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Effect of leaf extract of *Hydnocarpus* on control of anthracnose of Chinese cabbage caused by *Colletotrichum higginsianum*

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ABSTRACT

The dry leaf powder of hydnocarpus (*Hydnocarpus anthelminthicus* Pierre ex Lecomte) was extracted by boiling, autoclaving and shaking methods with water or 70% ethanol, adjusted to the concentrations of 0.25, 0.5, 1.0 and 2.5% (w/v), and tested for inhibitory effect on conidial germination and appressorial formation of *Colletotrichum higginsianum*, the causative agent of anthracnose of Chinese cabbage. The results showed that germination of conidia of *C. higginsianum* was not suppressed by the leaf extracts at concentration of 1.0% (w/v) or lower, whereas the formation of appressoria was significantly (P<0.05) suppressed by the extracts at concentrations of 1.0 and 2.5% (w/v). The leaf extracts in ethanol had higher suppressive effect on the formation of appressoria of *C. higginsianum*, as compared with the treatments of leaf extracts in water. Among the three extraction methods used, plant extract prepared by autoclaving showed the best ability to suppress the formation of appressoria, especially when ethanol was used as solvent for extraction of leaf samples. The results of a greenhouse test showed that spraying cabbage plants with leaf extract in ethanol at 0.5% (w/v) at 2 days prior to inoculation of *C. higginsianum* significantly (P<0.05) reduced the incidence and severity of anthracnose of Chinese cabbage. Whereas, spraying cabbage plants with leaf extract in ethanol at 0.5% (w/v) at 2days after inoculation of the pathogen had no significant (P>0.05) reduction on incidence and severity of anthracnose. In addition, disease severity of Chinese cabbage anthracnose rated by lesion number and lesionarea (%) was suppressed when ethanol extracts of hydnocarpus leaves at 0.5% (w/v) were applied at 1 to 7 days prior to inoculation of the pathogen, but the extracts were ineffective when sprayed at 1 to 6 days after inoculation of the pathogen.

Key words: Hydnocarpus, *Hydnocarpus anthelminthicus,* Chinese cabbage, anthracnose, *Colletotrichum higginsianum,* plant extract, disease control.

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INTRODUCTION

Anthracnose caused by *Colletotrichum higginsianum* Sacc. Apud Higgins is a destructive disease of Chinese cabbage (*Brassica campestris* L.) in Taiwan (Lin and Huang, 2002). Diseased leaves show formation of small, dry, circular and pale-grey to straw coloured lesions and, in severe cases, causing death of the entire leaf. The disease is of particular importance in the production of Chinese cabbage by organic farming practices, as it caused severe economic losses to the organic farming industry in Taiwan in 1999 and 2000 (Lin and Huang, 2002).

Currently, there are no alternative methods for the control of anthracnose, other than the use of chemical fungicides such as "Maneb®" and "Zineb®" (Sherf and MacNab, 1986). However, some of these chemicals may be potentially harmful to growers and consumers, as well as the agricultural ecosystem (Hickey, 1986). Moreover,

numerous plant pathogens have also been observed to develop tolerance or resistance to chemical fungicides after intensive treatment (Siegel and Sisler, 1977). Thus, developing environmentally friendly methods for controlling plant diseases has become an important and urgent issue in modern agriculture (Hsieh, 2014).

Some plants species may contain antimicrobial phytochemicals (secondary metabolites), such as β-glucan, alkaloids, terpenoids, phenols, phenolic acids, quinones, flavones, flavonoids, flavonols, saponins, tannins and coumarins (Cowan, 1999; Enyiukwu et al., 2014). Thus, plant materials with antifungal properties could be of potential for making natural plant products for controlling fungal diseases(Copping, 2004; Yoon et al., 2013). In a previous study, 67 plant extracts belonging to 33 Family, including ethanol extracts from leaves of *Hydnocarpus anthelminthicus* Pierre ex Lecomte, were effective in the suppression of spore germination of *C. higginsianum* (Hsieh et al., 2005). The objective of this study was to determine the effectiveness of leaf extracts of *H. anthelminthicus* on the control of anthracnose of Chinese cabbage.

