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Research Article

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Antifungal and antiaflatoxigenic mechanism activity of freeze-dried culture filtrate of *Streptomyces philanthi* RL-1-178 on the two aflatoxigenic fungi and identification of its active component

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Abstract

Aims: The study reports the antifungal and antiaflatoxigenic mechanism activity of freeze-dried culture filtrate of *Streptomyces philanthi* RL-1-178 (DCF RL-1-178) against two aflatoxigenic strains (*Aspergillus parasiticus* and *A. flavus*) and identification of its active component.

Methods and Results: Significant inhibition in ergosterol biosynthesis by the DCF RL-1-178 appeared on the plasma membrane. Moreover, the DCF RL-1-178 showed dose-dependent inhibition of methylglyoxal (MG) (an aflatoxin inducer) biosynthesis and exhibited a novel antiaflatoxigenic action mechanism. Significant impairments in enzymatic [superoxide dismutase (SOD) and catalase (CAT)] and nonenzymatic [oxidized and reduced glutathione (GSH) and ratio of oxidized and reduced glutathione (GSSG)] anti-oxidative defense molecules were observed in the two aflatoxigenic cells. The active component of the DCF RL-1-178 was identified as natamycin. The natamycin exhibited against *A. parasiticus* and *A. flavus* with the minimum inhibitory concentration (MIC) values of 0.5 and 1.0 μg ml^{-1}, respectively, while the minimum fungicidal concentration values were the same (4.0 μg ml^{-1}).

Conclusions: The DCF RL-1-178 containing natamycin exhibited the following effects: (1) inhibition of cellular ergosterol biosynthesis on plasma membrane, (2) reduction in MG (aflatoxin inducer) confirmed novel antiaflatoxigenic mechanism of action, and (3) caused remarkable debasement in antioxidant defense enzymes (SOD and CAT) and nonenzymatic defense molecules (GSH and GSSG) revealing biochemical mechanism of action.

Significance and Impact of the Study

This is the first report on antifungal and antiaflatoxigenic mechanism activity of *Streptomyces philanthi* RL-1-178 culture filtrate containing natamycin antibiotic. This new findings will broaden its applications as antifungal medicine and natural preservative in food.

Keywords: *Streptomyces philanthi*, ergosterol, methylglyoxal, natamycin, aflatoxin-producing fungi, antifungal mechanism

Introduction

Members of the genus *Aspergillus* are ubiquitous filamentous fungi found anywhere on earth. Among the *Aspergillus* species, *A. flavus* and *A. parasiticus* have been reported to cause harmful infections in humans, animals, and plants (Tian et al. 2012, Al-Ouqaili et al. 2018). In addition, they are frequently the main cause of food contamination and the key source in the production of aflatoxin B_1 (AFB_1) (Squire 1981). AFB_1 also exhibited hepatotoxic, teratogenic, and mutagenic properties, causing damage such as toxic hepatitis, hemorrhage, edema, immunosuppression, and hepatic carcinoma (Reddy et al. 2009). Methylglyoxal, a cytotoxic byproduct of respiratory pathways, has been reported to modify nucleic acids, proteins synthesis, and generation of advanced glycation products, and induce the secretion of aflatoxins in stored foods (Tian et al. 2012).

Despite the recent introduction of new antifungal drugs and synthetic preservatives, the application of synthetic antifungal agents has led to a notable increase in drug resistance (White et al. 1998). In this context, investigators are looking for effective antimicrobial agents to control disease and food contamination. The antimicrobial properties of natural products have been recognized and used as antifungal agents worldwide (Farzaneh et al. 2012, Sangare et al. 2014; Rao et al.2017; Afsharmanesh et al. 2018, Shu et al. 2018, Quinn et al. 2020, Campos-Avelar et al. 2021). Natural products, often called secondary metabolites, are low molecular weight organic molecules made of living organisms such as bacteria and fungi (Davies 2006). These metabolites, otherwise known as bioactive substances, are profoundly used as antibiotics and may be effective against *Aspergillus* species (Patil et al. 2017, Kim et al. 2021, Meena et al. 2021, Chang et al. 2022). In addition, the bioactive components may lead to mitochondrial system dysfunction and modulate the oxidative enzymes such as superoxide dismutase (SOD), catalase (CAT), and nonenzymatic defense molecules like glutathione causing inhibition in fungal growth and mycotoxin secretion (Sun et al. 2016).

Many species of actinomycetes, particularly those belonging to the genus *Streptomyces* are largely researched for their

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ability to produce numerous molecules of interest, namely, antibiotics (Igarashi et al. 2005, Quinn et al. 2020), antifungal compounds (Li et al. 2011, Boukaew et al. 2014, 2017, 2020a, b, Chen et al. 2016, Shakeel et al. 2016, 2021), and hydrolytic enzymes (glucanase, chitinase) (Prapagdee et al. 2008, Boukaew et al. 2016, VazJauri et al. 2016), which provide them with strong antagonistic capacities against fungal development. In a previous study, the antifungal metabolites produced in tuna condensate waste medium of *S. philanthi* RL-1-178 has shown a good efficacy to inhibit mycelial growth and AFB_1 production (Boukaew et al. 2020c) and aflatoxin B_1 degradation of *A. flavus* and *A. parasiticus*. However, to our best knowledge and according to a literature survey, there are no available reports on the underlying mechanism of antifungal action against the two aflatoxin-producing fungi. Thus, a further exploration of the subject is necessary.

