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The Efficacy of Ethyl Acetate Extract of *Trichoderma* Culture Broth on Growth Inhibition and Aflatoxin Production by *Aspergillus flavus* IMI 242684

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Abstract

Antifungal metabolites from *Trichoderma* spp. isolates TISTR 3167, KMC 5, SRS 4 and SCP III were extracted from cell pellets by methanol and also from culture filtrates by three different solvents, i.e. hexane, ethyl acetate and n-butanol and tested for growth inhibition of *A. flavus* IMI 242684 using paper disc diffusion assay on potato dextrose agar. 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. When ethyl acetate extracts of the 3 isolates at 50 mg/ml were further applied to peanut grains, the growth and aflatoxin (B_1 and B_2) production by *A. flavus* IMI 242684 were inhibited during 21 days of storage at room temperature.

Keyword: Antifungal metabolites, *Aspergillus flavus*, *Trichoderma* spp., aflatoxins, crude extract, peanut grains

1. Introduction

Aflatoxins are a group of highly toxic, mutagenic and carcinogenic polyketide compounds produced by certain strains of *Aspergillus flavus*, *A. parasiticus*, *A. nomius*, *A. tamarii*, *A. bombycis* and *A. pseudotamarii* [1-6]. These molds can infect crops such as corn, cottonseed, peanuts and tree nuts in the field and during storage and their contamination of these economic crops is a major problem all over the world [7]. When these fungi invade and grow in commodities such as peanuts, corn and cottonseed, the resulting contamination with aflatoxins often makes the commodities unfit for consumption [8]. Aflatoxins have been shown to cause hepatotoxicity, teratogenicity, immunotoxicity, and even death [9]. Numerous strategies have been proposed for the elimination, detoxification or reduction of aflatoxins in foods and feeds.

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Peanut or groundnut (*Arachis hypogaea*) is an important legume cultivated and utilized in at least 82 countries worldwide and is an important source of protein [10,11]. Contamination by aflatoxins is the major limiting factor in the production and marketing of peanuts [12] as well as posing public health risks [13]. Current measures for the control of aflatoxin contamination include the use of non toxigenic Aspergilli in pre-harvest crops and various chemicals and plant extracts in post-harvest crops. The ability of spore suspensions of *Trichoderma harzianum* and *T. viride* to efficiently suppress the growth of *A. flavus* and *A. parasiticus* and also to reduce aflatoxin production in peanut kernels have been demonstrated by Gachomo *et al*. [14]. Our previous investigation showed that *Trichoderma* isolates TISTR 3167 and C1-1 selected from 16 isolates gave the best inhibitory effect on *A. flavus* IMI 242684 and aflatoxin production [15]. Some strains of *T. harzianum* have been commercially applied in the form of spores as biological agents for crops such as strawberry, lettuce, tomato and outdoor ornamental plants. However, their efficacy is largely affected by weather and soil conditions as well as other chemical fungicides used on the crops [16-18]. Sivasithamparam and Ghisalberti [19] found that *Trichoderma* spp. produce over 40 different secondary metabolites that may contribute to their mycoparasitic and antibiotic action. Trichodermin was a secondary metabolite isolated from the fermented broth of *T. harzianum* and showed antifungal activity against mycelia growth of phytopathogenic fungi such as *Botrytis cinerea* [20, 21].

Since *Trichoderma* sp. can produce secondary metabolites against fungi, thus the objective of this study was to investigate the potential effects of secondary metabolites from *Trichoderma* isolates TISTR 3167, KMC 5, SRS 4 and SCP III on the growth inhibition and aflatoxin production of *A. flavus* IMI 242684 in peanuts.