MATERIALS AND METHODS

Inoculum of *Colletotrichum higginsianum*

A single-spore isolate PA-01 of *C. higginsianum*, courtesy of Dr. J. W. Huang, National Chung Hsing University, Taichung, Taiwan, was used in this study. Stock cultures were maintained on potato dextrose agar (PDA) (Difco Laboratory, Detroit,MI, USA) at room temperature (22-25°C) under 12 h diurnal illumination.

Inoculum of *C. higginsianum* was prepared from 14-day-old PDA slant cultures by adding 10 ml sterile distilled water to each culture and rubbing the surface of the colony with a scalpel to make a conidial suspension. Conidial suspensions collected from slant cultures were filtered through a two-layer cheese cloth to remove hyphae and agar fragments. The number of conidia in each conidial suspension was determined using a hemacytometer. Sterile distil water was used to adjust conidial concentrations required for the experiments.

Plant extracts and plant materials

Dry leaf powder of hydnocarpus (*H. anthelminthicus*) was prepared sing freeze-drying method (Murgatroyd, 1997). Dry leaf powder was added to water or 70% ethanol at a ratio of 1 : 3 (w/v). The samples were then processed using the extraction method of boiling water for 30 min, autoclaved for 20 min, or in a shaker at 100 rpm for 24 h. The extracted solutions were collected after centrifugation at 5,000 rpm for 10 min and diluted to 10, 25, 50 and 100 folds using sterile distill water to make the concentrations of 2.5, 1.0, 0.5 and 0.25% (w/v), respectively and used for testing spore germination and appressorial formation of *C. higginsianum*.

Seeds of Chinese cabbage, *Brassica rapa* L., were kept on a moistened filter paper (Whatman No. 1, 9-cm in diameter; Toyo Roshi Co., Japan) in a Petri dish and incubated at room temperature (22-25°C) for 1 day. Germinated seeds were sown in peat moss in plastic trays, 128 cells/tray and 1 seed/cell. After one week, seedlings were transplanted to Stender Peat Substrate (Known-You Seed Co., Ltd.Kaohsung, Taiwan) in plastic pots (18-cm in diameter), 3 plants/pot, kept in a growth chamber at 24°C or in green house at 28±4°C and watered daily for four weeks.

Effect of *Hydnocarpus* extracts on germination of conidia of *Colletotrichum higginsianum*

A glass slide method was used to evaluate the effect of water or ethanol extracts of leaves of Hydnocarpus on germination of conidia of *C. higginsianum,*isolate PA-01. This was done by placing four separated drops (10 µl/droplet) of conidial suspension (10^4 conidia/ml) of *C. higginsianum* on each of the glass slides. Ten microliter of 0.5, 1.0, 2.0 and 4.0 % (w/v) *Hydnocarpus* extracts were added to each drop of the spore suspension to make the final concentrations of 0.25, 0.5, 1.0 and 2.0% (w/v). For controls, 10 µl of sterile distill water or 70% ethanol by relative concentration (dilution fold) was added to each drop of the spore suspension. Each glass slide was placed on a moist filter paper in a Petri dish, incubated at 28°C for 15 h, and the sample was stained with cotton blue, approximately 20 µl/drop. One-hundred conidia per drop were examined for germination of conidia and formation of appressoria using a light microscope (model CH-2, Olympus, Optical Co., LTD. Japan). The experiment was repeated two times with four replicates per treatment.

Effect of *Hydnocarpus* extracts oncontrol of anthracnose of Chinese cabbage

To determine effect of *Hydnocarpus* extracts on the control of anthracnose of Chinese cabbage, 4-week-old plants were sprayed with water- or ethanol-extract of *Hydnocarpus* prepared using boiling, autoclaving and shaking extraction methods at concentration of 0.5% (w/v) until all leaves were covered with the solution at two days before or after inoculation of *C. higginsianum* PA-01. Plants sprayed with sterile distilled water or 70% ethanol plus 50 volume of distilled water were used as controls. Spore suspensions of *C. higginsianum* were inoculated on each plant at 8 ml/plant and10^5 conidia/ml, using a compressed air-sprayer (SIL-AIR, Werther International, Italy). All pots were placed in moist plastic bags, kept in a growth chamber at 24°C under 12 h diurnal illumination for 1-day, and then plastic bags

were removed. After further incubation for 7 days in the growth chamber, plants were examined for number of lesions per leaf and lesion area (%) in 3 cm diameter of leaf surface. The experiment was repeated two times with three replicates (pots) per treatment.