In the present study, the plasma membranes in *A. flavus* and *A. parasiticus* were taken as potential targets for antifungal activity. To investigate the exact target of antifungal compounds of *S. philanthi* RL-1-178 in the plasma membrane, the effect of the antifungal compounds on a lesion in the plasma membrane, and the content of ergosterol, cellular MG, cellular reactive oxygen species (ROS), CAT, SOD, and glutathione (reduced and oxidized) were determined. Additionally, the production, purification, structure elucidation, and bioactivity of the isolated antimicrobial metabolite have also been discussed.

Materials and methods

Microorganisms and preparation of freeze-dried culture filtrate

The antagonistic strain of *S. philanthi* RL-1-178 was isolated from the rhizosphere of chili pepper (*Capsicum annuum* L.) in southern Thailand (Boukaew et al. 2011). The culture filtrate of *S. philanthi* RL-1-178 was prepared by inoculating 10% (v/v) aliquots of the seed culture into a 5 l bioreactor containing 4 l tuna condensate medium (pH was adjusted to 7.0 before autoclaving) and stirred (150 rpm) at 30°C for 10 days (Boukaew et al. 2020c). Then, the culture broth was centrifuged (8880 × *g* for 20 min) and filtered through a 0.45 μm Millipore membrane. After that, the culture filtrate was freeze-dried by vacuum freeze-dryer (at 0.7 mbar, 4°C for 30 h, CHRIST Delta 2–24 Isc., Germany) at the Office of Scientific Instrument and Testing, Prince of Songkla University (PSU) to obtain the freeze-dried culture filtrate from *S. philanthi* RL-1-178 (so called DCF RL-1-178).

Fungal strains and spore suspension

Aspergillus flavus PSRDC-4 was isolated from contaminated maize grain and provided by Phitsanulok Seed Research and Development Center, Department of Agriculture, Thailand. The strain was identified based on macroscopical and microscopical characteristics following the identification keys of Klich and Pitt (1988) and Klich (2002). *Aspergillus parasiticus* TISTR 3276 was obtained from the Culture Collection of the Thailand Institute of Scientific and Technological Research (TISTR), Phathumthani, Thailand. The two strains were previously found to produce highly aflatoxin production (Boukaew et al. 2020b, c). They were cultivated on potato dextrose agar (PDA) (39 g l^{-1}; Difco Laboratory) at 30°C. For preparation of spore suspension, spores from Petri plate of 10 -day-old culture were scrapped with a sterilized spatula and diluted in sterilized distilled water followed by filtration through sterilized cotton.

Evaluation of the antifungal activity of the DCF RL-1-178 against the two aflatoxin-producing fungi by agar well diffusion assay

The agar well diffusion method (Magnusson and Schnürer 2001) was used to detect antifungal activity. *Aspergillus flavus* PSRDC-4 and *A. parasiticus* TISTR 3276 were used as sensitive molds. Plates were prepared by adding each aflatoxigenic fungal strain at 100 μl of spore inoculum (10^6 spore ml^{-1}) to 10 ml of molten PDA (45°C), and 5.0 -mm-diameter wells were punched out from the agar plate. The DCF RL-1-178 was dissolved in dimethyl sulfoxide (DMSO) to achieve a concentration of 50 mg ml^{-1} and filtered through a 0.45 μm Millipore membrane before addition (100 μl) to diffuse into the agar during a 1 h preincubation period at room temperature, followed by a 48 h incubation period at 30°C. The DMSO was used as a control treatment. Diameters of inhibition zones were measured with a vernier caliper. All treatments were performed in three replicates.

Antifungal and antiaflatoxigenic action mechanism of the DCF RL-1-178 against the two aflatoxin-producing fungi

Effect of the DCF RL-1-178 on the ergosterol content in the plasma membrane of the two aflatoxin-producing fungi cells

One milliliter of the DCF RL-1-178 (at 0, 5.0, 10, 25, 50, and 100 μg ml^{-1}) was incorporated into 49 ml melted sterile potato dextrose (PDB) broth. The control sets did not contain the DCF RL-1-178 solution. A volume of 50 μl of spore inoculum (10^5 spore ml^{-1}) of each aflatoxigenic fungal strain was transferred into each flask and incubated on a rotary shaker (150 rpm) at 30°C for 5 days. The wet weight of autoclaved mycelial mat was prepared for ergosterol content assay as described by Das et al. (2020). Methodology of Tian et al. (2012) was utilized for the estimation of ergosterol content in the two aflatoxin-producing fungal cells. For each treatment, three replicates were conducted.

Effect of the DCF RL-1-178 on cellular MG of the two aflatoxin-producing fungi

A volume of 50 μl of spore inoculum (10^5 spore ml^{-1}) of each aflatoxigenic fungal strain was aseptically transferred into 49 ml of PDB medium and treated with 1 ml of different concentrations (at 0, 5.0, 10, 25, and 50 μg ml^{-1}) of the DCF RL-1-178. The control sets were devoid of the DCF RL-1-178. All the sets were incubated on a rotary shaker (150 rpm) at 30°C for 5 days. Collected biomass was crushed in 3 ml 0.5 M $HClO_4$ followed by centrifugation (8880 × *g* for 20 min) (Hitachi Koki Co., Ltd., Japan). Neutralization of the supernatant was done by drop-wise addition of K_2CO_3 solution followed by centrifugation. The collected supernatant was utilized for the estimation of cellular MG as described by Yadav et al. (2005). Evaluation of MG was done by sequential addition of diaminobenzene, $HClO_4$, and supernatant and eventually optical density measurement at 341 nm (Upadhyay et al. 2018). The content of MG was calculated through a standard calibration curve of MG.

