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Antifungal activities of endophytic fungi isolated from orchids against *Colletotrichumgloeosporioides*caused anthracnose in orchids

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Crude extracts from endophytic fungi, *Daldiniaeschscholtzii*, *Chaetomiumcochliodes* and *Chaetomiumcupreum*, were isolated from orchids showed ability to inhibit the mycelia growth and spore production of *Collectotrichumgloeosporioide*s caused antracnose in orchids. Crude hexane, ethyl acetate and methanol from *Daldiniaeschscholtzii*at the concentrations 1,000µg/mlshowed the ability to inhibit mycelia growth of *Colletotrichum* sp. which was 75.5%, 61.75% and 41.75% respectively, inhibited spore productions which were 65.5%, 69.45% and 33.09% respectively which the ED_{50} values as 220, 104 and 2971µg/ml, respectively. Crude extract from *Chaetomiumcochliodes* at the concentrations 1,000µg/ml can inhibit the mycelia growth of *Colletotrichum* sp. which was 53.75%, 35.5% and 60.25% respectively, inhibited spore production of 46.12%, 51.77% and 60.87% respectively and the ED_{50} values as 1754, 1879 and 712µg/ml,respectively. Crude extract from *Chaetomiumcupreum* also showed ability to inhibit *Colletotrichum* sp., at the concentrations 1,000µg/ml that inhibited the growth of mycilia as 40.5%, 67.25% and 37.75%, inhibited spore productions as 51.35%, 51.27% and 58.65%, respectively and the ED_{50} value 794, 624 and 879µg/ml,respectively.

Keywords: endophytic fungi, orchid, *Daldiniaeshscholtzii*, *Chaetomiumcochliodes*, *Chaetomiumcupreum,Colletotrichum* sp.

Introduction

Resently, there are many works that studying about endophytic fungi from many plants, both fungi and bacteria. Endophytic microorganisms are those that colonize the healthy plant internal tissue (Stone, *et al*., 2004). The meaning of term "endophyte" is as broad as its literal definition and spectrum of potential hosts and inhabitants. Endophytes are used for both bacteria and fungi (Schulz. *et al*., 2006). The endophytic fungi have three major roles as saprophyte on dead or senescing tissue, Mutualism that can protect the host

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plant from pathogen or can promote plant growth and probably expressed as latent pathogens and avirulent pathogens at early stages of infection. Recently endophytic fungi have attracted the attention from many scientists in the world as estimated that such species may useful as sources of anticancer, antidiabetic, insecticidal and immune-suppressive compounds (Strobel and Daisy, 2003). Orchids are among the plant groups that have aroused most widespread interest among scientists and horticulturists, both for their study and use. They have been subjected to particularly high commercial demand over the past 40 years for the beauty of their structure and their vividly colored flowers, steeped in symbolism and mystery. Some countries have declared orchid species as their national flowers (Clemente, 2009).The orchid cultivation has many problems specially, fungal diseases. Among these, anthracnose caused by *Colletotrichumgloeosporioides* is a most destructive disease and known to cause great losses to the orchid growers in terms of bothquality and quantity (Duff and Daly, 2002). *C. gloeosporioides* can attack leaves, petioles and blooms during periods of prolonged leaf moisture and high humidity (Bailey, *et al*., 1992). The ability to cause latent or quiescent infections has grouped *Colletotrichum* among the most important post-harvest pathogens (Freeman, 1998). *C. gloeosporioides*, known as one of the world's most important pathogens, is a species complex comprising morphologically indistinguishable but genetically isolated species and has been reported on broad range of hosts (Cai, 2009).

The objectives of research findings were to tested the isolated endophytic fungi, *Chaetomiumcochliodes*, *Chaetomiumcupreum* and *Daldiniaeschscholtzii* from orchid varieties against*C. gloeosporioides* causing anthracnose in orchids.

Materials and methods

Isolates of endophytic fungi

The endophytic fungi were isolated and reported as previous study (Sour, *et al.*, 2015).