In addition, the treatments of 0.5% (w/v) ethanol extracts of *Hydnocarpus* prepared using autoclaving method, which showed significant inhibitory effects on anthracnose of Chinese cabbage in the growth cabinet, were selected for further experiments conducted in a greenhouse (28±4°C). The greenhouse experiment was carried out using the same procedure described above. One volume of 70% ethanol plus 50 volume of distilled water was used as control. The experiment was repeated two times with three replicates (pots) per treatment.

Effect of time of application of plant extract on controlling anthracnose of Chinese cabbage

To determine the effect of time of application of ethanol extracts of *Hydnocarpus* leaves on the control of anthracnose of Chinese cabbage, ethanol extract of *Hydnocarpus* leaves (0.5%; w/v) was sprayed on 4-week-old Chinese cabbage plants at 0, 1, 2, 4 and 7 days prior to the inoculation of *C. higginsianum*, or at 1, 2, 4 and 6 days after inoculation of the pathogen. The amount of ethanol plant extract was 8 ml per plant and the amount of conidial suspension (1×10^5 conidia/ml) of *C. higginsianum* was 8 ml per plant. Plants sprayed with conidial suspension of *C. higginsianum* alone were used as the control. Inoculated and control plants were kept in a growth chamber at 24°C for one week and examined for lesion number and infection area on leaves by the same method described previously. The experiment was repeated two times with three replicates (pots) per treatment.

Statistical analysis

Data collected from each experiment were analyzed by analysis of variance (ANOVA) with the SAS system for personal computers (SAS Institute, Cary, NC). Separation of means for the treatments of each experiment was accomplished using Duncan's multiple range test at P = 0.05.

RESULTS

Effect of *Hydnocarpus* extracts on germination of conidia of *Colletotrichum higginsianum*

Among the three extraction methods used in this study, the autoclaving and shaking methods were more effective than the boiling water method for extraction of ethanol extracts of *H. anthelminthicus* with strong inhibitory effect on conidial germination and appressorial formation of *Colletotrichum higginsianum*. For example, germination of conidia of *C. higginsianum* was completely inhibited by treatment with 2.5% (w/v) ethanol extracts of *Hydnocarpus* extracted using autoclaving and shaking methods, but not by the extraction method of boiling water (Table 1). Moreover, only the ethanol extracts of *Hydnocarpus* were effective, as the treatments with 2.5% (w/v) water extracts of *Hydnocarpus* failed to inhibit germination of conidia of *C. higginsianum* in any of the three extraction methods. Ethanol extracts at concentration of 1.0% (w/v) or lower, as well as water-extracts prepared by the three extraction methods failed to suppress germination of conidia of *C. higginsianum.* However, the formation of appressoria was significantly (P<0.05) suppressed by treatments with 1.0% (w/v) ethanol extract or water extract of *Hydnocarpus*. The hydnocarpus solutions derived from ethanol extraction had higher suppressive effect on the formation of appressria of *C. higginsianum*, as compared with the leaf extracts in water. For example, in the use of autoclaving method for extraction, the formation of appressoria of *C. higginsianum* was 0 and 26% by treatment with 1% (w/v) ethanol extract of *Hydnocarpus* and 1% (w/v) water-extract of *Hydnocarpus*, respectively (Table 1). Among the three extraction methods, plant extracts prepared using the method of autoclaving was superior to the extracts prepared using the boiling water method or shaking method in the inhibition of appressorial formation of *C. higginsianum*, especially when ethanol was used as a solvent for extraction.

Effect of *Hydnocarpus* extracts oncontrol of anthracnose of Chinese cabbage

The experiment in the growth cabinet showed that the incidence of anthracnose of Chinese cabbage was significantly (P<0.05) reduced, when the 4-week-oldplants were sprayed with ethanol extracts of *Hydnocarpus*, prepared using boiling water method or autoclaving method or shaking method and applied at 2-day prior to inoculation of *C. higginsianum* (Table 2). The number of leaf lesions on plants sprayed with ethanol extracts was 7.0, 11.2 and 12.1 lesions/leaf for the extraction methods of autoclaving, shaking and boiling, respectively as compared with 17.8 lesions/leaf in the treatment of ethanol control. However, all water extracts of *Hydnocarpus* prepared by boiling water or autoclaving or shaking method and sprayed on cabbage plants at 2-day prior to inoculation of *C. higginsianum* showed no significant (P>0.05) reduction in the number of anthracnose lesions (Table 2). The experiment in the growth cabinet also showed no significant (P>0.05) difference in disease severity when the plant extracts prepared by ethanol or water, were sprayed on plants at 2days after inoculation of *C. higginsianum*