Effect of the DCF RL-1-178 on *in vivo* enzymatic and nonenzymatic defense system of the two aflatoxin-producing fungal cells

Fungal biomass and enzyme extract preparation

Biomass of each aflatoxin-producing fungi treated with different concentrations (at 0, 5.0, 25, and 50 μg ml^{-1}) of the DCF RL-1-178 were crushed in 3 ml phosphate buffer saline (100 mM, pH 7.4) followed by centrifugation (8880 × *g* for 20 min) (Hitachi Koki Co., Ltd., Japan). The control set was devoid of the DCF RL-1-178. The supernatant obtained after extraction was used for biochemical tests.

Determination of cellular ROS, CAT, SOD, and glutathione (reduced and oxidized)

Intracellular ROS production was estimated by dichloro dihydro flurescein diacetate fluorescent dye assay (Keston and Brandt 1965). Fluorescence intensity was measured at excitation and emission wavelengths of 485 and 530 nm, respectively. Dichloroflurescein concentration was determined by using the standard curve. The protocol of Beers and Sizer (1952) was used for the determination of CAT activity. CAT activity was measured as unit activity/min/mg protein based on the molar extinction coefficient (43.6 M^{-1} cm^{-1}). SOD activity was determined by the quercetin auto-oxidation method (Kostyuk and Potapovich 1989). Reaction mixture was prepared from phosphate buffer (0.016 M, pH 10), EDTA (0.08 mM), *N–N–N–N*-tetramethylenediamine, and 100 μl quercetin solution (1.5 mg10 ml^{-1} DMSO). Inhibition of quercetin autooxidation was noted at 406 nm at 0 min and after 20 min. Enzyme activity was expressed as inhibition/mg protein. For the estimation of cellular glutathione, the seven-day-old biomass of each aflatoxin-producing fungi was homogenized in phosphate buffer (0.1 M, pH 8) + EDTA (0.005 M) + metaphosphoric acid (25%) solution mixture followed by centrifugation (8880 × *g* for 10 min at 4°C). For glutathione estimation, the fluorescence of the supernatant was measured at 420 nm with the activation at 350 nm (Hissin and Hilf 1976). For oxidized glutathione assay, the reaction mixture contained 500 μl supernatant and 200 μl 0.04 M NEM followed by mixing and incubation at ambient temperature for 20 min. Finally, *ortho*-phthalaldehyde and NaOH solution were added and incubated for 30 min. Fluorescence intensity was observed at 420 nm. The methodology of Lowry et al. (1951) was utilized for the estimation of total protein content.

Isolation and structural elucidation of the antifungal compound in the DCF RL-1-178

General experimental procedures

The general experimental procedures for the structural elucidation of the DCF RL-1-178 compounds were carried out according to the procedures previously described by Zhang et al. (2022).

Extraction and isolation of the DCF RL-1-178 active compounds

A 100 ml of methanol was added in a 250 ml Duran bottle containing 5.0 g of the DCF RL-1-178 and agitated on a rotary shaker (Orbital Shaker SK3-PO, Germany) for 1 h. The organic solvent was filtered through sterilized cotton. Evaporation of the solvent gave 1.0 g of an extract from 5.0 g of the DCF RL-1-178. The extract was fractionated by silica gel column chromatography eluted with a step gradient of $CHCl_3$–MeOH (1:0, 20:1, 10:1, 4:1, 2:1, 1:1, and 0:1 v/v). The last fraction eluted with $CHCl_3$ – MeOH (0:1 v/v) was concentrated to provide 178.1 mg of dark brown compound. Semipure compound was further purified by preparative high performance liquid chromatography (HPLC; Agilent HP1200, Model G1364C, Germany) (XTerra PrepRP18, 10 × 250 mm, 4 ml min^{-1}, UV detection at 254 nm) with 24% MeCN in 10 mM NH_4HCO_3 solution yielded DCF RL-1-178 compound (4.0 mg, t_R 20.1 min).

Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of the antifungal compound

The MIC and MFC values were determined by using broth microdilution assays performed in 96-well microtiter plate according to the National Committee for Clinical Laboratory Standards (2002) and Sugar and Liu (1995) with some modification. Twenty microliters of fungal spore suspension (culture and diluted as mentioned above to yield 10^6 spores per milliliter) was added to 180 μl PDB to achieve a final concentration of 10^5 spores ml^{-1} in each well. The pure isolated compound was added to create a twofold dilution series to achieve the final concentrations ranging from 0.0625 to 32 μg ml^{-1}. The plate was then incubated at 30°C, and fungal growth was determined visually at 48 h of incubation. The lowest concentration of antibiotic compound required to completely inhibit fungal growth after incubation for 48 h was reported as the MIC value defined by the National Committee for Clinical Laboratory Standards (2002). Fungal viability in the well, which showed no growth, was determined by transferring and spreading 100 μl of the culture broth on PDA and incubating it at 30°C for 48 h. The lowest concentration of antibiotic compound required to completely destroy the test fungi (no growth on the agar plate) after incubation at 30°C for 48 h was defined as the MFC. The antibiotic compound was evaluated for its antimicrobial activity in triplicate on two separate runs.