Isolation of pathogen

The infected leaf anthracnose in orchid was isolated by using transplanting technique (Agrios, 2005). The leaf was cut in to small pieces, washed by distilled water for one time, put in 5 % ethanol for 3 minutes, pour in 0.5% sodium hypochlorite solution 2 minutes and washed in distilled water thrice, dried with autoclaved tissue paper, thentransferred to water agar (WA) and

incubated at room temperature until mycelia emerge. The mycelia weretransferred to Potato Dextrose Agar (PDA) for pure culture isolation.

Pathogenicity test

The healthy leaf of *Dendrobriumspeciosum*were used to test for pathogenicity. The experiment was performed as Completely Randomized Design (CRD) with four replications by deatached leaf method. Treatments were set up as inoculated leaves and non-inoculated leaves which served as the controls. The leave sampleswere washed by distilled water for one time, dried with autoclaved tissue paper. The pathogen was cultured on PDA and collected as spore suspension at concentration of 1 X 10^6 spores/ml, then dropped onto wounded leaves. The controls were dropped only sterilize distilled water. All treated leaves were put into plastic box as moist chamber and incubated at room temperature fpr gathering data of disease level.

Extraction method

Crude extracts from endophytic fungiact as the promising antagonistswere done which followed the method of Kanokmedhakul *et al*. (2006). The fungi wereseparately cultured in potato dextrose broth at room temperature for one month. The dried fungal biomass of antagonistic fungus was ground with the electrical blender and dissolved with hexane for 7 days before running in vacuo to yield crude hexane extract. The marc was then continued to dissloved in ethyl acetate (EtOAC) as the same manner to get crude ethylt aceatate and crude methanol extract, respectively. Crude extracts were kept in refigerator umtil use.

Bioactive tests of crude extracts against pathogen

The experiment was conducted by using two factors factorial experiment in CRD with four replications. Factor A represented the crude extracts: A1 = crude hexane extract, A2 = crude ethyl acetate extract and A3 = crude methanol extract. Factor B represented the different concentrations: B1 = 0µg/ml(control), B2 = 10µg/ml, B3 = 50 µg/ml, B4 = 100 µg/ml B5 = 500 µg/ml and B6 = 1,000µg/ml. Each crude extract was dissolved in 2% dimethyl sulfoxide and added to PDA before autoclaving at 121°C (15 psi) for 20 minutes. A sterilized 3-mm diameter cork borer was used to cut at the colony peripheral to get culture agar plug, then transferred to the center of 5 cm diameter Petri dishes of PDA containing each crude extract at each concentration and incubated at room temperature until the pathogen on the control plates growing full plate. The

pathogen cells in each treatment were observed under compound microscope and taken phoyograph for comparison. Data were collected regarding the mycelia growth andnumber of spore produced by the pathogen and calculated the percentage of conidia inhibition. Data were subjected analysis of variance (ANOVA) and treatment means were compared using Duncan Multiple's Range Test (DMRT) at P=0.05 and 0.01. The effective dose (ED_{50})werecalculated using Probit analysis.

Results

Isolation of pathogen

Colletotrichumgloeosporioides was isolated from leave of orchid, *Grammatophylumspecinocum*.The detail characteristic were observed under compound microcope to see conidiophores and conidia, then measured (data not shown) as seen in Fig.1.

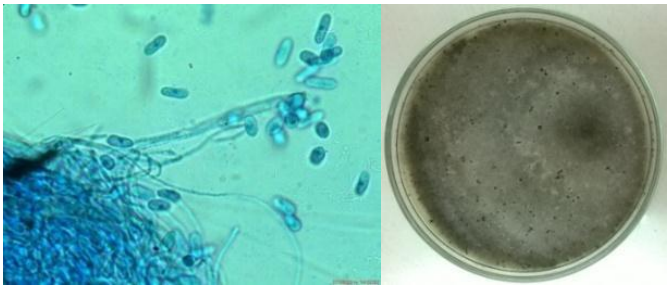
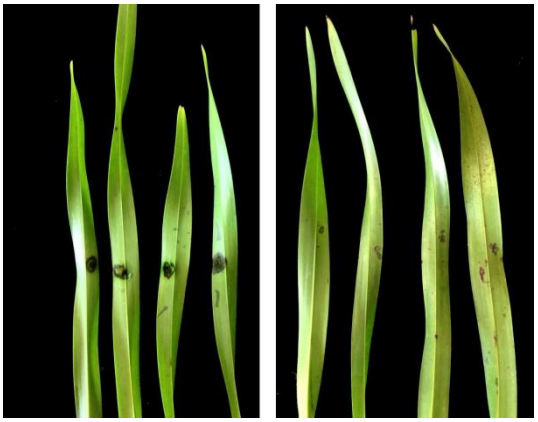


