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Effect of Quarum Sensing Molecules on *Aspergillus fumigatus*

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Abstract

Aspergillus fumigatus is an opportunistic fungal pathogen to which immunocompromised patients are especially susceptible. *A. fumigatus* can form biofilms both *in vitro* and *in vivo*. Quorum sensing molecules (QSMs) have activity against some fungi. This study aimed to determine the activity of the QSMs farnesol, tyrosol, phenylethanol and tryptophol against the growth *A. fumigatus* on solid media, and against its ability to form biofilms. The activity of each QSM against planktonic *A. fumigatus* growth was assessed using the CLSI M38-A2 broth microdilution assay, while QSM inhibition of *A. fumigatus*'s biofilm formation was measured in crystal violet, and 2, 3-bis (2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-caboxanilide (XTT) assays. The QSMs reduced the colony diameter of the studied strains in a QSM-dependent pattern. Tryptophol showed the best effect and tyrosol showed the poorest effect. The minimum inhibitory concentrations (MICs) for farnesol, tyrosol, phenylethanol and tryptophol tested against *A. fumigatus* were > 32, > 32, 16 and 8 mM, respectively. The effective concentration each QSM required to inhibit *A. fumigatus* biofilm formation were higher than the planktonic MICs. In this study, the performance of QSMs against *A. fumigatus* ranked from best to worst as follows: tryptophol, phenylethanol, farnesol and tyrosol. Because of phenylethanol and tryptophol showed the strongest effect to the growth and biofilm formation of *A. fumigatus*. Therefore, the cytotoxic activities of phenylethanol and tryptophol in A549 cells (lung alveolar epithelial cells) were determined. However, phenylethanol and tryptophol induced A549 cell damage (at MIC level), as demonstrated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) and lactate dehydrogenase (LDH) assays.

Keywords: Quorum sensing molecules, *Aspergillus fumigatus*, biofilms

Introduction

Eukaryotic quorum sensing has extensively beenstudied in *C. albicans*. This fungus has the greatest number of quorum sensing molecules (QSMs) identified to date [1,2]. Farnesol, tyrosol, phenylethanol, and tryptophol were QSMs identified in *C. albicans* [3].

Farnesol is the most studied fungal QSM. Previous studies have already shown that farnesol blocks the morphological transition from yeast to hyphae, prevents biofilm formation and alters fungal gene expression in *C. albicans* [4-9]. In filamentous fungi, there are some evidence that farnesol can induce cell death in *Aspergillus nidulans* [10,11], *Aspergillus niger* [12] and *Aspergillus flavus* [13]. Ditchl *et al*. showed that the growth rate of *Aspergillus fumigatus* wild-type strain D141 was inhibited and colony morphology was changed from powdery and cottony to a fluffy phenotype when they were grown on solid agar containing high concentrations of farnesol (1 mM) [14]. Tyrosol is another QSM produced by *C. albicans* and play a role in stimulation germ tube formation, pseudohyphae formation, and biofilm formation of *C. albicans* [1,15]. Although, tyrosol has an opposite effect to several fungi when exogenously treated as antifungal agents. Cordeiro *et al*. evaluated the susceptibility of tysosol to planktonic cells and biofilm of *Candida tropicalis* [16]. Tyrosol showed the synergistic effect with

amphotericin B. Moreover, tyrosol (80 μM) combined with amphotericin B (4 μg/ml) also reduced the biofilm formation of *Pichia krudievsevii* (formerly *Candida krusei*) [17].

More recently, phenylethanol and tryptophol were also identified in *C. albicans* [18,19] and *Saccharomyces cerevisiae* [20]. Phenylethanol inhibits germ tube formation and growth of *C. albicans* [3]. In *S. cerevisiae*, phenylethanol has an opposite effect by promoting pseudohyphal growth [3,20]. The role of tryptophol in fungi remain not completely understood. Tryptophol decreased the adhesion to polystyrene surface of *Debaryomyces hansenii* at concentration ≥ 500 μM and increased filamentous form at concentration 1,000 μM [21].

Several studies have investigated the capacity of *A. fumigatus* to form biofilm [22-27]. *A. fumigatus* forms hyphal nets in a parallel-packed hyphal network on plastic surfaces or epithelial cells embedded in an extracellular matrix [24,25]. *In vivo* biofilm formation was first reported in patients with an aspergilloma [23]. Four developmental stages of biofilm development of *A. fumigatus* have been proposed: initial conidial seeding (conidial adhesion); germination (6 - 8 h); filamentation and formation of a monolayer (12 h); followed by increased structural complexity, extracellular matrix production and maturation (24 h) [27]. Mowat *et al*. investigated the phase-dependent antifungal activity against developing multicellular filamentous biofilms of *A. fumigatus* and found that treatment of actively growing *A. fumigatus* cells with antifungal agents is more efficacious than treating mature structures *in vitro* [25].

