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Original Article

Biological activities of novel entomopathogenic fungi, *Polycephalomyces phaothaiensis* BCC78485 and BCC78486

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Abstract

Polycephalomyces phaothaiensis is a novel entomopathogenic fungi species found in Phitsanulok of Thailand and its biological activities were investigated. The mycelia of two strains, BCC78485 and BCC78486, were extracted with methanol and the media were partitioning extracted with ethyl acetate. The antimicrobial activities were determined by disc diffusion and broth dilution, and the antioxidant activity was tested by DPPH scavenging assay. The larvicidal bioassay was performed against mosquito, *Aedes aegypti*, blow fly, *Chrysomya megacephala*, and common house fly, *Musca domestica*. Results showed that ethyl acetate extracts exhibited antimicrobial activities on five tested organisms, while methanol extracts did not. All extracts had low antioxidant activity with IC50 >500 □g/ml, and no activity against larvae and adult of both fly species at 2,500 ppm. The BCC78486 methanol extract showed the highest mosquito larvicidal activity with LC_{50} at 593.41 ppm. This study shows that the extracts from *P. phaothaiensis* are a potential source for further study on its antimicrobial and mosquito larvicidal activity.

Keywords: entomopathogenic fungi, *Polycephalomyces phaothaiensis*, larvicidal activities, antimicrobial activities

1. Introduction

Entomopathogenic fungi play a vital role in ecosystems. They infect insects, one of the most diverse class of animals, by penetrating the insect body cavity and attacking the insect's immune system. The fungi then grow inside the insect, using it as a nutrient source and in the process killing it, before exiting to infect another insect. Entomopathogenic fungi are also well known for their ability to produce a variety of secondary metabolite compounds during their life cycle. These compounds hold potential as a new resource for biotechnology and pharmaceutical products. Some of these compounds are already being used for health benefits and as alternatives to chemical insecticides (Bawadekji, Al Ali, & Al Ali, 2016; Jurado, Sanchez-Moral, & Saiz-Jimenez, 2008).

Recently, the strains *Polycephalomyces phaothaiensis* BCC78485 and BCC78486 were collected from Ban Phaothai Community Forest in Phitsanulok Province, Thailand. Both strains were found as hyperparasitic fungi on the stromata of *Ophiocordyceps* sp. that had already infected Coleoptera larvae. Later the two strains were identified as novel species and given the name *P. phaothaiensis* (Crous *et al.*, 2017). Though few studies have examined the biological activities of *Polycephalomyces* spp. (*Ophiocordycipitaceae*, *Hypocreales*), existing studies indicate that *Polycephalomyces* spp. are potential sources of natural biological compounds.

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For example, cordypyridones, a potent antimalarial compound with weak cytotoxicity, was isolated from *P. nipponicus* BCC1389 (Isaka, Tanticharoen, Kongsaeree, & Thebtaranonth, 2001). The ethyl acetate extract of *P. nipponicus* Cod-MK1201, a fungus isolated from a dead cicada nymph, was also found to possess antibacterial activity against twelve kinds of bacteria, particularly against *Bacillus cereus* and *Vibrio cholera*. That extract also inhibited growth of breast cancer cells (Sangdee, Seephonkai, Buranrat, Surapong, & Sangdee, 2016). In another study, the methanol mycelia extract of *P. nipponicus* TBRC 6537 exhibited antimicrobial activity against *Escherichia coli*, *Staphylococcus epidermidis*, *Bacillus cereus*, and *Candida albicans*. That methanol extract exerted the strongest DPPH radical scavenging activity, compared to extracts made using other solvents (Somsila, Sakee, Srifa, & Kanchanarach, 2018). Biological activities, including insecticidal activity, have also been reported in family Cordycipitaceae such as *Isaria* sp., *Beauveria* sp., and *Paecilomyces* sp. In addition, other fungi in the family Ophiocordycipitaceae, such as *Tolypocladium* sp., possess compounds with mosquito larvicidal activity (Gibson, Donzelli, Krasnoff, & Keyhani, 2014; Wang & Xu, 2012; Xu *et al.*, 2009).

The purpose of this study is to investigate the antimicrobial, antioxidant, and insecticidal activities of extracts from *Polycephalomyces phaothaiensis* BCC78485 and BCC78486, a novel species which is the predominant entomopathogenic fungus found in Ban Phaothai Community Forest in Phitsanulok Province, Thailand. This study can also provide useful background information for future research on these and other entomopathogenic fungi.