Fig.1. *Colletotrichumgloeosporioides*. Left = conidiophores and conidia; right = pure culture on PDA

Pathogenicity test

The pathogenicity test was successful done by inoculated the spore suspension to woumded leaves of *Grammatophylumspecinocum*. The inoculated pattrenshowed cleaelysymptom and no symptom showing in the uninoculated controls as seen in Fig.2.



Inoculated *Colletotrichum* sp. Un-inoculated with *Colletotrichum* sp.

Fig.2. Show the infection and un-infection of pathogen on leaves of *Grammatophylumspecinocum*

Bioactive tests of crude extracts against pathogen

Hexane crude extract from *Daldiniaeschscholtzii* at the concentrations 500 and 1,000µg/ml gave significantly different in colony diameters of *Colletotrichum* sp. which were 1.9 and 1.22 cm, respectively when compared to the control (0µg/ml) 5cm. Ethyl acetate crude extract at the concentration of 100, 500 and 1,000µg/ml which were 3.98, 3.33 and 2.01cmrespectively gave significantly different when comparedwith the control (0µg/ml). Methanol crude extract at the concentrations 50, 100, 500 and 1,000µg/ml also gave significantly different which were 3.91, 4.46, 3.35 and 2.63cm respectively when compared to the control (0µg/ml).

Hexane crude extract from *Chaetomiumcochliodes*at all the concentrations of 10, 50, 100, 500 and 1,000µg/ml showed colony diameters of 4.18, 4.09, 3.94, 3.82 and 2.31cm respectivelywhich gave significantly different when compared to the control (0µg/ml). Ethyl acetate crude extract all the concentrations of 10, 50,100,500 and 1,000µg/mlshowing colony diameters of 4.39, 4.34, 4.11, 3.25 and 3.23cm, respectively which gave significantly different when compared to the control (0µg/ml). Methanol crude extract at the concentrations 50, 100, 500 and 1,000µg/ml, which were 4.19, 3.48, 3.45 and 1.99cm, respectively gave significantly different when compared to the control (0µg/ml) 5cm.

Hexane crude extract from *Chaetomiumcupreum*at all the concentrations of 10, 50, 100, 500 and 1,000µg/ml showing colony diameters of4.21, 4.15, 3.46, 2.85 and 2.73cm, respectively which significantly differed to the control (0µg/ml). Ethyl acetate crude extract at all the concentrations of 10, 50, 100,

500 and 1,000µg/ml, showing colony diameters of 4.21, 4.17, 4.08, 3.16 and 1.64cm, respectively which significant differed when compared to the control (0µg/ml). Methanol crude extract at all the concentrations of 10, 50,100, 500 and 1,000µg/mlshowing colony diameters of 4.3, 4.04, 3.01, 3.34 and 2.86cm, respectively which significantly differed when compared to the control (0µg/ml) as seen in Table 1.

The highest inhibition to tested pathogen showed that crude hexane extract at 1,000 µg/ml of from *Daldiniaeshscholtzii*was 75.5%, followed by crude hexane extract at the concentrations 500µg/ml which was 62.25% and crude ethyl acetate at the concentrations 1,000µg/ml which was 61.75%.

Crude extracts from *Chaetomiumcochliodes*inhibited mycelium growth of tested pathogen at different level of inhibition. The highest inhibition was shown in crude methanol extract at the concentration 1,000µg/ml which was 60.25%, and followed by crude hexane extraxctat the concentration 1,000µg/ml which was 53.75% and crude ethyl acetate extract at the concentrations of 1,000µg/ml which was 35.5%.

Table 1: Effect of crude extracts from antagonistic fungi on mycelia growth of *Colletotrichum* sp.

