

### ANTIFUNGAL ACTIVITY OF REUTERIN AGAINST AFLATOXIGENIC *ASPERGILLUS FLAVUS*

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#### ABSTRACT

Reuterin is a secondary metabolite of the lactic acid bacteria *Limosilactobacillus reuteri* having broad antimicrobial activity. This study aimed to determine the antifungal activity of reuterin against aflatoxigenic *Aspergillus flavus* (*A. flavus*). The aflatoxigenic *A. flavus* BIO 33212 isolated from Indonesian nutmeg was used in this study. The minimum inhibitory concentration (MIC) of reuterin based on radial growth and colony-forming assays in solid and liquid fat-enriched media against *A. flavus* was determined. The effect of reuterin on the morphological structure of *A. flavus* was investigated by scanning electron microscopy (SEM). The aflatoxin B1 (AFB1) produced by *A. flavus* was evaluated by enzyme-linked immunosorbent assay. Reuterin effectively prolonged the lag phase and decreased the growth rate of *A. flavus*. At 2 and 4 mM, reuterin demonstrated radial growth inhibition by 5.59% and 35.40%, respectively, while the reductions in colony numbers were 18.36% and 69.51%, respectively. At higher concentrations (6 and 8 mM), the fungal growth was inhibited and reduced completely (100%). SEM revealed the disruption of hyphae and conidiophore development and conidial damage. However, a contradictory phenomenon was found when reuterin at < 6 mM triggered a higher production of AFB1.

**Keywords:** aflatoxigenic, aflatoxin B1, antifungal, *Aspergillus flavus*, reuterin

#### INTRODUCTION

*Aspergillus flavus* is a saprophytic fungus distributed widely in soils (Amaike and Keller, 2011). Its contamination in agricultural products occurs globally and becomes a severe problem for developing countries due to its impact on health and economic losses (Kumar et al., 2021). Aflatoxigenic *A. flavus* can produce toxic compounds called aflatoxin, where aflatoxin B1 (AFB1) is the most carcinogenic (Amaike and Keller, 2011). Aflatoxin B1 is reported to be the leading cause of hepatocellular carcinoma (HCC) in the African and Southeast Asian regions (Ferreira et al., 2019; Swetha and Girish, 2022). The *A. flavus* spores could also cause aspergillosis, especially in populations with poor immune systems (Hedayati et al., 2007). Salehi et al. (2021) reported the increasing cases of aspergillosis complications in acute COVID-19 patients, called COVID-19-associated pulmonary aspergillosis (CAPA).

Various measures have been developed to control the growth of aflatoxigenic *A. flavus* and aflatoxin contamination in agricultural products. One of which is the use of natural antifungal compounds, which is currently studied widely due to public concern about the adverse impacts of fungicide chemicals on human health and the environment while their uses are still widespread (Kim et al., 2017; Rani et al., 2021).

Several studies have reported antifungal compounds synthesized by lactic acid bacteria (LAB), including reuterin (Crowley et al., 2013). It is derived from the anaerobic glycerol metabolism by a specific strain of *Limosilactobacillus reuteri*, which has a gene cluster of *pdu-cbi-hem-cob* (Morita et al., 2008; Spinler et al., 2014). Reuterin is a 3-hydroxypropionaldehyde (3-HPA) compound containing functional groups of aldehyde and alcohol that can trigger the formation of reactive oxygen species (ROS) in prokaryotic and eukaryotic organisms (Bell et al., 2020). Reuterin has gained much attention due to its broad antimicrobial activity. Its antifungal activity was first time reported by Chung et al. (1989) and subsequently by Vimont et al. (2019), Ju et al. (2021), and Pilote-Fortin et al. (2021). Nevertheless, its activity in inhibiting aflatoxin-producing fungal growth has yet to be reported. For this reason, the aim of this study was to determine the antifungal activity of reuterin against aflatoxigenic *A. flavus*. The *A. flavus* were grown in the fat-enriched media, a superior substrate for fungal growth and aflatoxin production (D'Mello et al., 2014).

One of the advantages of using LAB metabolites, specifically reuterin, is that it comes from bacteria generally recognized as safe (GRAS); therefore, it would be readily accepted by the public and authorities. Moreover, it can be produced biotechnologically on a large scale which promises cost efficiency (Stevens et al., 2011). These all suggested that reuterin is promising and needs to be further explored.

#### MATERIAL AND METHODS

##### Fungal isolate and conidial suspension

The isolate of *A. flavus* BIO 33212 used in this study was obtained from Phytopathology Laboratory, SEAMEO BIOTROP, Indonesia. It was isolated from Indonesian nutmeg and confirmed as aflatoxigenic *A. flavus*, producing only AFB1 (Anidah et al., 2019). The fungal isolate was cultured on a PDA medium (OXOID, UK) and incubated for five days at 30°C. The conidia (spores) were harvested by scrapping off it using a sterile inoculation loop after adding 10 mL of sterile distilled water containing Tween 80 (0.05%). Conidial concentration was counted using a hemacytometer and diluted to obtain a final concentration of  $1.0 \times 10^6$  CFU/mL.

