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ORIGINAL ARTICLE

The antimicrobial and antibiofilm properties of allicin against *Candida albicans* and *Staphylococcus aureus* – A therapeutic potential for denture stomatitis

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KEYWORDS

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Abstract The objective of this study is to determine the therapeutic efficacy of allicin against *Candida albicans (C. albicans)* and *Staphylococcus aureus* (*S. aureus*), the common etiological agents for denture stomatitis (DS). The minimum inhibitory concentration (MICs), minimum bactericidal concentrations (MBCs) and minimum fungicidal concentration (MFCs) of allicin were determined by the broth microdilution method followed by checkerboard microdilution method for a synergistic interaction between allicin + nystatin and allicin + CHX. The potential of allicin to eradicate *C. albicans* and *S. aureus* biofilms was assessed by treating biofilm formed on self- polymerized acrylic resin with allicin at a sub-MIC concentration for 5 min. The commercial denture cleanser (brand X) was used as a positive control. A Kruskal-Wallis test followed by the post-hoc Mann-Whitney *U* test was applied (SPSS 20.0), and the level of significance was set at $P < 0.05$. Allicin exhibited antimicrobial activity against *C. albicans* (MIC:8 µg/ml and MFC:16 µg/ml) and *S. aureus* (MIC:8 µg/ml and MBC:8 µg/ml). A synergistic interaction was observed between allicin + nystatin and allicin + CHX (FICI $\leq$ 0.5). Allicin exhibited significant biofilm eradication against *C. albicans* and *S. aureus* biofilms with percentages of 50.0% and 52.6%,

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respectively. The results of this study suggest a possible application of allicin in treating *C. albicans* and *S. aureus* infection in DS.

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1. Introduction

Denture stomatitis (DS) or also known as chronic erythematous candidiasis, is a type of disease that mostly affects the palatal mucosa. (Akpan and Morgan, 2002; Aoun and Berberi, 2017). The DS is characterized by inflamed mucosa, burning sensation, and pain, particularly under the upper denture and alteration in taste sensation. The risk of developing DS in those who are wearing a denture is up to 36.7% (Emami et al., 2012) and increase up to 65% in those who wear complete dentures (Frenkel, 2000, Lemos et al., 2003). The predisposing factors are continuous denture wearing, dental trauma, decreased salivary flow, denture hygiene, type of denture base material, denture age, cellular immunity of the wearer, smoking, dietary factors and changes in oral microbiota (Coco et al., 2008, Gasparoto et al., 2009).

One of the etiological factors that trigger DS is the presence of biofilm on the surface of the denture. Denture biofilm composed of a complex mixture of pathogenic and opportunistic bacteria, fungi, desquamated epithelial cells and biofilm matrix (Glass et al., 2001). This biofilm matrix created a physical barrier and acted as a reservoir of protection for oral microorganisms like *C. albicans* that is considered to be the primary etiological agent in the pathogenesis of DS (Ramage et al., 2004). The *C.albicans* has an adhesive property towards the acrylic resin (Salerno et al., 2011). Furthermore, the anaerobic microenvironment between the denture and palatal mucosa further supplements the growth of Candida (Budtz-Jörgensen, 1990). The other significant colonization factors of Candida are due to coaggregation with pre-attached microorganisms on the denture and oral mucosa, such as *S. aureus,* which also known as bacterial-yeast interaction (Coco et al., 2008). *S. aureus* was usually isolated together with *Candida* from dental prostheses of individuals with DS (Monroy et al., 2005; Pereira et al., 2013). The *C.albicans* and *S.aureus* will not only colonize the surface that has contact with oral mucosa but also adhere to the cracks and imperfections of the denture material (Ramage et al., 2004). Ultimately, dentures may act as a pathogen reservoir and provide a source of continued mucosal exposure to microorganisms (Gendreau and Loewy, 2011).

