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In vitro and *in situ* antifungal properties of a *Trichoderma asperelloides* SKRU-01 against aflatoxigenic *aspergillus* species

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ABSTRACT

This research investigates the potential of using the culture filtrates of *Trichoderma asperelloides* SKRU-01 as a peanut preservative and/or fungicide against two mycotoxin-producing fungi, *Aspergillus flavus* and *A. parasiticus*. The study assessed the effectiveness of culture filtrates SKRU-01 compared to two commercial preservatives and chemical fungicides, both *in vitro* and in peanuts. In addition, the study investigated the antifungal and antiaflatoxigenic mechanisms of culture filtrates SKRU-01 against mycotoxin-producing strains. The *in vitro* results demonstrated that culture filtrates SKRU-01 had a significantly stronger inhibitory effect on the growth and spore germination of the two mycotoxin-producing strains compared to sodium benzoate, sodium propionate, and metalaxyl®, but less inhibition than prochloraz®. The study also explored the mode of action of culture filtrates SKRU-01 and found that it impeded the biosynthesis of ergosterol, indicating its antifungal activity was directed towards the plasma membrane as a potential target site. Furthermore, the culture filtrates SKRU-01 inhibited the biosynthesis of methylglyoxal (AFB_1 inducer) in a manner that was dependent on the concentration, which represents a novel mechanism of action against aflatoxins. Additionally, the study observed significant impairment of both enzymatic and non-enzymatic antioxidant defense molecules in aflatoxigenic cells, indicating a potential biochemical mechanism of action. The study tested the potential application of the culture filtrates SKRU-01 in eliminating fungal spoilage in peanuts as a model. The results showed that it had a significant effect in preventing both mold density and AFB_1 production, compared to commercial preservatives and chemical fungicides. The antiaflatoxigenic property with AFB_1 production level was below the public health concern threshold of 20 μg/kg. Overall, the findings suggest that the culture filtrate of *T. asperelloides* SKRU-01 contained effective biological control agents possessing preservative and fungicidal properties.

1. Introduction

Peanut cultivation is globally widespread due to their high nutritional content and digestible protein, making them essential for oil production and industrial uses (Sun et al., 2021). However, peanuts are highly vulnerable to contamination by food spoilage fungi, notably *Aspergillus flavus* and *Aspergillus parasiticus*, throughout the entire supply chain (Hertwig et al., 2020). These fungi produce aflatoxins, which are highly mutagenic and carcinogenic, resulting in severe economic consequences and posing a significant threat to human health, especially in Asian and African countries (Gong et al., 2019).

To combat the health hazards associated with harmful aflatoxins, countries worldwide have implemented strict regulations and guidelines governing the production, importation, and sale of food and feed contaminated with these toxins. These regulations aim to safeguard against aflatoxin exposure. The permitted levels of aflatoxins in food products may vary based on the food type and country, following guidelines established by authorities like the United States Food and Drug Administration (FDA). For instance, Thailand has set a maximum allowable level of 20 μg/kg for total aflatoxins in food products

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(Panrapee et al., 2016). In comparison, the European Union has even more stringent restrictions, with limits of 4 μg/kg for total aflatoxins and 2 μg/kg for aflatoxin B_1 in food products (European Commission, 2010a).

Efforts are being made to address the presence of mycotoxins, harmful substances that can contaminate food grains. While chemical pesticides can effectively control mycotoxins, their use can lead to negative consequences, including the development of fungicide-resistant pathogenic fungi and the presence of fungicide residues in edible products (Bamisile et al., 2021). Such impacts compromise food safety, harm the environment, and pose risks to human health (Droby, 2006). Consequently, the development of reliable and efficient biological control agents has become a major focus for agricultural researchers, as they are presumed to carry lower risks to human health and the environment, given the increasing recognition of the potential hazards associated with the use of chemical agents in pest management.

Beneficial microorganisms are ecologically friendly bio-control agents for managing plant pathogens (Mahmud et al., 2021; Zhang et al., 2018). Various species, including *Trichoderma* spp. (Rodrigues et al., 2023), *Streptomyces* spp. (Boukaew & Prasertsan, 2020a; Boykova et al., 2023), *Bacillus* spp. (Iqbal et al., 2023), *Pseudomonas* spp. (de Freitas & Taylor, 2023), yeasts (Podgórska-Kryszczuk, 2023) and non-aflatoxigenic *Aspergillus* spp. (Accinelli et al., 2018; Ehrlich, 2014), have received significant attention in minimizing the impact of plant pathogens. Among them, *Trichoderma* spp. Is widely recognized as a promising biocontrol agent, extensively evaluated and implemented globally (Ren et al., 2022; Woo et al., 2014). They produce secondary metabolites with bioactive properties, such as cell wall-degrading enzymes (Qualhato et al., 2013) and fungitoxic antibiotics (Vinale et al., 2014). *Trichoderma* has shown potential in combating plant diseases, reducing reliance on synthetic pesticides, and stimulating plant growth (Lorito et al., 2010). Additionally, Trichoderma's secondary metabolites have emerged as a source of novel bioactive compounds applicable in various industries. However, its application for controlling food spoilage mycotoxins in peanuts remains limited, with only a few studies conducted on the subject (Ren et al., 2022; Thanh et al., 2014). Therefore, this paper investigates the potential of *Trichoderma* metabolites as biocontrol agents against mycotoxins on peanuts, providing updated information. This study aimed to investigate the potential of *T. asperelloides* SKRU-01 metabolites against food spoilage-causing fungi *A. flavus* and *A. parasiticus*, both *in vitro* and in peanuts, and compare their effectiveness to that of conventional commercial preservatives and chemical fungicides. Moreover, in this study, we examined the specific target of antifungal metabolites of culture filtrates SKRU-01 on the plasma membrane, evaluated their impact on plasma membrane integrity, and analyzed their effects on the levels of ergosterol, cellular methylglyoxal (MG), cellular reactive oxygen species (ROS), catalase (CAT), superoxide dismutase (SOD), and glutathione (reduced and oxidized) in mycotoxin cells.

2. Materials and methods

2.1. Molecular identification of the strain SKRU-01

The strain SKRU-01, was isolated from loam soil samples collected from cultivation plots at the Faculty of Natural Resources, Prince of Songkla University, Hatyai, Thailand. The fungal isolate was cultivated on potato dextrose agar (PDA) plates at 30 °C for 7 days. The spores were harvested from the agar surface and suspended in a solution of 5 mL sterile distilled water and 0.1% (v/v) Tween 80 solution. The suspensions were filtered through layers of sterile cheesecloth to remove any residual mycelia, and the spore count was determined using a hemocytometer. The resulting concentration was then adjusted with sterilized water to achieve a final concentration of 10^5 spores/mL.

2.1.1. DNA extraction, amplification, and sequencing

Genomic DNA was extracted from 5 day-old culture of strain SKRU on PDA using FavoPrep® DNA Extraction Mini Kit (Taiwan). To amplify the internal transcribed spacers (ITS), the second largest subunit of RNA polymerase II (*rpb*-2), and the translation elongation factor 1-alpha (*tef*-1) genes, the following primers were utilized: ITS4/ITS5 (White et al., 1990), fRPB2-5f/fRPB2-7cr (Liu et al., 1999), and EF1-728 F/EF1-986 R (Carbone & Kohn, 1999), respectively. The PCR programs for amplifying the ITS, *rpb*-2, and *tef*-1 genes were developed using the method described by Nuangmek et al. (2021). PCR products were subjected to analysis through 1% agarose gel electrophoresis and purified with the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, Germany). The purified products were sent to a commercial sequencing provider (1 S T BASE Company, Kembangan, Malaysia) for sequencing. To identify the resulting sequences, a BLASTn search was performed against the GenBank database (http://blast.ddbj.nig.ac.jp/top-e.html).

2.1.2. Phylogenetic analyses

After performing the multiple sequence alignment using MUSCLE (Edgar, 2004) and manual adjustments in BioEdit v.6.0.7 (Hall, 2022), a phylogenetic tree was constructed using both maximum likelihood (ML) and Bayesian inference (BI) methods. ML analysis was conducted using RAxML v7.0.3 under the GTRCAT model of substitution with 25 categories and 1000 bootstrap replications (Felsenstein, 1985; Stamatakis, 2006). Furthermore, the BI analysis was performed by estimating the best-fit model of substitution using jModel Test 2.1.10 (Darriba et al., 2012) individually for each region. The optimal substitution models were identified as GTR + I + G for ITS and *tef*-1, and HKY + I + G for *rpb*-2 using the Akaike Information Criterion (AIC). BI analysis was conducted using MrBayes v3.2.6 (Ronquist et al., 2012) for Markov chain Monte Carlo sampling (MCMC), running six chains for one million generations from random trees. Trees were sampled every 100th generation. The first 25% of trees were discarded and the remaining trees were used for calculating posterior probability value in the majority rule consensus tree. To visualize the resulting tree topologies, FigTree v1.4.0 (Rambaut, 2012) was used.

2.2. In vitro antagonistic effect of the strain SKRU-01 on the mycelial growth of the two aflatoxin-producing strains

2.2.1. Aflatoxin-producing fungi and spore inoculum preparation

The two strains of aflatoxigenic fungi, *Aspergillus parasiticus* TISTR 3276 and *A. flavus* PSRDC-4, were identified as producers of high levels of aflatoxin (Boukaew et al., 2020b). To collect spores for inoculation, the fungi were grown on PDA plates at 30 °C for 7 days, and the spores were collected using the method described above.

2.2.2. In vitro antagonistic effect of the strain SKRU-01

In vitro confrontation tests were performed in accordance with the method outlined by Amira et al. (2017). Two mycelial plugs with a diameter of 5 mm were taken from the actively growing edges of cultures of *T. asperelloides* SKRU-01 and each mycotoxin-producing fungus, respectively. They were placed 1 cm apart on the new PDA plates and incubated at 30 °C. Control cultures of each of the two strains of aflatoxigenic fungi alone were also included. Experiments were performed in triplicate. The radial growth of each mycelial colony was monitored daily using a vernier caliper, and the percentage of mycelial growth inhibition caused by *T. asperelloides* SKRU-01 was calculated at 3 days using the formula described by Parizi et al. (2012). The inhibition of fungal radial mycelial growth was calculated using the formula: $(R_1 \times R_2/R_1) \times 100$, where R_1 represents the radial mycelial growth in the control treatment and R_2 represents the radial mycelial growth in the strain SKRU-01 treatment.

2.3. Antifungal activity of the culture filtrates SKRU-01 against two mycotoxin-producing strains

2.3.1. Preparation of the culture filtrates SKRU-01

To prepare the culture filtrates, 1 mL of 10^5 spores/mL of strain SKRU-01 was transferred into 100 mL of potato dextrose broth (PDB) medium in 250 mL flasks. After an additional 10 days of incubation at 30 °C on a rotary shaker at 150 rpm, the mycelial mats were removed from the culture broth by filtering it through a Whatman No. 1 filter paper. The resulting filtrate was then passed through a 0.45 μm Millipore membrane to obtain the culture filtrates. The culture filtrates SKRU-01 was then used to study their antifungal and food preservative activities in the following bioassays.