2. Materials and Methods

2.1 Fungal materials and culture condition

The *Polycephalomyces phaothaiensis* (BCC78485 and BCC78486) were found as a hyperparasitic fungus on the stromata of *Ophiocordyceps* sp. infecting on Coleoptera larvae. They were isolated and deposited at BIOTEC Culture Collection (BCC). The cultures were grown on potato dextrose agar and incubated at 25 °C for 7 days. After that, the 0.7 cm agar plug was sterile cut from the outermost zone of the fungal colony and then transferred to 300 ml potato dextrose broth and incubated at 25 °C without shaking for 14 days.

2.2 Extract preparation

The fungal mycelia were harvested by filtration through sterile cloth. The extracts were prepared according to the respective method used for extraction of bioactive agents from entomopathogenic fungi (Kuephadungphan, Phong paichit, Luangsa-ard, & Rukachaisirikul, 2014). The mycelia were macerated with methanol for three days before evaporated drying to get BCC78485M and BCC78486M extracts. The separated media were partitioning extracted with equal volume of ethyl acetate for three times before evaporated drying to get BCC78485E and BCC78486E extracts.

2.3 Biological activity testing

The four extracts (BCC78485M, BCC78486M, BCC78485E, and BCC78486E) were tested for their antimicrobial activity, antioxidant activity and insect larvicidal activity in mosquito and flies. All four extracts were used in these experiments, with the exception that only the methanol extracts were used in fly larvicidal assay, because the ethyl acetate extracts had low solubility in ethanol. All experiments were performed in triplicate. The larvicidal activity in both mosquitos and flies was tested in an insect habitat facility under controlled conditions of 25±2 °C, 70-80% relative humidity, and a photoperiod of 10:14 (light:dark). The specific range of extract concentrations in each test was determined by preliminary test results and existing reference protocols.

2.4. Antimicrobial activity testing

The antimicrobial activity of the extracts was assessed following the method by Trinh *et al.* (2018). The extracts were screened by disc diffusion method against *Staphylococcus aureus* ATCC 25923, methicillin resistant *S. aureus* (MRSA) DMST 20651, *Escherichia coli* ATCC 25922, *Candida albicans* ATCC10231 and *Propionibacterium acnes* DMST 14916. The inoculum was prepared to a turbidity equivalent to McFarland no. 5 before plating on surface of Muller Hinton agar or on surface of brain heart infusion agar for *P. acnes*. The 6 mm diameter-paper disc impregnated with 2 mg/disc of the extracts were then placed on the surfaces. The standard commercially prepared discs of clindamycin (2 µg/disc) gentamycin (10 µg/disc) and nystatin (100 unit/disc) were used as a positive control. Plates were incubated at 37 °C for 24 hrs or 37 °C for 48 hrs under anaerobic conditions in case of *P. acnes*. Finally, the inhibition zone diameters were measured.

Extracts exhibited zones of inhibition were further evaluated for its minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) or minimum fungicidal concentration (MFC) by micro-broth dilution method or by macro-broth dilution method for *P. acnes*. Briefly, the extracts were dissolved in culture media containing 2% dimethyl sulfoxide (DMSO) and 2% tween 80 before serial dilution to the final concentration ranged from 0 to 8 mg/ml. The microbial cell suspension was added to the final density equal to 10^5 cfu/ml. The media containing 2% DMSO and 2% tween 80 were used as a growth control. Turbidity of the suspension were measured at 600 nm before and after incubated. MIC defined as the lowest concentration exhibited at least 90% growth inhibition when compared to the growth control. Then, the MBC values were determined by transfer of the suspensions on agar surfaces. MBCs defined as the lowest concentrations that no microbial colony observed on the agar surfaces after incubation.

2.5 Antioxidant activity testing

The antioxidant activity of the extracts was determined by DPPH radical scavenging assay as reported previously (Irshad, Zafaryab, Singh, & Rizvi, 2012). In brief,

the extracts and the standard compond, gallic acid, were prepared in methanol at various concentrations. The test solution (75 µl) was mixed with 150 µl of 0.2 mM DPPH (Sigma-Aldrich®, Steinheim, Germany) solution. The reaction mixtures were incubated in the dark at room temperature for 30 min, and the absorbance was measured at 515 nm. A percentage of DPPH radical scavenging activity was calculated and the percentage of inhibition was plotted against the concentration of the solution of the test solutions. The concentration yielding 50% radical scavenging (IC_{50}) was calculated using the software package Prism6.