##### Preparation of fat-enriched media

Fat-enriched media was used in this study to promote the growth of *A. flavus* and the production of AFB1. The media were prepared in two types: coconut agar medium (CAM) as solid media and coconut broth (CB) as liquid media to measure the different forms of fungal growth. Both media were prepared using a method described by Anidah et al. (2019). Preparation of CAM was performed by dissolving pure coconut milk in distilled water as much as 10% of the total volume, adjusting the pH of the solution to 7.0 using 2 N NaOH, adding 20 g/L of Bacto agar (DIFCO, USA), then heating the solution until the Bacto agar dissolved. The preparation of CB was like CAM but without the addition of Bacto agar. The media were then sterilized in an autoclave at 121°C for 15 min.

## Preparation of reuterin solution

In this study, pure commercial reuterin was used for the treatment. It was purchased from BLD Pharmatech Ltd., China, with a purity level of 98%. The reuterin solution was prepared by dissolving 50 mg of reuterin (MW = 74.08 g/mol) in double distilled water in a 10 mL volumetric flask. The reuterin solution with a concentration of 66.14 mM was kept at -18°C prior to use.

## Radial growth assay

The radial growth assay was performed in the solid media (CAM) by culturing the fungal conidia on media with and without reuterin to evaluate the antifungal activity of reuterin. Reuterin at different concentrations of 2, 4, 6, and 8 mM was applied as the treatment in this study. The radial growth assay was performed using a method described by Tian et al. (2011) with slight modifications on the inoculum concentration and incubation condition. Ten milliliters of CAM with or without reuterin was poured into a petri dish (85 × 10 mm) and allowed to solidify. Five microliters of conidial suspension were point-inoculated at the center of the medium and incubated for ten days at 30°C. The medium without reuterin was used for negative control (NC), while the medium containing 5 mg/mL ketoconazole (a well-known antifungal drug) was used for the positive control (PC). Each colony diameter (mm) was measured using a caliper every 24 h for ten days. All tests were performed in triplicate.

In this study, the percentage of radial growth inhibition was calculated using the colony diameter of day seven since the colony of NC nearly attained the petri dish diameter after seven days of incubation. The percentage of radial growth inhibition was calculated using the following formula:

$$\text{Radial growth inhibition (\%)} = \left[ \frac{dc - dt}{dc} \right] \times 100$$

where *dc* is the colony diameter of NC, and *dt* is the colony diameter of treatment. The lowest concentration that completely (100%) inhibited the growth of fungi was considered as MIC (Tian et al., 2011). In order to compare the growth rate between NC and treatment, a linear regression equation was determined by plotting the colony diameter against time during the exponential growth phase. The exponential growth phase begins when the colony appears on the medium and ends before it attains the petri dish diameter (85 mm).

## Colony-forming assay

The colony-forming assay was performed in the liquid media (CB) by culturing the fungal conidia in reuterin-treated and -untreated media to evaluate the antifungal activity of reuterin. Reuterin at different concentrations of 2, 4, 6, and 8 mM was applied as the treatment in this study. The colony-forming assay was performed using a method described by Omidbeygi et al. (2007) with slight modifications on the inoculum concentration and incubation condition. Five microliters of conidial suspension were inoculated into a test tube containing 10 mL of CB with or without the addition of reuterin. The medium without reuterin was used for NC, while the medium containing 5 mg/mL ketoconazole was used for PC. Incubation was carried out for six days at 30°C. The colony numbers (CFU/mL) were calculated after subculturing on PDA medium (OXOID, UK) and incubated for three days at 30°C. Serial dilutions up to 1/100 000 were carried out for NC, and each treatment showed fungal growth. All tests were performed in triplicate. The percentage of colony numbers reduction was calculated using the following formula:

$$\text{Colony numbers reduction (\%)} = \left[ \frac{nc - nt}{nc} \right] \times 100$$

where *nc* is the number of colonies of NC and *nt* is the number of colonies of treatment. The lowest concentration that completely (100%) inhibited the growth of fungi was considered as MIC (Tian et al., 2011).

## Scanning electron microscopy assay

Scanning electron microscopy (SEM) assay was performed to investigate the effect of reuterin on the microscopic morphology of *A. flavus* mycelia and conidia. The microscopic morphology of *A. flavus* mycelia was observed after seven-day-old fungal growth at 30°C in a solid medium (CAM) containing 5 mM reuterin. This reuterin concentration was chosen for treatment since it resulted in more than 60% inhibition of radial growth (data was not shown). The *A. flavus* grown on a medium without reuterin was used for NC. The microscopic morphology of *A. flavus* conidia was observed after the conidial suspension was treated with 6 mM reuterin and incubated for 3 h at 30°C. This reuterin concentration was chosen for treatment since it showed conidial inactivation. The treatment was performed using a method described by Shen et al. (2016) with a slight modification on the incubation condition. The untreated conidial suspension was used for NC.