Crude extract	**Mycelia growth (cm) of *Colletotrichum sp. At each concentration (µg/ml)***					
	0	**10**	**50**	**100**	**500**	**1,000**
Daldiniaeschscholtzii						
Hexane	5a[1]	4.75ab	4.45abc	4.5abc	1.9fg	1.22g
EtOAc	5a	4.7abc	4.23abc	3.98bcd	3.33de	2.01f
MeOH	5a	4.21abc	3.91cd	4.46abc	3.35de	2.63ef
Chaetomiumcochlioedes						
Hexane	5a	4.18bcd	4.09bcd	3.94cde	3.82de	2.31g
EtOAc	5a	4.39bc	4.34bcd	4.11bcd	3.25f	3.23f
MeOH	5a	4.58ab	4.19bcd	3.48ef	3.45ef	1.99g
Chaetomiumcupreum						
Hexane	5a	4.21b	4.15b	3.46c	2.85d	2.73d
EtOAc	5a	4.21b	4.17b	4.08b	3.16cd	1.64e
MeOH	5a	4.3b	4.04b	3.01cd	3.34c	2.86d

[1]Average of four replications. Means followed by the same letters in each column were not
significantly different by DMRT at P=0.01.

Fig.3. Crude extract test of *Daldiniaeshscholtzii* against *Colletotrichum* sp.

Fig.4. Crude extract test of *Chaetomiumcochliodes* against *Colletotrichum* sp.

Fig.5. Crude extract test of *Chaetomiumcupreum* against *Colletotrichum* sp.

Crude extracts from *Chaetomiumcupreum*inhibited mycelium growth of the tested pathogen at different levels. The highest inhibition to tested pathogen was crude ethyl acetate extract at the concentration 1,000µg/ml which was 67.25µg/ml, and followed by crude methanol extract at the concentration 1,000µg/ml which was 42.75%, and crude hexane extract at the concentrations 1,000µg/ml which was 40.4% as seen in Table 2.

Table 2: The percentages effected of crude extracts from antagonistic fungi on mycelia growth of *Colletotrichum* sp.

Crude extracts	**Percentage Colony inhibition of *Collectotrichum* sp.**				
	10	**50**	**100**	**500**	**1,000**
Daldiniaeschscholtzii					
Hexane	5e[1]	11e	10e	62.25ab	75.5a
EtOAc	6e	15.75de	20.5de	33.35cd	61.75ab
MeOH	15.75de	21.75de	9.25e	33.1cd	47.5bc
Chaetomiumcochlioedes					
Hexane	16.5de	18.25de	21.25cd	23.5bcd	53.75a
EtOAc	12.25de	13.5de	17.75de	35b	35.5b
MeOH	8.5e	14.25de	30.5bc	31bc	60.25a
Chaetomiumcupreum					
Hexane	15.75d	16.5d	31.25bc	38.5b	40.5b
EtOAc	15.75d	15.75d	18cd	36.75	67.25a
MeOH	13.25d	19.25cd	39.75b	33.1b	42.75b

[1]Average of four replications. Means followed by the same letters in each antagonist were not significantly different by DMRT at P=0.01.

Hexane crude extract of *Daldiniaeschscholtzii* at the concentrations 50, 100, 500 and 1,000µg/mlsignificantly inhibited spore production which were $2.74x10^7$, $2.67x10^7$, $1.46x10^7$ and $1.24x10^7$ spores/ml, respectively when compared to the control ($3.6x10^7$spore/ml). Ethyl acetate crude extract at all the concentrations of 10, 50, 100, 500 and 1,000µg/ml inhibited spore production which were $2.65x10^7$, $2.59x10^7$, $2.45x10^7$, $1.8x10^7$ and $1.15x10^7$spres/ml respectively, when compared to the control ($3.78x10^7$spores/ml). Methanol crude extract at the concentrationsof 50, 100, 500 and 1,000µg/mlinhibited spore production which were $2.89x10^7$, $2.59x10^7$, $1.8x10^7$ and $1.65x10^7$spores/ml which significantly differed from the controls($3.76x10^7$spores/ml). Crude extract of methanol, hexane and ethyl acetate inhibited spore production showed the ED_{50} values of 2971, 220 and 104 µg/ml,respectively.