2. Materials and methods

2.1 Fungal strains

A. flavus IMI 242684 obtained from the International Mycological Institute, England was grown on potato dextrose agar at room temperature for 7 days. Spores harvested in sterile distilled water plus 0.1% Tween 80 were pooled in a sterile bottle and the number of spores counted using a Hemacytometer. *Trichoderma* sp. TISTR 3167 was obtained from Thailand Institute of Scientific and Technological Research (TISTR), Thailand; *Trichoderma* sp. KMC 5 was isolated from a corn field, the Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand; isolate SRS 4 was isolated from a pineapple field, Khao Sok district, Surat Thani province and isolate SCP III was isolated from lotus field in Donjedee district, Suphanburi Province. They were grown on potato dextrose agar at room temperature for 7 days.

2.2 Extraction of Antifungal Metabolites from *Trichoderma* spp.

Seven mm mycelial discs from 7 days old cultures of *Trichoderma* spp. isolates TISTR 3167, KMC 5, SRS 4 and SCP III were each inoculated into 500 ml flasks containing 250 ml of 1/5 strength potato dextrose broth (PDB) and then, incubated at room temperature. Twenty-eight days after inoculation, spores and mycelia of *Trichoderma* spp. strains were removed from broth culture by filtration. Antifungal metabolites were extracted from cell pellets with methanol and also from culture filtrates by three different solvents, i.e. hexane, ethyl acetate and n-butanol. The various extracts were dried at 40^{o}C using a rotary evaporator and the dry weights of each extract were recorded [22, 23].

2.3 Effect of various solvent extracts (50 mg/ml) from the 4 *Trichoderma* isolates on growth of *A. flavus* IMI 242684 using Paper Disc Diffusion Assay

The dried extracts (obtained from 4 different solvents: methanol, hexane, ethyl acetate and n-butanol) were dissolved in dimethyl sulfoxide (DMSO), to a final concentration of 50 mg/ml. Aliquots (1ml) of spores of *A. flavus* IMI 242684 (10^6 spores/ml) were homogeneously mixed with PDA using the pour plate technique in 14 mm petri dishes. Whatman paper discs (6 mm) were impregnated with 50 mg/ml of each extract from *Trichoderma* spp. and were left to dry in laminar flow cabinet. Discs were placed on the surface of the media and incubated at room temperature for 5 days. After the incubation period, the inhibition zones around the discs were measured. Two control discs were used in the test: nystatin was used as a positive control and dimethyl sulfoxide (DMSO) as a negative control [24, 25]. The experiments were repeated three times. Since the ethyl acetate extract from all 4 isolates of *Trichoderma* demonstrated the best inhibitory effect on growth of *A. flavus* IMI 242684, this extract was selected for further experiments.

2.4 Determination of optimal concentration of ethyl acetate extracts in growth inhibition of *A. flavus* IMI 242684 using Paper Disc Diffusion Assay

Ethyl acetate extracts from all 4 *Trichoderma* isolates were dissolved in dimethyl sulfoxide (DMSO), to final concentrations of 1, 25, 50 and 100 mg/ml. Aliquots (1ml) of spores of *A. flavus* IMI 242684 (10^6 spores/ml) were homogeneously mixed with PDA using the pour plate method in 14 mm petri dishes. Whatman paper discs (6 mm) were impregnated with 1, 25, 50 and 100 mg/ml of the ethyl acetate extracts from the 4 *Trichoderma* isolates were left to dry in laminar flow cabinet and placed on the surface of the media and incubated at room temperature for 5 days. After the incubation period, the inhibition zones around the discs were measured. Two control discs were used in the test: nystatin was used as a positive control and dimethyl sulfoxide (DMSO) as a negative control [24, 25]. The experiments were repeated three times.

2.5 Peanuts and method of inoculation

Fifty peanut grains (*Arachis hypogaea* cultivar Khonkaen 6) were sterilized with 1% NaOCl solution and washed twice with sterile distilled water [26]; the peanut grains were immersed in 50 mg/ml of ethyl acetate extracts from the various *Trichoderma* spp. for 20 min, dried in a laminar flow hood for 30 min and were inoculated with spores of *A. flavus* IMI 242684 (10^6 spores/g). The peanut grains not immersed in ethyl acetate extracts were used as control. Analysis of aflatoxin B_1 and B_2 production was performed after 7, 14, 21 and 28 days of incubation at room temperature [27]. The experiment was repeated three times.

