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A novel, quorum sensor-infused liposomal drug delivery system suppresses *Candida albicans* biofilms

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Abstract

In contrast to the plethora of antibacterial agents, only a handful of antifungals are currently available to treat *Candida albicans* biofilm-associated infections. Additional novel antibiofilm strategies to eliminate *C. albicans* biofilm infections are needed. This study aims to improve the efficacy of a widely used azole, fluconazole by co-delivering it with a *Pseudomonas aeruginosa* quorum sensing molecule (QSM), N-3-oxo-dodecanoyl-L-homoserine lactone (C12AHL) in a liposomal formulation. C12AHL is known to inhibit *C. albicans*' morphological transition and biofilm formation. Four different formulations of liposomes with fluconazole (L-F), with C12AHL (L-H), with fluconazole and C12AHL (L-HF), and a drug-free control (L-C) were prepared using a [thin-film hydration followed by extrusion method](#), and characterised. The effect of liposomes on colonising (90min-24h) and preformed (24h) *C. albicans* biofilms were assessed using a standard biofilm assay. Biofilm viability (XTT reduction assay), biomass (Safranin-O staining) and architecture (confocal laser scanning microscopy, CLSM) were determined. Similar efficiencies of fluconazole entrapment were noticed in L-HF and L-F (11.74% vs 10.2%), however, L-HF released greater quantities of fluconazole compared to L-F during 24h (4.27% vs 0.97%, $P < 0.05$). The entrapment and release of C12AHL was similar for L-H and L-HF liposomes ([33.3% vs 33% and 88.9% vs 92.3% respectively](#)). L-HF treated colonising, and preformed biofilms exhibited >80%, and 60% reduction in their respective viabilities at a fluconazole concentration as low as 5.5µg/mL compared to 12% and 36%, respective reductions observed in L-F treated biofilms ($P < 0.05$). CLSM confirmed biofilm disruption, lack of hyphae, and reduction in biomass when treated with L-HF compared to other liposomal preparations. Liposomal co-delivery of C12AHL and fluconazole appears to suppress *C. albicans* biofilms through efficacious disruption of the biofilm, killing of constituent yeasts, and diminishing their virulence at a significantly lower antifungal dose. Therefore, liposomal co-formulation of C12AHL and fluconazole appears to be a promising approach to improve the efficacy of this common triazole against biofilm-mediated candidal infections.

Key words: *Candida albicans*; fluconazole; N-(3-oxo-dodecanoyl)-L-homoserine lactone; biofilms; antifungal resistance; liposomes

1. Introduction

Naturally existing complex microbial communities, known as biofilms, have a significant impact on human health and disease. Biofilms are thought to provide an important first line defence against the host from invasive pathogens, although, their transformation from an eubiotic commensal state to a dysbiotic state is estimated to trigger over 65% of all human infections (NIH and CDC)(Lewis, 2001).

Candida, a major fungal component of the human microbiome, is a dimorphic yeast that inhabits human oral cavities, gastrointestinal tract, skin and genital tract as a commensal organism (Kim and Sudbery, 2011). Such ubiquitous presence of *Candida* in a variety of host niches is testament to its versatility and adaptability to co-habit within an adversarial human host (Calderone and Fonzi, 2001). When the host immunity is impaired, *Candida* becomes pathogenic and causes chronic superficial mucosal infections such as oral candidiasis, to invasive and life-threatening systemic candidiasis (Bandara and Samaranayake, 2019; Sardi et al., 2013). The pathogenicity of *Candida* spp. is attributed to its diverse virulence factors such as the ability to adhere to host tissues, the formation of hyphae (filamentation), the development of complex and robust biofilms, and the production of tissue-damaging enzymes (Silva et al., 2009).

Eradication of pathogenic biofilms has become an overwhelming economic burden as well as persistent healthcare challenge due to their remarkable resistance to conventional antimicrobial agents (Hall and Mah, 2017; Silva et al., 2017). For instance, even with contemporary antifungals, *Candida* biofilm infections carry an alarming crude mortality, as high as 40%, in systemic candidiasis due to incomplete elimination of the yeast and the emergence of resistant phenotypes (Crump and Collignon, 2000). Despite ongoing advances in drug development, only a handful of antimicrobials are currently approved for the treatment of fungal infections (*i.e.* polyenes, pyrimidines, azoles and allylamines) (Pappas et al., 2004). Of these, azoles (*e.g.* fluconazole, miconazole) are the most widely used due to their higher margin of safety and lower incidence of adverse effects when compared to other antifungals. However, the emergence of azole resistant yeasts in many parts of the world is a grave concern (CDC, 2018; Whaley et al., 2016). It is thought that such recalcitrance to antifungals is partly due to the biofilm life style of many *Candida* species (Bandara et al., 2017; Thein et al., 2009).

Biofilm antimicrobial resistance is attributed but not limited to poor antimicrobial penetration into the biofilm, slow microbial growth and diminished metabolic rates, the heterogeneity and stress response of biofilm microbes, microbial quorum sensing (QS), the induction of biofilm specific microbial-phenotypes and the generation of 'persisters' (Mah and O'Toole, 2001). The azole resistance in *Candida* biofilms, in particular, is mainly linked to the poor penetration of the

drug to the biofilm and nonspecific binding of the drug into the biofilm matrix (Saag and Dismukes, 1988).

One approach to enhance the efficacy of antimicrobials against biofilms is to explore novel delivery mechanisms that facilitate biofilm drug penetration. Such novel approaches would be particularly beneficial for the wider use of highly efficacious azoles that are far less toxic than their counterparts, the pyrimidines and allylamines. Recent studies have shown that the penetration of drugs into biofilms can be significantly enhanced when accompanied with delivery vehicles such as liposomes (Smith, 2005). Liposomes are small, spherical vehicles that are increasingly used in drug delivery applications, as carriers of small molecules and drugs in disease states such as respiratory infections (Akbarzadeh et al., 2013). Despite several liposomal antibacterial preparations are reported, to date, amphotericin B is the only antifungal agent available as a liposomal formulation for clinical use.