2.6 Mosquito larvicidal bioassay

Mosquito used in this study was *Aedes aegypti* laboratory strain. Colonization of *Ae. aegypti* mosquito in the laboratory followed the procedure of Thongwat, Chokchaisiri, Ganranoo, and Bunchu (2018). The *Aedes* larval bioassay of the extracts followed the protocol of the World Health Organization (2005). Only the 3rd instar larvae, the strongest and most active stage, were kept for bioassay. In brief, 1% w/v stock solutions of the extracts were prepared using DMSO as a diluent. After that, 200 ml of various concentrations (300-1,300 µg/ml) of each extract was put into a plastic bowl, and then twenty-five of the 3rd instar larvae were transferred into the solutions. The larval mortality rates were determined after 24-hour exposing time. The larvae were considered dead when they were unable to normally move after gentle touching with a fine needle. The experiments were performed with 100 larvae for each concentration of each extract. The control group was set up simultaneously and consisted of the maximum concentration of the DMSO and twenty-five of the larvae. The mortality data were analyzed by using a computerized Probit analysis for the 50% lethal concentration (LC_{50}) value determination (Finney, 1971). The computerized program is commercial LdP Line® software (Cairo, EGY: Plant Protection Research Institute). The 95% Confidence Intervals (CI) of upper and lower fiducial limits (UCL and LCL) were also calculated.

2.7 Fly larvicidal bioassay

Flies used in this study were blow fly, *Chrysomya megacephala*, and common house fly, *Musca domestica*. Adults of each fly species used in this study were maintained in a rearing cage (30x30x30 cm) and fed with two kinds of food: a mixture of 10% (w/v) sucrose solution with 5% (v/v) multivitamin syrup solution (SEVEN SEA, England) and fresh pork liver. Fresh pork liver (40 g) was provided as larval food. Each rearing box was contained about 100-200 larvae then kept in a rearing cabinet. Livers were replaced daily until some early larvae (L1) developed into the third stage larvae (L3) and were added the saw dusts into the rearing box to reduce humidity. The third stage larvae (L3) of each species were kept for bioassay.

The third instar *C. megacephala* and *M. domestica*, used in this experiment were 3-day-old after hatching from the same egg batch as mentioned in Sukontason, Boonchu, Sukontason, and Choochote (2004). Each species was randomized into 6 groups (20 larvae/group) and reared in separate rearing boxes. The methanol extracts were used in this experiment since the ethyl acetate extracts had low solubility in ethanol. Each extract concentration was immediately prepared in a ceramic bowl by the serially 2-fold dilution method using absolute ethanol as the solvent to get final concentration ranging from 500-2500 ppm (0.5–2.5 mg/ml). A bowl containing each concentration of the fungal mycelia methanol extracts, BCC78485M and BCC78486M was tightly covered with the lid until they were used for the dipping method. For the experiment, the larvae of each group were wrapped in a voile cloth and gently dipped into solution, whereas those of the controls were dipped in absolute ethanol. After being dipped for exactly 30 sec, the larvae were transferred to the rearing box containing food. The mortality of each larva was assessed at 24 hrs by touching each one with a paint brush (no. 0), and non-responding larvae were considered as dead. Living larvae were studied further for their adult emergence after being tested with the extracts. Living larvae were reared in each rearing box after being dipped in each concentration of extractions or absolute ethanol (control), and the maintenance of the larvae was conducted in the same manner as previously described until emergence. Once emergence occurred, the adult flies were counted.

3. Results and Discussion

3.1 Antimicrobial activity of the fungal mycelia crude extracts

The methanol and ethyl acetate extract of BCC78485 and BCC78486 mycelia were screened for antimicrobial activity using the disc diffusion method. In the results, both ethyl acetate extracts, BCC78485E and BCC78486E, exhibited inhibition zones against all the tested microorganisms, with the largest zones observed against *P. acnes*, while the methanol extracts had no inhibition zones (Table 1). Thus, only the ethyl acetate extracts were further investigated for their MIC, MBC and MFC values.

The MIC values of the BCC78485E and BCC78486E extracts against *S. aureus*, MRSA, *E. coli and C. albicans* were determined by micro-broth dilution methods while the MIC values against *P. acnes* were determined by macro-broth dilution methods. Results indicate that both extracts inhibited all the tested microorganisms, with MICs from 1 to 4 mg/ml. These two extracts also killed *C. albicans* and *P. acnes* at MBCs of 8 mg/ml (Table 2).

