In vivo antifungal activity of five plant extracts against Chinese Kale leaf spot caused by *Alternaria brassicicola*

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ABSTRACT

Pathogens associated with Chinese kale leaf spot disease were recovered from necrotic lesions on the Chinese kale leaves and identified as *Alternaria brassicicola* (Schw.) Wiltshire. This disease is one of the most significant destructive pathogens to vegetable crops in Thailand and Southeast Asia. Disease management of these pathogens is done by using synthetic fungicides which are expensive and harmful to the environment. This study aimed to investigate the efficacy of the extracts from five plants, *Hydnocarpus anthelminthicus* Pierre ex Laness., *Crateva magna* (Lour.) DC., *Caesalpinia sappan* L., *Xanthophyllum lanceatum* J. J. Sm., and *Carallia brachiata* (Lour.) Merr. to control the Chinese kale leaf spots caused by *A. brassicicola* in *in vivo* condition. In an *in vivo* test, we found that the plant extracts of *C. brachiata, H. anthelminthicus, X. lanceatum* and *C. magna* showed a potential in control efficacy against brassica dark leaf spots caused by *A. brassicicola*. The four plant extracts effectively suppressed the development of leaf spots at a concentration of 10,000 ppm and 50,000 ppm at 30 DAT. However, at 40 DAT the *C. magna* and *H. anthelminthicus* extracts strongly inhibited *A. brassicicola* at concentrations of 10,000 ppm and 50,000 ppm respectively when compared with the water control. This is the first report demonstrating that the plant extracts collected from riparian forest can provide control against brassica dark leaf spots disease. Based on our study, we demonstrated that the ability of plant crude extracts can be used as natural fungicides to control Chinese kale leaf spots and replace synthetic fungicide.

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Keywords: Chinese kale leaf spots, *Alternaria brassicicola*, plant extracts, antifungal activity, plant disease control.

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INTRODUCTION

Plant species of the genus *Brassica* including Chinese kale are important vegetable crops in Thailand and Southeast Asia. Chinese kale leaf spots are caused by *Alternaria brassicicola* (Schw.) Wiltshire is a common disease of the genus *Brassica* worldwide, which causes significant reduction in the quality and quantity of Chinese kale crops. The disease caused by *Alternaria* fungi are serious and spreads throughout all parts of the plant and results in severe damage during all stages of plant growth development (Abo-Elyousr, 2012). The leaf spot diseases are estimated to cause about 20-50% yield loss found in infected crop plants. The disease displays itself visibly on the leaves where it appears as black, necrotic lesions on leaves with yellow halos around the chlorotic zone. It is spread during the growing season by wind-blown or rain-splashed spore. During the attack on the plant, the pathogen can produce bassicicolin A toxin that has been associated with *A. brassicicola*'s pathogenesis (Gloer *et al.,* 1988). Pattanamahakul and Strange (1999) also reported that the Thai isolates of *A.*

brassicicola produced toxins to the cell of different brassica species.

Previously, in Thailand, this disease was controlled mainly by the application of benomyl chemicals. However, there are concerns regarding the use of synthetic fungicides because of their potential harmful characteristics to human health and the environment, and they also cause disease resistance, and damage to the ecological balance of microorganisms. Presently, research is trending towards the control of plant diseases in sustainable agriculture by reducing the use of synthetic fungicides. The antifungal activity from the extracts of *Hydnocarpus anthelminthicus* Pierre ex Laness and *Xanthophyllum lanceatum* J. J. Sm.which were collected from the riparian forest have been reported to be effective against *P. oryzae, P. palmivora, R. solani* and *S. rolfsii* (Jantasorn *et al.,* 2016a). In recent years, biological control methods attempt to modify the management of plant diseases (Kagale *et al.,* 2004). Biological control using plant extracts and antagonistic microorganisms have emerged as viable options (El-Ghaouth, 1997; Duru and Onyedineke, 2010; Tapwal *et al.,* 2011; El-Gremi *et al.,* 2017). The use of biopesticides as a biological control is cheap, locally available, non-toxic, and easily degradable options. There can be a decrease in the use of synthetic pesticides which have an undesirable effect on other organisms present in the environment and humans through the food chain. Therefore, the use of natural products for fungal disease management is considered one of the better alternatives (Hadizadeh *et al.,* 2009; Aslam *et al.,* 2010; Gujar *et al.,* 2012). The objective of this study is to investigate the potential efficacy of five plant extracts collected from the riparian forest, *viz., Hydnocarpus anthelminthicus* Pierre ex Laness., *Crateva magna* (Lour.) DC., *Caesalpinia sappan* L., *Xanthophyllum lanceatum* J. J. Sm., and *Carallia brachiata* (Lour.) Merr. in their control of Chinese kale leaf spots caused by *Alternaria brassicicola* (Schw.) Wiltshire under *in vivo* conditions.