Hexane crude extract of *Chaetomiumcochliodes*at all the concentrations of 10, 50, 100, 500 and 1,000µg/mlinhibited spore production which were

$2.96x10^7$, $2.93x10^7$, $2.35x10^7$, $2.18x10^7$ and $2.05x10^7$spores/ml, respectively which significant differed when compared to the control ($3.44x10^7$spores/ml). Ethyl acetate crude extract at all the concentrations 10, 50, 100, 500 and 1,000µg/mlinbited spore production which were $2.41x10^7$, $2.19x10^7$, $2.07x10^7$, $1.84x10^7$ and $1.48x10^7$spores/ml, respectivelywhich significant differed when compared to the control ($3.08x10^7$spores/ml). Methanol crude extract at the concentrations 100, 500 and 1,000µg/mlinhibited spore production which were 2.21x107, 1.81x107 and $1.19x10^7$spores/ml, respectively which significantly differed to the control (3.03x10*7*spores/ml).Crude extract of ethyl acetate, hexane and methanol inhibited spore production of tested pathogen which the ED_{50} values of 1879, 1754 and 712µg/ml, respectively.

Hexane crude extract of *Chaetomiumcupreum*at all concentrations of 10, 50, 100, 500 and 1,000µg/ml inhibited spore production which were $3.38x10^7$, $3.17x10^7$, $3.11x10^7$, $2.37x10^7$ and $2.05x10^7$spores/ml, respectively which significantly differed when compared to the control ($4.27x10^7$). Ethyl acetate crude extract at the concentrations of 10, 50, 100, 500 and 1,000µg/mlinhibited spote production which were $3.38x10^7$, $3.23x10^7$, $3.11x10^7$, $2.31x10^7$ and $2.05x10^7$spores/ml respectively gave significantly different when compare to the control (0µg/ml) $4.26x10^7$spores/ml. Methanol crude extract at the concentration of 100, 500 and 1,000µg/mlinbibited spore production which were $2.64x10^7$, $2.49x10^7$ and $1.69x10^7$spores/ml that significantly differed when compared to the control ($4.07x10^7$spores/ml). The ED_{50}values of crude methanol, hexane and ethyl acetateti inhibit spore production of tested pathogen were 879, 794 and 624µg/ml,respectively as seen in Table 3.

Table 3: Effect of crude extracts from antagonistic fungi on spore number

Crude extract	Spore numbers($x10^7$) of *Colletotrichum sp. at each concentration (spores/ml)*						ED50
	0	10	50	100	500	1,000	
Daldiniaeschscholtzii							
Hexane	3.6a[1]	3.38ab	2.74c	2.67c	1.46e	1.24e	220.9214
EtOAc	3.78ab	2.65c	2.59c	2.45cd	1.35e	1.15e	104.7066
MeOH	3.76a	3.17ab	2.89b	2.59c	1.8c	1.65d	2971.033
Chaetomiumcochlioedes							
Hexane	3.44a	2.96b	2.93b	2.35cd	2.18cd	2.05de	1754.045
EtOAc	3.08b	2.41c	2.19cd	2.07de	1.84e	1.48f	1879.879
MeOH	3.03b	2.97b	2.93b	2.21cd	1.81e	1.19g	712.0882
Chaetomiumcupreum							
Hexane	4.27a	3.38bcde	3.17cdef	3.11def	2.37gh	2.05gh	794.8441
EtOAc	4.26a	3.38bcde	3.23cdef	3.11def	2.31gh	2.05gh	624.2459
MeOH	4.07ab	3.9abc	3.72abcd	2.64egf	2.49fg	1.69h	879.0221

[1]Average of four replications. Means followed by the same letters in each antagonist were not significantly different by DMRT at P=0.01.