2.6 Determination of fungal biomass content in peanut grains

The colonization of the grains was assessed as CFU/g of peanut grains after 7, 14, 21 and 28 days of incubation. A sample (10 g) was taken from each treatment and shaken for 30 minutes with 90 ml of 0.1% peptone plus 0.1 % tween 80 [28]. The spore suspensions from each treatment were filtered and pooled in a sterile bottle. The spore suspensions were diluted 10-fold to 10^6 spores/ml with 0.1% peptone plus 0.1% tween 80. A 0.1 ml aliquot of the three last serial decimal dilutions of each treatment was spread on the surface of five PDA media.

2.7 Determination of Aflatoxin content in peanut grains

The samples were extracted for aflatoxins by the Sep Pak method [29]. After 7, 14, 21 and 28 days of incubation, 50 g of peanut samples were extracted for aflatoxin B1 and B2 by placing in 100 ml methanol and shaken in an orbital shaker at 250 rpm for 20 minutes and then filtered using Whatman No.1. Forty ml of filtrate were extracted with 40 ml hexane and 80 ml $(NH_4)_2S0_4$ (20%) and warmed at 40-50^{0}C in a separatory funnel. The upper layer was discarded and the lower layer was then extracted twice with 50 ml of chloroform. The chloroform extracts were evaporated under vacuum until dryness. The dried extracts were dissolved with 10 ml of chloroform: hexane (3:7) and then added to the Lichrolut Chromatography Column. The column was drained and eluted with 10 ml of hexane followed by 10 ml of benzene: acetic acid (95.5: 4.5) and 10 ml of ethyl ether: hexane (60:40). The eluate was discarded and aflatoxins were eluted with 15 ml of methylene chloride: acetone (9:1) and evaporated to dryness. The aflatoxins were dissolved with 1 ml of methanol, filtered through 0.45 µm membrane filter (Millipore) and 10 µl injected to the HPLC (Shimadzu) using the following conditions: flow rate of 1 ml/min., UV spectrophotometric detector at 365 nm, reverse phase column C18 and mobile phase solvent (methanol: water: acetic acid (30:63:7). The amount of aflatoxins was calculated from the chromatogram by comparison with the standards.

3. Results

3.1 Inhibitory effects of various solvent extracts (50 mg/ml) from 4 *Trichoderma* isolates on growth of *A. flavus* IMI 242684

Of the 4 solvent extracts, ethyl acetate extracts from culture filtrate of isolates TISTR 3167, KMC 5, SRS 4 and SCP III showed the greatest activity against *A. flavus* IMI 242684 with inhibition zones ranging from $\simeq$ 34-37 mm. Of the 4 *Trichoderma* isolates, the various extracts obtained from SCP III demonstrated little or no activity against *A. flavus* IMI 242684. Similarly the n-butanol extracts of culture broths from TISTR 3167, KMC 5 and SRS 4 showed minimal activity (zones of inhibition ranging from $\simeq$ 8-9 mm) whereas the methanol extracts derived from the mycelia of the 3 isolates demonstrated moderate activity (inhibitory zone ranging from $\simeq$ 13-15 mm)(Figure 1 and Table 1). The ethyl acetate extracts from isolates TISTR 3167, KMC 5 and SRS 4 showed higher inhibitory effect against *A. flavus* IMI 242684 than the positive control, nystatin, at 20 µg/ml. Therefore the ethyl acetate extracts of the *Trichoderma* isolates TISTR 3167, KMC 5 and SRS 4 were used in further experiments.

3.2 Determination of optimal concentration of ethyl acetate extracts in growth inhibition of *A. flavus* IMI 242684

The ethyl acetate extracts of *Trichoderma* isolates TISTR 3167, KMC 5 and SRS 4 demonstrated similar activity against *A. flavus* IMI 242684 at the various concentration used. There was increased inhibitory activity with increase in concentrations (inhibition zone of $\simeq$ 13 mm at 1 mg/ml, 25 mm at 25 mg/ml) and maximal inhibitory activity i.e. inhibition zone of $\simeq$ 31 mm at 50 mg/ml and 100 mg/ml) (Figure 2 and Table 2).