Table 1: Effect of extraction methods and concentration of leaf extracts of Hydnocarpus on germination of conidia and formation of appressoria of *Colletotrichum higginsianum.*

Extraction method[1]	Concentration (%, w/v)	Ethanol-extract		Water-extract	
		Spore germination[2] (%)	Appressorial formation[2] (%)	Spore germination (%)	Appressorial formation (%)
Control	2.5	67^{d3}	65^{c}	95^{a}	93^{a}
(Ethanol or water)	1.0	85^{c}	78^{b}	-	-
	0.5	95^{ab}	94^{a}	-	-
	0.25	96^{a}	95^{a}	-	-
Boiling	2.5	94^{ab}	0^{g}	93^{b}	8^{g}
	1.0	96^{a}	2^{g}	95^{a}	43^{e}
	0.5	96^{a}	19^{e}	95^{a}	75^{c}
	0.25	96^{a}	52^{d}	95^{a}	84^{b}
Autoclaving	2.5	0^{e}	0 ^{g}	95^{a}	3^{gh}
	1.0	93^{b}	0 ^{g}	96^{a}	26^{f}
	0.5	96^{a}	0 ^{g}	96^{a}	52^{d}
	0.25	95^{ab}	8 ^{f}	95^{a}	82^{b}
Shaking	2.5	0^{e}	0^{g}	97^{a}	0^{h}
	1.0	94^{ab}	0^{g}	96^{a}	8^{g}
	0.5	95^{ab}	2^{g}	95^{a}	46^{e}
	0.25	95^{ab}	18^{e}	96^{a}	83^{b}

[1]One hundred grams of dry powder of *Hydnocarpus anthelmintica* leaves were added with 300 ml of distilled water or 70% ethanol and extracted by boiling for 30min., autoclaving for 20 min., and shaking for 24hrs, respectively. Each stock solution was further added with 10, 25, 50 and 100 volumes of distilled water before use. One volume of 70% ethanol plus 10, 25, 50 and 100 volumes of distilled water was used as control. [2]Percentage of spore germination and appressorial formation were recorded 16 h after treatment. [3]Data presented are the average of two experiments. Means followed by the same letter in each column are not significantly (P>0.05) different at according to Duncan's multiple range test.

Table 2: Effect of leaf extracts of Hydnocarpusprepared by different extractionmethods on control of anthracnose of Chinese cabbage caused by *Colletotrichum higginsianum* in a growth chamber[2].

Extraction method[1]	Solvent	2 days before inoculation		2 days after inoculation	
		Lesion no.	Lesion area (%)	Lesion no.	Lesion area (%)
Control	Water	19.1^{a3}	24.4^{a}	20.4^{a}	23.9^{a}
	Ethanol	17.8^{a}	22.9^{a}	18.9^{a}	23.9^{a}
Boiling	Water	18.3^{a}	22.2^{a}	18.6^{a}	23.8^{a}
	Ethanol	12.1^{b}	14.6^{b}	18.8^{a}	24.4^{a}
Autoclaving	Water	18.6^{a}	22.4^{a}	18.3^{a}	23.5^{a}
	Ethanol	7.0^{c}	9.1^{c}	17.9^{a}	24.8^{a}
Shaking	Water	18.4^{a}	22.5^{a}	19.4^{a}	24.4^{a}
	Ethanol	11.2^{b}	13.5^{b}	18.0^{a}	23.7^{a}

[1]One hundred grams of dry powder of *Hydnocarpus anthelmintica* leaves were added with 300 ml of distilled water or 70% ethanol and extracted by boring for 30min., autoclaving for 20 min., and shaking for 24hrs, respectively. Each stock solution was further added with 50 volume of distilled water before use. One volume of water or 70% ethanol plus 50 volume of distilled water was used as control. [2]Trial was conducted in growth chamber (24±2℃). The 0.5% (w/v) plant extracts were used. Data were recorded 7 days after inoculation. Each treatment had 10 plants.n[3]Data presented are the average of two runs of experiment. Means followed by the same letter in each column are not significantly (P>0.05) different according to Duncan's multiple range test.