Statistical analysis

All the experiments were done in replicates ($n = 3$), and the data were subjected to Analysis of Variance (ANOVA), SPSS, version 21; IBM Corp, Armonk, NY). The mean values and their significant difference were compared using Tukey's HSD (Honestly Significant Difference) test at $P < 0.05$.

Results

Evaluation of the antifungal activity of the DCF RL-1-178 against the two aflatoxin-producing fungi by agar well diffusion assay

The strong antifungal activity of the DCF RL-1-178 against the aflatoxin producing *A. flavus* PSRDC-4 and *A. parasiticus* TISTR 3276 is shown in Fig. 1. Its antifungal levels measured by agar well diffusion assay against *A. flavus* PSRDC-4 and *A. parasiticus* TISTR 3276 were 13.3 ± 1.3 mm and 13.0 ± 1.7 mm, respectively.

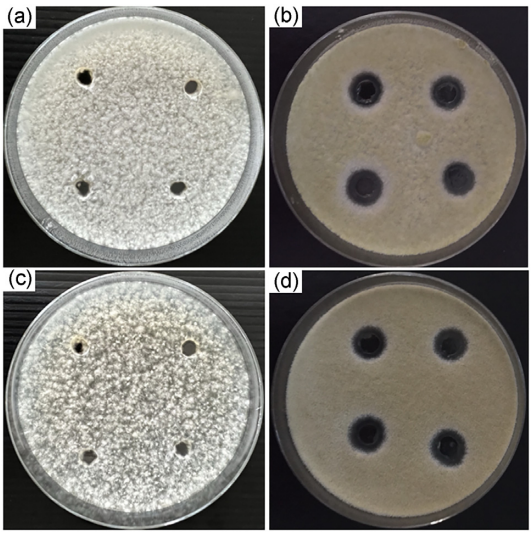

Figure 1. Inhibitory zone formed around the DCF RL-1-178 against the two aflatoxigenic fungi after 2 days of incubation at 30°C. (a) *Aspergillus flavus* PSRDC-4 + DMSO (control), (b) *A. flavus* PSRDC-4 + DCF RL-1-178, (c) *A. parasiticus* TISTR 3276 + DMSO (control), and (d) *A. parasiticus* TISTR 3276 + DCF RL-1-178.

Antifungal and antiaflatoxigenic action mechanism of the DCF RL-1-178 against the two aflatoxin-producing fungi

Effect of the DCF RL-1-178 on the ergosterol content in the plasma membrane of the two aflatoxin-producing fungi

The efficacies of the DCF RL-1-178 on the ergosterol content in the plasma membrane of *A. flavus* PSRDC-4 and *A. parasiticus* TISTR 3276 are shown in Table 1. Ergosterol content (at 280 nm) in the plasma membrane of the two aflatoxin-producing fungi was significantly inhibited by the different concentrations of the DCF RL-1-178. A dose-dependent decrease in ergosterol production was observed when isolates were grown in the presence of the DCF RL-1-178. The percentage inhibition of ergosterol production of *A. parasiticus* TISTR 3276 and *A. flavus* PSRDC-4 was significantly increased from 19.58% to 100%, and 10.61% to 100%, respectively, as the DCF RL-1-178 concentration increased from 5 to 100 μg ml^{-1}. The minimum concentration of the DCF RL-1-178 that showed > 70% reduction of the ergosterol content in the plasma membrane of the two aflatoxin-producing fungi was at 50 μg ml^{-1}. In fact, at the concentration of 100 mg ml^{-1}, the DCF RL-1-178 exhibited a complete (100%) reduction of the ergosterol content in the plasma membrane of the two aflatoxin-producing fungi.

Effect of the DCF RL-1-178 on cellular MG of the two aflatoxin-producing fungi

The effect of different concentrations of the DCF RL-1-178 on the cellular MG level of the two aflatoxin-producing fungi was investigated. The increase of the DCF RL-1-178 caused a significant reduction of MG level (Fig. 2). In the control set, the MG levels of *A. flavus* PSRDC-4 and *A. parasiticus* TISTR 3276 were found to be 1746 μM g^{-1} FW and 1578 μM g^{-1} FW, respectively, in contrast to the DCF RL-1-178 treatment at 100 μg ml^{-1}, the MG levels were 240 μM g^{-1} FW and 327 μM g^{-1} FW, respectively. These resulted in the 86.24% and 79.22% MG reduction of *A. flavus* PSRDC-4 and *A. parasiticus* TISTR 3276, respectively.

Effect of the DCF RL-1-178 on *in vivo* enzymatic and nonenzymatic defense system of the two aflatoxin-producing fungal cells

The impact of the DCF RL-1-178 at various concentrations (0, 5, 25, and 50 mg ml^{-1}) on different biochemical oxidative defense markers viz. ROS, SOD, CAT, and cellular glutathione of *A. flavus* PSRDC-4 and *A. parasiticus* TISTR 3276 cells was studied (Fig. 3). After exposed to different concentrations of the DCF RL-1-178, the production of intracellular ROS, SOD, CAT, GSH, and GSH/GSSG increased obviously in comparison to the control. ROS level at the control set of *A. flavus* PSRDC-4 and *A. parasiticus* TISTR 3276 was found as 0.09 and 0.07 unit min^{-1} mg^{-1} protein, respectively, while at 50 μg ml^{-1} dose of the DCF RL-1-178, the ROS level considerable increased up to 0.24 and 0.21 unit min^{-1} mg^{-1} protein, respectively (Fig. 3a).

