soaked in distilled water, 0.5% DMSO, 1,000 ppm Chi-der and 1.2 ppm Nano-Pt for 6 hrs before washing by distilled water. The 1,000 ppm Chi-der and 1.2 ppm Nano-Pt significantly reduced seed infection but enhanced seed germination compared to distilled water and 0.5% DMSO. Prasoetsang and Subtang (2012)[16] studied the effect of solvent on the antimicrobial activity of medicinal plant extraction using 1% DMSO as the negative control. The result of broth microdilution assay showed that 1% DMSO did not inhibit the bacteria. Medicinal plant extraction and essential oil treatment for *Colletotrichum* sp. to control anthracnose disease of *Dendrobium* 'Earsakul' before or after disease

Table 2. Efficiency of plant crude extracts and essential oils for control o f *Colletotrichum* sp. by inoculation modified detached leaf technique *in vitro*

Treatment	F-test inhibition percentage (%) for seven days						
	1	2	3	4	5	6	7
Factor A (type of substance)	**	**	**	**	**	**	**
Factor B (before-after infection)	ns	ns	ns	ns	ns	ns	ns
A×B	**	**	**	**	**	**	**
Control (A1)	0.00e	0.00e	0.00d	0.00d	0.00e	0.00d	0.00d
Mancozeb (A2)	100.00a	100.00a	100.00a	100.00a	100.00a	100.00a	100.00a
DMSO (A3)	0.00e	0.00e	0.00d	0.00d	0.00e	0.00d	0.00d
10,000 ppm Galangal extract (A4)	73.50b	69.75b	50.00b	49.75b	47.75b	24.13b	24.13b
80,000 ppm Garlic extract (A5)	50.00c	49.75c	49.75b	24.69c	24.44c	24.00b	24.13b
500 ppm Lemongrass (A6)	24.75d	24.63d	24.38c	24.25c	19.63d	17.25c	13.63c
8,000 ppm Ginger essential oil (A7)	100.00a	100.00a	100.00a	100.00a	100.00a	100.00a	100.00a
before pathogen infection (B1)	50.000	50.000	46.429	42.857	41.857	38.286	37.714
after pathogen infection (B2)	49.500	48.321	46.179	42.482	41.518	37.536	37.107
A1B1	0.00e	0.00e	0.00	0.00d	0.00	0.00d	0.00d
A2B1	100.00a	100.00a	100.00a	100.00a	100.00a	100.00a	100.00a
A3B1	0.00e	0.00e	0.00d	0.00d	0.00e	0.00d	0.00d
A4B1	75.00b	75.00b	50.00b	50.00b	48.00b	25.00b	25.00b
A5B1	50.00c	50.00c	50.00b	25.00c	25.00c	25.00b	25.00b
A6B1	25.00d	25.00d	25.00c	25.00c	20.00d	18.00c	14.00
A7B1	100.00a	100.00a	100.00a	100.00a	100.00a	100.00a	100.00a
A1B2	0.00e	0.00e	0.00d	0.00d	0.00e	0.00d	0.00d
A2B2	100.00a	100.00a	100.00a	100.00a	100.00a	100.00a	100.00a
A3B2	0.00e	0.00e	0.00d	0.00d	0.00e	0.00d	0.00d
A4B2	72.00b	64.50b	50.00b	49.50b	47.50b	23.25b	23.25b
A5B2	50.00c	49.50c	49.50b	24.38c	23.88c	23.00b	23.25b
A6B2	24.50d	24.25d	23.75c	23.50c	19.25d	16.50c	13.25c
A7B2	100.00a	100.00a	100.00a	100.00a	100.00a	100.00a	100.00a
CV (%)	14.70	9.50	10.80	8.80	12.04	6.02	8.40

ns = not significant, ** = significant difference at p = 0.01. Means within a column followed by the same letter do not differ significantly according to DMRT

infection showed that essential oil of ginger (8,000 ppm) inhibited the growth of *Colletotrichum* sp. by 100% similar to the chemical Mancozeb. Medicinal constituents in ginger essential oil are zingiberene, zingiberol, bisabolene and camphene in high quantities, and extract of ginger also contains phenolic compounds with antibacterial, rancid and preservative properties[17,18,19]. Jamkratoke et at. (1996)[20] studied the effect of *Boesenbergia rotunda*, *Curcuma longa* and *Zingiber officinalis* extracts on postharvest disease fungi. Results demonstrated that the inhibition characteristics of the tested herbs were significant at 10,000 ppm for crude extracts and 1,000 ppm for volatile extracts composed in PDA. Radial growth of almost all the tested fungi was inhibited by the tested herb extracts with various sensitivity. An identical inhibition (83.67 - 87.60%) was determined on fungal colonies formed by *Colletotrichum* spp. isolated on PDA supplemented with *Z. officinalis* crude extract. Shovan et al. (2008)[21] studied the effect of plant extracts on the growth of *Colletotrichum dematium* which causes anthracnose in soybean by fungicides, plant extracts and *Trichoderma* sp. in the laboratory. Results showed that the most effective material was garlic followed by onion, ginger and neem.

CONCLUSION

Results in the laboratory and under greenhouse conditions showed that essential oil of ginger at 8,000 ppm inhibited the growth of *Colletotrichum* sp. both before and after disease infection at 100% similar with the chemical compound Mancozeb.

Table 3. The efficiency of plant crude extracts and essential oils for control anthracnose disease of *Dendrobium* 'Earsakul' *in vitro*

Treatment	F-test of disease index for 21 days		
	7	14	21
Factor A (type of substance)	-	**	**
Factor B (before-after infection)	-	ns	ns
A×B	-	**	**
Control (A1)	0.00	2.25a	3.00a
Mancozeb (A2)	0.00	0.00b	0.00d
DMSO (A3)	0.00	2.13a	2.38b
10,000 ppm Galangal extract (A4)	0.00	0.13b	1.00c
80,000 ppm Garlic extract (A5)	0.00	0.25b	1.25c
8,000 ppm Ginger essential oil (A7)	0.00	0.00b	0.00d
before pathogen infection (B1)	0.00	0.67	1.17
after pathogen infection (B2)	0.00	0.92	1.38
A1B1	0.00	2.00a	3.00a
A1B2	0.00	2.50a	3.00a
A2B1	0.00	0.00b	0.00d
A2B2	0.00	0.00b	0.00d
A3B1	0.00	2.00a	2.00b
A3B2	0.00	2.25a	2.75b
A4B1	0.00	0.00b	1.00c
A4B2	0.00	0.25b	1.00c
A5B1	0.00	0.00b	1.00c
A5B2	0.00	0.50b	1.50c
A6B1	0.00	0.00b	0.00d
A6B2	0.00	0.00b	0.00d
CV (%)	-	10.08	12.70

ns = not significant, ** = significant difference at $p < 0.01$. Means within a column followed by the same letter do not differ significantly according to DMRT

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