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***In vitro* Evaluation of Antimicrobial Activity of 1-Lauroyl-rac-glycerol on *Candida albicans* Biofilms**

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Monolaurin (also known as glycerol monolaurate) is a natural compound found in coconut oil and is known for its protective biological activities as an antimicrobial agent. The nature of oral candidiasis and the increased antifungal resistance, culminate the need for investigating novel antifungal therapeutic agents. In this study, we examine the antifungal activity of monolaurin against *Candida albicans* biofilms (strain ATCC: SC5314/MYA2876) in vitro and how monolaurin may alter specific host inflammatory markers, such as gene expression of inflammatory cytokines IL-1 α and IL-1 β , as illustrated in co-culture models. The results from three groups were compared: 1- monolaurin (in the range of 3.9-2500 μ M), positive control fluconazole (322 μ M), and vehicle control group 1% Ethanol (v/v) The MIC and MFC of monolaurin were in the range 62.5-125 μ M and 125-250 μ M, respectively. The results show significant reduction in Log (CFU/ ml) of biofilms treated with 1250 and 2500 μ M of 1- monolaurin when compared to the control groups. There was also a significant down-regulation of IL-1 α and IL-1 β in the biofilms treated with monolaurin. It can be concluded that monolaurin has a potential antifungal activity against *C. albicans* and can modulate the host's pro-inflammatory response.

1 ***In vitro* Evaluation of Antimicrobial Activity of 1-Lauroyl-rac-glycerol on *Candida albicans***
2 **Biofilms**

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25 culture model, host inflammatory response.

26 **ABSTRACT**

27 Monolaurin (also known as glycerol monolaurate) is a natural compound found in coconut oil
28 and is known for its protective biological activities as an antimicrobial agent. The nature of oral

candidiasis and the increased antifungal resistance, culminate the need for investigating novel antifungal therapeutic agents. In this study, we examine the antifungal activity of monolaurin against *Candida albicans* biofilms (strain ATCC: SC5314/MYA2876) *in vitro* and how monolaurin may alter gene expression of host inflammatory cytokines, *IL-1 α* and *IL-1 β* . In a co-culture model, oral fibroblast cells were cultured simultaneously with *C. albicans* for 24 hrs followed by the exposure to treatments of monolaurin (3.9-2500 μ M), positive control fluconazole (32.2 μ M), and vehicle control group 1% Ethanol (v/v), which was used to evaluate cytotoxicity of monolaurin on fibroblasts, as well as analyze morphological characteristics of biofilms through fluorescence microscopy. In addition, the co-culture model was used for RNA extraction from oral fibroblasts and gene expression of host inflammatory cytokines were analyzed using quantitative real-time PCR. Our results show the MIC and MFC of monolaurin were in the range 62.5-125 μ M and 125-250 μ M, respectively. The results show significant reduction in Log (CFU/ ml) of biofilms treated with 1250 and 2500 μ M of 1- monolaurin when compared to the control groups. There was also a significant down-regulation of *IL-1 α* and *IL-1 β* in the co-culture treated with monolaurin. It can be concluded that monolaurin has a potential antifungal activity against *C. albicans* and can modulate the host's pro-inflammatory response.

Keywords: monolaurin, antimicrobial agent, oral candidiasis, *Candida albicans*, *in vitro*, virulence factors, proteolytic enzymes, host inflammatory response, MIC/MFC, biofilms.

INTRODUCTION

Oral candidiasis is the most common fungal opportunistic infection to affect the oral cavity. *Candida albicans* is the fungal organism most responsible for causing denture stomatitis, thrush, and urinary tract-infections, but can also provoke more severe systemic infections, which are frequently life-threatening, in particular among immunocompromised individuals, whose numbers are constantly increasing due to organ transplant, chemotherapy, and the prevalence of AIDS and Hepatitis (Zaoutis et al. 2005). The limited numbers of suitable and effective antifungal drugs, together with increasing drug resistance of the pathogens to azole antifungal agents, demand the search for new antimicrobials (Hunter et al. 1998; White et al. 1998). The development of therapeutic agents with the ability to inhibit the biofilm formation and modulate host inflammatory response can promote major impact in the prevention and treatment of oral candidiasis.