The mutualistic *C. albicans–S. aureus* relationship was reported to alter phenotypic of the microorganisms, induce drug tolerance (Todd and Peters, 2019; Kean et al., 2017), and form a unique biofilm architecture (Peters et al., 2010), which enhance the disease severity. It was reported that the *C. albicans-S.aureus* biofilm displayed synergistic pathogenicity and also reduced the sensitivity to antifungal miconazole (Kean et al., 2017). The mechanism for the increased tolerance to the antibiotic was reported to cause by the upregulation of drug efflux pumps in *S.aureus* following sequential exposure to farnesol the quorum sensing (QS) molecule of *C. albicans* (Todd and Peters, 2019). Zago et al., (2015) also reported the enhanced secretion of hydrolytic enzymes, secreted aspartyl proteinase (SAP) in co-culture of *C.albicans* and *S.aureus* compared to the monospecies culture of *C. albicans*. SAP is one of the *C. albicans*' virulence factors that contribute to their proteolytic activity and play an essential role in host tissue adherence and colonization (Kumar et al., 2015).

DS has been recognized for long periods; however, no treatments have been suggested as the best treatment of choice due to the reported relapse cases (Martins and Gontijo, 2017). DS has a multifactorial aetiology; thus, treatments are done by elimination of predisposing factors. For DS caused by Candida infection, brushing, washing with commercial cleanser products and using disinfectants such as chlorhexidine and sodium hypochlorite are highly recommended for denture cleaning procedures. However, the efficacy of commercial denture cleansers in the removal of fungi is doubtful (Felton et al., 2011). Failure in ensuring denture hygiene contributes to DS recurrent case even following the treatment regimen. The specific antifungal agents such as amphotericin B, nystatin, miconazole or clotrimazole also used locally or systemically to cease candida from developing. (Walsh et al., 2015).

Nystatin is the most commonly prescribed antifungal agent for the treatment of oral candidiasis and DS (Tay et al., 2014) besides other antifungals such as miconazole and fluconazole. However, there have been some side effects related to the use of anti-fungal agents, such as nausea, vomiting, diarrhoea (Walsh et al., 2015), fungal resistance, drug toxicity (Tay et al., 2014, Lyu et al., 2016) and relapse cases (Martins and Gontijo, 2017). Meanwhile, CHX is the common antimicrobial agent that has been widely prescribed in dentistry as an antiseptic mouthwash (Ellepola and Samaranayake, 2000). CHX drug prescription is available in the dental clinic or can be purchased over-the-counter (OTC) at the pharmacies (Ingram et al., 2008). The formulation of mouth rinse, which consists of 0.2% chlorhexidine gluconate, was successfully used to treat Candida-associated denture stomatitis as well as acute pseudomembranous candidiasis. CHX with higher concentration which is 2%, is recommended for overnight denture disinfectant (Ellepola and Samaranayake, 2000).

Researchers are actively investigating the alternative therapeutic agents for management and treatment of DS derived from natural sources such as plant extract or phytocompounds. Consideration of the natural products to be investigated are; elicit the same therapeutic effects as commercial drugs, safe and fewer side effects to the patients (Ramage et al., 2004, Bakri and Douglas, 2005). Garlic (*Allium sativum L.*) is one of herbal medicine which is known to have medication benefits, such as antibacterial, anti-fungal, and antiviral properties (Bakri and Douglas, 2005). For this study we used, allicin a phytocompound derived from garlic, which was reported to exhibit prominent antifungal effects (Kim et al., 2012).

This study aimed to assess the synergistic/additive antimicrobial effect of allicin with nystatin on *C. albicans* and allicin with CHX on *S. aureus*. We also investigated the therapeutic efficacy of allicin in removing *C. albicans, and S. aureus*

preformed biofilm on self-cured resin cubes within 5 min exposure to mimic the clinical application of using denture cleanser (express formula) as denture disinfectant.

2. Material and methods

2.1. Preparation for standard *C. albicans* and of *S. aureus* inoculum

The stock culture of *C. albicans* ATCC 14053 was inoculated initially into 30 mL of sterilized Yeast Peptone Dextrose (YPD) broth (Oxoid Ltd, Basingstoke, Hampshire, UK), accordingly, to form the *C. albicans* suspension. The concentration of the *C. albicans* in the study was adjusted to a final concentration of 0.5 McFarland (1.5×10^6 CFU/mL) before the experiment.