Table 3: Effect of ethanol extract of Hydnocarpus leaves prepared by autoclave extraction method on control of anthracnose of Chinese cabbage in a greenhouse.

Treatment[1]	Inoculation[2]	Lesion no.	Lesion area (%)
Check (ethanol dilution)	Pre-inoculation	16.4[a3]	24.4[a]
	Post-inoculation	15.8[a]	24.6[a]
0.5% (w/v) Hydnocarpus	Pre-inoculation	7.2[b]	10.6[b]
	Post-inoculation	15.4[a]	23.8[a]

[1]One hundred grams of dry powder of *Hydnocarpus anthelmintica* leaves were added with 300 ml of 70% ethanol and extracted by autoclaving for 20 min. The stock solution was further added with 50 volume of distilled water before use. One volume of 70% ethanol plus 50 volume of distilled water was used as control. [2]The plants were sprayed with diluted plant extract 2 days before (pre-) or after (post-) inoculation. [3]Data were average of two experiments. Data followed by the same letter in each column are not significantly (P>0.05) different according to Duncan's multiple range test.

(Table 2). Also, there was no significant (P>0.05) difference in disease severity among the three methods used for extraction of plant leaves in the form of ethanol extracts or water extracts.

The experiment in the greenhouse showed that 4-week-old, Chinese cabbage plants sprayed with 0.5% (w/v) ethanol-extract of *Hydnocarpus* at 2 days before inoculation of *C. higginsianum* significantly (P<0.05) reduced the incidence and severity of anthracnose on the treated plants (Table 3). The lesion number per leaf and lesion area (%) were 7.2 and 10.6, respectively for the pre-inoculation treatment of 0.5% (w/v) ethanol-extract, as compared with 16.4 and 26.4, respectively for the pre-inoculation treatment of 0.5% (w/v) ethanol control (Table 3). The experiment in the greenhouse also showed no significant (P>0.05) difference in disease severity when the 0.5% (w/v) ethanol extract of *Hydnocarpus* leaves was sprayed on plants at 2 days after inoculation of *C. higginsianum* (Table 3).

Effect of time of application of plant extract on the control of anthracnose of Chinese cabbage

Days of application of leaf extracts of *Hydnocarpus* prepared in ethanol using the extraction method of autoclaving affected both lesion number and lesion area (%) of anthracnose on cabbage plants. Autoclaved ethanol extracts of *Hydnocarpus* sprayed on cabbage plants at 0, 1, 2, 4 and 7 days prior to inoculation of *C. higginsianum* reduced lesion number to lower than 12 lesions/leaf and lesion area of 12%, as compared with 17 lesions/leaf and lesion area of 25% in the treatment of control (Figure 1). However, the application of ethanol extracts of *Hydnocarpus* at 1, 2, 4 and 6 days after inoculation of C. *higginsianum* showed no significant reduction in lesion number and lesion area, as compared with the concurrent application (0 day) of ethanol extracts of *Hydnocarpus* and the pathogen (Figure 1).

DISCUSSION

H. anthelminthicus is a species of medicinal plants widely cultivated in the Southeast Asia region, mainly in China, Thailand, Indonesia, Malaysia, and Taiwan (Padua et al., 1999). It is one of the 50 fundamental herbs used in traditional Chinese medicine (Wong, 1976) for the treatment of leprosy, skin disorders and other types of dermatitis and tuberculosis (Saboo et al., 2014). In addition to the medicinal value, Jantasorn et al. (2016) reported that fruit extracts of *H. anthelminthicus* contain antifungal substances against plant pathogens such as *Pyricularia oryzae*, *Phytophthora palmivora*, *Rhizoctonia solani* and *Sclerotium rolfsii,* and, therefore, they may be of potential for use in developing natural products for control of these plant pathogenic fungi. Beside fruit extracts (Jantasorn et al. 2016), present study indicates that ethanol extracts from leaves of *H. anthelminthicus* also have strong inhibitory effects on conidial germination and appressorial formation of *C. higginsianum*, the causative agent of anthracnose of Chinese cabbage. Therefore, the potential of leaves and fruits of *H. anthelminthicus* for practical use in the control of fungal diseases of plants needs further investigations.

