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Growth Inhibition of *Aspergillus flavus* IMI 242684 by Crude Extract of *Penicillium* sp.

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Abstract

Crude extract of *Penicillium* sp. isolate 480519 I06 (3) was tested for an ability to inhibit the growth of *Aspergillus flavus* IMI 242684 using paper disc diffusion assay on Sabouraud Dextrose Agar (SDA). The effectiveness of extracts from culture filtrates and mycelia by different solvents, hexane, ethyl acetate, butanol and methanol, using liquid-liquid extraction were compared. The results show that hexane and ethyl acetate extracts from culture filtrates were most active against *A. flavus* IMI 242684. Hexane extract at 100 mg/ml gave the highest inhibitory zone of ≃ 17.50 mm followed by ethyl acetate extract at 100 mg/ml (inhibitory zone of ≃ 9.87 mm) and hexane extract at 50 mg/ml (inhibitory zone of ≃ 9.75 mm).

Keywords: aflatoxin, *Aspergillus flavus*, *Penicillium*, crude extract

1. Introduction

Aflatoxins produced by certain strains of *Aspergillus flavus*, *A. parasiticus*, *A. nomius*, *A. tamarii*, *A. bombycis* and *A. pseudotamarii* [1-6], are one of the most potent groups of toxic substances that occur naturally [7] and their contamination in economic crops is a major problem all over the world [8]. Ingestion of aflatoxins in contaminated food or feed can cause aflatoxicosis. Experiments conducted in different areas especially in China and in the African countries have shown high incidence of liver cancer [9-10] attributable to mycotoxicoses. Many countries have limitation of aflatoxin residue in agricultural products for safety of consumer. Legislative level of aflatoxins permissible in Thailand is 20 ng/g or 20 ppb [11]. The contamination of aflatoxins ranging from 0.01-17.3 µg/kg was found in one hundred and twenty noodle dishes (51%) consumed in 3 areas of Bangkok [12] and 5 herbal medicinal products out of 28 products were also found to be contaminated with aflatoxins ranging from 1.7-14.3 ng/g [11].

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Physical, chemical and biological methods have been investigated in order to prevent the growth of aflatoxigenic fungi, to reduce or eliminate the toxin levels, to degrade or detoxify the toxins in foods and feeds [13]. The use of biocontrol agents as an alternative control strategy for agriculture has been focused on due to their being safe and eco-friendly substances [14]. Many microorganisms have mechanisms to compete with others for nutrients and growth development. Fungi have many mechanisms such as growth competition, antibiotic production and endolysis, mycoparasitism and exolysis [15]. Many groups of fungi are well known as biocontrol agents. For example, *Trichoderma viride* and *T. harzianum* can produce amylolytic, pectinolytic, proteolytic and cellulolytic enzymes for growth inhibition of *A. flavus* and *Fusarium moniliforme* [16]. An extract from *Penicillium oxalicum* showed fungicidal activity which affected *A. candidus*, *A. versicolor*, *P. griseofulvum* , *Curvularia trifolii* and *Botrytis cinerea* [17]. Our previous study showed that culture filtrate of *Penicillium* sp. isolate 480519 I06 (3) could inhibit the growth of *A. parasiticus* IMI 102566 [18]. Thus the objective of this study is to compare the inhibitory effect of crude extracts of *Penicillium* sp. isolate 480519 I06(3) made with different solvents on the growth of *Aspergillus flavus* IMI 242684 on Sabouraud Dextrose Agar (SDA).

2. Materials and Methods

2.1 Fungi

Aspergillus flavus IMI 242684 obtained from International Mycological Institute, U.K. and *Penicillium* sp. isolate 480519 I06 (3) previously isolated from cultivated soil and irradiated with gamma ray by Wongsariya *et al.* [19] were cultured on Potato Dextrose Agar (PDA) for 7 days at room temperature.

2.2 Preparation of spore suspension

Spores of *A. flavus* IMI 242684 were prepared by growing this fungus on PDA for 10-14 days at room temperature before harvesting in sterile distilled water plus 0.05 % tween 80 and filtering through sterile cotton wool. The numbers of spores were counted by haemacytometer [20].

