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# Anti-hyphal formation property of allicin in suppression of *Aspergillus fumigatus* growth

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## ABSTRACT

**Aims:** The aim of this study was to examine whether allicin, a compound derived from fresh garlic, leads to growth inhibition and changes in the ultrastructure of the cell surface on medically important filamentous fungi, particularly *Aspergillus fumigatus*.
**Methodology and results:** The minimum inhibitory concentration (MIC) of allicin in *A. fumigatus* ATCC 36607 was determined by broth microdilution method according to the CLSI M38-A2 documents whereby the minimal fungicidal concentration (MFC) was determined by plating suspensions from visibly clear wells onto Sabouraud dextrose agar (SDA). Morphological changes on cell surface were observed through scanning electron microscopy (SEM) after 48 h incubation with allicin. In addition, time kill assay was conducted by incubating *A. fumigatus* at selected time points within 24 h period. Our finding indicated that the MIC and MFC for allicin were both 3.2 µg/mL. Quantitative data for optical density obtained through microplate reader indicated that $p<0.05$ at MIC value in comparison with untreated control. Observation of allicin-treated cells through SEM demonstrated complete abrogation of hyphae formation at 3.2 µg/mL and reduced mycelial growth at 1.6 µg/mL of allicin. This finding revealed anti-hyphal activity of allicin at 3.2 µg/mL. When *A. fumigatus* was incubated with 3.2 µg/mL allicin in the time course assay, the inhibitory effect of allicin was evident after 12 h incubation.
**Conclusion, significance and impact of study:** Our finding strongly implied that allicin exerts its antifungal activity against *A. fumigatus* via inhibiting the fungal cell proliferation as well as hindering transformation of the conidia into hyphae. Thus, this study depicted potential antifungal property of allicin to be used as alternative therapy to alleviate invasive fungal infection caused by *A. fumigatus*.

*Keywords: Aspergillus fumigatus*, allicin, minimum inhibitory concentration, minimum fungicidal concentration, time kill assay, scanning electron microscopy

## INTRODUCTION

*Allium sativum* or commonly known as garlic has been recognised worldwide as a traditional medicine for treating various kinds of diseases. The historical perspective of its usage has been documented (Rivlin, 2001). As far as antimicrobial activities of garlic are concerned, Cavallito and Bailey had discovered as early as in 1944 that allicin or allyl 2-propene thiosulfinate, a pure compound from garlic possessed antibacterial activity (Cavallito and Bailey, 1944). Subsequently, other researchers had also found that allicin exhibited antifungal property (Yamada and Azuma, 1977; Shadkchan *et al*., 2004; Khodavandi *et al*., 2011), antiparasitic potential (Mirelman *et al*., 1987) and antimalarial activities (Coppi *et al*., 2006). It has been suggested that the antimicrobial properties of allicin are attributed to inhibition of sulfhydryl metabolic enzymes (Willis, 1956). Production of allicin involves a reaction between alliin which acts as a stable precursor with an enzyme called alliinase when garlic is crushed (Ellmore and Feldberg, 1994). Previous research has also reported several promising effects of allicin, not only in terms of its antimicrobial properties, but also protection against atherosclerosis onset (Lu *et al*., 2012).

*Aspergillus fumigatus*, a fungal mould characterised by mycelia formation, is the most common causative agent of invasive aspergillosis (Chakrabarti *et al*., 2011). Aspergillosis remains a critical illness among patients with debilitating immune systems (Dagenais and Keller, 2009).

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Patients with prolonged neutropenia, advance acquired immunodeficiency syndrome or AIDS, as well as those infected with chronic granulomatous disease and recipients of hematopoietic stem-cell transplants or solid organ transplantation are high risk groups of acquiring invasive aspergillosis (Segal and Walsh, 2006). Various commercial antifungal preparations have been widely used to treat aspergillosis. Amphotericin B has been regarded as a gold standard for treatment of invasive fungal infections since the 1960s (Ostrosky-Zeichner *et al*., 2003; Gibbs *et al*., 2005). However, the adverse effects of amphotericin B including various mechanisms of nephrotoxicity have been described; consequently, impede the usage of this drug for antifungal therapy (Laniado-Laborín and Cabrales-Vargas, 2009).