The two strains of *P. phaothaiensis* exhibited antimicrobial activity of similar potency. The antimicrobial activity was observed from only the two ethyl acetate extracts. The methanol extracts had no antimicrobial activity. These results are consistent with a previous report that only the mycelial extract of *P. nipponicus* Cod-MK1201 obtained with ethyl acetate exhibited antibacterial activity, and the methanol extracts did not inhibit any bacteria tested. Similarly, the *P. nipponicus* Cod-MK1201 methanol extracts also exhibited activity against the antibacterial resistant bacteria MRSA (Thammawat, Sangdee, & Sangdee, 2017). In contrast, the methanol extract of *P. nipponicus* TBRC6537 inhibited all tested bacteria but it had no activity on *Candida albicans* (Somsila *et al.*, 2018). The methanol extract from the fruiting body of *Cordyceps militaris* also had broad spectrum of activity against all bacteria and fungi tested, while the mycelial extract exhibited selective activity that it did not inhibit *P. aeruginosa*, *Bacillus subtilis*, *Aspergillus flavus* and

Table 1. Inhibition zone diameters of the fungal mycelia crude extracts (2 mg/disc)

Microorganisms	Diameter of inhibition zone (mm)*				
	BCC78485M	BCC78486M	BCC78485E	BCC78485E	Positive control
S. aureus	-	-	19.30±1.01	11.80±2.60	22.79±0.44[a]
MRSA	-	-	13.54±1.67	9.81±1.34	14.25±0.65[b]
E. coli	-	-	9.89±1.32	11.55±0.89	14.22±0.88[a]
C. albicans	-	-	8.55±2.34	9.85±1.04	18.18±0.63[c]
P. acnes	-	-	34.28±0.97	28.23±1.07	26.35±0.48[d]

[a] Gentamicin (10 µg/disc) [b] Vancomycin (30µg/disc) [c] Nystatin (100 unit/disc), [d] Clindamycin (2 µg/disc), *Values are mean±SD from triplicate test.

Table 2. Quantitative analysis of antimicrobial activity of the fungal mycelia crude extracts

Microorganisms	BCC78485E (mg/ml)		BCC78486E (mg/ml)	
	MIC	MBC/MFC	MIC	MBC/MFC
S. aureus	1	8	1	8
MRSA	1	>8	1	>8
E. coli	4	8	4	>8
C. albicans	2	8	2	8
P. acnes	2	8	2	8

C. albicans (Dong, Yang, & Lian, 2014). The difference between these results can be attributable to various factors such as different entomopathogenic fungi species, extract preparation methods, and strains of tested microorganisms.

3.2 Antioxidant activity of the fungal mycelia crude extracts

The antioxidant activity of the mycelial extracts was found to be low, with all four extracts having IC_{50} values higher than 500 µg/ml. In comparison, the IC_{50} of the positive control gallic acid was 1.85±0.45 µg/ml (Table 3). The previously mentioned study on *P. nipponicus* TBRC6537 found that its mycelial methanol extract also had low DPPH scavenging activity with an IC_{50} of 697 µg/ml, while its aqueous extract had stronger activity, with an IC_{50} of 228 µg/ml (Somsila *et al.*, 2018). One limitation of the current study is that, due to limited solubility of the extracts in ethanol; 500 µg/ml was the highest concentration used for testing.

3.3 Mosquito larvicidal activity of the fungal mycelia crude extracts

After the 24-hour mosquito larvicidal bioassay, the most toxic activity was found from the BCC78486M extract with an LC_{50} value of 593.41 µg/ml, while significantly lower larvicidal activity was found from the BCC78485E and BCC78485M extracts, with respective LC50 values of 863.37 µg/ml and 851.41 µg/ml (Table 4). The BCC78486E extract did not exhibit any larvicidal activity against the 3rd instar *Ae. Aegypti*, causing only 12% larval mortality rate even at a very high concentration (1,000 µg/ml).

There are few previous studies researching mosquito larvicidal metabolites from entomopathogenic fungi. Most of the research has explored pathogenic efficacy of various fungi on both adult and larval stages of the mosquito (Scholte, Knols, Samson, & Takken, 2004). There has been particular focus on the larvicidal activity of metabolites from *Metarhizium anisospliae* against the *Ae. aegypti* mosquito (Vyas, Dua, & Prakash, 2015). Studies have reported that extracellular metabolites of *M. anisospliae* displayed efficacy against the 3rd stage *Aedes* larvae with an LC_{50} value of 29.51 ppm (µg/ml). It was more than ten times greater efficacy than the *Polycephalomyces* (BCC78486M) of our study (593.41 µg/ml). Moreover, recently, the study on *Beauveria bassiana* extract revealed an extremely larvicidal efficacy on the *Ae. aegypti* with <0.1 ppm LC_{50} value (Daniel *et al.*, 2017). The active substance that displayed a very high toxic to the *Aedes* larvae was identified as beauvericins. Compared to this study, *Polycephalomyces*, a hyperparasitic fungus on *Ophiocordyceps* sp. that naturally infected on Coleoptera insect, both *M. anisospliae B. bassiana* are widely known as the entomopathogenic fungi of various insects, including, the mosquitoes. Then, finding of great mosquito larvicide from them than the *Polycephalomyces* are reasonable. In addition, the strain used in this study are the hyperparasitic fungus which may have different secondary metabolites, mechanisms of action, and fungal pathogensis from on the mosquitoes from the *Polycephalomyces* spp. that found on the infected insects.