The DCF RL-1-178 exhibited significant enhancement of cellular antioxidant enzymes such as SOD and CAT. In control, cellular levels of SOD and CAT of *A. flavus* PSRDC-4 and *A. parasiticus* TISTR 3276 were 0.93 and 0.81 unit/min/mg protein (SOD) and 23.54 and 23.77 unit/min/mg protein (CAT) respectively, while at 50 μg ml^{-1} concentration of the DCF RL-1-178, the levels were found to be 2.08 and 1.79 unit/min/mg protein (SOD) and 41.23 and 51.78 unit/min/mg protein (CAT), respectively (Fig. 3b and c).

Furthermore, significant alteration in reduced glutathione (GSH), oxidized glutathione (GSSG), and GSH/GSSG levels in *A. flavus* PSRDC-4 and *A. parasiticus* TISTR 3276 cells were observed after treatment with the DCF RL-1-178. The DCF RL-1-178 sets exhibited significant enhancement in GSH (Fig. 3d), and GSH/GSSG (Fig. 3f) levels as compared to control sets; GSH (from 2.0 to 5.07 μM/mg protein of *A. flavus* PSRDC-4 and from 2.03 to 4.81 μM/mg protein of *A. parasiticus* TISTR 3276, for control and 50 μg ml^{-1} treatment, respectively) and GSH/GSSG (from 0.48 to 1.19 μM mg^{-1} protein of *A. flavus* PSRDC-4, and from 0.47 to 0.85 μM/mg protein of *A. parasiticus* TISTR 3276, for control and 50 μg ml^{-1} treatment, respectively). Dose-dependent increment of GSH and GSH/GSSG levels suggests a significant rise of cope up mechanism to alleviate oxidative stress (Das et al. 2020). Unlike the other enzyme activities mentioned above, the cellular GSSG activity decreased with the increase of the DCF RL-1-178 concentration (Fig. 3e).

Isolation and structural elucidation of antifungal compound in the DCF RL-1-178

The DCF RL-1-178 component that showed strong antifungal activity against *A. flavus* PSRDC-4 and *A. parasiticus* TISTR 3276 was extracted with methanol. The extract was subjected to consecutive fractionation using silica gel chromatography. Among the seven fractions collected, the seventh fraction showed antifungal activity against *A. flavus* PSRDC-4 (12.5 mm) and *A. parasiticus* TISTR 3276 (11.0 mm). Hence, the seventh fraction compound was selected for structure elucidation as follows: firstly, the crude compound was purified by preparative high-performance liquid chromatography (HPLC; Agilent HP1200, Model G1364C, Germany), and finally, the pure compound was elucidated and characterized on the basis of UV, one- and two-dimensional NMR data,

Table 1. Effect of the DCF RL-1-178 concentration (0–100 μg ml^{-1}) on % inhibition of ergosterol production of *A. flavus* PSRDC-4 and *A. parasiticus* TISTR 3276 in potato dextrose broth and incubated at 30°C for 5 days.

Concentrations of the DCF RL-1-178 (μg ml^{-1})	Mean of percentage inhibition of ergosterol production ± SD	
	A. flavus PSRDC-4	*A. parasiticus* TISTR 3276
0	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}
5	10.61 ± 1.94^{d}	19.58 ± 0.69^{d}
25	62.08 ± 2.85^{c}	41.97 ± 1.22^{c}
50	72.31 ± 3.66^{b}	72.08 ± 0.72^{b}
100	100 ± 0.00^{a}	100 ± 0.00^{a}

The values are means of three replicates and their standard deviation (SD). Values followed by the same letter within each column are not significantly different (ANOVA and Tukey's multiple comparison tests, $P < 0.05$).

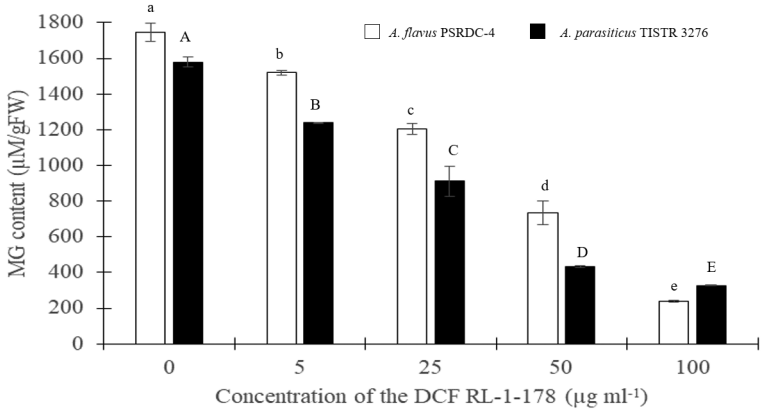

Figure 2. Effect of the DCF RL-1-178 on cellular MG of *A. flavus* PSRDC-4 and *A. parasiticus* TISTR 3276 cells in potato dextrose broth (PDB) and incubated at 30°C for 5 days. Values are means of three replications (± SD). Different letters above the bars indicate significant differences (ANOVA and Tukey's multiple comparison tests, $P < 0.05$) among different concentration of the DCF RL-1-178.