Although there is a major shift in polyene therapy, with amphotericin B lipid preparations becoming the new "gold standard" in place of amphotericin B (Ostrosky-Zeichner *et al*., 2003), a number of reasons have hindered the use of lipid formulations as a drug of choice. These include cost and scarcity of published safety data to guide clinicians (Kleinberg, 2006). Thus, these issues presented a strong justification to search for an alternative therapeutic candidate which has fewer side effects as a substitute for commercially prepared antifungal drugs. To date, there are less than five reports in the literature on the potential antifungal activity of allicin against *A. fumigatus*, and the mechanism through which allicin suppresses the growth of this pathogenic mould has not been investigated previously. In view of the antifungal properties of allicin, and the lack of study on allicin in its ability to interfere with the growth of *A. fumigatus*, this study was carried out to determine the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of allicin against *A. fumigatus*, and to observe the effects of allicin on the fungal cell surface changes through scanning electron microscopy (SEM).

## MATERIALS AND METHODS

### Fungal strain

*A. fumigatus* ATCC 36607 was obtained from American Type Culture Collection (ATCC). This strain was maintained on potato dextrose slant agar (PDA) and subcultured every 6 months to ensure viability.

### Antifungal agent

Allicin was purchased from LKT laboratories, St Paul, USA (purity ≥ 98%, cat no: LKT-A4440-M005) and allicin was dissolved at the concentration of 4 mg/mL in a solvent mixture of methanol, water and formic acid (60:40:1) as described previously (Khodavandi *et al*., 2011).

### Inoculum preparation

Inoculum was prepared according to CLSI M38-A2 document (2008). *A. fumigatus* ATCC 36607 was grown on potato dextrose agar (Becton Dickinson, USA) for 48 h at 35 °C. Sporulating colonies were harvested by gently probing the colonies with pipette tips using a micropipette in the presence of 0.85% saline and three drops of Tween 20 (Nacalai Tesque, Japan) to facilitate the process. The resulting mixture of conidia and hyphal fragments were then transferred into a sterile tube and subsequently the density was adjusted at optical density ($OD_{530nm}$) of 0.09 to 0.13 (T60 UV Visible Spectrophotometer, USA). A further 1:50 dilution was made in RPMI medium (Sigma-Aldrich, USA).

### Broth microdilution method

About 0.1 mL of prepared allicin in a range of doubling concentration from 0.05-25.6 µg/mL was dispensed into a 96-well microtiter plate (Greiner Bio-One GmbH, German) in ascending concentration. Subsequently, 0.1 mL of standardised inoculum were dispensed into wells containing the allicin with final concentration of 0.4 to $5\times10^4$ CFU/mL which makes the final concentration of allicin ranging from 0.025-12.8 µg/mL. Growth control well contained standardised diluted inoculum and the drug diluents without allicin, whereas negative control contained only RPMI 1640 medium. The plate was incubated for 48 h at 35 °C. MIC was determined with unaided eye and quantitative data was reported spectrophotometrically at $OD_{530nm}$ using microplate reader (Dynex Technologies, USA). The percentage of growth inhibition at MIC was determined using the formula (Patton *et al*., 2006): Percent of inhibition = $1 - (OD_{\text{test well}} / OD_{\text{untreated control well}}) \times 100$.

### Determination of minimum fungicidal concentration (MFC)

Fungicidal activity of allicin on *A. fumigatus* was determined according to the method described by Espinel-Ingroff (1998), with slight modification. Twenty microlitres of aliquots were pipetted from optically clear wells that exhibited complete inhibition (growth similar to growth control well) and from growth control well. These suspensions were subcultured onto Sabouraud dextrose agar plates. The plates were incubated for 2 days at 35 °C or until growth was observed on growth control plates. The lowest drug concentration that demonstrated in either no growth or fewer than three colonies which inferred 99.9% of killing was term as MFC (Espinel-Ingroff, 1998).