3.4 Blow fly and house fly larvicidal activity of the fungal mycelia crude extracts

Results on the larvicidal activity of the BCC78485M and BCC78486M methanol extracts against the third stage larvae of the blow fly, *C. megacephala*, and the common house fly, *M. domestica*, as evaluated using the dipping method are shown below in Table 5. In short, the two extracts did not exhibit any larvicidal activity against these two species of fly larvae.

All four extracts had low antioxidant activity. These results were in the same way with larvicidal activity in flies that the methanol extracts, BCC78485M and BCC78486M, had no activity. Unfortunately, the ethyl acetate extracts, BCC78485E and BCC78486E, were not determined for their fly larvicidal activity due to its insolubility in ethanol. Therefore, these extracts may be screened for fly larvicidal activity in the future to clarify this point. However, no

Table 3. Concentrations of the fungal mycelia crude extracts and positive control providing 50% radical scavenging (IC_{50})

DPPH radical scavenging assay	Extracts [a]				
	BCC78485M	BCC78486M	BCC78485E	BCC78486E	Gallic acid [b]
IC_{50}±S.D. (µg/ml)	>500	>500	>500	>500	1.85±0.45

[a] Values are means of triplicate. [b] Positive control

Table 5. Larvicidal activity of the fungal mycelia crude methanol extracts, BCC78485M and BCC78486M, against the third stage larvae of blow fly, *Chrysomya megacephala*, and common house fly, *Musca domestica*, using dipping method.

Extract	Concentration (ppm)	*Chrysomya megacephala*		*Musca domestica*	
		%Mortality	%Emergence	%Mortality	%Emergence
BCC78485M	500	0	89.33	0	86.66
	1000	0	88	0	90.66
	1500	0	92	0	89.33
	2000	0	92	0	96
	2500	0	93.33	0	92
BCC78486M	500	0	86.66	0	93.33
	1000	0	90.66	1.33	90.66
	1500	0	86.66	0	88
	2000	1.33	88	0	96
	2500	0	92	0	93.33
Control		0	90.66	0	92

Table 4. Larvicidal activity of the four fungal mycelia crude extracts against the 3rd stage *Ae. aegypti* larvae after 24-hours of exposure

Extracts (µg/ml)	%Mortalities[a] (mean±SE)	Larvicidal activity (µg/ml)	
		LC_{50} values[b]	95% CI (UCL - LCL)
BCC78485M			
400	13.00±4.12		
600	27.00±6.61		
800	43.00±12.79	851.41*	790.64 - 923.56
1,000	63.00±8.23		
1,200	71.00±8.54		
BCC78485E			
500	17.33±9.61		
700	25.33±11.85		
900	54.67±8.11	863.37*	805.97 - 923.97
1,100	66.67±1.33		
1,300	84.00±2.31		
BCC78486M			
300	5.00±1.00		
500	52.00±5.89		
700	58.00±7.39	593.41**	426.72 - 760.32
900	77.00±2.53		
1,100	84.00±1.63		
BCC78486E			
1,000	12.00±0.00	-[c]	-[c]

[a] Nil mortality rates were observed from all the control groups; [b] Statistically significant differences are indicated by different numbers of asterisks following the LC_{50} values; [c] Very low mortality rates were observed from BCC78486E at 1,000 µg/ml, so the LC_{50} could not be calculated.

previous evidence demonstrated that metabolites of entomopathogenic fungi affected to larval stage of fly. Only spores of some entomopathogenic fungi showed larvicidal activity against fruit fly (Aemprapa, 2007). In addition, species-specific activity of entomopathogenic fungi to insect species has been previously reported (Wichadakul *et al.*, 2015; Xu, Luo, Li, Shang, & Wang, 2016).

4. Conclusions

This preliminary study indicates that the extracts from *Polycephalomyces phaothaiensis* BCC78485 and BCC78486 possess interesting biological activities, especially antimicrobial and mosquito larvicidal activities. The novel species, *P. phaothaiensis*, is a new potential source for development of alternative medicine. Future investigation focused on their active metabolites by activity guided separation and identification should be further studied.

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