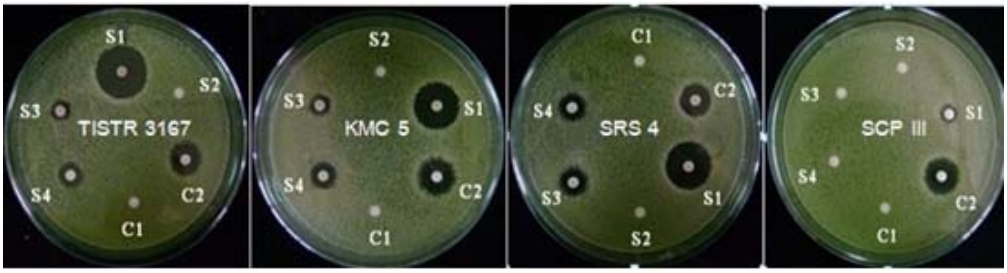

Figure 1 Effect of extracts from 4 solvents at 50 mg/ml (derived from *Trichoderma* isolates TISTR 3167, KMC 5, SRS 4 and SCP III) on the growth of *A. flavus* IMI 242684 on PDA, incubated for 5 days using paper disc diffusion method (Diameter of paper disc 6 mm),
(C1) Negative control (DMSO) (C2) Positive control (nystatin) (S1) ethyl acetate extract
(S2) hexane extract (S3) butanol extract (S4) methanol extract

Table 1 Effect of extracts from 4 solvents at 50 mg/ml (obtained from *Trichoderma* isolates TISTR 3167, KMC 5 and SRS 4) on the growth of *A. flavus* IMI 242684 on PDA, incubated for 5 days using paper disc diffusion method (Diameter of paper discs 6 mm).

Extracts	Inhibition zones (mm)*			
	TISTR 3167	KMC 5	SRS 4	SCP III
Hexane extract	06.00±0.00^{e}	06.00±0.00^{e}	06.00±0.00^{e}	06.00±0.00^{b}
Ethyl acetate extract	37.00±1.87^{a}	36.80±0.84^{a}	34.60±1.34^{a}	07.60±0.55^{a}
n-butanol extract	09.20±0.71^{d}	09.40±0.89^{d}	08.80±0.29^{d}	06.00±0.00^{b}
Methanol extract	15.00±0.00^{c}	14.00±0.00^{c}	13.80±0.37^{c}	06.00±0.00^{b}
Negative control (DMSO)	06.00±0.00^{d}	06.00±0.00^{d}	06.00±0.00^{d}	06.00±0.00^{b}
Positive control (Nystatin)	20.60±1.14^{b}	20.60±1.14^{b}	20.60±1.14^{b}	20.60±1.14^{b}

*= Mean ±SD; Mean of three replications
Means followed by different letters in each column differ significantly at P≤ 0.05 (DMRT)

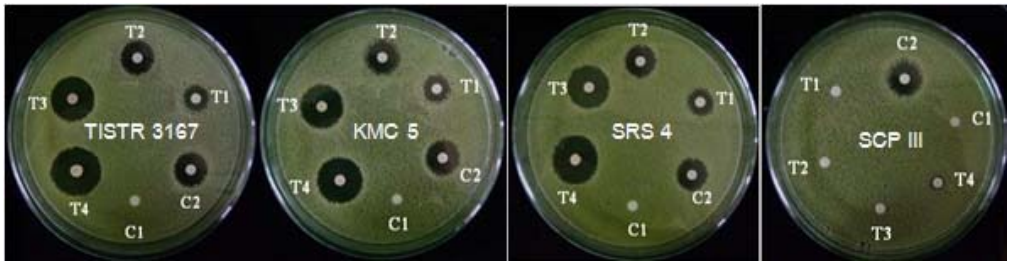

Figure 2 Effect of different concentrations of ethyl acetate extracts from *Trichoderma* isolates TISTR 3167, KMC 5, SRS 4 and SCP III on the growth of *A. flavus* IMI 242684 on PDA, incubated for 5 days by paper disc diffusion method (Diameter of paper disc 6mm)**.**
(C1) Negative control (DMSO) (C2) Positive control (nystatin) (T1) 1 mg/ml
(T2) 25 mg/ml (T3) 50 mg/ml (T4) 100 mg/ml