### Scanning electron microscopy

*A. fumigatus* ATCC36607 inoculum was prepared as described previously (CLSI, 2008) with modifications in the total volume. One millilitre of standardised inoculum was treated with 1 mL of ¼ × MIC, ½ × MIC, 1 × MIC and 2 × MIC of allicin respectively. Untreated inoculum served as a growth control. Samples were incubated at 35 °C for 48 h. Each sample was placed into separate vials and fixed with 4% glutaraldehyde (Agar Scientific, UK) for 4 h at 4 °C. Samples were rinsed with 0.1 M sodium

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cacodylate buffer for 3 times, 10 min each. The samples were post-fixed in 1% osmium tetroxide

**Table 1**: Effect of allicin on *A. fumigatus* ATCC 36607 based on OD differences and its percentage of inhibition at MIC calculated normalize to growth control. Results are reported as means of three independent experiments done in triplicate.

| Fungal strain | OD | | | Percentage of inhibition (%) | Minimum fungicidal concentration |
|---|---|---|---|---|---|
| | Growth control[a] | Control[b] | Allicin[c] (3.2 mg/L) | | |
| *A. fumigatus* ATCC36607 | 0.349 ± 0.058 | 0.086 ± 0.001 | 0.079 ± 0.001[d] ($p$=0.046) | 77.4%[e] | 3.2 µg/mL |

[a] Fungus+broth
[b] Broth only
[c] Treated with allicin at 1×MIC
[d] Mean±SEM is significantly different ($p$<0.05) at 3.2 µg/mL allicin-treated cells in comparison with growth control
[e] Percentage of inhibition at MIC = [1-(0.079/0.349)] × 100

(Agar Scientific, UK) for 2 h at 4 °C. Samples were rinsed again with 0.1 M sodium cacodylate buffer followed by dehydrating in a series of different concentrations of alcohol starting with 20% until 100% (v/v) (ChemAR ® Systerm, Malaysia) and finally with 100% (v/v) acetone (Merck, USA). Samples were transferred into specimen basket and put into critical point dryer (Bal-tec, Germany) for 30 min. Coverslips with samples attached to it were then adhered onto the stub and coated with gold in sputter coater (Bal-tec, Germany) prior to viewing under SEM (LEO 1455 VPSEM) attached with energy dispersive X-ray (EDX).

**Time kill assay**

Effect of allicin towards *A. fumigatus* within 24 h was observed through time kill assay following the method described by Shadkchan *et al*., (2004), with slight modifications. *A. fumigatus* ATCC36607 was grown in the presence of 3.2 µg/mL allicin. This concentration was chosen because it was the concentration that inhibits the growth of *A. fumigatus* as determined by broth microdilution method (CLSI, 2008). Conidia was serially diluted in tenfold dilution after 0, 2, 4, 6, 8, 10, 12 and 24 h incubation before plated onto Sabouraud dextrose agar. The plates were incubated for 48 h at 35 °C. Colonies were counted and compared with untreated control at 0 h. Percentage of survival was plotted against incubation with allicin using GraphPad Prism Version 5.02.

**Statistical analysis**

Each experiment was performed in triplicate. Values were expressed as mean ± standard error of mean (SEM) and mean difference for each OD reading for the respective concentrations were compared with growth control by performing Mann-Whitney U for two independent samples test, using Statistical Package for the Social Sciences (SPSS) version 16.0 with $p$<0.05 deemed as statistically significant.

## RESULTS AND DISCUSSION

**Antifungal susceptibility testing and minimum fungicidal concentration**

The minimum inhibitory concentration of allicin was determined to be 3.2 µg/mL against *A. fumigatus* ATCC 36607, as observed visually. This was further supported by quantitative values which were based on OD reading. In comparison with growth control which did not contained allicin, there was a significant difference ($p$<0.05) in the OD reading in fungal cells treated with allicin at MIC concentration as shown in Table 1. At this concentration, percentage of inhibition was 77.4%. However, the MFC was also found to be 3.2 µg/mL.

Previous study by Shadkchan *et al*., (2004) reported both MIC and MFC for allicin on *A. fumigatus* was determined to be 8 µg/mL. Differences in MIC and MFC values between previous study and present study could be due to variation in the allicin preparations used in both studies which involved different synthesis and purification procedures. Indeed, various methods of synthesizing allicin had been reported previously, and this has contributed to differing grades and purities of allicin obtained (Dušica *et al*., 2011). Several techniques of allicin synthesis involve oxidation of allyl sulphide by hydrogen peroxide in acid medium by ESR spin trap method (Nikolić *et al*., 2004) and processing of dichloromethane solution of allyl disulfide by magnesium monoperoxy hydrate in the presence of ammonium-butyl sulphate (Cruz-Villalon, 2001).