The *S.aureus* suspension was prepared by inoculating the stock culture of *S. aureus* ATCC 29213 into 30 mL of sterilised Brain Heart Infusion (BHI) broth (Oxoid Ltd, Basingstoke, Hampshire, UK). The concentration of the *S. aureus* in the study was adjusted to a final concentration of 0.5 McFarland (1.5×10^8 CFU/mL) before the experiment.

2.2. Preparation of allicin and other reagents

Allicin was purchased from AK Scientific, Inc (US). A stock solution of 2048 μg/ml of allicin was prepared in 5% dimethyl sulfoxide (DMSO) and stored at 4°C until further use. The 5-Min Express Denture Cleanser (Brand X) in the form of a tablet was purchased from the local pharmacy (OTC) and was freshly prepared according to manufacturer's instruction on the packaging, 10 min before the test. The Brand X denture cleanser contained potassium monopersulfate as one of the active ingredients which also acts as a disinfectant. The CHX $\leq$ 99.5% and nystatin were purchased from Sigma-Aldrich and were diluted for the application in the experiment.

2.3. Minimum inhibitory concentration (MIC), minimum fungicidal concentration (MFC) and minimum bactericidal concentration (MBC)

The MIC, MBC, and MFC of allicin against *C. albicans* and *S. aureus* were determined using the broth microdilution method outlined by the Clinical and Laboratory Standard Institute (CLSI) with minor modifications (CLSI 2008, CLSI 2010).

2.4. Checkerboard microdilution assay

The synergism activity between allicin + nystatin and allicin + CHX was assessed using a checkerboard microdilution assay according to the method described elsewhere (Cuenca-Estrella, 2004; Haroun and Al-Kayali, 2016). The final concentration after the addition of 100 μL of inoculum in 96-well U-bottom microplates ranged between 0.03% and 1.92% for CHX, 2 μg/mL to 128 μg/mL for nystatin and 1 μg/mL to 128 μg/mL for allicin. The inoculum was prepared at a final concentration of 5×10^6 CFU/mL per well. The cells were incubated at 35°C for 24–48 h. The effects of the combinations of antimicrobial agents were interpreted by the fractional inhibitory concentration index (FICI) based on Loewe additivity (LA) theory as synergistic (FICI $\leq$ 0.5), additive or indifferent ($0.5 <$ FICI ≤ 4.0) or antagonistic (FICI > 4.0).

$$\text{FICI equation}: \text{FICI} = \text{FICA} + \text{FICB} = (\text{MIC}_A{}^{\text{comb}}/\text{MIC}_A{}^{\text{alone}}) + (\text{MIC}_B{}^{\text{comb}}/\text{MIC}_B{}^{\text{alone}})$$

2.5. Biofilm eradication assay

2.5.1. Fabrication of self-cured acrylic resin cubes

The self-curing (SC) acrylic resin cubes used in this study were also used by Lee et al. (2009). Ninety acrylic resin cubes (8 mm square by 2 mm thick) were prepared for biofilm eradication assay of *C. albicans* (n = 45) and *S. aureus* (n = 45). The SC acrylic resin cubes were prepared following the manufacturer's recommendations ((PalaXpress auto polymerising acrylic resin, Heraeus Kulzer). The resins were allowed to polymerise in a pressure pot at temperature 55°C (131°F), pressure: 200 kPa for 30 min. After polymerization, the cubes were removed from the matrix mould and were polished using 150-, 300-, 600-, and 1200-grit sandpapers (3 M/ESPE, Sumaré, Brazil). The acrylic resin cubes were then stored in distilled water at 37°C for 48 h to remove excess monomer. After storage for 48 h, the quality of the polishing of acrylic resin cubes was checked by a profilometer (Ambios Technology) and specimens size were measured by digital calliper to standardize the specimens and to homogenize the groups. The cubes that had roughness between 2.7 and 3.7 μm were selected for the following tests (Panariello et al., 2015). Immediately before use in the experiment, cubes were sterilized using an ultraviolet light unit for 10 min.