Table 2 Effect of different concentrations of ethyl acetate extracts (obtained from *Trichoderma* isolates TISTR 3167, KMC 5, SRS 4 and SCP III) on growth of *A. flavus* IMI 242684 on PDA, incubated for 5 days using paper disc diffusion method (Diameter of paper discs 6 mm).

Concentrations (mg/ml)	Inhibition zones (mm)*			
	TISTR 3167	KMC 5	SRS 4	SCP III
1	13.80±0.44^{d}	13.40±0.54^{d}	13.00±0.00^{d}	06.00±0.00^{c}
25	25.00±0.00^{b}	25.20±0.44^{b}	24.60±0.54^{b}	06.00±0.00^{c}
50	31.60±0.54^{a}	31.00±0.00^{a}	30.80±0.83^{a}	06.00±0.00^{c}
100	31.80±0.44^{a}	31.60±0.54^{a}	31.60±0.54^{a}	08.00±0.00^{b}
Positive control(nystatin)	21.00±1.00^{c}	21.00±1.00^{c}	21.00±1.00^{c}	21.00±1.00^{a}
Negative control(DMSO)	06.00±0.00^{e}	06.00±0.00^{e}	06.00±0.00^{e}	06.00±0.00^{c}

*= Mean ±SD; Mean of three replications
Means followed by different letters in each column differ significantly at P≤ 0.05 (DMRT)

3.3 Inhibitory effects of ethyl acetate extracts derived from TISTR 3167, KMC 5 and SRS 4 on growth of *A. flavus* and aflatoxin production in peanut grains

Ethyl acetate extracts from TISTR 3167, KMC 5 and SRS 4 all at 50 mg/ml could inhibit the growth of *A. flavus* IMI 242684 on peanut up to 21 days of storage. At day 28 the spores of *A. flavus* IMI 242684 were detected but the numbers were lower than the number of spores found on untreated peanut grains by $\simeq 10^8$ CFU/g (Table 3). Similarly the total aflatoxins (B_1+B_2) extracted

from the peanuts treated with the ethyl acetate extracts after 28 days of storage were about 300 times lower than the total aflatoxins extracted from untreated peanuts (Table 4).

Table 3 Effect of ethyl acetate extracts (50 mg/ml) from *Trichoderma* isolates TISTR 3167, KMC 5 and SRS 4 on the growth of *A. flavus* IMI 242684 on peanut grains.

Ethyl acetate extracts from *Trichoderma* spp. (50 mg/ml)	Spore formation (CFU/g) *			
	Days of Incubation			
	7	14	21	28
Control	$3.69x10^{9}$	$1.17x10^{10}$	$9.48x10^{10}$	$1.84x10^{11}\pm6.02^{a}$
Trichoderma sp. TISTR 3167	NG	NG	NG	$1.60x10^{3}\pm11.00^{b}$
Trichoderma sp. KMC 5	NG	NG	NG	$1.88x10^{3}\pm6.57^{b}$
Trichoderma sp. SRS 4	NG	NG	NG	$3.17x10^{3}\pm3.78^{b}$

NG= No Growth
*= Mean ±SD; Mean of three replications
Means followed by different letters differ significantly at P≤ 0.05 (DMRT)

Table 4 Effect of ethyl acetate extracts (50 mg/ml) from *Trichoderma* isolates TISTR 3167, KMC 5 and SRS 4 on aflatoxin production by *A. flavus* IMI 242684 on peanut grains after 28 days of incubation.