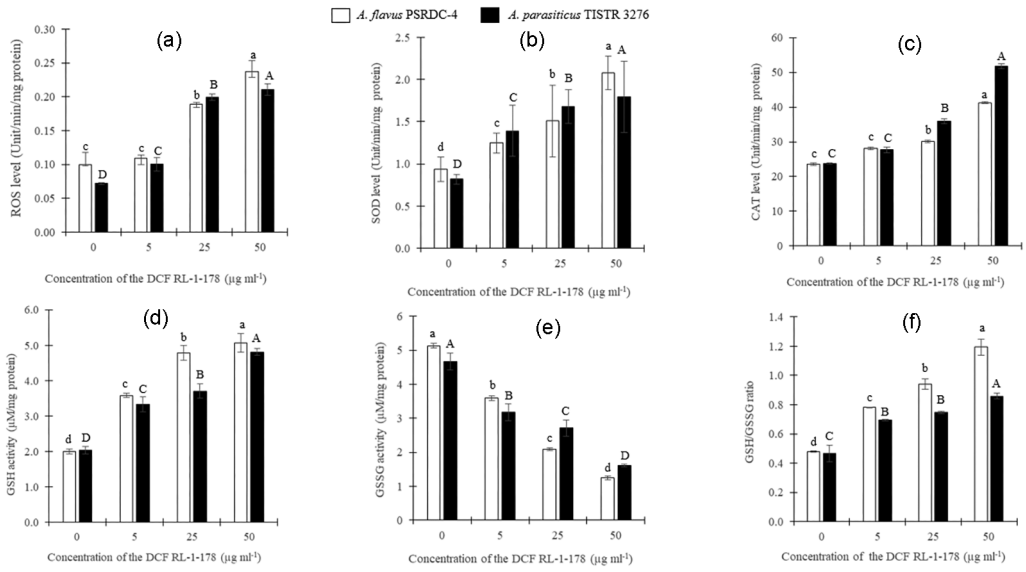

Figure 3. Effect of the DCF RL-1-178 on cellular ROS level (a), SOD level (b), CAT level (c), reduced glutathione (GSH) activity (d), oxidized glutathione (GSSG) activity (e), and GSH/GSSG ratio (f) of *A. flavus* PSRDC-4 and *A. parasiticus* TISTR 3276 cells in potato dextrose broth (PDB) and incubated at 30°C for 5 days. Values are means of three replications (± SD). Different letters above the bars indicate significant differences (ANOVA and Tukey's multiple comparison tests, $P < 0.05$) among different concentration of the DCF RL-1-178.

coupled with MS analytical data to identify the DCF RL-1-178 compound.

The pure DCF RL-1-178 compound was obtained as a yellow amorphous solid. The molecular formula was determined to be $C_{33}H_{47}O_{13}$ based on the HR-ESITOFMS analysis ([M + Na]+ m/z 688.2933, Δ-0.7 mmu) (Fig. 4). In addition, the UV (MeOH) maxima absorptions was λ_{max} (log ε) 288 (3.97), 300 (4.11), and 315 (4.04) nm was indicative of the tetraenechromophore. Combined analysis of ^{1}H and ^{13}C NMR and HSQC spectral data revealed the presence of

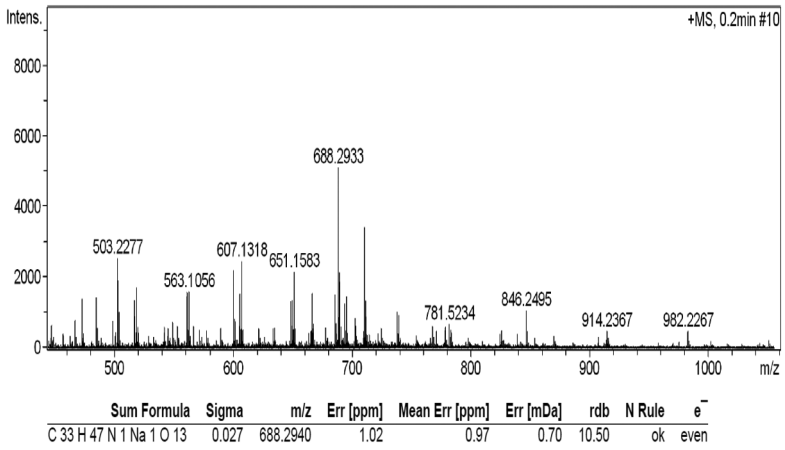

Figure 4. HR-ESITOFMS spectrum of purified the DCF RL-1-178 compound.

eight sp^2 methine groups, thirty sp^3 methine protons, four sp^3 methylene groups, and two methyl groups shown in Table 2. COSY analysis clarified three fragments C2–C8, C10–C26, and C5′–C6′. Linkage these fragments were deduced from the HMBC correlations shown in Fig. 5. Finally, the structure of compound DCF RL-1-178 was confirmed to be the same as natamycin (pimaricin) on the basis of almost the same ^1H HMR spectral data shown in Table 2.

MIC and MFC of antibiotic natamycin

MIC and MFC values of natamycin produced by *S. philanthi* RL-1-178 against the two aflatoxin-producing fungi were determined. The MIC values were exhibited against *A. flavus* PSRDC-4 (1.0 μg ml^{-1}) and *A. parasiticus* TISTR 3276 (0.5 μg ml^{-1}). The MFC of the natamycin against the two aflatoxin-producing fungi exhibited the same values (4.0 μg ml^{-1}) (Table S1; Supplementary data).