2.5.2. Biofilm eradication assay

The subMIC concentration allicin (4 μg/mL) was used in the biofilm eradication assay. The cubes were randomly divided

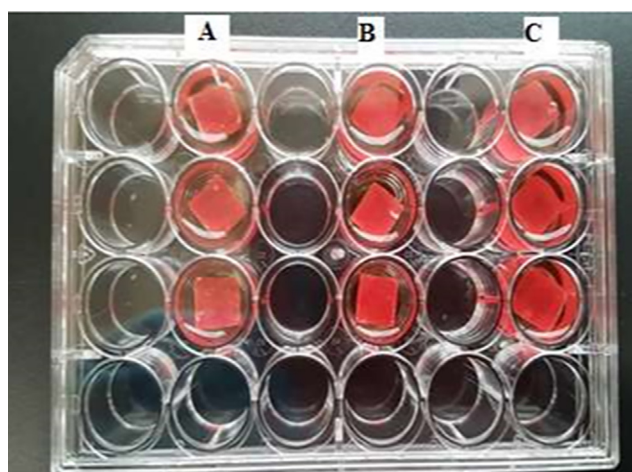

Fig. 1 Self-cured acrylic resin cubes (8 mm × 8 mm × 2 mm) with *S. aureus* biofilm were treated for 5 min in 24-well flat-bottom microplate containing 1 mL of sterile distilled water (A), 1 mL of 4 μg/mL allicin (B) and 1 mL of denture cleanser (Brand X) (C). Similar steps were repeated for *C. albicans*.

into three groups, and fifteen cubes were subjected to each group. For the formation of preformed biofilm, cubes were placed in 6-well flat-bottom plate and immersed with 6 mL of standard *S. aureus* suspension (0.5 McFarland) and incubated at 37°C for 24 h. After 24 h, using a sterile forceps, the cubes with preformed biofilm were taken out from the incubation well. Remaining broth and loosely attached bacteria were removed by gently tapping on a sterile absorbent paper in an empty well. The resin cubes were then placed in a new 24-well flat-bottom plate (one cube per well). 1 mL of allicin, denture cleanser (brand X) and sterile distilled water (control) were pipetted into the well according to the group, and the cubes were incubated for 5 min (Fig. 1). The solutions were then removed, and 1 mL of 0.1% crystal violet was pipetted into the wells and left for 15 min. After staining, the cubes were slowly immersed in distilled water to remove loosely-bound cells and placed on a paper towel to be air-dried for 15 min or more. Next, the stained cubes were transferred into a new 24 wells plate, and the bound dye was extracted from the stained cells using 1 mL of 95% ethanol. 700 μL of the de-staining solution was then transferred to the next well. By using the crystal violet (CV) assay, biofilm disruption was determined and quantified by measuring the absorbance of the solution at 550 nm in a microplate reader (Tecan′s *Infinite ® 200 PRO*).

The percentage of biofilm eradication (on established biofilm) was calculated as:

$$\text{Biofilm eradication (\%)} = [[\text{Control OD}_{550nm} - \text{Test OD}_{550nm}] / \text{Control OD}_{550nm}] \times 100$$

The similar procedures were repeated for *C. albicans*. Each species was tested for biofilm eradication in triplicates, and the assay was repeated five times.

2.6. Statistical analysis

Statistical analysis was carried out using the Statistical Package for Social Sciences Software (SPSS version 20). The differences between groups were evaluated using Kruskal Wallis and Mann Whitney test. Data obtained was expressed as the mean ± standard deviation. The significance level was set up at $p < 0.001$.

3. Results

3.1. Minimum inhibitory concentration (MIC), minimum fungicidal concentration (MFC) and minimum bactericidal concentration (MBC)

The antimicrobial activity of allicin against *C. albicans* and *S. aureus* was shown in Table 1. The MIC or the lowest concentration of allicin, which prevents visible growth of *C. albicans* after 24 h incubation in microwell culture plates were 8 μg/mL. The MFC or lowest allicin dilutions causing approximately 99–99.5% killing activity of *C. albicans* was 16 μg/mL. The MIC and MBC of allicin against *S. aureus* were recorded at the same concentration, 8 μg/mL. Thus, the MIC/MBC ratio of allicin against *S. aureus* was calculated as 1.