During the process of biofilm development, biofilm microbes communicate with neighbourhood organisms *via* a chemical signalling mechanism termed QS. QS is defined as cell-density linked, coordinated gene expression in microbial populations in response to threshold signal concentrations followed by induction of a synchronized response within the population (Bandara et al., 2012b). QS permits the microbes to optimize their survival, defending themselves not only against adverse environmental/nutritional conditions, but also against competing organisms (McAlester et al., 2008). Recent studies have shown that the morphogenesis of *C. albicans* yeast-to-hyphal transition, a major virulence attribute, and a key requisite for host tissue invasion, is inhibited by a quorum sensing molecule (QSM) secreted by *Pseudomonas aeruginosa*, *N*-3-oxo-dodecanoyl-L-homoserine lactone (C₁₆H₂₇NO₄, C12AHL) (Hogan et al., 2004). QS is increasingly gaining attention as an antimicrobial target (Jiang et al., 2019), however, these naturally existing signalling molecules have received little attention as potential therapeutic agents. Recently, our group have shown the significant potential of using fungal QSM in augmenting the antibacterial activity of an antibiotic against *Pseudomonas aeruginosa* biofilms (Bandara et al., 2016). Interestingly, despite its inhibitory properties, no studies have been conducted to evaluate the potential of C12AHL as an antifungal agent against *Candida* biofilm infections. Thus, the aim of the current study was to develop a novel, liposomal mediated, co-delivery system of fluconazole and C12AHL, and thus to better suppress *C. albicans* biofilms.

2. Materials and methods

2.1 Preparation of liposomes

Four different formulations of liposomes were freshly prepared for each experiment including liposome only (L-C), liposome containing fluconazole (L-F), liposomes containing C12AHL (L-H)

and liposome containing both fluconazole and C12AHL (L-HF). Liposomes were prepared as described by Mezei *et al*, 1984 (Mezei and Nugent, 1984) and El-Nesr *et al*, 2010 (El-Nesr et al., 2010) with some modifications. Briefly, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC, Avanti Polar Lipids, Inc., AL, USA) cholesterol (Sigma Aldrich, St. Louis, USA) and α -tocopherol acetate (Sigma Aldrich, St. Louis, USA) were added in a 1:0.6:0.1 molar ratio and dissolved in chloroform (1:1 w/v, HPLC grade, Fisher Scientific, USA). α -tocopherol acetate was used as a liposomal membrane stabilizer (Hernandez-Caselles et al., 1990; Hinch, 2008; Honary and Zahir, 2013; Urano et al., 1990). Fifty μ L of 60 mM C12AHL solution (in DMSO, Sigma Aldrich, St. Louis, USA) were added to the lipid solution for L-H and L-HF preparations while a similar volume of DMSO was added to L-F and L-C preparations; 20mg of fluconazole (Sigma Aldrich, St. Louis, USA) was added to L-F and L-HF preparations. The molar ratio of fluconazole: C12AHL in the L-HF was 6.5:0.3. The lipid solution was added to a round bottom flask containing a defined number of glass beads (5 mm diameter) and evaporated to form a thin layer of lipids using a rotary evaporator at 100 rpm for 5 min at 55°C (Heidolph Instruments GmbH & Co, Germany). The lipid layer was hydrated with the addition of 10 mL of phosphate buffered saline (PBS). After a vigorous shake for 1 min, the flask was rotated for another 30 min at 55°C and 100 rpm for further hydration of the lipid layer. After 30 min of hydration, the liposomal preparations were transferred to glass vials and incubated at either room temperature for 1h or at 4°C for 24 h to assist liposomal maturation.

After the maturation period, the multilamellar liposomes were extruded through 0.2 μ m polycarbonate membranes using a mini extruder (Avanti Polar Lipids, Inc., AL, USA) and were centrifuged at 1500 $\times g$ for 10 min at 4°C. The supernatant was separated, and the pellet was resuspended in PBS, washed twice and the washes collected. The resultant liposome pellets were resuspended in PBS as necessary and used in the characterization and microbiological studies.

2.2 Determination of drug entrapment efficiency

2.2.1 Fluconazole entrapment

The absorbance values of the liposome-free supernatants and washes collected during liposomal preparations were read using a spectrophotometer (Infinite M200, Tecan Systems Inc., CA, USA) at 260 nm and fluconazole concentrations were calculated using a standard curve (Bandara et al., 2016).

The entrapment efficiency was calculated as below:

(Total quantity of fluconazole added – the quantity of unencapsulated fluconazole)×100

(Total quantity of fluconazole added)

2.2.2 C12AHL entrapment

Defined volume of liposomes pelleted after mini extrusion was lysed in ethanol for quantification of encapsulated C12AHL. An HPLC-MS-MS (high performance liquid chromatography-tandem mass spectrometry) method was developed to determine the concentrations of C12AHL. The separation of compounds was carried out using an Agilent 1100 LC binary pump and Agilent 1100 autosampler (Agilent Technologies, Santa Clara, CA, USA). The HPLC column used was a Synergy 2 µm Fusion RP 100Å (2.0 x 20 mm, Phenomenex, Torrance, CA, USA). For detection and quantification, an API 3000 tandem mass spectrometer with a turbo ion spray interface and Analyst 1.5 software (Applied Biosystems, Foster City, CA, USA) was used. Multiple Reaction Monitoring (MRM) was used in positive ion mode. The transitions of the 298 m/z ion → 102 m/z ion (for C12AHL) and 301 m/z ion → 102 m/z ion (for labelled internal standard d_3 -C12AHL see below) were monitored for each chromatographic run. The optimized parameter values for C12AHL to obtain the highest sensitivity are listed below:

An ion spray voltage (IS) of 5000V and entrance potential (EP) of 10V were used. Orifice/ declustering potentials (DP) was 101V. Ring/ focusing potentials (FP) was 370V. The collision energy (CE) was 19V, and the Collision exit potential (CXP) was 8V. Curtain gas (CUR), nebulizer gas (NEB) and the collision gas (CAD) flows were maintained at 12, 8 and 8 L/min respectively. The temperature of the ion spray was maintained at 400°C. A dwell time of 1000 msec was used for all transitions. Resolution of both Q1 and Q3 were 1amu.