Additionally, variation in the assay methods in determining the purity of the compound could be one of the key factors for the varying activities of allicin reported in different studies. In our study, the purity of allicin was ≥ 98% according to the manufacturer, which was assayed using UPLC (ultra performance liquid chromatography). In contrast, previous studies described that their source of allicin was produced by a reaction between the synthetic

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substrate of alliin with allinase through an immobilised allinase column and assayed using high performance liquid chromatography (HPLC). UPLC provides significant advances compared to conventional HPLC due to its rapid operation time, higher sensitivity and resolution (Srivastava *et al*., 2010).

**Morphological changes**

Apart from the antifungal susceptibility data obtained, the effect of allicin against *A. fumigatus* ATCC 36607 was also examined morphologically through SEM at ¼ × MIC, ½ × MIC, 1 × MIC and 2 × MIC. Perturbation to the cell surface morphology could be seen when *A. fumigatus* ATCC 36607 was treated with various concentrations of allicin. Scanning electron microscopy images captured at 10,000X and 1000X magnification exhibited significant changes in treated *A. fumigatus* hyphae and conidial morphology compared with growth control.

Untreated growth control of *A. fumigatus* was characterised by smooth cell surface of septate hyphae with a few branches and linear, elongated mycelia with apparently consistent diameter throughout the length of the hyphae as shown in Figures 1 (a) and 2 (a). However, allicin-treated *A. fumigatus* in Figure 1(b) exhibited uneven and rough surface which denote the effect of treatment at ¼ × MIC. In marked contrast, allicin-treated *A. fumigatus* in Figure 1 (c) shows irregular shape of hyphae with irregular constriction and bulging along the length of the hyphae. In addition, the cell surface of hyphae was observed to be crinkled, patchy and uneven in which it might indicate possible cell wall or cell membrane disruption when *A. fumigatus* was treated with allicin at this concentration (½ × MIC). When examined under lower magnification at 1000X, fewer mycelia were observed as shown in Figures 2 (b) and 2 (c), with distorted and wavy shape at certain areas of the hyphae. Complete inhibition of hyphal growth was observed in *A. fumigatus* when treated with both 1 × MIC and 2 × MIC as illustrated in Figures 1(d), (e) and Figure 2 (d), (e). Complete disruption in morphological of conidia could be seen in Figures 1(e) and 2 (e).

Scanning electron microscopy observation indicated that treatment of *A. fumigatus* at 3.2 µg/mL (1 × MIC) and 6.4 µg/mL (2 × MIC) of allicin has impeded the transformation of hyphae from conidia. This could be due to anti-hyphal properties of allicin at these concentrations, which subsequently led to complete absence of formation of hyphae. Hyphae formation and penetration into host tissue are primarily associated with pathogenesis of invasive aspergillosis. When conidia are being inhaled by immunocompromised patients or those with weakened immune system, the conidia will initially colonise and invade pulmonary epithelium, which will eventually germinate into invasive hyphae. Subsequently, angioinvasion takes place in which hyphae penetrate blood capillaries. Simultaneous dissemination of hyphal fragment as well as other toxic molecule finally leads to dissemination in deep organ (Askew, 2008; Abad *et al*., 2010). Hence, suppression of hyphae formation by allicin highlights the potent anti-hyphae activity of this compound and thus underlies its potential use for preventing the development of organ-invasive aspergillosis. It would be interesting to investigate whether allicin could exhibit a similar degree of anti-hyphae property against *A. fumigatus* in an in vivo model utilising a mouse model of invasive aspergillosis.

In relation to this finding, Yoshida *et al*. (1987) has reported the effect of ajoene, one of the bioactive compounds derived from garlic on the ultrastructure of *Aspergillus niger*. Treatment with ajoene at the inhibitory concentration of 20 µg/mL demonstrated surface depression or flat ribbon-like structure. Our present study found that allicin at a lower inhibitory concentration than that reported for ajoene, which is 3.2 µg/mL, was able to exert complete inhibition of hyphae formation in *A. fumigatus*. Hence, this finding underscores the potent activity of allicin on the filamentous fungus. In this study, the irregular shape of hyphae with uneven surface featuring constriction and bulging appearance along the hyphae were clearly seen in *A. fumigatus* treated with ¼ × MIC and ½ × MIC. However, the underlying mechanism through which allicin acts to cause these morphological changes is still unclear. It could possibly be due to a disruption in the homeostasis process which leads to a defect in hyphae morphology, or it could also be attributed to the ability of allicin to target a hypha-specific cell wall components or inhibition of sulfhydryl metabolic enzymes as previously reported (Willis, 1956).