2.3.2. Antifungal activity of the culture filtrates SKRU-01

The antifungal activity of culture filtrates SKRU-01 was evaluated against the mycelial growth of the mycotoxin-producing fungal strains on PDA plates, using the method described by Droby et al. (2003) with some modifications. Briefly, 10 μL of a spore suspension containing 10^5 spores/mL of each of the two fungi was inoculated in the center of a Petri dish (9-cm-diameter) containing PDA with different concentrations (0.5–5.0 mL/10 mL PDA) of the culture filtrates SKRU-01. The PDA medium was treated with sterile distilled water as a control. After incubating at 30 °C for 3 days, the radial growth of each mycotoxin-producing fungus was monitored by measuring the colony size using a vernier caliper. The percentage of mycelial growth inhibition was determined using the equation described earlier.

2.4. Comparison of the antifungal activity of culture filtrates SKRU-01 with commercial preservatives or chemical fungicides on mycelial growth inhibition of two mycotoxin-producing strains

2.4.1. Commercial preservatives and chemical fungicides

Two commercial preservatives, sodium benzoate, and sodium propionate, were obtained from Aldrich (Milwaukee, WI, USA). Sodium benzoate, which is FDA-approved for use as a food preservative, is typically used in the food industry at concentrations ranging from 0.05% to 0.1% (w/v) (Jay, 1992). Sodium propionate was typically used at concentrations ranging from 0.1% to 0.2% (w/v). Both preservatives were dissolved in sterile distilled water at various concentrations, including 0.05%, 0.1%, 0.15%, and 0.2% (w/v). Two chemical fungicides were used: 45% (w/v) prochloraz (PESTANAL® analytical standard, Sigma-Aldrich) and 25% (w/v) metalaxyl (PESTANAL® analytical standard, Sigma-Aldrich).

2.4.2. Comparison of the antifungal activity of culture filtrates SKRU-01 with commercial preservatives or chemical fungicides

The antifungal activity of culture filtrates SKRU-01 was compared to that of commonly used antifungal preservatives by employing PDA plates with some modifications to the method described by Droby et al. (2003). Specifically, a spore suspension with a concentration of 10^5 spores/mL of each mycotoxin-producing fungus was added in the center of a Petri dish (9-cm-diameter) containing PDA medium and treated with either culture filtrates SKRU-01 or various concentrations (0.05%, 0.1%, 0.15%, and 0.2% (w/v)) of sodium benzoate and sodium propionate. The culture filtrates SKRU-01, sodium benzoate, and sodium propionate solutions were sterilized by filtering through a 0.45 μm Millipore filter (Sartorius®) before being added to the autoclaved PDA medium, which was approximately 60 °C. As a control, sterile distilled water was used to treat the PDA medium. To perform the incubation and calculation of the percentage inhibition of mycelial growth, we followed the method that was previously mentioned.

The antifungal activity of culture filtrates SKRU-01 was compared to that of chemical fungicides on PDA plates, according to decried Droby et al. (2003) with minor modification. Ten mL of melted sterile PDA was mixed with culture filtrates SKRU-01, prochloraz® (45% (w/v)), or metalaxyl® (25% (wp)) and poured onto a Petri dish (9-cm-diameter). The PDA medium was treated with sterile distilled water as a control. Next, a spore suspension of 10^5 spores/mL of each mycotoxin-producing fungus was dropped onto the center of the test PDA plates. Finally, the percentage inhibition of mycelial growth was calculated following the incubation period, using the method previously mentioned.

2.5. Inhibitory effects of culture filtrates SKRU-01, commercial preservatives, and fungicides on germination of two mycotoxin-producing fungi

To assess the inhibitory effect of culture filtrates SKRU-01, commercial preservatives (0.2% (w/v) sodium benzoate and sodium propionate), and chemical fungicides (45% (w/v) prochloraz® and 25% (wp) metalaxyl®) on the germination of two strains of mycotoxin-producing fungi spores, we employed a method described by Li et al. (2019) with minor modifications. Briefly, 100 μL of spore suspensions (10^5 spores/mL) of each fungus strain were added to wells in a 24-well microtitration plate. Each well was supplemented with 800 μL of PDB medium and 100 μL of the test antifungal agent. As a control, sterile distilled water was utilized. The microtitration plate was placed on a rotary shaker and incubated at 30 °C and 100 rpm. After 6, 12, and 24 h of incubation, approximately 200 spores were examined microscopically to determine their germination rate, which was defined as the emergence of a germ tube equal to or greater than the diameter of the spore. The percentage of inhibition on spore germination was determined using the formula: Inhibition index (%) = $[(R_1–R_2)/R_1] \times 100$, where Control is the spores treated with sterile distilled water (R_1), and Treatment is the spores treated with culture filtrates SKRU-01, commercial preservatives, and chemical fungicides (R_2).

2.6. Mechanisms of antifungal and antiaflatoxigenic action of culture filtrates SKRU-01 against two mycotoxin-producing fungi

After adding the culture filtrates SKRU-01 at concentrations of 5% and 10% to PDB, their antifungal properties were evaluated. The control group did not receive culture filtrates of SKRU-01. Following inoculation with a 50 μL spore inoculum (10^5 spores/mL) of the corresponding mycotoxin-producing fungus strain, the flasks were incubated for 5 days at 30 °C on a rotary shaker (150 rpm). After incubation, the culture broth was divided into two equal batches. One batch was used to determine the mycelial mats of two mycotoxin-producing strains according to the method of Li et al. (2011), while the other was used to estimate the mechanism of the antifungal and antiaflatoxigenic action.

2.6.1. Effect of culture filtrates SKRU-01 on plasma membrane ergosterol levels in two mycotoxin-producing strains

The ergosterol content of two mycotoxin-producing strains was determined using the procedure outlined by Das et al. (2020) to measure the wet weight of the sterilized mycelial mat. The ergosterol content of each mycotoxin-producing strain was determined using the method described by Tian et al. (2012). Three replicates were performed for each treatment.

2.6.2. Effect of culture filtrates SKRU-01 on cellular methylglyoxal levels in two mycotoxin-producing strains

Fifty μL of spore inoculum at a concentration of 10^5 spores/mL of each mycotoxin-producing fungus strain were added to 10 mL of PDB medium, followed by the addition of culture filtrates SKRU-01 at volumes ranging from 5 to 10 mL. The control group did not receive culture filtrates of SKRU-01. The biomass was collected and ground in 3 mL of 0.5 M $HClO_4$ after incubation on a rotary shaker (150 rpm) at 30 °C for 5 days. The resulting mixture was centrifuged at 8880×*g* for 20 min, and the supernatant was neutralized by adding K_2CO_3 solution drop-wise, followed by another round of centrifugation.

Methylglyoxal levels were estimated from the supernatant by

sequentially adding diaminobenzene, $HClO_4$, and the supernatant, followed by measuring the optical density at 341 nm, using the method outlined by Yadav et al. (2005). The level of methylglyoxal was determined using a standard calibration curve of methylglyoxal. Each treatment was performed in triplicate.

2.7. The effect of culture filtrates SKRU-01 on the enzymatic and non-enzymatic defense systems of two mycotoxin-producing fungi in vitro

2.7.1. Preparation of fungal biomass and enzyme extracts

After treating each mycotoxin-producing fungus with 5 and 10 mL of culture filtrates SKRU-01, the resulting biomass was homogenized in 3 mL of phosphate-buffered saline (PBS, 100 mM, pH 7.4), followed by centrifugation at 8880×*g* for 20 min. The control group did not receive any culture filtrates SKRU-01. Biochemical tests were performed using the obtained supernatant after extraction of the treated and untreated samples.

2.7.2. Measurement of intracellular ROS, CAT, SOD, and glutathione (reduced and oxidized) levels

To measure intracellular ROS production, we followed the method described by Keston and Brandt (1965) and used the dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescent dye assay. We excited the samples at a wavelength of 485 nm and measured the emitted fluorescence at a wavelength of 530 nm to determine the fluorescence intensity. The concentration of dichlorofluorescein was determined by utilizing the standard curve. We determined the CAT activity using the method of Beers and Sizer (1952), and calculated the unit activity per minute per milligram of protein using the molar extinction coefficient of 43.6 M^{-1} cm^{-1}. To measure the SOD activity, the quercetin auto-oxidation method described by Kostyuk and Potapovich (1989) was utilized. The reaction mixture consisted of phosphate buffer (0.016 M, pH 10), EDTA (0.08 mM), N–N–N–N-tetramethylenediamine, and 100 μL of quercetin solution (1.5 mg/10 mL DMSO). The degree of inhibition of quercetin autooxidation was measured at 406 nm at the beginning and after 20 min, and the enzyme activity was expressed as inhibition per milligram of protein. The cellular glutathione content was assessed by homogenizing the 7-day-old biomass of each mycotoxin-producing fungus in a solution containing phosphate buffer (0.1 M, pH 8), EDTA (0.005 M), and metaphosphoric acid (25%). The homogenate was then centrifuged at 8880×*g* for 10 min at 4 °C. The level of both reduced and oxidized glutathione was determined by measuring the fluorescence of the supernatant at 420 nm with an excitation at 350 nm, using the method outlined by Hissin and Hilf (1976). The level of GSSG was determined by incubating 500 μL of supernatant and 200 μL of 0.04 M NEM in a reaction mixture for 20 min at room temperature, followed by the addition of *ortho*-phthalaldehyde and NaOH solution and incubation for 30 min, and then measuring the fluorescence intensity at 420 nm. Total protein content was quantified using the protocol described by Lowry et al. (1951).

2.8. Application of culture filtrates SKRU-01 as biofungicide to inhibit the growth of two mycotoxin-producing strains in peanuts

2.8.1. Sample preparation and survey of molds on peanuts

To examine the effect of SKRU-01 culture filtrates on peanuts inoculated with two types of mycotoxin-producing fungal spores, ten peanut kernels weighing approximately 4.88 g were obtained from a supermarket and kept in storage at −20 °C until they were utilized. Before testing, the peanuts underwent surface sterilization by being soaked in a sodium hypochlorite solution with a concentration of 3% (v/v) for 3 min. They were then rinsed multiple times with sterile distilled water as described by Abd-Alla (2005) and dried using aseptic procedures in a laminar airflow chamber for 30 min (Krusong et al., 2015). To prevent contamination by *Aspergillus* spp. Or other molds, the peanuts were heated at 121 °C for different periods, including 0 (control), 15, 20, 25, or 30 min.

Whole peanuts, which had been sterilized on the surface, were placed onto Petri dishes containing PDA and incubated at 30 °C for 5 days. The growth of the fungal colony was observed after incubation, with three replicates per treatment and 10 peanuts per treatment.