The antifungal property of extracts of medicinal plants is highly related to the method of extraction and the type of solvents used in the preparation of plant extracts. The present study evaluates the preparation of crude extracts from leaves of *Hydnocarpus* for their antifungal activities using 70% ethanol and water as solvents and preparing extracts using the method of boiling water, autoclaving and shaking. Among the three extraction methods, ethanol extracts prepared from leaves of *Hydnocarpus* by autoclaving had the strongest suppression effect on the formation of appressoria of *C. higginsianum*, whereas water extracts from leaves of *Hydnocarpus* were ineffective in any

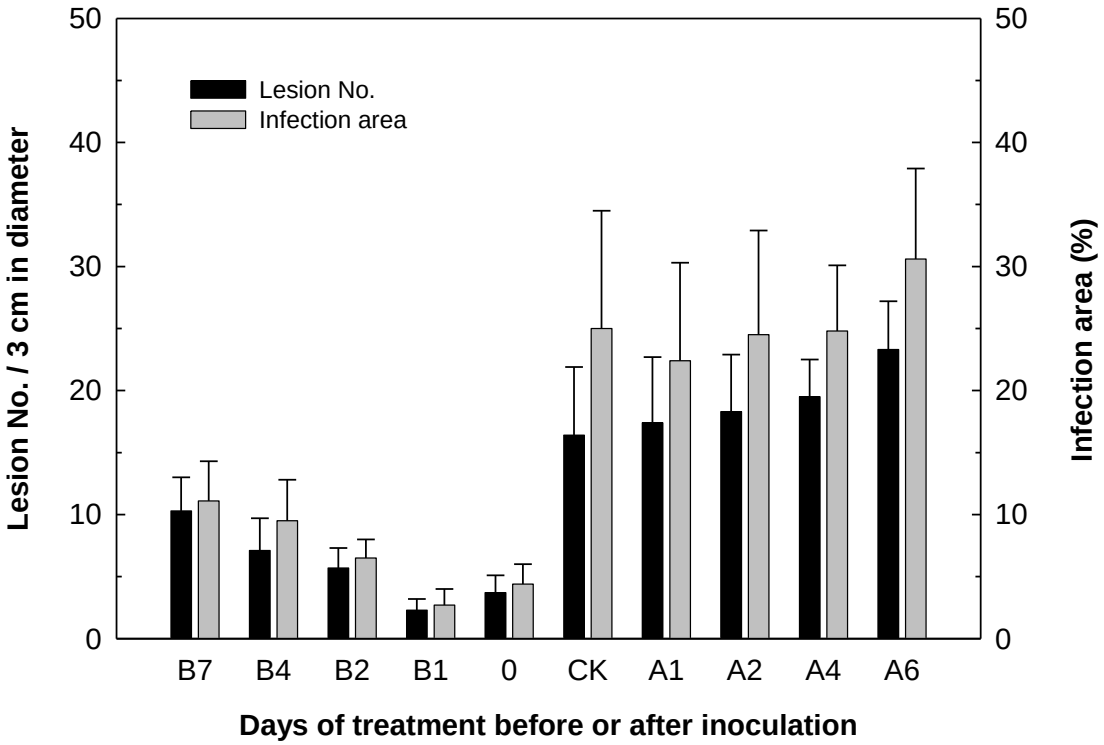

Figure 1: Effect of days of application of ethanolleaf extract of hydnocarpus preparedby autoclaving methodon lesion number and lesion area of anthracnose of Chinese cabbage cause by *Colletotrichum higginsianum* in a growth chamber. 0 = leaf extract and pathogen were applied on plants simultaneously; B1, B2, B4 and B7 = leaf extracts were sprayed on plants at 1, 2, 4 and 7 days before inoculation of the pathogen; A1, A 2, A 4 and A 6 = leaf extracts were sprayed on plants at 1, 2, 4 and 6 days after inoculation of the pathogen.

of the three extraction methods used in this study (Table 1). Our study confirms the report of Azwanida (2015) that the pre-extraction and the extraction procedures are important steps in the processing of bioactive constituents from medicinal plant materials. However, no universal extraction methods and extraction procedures are ideal and unique for the extraction of all medicinal plants (Azwanida, 2015). Pandey and Tripathi (2014) reported that successful extraction of biologically active compounds from plant materials is mainly dependent on the type of solvent used in the extraction procedure. They also reported that ideal solvents used for active component extraction are water, ethanol, methanol, chloroform, ether, and acetone (Pandey and Tripathi, 2014). Water and ethanol were used as the solvents in the present study in order to minimize negative residual effects of solvent in the leaf extracts of *Hydnocarpus*.