Specimen preparation for SEM assay was performed using a method described by Goldstein et al. (1992). Pieces of *A. flavus* mycelia (1 cm in diameter, taken from the periphery of the colony) and conidial suspension were fixed in 2.5% glutaraldehyde and 6% tannic acid for 24 h at 4°C sequentially, then washed with cacodylate buffer (4 × 5 min). The specimens were dehydrated in ethanol with gradual concentrations (50% ethanol for 4 × 5 min; 70%, 85%, 95% ethanol for 20 min, sequentially; and 100% ethanol for 2 × 10 min). Specimens drying was carried out in a vacuum drier after soaking in t-butanol for 2 × 10 min. The specimens were attached to a stub and coated with gold using Ion Coater (IB-2, Eiko Engineering, Japan), then analyzed using SEM (JSM-IT200, Jeol USA, Inc.).

## Aflatoxin B1 production assay

Aflatoxin B1 (AFB1) produced by *A. flavus* after ten days of growth at 30°C was measured to evaluate the effect of reuterin on AFB1 production. In this study, the fungal growth was only observed from 2 and 4 mM reuterin treatment. Therefore, AFB1 production was simulated in CAM and CB with addition of 2 and 4 mM reuterin, respectively. All tests were performed in triplicate. The concentration of AFB1 in the samples was measured by the enzyme-linked immunosorbent assay (ELISA) using a direct competitive ELISA kit developed by the Indonesian Research Center for Veterinary Sciences (IRCVS). The AFB1 extraction and determination by ELISA were performed according to the AOAC Official Method 990.32 (2012) and procedure developed by IRCVS. The growth medium with *A. flavus* mycelia was extracted with 50 mL of methanol 70% in a glass jar, using a hand blender for 2 min, then filtered using Whatman No. 1 filter paper. One hundred microliters of standards or samples were pipetted into the mixing wells, added with 100 µL of enzyme conjugate, and mixed with a multichannel pipette. A 75 µL of each mixture was transferred into antibody-coated wells and incubated for 1 h at 25°C in an unlighted condition. Afterwards, the solution in the wells was removed, then washed three times with distilled water and air-dried. One hundred microliters of substrate solution were added into the well and incubated for 30 min at 25°C to form a blue color. The reaction was stopped by adding 50 µL of stop solution into the well to change the color to yellow. The absorbance was measured at 450 nm using Microplate Spectrophotometer (Thermo Scientific™ Multiskan™ GO, Fisher Scientific, UK), and then the inhibition percentage of standards and samples was calculated. A standard curve was built by plotting the inhibition percentage against AFB1 concentration to obtain a linear equation  $y = a \ln x + b$ , where *x* and *y* are AFB1 concentration and inhibition percentage, respectively, while *a* and *b* are slope and intercept, respectively. The AFB1 concentration in the sample solution was calculated using the following formula:

$$\text{AFB1 concentration (ng/mL sample solution or ppb)} = 2.718 \times \left[ \frac{\% \text{ inhibition} - b}{a} \right] \times \text{dilution factor}$$

## Statistical analysis

All data are presented as mean value ± standard deviation (SD). The significant differences between mean values were analyzed statistically by Analysis of Variance (ANOVA) using IBM SPSS Statistics 26. Further analysis by Duncan's Multiple Range Test (DMRT) was performed for significant differences at a significance level of 95% ( $p < 0.05$ ).

## RESULTS AND DISCUSSION

### Activity of reuterin in inhibiting the radial growth of *A. flavus* BIO 33212

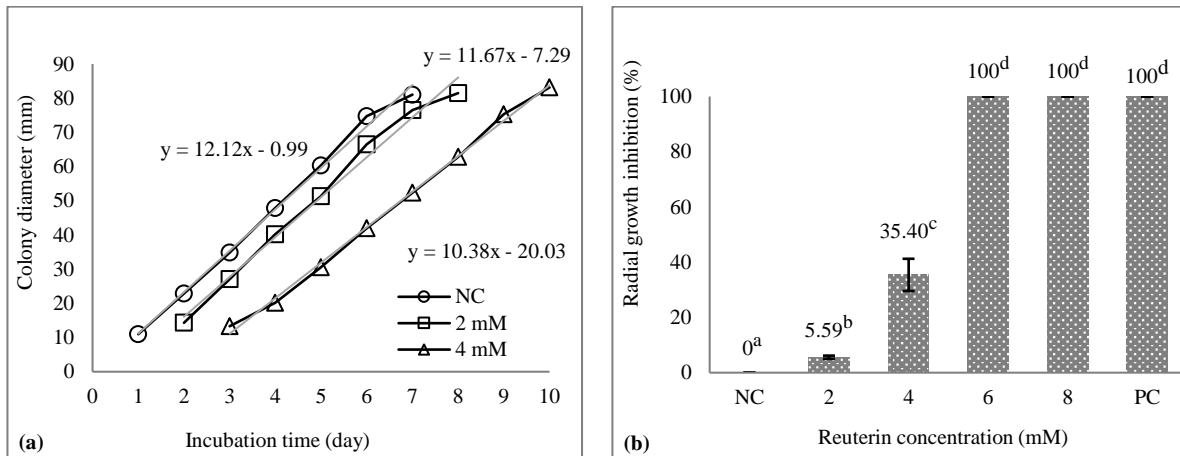
The radial growth of *A. flavus* during ten days of incubation on CAM with different concentrations of reuterin is shown in Table 1. The results indicated that reuterin effectively prolonged the *A. flavus* lag phase. Compared to the NC, the treatment with 2 and 4 mM reuterin prolonged the lag phase for one day and two days, respectively. Meanwhile, treatment with 6 and 8 mM reuterin inhibited the fungal growth completely until the end of incubation time, as well as the PC. The exponential growth phase of *A. flavus* treated with reuterin is illustrated in Figure 1a. The slope values indicated that the growth rate of the NC was 12.12 mm/day, while the growth rate of the treatment with 2 and 4 mM reuterin were 11.67 and 10.38 mm/day, respectively. The results suggested that reuterin was not only effective prolonged the lag phase, but also decreased the growth rate of *A. flavus* during the exponential phase.