In addition, the effects of micafungin, a commercially prepared antifungal drug on *A. fumigatus* have been described by Nishiyama *et al*. (2005). Treatment of *A. fumigatus* with various concentrations of micafungin ranging from 0.001 - 0.1 µg/mL, within 2 - 5 h incubation period has contributed to changes in hyphal morphology. Short branches, with rough, wrinkled surface and subsequently disruption of hyphae were observed at various concentrations of treatment within the incubation period. Although the study demonstrated that lower micafungin concentration resulted in defects in hyphal morphology, direct suppression of hyphae formation by this drug against *A. fumigatus* was not unambiguously portrayed. This could be due to the nature of the experiment that was being carried out after most of the conidia had germinated into hyphae. Germination of conidia into hyphae is the main culprit in pathogenesis of invasive aspergillosis and hence treatment should be done before the germination process occur. Thus, the previous finding was dissimilar with our study which pinpoints the effect of allicin on conidia treated with allicin before they had germinated into hyphae.

**Time kill assay**

After 10 h incubation with allicin, the growth of *A. fumigatus* ATCC36607 had decreased to half as shown in the time kill curve in Figure 3, and the growth diminished gradually after 12 h and began to plateau after 24 h. However, there was a small fluctuation in the decreasing trend of growth within the first 12 h of incubation.

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Phenotypically, colonies that survived after the cells had been treated with allicin appeared smaller in contrast with