C12AHL was purchased from Sigma (St Louis, MO, USA). The stable isotope analogue of C12AHL, N(12,12,12- d_3 -3-oxododecanoyl)- L-Homoserine Lactone; D_3 -C12AHL, was synthesized by Dr S. R. Chhabra (Centre for Biomolecular Sciences, University of Nottingham, Nottingham, UK)(Cámara et al., 1998). The stable isotope analogue was used as a co-eluting internal standard in order to correct for matrix effects (Hewavitharana, 2011). Stock solutions of both compounds were prepared in acetonitrile and stored at -20°C. Standard solutions for the six-point calibration curve were prepared to match analyte concentrations in 5x dilution of samples (as described below), using 100 µL of internal standard solution (300µM D_3 -C12AHL in 10% acetonitrile), appropriate volumes of 100 µM and 3.36 mM C12AHL, and 10% acetonitrile to make the final volume up to 1000µL. The concentrations of calibration standards were 10, 20, 30, 40, 50, 60 µM C12AHL; and 30 µM D_3 -C12AHL (in all six standards) in 10% acetonitrile.

Samples were prepared as above for the 5x dilutions. To 20 µL of the sample, 10 µL of internal standard solution (300 µM *D*₃-C12AHL) and 70 µL of 10% acetonitrile were added to make the volume up to a total of 100 µL.

Mobile phase A consisted of deionized water containing 0.1% (v/v) formic acid, and mobile phase B consisted of acetonitrile with 0.1% (v/v) formic acid. Both solutions were filtered through a 0.45µm polytetrafluoroethene (PTFE) filter (Millipore, Bedford, MA, USA) before use. Chromatography was carried out at an ambient temperature of approximately 25°C. The flow rate was 200 µL/min with an injection volume of 10 µL. Following injection, analytes were chromatographed using a gradient elution: the mobile phase composition was maintained at 12% B for the first 6 min, changed from 12% B to 55% B during the next 3 min, held at 55% B for the next 6 min, then changed to 100% over the next 2 min, held at 100% for 1 min before returning to 12% B over 2 min. The original composition of 12% B was maintained for a final 6 min period to equilibrate the column prior to the next injection (total of 26 min). Co-elution of both the analyte and the internal standard was achieved with this gradient, with a retention time of 12 min for both compounds. Calibration curves (six-point) were prepared by plotting the peak area ratio of C12AHL /*D*₃-C12AHL against the concentration of C12AHL and used to determine the accurate concentrations of C12AHL in samples.

The entrapment efficiency was calculated as below;

$$\frac{(\text{The quantity of encapsulated C12AHL}) \times 100}{(\text{Total quantity of C12AHL added})}$$

2.3 Characterization of liposomes

2.3.1 Particle size and zeta potential

The size, polydispersity index and the zeta potential of liposomes were determined by Dynamic Light Scattering Zetasizer Nano ZS (Malvern instruments Ltd, UK) for a total period of 1 month (immediately after preparation, 24 h, 48 h, 7 days, 14 days, 21 days and 1 month at 4°C).

2.3.2 Morphology of liposomes

The morphology of freshly prepared liposomes was visualized under a transmission light microscope (TLM, Nikon C2+ Confocal Microscope System, Nikon Instruments, Japan).

2.4 Drug release

Sample and Separate technique with some modifications was used to quantify drug release (D'Souza, 2014). Briefly, defined volumes (1 ml) of liposomal preparations at sink conditions were added to 10mL of PBS and incubated for 24h at 37°C in a shaker (250 rpm). At the end of incubation, the liposomal suspensions were centrifuged (1500 *xg* at 4°C for 10 min), the supernatant was collected, centrifuged for 10 min at 15800 *xg* at 4°C and the absorbance of the supernatant was read at 260 nm (Infinite M200, Tecan Systems Inc., CA, USA) to quantify the released fluconazole. The supernatant was filtered (0.22 μ m) and released C12AHL was quantified by HPLC-MS-MS assay as described above.

2.5 Microorganisms and growth conditions

Candida albicans SC5314 was used throughout the study. The identity of *C. albicans* was confirmed with the commercially available API 32 C kit (Biomérieux, Mercy l'Etoile, France) and stored in multiple aliquots at -80°C. Sabouraud Dextrose Agar (SDA, Sigma Aldrich, USA), Sabouraud Dextrose Broth (SDB, Sigma Aldrich, USA) and Yeast Nitrogen Base solution supplemented with 100 mM glucose (YNB) were used for culturing *C. albicans*.

Freshly grown *C. albicans* cultures were used in each experiment. Briefly, *C. albicans* was sub-cultured on SDA for 18 h at 37°C. A loopful of the yeast growth was inoculated into SDB, incubated for 18 h in an orbital shaker (80 rpm) at 37°C. The resultant growth was washed twice in Phosphate Buffered Saline (PBS, pH 7.2), resuspended in YNB and *C. albicans* concentration was adjusted to 1 $\times$ 10⁷ cells/mL by spectrophotometry and confirmed by hemocytometric counting.

2.6 Biofilm development

C. albicans biofilms were developed as described by Bandara *et al.* (Bandara et al., 2010a) with some modifications. Commercially available tissue culture treated, sterile, polystyrene, flat bottom 96-well microtiter plates (Corning, NY, USA) were used. An aliquot of 100 μ L of freshly prepared *Candida* suspension was transferred into the wells of a microtiter plate and incubated for 1.5 h (37°C, 80 rpm) to promote microbial adherence to surface of the wells. After the initial adhesion phase, the cell suspensions were aspirated, and wells were carefully rinsed twice with PBS to remove non-adherent and loosely adherent cells. A total of 200 μ L of YNB was transferred to each well, incubated for 24 h (37°C, 80 rpm), and the wells washed twice with PBS to eliminate traces of the medium. The resultant biofilms were used for further investigations.

2.7 Determination of minimum inhibitory concentration (MIC)

2.7.1 Planktonic phase

MIC was determined by a broth microdilution assay in accordance with the CLSI guidelines (CLSI, 2017). Briefly, *Candida* suspensions (5×10^3 Cells/mL) were treated with serially diluted (two-fold) fluconazole or C12AHL and incubated in a 96 well microtiter plate for 24 h at 35°C. The optical densities of the suspensions were measured by a spectrophotometer at 595 nm at the end of the incubation. The lowest concentration of the fluconazole or C12AHL at which the bacteria demonstrated visible growth inhibition compared to the solvent control was considered the MIC of the drug or the QSM against *C. albicans*. The assay was performed in quadruplicate at three time points.