MATERIALS AND METHODS

Plant materials and extraction

H. anthelminthicus (fruits), *C. magna* (fruits), *C. sappan* (bark), *X. lanceatum* (fruits) and *C. brachiata* (bark) were collected from the riparian forest at Bodhivijjalaya College, Srinakharinwirot University, Sakaeo campus. The plant extraction was performed as described previously (Jantasorn *et al.,* 2016a), in which 100 g of each dry plant sample was macerated with 900 ml of 70% ethanol and incubated for 7 days at room temperature and then filtered through Whatman No.1 filter paper. Next, the solutions were evaporated under reduced pressure to furnish the crude ethanol extracts of each plant.

Preparation of spore suspension

The spore suspension of *A. brassicicola* was prepared in sterile distilled water. The spore of *A. brassicicola* was obtained from 10 day cultures and then centrifuged. A hemocytometer was used to obtain a homogenous *A. brassicicola* spore suspension of 1 x 10^6 spore/mL. To prepare the stock solutions of plant extract, the extracts were dissolved in dimethyl sulfoxide (DMSO) and adjusted for their final concentrations of plant extract to 10,000 ppm and 50,000 ppm concentrations for further study.

In vivo antifungal activity assay

The antifungal activity of plant extracts were evaluated against *A. brassicicola* on Chinese kale plant. The *in vivo* antifungal assay were performed and a slight modification was made as described previously (Yoon *et al.,* 2011). Briefly, Chinese kale plant was grown in plastic pots (18 cm diameter) in a greenhouse at 28 ±2 °C for 4 weeks. The Chinese kale seedling were sprayed twice at 30 day and then again 40 day intervals with diluting plant extract containing a surfactant Tween 20 (200µg/mL) at 10,000 ppm and 50,000 ppm concentrations as foliar application. After 24 h the treated Chinese kale seedlings were inoculated with *A. brassicicola* by spraying

Table 1. Effect of plant extracts on disease incidence of Chinese kale leaf spot caused by *Alternaria brassicicola* at concentration of 10,000 ppm and 50,000 ppm as foliar application.

Treatments/Conc. of plant crude extracts	% Disease Incidence			
	30 DAT[1/]		40 DAT[1/]	
	10,000 ppm	50,000 ppm	10,000 ppm	50,000 ppm
Hydnocarpus anthelminthicus	22±2.2 b	18±2.2c	51±2.2b	22±2.2c
Xanthophyllum lanceatum	20±11.5b	20±0.0c	60±0.0a	38±2.2b
Crateva magna	33±6.7 b	20±0.0c	38±2.2c	38±2.2b
Caesalpinia sappan	42±13.5ab	38±9.7b	58±2.2a	58±2.2a
Carallia brachiata	20±0.0b	16±2.2c	60±0.0a	38±2.2b
Distilled water (Control)	58±2.2a	58±2.2a	60±0.0a	60±0.0a
LSD	24.207	13.112	4.8442	6.2408

[1/]DAT=Date after transplanting

*Mean values along with their Standard error (±) are given in the table. Means followed by the same letter in each column do not significantly different, when analysed using Least Significant Difference test (LSDs) at $P<0.05$.