A. fumigatus is an opportunistic fungal pathogen found especially in immunocompromised patients [28]. It can form filaments within the lungs, forming dense intertwined mycelium balls [29]. *A. fumigatus* virulence is caused by proteins that promote mycelium growth in the lung parenchyma and by features of the conidia that confer resistance to host antifungal defense mechanisms [22]. Some strains of *A. fumigatus* are resistant to azole anti-fungal agents, leading to treatment failure. It is important to seek other anti-fungal agents.

The biological effect of QSMs other than farnesol, such as tyrosol, phenylethanol, and tryptophol, on *A. fumigatus* are not well studied. Therefore, the present study aimed to determine the effect of QSMs (farnesol, tyrosol, phenylethanol, and tryptophol to the growth phenotypes on solid agar, QSMs susceptibility testing and biofilm formation of *A. fumigatus* using a phenotypic approache.

Materials and methods

Fungal strains and reagents

The strains of *A. fumigatus* used in this study were *A. fumigatus* AF293, *A. fumigatus* BUU-001, *A. fumigatus* CL-262 (an azole resistant clinical strain carrying the M220I mutation in Cyp51A gene), and *A. fumigatus* ENV-004 (an azole resistant environmental strain carrying the M220V mutation in Cyp51A gene) which were kindly provided by Prof. Oliver Bader (Institute for Medical Microbiology and German National Reference Center for Systemic Mycoses, University Medical Center Gottingen, Germany) and Asst. Prof. Dr. Marut Tungwattanachuleeporn (Faculty of Allied Health Sciences, Burapha University, Chonburi, Thailand).

For the study, the QSMs used were farnesol, tyrosol, phenylethanol and tryptophol (Sigma-Aldrich, St. Louis, MO, USA) prepared immediately as 2 M stock solutions in ethanol prior to each experiments [21].

Effect of studied QSMs to the growth phenotypes of *A. fumigatus* on solid media

A. fumigatus conidia were harvested from 5 day old cultures grown on Sabouraud Dextrose Agar (SDA) slant. The slant surface was flooded with 5 ml PBS containing 0.025 % Tween 20 (v/v) while gently rocking [30]. The conidia were then recovered and counted using a counting chamber. Ten microliters of the solution containing the *A. fumigatus* conidia at concentration of 10^4 conidia/ml were spotted onto Sabouraud Dextrose Agar (SDA) plates containing 0.1, 1 or 10 mM each of farnesol, tyrosol, phenylethanol, tryptophol, ethanol (solvent control) or SDA alone. The plates were then sealed with paraffin and incubated at 37 ºC for 3 days and the colony diameters were measured daily.

***In vitro* susceptibility testing**

In vitro susceptibility testing was performed using broth microdilution method, according to the Clinical and Laboratory Standards Institute (CLSI) protocol M38-A2 [31]. Each isolate was tested against the two-fold dilution ranges tested for each QSM from 0.06 to 32 mM. The conidial suspensions were prepared in 165 mM morpholinepropanesulfonic acid-buffered RPMI 1640 to give a final concentration of 0.4 - 5×10^5 conidia/ml and 100 µl of this was added to each well of a 96-well plate containing 100 µl of each QSM. The plates were then incubated for 48 h at 37°C. We determined the minimum inhibitory concentration (MIC) for of each QSM against *A. fumigatus*. The MIC for each QSM was defined as the lowest concentration of QSM that produced complete visible inhibition of growth using a microtiter plate reading mirror (Cooke Engineering, Alexandria, VA).

Effect of studied QSMs on biofilm formation of *A. fumigatus*

Effect of QSMs on biofilm formation in a 96-well polystyrene plate of the studied strains was performed following to Mowat *et al*. [24] and Pierce *et al*. [30]. Two hundred microliters of conidia at a concentration of 10^5 conidia/ml of RPMI 1640 without sodium bicarbonate, supplemented with L-glutamine and buffered with 165 mM morpholinepropanesulfonic acid were seeded into each well of a 96-well plate.

At 24 h, the medium was aspirated and the plates washed three times with PBS pH 7.4 to remove non-adherent cells. Two hundred microliters of farnesol, tyrosol, phenylethanol or tryptophol of the twofold dilution ranges tested for each QSM from 0.25 to 128 mM were then added to each well. Plate was incubated for a 24 h. After treatment with each QSM, the supernatant was aspirated from each well. The non-adherent cells were removed and washed with PBS (three times). The plates were air-dried and the biofilm mass were stained with 100 µl 0.5 % (w/v) crystal violet solution for 5 min. Excess stain was removed by rinsing the biofilms using running water. Subsequently, 100 µl 95 % ethanol was added to each well to dissolve the stain attached to the biofilms. Then, 80 µl of the resulting colored supernatant from each well was removed and transferred into the new microtiter plate. Finally, the absorbance value was measured at 570 nm using a spectrophotometer (Tecan Sunrise, Crailsheim, Germany) [24]. The absorbance values were assumed to be proportional to the quantity of the biofilm mass, comprised of hyphae and extracellular polymeric material. The experiments were done in triplicate.