2.7.2 Biofilm phase

Biofilm colonisation phase: After the initial adhesion phase (90 min), washed wells containing attached *C. albicans* were treated with fluconazole or C12AHL and incubated for 24 h at 37°C and 80 rpm to allow *C. albicans* biofilm colonisation.

Mature biofilms: *C. albicans* biofilms grown in tissue culture treated sterile 96 well plates for 24 h (BD Biosciences, USA) were used in determining the minimum biofilm inhibitory concentrations (MBIC) of the active agents used in this study. Biofilms were rinsed with PBS and treated with a concentration gradient (two-fold) of fluconazole and/or C12AHL and the plates were incubated for 24 h at 37°C and 80 rpm.

At the end of each incubation period, an XTT (sodium 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium inner salt) reduction assay was performed to quantify the viability of biofilms as described in following sections (Bandara et al., 2009). The lowest concentration of the antifungal or QSM at which the fungal biofilm exhibited 90% or 50% of reductions of the viability compared to the solvent control is considered as respective Minimum Biofilm Inhibitory Concentration (MBIC90 or MBIC50) of the active agent against *C. albicans*. The MBIC assay was conducted in quadruplicates on three different occasions.

2.8 Treatment of biofilms with liposomes

C. albicans biofilms were treated with a concentration gradient of freshly prepared liposomes (L-C, L-H, L-F and L-HF) in two different time points during the process of biofilm development. First, liposomes and YNB were added immediately after 90 min adhesion phase and incubated for another 24 h (37°C, 80rpm) to assess the effect of antifungal preparations on the *C. albicans* biofilm colonisation process. Second, liposomes with YNB were added to 24h preformed biofilms and incubated for further 24 h at 37°C at 80 rpm to assess the effect of the antifungal preparation on preexisting mature biofilms. At the end of incubation, biofilms were washed twice with PBS and the XTT reduction and safranin-O assays were performed to assess biofilm viability and

biomass respectively. In addition, test and control biofilms were visualized under confocal laser scanning microscopy. Each experiment was conducted in quadruplicate on three different occasions.

2.9 XTT reduction assay

At the end of incubation, a standard XTT reduction assay was performed in determining biofilm viability by means of the assessing fungal metabolic activity (Bandara et al., 2010b). Succinctly, commercially available XTT powder (Cayman Chemical, MI, USA) was dissolved in PBS (1 mg/mL), filter-sterilized (0.22 µm pore size filter), and stored at -70°C. Immediately before the assay, PBS, thawed XTT solution and a freshly prepared 0.4 mM menadione (Sigma Aldrich, MO, USA) solution were mixed in a 79:20:1 ratio and added into each well (200 µL in total) containing biofilms and incubated in the dark for 3 h at 37°C. The changes in the colour/absorbance were quantified by microtiter plate reader (Infinite M200 microplate reader, TECAN US Inc, NC, USA) at 492 nm.

2.10 Safranin-O assay

At the end of incubation of both test and control biofilms, a safranin-O assay was performed to quantify biofilm biomass. Biofilms were carefully washed twice with PBS and stained with a 0.1% safranin-O (3,7-diamino-2,8-dimethyl-5-phenylphenazin-5-ium chloride, Sigma Aldrich, MO, USA) aqueous solution for 15 min at 25°C without shaking. Wells were carefully washed three times with PBS to remove excess stain and air dried at room temperature. Aqueous acetic acid solution (10% v/v) was added to the wells containing stained biofilms and incubated for 20 min at 25°C to de-stain. The solution containing released safranin-O was transferred to new wells and the absorbance was measured at 490 nm.

2.11 Confocal Laser Scanning Microscopy (CLSM)

Tissue culture treated, sterile, plastic cover slips (Thermanox plastic cover slips, Nunc, NY, USA) placed in tissue culture treated, sterile, flat bottom six well plates (Corning, NY, USA) were used for CLSM imaging. Adherent *C. albicans* cells or 24 h *C. albicans* biofilms formed on cover slips were exposed to all four different liposomal preparations or free drugs and incubated for another 24 h at 37°C in a shaker (80 rpm). At the end of incubation, prewashed coverslips were stained with Live and Dead stain (Live/Dead BacLight Bacterial Viability kit, Invitrogen, Eugene, USA) (Bandara et al., 2010a) and analysed using confocal laser scanning microscopy (Nikon C2+ Confocal Microscope System, Nikon Instruments, Japan and Leica SP5, Leica Microsystems, Germany).

2.12 Statistical analysis

Quantitative data collected were statistically analyzed using SPSS software (version 16.0). Mann—Whitney U-tests were performed to compare the significant differences between corresponding controls and test samples of the *C. albicans* biofilms. A P-value of less than 0.05 was considered statistically significant.

3. Results

3.1 Liposomal characteristics

3.1.1 Entrapment efficiencies of active agents

The efficiency of the entrapment of fluconazole in liposomes was 11.8% in L-HF and 10.2% in L-F immediately after preparation whereas it was 12.3% in L-HF and 3.8% in L-F when the liposomes were prepared, stored at 4°C for 24 h for maturation, and then processed (Table 1).

The entrapment efficiency of C12AHL was 33% in L-HF and 33.3% in L-H immediately following preparation whereas it was 48.9% in L-HF and 44% in L-H when prepared liposomes were stored at 4°C for 24 h to mature before processing (Table 1).

The size and the zeta potential of the liposomes were measured over a period of 30 days (immediately after preparation, 1, 4, 7, 14, 21 and 30 days). The diameters of all four different preparations ranged from 138.4 nm to 164.2 nm. The average diameters measured over a period of one month were 141.8 ± 2.6 nm, 147.9 ± 1.4 nm, 161.7 ± 2.1 nm and 141.0 ± 1.8 nm for L-HF, L-H, L-F and L-C respectively (Figure 1A). There was no significant difference between the liposome sizes of L-C and L-HF, however, average diameters of both L-H and L-F were significantly larger than those for L-C and L-HF ($p < 0.05$). L-F was significantly larger than the other three liposomal preparations ($p < 0.05$).