2.8.2. Effect of culture filtrates SKRU-01 on peanuts inoculated with two mycotoxin-producing fungal spores

2.8.2.1. Methods validation. The method was validated in accordance with the SANCO/12,571/2013 guidelines, which confirms that the analytical performance satisfies the requirements of regulation (EC) no. 178/2010 (European Commission, 2010b). These recommendations stipulate a validation procedure for evaluating linearity, specificity, limit of detection (LOD), limit of quantitation (LOQ), accuracy, and precision. In this investigation, Thailand's Animal Feed Quality Control Act B.E. 2525's maximum permitted levels (MPLs) for mycotoxin in animal feed served as a benchmark (Choochuay et al., 2018). In accordance with these MPLs, the maximum fortified concentration for the samples was set at 100 μg/kg.

An external standardization approach was used to evaluate linearity. Using AFB_1 standard solutions at six distinct concentrations encompassing the range of 5–100 μg/kg (specifically, 5, 10, 20, 40, 60, and 100 μg/kg), calibration curves for matrices were generated. By plotting the peak areas, which served as the analytical signal response (y), against the corresponding AFB_1 concentrations (x), analytical curves were constructed.

The specificity of the method was evaluated by comparing the retention periods of blank sample matrices to those of samples containing 100 μg/kg AFB_1. This analysis ensured that the retention time of the analyte of interest remained unaffected and unimpeded (Choochuay et al., 2018).

The sensitivity of the method was evaluated based on the LOD and LOQ. The LOD was determined as the lowest concentration of AFB_1 that produced a signal response three times greater than the average baseline noise from 10 independent blank samples of each matrix (S/N: 3:1). Alternatively, the LOQ was defined as the AFB_1 signal response that was ten times greater than the average baseline noise derived from ten independent blank samples of each matrix (S/N 10:1) (Xie et al., 2018).

Using a spiked AFB_1 standard solution at three distinct concentration levels (equivalent to 20, 40, and 100 μg/kg) in blank sample matrices, the accuracy of the test was determined. Six replicates of each concentration were prepared for each matrix. The precision level was determined by calculating the intraday repeatability (RSD_r) and interday reproducibility (RSD_R) relative standard deviations (RSDs). This was accomplished by adding AFB_1 standard solution at three concentration levels (20, 40, and 100 μg/kg) to blank samples. For each matrix, six replicates of each concentration were also prepared (Choochuay et al., 2018).

2.8.2.2. Biocontrol potential of culture filtrates SKRU-01 against two mycotoxin-producing fungi on peanuts. The peanuts (prepared under the optimum condition as above) were soaked in culture filtrates SKRU-01, commercial preservatives (0.2% (w/v) each of sodium benzoate and sodium propionate) and chemical fungicides 45% (w/v) prochloraz® and 25% (wp) metalaxyl® for 8 h at room temperature (Yang & Chang, 2010). Peanuts soaked in sterile distilled water were used as a control. They were dried in a laminar airflow for 30 min before being transferred to Petri dishes. A spore suspension of the two strains of aflatoxigenic fungi was prepared (10^5 spores/mL), and approximately 10 μL of the suspension was directly dropped into each peanut. The peanuts were incubated at 30 °C for 5 days. After 5 days, the peanuts inoculated with the two strains of aflatoxigenic fungi were divided into two equal batches; one was used to determine mold density, and the other was used to estimate aflatoxin production.

To determine the mold density in each peanut sample, 5 g of the sample were mixed with 45 mL of saline solution (0.85% NaCl) and agitated in a rotary shaker at 200 rpm for 2 h at 30 °C. The resulting suspension was serially diluted two-fold in saline solution (0.85% NaCl), and 0.1 mL of the diluted suspension was spread onto potato dextrose agar (PDA) plates and incubated at 30 °C for 48 h. The mold density was calculated as the logarithm of spores per gram of peanuts. The percentage of spore reduction was determined as previously described. Each treatment was conducted in triplicate.

To determine the concentration of aflatoxin AFB_1, the extraction of aflatoxin from peanuts was performed using the modified method of Sidhu et al. (2009). The ground peanut powder (10 g) was extracted for 3 min in 25 mL of 70% aqueous methanol using a laboratory homogenizer and then filtered through Whatman No. 1 filter paper. The sample was cleaned, and the concentration of AFB_1 was quantified using High-Performance Liquid Chromatography (HPLC) following the method described by Choochuay et al. (2018). The experiment was conducted in triplicate for each treatment.

2.9. Statistical analysis

The experimental procedures were carried out in triplicate, and the resulting data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 26 (IBM Corp; IBM SPSS Statistics for Windows, ver. 26.0, Armonk, NY), and subjected to analysis of variance (ANOVA). Whenever necessary, means were compared using Tukey's HSD (Honestly Significant Difference) test, with a significance level of $p < 0.05$.

3. Results

3.1. Molecular identification of the strain SKRU-01

The partial ITS, *rpb*-2, and *tef*-1 sequences of strain SKRU-01 were deposited in GenBank as OQ568184, OQ572416, and OQ572417, respectively. The dataset analyzed in this study included 31 taxa and aligned sequences of ITS, *rpb*-2, and *tef*-1, with a total length of 2329 characters, including gaps (ITS: 1–637, *rpb*-2: 638–1725, and *tef* 1-α: 1726–2329). The phylogenetic trees obtained from both ML and BI analyses were congruent in topology, thus we only presented the ML tree (Fig. 1). Consistent with previous phylogenetic studies (Cai &

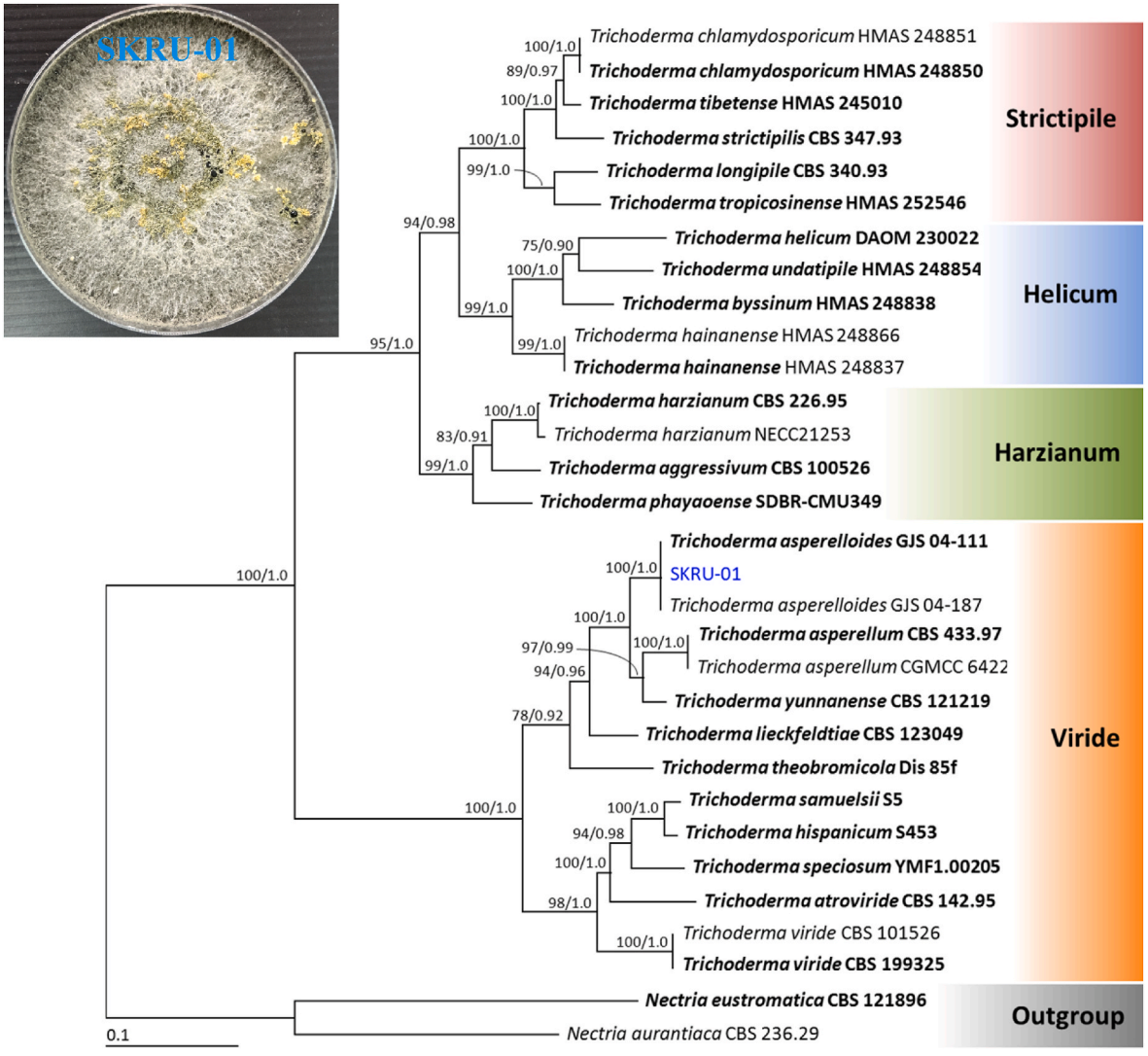

Fig. 1. A phylogenetic tree was generated using the maximum-likelihood analysis of combined ITS, *rpb*-2, and *tef*-1 genes of 31 taxa, with *Nectria aurantiaca* and *N. eustromatica* as the outgroup. The numbers above branches indicate the maximum-likelihood bootstrap percentages on the left and Bayesian posterior probabilities on the right. Only values ≥ 70% for bootstrap and ≥0.90 for Bayesian posterior probabilities are shown. The scale bar represents 10 substitutions per nucleotide position, and the strain obtained in this study is marked in blue, while the type species are in bold.

Druzhinina, 2021; Ma et al., 2020; Qiao et al., 2018), four major clades were identified, namely Harzianum, Helicum, Strictipile, and Viride. The phylogenetic tree assigned the fungal strain SKRU-01 in this study into the same clade of *T. asperelloide*s, containing the type species (GJS 04–111) with high supports (BS 100%, PP 1.0) in the Viride clade. Therefore, the strain SKRU-01 was identified as *T. asperelloides*.

3.2. *In vitro antagonistic effect of T. asperelloides SKRU-01 on the mycelial growth of two mycotoxin-producing strains*

Given the biological facts presented, an *in vitro* experiment was conducted to further investigate the competitiveness and biocontrol potential of strain SKRU-01 against the two strains of aflatoxigenic fungi. In dual culture experiments conducted on Petri plates, the strain SKRU-01 rapidly stopped the growth of two mycotoxin-producing strains, and completely covered their colonies (Fig. 2). The strain SKRU-01 extensively colonized the culture medium due to its faster mycelial growth rate compared to the strain TISTR 3276 (Fig. 2a) and PSRDC-4 (Fig. 2c). A thin zone of growth inhibition for the strain SKRU-01 can be observed for several hours before physical contact between hyphae, after 2 days-PM and 3 days growth of the stain TISTR 3276 and PSRDC-4, respectively. After a 3-day incubation period during which the fungi underwent cross-signaling via pre-contact chemical communication, the growth of the strain TISTR 3276 (Fig. 2a) and PSRDC-4 (Fig. 2c) hyphae was significantly inhibited, with a maximum mycelial inhibition of 63% and 61%, respectively, as compared to the control treatment. The results of the plate confrontation assay showed that after 10 weeks of inoculation at 30 °C, *T. asperelloides* SKRU-01 mycelial overgrew and produced spores on both strains TISTR 3276 (Fig. 2b) and PSRDC-4 (Fig. 2d).