The greenhouse study showed that, beside the type of solvent, concentration and time of application of leaf extract of *Hydnocarpus* are critical for effective control of anthracnose of Chinese cabbage. The application of ethanol extracts of *Hydnocarpus* leaves on each plant, at a concentration of 0.5% and at 2 days prior to inoculation of the pathogen, significantly reduced incidence and severity of anthracnose of Chinese cabbage (Table 3). In addition, the result of the application time of ethanol extracts on controlling anthracnose of Chinese cabbage showed that the disease was suppressed by spraying extracts in the simultaneous inoculation (0 day) and 1 to 7 days prior to inoculation of the pathogen (Figure 1). Several reports have shown that the application of plant extract on target plants before inoculation of pathogens results in best disease-reduction. The greatest reduction in the incidences or severity of pearl millet midrib spot caused by *Curvularia eragrostidis* (Zarafi and Moumoudou, 2010), blast disease caused by *Pyricularai oryzae* (Amadioha, 2000), and cowpea anthracnose caused by *Colletotrichum lindemuthianum* (Falade, 2017) were observed in targeted plants treated with the selected extracts 2 days before inoculation. Lowest percent anthracnose caused by *Colletotrichum capsici* was observed in the fruits sprayed with clerodendrum extract (*Clerodendrum infortunatum* L.) before inoculum (Choudhury et al., 2017). Khoa et al. (2011) evaluated the spraying time of *Chromolaena odorata* fresh or dry leaf extract on control of rice sheath blight caused by *R. solani* and revealed that the effect of disease reduction was no differences between the spraying time points of 3, 5, and 7 day before inoculation. The greenhouse study suggests that ethanol extracts of *Hydnocarpus* prepared using autoclaving method may be of potential for

the control of anthracnose of Chinese cabbage in commercial production and, thus, requires further investigations.

Seed oil contained a characteristic class of compounds as cyclopentenyl fatty acids from species of the *Hydnocarpus* genus is used for medicinal purposes, predominantly for the treatment of various skin disorders (Sahoo et al., 2014). Furthermore, seeds of this genus have been reported to contain triglycerides of fatty acids, sterols, flavonoids, and flavonolignans. Hydnocarpin, a flavonolignan, has been reported to have antimicrobial and anticancer activity (Sahoo et al., 2014). The present study showed that *Hydnocarpus* leaf extracts contain a heat-resistant secondary metabolite which is highly inhibitory to the formation of appressoria from conidia of *C. higginsianum*. Further, phytochemical investigations are needed to identify the active ingredient of *Hydnocarpus* responsible for antifungal plant pathogens.

So far, only a few botanical fungicides derived from plant extracts were developed and commercialized in the world. The low number of products registered as botanical fungicides might be due to the fact that most of researches on antifungal activities of medicinal plants were confined to *in vitro* studies, not *in vivo* or greenhouse and/or field studies (Hsieh, 2014). Thus, besides *in vitro* studies, it is also of paramount importance to conduct commercial greenhouse trials or field trials to determine the efficacy and practicality of control fungal plant diseases by plant extracts.

Conclusion

This study showed that ethanol extract of leaves of *H. anthelminthicus* contain substances with strong inhibitory effects on appressorial formation of *C. higginsianum*, the causative agent of anthracnose of Chinese cabbage. Among the three extraction methods used, the ethanol extracts of *Hydnocarpus* prepared using autoclaving method is the most promising method for the control of anthracnose of Chinese cabbage as application of 0.5% (w/v) leaf extract in ethanol on 4-week-old plants at 2-day before inoculation of *C. higginsianum* significantly reduced incidence and severity of anthracnose of Chinese cabbage. However, the application of 0.5% (w/v) leaf extract of *Hydnocarpus* in ethanol at 2-day after inoculation of *C. higginsianum* failed to control anthracnose of Chinese cabbage. Thus, further studies are required to determine the practicality of the application of 0.5% (w/v) leaf extract of *Hydnocarpus* to ethanol for protection of Chinese cabbage plants from outbreak of anthracnose.

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