The average zeta potentials of L-HF, L-H, L-F and L-C preparations were -1.00 ± 0.4 mV, -0.57 ± 0.3 mV, -0.57 ± 0.3 mV and -0.51 ± 0.4 mV over one month of observation period (Figure 1B). The average zeta potential of L-HF was significantly higher than that of the remaining three preparations ($p < 0.05$). The average polydispersity indices of respective liposomes during the period of observation were 0.21 ± 0.1 , 0.24 ± 0.1 , 0.26 ± 0.1 and 0.20 ± 0.1 (Figure 1C).

3.1.2 Morphology of liposomes

Transmission light microscopic assessments further confirmed DLS findings. As shown in Figure 2, all four preparations of liposomes appeared uniform and spherical and evenly distributed in the microscopic field.

3.1.3 Drug release

After a total of 24h of the drug release period, 4.26% (13 µg) of encapsulated fluconazole (305 µg) was released from L-HF, however, only 0.97% (2.46 µg) of the total encapsulated fluconazole (254 µg) was released from L-F.

Whereas 92.3% (48.8 µM) of total encapsulated C12AHL (52.9 µM) was released from L-HF, and 88.9% (45.6 µM) of total C12AHL encapsulated (51.3 µM) was released from L-H.

3.2 Minimum inhibitory concentrations (MIC)

The planktonic MIC₈₀ of fluconazole was 0.25 µg/mL for *C. albicans* and MBIC₅₀ was >512 µg/mL for both colonising biofilms and 24 h mature *C. albicans* biofilms.

The planktonic MIC₅₀ of C12AHL was >300 µM for *C. albicans* (45% reduction of *C. albicans* growth compared to solvent controls was observed at 300 µM C12AHL, $P < 0.05$). The MBIC₅₀ of C12AHL for *C. albicans* colonising biofilms and 24h mature biofilms were >600 µM (43% and 13% reduction of *C. albicans* biofilm metabolic activity measured by XTT reduction assay were observed for colonising and 24 h mature biofilms at 600 µM of C12AHL respectively).

3.3 Liposomal effect on biofilm metabolism: XTT reduction assay

3.3.1 Effect of liposomes on the viability of colonising *C. albicans* biofilms

When treated with various liposomal preparations, colonising *C. albicans* biofilms exhibited significant differences in their metabolic activities when compared to controls (Figure 3). The biofilms treated with L-HF showed a significantly lower metabolic activity when compared to L-F and free fluconazole at the same final concentration released within 24 h. There was a >80% reduction in the colonised *C. albicans* biofilm ($P < 0.05$) when ≥ 5.5 µg/mL of fluconazole concentration was delivered from L-HF compared to controls. However, similar concentrations of fluconazole delivered from L-F resulted only a 12% reduction in the biofilm metabolism ($P > 0.05$) (Figure 3A), and free fluconazole at similar concentrations suppressed *C. albicans* colonised biofilm metabolic activity by 13% compared to controls ($P > 0.05$) (Figure 3A).

L-HF that released above mentioned concentration of fluconazole (5.5 µg/mL), released 144 µM of C12AHL and resulted >80% reduction in the colonising *C. albicans* biofilm when compared to

the untreated control, as mentioned above ($P<0.05$). However, L-H, that delivered similar C12AHL concentrations did not elicit any changes in colonised *C. albicans* biofilms when compared to controls ($P>0.05$) (Figure 3B); free C12AHL at similar concentrations suppressed colonised biofilm metabolism by 41% ($P<0.05$) (Figure 3B).

The combined exposure to free fluconazole 5.5 $\mu\text{g/mL}$ and free C12AHL 144 μM resulted 40% reduction in metabolism in colonising biofilms when compared to untreated controls (Figure 3A and 3B, $P<0.05$). The exposure of colonising biofilms to L-C did not elicit any significant changes in the biofilm metabolism ($P>0.05$).

When the percentage suppression data among the various treatment groups were compared, the inhibition exerted by L-HF on developing *C. albicans* biofilms was significantly greater than that of L-F, L-H, free fluconazole, free C12AHL or their combinations ($P<0.05$). Free fluconazole + free C12AHL exposure exhibited greater inhibition when compared to L-F, L-H, free fluconazole or free C12AHL treated samples ($P<0.05$). No significant changes were observed in the comparisons of L-F vs free fluconazole and L-H vs free C12AHL.

3.3.2 Effect of liposomes on the viability of preformed *C. albicans* biofilms

The preformed 24h *C. albicans* biofilms exposed to L-HF demonstrated a significant reduction in biofilm metabolism (60%, $P<0.05$) compared to untreated controls. However, L-F and free fluconazole (at similar concentrations) demonstrated only 36% ($P<0.05$) and 2% ($P>0.05$) reduction in the metabolism, respectively, compared to controls (Figure 3A).

L-H and free C12AHL, at the concentration of C12AHL released by the L-HF (144 μM) which suppressed the metabolism by 60%, lowered *C. albicans* biofilm metabolism by 47% and 14% respectively (Figure 3B, $P<0.05$).

The combined exposure of 24h preformed *C. albicans* biofilms to free fluconazole (5.5 $\mu\text{g/mL}$) and free C12AHL (144 μM) resulted 29% reduction in the biofilm metabolism (Figure 3A and 3B $P<0.05$). The exposure of preformed biofilms to L-C did not elicit any significant changes in the biofilm metabolism ($P>0.05$).

Similar to colonising biofilms, the suppression of preformed biofilms was significantly greater when treated with L-HF compared to L-F, L-H, free fluconazole, free C12AHL or their combination ($P<0.05$). The metabolic reduction resulted by L-F exposure was significantly greater than that of free fluconazole ($P<0.05$) but was insignificant compared to free fluconazole + free C12AHL

($P>0.05$). In contrast to colonising biofilms, L-H treated biofilms showed a significant reduction of metabolism compared to free C12AHL, free fluconazole + free C12AHL treated samples ($P<0.05$). The latter combination of free QSM and the antifungal agent performed significantly better than the free QSM ($P<0.05$).