3.3. *Antifungal activity of culture filtrates SKRU-01 against two mycotoxin-producing strains*

The inhibition of mycelial growth in the PDA medium for both TISTR 3276 and PSRDC-4 strains was found to be directly proportional to the concentration of SKRU-01 culture filtrates (Fig. 3). The study revealed that the growth inhibition of both fungal strains, TISTR 3276 and PSRDC-4, was directly proportional to the concentration of culture filtrates SKRU-01 in the PDA medium (Fig. 3). The results indicated a significant ($p < 0.05$) increase in the percentage of growth inhibition for both strains when the concentration of culture filtrates SKRU-01 was increased from 0.5 mL to 2.0 mL (per 10 mL of PDA), from 35.36% to 55.0% for TISTR 3276 and from 28.52% to 54.23% for PSRDC-4. Furthermore, with a further increase in the concentration of culture filtrates SKRU-01 to 5.0 mL per 10 mL of PDA, a significant ($p < 0.05$) increase in the growth inhibition of TISTR 3276 and PSRDC-4 to 76.62% and 73.05%, respectively, was observed.

3.4. *Comparison of the antifungal activity of culture filtrates SKRU-01 with commercial preservatives or chemical fungicides on mycelial growth inhibition of two mycotoxin-producing strains*

Fig. 4 presents the comparison of the antifungal activity of culture filtrates SKRU-01 with commercial preservatives or chemical fungicides, regarding their inhibition of mycelial growth in both TISTR 3276 and PSRDC-4 strains. As shown in Fig. 4a and b, the ability of the culture filtrates SKRU-01 in preventing the growth of two mycotoxin-producing strains in the PDA medium was compared to those of sodium benzoate and sodium propionate. The results showed that culture filtrates SKRU-01 were significantly ($p < 0.05$) more effective at inhibiting the growth of two mycotoxin-producing strains compared to any of the tested concentrations (0.05–0.20% (w/v)) of the two commercial preservatives. The culture filtrates SKRU-01 had stronger antifungal activity than both 0.2% (w/v) of sodium benzoate (Fig. 4a) and sodium

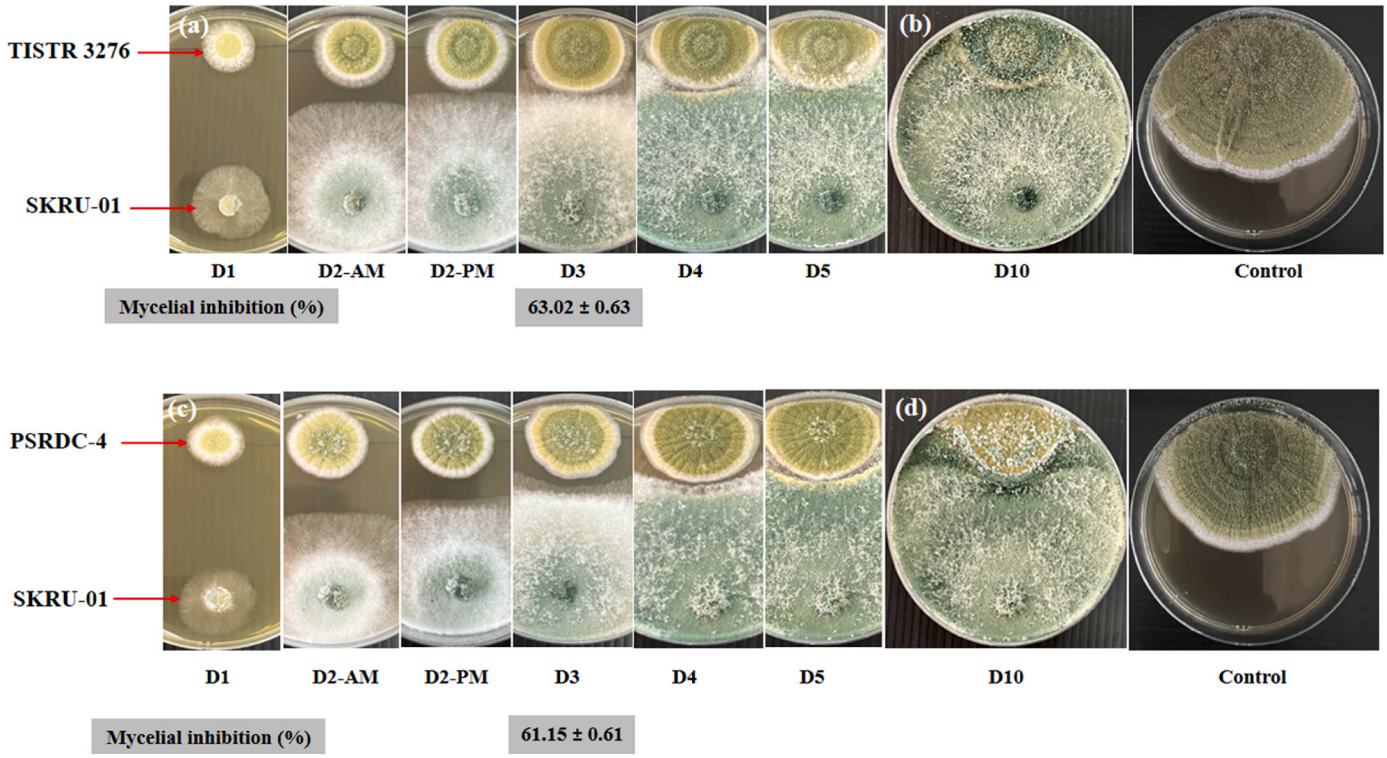

Fig. 2. Development of the mycoparasitic reaction of *Trichoderma asperelloides* SKRU-01 against *Aspergillus parasiticus* TISTR 3276 and *A. flavus* PSRDC-4. Growth kinetic of *T. harzianum* SKRU-01 and *A. parasiticus* TISTR 3276 **(a)** and *A. flavus* PSRDC-4 **(c)** on PDA medium for 5 days. Plate confrontation assays where *T. asperelloides* SKRU-01 mycelial overgrew and sporulated on *A. parasiticus* TISTR 3276 **(b)** and *A. flavus* PSRDC-4 **(d)** ten days after inoculation 30 °C.

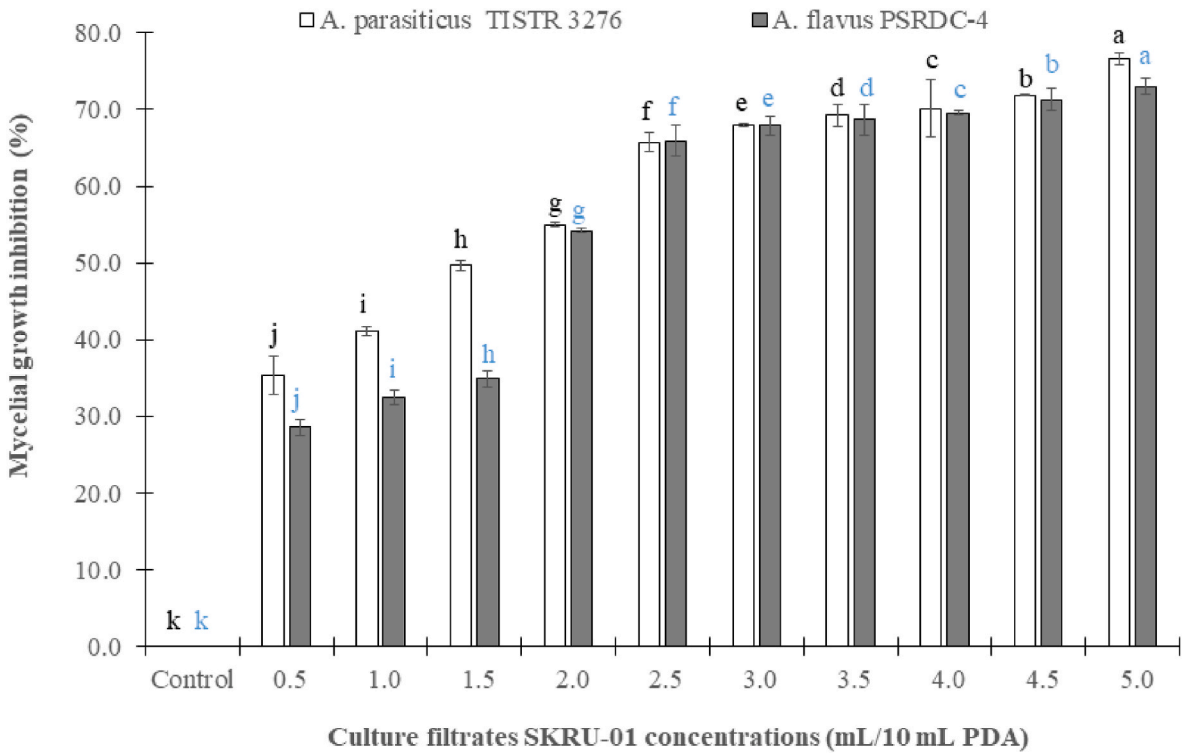

Fig. 3. Investigating the impact of varying concentrations of culture filtrates SKRU-01 (0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, and 5.0 mL/10 mL PDA) on the mycelial growth of *Aspergillus parasiticus* TISTR 3276 and *A. flavus* PSRDC-4 in PDA medium after three days incubation at 30 °C. The values presented are the means of three replicates (±SD). Values with the same letter are not significantly different (ANOVA and Tukey's HSD comparison tests, $p < 0.05$).