Discussion

The efficacy of antifungal metabolites produced in the tuna condensate waste medium of *S. philanthi* RL-1-178 against mycelial growth and aflatoxin B_1 (AFB_1) production of *A. flavus* PSRDC-4 has been reported (Boukaew et al. 2020c). Nevertheless, to date, little is known about the mechanism of antifungal action against the two aflatoxin-producing fungi.

Generally, the basic mechanisms of antibiotic action are six major modes of action as follows: (1) interference with cell wall synthesis, (2) inhibition of protein synthesis, (3) interference with nucleic acid synthesis, (4) inhibition of a metabolic pathway, (5) inhibition of membrane function, and (6) inhibition of ATP synthase (Etebu and Arikekpar 2016). In this study, the impact of freeze-dried culture filtrate of *S. philanthi* RL-1-178 (DCF RL-1-178) on membrane ergosterol biosynthesis in *A. flavus* PSRDC-4 and *A. parasiticus* TISTR 3276 cells was determined. Membrane ergosterol is a unique sterol maintaining growth, enzymatic functions, and major sterol component in fungal cells (Kishimoto et al. 2005, Tao et al. 2014, Das et al. 2020; Xu et al. 2019). DCF RL-1-178 exhibited dose-dependent ergosterol inhibition in all the treatment sets suggesting a direct interaction of the DCF RL-1-178 components on key enzymes of ergosterol biosynthesis. A similar report on ergosterol reduction by antifungal metabolite from *S. corchorusii* stain AUH-1 in *Fusarium oxysporum* f. sp. *niveum* was previously described by Yang et al. (2019). Our result corroborated with the recent findings of Xu et al. 2019, who suggested significant the ergosterol content in *Magnaporthe oryzae* cells declined dose-dependently in the presence of culture filtrate produced by *S. hygroscopicus* OsiSh-2. Moreover, silver nanoparticles synthesized by *Streptomyces* sp. inhibit the growth of *F. verticillioides* could be due to the inhibition of ergosterol biosynthesis and membrane damage (Marathe et al. 2021). This result indicated leakage of cellular electrolytes from the fungi due to the loss of cell membrane integrity.

MG is a short-chain alpha-keto-aldehyde intermediate of glycolytic pathway, synthesized in cells as a byproduct of lipid peroxidation, oxidative degradation of carbohydrates as well as through enzymatic action of MG synthetase (Das et al. 2020). A higher level of cellular MG accumulation effectively alters the protein and nucleic acid profile of fungal cells (Thornalley 2008). DCF RL-1-178 caused a significant reduction of MG level with respect to different doses used, suggesting that the DCF RL-1-178 may affect in alters the protein and nucleic acid profile of the two aflatoxin-producing fungi cells. In aflatoxin-producing fungi, MG acts as a cellular stress marker in the food system by promoting the AFB_1 secretion through the upregulation of key regulatory genes such as afl R and nor 1 (Chen et al. 2004). Dose dependent reduction of MG by the DCF RL-1-178 may be correlated with significant decrement in MG level (Upadhyay et al. 2018, Das et al. 2019) by altering the afl R and nor 1 gene expression.

Different biochemical oxidative defense markers viz. ROS, SOD, CAT, and cellular glutathione were assessed to study the changes in the biochemical profile of *A. parasiticus* TISTR 3276 and *A. flavus* PSRDC-4 cells to reveal the antifungal action of the DCF RL-1-178. ROS accumulation is considered an indicator of apoptosis, and the oxygen metabolism of cells is easily disturbed by ROS (Hwang et al. 2012). Thus, we hypothesized that ROS accumulation might be involved in the induction of cell apoptosis in the DCF RL-1-178-treated *A. parasiticus* TISTR 3276 and *A. flavus* PSRDC-4. The DCF RL-1-178 caused an increased level of ROS with respect to the different doses used. The DCF RL-1-178 at a concentration > 25 μg ml^{-1} had significantly the ability to increase ROS compared to the control and also exhibited significant enhancement of cellular antioxidant enzymes such as SOD and CAT. The increased SOD and CAT activity in treated

Table 2. Comparison of ^{1}H chemical shifts of known natamycin and the DCF RL-1-178 compound.

No.	Natamycin	DCF RL-1-178[a]	Proton?	Natamycin	DCF RL-1-178[a]
2	6.02	6.06	17	6.11	6.14
3	6.38	6.42	18	6.43	6.47
4	3.12	3.15	19	6.18	6.26
5	2.79	2.84	20	6.15	6.26
6a	2.00	2.04	21	6.15	6.26
6b	1.16	1.20	22	6.10	6.14
7	4.28	4.34	23	5.56	5.60
8a	1.55	1.60	24a	2.20	2.25
8b	1.66	1.69	24b	2.36	2.39
10a	1.23	1.27	25	4.68	4.73
10b	1.95	1.99	$26CH_3$	1.28	1.32
11	4.25	4.28	1′	4.53	4.57
12	1.95	2.00	2′	3.98	4.03
13	4.34	4.39	3′	3.16	3.19
14a	1.57	1.62	4′	3.35	3.38
14b	2.28	2.31	5′	3.28	3.32
15	4.38	4.42	$6'CH_3$	1.24	1.29
16	5.95	5.99			

[a]Recorded at 500 MHz.