After seven days of incubation, we observed that the colony of NC nearly attained the petri dish diameter, causing the increase of colony diameter afterward got limited and could not illustrate the actual increase. Therefore, we use the colony diameter of day seven to calculate the percentage of radial growth inhibition (Figure 1b). The results indicated that reuterin at concentrations of 2 and 4 mM inhibited the *A. flavus* radial growth by 5.59% and 35.40%, respectively. In comparison, reuterin at concentrations of 6 and 8 mM as well as the PC, resulted in 100% growth inhibition. Therefore, the concentration of 6 mM was considered as MIC.

**Table 1** Effect of different concentrations of reuterin on the radial growth of *A. flavus* BIO 33212

Incubation time (day)	Colony diameter (mm)					
	NC	2 mM reuterin	4 mM reuterin	6 mM reuterin	8 mM reuterin	PC
1	10.97 <sup>ab</sup> ± 0.30	0.00 <sup>aA</sup> ± 0.00	0.00 <sup>aA</sup> ± 0.00	NG	NG	NG
2	22.85 <sup>bc</sup> ± 0.37	14.30 <sup>bb</sup> ± 0.39	0.00 <sup>aA</sup> ± 0.00	NG	NG	NG
3	34.81 <sup>cc</sup> ± 0.67	27.10 <sup>cb</sup> ± 0.61	13.30 <sup>bA</sup> ± 2.82	NG	NG	NG
4	47.83 <sup>dc</sup> ± 0.39	40.15 <sup>db</sup> ± 0.25	20.13 <sup>cA</sup> ± 3.17	NG	NG	NG
5	60.29 <sup>ec</sup> ± 0.60	51.34 <sup>eb</sup> ± 0.19	30.55 <sup>dA</sup> ± 2.64	NG	NG	NG
6	74.73 <sup>fc</sup> ± 0.17	66.54 <sup>fb</sup> ± 0.09	42.00 <sup>eA</sup> ± 3.90	NG	NG	NG
7	81.04 <sup>gb</sup> ± 0.29	76.51 <sup>gb</sup> ± 0.20	52.34 <sup>fA</sup> ± 4.58	NG	NG	NG
8	85.00 <sup>hb</sup> ± 0.00	81.48 <sup>hb</sup> ± 0.33	62.82 <sup>gA</sup> ± 3.49	NG	NG	NG
9	85.00 <sup>hb</sup> ± 0.00	85.00 <sup>hb</sup> ± 0.00	75.34 <sup>hA</sup> ± 3.40	NG	NG	NG
10	85.00 <sup>hb</sup> ± 0.00	85.00 <sup>hb</sup> ± 0.00	83.15 <sup>iA</sup> ± 1.32	NG	NG	NG