3.2. Checkerboard microdilution assay

The synergism between allicin and nystatin against *C. albicans*, allicin, and CHX against *S. aureus* was assessed via checkerboard microdilution assay. The following standard classified the effect of the combinations of antimicrobial agents (1) FICI ≤ 0.5, synergistic effect; (2) 0.5 < FICI ≤ 4.0, additive or indifferent; and (3) FICI > 4.0, antagonistic. The results show that allicin and nystatin, in combination, expressed a synergistic activity against *C. albicans* with FICI = 0.5. (Table 2) The synergistic activity was also recorded between allicin and CHX against *S. aureus* with FICI = 0.375. (Table 2)

3.3. Biofilm eradication assay

As for the biofilm eradication assay, the results are shown in Fig. 2. The percentage of *C. albicans* biofilm eradication by allicin (4 μg/mL) was 50.00 ± 1.45%, whereas for *C. albicans*

Table 1 The antimicrobial activities (MIC, MBC, and MFC) of allicin against *S. aureus* and *Candida albicans*.

Organism	*C. albicans* ATCC 14053		*S. aureus* ATCC 29213	
Measurement parameter	MIC	MFC	MIC	MBC
Antimicrobial concentration (μg/mL)	8	16	8	8

Table 2 Antimicrobial activity of allicin, nystatin, and CHX and combination of allicin/nystatin against *C. albicans* and combination of allicin/CHX against *S.aureus* showing minimum inhibitory concentration (MIC), fractional inhibiting concentrations (FIC) and FIC index (FICI).

Microorganism	Agent	The MIC of each agent (μg/mL)		FIC	FICI	Outcome
		Alone	Combination			
S. aureus	CHX	0.96	0.12	0.125	0.375	Synergism
	allicin	8	2	0.25		
C.albicans	nystatin	8	2	0.25	0.5	Synergism
	allicin	32	8	0.25		

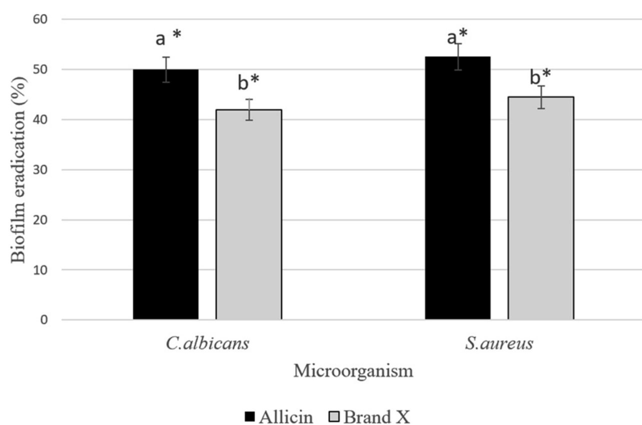

Fig. 2 The efficacy of allicin to eradicate *C. albicans and S. aureus* biofilm at 4 µg/mL concentration (sub-MIC). The biofilm eradication percentage was denoted as the mean ± standard deviation (SD). An asterisk indicates significant differences in mean percentages were compared to the negative control ($p < 0.001$) according to the non-parametric Kruskal Wallis test with Mann Whitney. Different letters indicate statistically significant differences between groups.

biofilm treated with Brand X was 41.99 ± 1.69%. For *S. aureus*, the biofilm eradication following 5-minutes exposure to allicin and Brand X was 52.56 ± 0.9% and 44.46 ± 1.1%, respectively. Similar to the activity against *C. albicans* biofilm, allicin also significantly dispersed *S. aureus* biofilm on self-cured acrylic resin cubes compared to a positive control (brand X denture cleanser).

4. Discussion

In this study, an approach was made to determine the therapeutics value of allicin, one of the phytocompounds in garlic (*Allium sativum L.)*. The use of plant extracts as antimicrobial agents was widely reported; however, there is still limitations of studies using phytocompounds against *C.albicans* and *S.aureus*. Among the phytocompounds reported exhibiting antimicrobial activity against oral microorganisms were thymol (Belato et al., 2018), glabridin and licochalcone A (Messier and Grenier, 2011).