The metabolic activity was assessed by adding 100 µl of XTT [2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-caboxanilide/menadione solution (24 h after treatment and pre-washed biofilm as described above). The plate was incubated in the dark for 2 h at 37 °C. After that, 80 µl of the resulting colored supernatant from each well was removed and transferred into the wells of a new microtiter plate and measuring absorbance at 490 nm using a spectrophotometer (Tecan Sunrise, Crailsheim, Germany) [30]. The colorimetric change in the XTT reduction assay directly correlates with the metabolic activity of the biofilm. The experiments were done in triplicate. The biofilm minimum inhibitory concentration for each QSM was defined as the lowest concentration of QSM that completely inhibition (100 %) by using this XTT technique.

MTT proliferation assays of A549 human pulmonary epithelial cells in the presence and absence of phenylethanol and tryptophol

A549 cells (ATCC® CCL-185™) were purchased from ATCC. These cells were routinely grown in T25-cm^2 flasks (Corning®) containing Ham's F-12K (Kaighn's) Medium (Gibco® by life invitrogen™, Carlsbad, USA) supplemented with 10 % fetal bovine serum and 100 units/ml of penicillin-streptomycin (Gibco® Invitrogen™) and were incubated under humidified conditions containing 5 % carbon dioxide at 37 °C. These cells were subcultured using 0.25 % (w/v) Trypsin-EDTA (Gibco® by life Invitrogen™) after washing with PBS (Gibco® by life Invitrogen™) and were observed using an inverted microscope until the cell layers were dispersed. Subsequently, 5 ml of complete growth medium was added, and cells were aspirated by gently pipetting. Appropriate volumes of cell suspensions were then divided into culture flasks.

Cell numbers and viability were assessed using Trypan blue exclusion and a counting chamber. Suspensions were then adjusted to 1×10^4 A549 cells and were grown to confluence on 96-well

polystyrene plates in Ham's F-12K with 10 % fetal calf serum (FCS). Phenylethanol and tryptophol were added at indicated concentrations after 48 h when cells had reached 80 - 90 % confluence. Confluent cells were then incubated with phenylethanol and tryptophol at 0.25 - 16 mM for 12, 24, or 48 h. Subsequently, 10-µl aliquots of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) stock solution were added and incubated for 2 h at 37 °C. After carefully removing media 100 µl aliquots of 2 % NP-40 were added to each well and mixed by repetitive pipetting. The absorbance of the MTT assay plates were then read using a spectrophotometer at 540 nm with a reference filter of 620 nm.

Lactate dehydrogenase assays of A549 cells after treatments with phenylethanol and tryptophol

Cell suspensions were prepared as described for MTT assays, and lactate dehydrogenase (LDH) activities were determined using LDH assay kits (CytoTox 96 Non-Radioactive Cytotoxicity Assay, Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, cell suspensions were adjusted to 1×10^4 and were grown to confluence on 96-well polystyrene plates containing Ham's F-12K supplemented with 10 % FCS. Phenylethanol and tryptophol were added after 48 h (80 - 90 % confluence) at 0.25 - 16 mM. After 12, 24, or 48 h incubation, 50 µl aliquots of media from test and control wells were added to fresh 96-well flat clear bottom plates with 50 µl of CytoTox 96® Reagent. The plates were then incubated in the dark for 30 min at room temperature, and 50 µl aliquots of stop solution were then added and the absorbance was read at 490 nm. Untreated A549 cells were used as negative controls, and the positive controls were provided in the assay kit. Data are presented as means ± standard deviations of three independent experiments, and percent cytotoxicity is calculated.

Statistical analysis

The means of the 3 value sets of the triplicate experiments and the standard deviations of these were then calculated. The different effect of each QSM at each concentration in the mean colony diameters and the effect of each QSM on biofilm formation of the studied strains compared to control were analyzed using One-way Anova. The statistical calculations were performed using the GraphPad® PRISM version 6.01. A p-value < 0.05 was considered statistically significant.

Result and discussion

The effect of QSMs has been widely studied against different fungi [8]. In this study, we investigated the effect of fungal QSMs (farnesol, tyrosol, phenylethanol, and tryptophol) to the growth phenotypes on solid media, QSMs susceptibility testing and biofilm formation of *A. fumigatus*.

Effect of farnesol, tyrosol, phenylethanol and tryptophol on the growth phenotypes of *A. fumigatus* on solid media and *in vitro* susceptibility testing

The growth rates and phenotype of the studied strains are presented in **Figures 1** and **2**. Farnesol at the concentrations of 0.1, 1, and 10 mM significantly caused the colony diameter of the studied strains to be smaller than controls (SDA without farnesol) in a concentration-dependent manner when observed at day 3 ($p < 0.05$). The colony phenotype was altered from powdery and cottony to fluffy by farnesol at the concentrations of 1 and 10 mM.