the plant with a spore suspension (1 x 10^6 spore/mL) of the fungus on to each pot. Control plants were treated with water containing Tween 20. Regarding the incubation, the seedlings were planted in moist plastic bags to maintain temperature and humidity for 24 h. The plastic bags were removed after one-day and the treated plants were kept in greenhouse. Disease incidence was determined as a percentage of infected leaf area 7 days after inoculation. Difenoconazole was applied as a positive control. The pots experiment was arranged in a Completely Randomized Design, with three replicate per treatment. The percentage of disease incidence was determined using the formula:

% Disease incidence = (Number of plants infected/Total number of plants) x (100/1)

Data analyses

Data from inoculation were evaluated by analysis of variance (ANOVA) and means were compared by Least Significant Difference (LSDs) ($P<0.05$) using the statistical program Statistix8 (analytical software, SXW, Tallahassee, FL, USA).

RESULTS

The different concentrations of the five plant extracts, *viz.*, *H. anthelminthicus, C. magna, C. sappan*, *X. lanceatum* and *C. brachiata* significantly reduced the Chinese kale leaf spots disease. When 10,000 ppm concentrations of the five plant extracts were sprayed at 30 days after planting (DAT), the *X. lanceatum*, *C. brachiata, C. magna* and *H. anthelminthicus* extracts effectively suppressed the development of leaf spot symptoms caused by *A. brassicicola* on Chinese kale seedlings (Fig. 1). The most effective treatments to reduce the development of leaf spot symptoms with plant extracts were *X. lanceatum*, *C. brachiata*, *H. anthelminthicus* and *C. magna* extracts in concentration of 10,000 ppm and 50,000 ppm at 30DAT and inoculation with the pathogen (Table 1), whereas the *H. anthelminthicus* extracts recorded a disease incidence at 22%

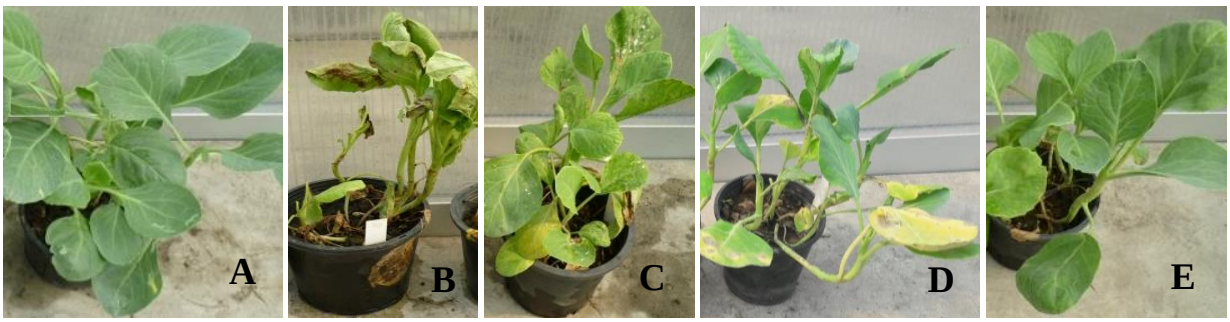

Figure 1. *In vivo* antifungal activities of plant extract against *A. brassicicola*. Chinese kale were inoculated with spore suspensions of the test organism 1 day after spraying with the various plant extracts at concentration of 10,000 ppm for 30 days after planting (A) untreated plant (healthy); (B) treated with distilled water; (C) treated with *H. anthelminthicus* extract; (D) treated with *X. lanceatum* extract and (E) treated with *C. brachiata* extract.