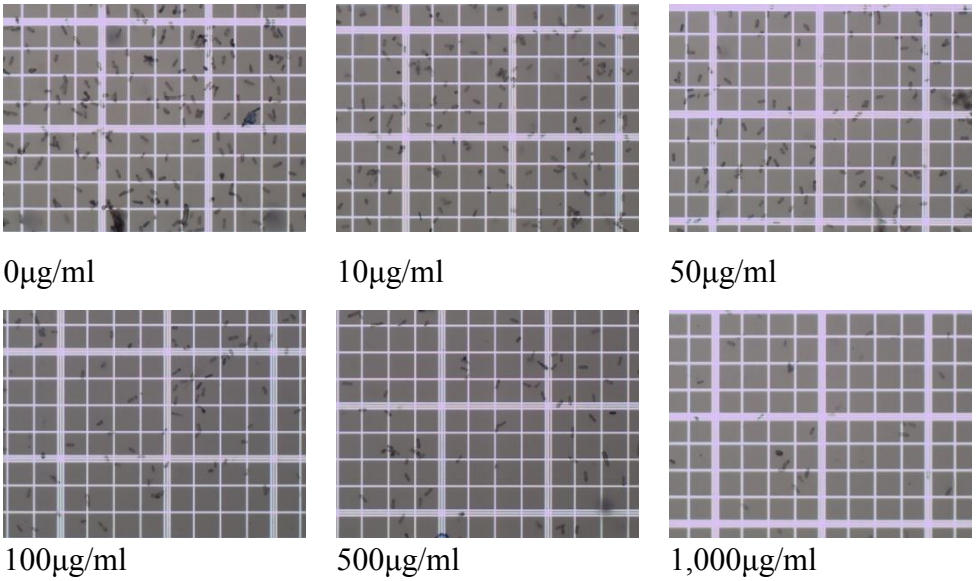


Fig.6. Spore production of *Colletotrichum*sp. at different concentrations

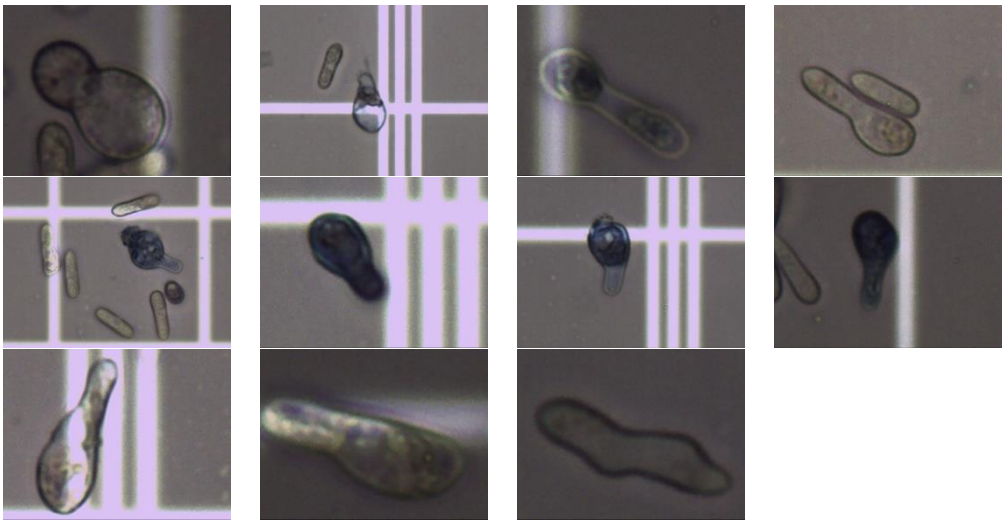


Fig.7. Abnormal spores of *Colletotrichum*sp. at

The spore production of *Colletotrichumgloeosporioides* was significantly inhibited by metabolites from *Daldiniaeshscholtzii*. The highest sporeinhibitonwas crude ethyl acetate at the concentrations 1,000µg/ml (69.45%), followed by crude hexane at concentration 1,000µg/mlwhich was 65.5%, crude ethyl acetate which was 64.34% and crude hexane at the concentration of 500µg/mlwas 59.32%.

Crude extracts from *Chaetomiumcochliodes*significantly inhibited spore production of *Colletotrichumgloeosporioides.* The highest spore inhibition was crude methanol at the concentrations 1,000µg/ml (60.87%), followed by crude ethyl acetate at the concentrations 1,000µg/ml (51.77%) and crude hexane at the concentration 1,000µg/ml(46.12%). The lowest percentages were crude methanol at the lowest concentrations 10 and 50µg/ml.