The most studied of fungal QSM is farnesol. Only one previous study has investigated the effect of farnesol on the *A. fumigatus*. The growth phenotype of *A. fumigatus* wild type strain was changed at a concentration of 1 mM [14]. In our study, we observe that farnesol caused the smaller colony diameter (at the studied concentrations of 0.1, 1, and 10 mM) than control in a concentration-dependent manner and altered the colony morphology at the concentrations of 1 and 10 mM (**Figures 1** and **2**). This finding supported the Dicthl *et al*. study [14]. Farnesol exhibited a relatively high MIC of > 32 mM for *A. fumigatus*. Therefore, the studied strains was not killed by farnesol. In comparison, the MIC of farnesol for *Paracoccidioides brasiliensis* is around 25 µM [32], *Coccidioides posadasii* ranges from 0.00171 - 0.01369 µg/mL [33]. The activity of farnesol also investigated in *Histoplasma capsulatum*. The range of MICs of farnesol were 0.0078 to 0.0156 and 0.0078 to 0.0312 µM against mold-form and yeast-form of

H. capsulatum respectively [34]. A number of studies have also demonstrated differences in MIC for treatment for *Candida* spp. [35] and *Cryptococcus neformans* [36]. Castelo-Branco *et al*. showed the MIC of fanesol against planktonic *Burkholderia pseudomaleii* (bacteria) in range 150 - 300 mM. These discrepancies in the MIC pattern could be because of differences depending on genus and species of each organism [37].

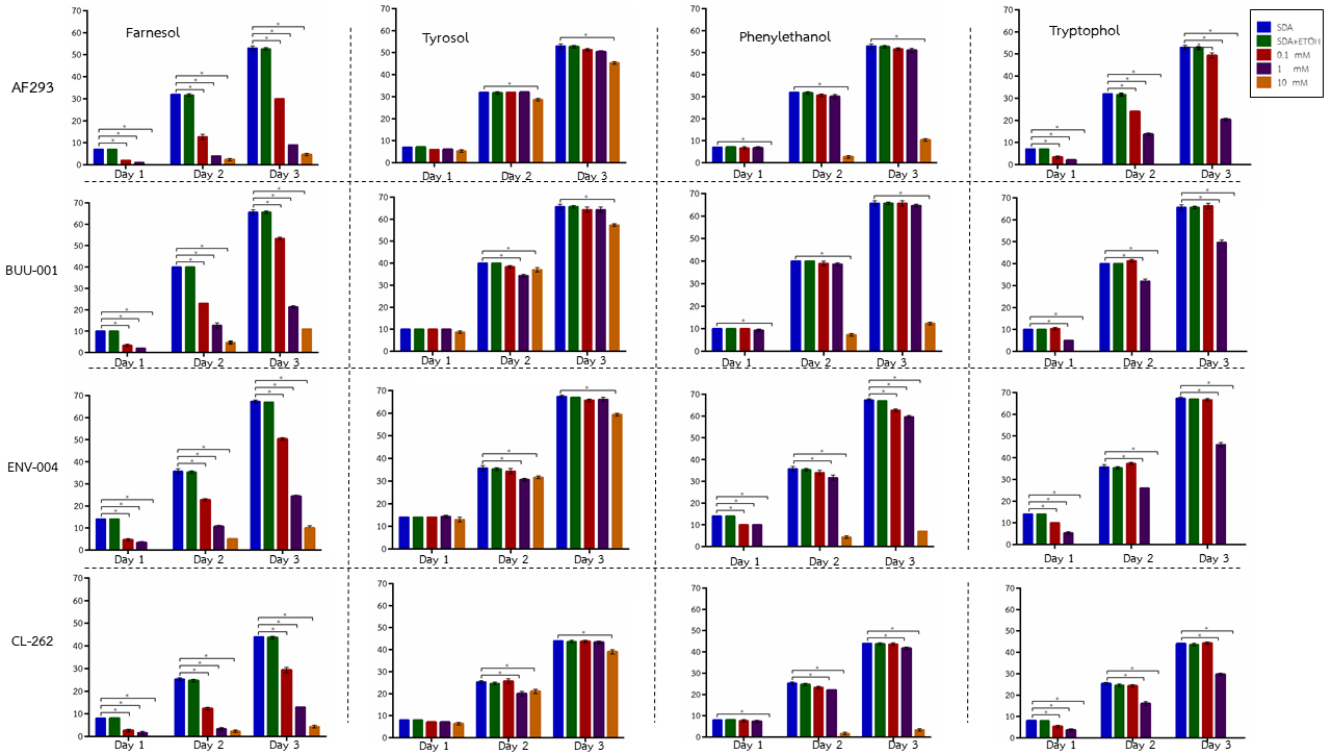

Figure 1 Growth curve of *A. fumigatus* on SDA containing 0.1, 1 or 10 mM farnesol, tyrosol, phenylethanol, tryptophol and control (ethanol (ETOH) and SDA alone). The colony diameter measured in millimeter (y-axis). * = $p < 0.05$ compared with the colony diameter of *A. fumigatus* grown in SDA alone.