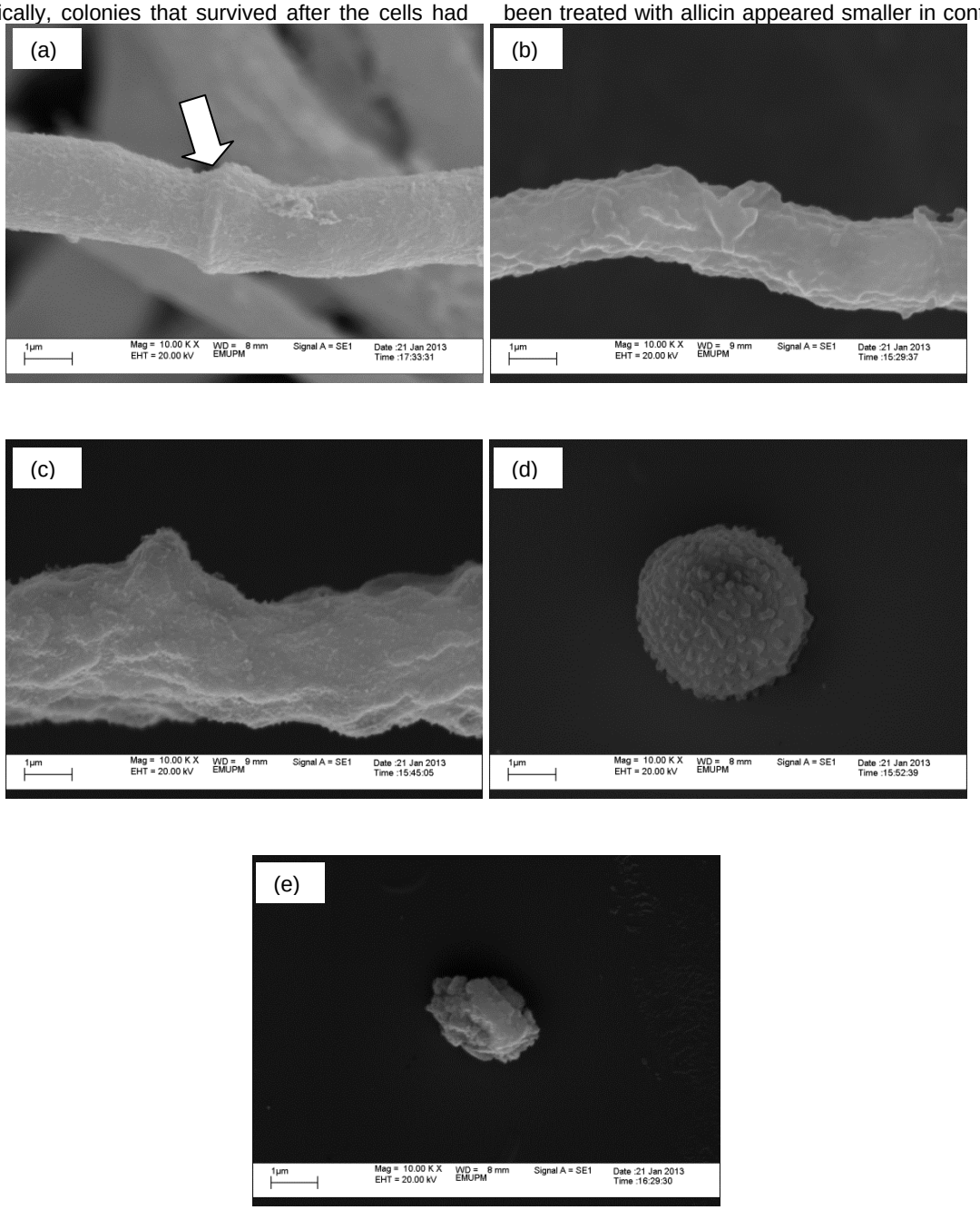


**Figure 1:** Scanning electron micrographs of *A. fumigatus* surface ultrastucture observed after 48 h incubation with allicin. (a) Growth control, (b) *A. fumigatus* treated at ¼ × MIC, (c) ½ × MIC and (d) 1 × MIC (e) 2 × MIC. Magnification × 10,000. Septate hyphae can be observed in growth control as indicated by an arrow symbol.

colonies in untreated control which appeared larger and were clumped together (data not shown). Colony counting was feasible post-treatment with allicin but not for the untreated control at the extended time points of growth due to the formation of clustered colonies.

On contrary, Shadkchan *et al*. (2004) reported that *A. fumigatus* started to display reduction in growth after 8 h incubation. This discrepancy in both findings could be due to the difference in fungicidal concentration of allicin

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treated upon *A. fumigatus* in which we observed the effect of 1 × MIC in present study whereas the previous study