The antimicrobial and antibiofilm activities of allicin, against *C. albicans* and *S. aureus* biofilm, were initially evaluated by determination MIC + MFC and MIC + MBC values. The broth microdilution method is a quantitative miniaturization concept with lower cost, yet still, it provides reproducible results (Reller et al.,2009). MIC is the gold standard method to measure the lowest concentration of antimicrobial components to kill animals and human pathogenic bacteria (Andrews, 2001).

The results showed that allicin exhibited excellent antimicrobial activity against *C. albicans* and *S. aureus* with a MIC of 8 µg/mL for both *C. albicans* and *S. aureus*, MBC of 8 µg/mL for *S. aureus* and MFC of 16 µg/mL for *C. albicans*. MIC and MBC values obtained indicate the bactericidal property of allicin against *S. aureus*. This is supported by Tripathi (2013). Furthermore, our results are in agreement with a previous study done by Khodavandi et al. (2010). As for MFC, the values obtained in this study are consistent with a study conducted by Shadkchan et al., (2004), the antifungal ability of allicin against *Aspergillus spp*. A previous study also documented the antibacterial activity of allicin against other bacterial species such as *Salmonella, Escherichia coli, Streptococcus* spp., certain parasites, and fungi (Ankri and Mirelman, 1999). Allicin has a chemical property to react with free thiol groups, through the thiol-disulfide exchange. Therefore, the antimicrobial activity of allicin is thought to be mainly due to interaction with thiol-containing enzymes, including alcohol dehydrogenases and cysteine proteases. These enzymes are essential for the nutrition and metabolism of bacteria.

Allicin's antifungal activity was thought to be due to the ability of allicin to invade the cellular membrane of *C. albicans* and organelles. Under electron microscope observation, this penetration results in host cell destruction and death (Lee et al.,2011). Apart from the cell-killing effect towards *C. albicans*, Shadkchan et al. (2004) reported the significant ($P < 0.001$) antifungal ability of allicin, which caused ten times inhibition of *Aspergillus fumigatus* in infected mice.

A synergistic interaction was recorded between allicin + nystatin and allicin + CHX. Allicin's synergistic effects with other antifungal agents were also reported in other studies such as azoles (Khodavandi et al.,2010) and amphotericin B (An et al.,2009).

In the present work, we have evaluated the performance of allicin to eradicate *S. aureus,* and *C. albicans* preformed biofilm in monospecies culture. Commercial denture cleanser (Brand X) was used as a positive control because it is easily purchased at the pharmacy and one of the common brands in the market. In this study, the self-cured resin was used because the attachment of Candida to self-cured acrylic resin was reported higher compared to heat-cured acrylic resin (Kalla et al.,2011)

The antibiofilm efficacy of allicin against *C. albicans* and *S. aureus* biofilm was confirmed because the compound had significantly ($p < 0.001$) dispersed >50% of the biofilm upon short exposure (5 min). Interestingly, in this study, from the post-hoc statistical analysis, we managed to demonstrate that biofilm eradication by allicin is significantly more effective than Brand X cleanser. The findings of another study also showed the ability of allicin to disperse biofilm and exhibited antibiofilm activity against *E. coli* (Barbieri et al.,2007). The concrete and exact modes of biofilm eradication by allicin are not yet understood. However, since allicin is a hydrophobic compound, it can easily penetrate the structure of exopolysaccharides (EPS) in the biofilm and interact with microbial molecules (Miron et al., 2000).

Based on the results, we determined the antimicrobial and antibiofilm activity of allicin, which might have beneficial effects on DS alternative treatment. Due to antimicrobial activity, allicin has the potential to be used as a topical treatment on the infected mucosa surface. It can also be included as one of the active ingredients in the denture cleanser due to its ability to remove the biofilm following short exposure. Further tests are required to explore the anti-biofilm property of allicin by employing a multi-species biofilm model and determination of toxicity dose against the oral mucosal cell. Future exploration could also include determination of safe dosage of allicin applicable for a clinical application through *in vivo* study.

5. Conclusions

In conclusion, our findings indicated the antimicrobial effects of allicin against *S. aureus* and *C.albicans* and the efficacy to disperse the preformed biofilms. Thus, their antimicrobial and antibiofilm activities could present them as a promising alternative for future clinical application of managing and treating oral candidiasis and *S. aureus* infection related to DS patients.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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