3.4 Liposomal effect on biofilm biomass: safranin-O assay

The changes in the biomasses of the colonising biofilms were assessed when exposed to various liposomal preparations. Colonising biofilms, when exposed to L-HF, exhibited the smallest biomass compared to other test and control biofilms (70% reduction vs control, $P<0.05$). There were 42%, 23% and 31% reductions in biomass when *C. albicans* colonising biofilms were treated with L-F, free fluconazole and free fluconazole + free C12AHL respectively (Figure 4A, $P<0.05$). L-H treated *C. albicans* colonising biofilms exhibited a significant 33% reduction in the biomass compared to untreated controls ($P<0.05$), while free C12AHL or L-C treated samples did not elicit significant changes ($P>0.05$).

When the reduction in the biomasses were compared among various treatment groups, the greatest reduction was observed when treated with L-HF followed by L-F > L-H/free fluconazole + free C12AHL > free fluconazole > free C12AHL (Figure 4A, $P<0.05$). There was no significant difference between biofilms treated with L-H and free fluconazole + free C12AHL ($P>0.05$).

L-HF treated 24h preformed *C. albicans* biofilms exhibited 46% reduction in the biomass compared to untreated control (Figure 4B, $P<0.05$). Conversely, compared to untreated controls, the reduction in the biomass observed when exposed to L-F (6%, $P>0.05$), L-H (16%, $P<0.05$), L-C (1% $P>0.05$), free fluconazole (12%, $P<0.05$), free C12AHL (10%, $P>0.05$) and free fluconazole + free C12AHL (12%, $P<0.05$) was noted to be lower (Figure 4B).

Similar to colonising biofilms, the greatest reduction of biomass was observed when preformed biofilms were exposed to L-HF compared to other test groups ($P<0.05$). There were no significant differences in the biomasses among L-F, free fluconazole or free fluconazole + free C12AHL treated preformed biofilms ($P>0.05$). Similarly, nonsignificant differences were noted among L-H, free C12AHL or free fluconazole + free C12AHL treated preformed biofilms ($P>0.05$).

3.5 Confocal laser scanning microscopy: effect of liposomes on *C. albicans* biofilms

Confocal microscopy further confirmed the significant effect of liposomal treatment on *C. albicans* biofilm colonisation, and preformed biofilms.

C. albicans colonising biofilms exposed to control liposomes (L-C) colonised in to a dense, three-dimensional, robust biofilm with long, intermingled hyphae and some scattered yeast cells (Figure 5A). L-H and L-F treated *C. albicans* samples also developed into dense biofilms (Figure 5B and 5C) similar to L-C treated control; fewer hyphae were, however, observed in L-H treated sample (Figure 5B). Some dead yeast cells were visible within the colonising biofilm exposed to L-F (Figure 5C) compared to those biofilms exposed to L-C or L-H. *C. albicans* exposed to free C12AHL (Figure 5F) or free fluconazole (Figure 5G) also developed into dense biofilms similar to untreated control (Figure 5E). Dead yeast cells and hyphae were sparingly visible in free C12AHL or free fluconazole exposed biofilms.

The most striking findings were noted in the colonising phase biofilm treated with L-HF, which showed structurally scant, amorphous biofilms that were haphazardly and unevenly arranged. Further, significant numbers of dead yeast cells and hyphae were noted in the colonised biofilm exposed to L-HF, compared to any other type of exposures (Figure 5D). Free C12AHL+fluconazole exposure (Figure 5H) resulted in higher quantities of dead yeast cells compared to respective controls (Figure 5E-G), however, biofilm architecture remained largely unaffected.

Preformed, mature biofilms also exhibited structural features akin to colonising biofilms when treated with various liposomal preparations. Thus, L-HF treated preformed *C. albicans* biofilms were strikingly different from others, devoid of a biofilm architecture and a scattering of yeast blastopores, implying possibly an aborted attempt at biofilm growth (Figure 6D). In contrast, the L-C, L-H or L-F treated preformed, mature *C. albicans* biofilms remained healthy and fully colonised with a few hyphal elements, blastopores and occasional dead cells. There were no discernible visual differences between the latter three biofilms exposed to L-C, L-H or L-F (Figure 6A, 6B and 6C). Similarly, free C12AHL (Figure 6F) and free fluconazole (Figure 6G) exposed preformed biofilms remained viable structurally sound compared to undisturbed control (Figure 6E). Although a higher quantity of dead yeast cells and hyphae were visible in preformed biofilms exposed to free C12AHL+ fluconazole (Figure 6H), biofilm structure remained unaffected compared to L-HF treated sample.

4. Discussion

Candida-associated infections have been steadily rising over the last few decades due to the increasing prevalence and incidence of immunological disorders including HIV disease, heavy use of immunosuppressive and cytotoxic drugs, and broad-spectrum antibiotics, systemic disorders (e.g. uncontrolled diabetes mellitus), and the extensive use of indwelling medical devices

(Bongomin et al., 2017). The management of these fungal infections remains a significant challenge due to the emergence of antifungal resistant candidal phenotypes, and new, virulent *Candida* species such as *Candida auris* (Kean and Ramage, 2019) dearth of exploitable fungal drug targets, and also the serious adverse effects of the existing drugs (Perlin et al., 2017). Consequently, new approaches to improve and expand the efficacy of the current armamentarium of antifungals are critical.

A recent exciting development in the understanding of the fungal eco-biology has been the discovery of QS mechanisms and signals that mediate communications between fungi and other neighbouring co-habitants in polymicrobial biofilm habitats (Bandara et al., 2012b; Diggle et al., 2007; Kuroda et al., 2007; McAlester et al., 2008; Peleg et al., 2010). In this context, inter-kingdom QS signaling between *C. albicans* and *P. aeruginosa* have been extensively studied as these species are frequently co-isolated in a variety of diseases including chronic wound infections, cystic fibrosis lung disease, and indwelling devices (El-Azizi et al., 2004; Fourie et al., 2016; McAlester et al., 2008). Two such major QS molecules that have been the focus of many investigations are C12AHL and farnesol (from *Candida*).