Ethyl acetate extracts from *Trichoderma* spp. (50 mg/ml)	Average of aflatoxin (µg/g)*		
	B_1	B_2	Total B_1+ B_2
Control	190.25 ± 0.81^{a}	119.25 ± 0.31^{a}	309.50 ± 0.45^{a}
Trichoderma sp. TISTR 3167	0.02 ± 0.04^{b}	0.06 ± 0.10^{b}	0.08 ± 0.14^{b}
Trichoderma sp. KMC 5	0.06 ± 0.10^{b}	0.03 ± 0.05^{b}	0.09 ± 0.15^{b}
Trichoderma sp. SRS 4	0.07 ± 0.13^{b}	0.04 ± 0.08^{b}	0.11 ± 0.20^{b}

*= Mean ±SD; Mean of three replications
Means followed by different letters in each column differ significantly at P≤ 0.05 (DMRT)

4. Discussion

When 4 solvents, i.e. hexane, ethyl acetate, n-butanol and methanol were used for extracting metabolites from culture filtrates of all *Trichoderma* isolates (TISTR3167, KMC 5, SRS 4 and SCP III), the ethyl acetate extracts showed the greatest ability against *A. flavus* IMI 242684. Yin *et al.* [30] 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 mould. The efficacy of the extract was equivalent or superior to the commercial dicarboximide fungicide, procymidone. They also found that the ethyl extract had better protective effects than theurapeutic effects. However, Febles *et al*. [31] reported that methanol extraction yielded higher antimicrobial activity than n-hexane and ethyl acetate. The methanol extract from culture filtrates of *T. asperellum* and *T. longibrachiatum* gave the best non-enzymatic antimicrobial profiles [32]. On the contrary, Sastry and Rao [33] found that chloroform was better than methanol. Different

isolates of the same species can produce different compounds [34]. Vizcaino *et al*. [32] explained that the variation detected for the antimicrobial activities at strain level within the different sections and species studied, thus the antimicrobial spectrum of a given strain must be evaluated individually.

Comparing the efficacy of ethyl acetate extract from 4 *Trichoderma* isolates on the growth inhibition of *A. flavus* IMI 242684 on PDA, the extract from SCP III showed the poorest inhibition. Further studies showed that isolates TISTR 3167, KMC 5 and SRS 4 could inhibit the growth of *A. flavus* IMI 242684 on peanuts for 21 days and little growth was observed on day 28. The total aflatoxin production (B_1 and B_2) was reduced by approximately 300 times on day 28. Our previous studies showed that culture filtrates of *Trichoderma* isolates KMC 5 and SRS 4 identified as *Trichoderma atroviride* produced varying degrees of inhibition against *A. parasiticus* IMI 102566 [35]. Culture filtrates of *T. viride* and *T. harzianum* showed inhibitory effect on *Fusarium moniliforme* and to a lesser extent, *A. flavus* [36]. Gachomo and Kotchoni [14] reported that the degree to which *Trichoderma* species suppressed the growth of peanut moulds such as *Fusarium* species, *A. niger*, *A. flavus*, *A. parasiticus* and *A. ochraceous* might involve the production of volatiles and/or production of extracellular enzymes. Doi and Mori [37] demonstrated that volatiles from *Trichoderma* species were able to arrest the hyphal growth of different fungal pathogens on agar plates. Most strains of *Trichoderma* can produce volatiles and non volatiles toxic metabolites such as harzianic acid, alamethicins and tricholin that hinder growth of other microorganisms [38]. Some aggressive *Trichoderma* spp. used as biocontrol agents are capable of producing either antibiotics or extracellular enzymes or both [39]. Aflatoxin reduction might be an indirect result of the suppressed growth of the aflatoxigenic fungi [14].

5. Conclusions

It could be concluded that ethyl acetate was the best solvent for extracting secondary metabolites from *Trichoderma* spp. isolates TISTR 3167, KMC 5 and SRS 4 for the control of *A. flavus* IMI 242684. Ethyl acetate extract of culture broth of *Trichoderma* sp. might be useful for controlling aflatoxin producing fungi in stored products. Further investigation has to be done.

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