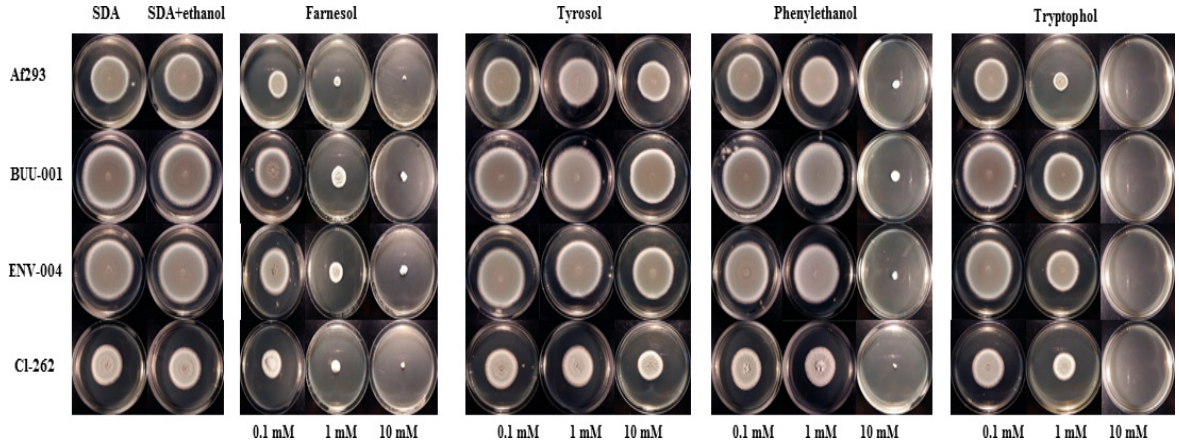

Figure 2 Colony morphology at day 3 of *A. fumigatus* on SDA containing 0.1, 1 or 10 mM farnesol, tyrosol, phenylethanol, tryptophol and control (ethanol (ETOH) and SDA alone).

Tyrosol at the concentrations of 0.1 and 1 mM did not change the colony phenotype and dimeter of the studied strains when compared to the control (SDA without tyrosol), Interestingly, colony diameter of the studied strains on SDA contained 10 mM tyrosol significantly smaller than controls ($p < 0.05$). Normally, tyrosol stimulated hyphal growth and increased biofilm biomass of *C. albicans* at early stages [15]. The role of tyrosol was also investigated in combination with antifungal agents. The exogenous tyrosol alone or combined with amphotericin B inhibited planktonic cells and *Candid*a biofilm growth [20]. As in tyrosol, Monteiro *et al*. they showed the MIC of tyrosol against *C. albicans* was 150 mM and *Candida glabrata* was 300 mM [38] but Arias *et al*. [39] showed the MIC of tyrosol against *C. albicans* was 50 mM and *C. glabrata* was 300 mM. The MIC pattern could vary because differences on the studied strains. In this study, tyrosol at the concentrations of 0.1 and 1 mM did not change the growth phenotypes and colony dimeter of the studied strains?. We observed that the colony diameter of the studied strains were smaller than control on SDA contained 10 mM tyrosol. Tyrosol MIC was a relatively high (> 32 mM) as well as farnesol.

The colony diameter at day 3 of the studied strains on the SDA presented of 10 mM phenylethanol were statistically significant smaller than colony on SDA without phenylethanol ($p < 0.05$). Tryptophol at a concentration of 1 mM cause the colony diameter of the *A. fumigatus* to be smaller than controls (SDA without tryptophol) at day 3 ($p < 0.05$). The colonies of the studied strains were not observed (no growth) at day 3 on SDA contained of 10 mM tryptophol ($p < 0.05$).

Previous investigations by Chen *et al*. suggest that phenylethanol and tryptophol are promoted in pseudohyphal growth in *S. cerevisiae* [20]. Also Martins *et al*. propose a role for phenylethanol in morphogenesis via signaling molecules of *C. albicans* and *Candida dubliniensis* [40]. Moreover, phenylethanol and tryptophol also showed activity in adhesion and sliding motility of *Debaryomyces hansenii* [21]. Here this study reports, for the first time, that the effects of phenylethanol and tryptophol on *A. fumigatus*. We found that phenylethanol and tryptophol showed the inhibitory effect on the growth of *A. fumigatus*, as evidenced by decreased colony diameter on agar contained 10 mM phenylethanol, 1 mM tryptophol, and the absence of colonies on agar containing 10 mM tryptophol when compared with control. The MICs of phenylethanol and tryptophol were 16 and 8 mM, respectively.

Table 1 Effect of farnesol, tyrosol, phenylethanol, and tryptophol on biofilm development by *A. fumigatus*. The biofilm biomass was quantified by crystal violet staining