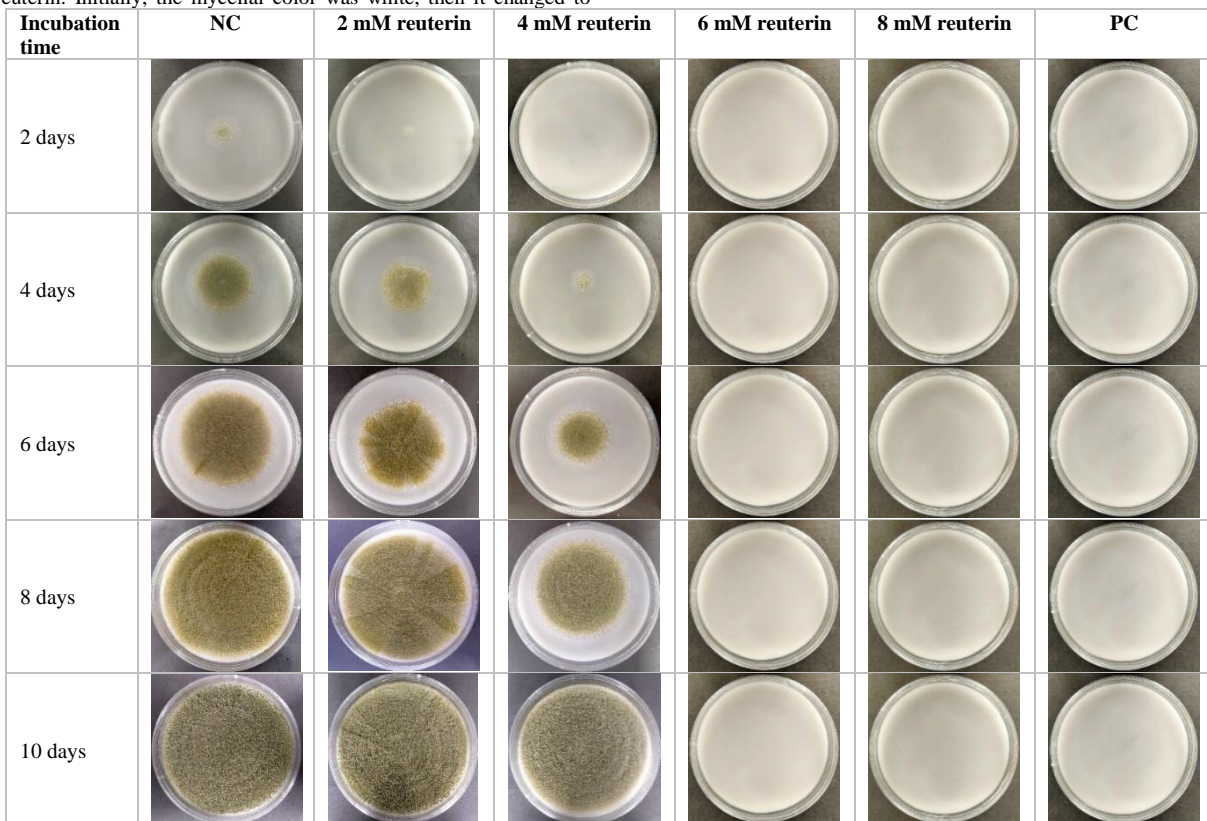
Note: The values represent the mean ± SD of triplicate tests. Mean values with different small letters in a column or capital letters in a row are significantly different ( $p < 0.05$ ). NC – negative control (colony growth on media without reuterin), PC – positive control (colony growth on media containing 5 mg/mL ketoconazole), NG – no colony growth on the media.



**Figure 1** Effect of different concentrations of reuterin on the growth rate (a) and the percentage of radial growth inhibition (b) of *A. flavus* BIO 33212. Mean values with different letters are significantly different ( $p < 0.05$ ). NC – negative control (colony growth on media without reuterin), PC – positive control (colony growth on media containing 5 mg/mL ketoconazole).

The macroscopic morphology of colony growth on CAM with different concentrations of reuterin is presented in Figure 2. Generally, there were no differences in the colony morphology between the NC and the treatment with 2 and 4 mM reuterin. Initially, the mycelial color was white, then it changed to

yellow-green after two days. This appearance indicated that *A. flavus* treated with 2 and 4 mM reuterin could still produce conidia, although the lag phase was longer and the growth rate was lower than NC.



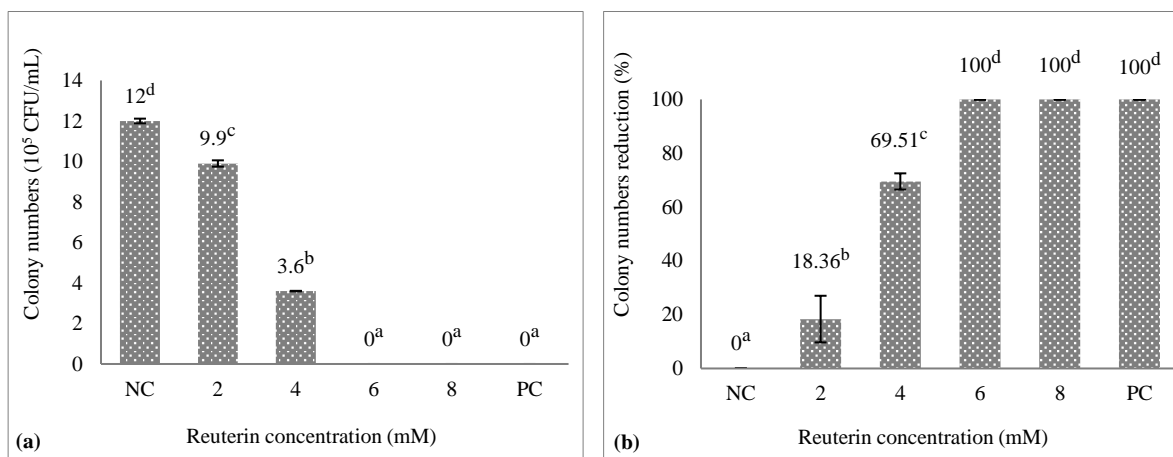
**Figure 2** Macroscopic morphology of *A. flavus* BIO 33212 grown on CAM with different concentrations of reuterin and different length of incubation.



**Activity of reuterin in reducing the colony-forming of *A. flavus* BIO 33212**

The colony numbers of *A. flavus* BIO 33212 cultured in CB containing different concentrations of reuterin are presented in Figure 3a. The results showed that the colony number of the NC was  $1.2 \times 10^6$  CFU/mL, while the colony numbers after

being treated with 2 and 4 mM reuterin were  $9.9 \times 10^5$  and  $3.7 \times 10^5$  CFU/mL, respectively. By contrast, no colony growth was observed from the treatment with 6 and 8 mM reuterin, likewise the PC.