QS interactions between *C. albicans* and the *P. aeruginosa* are reported to be bidirectional in nature. For instance, *C. albicans*' farnesol decreases the synthesis of *P. aeruginosa* quinolone signal, pyocyanin, as well as bacterial swarming in solid media (Cugini et al., 2007; Diggle et al., 2007; McAlester et al., 2008; Nickerson et al., 2006) whilst a QSM produced by *P. aeruginosa*, C12AHL (structural analog of farnesol) is known to inhibit *C. albicans* yeast to hypha morphological transition (Bandara et al., 2012a; Hogan et al., 2004). Farnesol inhibits hyphal development by repressing the activity of *Candida* adenylyl cyclase, Cyr1p, in the Ras1-cyclic AMP-protein kinase; a pathway, which positively regulates hyphal growth (Davis-Hanna et al., 2008).

Due to its structural resemblance C12AHL is also believed to suppress *C. albicans* filamentation by inhibiting the foregoing regulatory pathway (Hall et al., 2011; Hogan et al., 2004). Hyphal formation is a key virulent attribute of *Candida*, and play a crucial role in tissue adhesion and invasion, iron acquisition from the host, and immune evasion (reviewed in (Jacobsen et al., 2012)). Hyphae are also essential structural components of *C. albicans* biofilms as they strengthen its overall architecture and fabric by providing a scaffold for the biofilm matrix, and microbial growth (Nobile and Johnson, 2015). Due to the indisputable key role the hyphal elements play in yeast biofilm development and pathogenicity, perturbation of the yeast to hyphal transition phase of *C. albicans* is an attractive target for therapeutic interventions (Jacobsen et al., 2012).

In a previous study, we have convincingly demonstrated that *C. albicans* and *P. aeruginosa* QS interactions can be successfully exploited for possible therapeutic benefit (Bandara et al., 2016). We incorporated the QS farnesol, and the antibacterial, ciprofloxacin in to liposomes, and showed that the latter combination, with significantly lower antibiotic concentrations, effectively destroyed *P. aeruginosa* biofilms (Bandara et al., 2016). Similarly, we hypothesized in this study that the efficacious suppression of yeast to hypha transition by C12AHL could be exploited as a therapeutic target, in combination with a common antifungal agent fluconazole, whilst simultaneously achieving a reduction in the drug dosage. It is also noteworthy that no studies, to date, have been conducted, to evaluate the therapeutic potential of C12AHL as an anticandidal agent.

Liposomes have been used in pharmaceutical industry over a few decades as drug delivery vehicles as they are non-toxic, non-immunogenic, and completely biodegradable, with a high degree of biocompatibility and biosafety (Akbarzadeh et al., 2013). Additionally, their enhanced drug encapsulation potential, the slow and sustained drug release, their superior potential for entrapping multiple active agents and the consequent reduction in infusion-associated side effects makes them virtually ideal candidates for drug delivery (Salem et al., 2005; Wong-Beringer et al., 1998). Therefore, we have chosen this well-characterised and relatively easy to prepare nano vesicles as the drug carrier to study the forgoing hypothesis.

We experimented with different molar ratios of DPPC, cholesterol and α - tocopherol acetate in pilot experiments on liposomal synthesis and identified the 1:0.6:0.1 (DPPC: cholesterol: α - tocopherol acetate) molar ratio as optimal for encapsulating an antifungal agent and/or QSM (data not shown). In order to ascertain the consistency of the liposomes during preparation and storage, we evaluated four different liposomal preparations over a period of 30 days. Their particle size remained relatively uniform (138.4 - 164.2 nm) on repeated evaluation, and on light microscopic examination. Interestingly, the size of our liposomes appeared to be ideal for biofilm penetration, as according to previous data, nanoparticles up to 130nm in diameter could efficiently traverse bacterial biofilms unimpeded by their profuse extracellular matrix or the microbial cell surface appendages (Liu et al., 2019; Peulen and Wilkinson, 2011).

Another feature that needs to be considered in the pharmaceutical evaluation of nanoparticles in a suspension is their polydispersity index (PDI), an indicator of the particle size distribution within the mixture. Further, a PDI of ≤ 0.30 implies homogeneity of a phospholipid vesicle population and is considered pharmaceutically acceptable (Badran, 2014; Chen et al., 2011; Danaei et al., 2018; Putri et al., 2017). The PDI of our liposome preparations ranged between 0.19-0.27 throughout the 30-day observation period indicating their uniform distribution and acceptability for pharmaceutical use, particularly for administration via either topical,

transdermal or inhalational routes. Although the weak surface charges of the particulate drug carriers has been reported to affect their long-term stability and drug encapsulation (Jones et al., 1997), our liposomal preparations remained stable for 30 days, despite their weak negative surface charges (zeta potential of minimum -0.07 mV and maximum -1.47 mV over the 30-day period). This could be attributable to the incorporation of α -tocopherol acetate, a known stabilizer of vesicular membranes by reducing the breakdown of phosphatidylcholine (DPPC), in to liposomal membranes (Hernandez-Caselles et al., 1990; Hinch, 2008; Honary and Zahir, 2013; Urano et al., 1990). Temperature is a known factor affecting the long-term liposomal stability (Weiner et al., 1989). As it is known that liposomes stored at 4°C have optimal stability (El-Nesr et al., 2010; Hernandez-Caselles et al., 1990), we followed this protocol in our study. Hence, investigations under different temperatures and longer duration are however required to further extend the utility of our findings.