QSMs	Strain	Concentration of quorum sensing molecules										
		0 mM	0.25 mM	0.5 mM	1 mM	2 mM	4 mM	8 mM	16 mM	32 mM	64 mM	128 mM
Farnesol	AF293	4.23 ± 0.12	4.14 ± 0.32	3.97 ± 0.35	3.86 ± 0.08	3.93 ± 0.15	3.85 ± 0.33	3.38 ± 0.26*	3.21 ± 0.40*	2.54 ± 0.54*	2.40 ± 0.16*	2.36 ± 0.32*
	BUU-001	4.32 ± 0.14	4.13 ± 0.16	3.92 ± 0.05	3.91 ± 0.01	3.89 ± 0.02	3.87 ± 0.09	3.46 ± 0.05*	3.23 ± 0.20*	2.76 ± 0.27*	2.50 ± 0.19*	2.20 ± 0.09*
	ENV004	4.15 ± 0.01	4.14 ± 0.01	3.94 ± 0.04	3.82 ± 0.07	3.92 ± 0.05	3.86 ± 0.07	3.36 ± 0.05*	3.14 ± 0.08*	2.72 ± 0.22*	2.49 ± 0.12*	2.28 ± 0.12*
	CL262	4.11 ± 0.09	4.14 ± 0.04	3.88 ± 0.12	3.89 ± 0.08	3.91 ± 0.04	3.90 ± 0.05	3.36 ± 0.08*	3.31 ± 0.29*	2.77 ± 0.22*	2.51 ± 0.24*	2.38 ± 0.15*
Tyrosol	AF293	4.05 ± 0.34	4.01 ± 0.09	4.15 ± 0.10	4.13 ± 0.23	4.03 ± 0.06	3.94 ± 0.03	3.92 ± 0.14	3.88 ± 0.32	3.86 ± 0.16	3.52 ± 0.10	2.61 ± 0.16*
	BUU001	4.30 ± 0.25	4.22 ± 0.15	4.24 ± 0.07	4.19 ± 0.14	4.20 ± 0.13	3.94 ± 0.02	3.88 ± 0.11	3.84 ± 0.03	3.84 ± 0.03	3.73 ± 0.13	2.69 ± 0.02*
	ENV004	4.21 ± 0.05	4.17 ± 0.06	4.23 ± 0.11	4.16 ± 0.06	4.18 ± 0.12	3.91 ± 0.01	3.96 ± 0.05	3.91 ± 0.03	3.81 ± 0.06	3.79 ± 0.01	2.85 ± 0.13*
	CL262	4.04 ± 0.02	4.05 ± 0.06	4.05 ± 0.05	4.07 ± 0.96	4.10 ± 0.12	3.92 ± 0.07	3.86 ± 0.02	3.85 ± 0.07	3.73 ± 0.12	3.71 ± 0.06	2.88 ± 0.04*
Phenylethanol	AF293	4.29 ± 0.09	4.13 ± 0.10	4.21 ± 0.30	4.09 ± 0.22	3.95 ± 0.09	3.89 ± 0.05	3.90 ± 0.32	3.87 ± 0.21	1.84 ±0.17*	1.33 ± 0.11*	0.78 ± 0.04*
	BUU001	4.22 ± 0.08	4.25 ± 0.09	4.14 ± 0.04	4.06 ± 0.05	3.93 ± 0.10	3.85 ± 0.06	3.88 ± 0.54	3.83 ± 0.03	1.84 ± 0.03*	1.14 ± 0.02*	0.64 ± 0.07*
	ENV004	4.20 ± 0.09	4.22 ± 0.10	4.11 ± 0.09	4.12 ± 0.05	3.91 ± 0.07	3.89 ± 0.04	3.84 ± 0.05	3.81 ± 0.07	1.84 ±0.13*	1.13 ± 0.12*	0.69 ± 0.16*
	CL262	4.17 ± 0.01	4.12 ± 0.03	4.16 ± 0.02	4.11 ± 0.03	3.87 ± 0.06	3.78 ± 0.09	3.79 ± 0.05	3.75 ± 0.03	1.57 ± 0.44*	1.20 ± 0.73*	0.44 ± 0.13*
Tryptophol	AF293	4.29 ± 0.30	4.12 ± 0.12	4.29 ± 0.09	3.97 ± 0.31	3.97 ± 0.25	3.54 ± 0.07*	2.36 ± 0.09*	2.36 ± 0.14*	1.13 ±0.12*	0.81 ± 0.08*	0.45 ± 0.13*
	BUU001	4.29 ± 0.14	4.09 ± 0.04	4.13 ± 0.13	3.96 ± 0.02	3.95 ± 0.12	3.58 ± 0.05*	2.90 ± 0.55*	2.41 ± 0.03*	1.14 ± 0.01*	0.85 ± 0.04*	0.58 ± 0.11*
	ENV004	4.15 ± 0.13	4.17 ± 0.12	4.19 ± 0.10	3.94 ± 0.06	3.89 ± 0.16	3.59 ± 0.10*	2.94 ± 0.03*	2.41 ± 0.13*	1.19 ± 0.01*	0.86 ± 0.06*	0.58 ± 0.10*
	CL262	4.17 ± 0.10	4.17 ± 0.05	4.17 ± 0.04	3.89 ± 0.01	3.92 ± 0.02	3.49 ± 0.19*	2.79 ± 0.15*	2.44 ± 0.25*	1.50 ± 0.22*	0.74 ± 0.13*	0.36 ± 0.01*

* = $p < 0.05$ compared the OD value of *A. fumigatus* biofilm in RPMI alone, QSMs = quorum sensing molecules

Effect of QSMs on *A. fumigatus* biofilms

We investigated the effect of each QSM (at the concentrations of 0.06, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, and 32 mM) after treatment on fungal preformed biofilms for 24 h. We observed a decreased in both biomass and metabolic activity as performed by crystal violet staining (**Table 1**) and XTT reduction assay (**Figure 3**) (overall statistically significant at $p < 0.05$ for each QSM by One-way ANOVA).