**Figure 3** Effect of different concentrations of reuterin on the colony numbers (a) and the percentage of colony numbers reduction (b) of *A. flavus* BIO 33212. Mean values with different letters are significantly different ( $p < 0.05$ ).

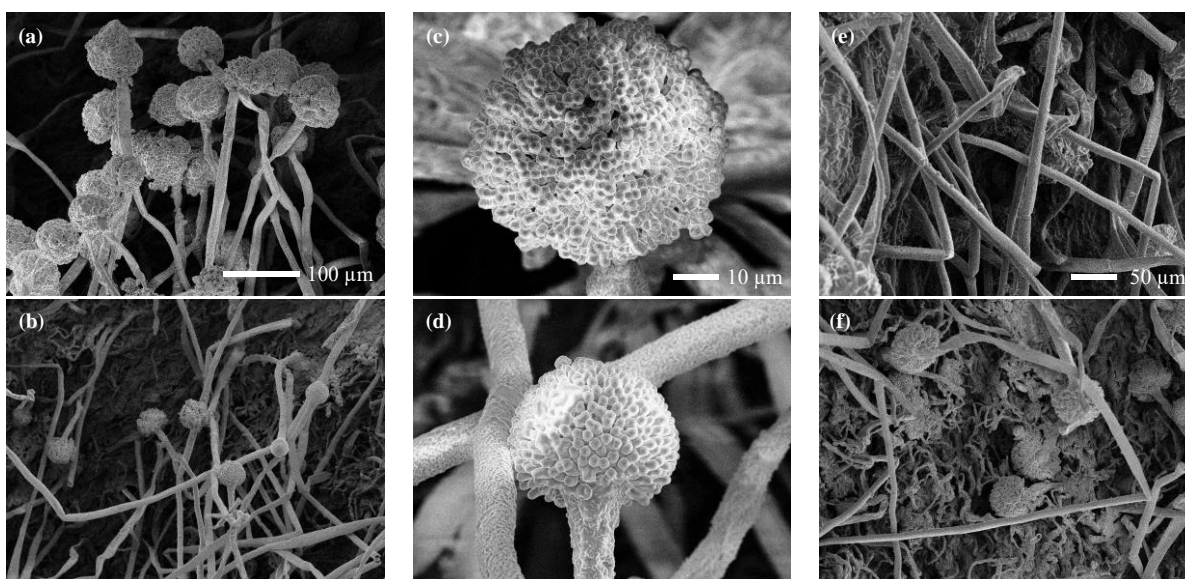
Compared to the NC, reuterin at concentrations of 2 and 4 mM demonstrated the reduction of colony numbers by 18.36 and 69.51%, respectively (Figure 3b), and resulted in a log reduction of 0.09 and 0.52, respectively. Meanwhile, the treatment with 6 and 8 mM reuterin as well as the PC, resulted in 100% colony numbers reduction (Figure 3b) and 6.08 log reduction. Herein, we found a similar result: reuterin at 6 and 8 mM concentrations, whether in liquid or solid media, resulted in 100% fungal growth inhibition. The findings suggested that reuterin with a minimum concentration of 6 mM could inactivate the fungal conidia to germinate. The results of this study deduced that reuterin has antifungal activity against aflatoxigenic *A. flavus*.

The antifungal activity of reuterin with the target of aflatoxigenic *A. flavus* was the first time reported in this current study. Chung *et al.* (1989) used *A. flavus* as the target fungi, but its toxigenicity was not stated. Their study reported that the MIC of reuterin against *A. flavus* was 8 units/mL (equivalent to 0.56 mM, converted by Stevens *et al.*, 2011) after two days of incubation in YNB broth containing reuterin. A study on the antifungal activity of reuterin against genera of *Aspergillus* was performed by Vimont *et al.* (2019). The study resulted in MIC values ranging from 0.1 mM to 1.0 mM for *A. niger* and *A. versicolor* after two days of incubation in RPMI 1640 media containing reuterin. Another study by Ju *et al.* (2021) reported that the MIC of reuterin against *A. niger* was 4 mM after three days of incubation in SDB containing reuterin.

Herein, we report for the first time the MIC value of reuterin against aflatoxigenic *A. flavus*. The MIC value resulted from our study was much higher than the MIC values reported by Chung *et al.* (1989), Vimont *et al.* (2019), and Ju *et al.* (2021). However, these results are not comparable due to the differences in fungal strains, media and methods used in the study. Different fungal strains tested possess

different susceptibility to reuterin; therefore, reuterin had a broad spectrum of antifungal activity (Pilote-Fortin *et al.*, 2021). The stability of reuterin is affected by many factors, including media (Sun *et al.*, 2022). A study by Lüthi-Peng *et al.* (2002) indicated that reuterin was more stable in aqueous solution than that in milk or MRS media. In our study, the media was enriched with pure coconut milk (10%), allowing the interaction of reuterin with the matrices that might diminish the stability of reuterin. Moreover, the incubation time used in this study was much longer, so there was possibility to observe the growth of *A. flavus* after having an extension of lag phase for up to three days.