Crude extracts from*Chaetomiumcupreum*also showed inhibition of spore production (*Colletotrichumgloeosporioides*). The highest spore inhibition was crude methanol extractat the concentration 1,000µg/ml (58.65%), followed by crude hexane and ethyl acetate extracts at concentration of 1,000µg/ml which were 51.35% and 51.27%, respectively.

Table 4: Effect of crude extracts from antagonistic fungi on spore inhibition of *Colletotrichumgloeosporioides*.

Crude extract of	**Percentages of spore inhibition of *Collectotrichum* sp.**				
	10	**50**	**100**	**500**	**1,000**
Daldiniaeschscholtzii					
Hexane	6.15g[1]	23.68defg	25.83def	59.32ab	65.5a
EtOAc	30cde	31.48cde	34.98cd	64.34ab	69.45a
MeOH	10.42fg	14.03efg	31.12cde	33.09cd	34.24bc
Chaetomiumcochlioedes					
Hexane	13.75ef	14.75ef	31.29cd	36.6bcd	46.12abc
EtOAc	25.5de	33.15cd	32.8cd	40.05bcd	51.77ab
MeOH	2.06f	3.88f	26.76de	40.05bcd	60.87a
Chaetomiumcupreum					
Hexane	20.96def	25.14cde	26.38cde	44.45abc	51.35ab
EtOAc	20.53def	23.59de	25.97cde	45.55ab	51.27ab
MeOH	4.19f	8.56ef	35.03bcd	38.72bcd	58.65a

[1]Average of four replications. Means followed by the same letters in each antagonist were not significantly different by DMRT at P=0.01.

Discussions

As the results, Itis showed that all crude extracts from *Daldiniaeschscholtzii*, *Chaetomiumcochliodes* and *Chaetomiumcupreum* can

ihibit the colony growth and spore production of *Colletotrichum gloeosporioides* causing anthracnose of orchid. The crude extracts from *Daldiniaeschscholtzii* expressed its ability to inhibit the colony growth and spore production of tested pathogen. There was no previous studied about crude extracts or fungal metabolites from theasespiecies against *Colletotrichumgloeosporioides* causing anthracnose of orchid. So, this is reported to be the first time. But *Daldiniaeschscholtzii* was reported to express a feature of many immunosuppressive substance(Zhang , et al.,2008) who discovered Dalesconol A and B polyketides with showing immunosuppressive activity which initially isolated from *Daldiniaeschscholtzii* and two years later, they discovered daeschol A, dalesconol C, 2, 16-dihydroxyl-benzo fluoranthene and dalmanol A which were isolated froim*D.eschscholtzii*(Zhang,*et al.*,2011). Moreover, helicascolide C,a new lactone with fungistatic activity against *Cladosporiumcucumerinum*was isolated together with helicascolide A froman Indonesian marine algicolous-associated *D. eschscholtzii*strain was reported by Tarman*et al.* (2012).

The crude extracts of *Chaetomiumcochliodes* can inhibit*Colletotrichum* sp. causing tea anthracnose was confirmed by Nguyen Van Thiep*et al*.(2014) who stated that crude extract from hexane, ethyl acetate and methanol at the concentration 1,000µg/ml can also inhibit the spore productions of *Fusariumroseum*causing wilt of coffee which were 60.87%, 78.16% and 74.57% respectively. Soytong (2014)reported that crude hexane, ethyl acetate and methanol from *Chaetomiumcochliodes* at the concentrations 50µg/ml can inhibit the spore productions of *Drechsleraoryzae*causing brown leaf spot in rice which were 54.99%, 63.14% and 48.96% respectively.

Crude extracts from *Chaetomiumcupreum*also showed ability to control *Collectotrichum* sp. Tathan, *et al.*(2012) reported that crude extract from this fungus can inhibit the growth of *Drechsleraoryzae* causing rice leaf spot. Hung PhungManh*et al*.(2014) also reported *Chaetomiumcupreum*was actively inhibited spore production of *Pythiumaphanidermatum* causing root rot of in pomelo.

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