Farnesol significantly decreased the biofilm biomass ($p < 0.05$) at the concentrations of 8, 16, 32, 64, and 128 mM when compare to the control (**Table 1**). Tyrosol significantly decreased the biofilm biomass ($p < 0.05$) at the concentration of 128 mM when compare to the control. Therefore, farnesol and tyrosol did not completely inhibit preformed biofilm of the studied strains.

Phenylethanol significantly decreased the biofilm biomass ($p < 0.05$) at the concentrations of 32, 64 and 128 mM when compare to the control, and tryptophol significantly decreased the biofilm biomass ($p < 0.05$) at the concentrations of 4, 8, 16, 32, 64, and 128 mM when compared to the control (**Table 1**). Similar to farnesol, they also reduced biofilm biomass and in a contentration-dependent.

The metabolic activity of biofilm was decreased in different pattern in each strain (**Figure 3**). Preformed biofilms of the studied strains were not inhibited by farnesol and tyrosol (the biofilm minimum inhibitory concentration of farnesol and tyrosol were more than 128 mM, determined by using the XTT reduction assay). The biofilm minimum inhibitory concentration of phenylethanol and tryptophol to the four studied strains were 128 and 32 mM, respectively. These results suggest that these QSMs, phenylethanol and tryptophol, showed the inhibitory effect of the growth and biofilm formation to the studied strains of *A. fumigatus*.

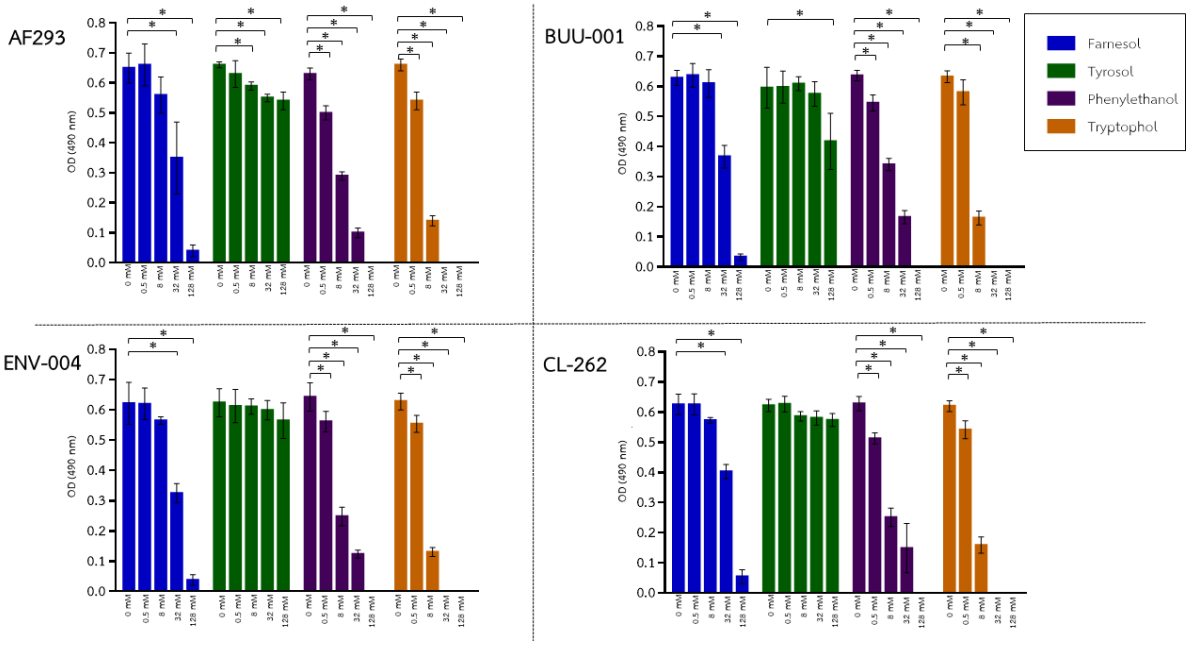

Figure 3 Effect of farnesol, tyrosol, phenylethanol, and tryptophol on biofilm development by *A. fumigatus*. The metabolic activity was quantified using XTT assay. * = $p < 0.05$ compared the OD value of *A. fumigatus* biofilm in RPMI alone.