The determination of MIC in this current study was carried out by radial growth and colony-forming assays exhibiting a corresponding result. The percentages of radial growth inhibition and colony numbers reduction were significantly concentration-dependent. The higher concentration of reuterin, the higher radial growth inhibition and colony numbers reduction occurred. Until now, the antifungal mechanism of reuterin remains unclear. However, several studies proposed that its functional groups induce oxidative stress via interaction with thiol groups in small molecules and protein, causing an imbalance of the cellular redox status and ultimately resulting in cell death. (Schaefer *et al.*, 2010; Vollenweider *et al.*, 2010). Bell *et al.* (2020) also proposed that reuterin triggers the formation of reactive oxygen species (ROS), leading to irreversible cysteine oxidation and enhancing cell death. Hence, we assume that oxidative stress levels resulting from 2 and 4 mM reuterin were still tolerable since conidial germination and mycelial development still occurred within this treatment. Meanwhile, after being treated with 6 mM reuterin, the conidia could no longer tolerate the oxidative stress and became inactive.

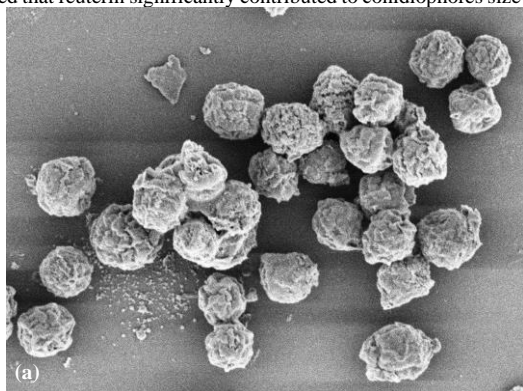


**Figure 4** SEM images of *A. flavus* BIO 33212 grown on CAM: (a-b) conidiophores at 250 $\times$  mag. (a: untreated, b: treated with 5 mM reuterin); (c-d) conidia at 1500 $\times$  mag. (c: untreated, d: treated with 5 mM reuterin); (e-f) hyphae at 300 $\times$  mag. (e: untreated, f: treated with 5 mM reuterin).

**Effect of reuterin on the microscopic morphology of *A. flavus* BIO 33212 mycelia and conidia**

In this study, the effect of reuterin on the microscopic morphology of *A. flavus* growth was revealed by SEM. The effect of reuterin on mycelial morphology was investigated using a concentration of 5 mM since it resulted in more than 60% radial growth inhibition (data was not shown). Meanwhile, the effect of reuterin on conidial morphology was investigated using a concentration of 6 mM since it resulted in conidial inactivation.

The microscopic morphology of *A. flavus* BIO 33212 after seven days of growth on CAM is depicted in Figure 4. The NC *A. flavus* produced abundant conidiophores with normal morphology (Figure 4a). Meanwhile, the *A. flavus* grown on CAM with 5 mM reuterin had sparse conidiophores with smaller and shorter stalks and smaller conidial heads (Figure 4b). As shown in Figure 4c, a normal conidiation in *A. flavus* resulted in abundant spherical conidia in long chains which wholly differentiated. Conversely, as shown in Figure 4d, the treatment with 5 mM reuterin resulted in fewer conidia with abnormal morphology, indicated by smaller conidia in short chains which not wholly differentiated. The hyphae of the NC were sizeable elongated tubes with a smooth appearance (Figure 4e). Meanwhile, the hyphae of *A. flavus* treated with 5 mM reuterin were smaller, and some were wrinkled (Figure 4f). A quantitative comparison of the conidiophores size of NC and treatment with 5 mM reuterin is presented in Table 2. The results indicated that reuterin significantly contributed to conidiophores size



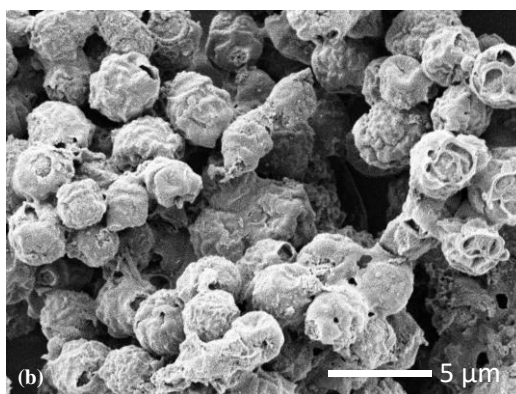
reduction. In the treated *A. flavus*, the diameter of stalks was reduced by 36.11%, while the reduction of conidial head and conidia diameter were 48.18% and 25.36%, respectively.

**Table 2** Effect of reuterin treatment on *A. flavus* BIO 33212 conidiophore size

	Size (µm)		p value	Size reduction (%)
	NC	Reuterin-treated		
Stalk (diameter)	15.98 ± 1.36	10.21 ± 1.43	0.007	36.11
Conidial head (diameter)	71.98 ± 2.97	37.30 ± 3.43	0.000	48.18
Conidia (diameter)	3.43 ± 0.30	2.56 ± 0.18	0.012	25.36

Note: The values represent the mean ± SD of triplicate tests. *p* < 0.05 means significant differences between NC and the treatment with 5 mM reuterin.