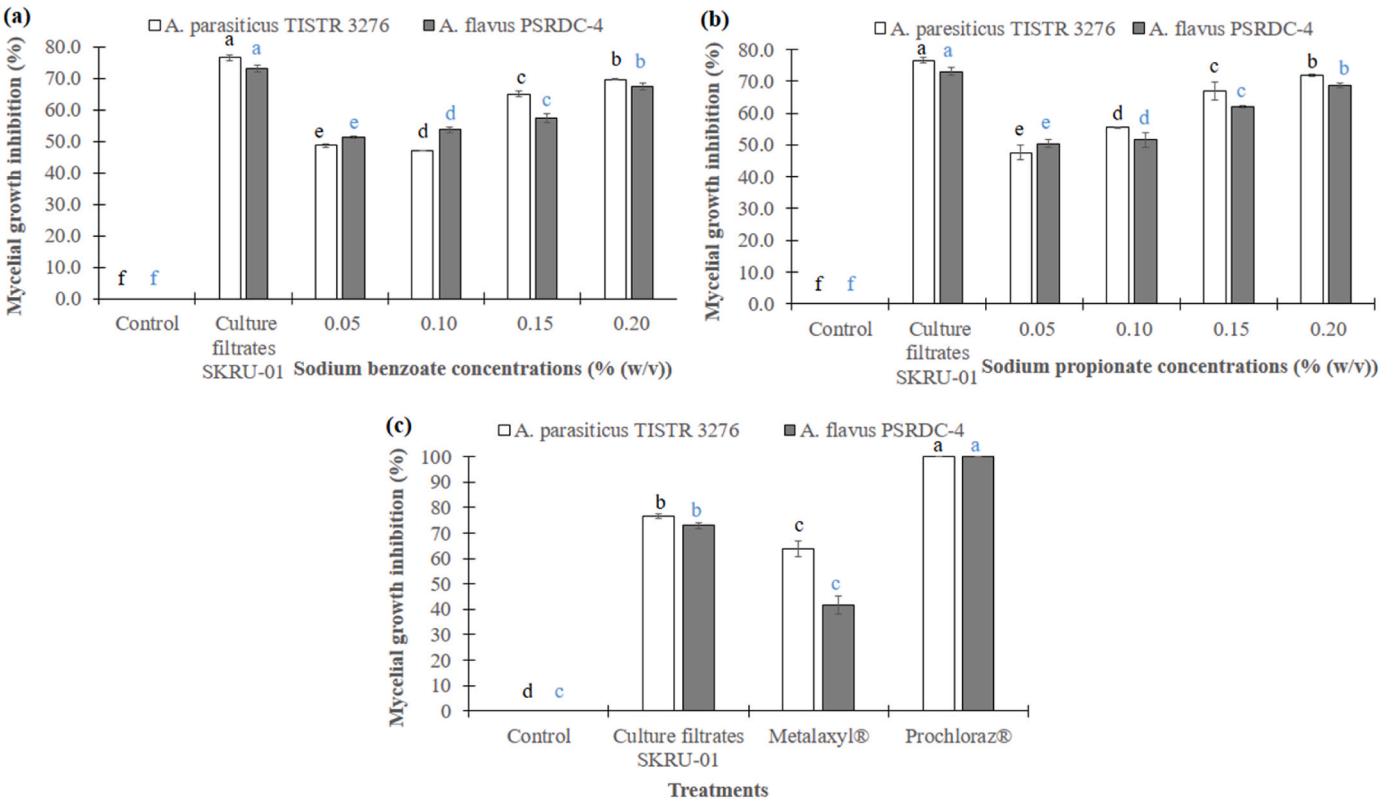

Fig. 4. Comparison the antifungal activity of culture filtrates SKRU-01 with commercial preservatives (sodium benzoate **(a)** and sodium propionate **(b)**) and chemical fungicides (prochloraz® and metalaxyl®) **(c)** on the mycelial growth of *Aspergillus parasiticus* TISTR 3276 and *A. flavus* PSRDC-4 in PDA medium after three days incubation at 30 °C. The values presented are the means of three replicates (±SD). Values with the same letter are not significantly different (ANOVA and Tukey's HSD comparison tests, $p < 0.05$).

propionate (Fig. 4b). It demonstrated a 76.62% and 73.05% inhibition of the strains TISTR 3276 and PSRDC-4, respectively, whereas sodium benzoate resulted in 69.67% and 67.32% inhibition while sodium propionate resulted in 72.0% and 68.69% inhibition, respectively.

The ability of the culture filtrates SKRU-01 and chemical fungicides to inhibit the growth of two mycotoxin-producing strains was compared as shown in Fig. 4c. The culture filtrates SKRU-01 demonstrated stronger antifungal activity against the strain TISTR 3276 (76.62% inhibition) and the strain PSRDC-4 (73.05% inhibition) than metalaxyl® (63.89% and 41.76% inhibition, respectively). However, its activity was weaker than that of prochloraz® which showed complete inhibition (100%) of the two strains of aflatoxigenic fungi.

3.5. Inhibitory effects of culture filtrates SKRU-01, commercial preservatives, and fungicides on germination of two mycotoxin-producing fungi

As shown in Table 1, the effectiveness of culture filtrates SKRU-01 in inhibition of the spore germination of the strain TISTR 3276 (Table 1a) and the strain PSRDC-4 (Table 1b) was compared with those of commercial preservatives and chemical fungicides. The use of SKRU-01 culture filtrates resulted in a noteworthy ($p < 0.05$) decrease in the spore germination percentage of both mycotoxin-producing fungi, in comparison to the chemical fungicides and commercial preservatives. The results showed that the inhibitory effect on spore germination of antifungal agents decreased as the incubation time increased. In particular, all treatments effectively inhibited the spore germination completely (100%) in the two strains of aflatoxigenic fungi during the initial 6 h of observation. After the 24 h incubation, the culture filtrates SKRU-01 (87.52%) demonstrated greater spore inhibition of the strain TISTR 3276 compared to metalaxyl® (79.89%), as well as sodium benzoate (36.22%) and sodium propionate (27.73%). Similarly, for the strain PSRDC-4, the culture filtrates SKRU-01 (85.03%) was more effective in inhibiting spore germination than metalaxyl® (80.28%), as well as sodium benzoate (33.27%) and sodium propionate (29.22%). However, prochloraz® had a stronger ability and completely inhibited spore germination (100%) during 24 h incubation of the two strains of aflatoxigenic fungi, compared to other antifungal agents.

Table 1
The impact of culture filtrates SKRU-01, commercial preservatives (sodium benzoate and sodium propionate), and chemical fungicides (metalaxyl® and prochloraz®) on the germination inhibition of **(a)** *Aspergillus parasiticus* TISTR 3276 and **(b)** *A. flavus* PSRDC-4 spores after incubation at 30 °C for 24 h.

(a)

Time (h)	Spore germination inhibition (%)				
	Culture filtratesSKRU-01	Sodium benzoate	Sodium propionate	Prochloraz®	Metalaxyl®
6	$100^{a} \pm 0$	$100^{a} \pm 0$	$100^{a} \pm 0$	$100^{a} \pm 0$	$100^{a} \pm 0$
12	$100^{a} \pm 0$	$85.92^{b} \pm 7.37$	$78.97^{c} \pm 3.05$	$100^{a} \pm 0$	$100^{a} \pm 0$
24	$87.52^{b} \pm 8.0$	$36.22^{d} \pm 7.37$	$27.73^{e} \pm 3.60$	$100^{a} \pm 0$	$79.89^{c} \pm 5.85$

(b)

Time (h)	Spore germination inhibition (%)				
	Culture filtrates SKRU-01	Sodium benzoate	Sodium propionate	Prochloraz®	Metalaxyl®
6	$100^{a} \pm 0$	$100^{a} \pm 0^{a}$	$100^{a} \pm 0$	$100^{a} \pm 0$	$100^{a} \pm 0$
12	$100^{a} \pm 0$	$81.80^{b} \pm 5.85$	$76.67^{c} \pm 6.24$	$100^{a} \pm 0$	$100^{a} \pm 0$
24	$85.03^{b} \pm 4.04$	$33.27^{d} \pm 3.72$	$29.22^{e} \pm 1.21$	$100^{a} \pm 0$	$80.28^{c} \pm 6.11$

Note: The presented data represents the mean of three replicates ± standard deviation (SD), and data with the same letter within each column are not significantly different based on ANOVA after Tukey's HSD test with a significance level of $p < 0.05$.

3.6. Mechanisms of antifungal and antiaflatoxigenic action of culture filtrates SKRU-01 against two mycotoxin-producing strains

The mycelial dry weights of the two strains of aflatoxigenic fungi were significantly reduced ($p < 0.05$) in the presence of culture filtrates SKRU-01. The antifungal activity of both concentrations of culture filtrates SKRU-01 was higher against strain TISTR 3276 as compared to strain PSRDC-4. In fact, at the concentration of 10% (v/v), the culture filtrates SKRU-01 displayed greater than 70% inhibition on the growth of the strain TISTR 3276 (89.92%) and the strain PSRDC-4 (73.36%) (Table 2).

The investigation of the impact of culture filtrates SKRU-01 on membrane ergosterol biosynthesis in cells of the two strains of aflatoxigenic fungi was carried out, as presented in Table 3. The culture filtrates SKRU-01 significantly inhibited ($p < 0.05$) ergosterol biosynthesis in a dose-dependent manner. The ergosterol reduction percentages were determined for the two strains of aflatoxigenic fungi treated with 5% and 10% (v/v) concentrations of the culture filtrates SKRU-01. The results showed that the reduction percentage of ergosterol in the strain TISTR 3276 was 39.30% and 52.40%, while the reduction percentage in the strain PSRDC-4 was 29.56% and 45.74%, respectively. Fig. 5 shows that the culture filtrates SKRU-01 caused a significant ($p < 0.05$) reduction in methylglyoxal (MG) levels in relation to different doses used. The MG level in the control set of the both strains TISTR 3276 and PSRDC-4 was found to be 1606.71 μM/gFW and 1676.09 μM/gFW, respectively. At the 10% (v/v) dose, a considerable reduction was observed, reaching 972.12 μM/gFW and 1186.12 μM/gFW, respectively.

3.7. The effect of culture filtrates SKRU-01 on the enzymatic and non-enzymatic defense systems of two mycotoxin-producing fungi in vitro

The impact of culture filtrates SKRU-01 on the enzymatic and non-enzymatic defense systems of two mycotoxin-producing fungi *in vitro* was investigated (Fig. 6). Treatment with varying concentrations of culture filtrates SKRU-01 led to a significant increase ($p < 0.05$) in the production of intracellular ROS, SOD, CAT, GSH, and GSH/GSSG as compared to the control. The control ROS level in the strains PSRDC-4 and TISTR 3276 were observed to be 0.59 and 0.44 unit/min/mg protein, respectively. The application of 10% (v/v) concentration of culture filtrates SKRU-01 resulted in a significant ($p < 0.05$) increase in the ROS

Table 2
The impact of culture filtrates SKRU-01 on growth of *Aspergillus parasiticus* TISTR 3276 and *A. flavus* PSRDC-4 cultured in potato dextrose broth (PDB) at 30 °C for five days.

Treatments	*A. parasiticus* TISTR 3276		*A. flavus* PSRDC-4	
	Mycelial dry weights (mg)	Mycelial growth inhibition (%)	Mycelial dry weights (mg)	Mycelial growth inhibition (%)
0 (Control)	$231.7^{a} \pm 2.27$	–	$297.37^{a} \pm 3.56$	–
Treated 5% (v/v) culture filtrates SKRU-01	$95.1^{b} \pm 4.10$	58.96	$127.80^{b} \pm 2.01$	56.97
Treated 10% (v/v) culture filtrates SKRU-01	$23.37^{c} \pm 1.16$	89.92	$79.23^{b} \pm 1.86$	73.36

Note: The presented data represents the mean of three replicates ± standard deviation (SD), and data with the same letter within each column are not significantly different based on ANOVA after Tukey's HSD test with a significance level of $p < 0.05$.