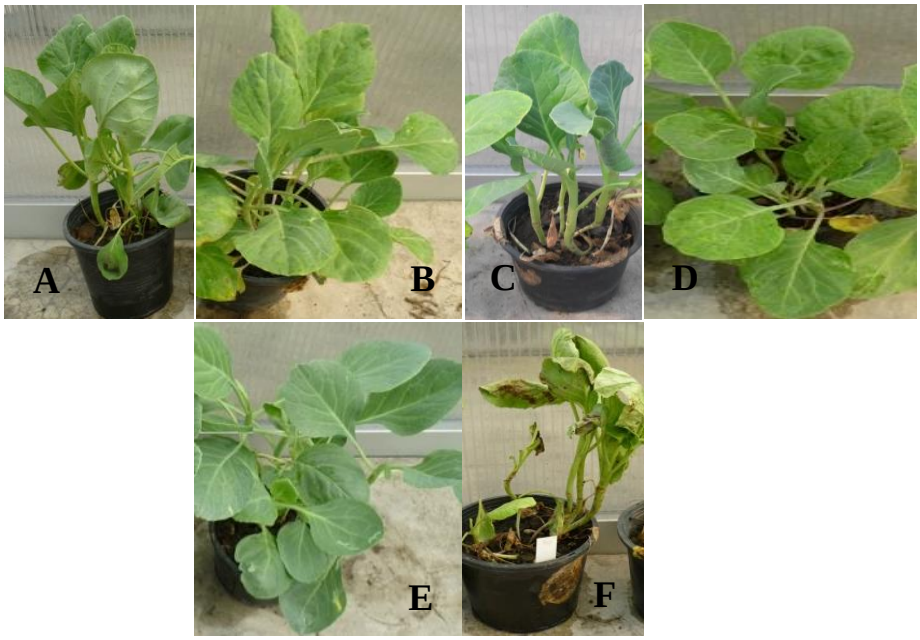

Fig. 2. *In vivo* antifungal activities of plant extract against *A. brassicicola*. Chinese kale were inoculated with spore suspensions of the test organism 1 day after spraying with the various plant extracts at concentration of 50,000 ppm for 30 days after planting (A) treated with *H. anthelminthicus* extract; (B) treated with X. *lanceatum* extract; (C) treated with *C. magna* extract; (D) treated with *C. brachiata* extract; (E) untreated plant (healthy) and (F) treated with distilled water.

Fig. 3. *In vivo* antifungal activities of plant extract against *A. brassicicola*. Chinese kale were inoculated with spore suspensions of the test organism 1 day after spraying with the various plant extracts at concentration of 50,000 ppm for 40 days after planting (A) untreated plant (healthy); (B) treated with distilled water and (C) treated with *H. anthelminthicus* extract.

in reducing the leaf spots disease, at 50,000 ppm for 40DAT and inoculation with the pathogen. The *in vivo* antifungal activity of plant extracts increased in parallel with an inverse in concentration. Fig. 2 displays the excellent inhibitory activity of *C. brachiata*, *H. anthelminthicus*, *C. magna* and *X. lanceatum* extracts to the development of typical leaf spot symptoms caused by *A. brassicicola* at a concentration of 50,000 ppm. On the other hand, the treatment of Chinese kale with the pathogen alone showed typical leaf spot symptoms. But no lesions were found on Chinese kale leaves treated with difenoconazole. The treatment of *C. brachiata* and *H. anthelminthicus* extracts controlled the leaf spots disease of Chinese kale by more than 80% at a concentration of 50,000 ppm. According to the results given in Table 1, the *C. brachiata*, *H. anthelminthicus*, *C. magna* and *X. lanceatum* extracts were found to inhibit the development of leaf spot symptoms on Chinese kale plants. At a concentration of 50,000 ppm, the disease incidence score was registered with *C. brachiata*, *H. anthelminthicus*, *C. magna* and *X. lanceatum* extracts on it at 16%, 18%, 20% and 20% respectively, 30 DAT and inoculation with the *A. brassicicola*.

Interestingly, the *H. anthelminthicus* extract was found to indicate a high degree of inhibition in leaf spots disease development at a concentration of 50,000 ppm, 40 DAT and inoculation with *A. brassicicola* (Fig. 3). However, the three plant extracts, namely *C. brachiata*, *H. anthelminthicus* and *X. lanceatum* were the most effective in suppressing the development of leaf spot symptoms on Chinese kale when compared with *C. sappan* extract. On the other hand, the three plant crude extracts did not appear to be phytotoxic to the Chinese kale plants even at a concentration of 50,000 ppm.