The microscopic morphology of *A. flavus* BIO 33212 conidia is depicted in Figure 5. The NC had a normal conidial morphology with an irregular spherical shape and intact cell wall (Figure 5a). Meanwhile, the treatment with 6 mM reuterin for 3 h resulted in conidial damage, indicated by pore formation in conidia (Figure 5b).



**Figure 5** SEM images of *A. flavus* BIO 33212 conidia at 5000 × mag: (a) untreated, (b) treated with 6 mM reuterin.

SEM images of treated mycelia confirmed the growth inhibition was due to the disruption on hyphae and conidiophore development. Meanwhile, SEM images of treated conidia confirmed the conidial inactivation was due to the pore formation in conidia. Similar effect has been reported in high hydrostatic pressure-treated *A. flavus* conidia (Hsiao et al., 2021). Study by Hsiao et al. (2021) revealed that pore formation in conidia led to the release of intracellular molecules, such as nucleic acids and proteins, that ultimately led to conidial death. Conidial death phenomenon was also reported by Shen et al. (2016) in thymol-treated *A. flavus* conidia due to the accumulation of ROS in conidia. Hence, we assume that the disruption of hyphae and conidiophore development as well as conidial damage found in this study were due to the imbalance of cellular redox status induced by reuterin. Conidial germination is early stage prior to mycelial development in *A. flavus* growth, where abundant of genes and proteins involved in these stages with their specific biological function (Pechanova et al., 2013; Tiwari et al., 2016; Baltussen et al., 2020; Li et al., 2022). The imbalance of the cellular redox status induced by reuterin might regulate these genes expression and alter protein stability (Jorgenson et al., 2013).

**Effect of reuterin on the production of aflatoxin B1 by *A. flavus* BIO 33212**

The concentrations of AFB1 produced by *A. flavus* after ten days cultured in CAM and CB are presented in Table 3. Surprisingly, the results showed a contradictory phenomenon from the previous results, where the treatment with 2 and 4 mM reuterin triggered a higher production of AFB1. In NC CAM, the AFB1 production reached 21.99 ng/mL sample solution (ppb) and increased by 9.32% and 22.56 % after being treated with 2 and 4 mM reuterin, respectively. Meanwhile, in NC CB, the AFB1 production reached 19.53 ng/mL sample solution and increased by 133.90% and 139.12 % after being treated with 2 and 4 mM reuterin, respectively.

**Table 3** Production of AFB1 by *A. flavus* BIO 33212 cultured in different types of media with different concentrations of reuterin

Treatment	AFB1 concentration (ng/mL sample solution)	
	CAM	CB
NC	21.99 <sup>a</sup> ± 2.65	19.53 <sup>a</sup> ± 10.46
2 mM reuterin	24.04 <sup>ab</sup> ± 1.06	45.68 <sup>b</sup> ± 2.71
4 mM reuterin	25.95 <sup>b</sup> ± 0.46	46.70 <sup>b</sup> ± 2.50

Note: The values represent the mean ± SD of triplicate tests. Mean values with different letters in a column are significantly different (*p* < 0.05). CAM – coconut agar medium, CB – coconut broth.

The effect of reuterin on AFB1 production was also reported for the first time in this study. We found that AFB1 production was increased in the treatment with reuterin at a concentration < MIC (< 6 mM). As mentioned before, reuterin induce oxidative stress in cell, a study by Jayashree and Subramanyam (2000) concluded that oxidative stress is required to produce aflatoxin. The study by Fountain et al. (2016) proposed that the role of aflatoxin production correlates with alleviating oxidative stress. The molecular mechanism of this phenomenon was due to the expression of genes involved in the aflatoxin biosynthesis and antioxidative system in *A. flavus* induced by oxidative stress (Fountain et al., 2016). The different increasing levels of AFB1 production in solid and liquid media found in our study were presumably due to the different expression levels of these genes. The study by Wang et al. (2019) deduced that aflatoxin biosynthesis in *A. flavus* was affected by different types of growth media (solid or liquid). Moreover, differentially expressed proteins were identified in *A. flavus* at different types of growth media.

Similar finding was reported by Masiello et al. (2020) where sub-lethal fungicides concentrations of boscalid and isopyrazam enhancing aflatoxin production in resistant strains of *A. flavus*. Although its molecular mechanism was not investigated, this enhanced aflatoxin production was explained by the activity of both fungicides in inhibiting fungal respiration that led to an increasing ROS production thus promoting aflatoxin production.

**CONCLUSION**

The current study confirmed that reuterin has antifungal activity against aflatoxigenic *A. flavus*. Reuterin effectively prolonged the lag phase and decreased the growth rate of *A. flavus*. The MIC of reuterin was 6 mM, inhibiting the fungal growth completely in solid and liquid fat-enriched media. Reuterin-treatment resulted in disruption of hyphae and conidiophore development as well as conidial damage. Reuterin was not considered anti-aflatoxigenic since it (at a concentration < 6 mM) could trigger the *A. flavus* to produce AFB1. Further study is needed to confirm the effectiveness of its application in agricultural products to prevent the aflatoxigenic *A. flavus* growth during storage.

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