The triazole, fluconazole is sparingly soluble in water but highly soluble in organic solvents. Hence, fluconazole needed to be entrapped into the lipid membranes of the liposome structure. Although the fluconazole entrapment efficacy remained relatively low, especially compared to water soluble drugs encapsulated in the liposome core, the total quantity of the entrapped drug was reasonable considering it's likely location in the membrane and that fact that it was greater than its minimum inhibitory concentration against *C. albicans*, for both L-HF and L-F preparations (305 μ g/mL and 254 μ g/mL, respectively). In line with our study, previous workers have also noted the variations in entrapment ratios of fluconazole in liposomes (El-Nesr et al., 2010; Mezei and Nugent, 1984). Others have reported that the use of other phospholipids such as soy lecithin instead of DPPC, or hydration of the lipid membranes and rehydration of freeze-dried liposomes with sugar solutions (i.e. 9% sucrose) may improve entrapment efficiencies of fluconazole (El-Nesr et al., 2010; Kulkarni et al., 1995; Mehrdad et al., 2016). It is notable that when the entrapment efficiencies were quantified immediately after preparation, there was no observed antagonistic effect between C12AHL and fluconazole confirming the two agents do not compete with each other for entrapment within the liposomes. The reasons for increased entrapment efficiency of C12AHL after 24 h maturation period is yet to be investigated.

Despite the noted similar entrapment efficiencies, fluconazole release was significantly greater for L-HF compared with L-F, over a 24h period. Antimicrobial release from liposomes is known to be impacted by various intrinsic factors (e.g. membrane composition, choice of drugs) and environmental factors (e.g. pH, temperature, the presence of enzymes) (Lindner and Hossann, 2010). It is, therefore, possible that the presence of C12AHL within the liposome, may have increased the fluconazole release from L-HF by altering the liposomal membrane dynamics in

some manner. In contrast, most of C12AHL entrapped was released during the 24h period in both L-H and L-HF irrespective of the presence or absence of fluconazole. Therefore, our data prove the concept, that despite similar entrapment efficiencies, L-HF is the preferred mode of *in vitro* antifungal delivery system compared to L-F. Though we quantified the drug release only after 24 h, future kinetic drug release studies would be beneficial for understanding the rate and the order of drug release (first-order vs zero-order), determining effective doses and dosing intervals to achieve desired degree of biofilm elimination and for optimisations of the liposomal formulations to achieve desired drug release profiles (e.g. controlled release).

More importantly, the superior antibiofilm properties of L-HF compared to all other liposomal formulations and free forms we evaluated were clearly substantiated by the microbial viability, the biomass assays, as well as the microscopic visualization. When exposed to L-HF, *C. albicans* colonising, and preformed biofilms exhibited >80% and 60% reductions in their respective viabilities at a fluconazole concentration as low as 5.5 µg/mL. Considering that the MBIC50 of free fluconazole is >512µg/mL against colonising and preformed *C. albicans* biofilms, and the failure of other tested liposomal and free forms to elicit a similar biofilm suppressive effect, our data imply that the L-HF preparations possesses superior functional attributes in suppressing *C. albicans* biofilms. As *C. albicans* biofilms did not elicit a biologically meaningful response to individual agents in either the free (free fluconazole or C12AHL) or the liposomal formulations (L-H or L-F), we did not calculate the combinatorial indices to assess the synergism between fluconazole and C12AHL in L-HF. However, it is tempting to speculate that the observed efficacy of L-HF could at least be partly attributable to the tandem synergistic effect of biomass reduction, and fungal killing. This hypothesis is further supported by the significant reduction in biomass (observed with safranin-O staining) and the scant biofilm structure, as well as the reduced hyphal content observed under CLSM.

It is well known that hyphal elements contribute substantially to the *C. albicans* biofilm volume (Chandra and Mukherjee, 2015), and the lack of this morphological form in L-HF treated biofilms was evident by a sparse, and rudimentary biofilm with scattered blastopores. The striking reduction in the hyphal content is highly likely to be associated with the hyphal inhibitory effect of C12AHL released from the liposomes. Interestingly, previous studies have shown that C12AHL also possesses biofilm ‘dispersal’ properties against bacterial species such as *P. aeruginosa*, *Serratia marcescens* and *Vibrio vulnificus* (Kaplan, 2010; Kim et al., 2013; Rice et al., 2005). Although there are no data appertaining to C12AHL mediated *C. albicans* biofilm dispersal, it can be speculated that C12AHL and fluconazole, in tandem, may act synergistically to suppress *C. albicans* biofilms by dispersing the biofilm cells and converting it into a less virulent and invasive form.

The current data imply that the C12AHL-fluconazole liposomal preparation suppresses *C. albicans* biofilms by a synergistic, trichotomous mechanism, of i) killing the organisms, ii) reducing yeast to hypha transformation rendering the biofilm less virulent and less invasive, and iii) dispersing the biofilm cells. Further studies including *in vivo* evaluations are, however, necessary to confirm this hypothesis and to evaluate biological compatibilities of the novel formulations.

5. conclusions

Due to the ever increasing emergence of resistance to the existing armamentarium of antifungal drugs, and the myriad challenges associated with new drug development, co-delivery of a lower drug dose and another active agent is an attractive option to improve the antimicrobial efficiency, lower the antimicrobial toxicity and improve drug release (Bandara et al., 2016). For this reason, combination therapy of fluconazole with various other agents ranging from antibacterial drugs, natural products to nanoparticles have been extensively studied (Jia et al., 2016; Liu et al., 2017; Longhi et al., 2016; Lu et al., 2018; Raut et al., 2017; Sun et al., 2019). This study provides novel insights into a possible exploitation of a liposomal-mediated tandem, low dose, delivery of a popular antifungal agent, and a QSM. The findings strongly indicate that relatively low dose, fluconazole, together with C12AHL a QSM, incorporated into liposomes possess superior, *in vitro*, biofilm suppression activity against *C. albicans*. This rationally designed novel drug delivery system is likely to breathe fresh life into azole drug therapy and extend their usefulness in managing candidal infections in general, and more specifically, oral and mucocutaneous candidiasis.

6. Author contribution

H.M.H.N. Bandara: Conceptualization, Methodology, Formal Analysis, Investigation, Resources, Writing-Original Draft, Visualization, Project Administration, Funding Acquisition. **A. K. Hewavitharana:** Methodology, Validation, Formal Analysis, Investigation, Resources, Writing-Original Draft. **P.N. Shaw:** Conceptualization, Methodology, Resources, Writing-Review and Editing, Supervision. **H.D.C. Smyth** Conceptualization, Methodology, Writing-Review and Editing, Supervision, Funding Acquisition. **L.P. Samaranayake:** Conceptualization, Methodology, Resources, Writing-Review and Editing, Supervision, Funding Acquisition

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