DISCUSSION

The results indicate that the five plant extracts, *viz., H. anthelminthicus, C. magna, C. sappan*, *X. lanceatum* and *C. brachiata* had different levels of inhibitory activity towards the development of leaf spot symptoms on Chinese kale plants and caused significant reduction in the growth of *A. brassicicola* in *in vivo*. At concentration of 50,000 ppm, the most effective plant extracts were found to be *C. brachiata*, *H. anthelminthicus*, *C. magna* and *X. lanceatum* which recorded suppression of the development of typical leaf spot symptom caused by *A. brassicicola* at 30DAT. The *H. anthelminthicus* extract at 50,000 ppm all strongly, significantly repressed disease expression, but the effect of *C. sappan* extract only was transient while the rest managed to reduce symptoms at 40DAT. In another study, the antifungal activity from the plant was tested on *Alternaria spp*. and has been reported by various researchers (Goussous *et al.,* 2010; Nashwa and Abo-Elyousr, 2012; Sasode *et al.,* 2012; Ganie *et al.,* 2015; Gupta *et al.,* 2015; Ahmad and Ashraf, 2016)

Our results revealed that the plant extracts of *C. brachiata* and *H. anthelminthicus* had a potent *in vivo* antifungal activity against *A. brassicicola* at a concentration 50,000 ppm. Both *C. magna* and *X. lanceatum* extracts displays a strong *in vivo* antifungal activity at the same concentration. The extracts effectively suppressed the development of leaf spot symptom by more than 80% when compared with the control at a concentration of 50,000 ppm. The efficacies of plant extracts and soil fungi extracts in inhibiting the growth of pathogenic fungi *in vitro* and *in vivo* have been reported earlier (Al-Reza *et al.,* 2010; Yoon *et al.,* 2011; Jantasorn *et al.,* 2016b; El-Gremi *et al.,* 2017). Jantasorn *et al.* (2016a) also reported that the plant extracts, namely *H. anthelminthicus* and *X. lanceatum* inhibited the growth of rice diseases caused by *P. grisea* and *R. solani*. On the other hand, the extract from *C. sappan* was more efficient in the growth inhibition of *P. palmivora* and *S. rolfsii*. The reduction of the disease development by plant extracts inducing systemic resistance has been reported earlier by Kagale *et al.,* 2004 and Amadioha (2000) who did an investigation on the suppression disease mechanism by plant extracts have suggested that the active principles present act on the plant pathogen directly.

The *in vivo* experiments indicated that the folia sprayed on Chinese kale plants with plant extracts resulted in a significant reduction in leaf spots infection. This study is the first to report on the inhibiting activity of the *C. brachiata*, *H. anthelminthicus*, *C. magna* and *X. lanceatum* extracts against *A. brassicicola* caused by Chinese kale in *in vivo*. All treatments with tested plant extracts reduce the development leaf spot symptoms in plants compared with the infected control. We could not observe any harmful effects from the plant extracts on the Chinese kale plants at a maximum concentration tested in our study. Based on our results, the *C. brachiata* and *H. anthelminthicus* extracts were effective in suppressing the development of Chinese kale leaf spots by more than 80% even at a concentration of 50,000 ppm, 30 days after planting and inoculation with *A. brassicicola*. The results strongly suggest that the crude extracts of *C. brachiata* and *H. anthelminthicus* can be used as new potential sources for the development of alternative bio-fungicide to replace synthetic fungicide. Moreover, it will be simpler in its application.

Our study demonstrated that three plant extracts, *viz.,* from *H. anthelminthicus*, *X. lanceatum* and *C. brachiata* can be used for the biological control of Chinese kale leaf spots disease. The three plant extracts can effectively control Chinese kale leaf spots in *in vivo*. The greatest reduction in the disease incidence of leaf spots was observed in Chinese kale plants treated with the *C. brachiata* and *H. anthelminthicus* extracts at concentration of 50,000 ppm, 30 days after planting and inoculation with the pathogen. Thus, the three plant extracts could be applied to control *A. brassicicola* caused by Chinese kale leaf spots disease and be employed as the alternative method in reducing synthetic fungicide use, especially on crops and vegetables produced for fresh consumption. There will be a reduction of hazards to human health and environment. However, further study is needed regarding plant extracts to identify the bioactive compounds responsible for their fungicidal activity in order to replace the use of synthetic fungicides.

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