Table 3
The impact of culture filtrates SKRU-01 on percent inhibition of ergosterol production of *Aspergillus parasiticus* TISTR 3276 and *A. flavus* PSRDC-4 cultured in potato dextrose broth (PDB) at 30 °C for five days.

Treatments	Mean of percentage inhibition of ergosterol production ± SD	
	A. parasiticus TISTR 3276	*A. flavus* PSRDC-4
0 (Control)	$0.00^{c} \pm 0.00$	$0.00^{c} \pm 0.00$
Treated 5% (v/v) culture filtrates SKRU-01	$39.30^{b} \pm 0.08$	$29.56^{b} \pm 0.25$
Treated 10% (v/v) culture filtrates SKRU-01	$52.40^{a} \pm 0.42$	$45.74^{a} \pm 0.12$

Note: The presented data represents the mean of three replicates ± standard deviation (SD), and data with the same letter within each column are not significantly different based on ANOVA after Tukey's HSD test with a significance level of $p < 0.05$.

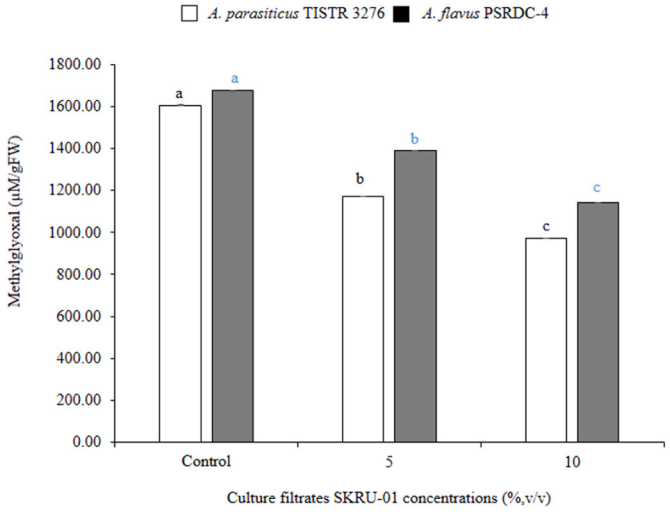

Fig. 5. The impact of culture filtrates SKRU-01 on cellular methylglyoxal (MG) of *Aspergillus parasiticus* TISTR 3276 and *A. flavus* PSRDC-4 cells cultured in potato dextrose broth (PDB) at 30 °C for five days. The values presented are the means of three replicates (±SD). Values with the same letter are not significantly different (ANOVA and Tukey's HSD comparison tests, $p < 0.05$).

level, which reached 1.98 and 1.38 unit/min/mg protein for PSRDC-4 and TISTR 3276, respectively (Fig. 6a).

Fig. 6b and c shows that the culture filtrates SKRU-01 were found to significantly increase the levels of cellular antioxidant enzymes such as SOD and CAT. In comparison, when no cultures filtrate was used (the control), the levels of SOD and CAT in the strain TISTR 3276 and PSRDC-4 were 0.08 and 0.06 unit/min/mg protein (for SOD) and 21.09 and 20.87 unit/min/mg protein (for CAT), respectively. However, when 10% (v/v) of the culture filtrates SKRU-01 was used, the levels of SOD and CAT were observed to be 1.51 and 0.69 unit/min/mg protein (for SOD) and 54.67 and 33.78 unit/min/mg protein (for CAT), respectively.

After exposure to the culture filtrates SKRU-01, the levels of reduced glutathione (GSH), oxidized glutathione (GSSG), and GSH/GSSG in two fungal cells capable of producing aflatoxin showed significant ($p < 0.05$) alterations. The GSH and GSH/GSSG levels in the treated sets were significantly higher compared to the control sets, as seen in Fig. 6d and f. For example, in the control and 10% (v/v) treatment, the GSH levels of the strain TISTR 3276 and PSRDC-4 increased from 17.60 to 31.07 µM/mg protein, and from 18.91 to 35.79 µM/mg protein, respectively. The observed dose-dependent increase in GSH and GSH/GSSG levels suggests that the fungal cells have an enhanced mechanism to combat oxidative stress (Das et al., 2020). However, unlike other enzyme activities, the cellular GSSG activity declined as the concentration of culture filtrates SKRU-01 increased (Fig. 6e).

3.8. Application of culture filtrates SKRU-01 as biofungicide to inhibit the two mycotoxin-producing strains in peanuts

3.8.1. Method performance

In a validation procedure, peanut samples that had been adulterated were used to evaluate the performance characteristics of the optimized method. The procedure involved linearity, specificity, accuracy, precision, LOD, and LOQ testing. The obtained results revealed exceptional linearity of the analytical curves within the working range of 5–100 µg/kg, with R^2 values exceeding 0.9995. Specificity was achieved by distinguishing AFB_1 from interfering compounds, while the absence of any signal response near the retention time of AFB_1 in all matrices indicated the absence of matrix interferences, despite the high complexity of the matrices (Xie et al., 2018).

To evaluate the method's sensitivity, the LOD and LOQ values were considered (Table 4). For all matrices, the calculated values indicated a LOD of 1.2 µg/kg and a LOQ of 1.6 µg/kg. The LOD and LOQ values for detecting AFB_1 in peanuts were deemed adequate because they were well below the maximum permitted levels (MPLs) mandated by law (2 µg/kg) (European Commission, 2010a).

In addition, the method's precision was evaluated via recovery experiments employing an AFB_1 standard solution spiked at three distinct concentration levels (20, 40, and 100 µg/kg). The average legume recoveries ranged from 102.65% to 106.57% (Table 4), demonstrating compliance with the recovery analysis range guidelines of 71–107% set by the Commission Regulation (EC) No 401/2006 (European Commission, 2006b). By calculating the relative standard deviations (RSDs) under RSD_r and RSD_R conditions, the method's precision was assessed. In this study, the RSD_r ranged between 1.05% and 5.06%, and the RSD_R ranged between 1.47 and 6.82% (Table 4). According to the performance criteria of the European Commission (2010b), these RSD values fall within an acceptable range of less than 20%. In addition, the obtained results comply with the regulations established by Thai authorities for the detection of AFB_1 in animal feed.

3.8.2. Biocontrol potential of culture filtrates SKRU-01 against two mycotoxin-producing fungi on peanuts

The results presented in Fig. 7 showed that while mold growth occurred on controlled peanuts (surface-sterilized but not heat-treated), it was absent on peanuts heated at 121 °C for 15 min. Therefore, it was concluded that heating surface-sterilized peanuts to 121 °C for 15 min was an effective way to ensure their suitability for use in these experiments.

As shown in Fig. 8 and Tables 5 and 6, the culture filtrates SKRU-01 effectively prevent the growth of the strain TISTR 3276 (Fig. 8a) and PSRDC-4 (Fig. 8b) on peanuts, compared to commercial preservatives and chemical fungicides. The control treatment resulted in dark green spores and abundant mold growth on peanuts, while peanuts treated with culture filtrates SKRU-01, metalaxyl®, sodium propionate, and sodium benzoate showed some degree of growth inhibition. No mold growth was found in peanuts treated with prochloraz® (Fig. 8).

As shown in Tables 5 and 6 after 5 days of incubation, control peanuts had a fungal population of 3.37 log spores/g and aflatoxin production of 133.80 µg/kg for the strain TISTR 3276, and 3.44 log spores/g and 164.07 µg/kg for the strain PSRDC-4, respectively. Culture filtrates SKRU-01 significantly ($p < 0.05$) reduced the fungal population (1.22 log spores/g) and aflatoxin production (18.23 µg/kg) in peanuts inoculated with the strain TISTR 3276, which was higher than the reductions achieved with metalaxyl® (1.30 log spores/g and 18.30 µg/kg), sodium propionate (1.57 log spores/g and 80.67 µg/kg), and sodium benzoate (1.64 log spores/g and 88.00 µg/kg), but lower than the reduction achieved with prochloraz® (0 log spores/g and 0 µg/kg) (Table 5). Similar growth of the strain PSRDC-4 was seen in both control peanuts

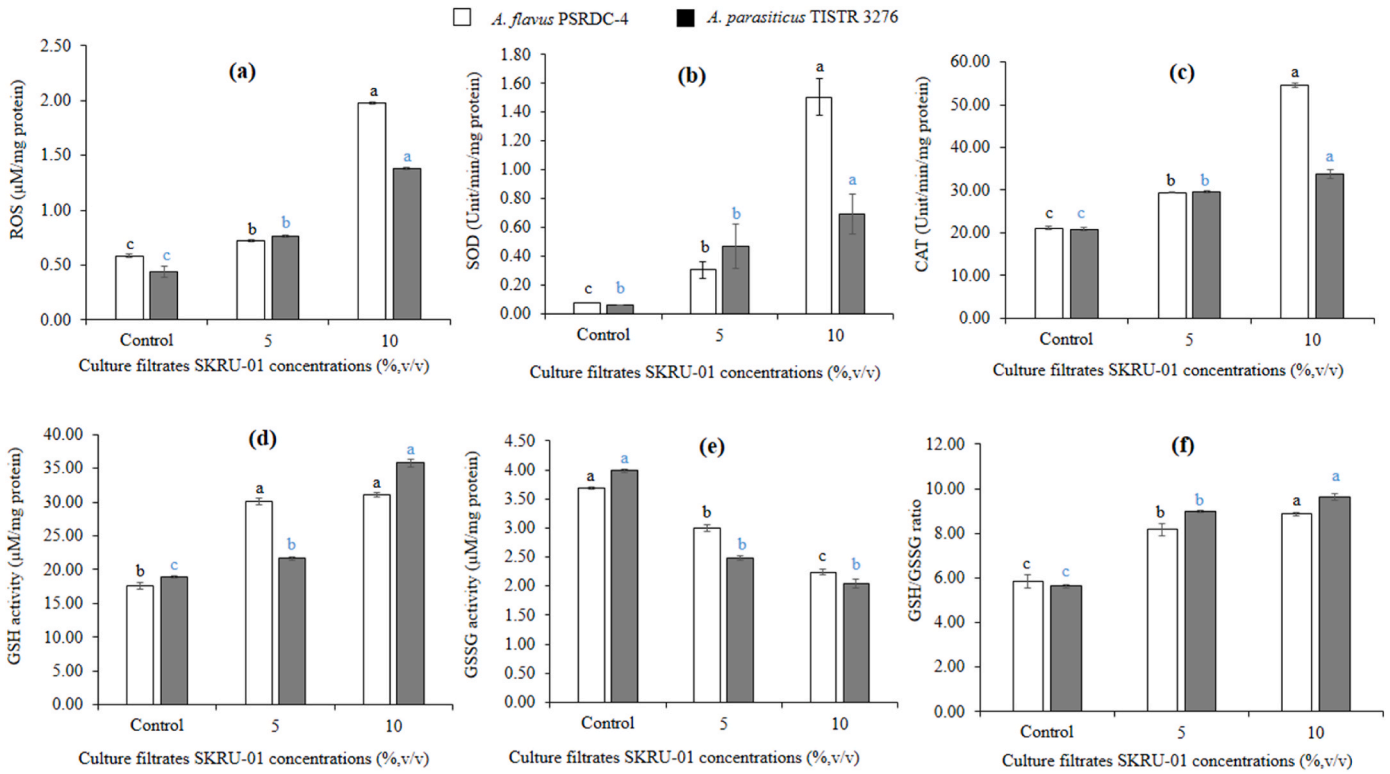

Fig. 6. The effect of culture filtrates SKRU-01 on the levels of reactive oxygen species (ROS) **(a)**, superoxide dismutase (SOD) **(b)**, catalase (CAT) **(c)**, reduced glutathione (GSH) activity **(d)**, oxidized glutathione (GSSG) activity **(e)**, and GSH/GSSG ratio **(f)** was assessed in *Aspergillus parasiticus* TISTR 3276 and *A. flavus* PSRDC-4 cells cultured in potato dextrose broth (PDB) at 30 °C for five days. The values presented are the means of three replicates (±SD). Values with the same letter are not significantly different (ANOVA and Tukey's HSD comparison tests, $p < 0.05$).