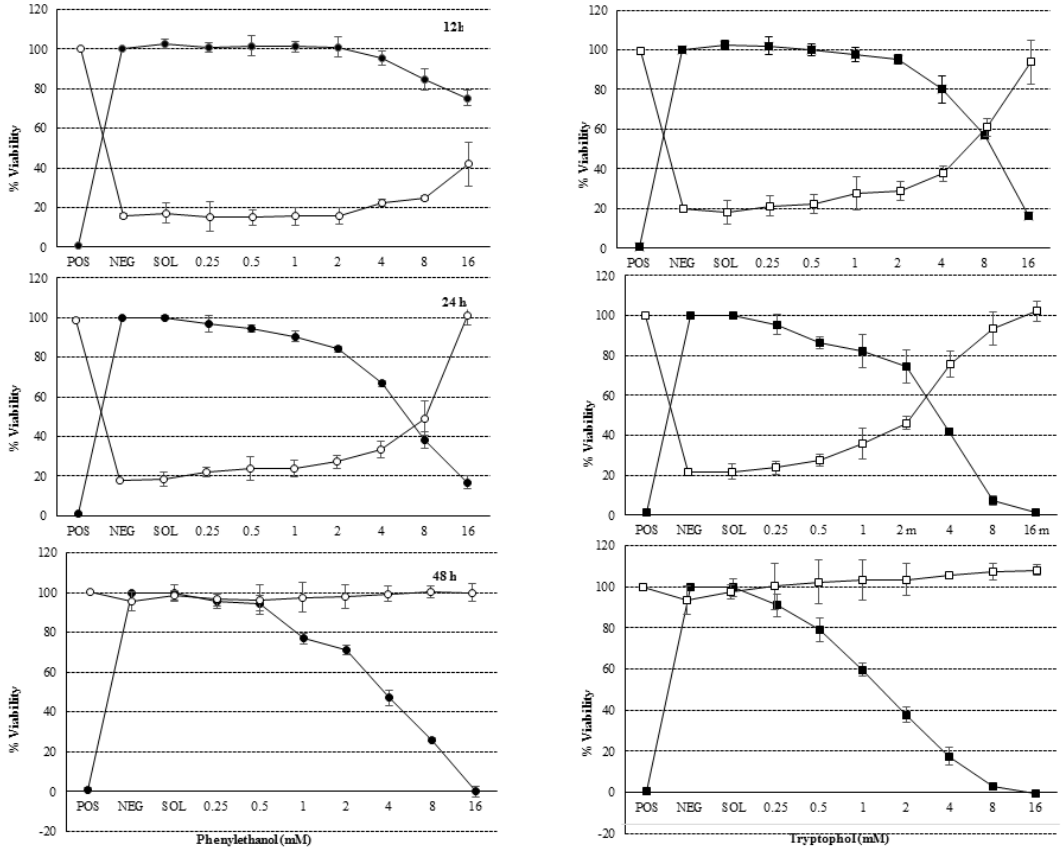

Figure 4 Effects of phenylethanol and tryptophol at selected concentrations in A549 cells after 12, 24, and 48 h; black circles and square represent MTT assays and white circle and square represents lactate dehydrogenase (LDH) assays. Percent viability is indicated on the Y-axis.
POS = positive control, NEG = negative control, SOL = control medium

Effects of phenylethanol and tryptophol on A549 cell viability using MTT and LDH assays.

Because of phenylethanol and tryptophol showed the strongest effect to the growth and biofilm formation of *A. fumigatus*. Therefore, the cytotoxic activities of phenylethanol and tryptophol in A549 cells (lung alveolar epithelial cells) were determined at varying concentrations using MTT assays. After 12 - 48 h treatments with phenylethanol and tryptophol at 0.25 - 16 mM, phenylethanol caused a decrease in cell viability in a dose dependent manner before 12 h. The cell viability decreased to lower than 80 % at 16 mM (12 h). The cell viability, as determined by MTT assay, was strongly affected between 12 h until 48 h (lower 80 % at 4 - 16 mM after 24 h and lower than 80 % at 1 - 16 mM after 48 h). For the LDH assay, corresponding as MTT during the first 12 h generation times (percent cytotoxicity more than 20 % at 8 mM and more than 40 % at 16 mM), phenylethanol caused release LDH in a dose dependent manner and showed strongly affected between12h (more than 40 % at 8 - 16 mM) until 48 h (LDH released 100 % at all concentration because of cell normally released LDH, therefore, 48 h is the long time and not optimum to detect LDH by this assay. Tryptophol caused a decrease in cell viability in a dose dependent manner and strongly affected at the 12, 24, and 48 h. The cell viability decreased to lower than 80 % at 4 - 16 mM (12 h), 2 - 16 mM (24 h), and 0.5 - 16 mM (48 h). For the LDH assay, corresponding as MTT during, tryptophol showed the strongly release LDH in dose dependent manner by released LDH more than 40 % at 8 - 16 mM (12h) and 2 - 16 mM (24) (**Figure 4**). Greater than 30 % reductions in cell viability are generally associated with toxic effects.

Conclusions

In this study, each QSM has differential activities on the growth and development of biofilms of *A. fumigatus*. Tryptophol and phenylethanol showed more inhibitory effect on the studied strains of *A. fumigatus* when compare with farnesol and tyrosol. *In vitro* and *in vivo* experiments are needed to study in-depth mechanism of fungal QSMs on *A. fumgiatus*. However, phenylethanol and tryptophol were moderately cytotoxic in MTT and LDH assays of A549 cells. Further studies may investigate the efficacy of these QSMs in combination with antifungal drugs.

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