2.3 Cultivation of *Penicillium* sp. isolate 480519 I06 (3)

Yeast extract sucrose (YES) medium was used for culturing *Penicillium* sp. isolate 480519 I06 (3). The medium was prepared by adding 90 ml broth into 250 ml flask and autoclaving. Mycelial disc (Ø 7 mm) of *Penicillium* sp. isolate 480519 I06(3) was inoculated into each flask and incubated at static condition for 14 days [21]. Culture broths were then filtered through Whatman paper no. 4. Culture filtrates and mycelial mass were collected and kept separately at 4°C for further extractions.

2.4 Extraction method

2.4.1 *Extraction of mycelia*

Antifungal metabolites from mycelia were extracted by grinding mycelial mass in liquid nitrogen and extracting with methanol by shaking at 1,500 rpm for 15 min before filtering through Whatman paper no.1 and evaporating to dryness at 40°C [22].

2.4.2 *Extraction of culture filtrates*

Culture filtrates were extracted successively with 3 solvents: hexane, ethyl acetate and butanol with the ratio of 1:1 (filtrate: solvent). Filtrate extracts in hexane and ethyl acetate were extracted three times before evaporating to dryness at 40°C using a rotary evaporator, whereas filtrate extracts in butanol were extracted three times and the extracts were then added with water with the

ratio of 1:3 (water: extracted solution) and evaporated to dryness at 60°C using method of Se-Gul [23]. The dry weights of each extract from 2.4.1 and 2.4.2 were recorded and the dried preparations were kept at -20°C prior to further analysis.

2.5 Disc diffusion assay

The efficiency of crude extracts were tested using modified method from Lorian [24]. The dried extracts from each solvent were dissolved in methanol to various concentrations (1, 12.5, 25, 50 and 100 mg/ml). Twenty ml of Sabouraud Dextrose Agar (SDA) was poured into a Petri dish (Ø 9 cm) and left to set and 20 ml of SDA inoculated with 2 ml of spores of *A. flavus* IMI 242684 (10^8 spores/ml) was poured over the surface of SDA agar plate after the agar had set. Sterile paper discs (Ø 6 mm) containing 20 µl of each extract at various concentrations were placed onto the same plates. Two control discs were used in the test: nystatin (50 µg/ml) was used as a positive control and methanol was used as a negative control. The plates were then incubated at 20° C for 10-20 min in order to let the extract diffuse into agar before further incubation at room temperature for 5 days. The inhibition zones were determined using vernier digital caliper. All experiments were repeated at least four times.

3. Results

Dry weight of crude methanol extract from mycelia of *Penicillium* sp. isolate 480519 I06(3) was 0.3227 g/l. Dry weights of crude extract from culture filtrate from hexane and ethyl acetate were 0.0949 g/l and 0.3389 g/l, respectively whereas the crude extract from butanol gave the highest dry weight (9.8732 g/l). The effects of crude extracts from mycelia and culture filtrates at concentrations of 1, 12.5, 25, 50 and 100 mg/ml on the growth of *A. flavus* IMI 242684 on SDA incubated for 5 days were compared and the results are shown in Table 1. It was found that crude extracts from culture filtrates inhibited the growth of *A. flavus* IMI 242684. On the contrary, the extracts from mycelium did not inhibit the growth of this fungus. Various solvent extracts from culture filtrates of *Penicillium* sp. isolate 480519 I06(3) were further compared and it was found that hexane extracts at 50 and 100 mg/ml and ethyl acetate extract at 100 mg/ml significantly inhibited the growth of *A. flavus* IMI 242684 whereas butanol extract at all concentrations could not inhibit fungal growth. Hexane extract at 100 mg/ml gave the highest inhibition with the inhibitory zone of $\underline{\sim}$ 17.50 mm followed by ethyl acetate extract at 100 mg/ml and hexane extract at 50 mg/ml with the inhibitory zones of $\underline{\sim}$ 9.87 mm and $\underline{\sim}$ 9.75 mm, respectively.