Table 4
The results of validation method.

Spiked level (μg/kg)	% Recovery[a]	Intra-day (%RSD_r)[a]	Inter-day (%RSD_R)[a]	LOD (μg/kg)	LOQ (μg/kg)
20	106.57	5.06	6.82	1.2	1.6
40	102.65	3.46	4.67		
100	106.50	1.05	1.47		

Note.
[a] The presented data represents the mean of six replicates, reflecting the accuracy and precision of the method.

and peanuts treated with culture filtrates SKRU-01 (1.34 log spores/g and 19.33 μg/kg), and commercial preservatives of sodium propionate (1.62 log spores/g and 84.67 μg/kg) and sodium benzoate (2.00 log spores/g and 92.57 μg/kg), as well as chemical fungicides metalaxyl® (1.55 log spores/g and 19.70 μg/kg) and prochloraz® (0 log spores/g and 0 μg/kg) (Table 6).

4. Discussion

Trichoderma asperelloides is known for controlling plant pathogens through the production of antifungal metabolites (Ruangwong et al., 2021; Sumida et al., 2018). This paper provides recent updates on antifungal metabolites produced by *T. asperelloides* SKRU-01 and their effectiveness in controlling food/feed spoilage caused by two aflatoxigenic *Aspergillus* species. The mechanism of action behind their antifungal properties was also investigated. The findings indicate that the antifungal metabolites from *T. asperelloides* SKRU-01 effectively mitigate the harmful effects of aflatoxigenic-producing strains. They disrupt key components of fungal cell membranes, such as ergosterol, inhibit the cellular methylglyoxal pathway, and impede both enzymatic and non-enzymatic defense systems *in vivo* (Das et al., 2020, 2021).

To combat plant pathogenic fungi, *Trichoderma* species employ various tactics, including generating inhibitory volatile and non-volatile compounds, antifungal enzymes through antibiosis, mycoparasitism,

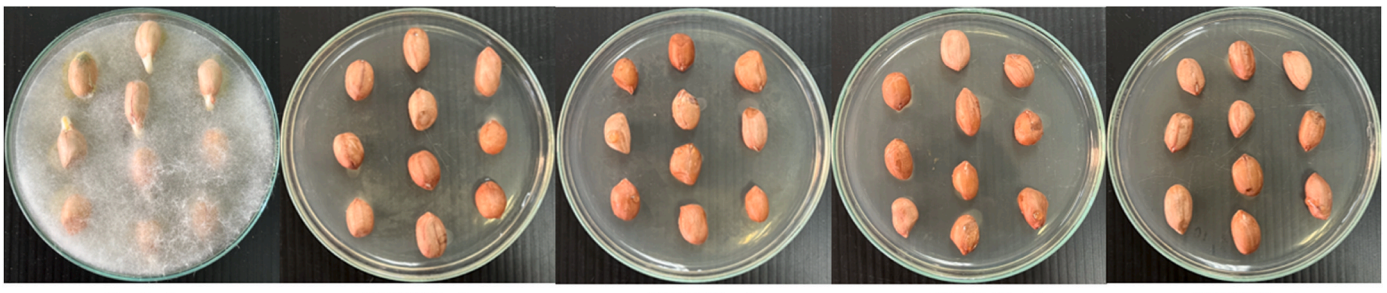

Fig. 7. The impact of heating (at 121 °C for varying times of 15, 20, 25, or 30 min) on the elimination of *Aspergillus* species remaining on surface-sterilized (3% sodium hypochlorite for 3 min) peanuts. Peanuts were incubated at 30 °C for five days.

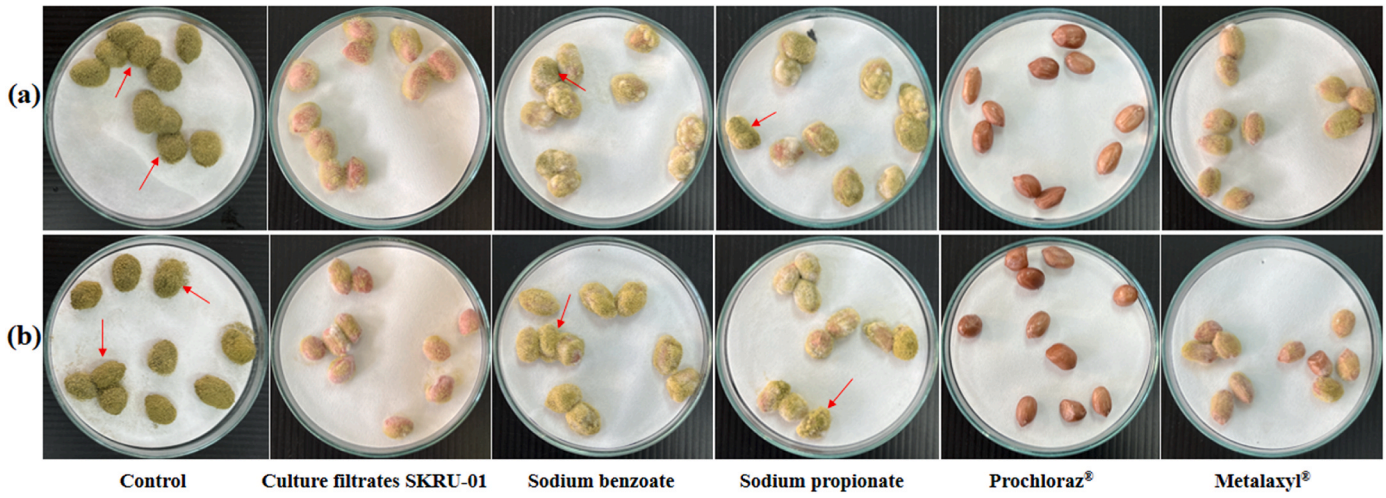

Fig. 8. Growth of *Aspergillus parasiticus* TISTR 3276 **(a)** and *A. flavus* PSRDC-4**(b)** in peanuts treated with sterile distilled water (control), culture filtrates SKRU-01, sodium benzoate (0.2% (w/v)) and sodium propionate (0.2% (w/v)) and prochloraz® (45% (w/v)) and metalaxyl® (25% (wp)) after storage at 30 °C for five days. The arrow indicates the dark green spores.

Table 5
Comparison the efficacy of culture filtrates SKRU-01 with commercial preservatives (sodium benzoate and sodium propionate) and chemical fungicides (prochloraz® and metalaxyl®) to inhibit spore and aflatoxin formation by *Aspergillus parasiticus* TISTR 3276 growing on peanuts after five days incubation at 30 °C.

Treatments	Viable spores (log spores/g)	Spore reduction (%)	AFB_1 production (μg/kg)
Control	$3.37^{a} \pm 0.29$	–	$133.80^{a} \pm 4.48$
Culture filtrates SKRU-01	$1.22^{e} \pm 0.12$	63.80	$18.23^{c} \pm 1.59$
Sodium benzoate	$1.64^{b} \pm 0.04$	51.34	$88.00^{b} \pm 2.65$
Sodium propionate	$1.57^{c} \pm 0.11$	53.41	$80.67^{b} \pm 5.69$
Prochloraz®	ND^{f}	100	ND^{d}
Metalaxyl®	$1.30^{d} \pm 0.18$	61.42	$18.30^{c} \pm 1.90$

Note: The presented data represents the mean of three replicates ± standard deviation (SD), and data with the same letter within each column are not significantly different based on ANOVA after Tukey's HSD test with a significance level of $p < 0.05$. ND = not detected.

Table 6
Comparison the efficacy of culture filtrates SKRU-01 with commercial preservatives (sodium benzoate and sodium propionate) and chemical fungicides (prochloraz® and metalaxyl®) to inhibit spore and aflatoxin formation by *Aspergillus flavus* PSRDC-4 growing on peanuts after five days incubation at 30 °C.

Treatments	Viable spores (log spores/g)	Spore reduction (%)	AFB_1 production (μg/kg)
Control	$3.44^{a} \pm 0.23$	–	$164.07^{a} \pm 5.80$
Culture filtrates SKRU-01	$1.34^{e} \pm 0.18$	61.05	$19.33^{c} \pm 3.06$
Sodium benzoate	$2.00^{b} \pm 0.15$	41.86	$92.57^{b} \pm 4.31$
Sodium propionate	$1.62^{c} \pm 0.27$	52.90	$84.67^{b} \pm 5.86$
Prochloraz®	ND^{f}	100	ND^{d}
Metalaxyl®	$1.55^{d} \pm 0.12$	54.94	$19.70^{c} \pm 0.90$

Note: The presented data represents the mean of three replicates ± standard deviation (SD), and data with the same letter within each column are not significantly different based on ANOVA after Tukey's HSD test with a significance level of $p < 0.05$. ND = not detected.

competition for nutrients and space due to rapid growth, and the activation of systemic resistance in plants (Zhang et al., 2018). In dual culture tests on Petri plates, *T. asperelloides* SKRU-01 effectively inhibited the mycelial growth of two mycotoxin-producing strains and completely covered their colonies. These results strongly suggest that *T. asperelloides* SKRU-01 has the potential to obstruct the growth of colonies from TISTR 3276 and PSRDC-4 strains. This is likely due to the production of diffusible antifungal agents by SKRU-01 and its competitive nutrient acquisition ability. Antibiosis is expected in the dual culture bioassay, as SKRU-01 likely releases extracellular antifungal agents into the growth medium. The antagonist's rapid growth rate gives it an advantage in competing for space, nutrients, and dominance over its host, as these factors are interconnected and crucial (Amira et al., 2017; Benitez et al., 2004). Furthermore, the ability of *T. asperelloides* SKRU-01 to overgrow the two mycotoxin-producing strains after 10 days of incubation in the dual culture suggests a contribution from mycoparasitism to its antagonistic behavior. *T. asperelloides*, like other *Trichoderma* species such as *T. harzianum*, *T. atroviride*, *T. asperellum*, *T. citrinoviride*, *T. inhamatum*, *T. parceramosus*, *T. polysporum*, and *T. viride*, employs multiple mechanisms to inhibit mycotoxins (Amira et al., 2017; Madbouly et al., 2023; Ren et al., 2022; Stracquadanio et al., 2020; Zhang et al., 2018).