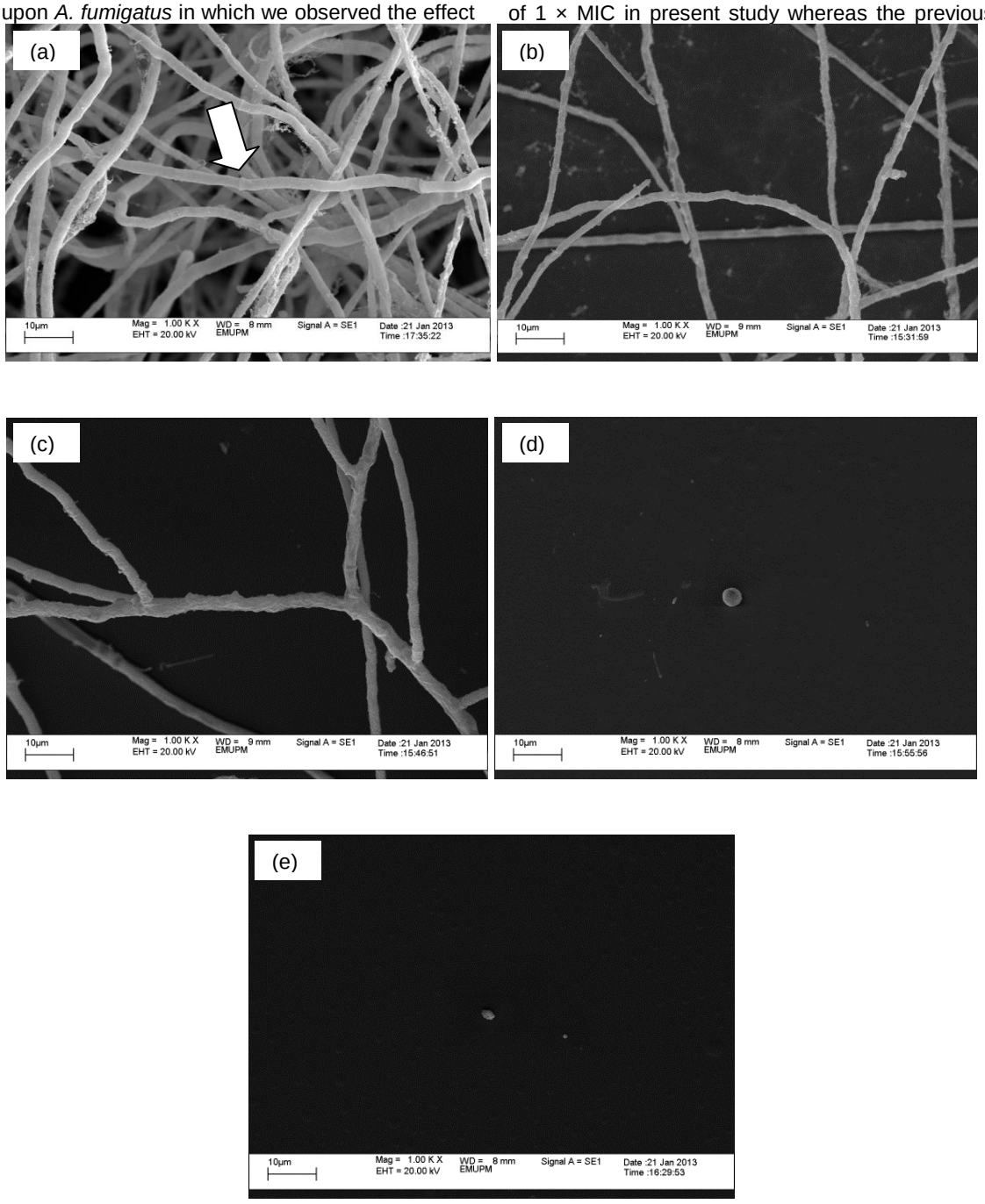


**Figure 2:** Scanning electron micrographs of *A. fumigatus* surface ultrastucture observed after 48 h incubation with allicin. (a) Growth control, (b) *A. fumigatus* treated at ¼ × MIC, (c) ½ × MIC and (d) 1 × MIC (e) 2 × MIC. Magnification × 1000. Septate hyphae can be observed in growth control as indicated by an arrow symbol.


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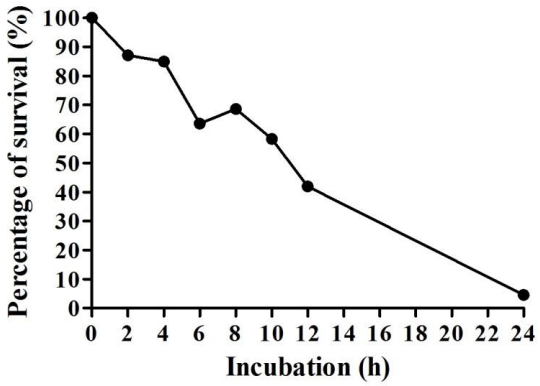


**Figure 3:** Percentage of *A. fumigatus* ATCC 36607 survival (%) against incubation time with allicin (h). Results are representative of three independent experiments performed in triplicate. Growth percentages were calculated by dividing average colonies counted for each selected time point with untreated control at 0 h.

examined the allicin concentration at two-fold above MFC which is 2 × MFC. Taken together, the findings from both studies denote that a higher concentration of allicin decreases the time taken for the fungicidal effect to be exhibited. In time kill assay, time exposure and ability of conidia to be permeated and subsequently eradicated by antifungal compound play a part in the fungicidal activity (Hammer *et al*., 2002).

## CONCLUSION

In conclusion, the anti-hyphal and growth inhibitory properties of allicin against *A. fumigatus* could be observed through broth microdilution test, SEM and time kill assay. More studies need to be done to establish the efficacious range of concentration for allicin *in vivo* and to rule out any possible toxicity to humans prior to any human clinical trial before it can be developed as an antifungal agent on par with commercial antifungal drugs such as amphotericin B and fluconazole. On the other hand, further fundamental investigations could include ultrastructural observation of internal components of cells under transmission electron microscopy which would denote changes in cytosolic components, cell membrane and cell wall structure post-treatment with allicin. In addition, elucidation of the mechanism of action of allicin through quantifying the expression level of targeted genes that are implicated in hyphae-specific cell well related genes could further bolster the premise of allicin as an alternative antifungal agent.

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