4. Discussion

Our study showed that hexane extract gave the highest inhibitory effect on the growth of *A. flavus* IMI 242684 on SDA incubated for 5 days followed by ethyl acetate extract at 100 mg/ml. However, when the production yields were compared, it showed that ethyl acetate extract gave higher yield than hexane extract. Thus, ethyl acetate extract might be more practical than hexane extract. From the previous result, it was found that the culture filtrate of *Penicillium* sp. isolate 480519 I06 (3) gave the best inhibitory effect on the growth of *A. parasiticus* IMI 102566 on PDA [18]. When antifungal metabolites from *Trichoderma* spp. isolates TISTR 3167, KMC 5, SRS 4 and SCP III were extracted from culture filtrates by hexane, ethyl acetate and n-butanol and tested for growth inhibition of *A. flavus* IMI 242684, ethyl acetate extracts from TISTR 3167, KMC 5 and SRS 4 were most active against *A. flavus* IMI 242684 and 50 mg/ml was the optimal concentration [25]. Yin *et al.* [26] also found that ethyl acetate extract from *T. harzianum*

fermented broth could inhibit mycelia growth, conidia germination and conidia production of *B. cinera*, tomato grey mold. They also found that the ethyl acetate extract had better protective effects than theurapeutic effects. However, Febles *et al*. [27] reported that methanol extraction yielded higher antimicrobial activity than n-hexane and ethyl acetate whereas the methanol extract from culture filtrates of *T. asperellum* and *T. longibrachiatum* gave the best non-enzymatic antimicrobial profiles [22]. Different isolates of the same species can produce different compounds [28]. Vizcaino *et al*. [22] explained that the antimicrobial spectrum of a given strain must be evaluated individually due to the variation detected for the antimicrobial activities at strain level within the different sections and species studied. *Penicillium oxalicum* strain PY-1

Table 1 The effect of crude solvent extracts of *Penicillium* sp. isolate 480519 I06 (3) at various concentrations on the growth of *A. flavus* IMI 242684 on SDA, incubated for 5 days using disc diffusion assay.

Type/Crude solvent extracts		**Concentrations (mg/ml)**	**Diameters of inhibition zone (mm)***
Culture filtrates	hexane	1	00.00 ± 0.00^{d}
		12.5	00.00 ± 0.00^{d}
		25	00.00 ± 0.00^{d}
		50	9.75 ± 1.70^{c}
		100	17.50 ± 1.91^{a}
	ethyl acetate	1	00.00 ± 0.00^{d}
		12.5	00.00 ± 0.00^{d}
		25	00.00 ± 0.00^{d}
		50	00.00 ± 0.00^{d}
		100	9.87 ± 1.31^{bc}
	butanol	1	00.00 ± 0.00^{d}
		12.5	00.00 ± 0.00^{d}
		25	00.00 ± 0.00^{d}
		50	00.00 ± 0.00^{d}
		100	00.00 ± 0.00^{d}
mycelia	methanol	1	00.00 ± 0.00^{d}
		12.5	00.00 ± 0.00^{d}
		25	00.00 ± 0.00^{d}
		50	00.00 ± 0.00^{d}
		100	00.00 ± 0.00^{d}
nystatin (positive control)		0.05	10.75 ± 0.95^{b}

* = mean ± S.D. Means followed by different letters in each column differ significantly at $P \leq 0.05$) and no growth on methanol used as negative control.

isolated from soil produced antifungal substances that effectively suppressed the mycelia growth of *Sclerotinia sclerotiorum* and other tested plant pathogenic fungi [29]. Ma *et al*. [30] isolated *P. striatisporum* Pst 10 from the rhizosphere of chilli peppers and tested for antagonistic effects on *Phytophthora* spp., *Cladosporium cucumerium* and *S. sclerotiorum.* They found that isolate Pst10 could produce antifungal substances in liquid culture in PDB or solid fermentation on wheat bran.

5. Conclusions

This preliminary study showed that the biological control mechanism of *Penicillium* sp. isolate 480519 I06 (3) against *A. flavus* IMI 242684 may be the production of antifungal substances. Hexane and ethyl acetate were the best solvents for extracting secondary metabolites from this fungus for the control of *A. flavus* IMI 242684. However, the use of this crude fungal extract might not be practical because of the high concentration required for effective suppression. Thus more work is required to further purify and characterize the active substance(s) present in the extract. This could then lead to screening and breeding work to obtain strains with enhanced inhibitor production.

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