Our experiments demonstrated that *T. asperelloides* SKRU-01 produces important metabolites that effectively combat two strains of aflatoxigenic fungi. These metabolites, released into the growth medium, successfully halt the growth of these fungi. In our *in vitro* experiment, we found that the culture filtrates of *T. asperelloides* SKRU-01 may contain fungitoxin metabolites that inhibit the growth of two mycotoxins. A key finding of our study is that these metabolites were more effective in inhibiting colony growth and spore germination of both mycotoxins compared to sodium benzoate, sodium propionate, and metalaxyl®, but less effective than prochloraz®. Our results align with those reported by Luo et al. (2014), indicating that potassium sorbate and sodium benzoate have weakened antifungal properties against *A. niger* and *Penicillium glaucum* food spoilage. The culture filtrates have the potential to control spore populations in various substrates and environments by strongly inhibiting spore germination or destroying fungal structures (Plascencia-Jatomea et al., 2003). The inhibitory mechanism of metabolites produced by *Trichoderma* species on mycelial growth is not yet fully understood (Ren et al., 2022). To investigate this, our study focused on the antifungal and antiaflatoxigenic actions of the culture filtrates SKRU-01 against both TISTR 3276 and PSRDC-4 strains. Furthermore, we analyzed the impact of culture filtrates SKRU-01 on the enzymatic and non-enzymatic defense systems of cells from the two fungi *in vivo* to enhance our understanding of the underlying mechanisms. These findings could have implications for the development of new antifungal and antiaflatoxigenic agents.

Membrane ergosterol is a target of many antibiotics, as it plays a crucial role in the growth, enzymatic functions, integrity, and actin fibril development that maintain asymmetry in fungal cells, which are necessary for fungal survival and virulence (Etebu & Arikekpar, 2016; Kishimoto et al., 2005). We observed a significant difference in the dry cell weight between the two mycotoxin growth tests using different concentrations of culture filtrates SKRU-01. Based on these results, we concluded that the culture filtrates SKRU-01 containing toxic secondary metabolites. Therefore, we investigated the inhibitory mechanism of the culture filtrates SKRU-01 on mycotoxin cells. *T. asperelloides*SKRU-01 culture filtrates contain metabolites that demonstrate ergosterol inhibition, which is dose-dependent. The percentage of inhibition of ergosterol biosynthesis ranged between 52.40% for the strain TISTR 3276 and 45.74% for PSRDC-4, indicating a direct interaction between the components of culture filtrates SKRU-01 and key enzymes of ergosterol biosynthesis. The culture filtrates SKRU-01 showed significant inhibition of ergosterol biosynthesis, leading to changes in membrane integrity, indicating that the primary target site for antifungal action could be the plasma membrane, which is consistent with the findings of Malmierca et al. (2015) who reported ergosterol reduction by *T. harzianum* through the targeted action of trichodiene, an enzyme essential for ergosterol biosynthesis.

Methylglyoxal (MG) is a natural byproduct of glucose breakdown in the body, which reacts with certain amino acids in cells, forming advanced glycation end products (AGEs) (Das et al., 2021). Elevated levels of MG can lead to changes in proteins and nucleic acids due to advanced glycation reactions on specific amino acids and nucleotide bases (Das et al., 2020; Thornalley, 2008). In our study, culture filtrates SKRU-01 demonstrated higher inhibitory activity on MG biosynthesis at 5% and 10% (v/v) doses compared to the control. The observed alterations in GSH and GSSG levels, along with decreased GSSG activity, suggest that culture filtrates SKRU-01 may impact the protein and nucleic acid profiles of TISTR 3276 and PSRDC-4 cells. During mycotoxin biosynthesis, MG serves as a cellular stress marker in the food system, leading to the upregulation of important regulatory genes like *afl*-R and *nor*-1, which promote AFB_1 production (Chen et al., 2004). The reduction in MG biosynthesis after treatment with culture filtrates SKRU-01 may be associated with a dose-dependent decrease in AFB_1 production through the downregulation of various aflatoxin regulatory genes, including *nor*-1 and *afl*-R. Furthermore, the inhibition of cellular MG by the bioactive components in the filtrates highlights their potential for developing aflatoxin-resistant food crops through the integration of green transgenic technology.

The accumulation of ROS is associated with apoptosis and can disrupt cellular oxygen metabolism (Hwang et al., 2012). Based on this, it was hypothesized that the accumulation of ROS might induce apoptosis in aflatoxigenic fungi cells treated with culture filtrates SKRU-01. The findings indicated that culture filtrates SKRU-01 at 5% and 10% (v/v) concentrations significantly elevated ROS levels compared to the control treatment. Elevated ROS levels, as observed after treatment with culture filtrates SKRU-01, can lead to various detrimental effects, including nucleic acid fragmentation, cellular tissue swelling, chromatin fibril condensation, depletion of ATP pools, inhibition of ATPase activity in the mitochondrial inner membrane, and degradation of the phosphatidylserine lipid layer, ultimately resulting in cellular death or apoptosis (Yoo et al., 2005). Additionally, we observed a significant increase in the activity of key cellular antioxidant enzymes, such as SOD and CAT, in the treated groups. This increase in antioxidant activity helped reduce oxidative stress, which was indirectly correlated with the dose-dependent reduction of AFB_1 content. Treatment with culture filtrates SKRU-01 led to an increase in cellular SOD levels, which may impact reactive hydroxyl radicals through dehydrogenase activity, leading to mitochondrial respiratory system dysfunction and inhibition of AFB_1 biosynthesis (Furukawa & Sakuda, 2019). Furthermore, treatment of mycotoxin cells with culture filtrates SKRU-01 resulted in noticeable changes in the levels of reduced GSH, GSSG, and the GSH/GSSG ratio. The interconversion of GSH and GSSG, mediated by cysteine residues thiol groups and their distribution among cellular organelles, can influence the cell's redox state and overall oxidative defense capacity (Sies & Jones, 2020). These findings are consistent with a previous study by Grintzalis et al. (2014), which suggested that reducing oxidative stress could inhibit AFB_1 biosynthesis by regulating GSH/GSSG activity.

Preventing the growth of mycotoxins in stored cereal grains is crucial for human and animal health, as well as the agricultural economy. To investigate the potential application of antifungal metabolites from *T. asperelloides* SKRU-01 in eliminating fungal spoilage in food and feed, peanuts were used as a model. Commercial preservatives (such as sodium propionate and sodium benzoate) and chemical fungicides (such as metalaxyl® and prochloraz®) were compared to the antifungal metabolites from *T. asperelloides* SKRU-01. Before the experiment, it was discovered that the peanut samples still contained mold spores, indicating potential mycotoxin growth. Surface-sterilized peanuts subjected to heat treatment effectively eliminated viable microbial contaminants, as no mold growth was observed after incubation. In contrast, peanuts that were only surface sterilized showed abundant mold growth, likely due to dormant spores resistant to sterilization.

Secondary metabolites produced by *Trichoderma* species have been extensively studied for their effectiveness against various microbial agents (Madbouly et al., 2023; Ren et al., 2022; Stracquadanio et al., 2020). In this study, the culture filtrates from *T. asperelloides* SKRU-01 were investigated for their ability to prevent mycotoxins in peanuts. The results showed that the culture filtrates SKRU-01 significantly prevented mold growth and aflatoxin production compared to commercial preservatives and chemical fungicides. They were more effective than sodium propionate, sodium benzoate, and metalaxyl®, but less effective than prochloraz®. These findings are consistent with those of other studies, which have shown that treatment of seeds with secondary metabolites produced by antagonistic microorganisms such as *Streptomyces* sp. (Boukaew et al., 2020b, 2023; Zucchi et al., 2008), *Lactobacillus plantarum* (Yang & Chang, 2010), *Rhodococcus erythropolis* (Reddy et al., 2009), *Pseudomonas fluorescens* (Reddy et al., 2009), *T. virens* (Reddy et al., 2009), and *Bacillus subtilis* (Reddy et al., 2009) can help prevent food spoilage caused by *Aspergillus* spp. In grains. The superior antifungal properties of culture filtrates SKRU-01 highlight their importance, surpassing the effectiveness of the tested commercial preservatives.

The study focused on analyzing the concentration of AFB_1, the most harmful toxin among aflatoxins, produced by aflatoxigenic strains such as *A. parasiticus* and *A. flavus*. AFB_1 poses a serious health risk to animals and humans (Dövényi-Nagy et al., 2020; Mutungi et al., 2008). The results demonstrated that the culture filtrates SKRU-01 effectively restricted AFB_1 production by the two strains of aflatoxigenic fungi on peanuts. All treatments, except those involving sodium benzoate and sodium propionate as commercial preservatives, exhibited *anti*-aflatoxigenic properties. AFB_1 levels remained below the public health concern threshold of 20 μg/kg. Different countries have varying maximum limits for total aflatoxin levels in food and feed. For instance, in the United States, the permissible levels of aflatoxins in food products vary, while in Thailand, the highest permissible level is 20 μg/kg (Panrapee et al., 2016). The European Union has established stringent standards, setting the maximum levels for total AFs and AFB_1 in peanuts at 4 μg/kg and 2 μg/kg, respectively (European Commission, 2010a). The findings highlight the significant antifungal and antiaflatoxigenic properties of *T. asperelloides* SKRU-01, effectively preventing food spoilage caused by mycotoxins both *in vitro* and in peanuts. This underscores the potential of *T. asperelloides* SKRU-01 as a promising natural alternative to chemical preservatives and fungicides.

In conclusion, the culture filtrates SKRU-01 exhibited significant inhibitory effects on the growth of *A. parasiticus* TISTR 3276 and *A. flavus* PSRDC-4, both *in vitro* and in peanuts. These effects may be attributed to the culture filtrates ability to target the plasma membrane

and inhibit cellular ergosterol biosynthesis. Moreover, the culture filtrates SKRU-01 reduced the level of methylglyoxal, which is known to induce aflatoxin production, revealing its mechanism of action in inhibiting aflatoxin production. The observed decrease in the activity of antioxidant defense enzymes (SOD and CAT) and non-enzymatic defense molecules (GSH and GSSG) shed light on the biochemical mechanisms involved. Overall, the culture filtrates SKRU-01 show promise as a natural and effective approach for managing fungal and aflatoxin contamination in food and feed products.

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Availability of data and materials

Not applicable.

CRediT authorship contribution statement

Sawai Boukaew: Conceptualization, Data curation, Supervision, Formal analysis, Funding acquisition, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Jaturong Kumla:** Investigation, Writing – original draft. **Poonsuk Prasertsan:** Funding acquisition, Writing – review & editing. **Benjamas Cheirsilp:** Writing – review & editing. **Wanida Petlamul:** Writing – review & editing. **Sirasit Srinuanpan:** Writing – review & editing.

Declaration of competing interest

The authors declare no competing interests regarding the publication of this study.

Data availability

The authors do not have permission to share data.

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