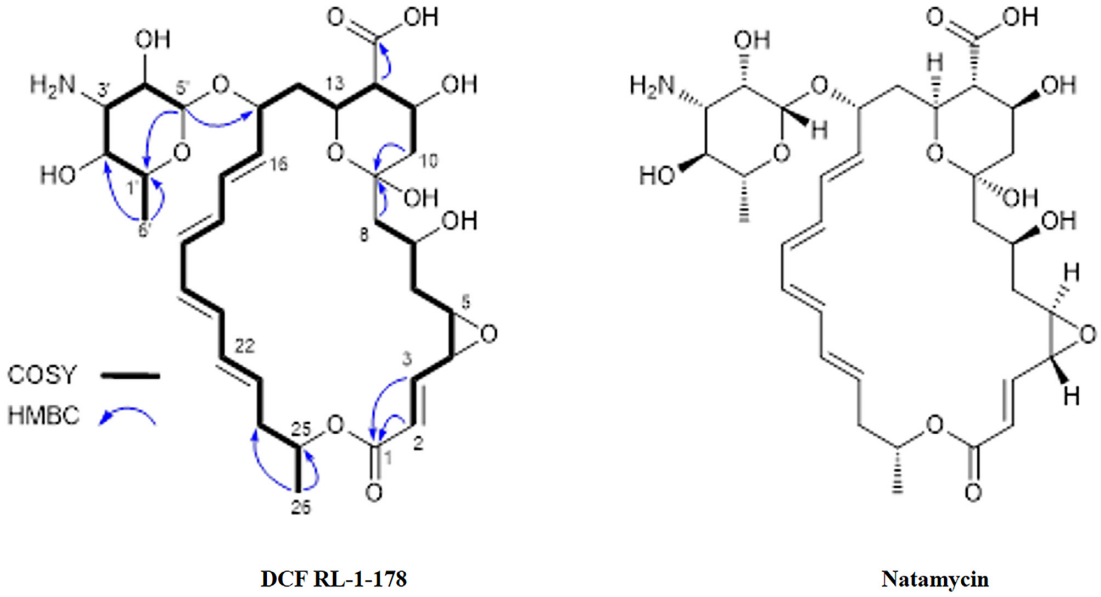

Figure 5. COSY and key HMBC correlations of the compound DCF RL-1-178 and the structure of natamycin.

sets is responsible for the mitigation of oxidative stress and is indirectly correlated with dose-dependent reduction of AFB_1 content. Elevation in the level of cellular SOD by the DCF RL-1-178 treatment may also cause modulation in reactive hydroxyl radicals through dehydrogenase activity which ultimately led to dysfunction in the mitochondrial respiratory system with subsequent inhibition of AFB_1 biosynthesis (Furukawa and Sakuda 2019). Furthermore, significant alteration in reduced glutathione (GSH), oxidized glutathione (GSSG), and GSH/GSSG level in the two aflatoxin-producing fungi cells was observed after treatment with the DCF RL-1-178. GSH and GSSG conversion depends on the thiol groups of cystein residues and the amount distributed in different cellular organelles. Our results are in accordance with the previous investigation of Grintzalis et al. (2014) suggesting modulation in GSH/GSSG activity with low oxidative stress that may reduce the AFB_1 biosynthesis.

The antifungal metabolites of the DCF RL-1-178 have an effect on *A. flavus* PSRDC-4 and *A. parasiticus* TISTR 3276 cells. The bioactive compounds of the DCF RL-1-178 was identified as natamycin with the molecular formula $C_{33}H_{47}O_{13}$. In fact, natamycin was reported to be responsible for the antifungal effect of crude preparation isolated from a strain of *S. natalensis* (Struyk and Waisvisz 1955). It was classified as a member of the polyene macrolide group (Dalhoff and Levy 2015). Nevertheless, this is the first report on natamycin produced by *S. philanthi* RL-1-178. Natamycin showing *in vitro* activity against *Aspergillus* spp. has been reported by many scientists (Patil et al. 2017, Kim et al. 2021, Meena et al. 2021, Chang et al. 2022). In this study, natamycin produced by *S. philanthi* RL-1-178 showed strong antifungal activity against aflatoxin-producing *A. parasiticus* TISTR 3276 and *A. flavus* PSRDC-4 with MIC values ranging from 0.5 to 1.0 μg ml^{-1}. This suggested that the DCF

RL-1-178 contained a natamycin antibiotic. Natamycin inhibits the growth of fungi by specifically binding to ergosterol present in fungal cell membranes (Van Leeuwen et al. 2009). Moreover, natamycin inhibits amino acid and glucose transport proteins leading to a loss of nutrient transport across the plasma membrane (Chervitz et al. 1999, Ozcan and Johnston 1999, Regenberg et al. 1999).

In conclusion, the strong inhibitory effect of the DCF RL-1-178 on cellular ergosterol biosynthesis suggests plasma membrane as the target site of action. Reduction in MG (aflatoxin inducer) by the DCF RL-1-178 treatment depicted the aflatoxin inhibitory mechanism of action. Besides, the DCF RL-1-178 caused remarkable debasement in antioxidant defense enzymes (SOD and CAT) and nonenzymatic defense molecules (GSH and GSSG) revealing biochemical mechanism of action. The DCF RL-1-178, possessed both antifungal and antiaflatoxigenic mechanism activity against the aflatoxigenic *A. parasiticus* and *A. flavus*.

Supplementary data

Supplementary data are available at *JAMBIO* online.

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Author contributions

Sawai Boukaew (Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing), Zhiwei Zhang (Writing – original draft), Poonsuk Prasertsan (Funding acquisition, Writing – review & editing) , and Yasuhiro Igarashi (Writing – review & editing)

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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