Monolaurin, also known as glycerol monolaurate or lauroyl, is a natural surfactant compound commonly used in cosmetics and in the food industry as an emulsifier and preservative. It is recognized as a GRAS (Generally Recognized as Safe) food additive by the FDA (Food and Drug Administration), with topical doses of up to 100 mg/ml (Title 21, Code of Federal Regulations, Part 184) (Peterson and Schlievert 2006). Monolaurin is a monoglyceride composed of lauric acid esterified with glycerol that is found in coconut oil (Carpo et al. 2007). More than 50% of coconut oil consists of lauric acid, a medium fatty acid chain (Hegde 2006). Studies have shown that monolaurin has broad bioactivity, such as antimicrobial, antiviral and antifungal properties (Bergsson et al. 2001; Carpo et al. 2007; Li et al. 2009; Peterson and Schlievert 2006). In an *in vitro* study by Schlievert et al. 2012 tested the antibacterial activity of glycerol monolaurate (GML) against the gram-positive bacteria, *Staphylococcus aureus*. GML prevented biofilm formation of *S. aureus* with no drug resistance developing at a sub-growth inhibitory concentration. It was concluded that GML has a broad-spectrum of antimicrobial activity and has the potential for future application as a topical therapeutic agent *in vivo*.

The aim of the present study was to primarily evaluate monolaurin's antifungal activity against *Candida albicans* biofilms *in vitro* and to study investigate whether monolaurin can modulate host immune response during fungal infections. Furthermore, we provide validation for the safety of monolaurin use in future *in vivo* study in the treatment of oral to candidiasis by examining its cytotoxicity on oral fibroblast cells. Finally, we attempted to identify whether monolaurin can disrupt biofilm formation by reducing critical virulence factors associated with *C. albicans*, such as its secretion of hydrolytic protease and phospholipase enzymes that can elicit tissue damage and invasion to the host mucosa.

MATERIALS & METHODS

Susceptibility Test. The antimicrobial activity of 1-Lauroyl-rac-glycerol (1-monolaurin) (Sigma) was tested *in vitro* against the following *Candida albicans* strains: fluconazole-resistant strain 96901, SC5314, ATCC: MYA2876, and ATCC 90028, according to the NCCLS guidelines (NCCLS 2002). The strains of *C. albicans* are proven virulent pathogens and were selected for their known genomic sequencing (Jones et al. 2004). The minimum inhibitory concentration (MIC) was determined using an inoculum of 5×10^3 CFU/ml *C. albicans* grown in RPMI-1640 (Lonza) in a 96-well plate. Serial dilutions in the range of 3.9-2000 μ M of 1-monolaurin (Sigma) were prepared. Based on the MIC found for the tested *C. albicans* strains, fluconazole (32 μ M) (Sigma) was used as the positive control in this experiment in comparison to the vehicle control 1% Ethanol (v/v). The plate was incubated for 24 hours at 37 °C in 5% CO₂. Minimum inhibitory concentration (MIC) was determined after 24 hours as the concentration at which *C. albicans* growth was visibly inhibited (NCCLS 2002; Pasetto et al. 2014). Minimum fungicidal concentration (MFC) was found by subculturing 20 μ l of each well with concentrations above the

95 MIC on Sabouraud Dextrose Agar (BD). After 24 hours of incubation, MFC concentration was
96 determined as the lowest concentration of 1-monolaurin, showing no visible *C. albicans* growth
97 on the agar plates (NCCLS 2002).

98 **Biofilm Assay.** An inoculum of 1×10^6 CFU/ml of *C. albicans* (ATCC: SC5314/MYA2876) was
99 grown for 24 hours in a sterile 24-well plate using Yeast Nitrogen Base Medium (Difco) with 50
100 mM of glucose for 24 hours at 37 °C in 5% CO₂ to establish initial biofilm growth. Total volume
101 of 1 ml of inoculum was pipetted in each well. After 24 hours of incubation, the biofilms were
102 treated once daily with 1-monolaurin at concentrations of 1250 µM and 2500 µM (equivalent to
103 10x MIC and 20x MIC; respectively). The vehicle control used was 1% ethanol while
104 fluconazole (1mg/1ml) was the positive control. Before each treatment, biofilms were washed
105 with Phosphate Buffer Solution (PBS) and replenished with fresh medium. After 72 hours of
106 treatments, biofilms were suspended in PBS and the solution was centrifuged at 10,000 rpm for 5
107 minutes (Santana et al. 2013). Colony formation unit (CFU) was determined by suspending each
108 sample of biofilm in 1 ml of PBS and plating 20 µl of the suspension on Sabouraud Dextrose
109 Agar plates (BD). After 24 hours of incubation, the number of *C. albicans* colonies was counted
110 and data was expressed in Log (CFU/ml).

111 **Proteinase and Phospholipase Enzyme Secretion Assay.** Proteinase and phospholipase enzyme
112 secretion assays were conducted as previously performed by Santana et al. 2013. Biofilms of *C.*
113 *albicans* were grown for 24 hours in Yeast Nitrogen Base Medium (Difco) with 50 mM of
114 glucose at 37 °C in 5% CO₂ and treated using 1-monolaurin concentrations of 1250 µM and 2500
115 µM (10x MIC and 20x MIC; respectively). Phospholipase A2 (for phospholipase assay) (Sigma)
116 and Trypsin (for proteinase assay) (Lonza) were used as standards. Vehicle control was 1%
117 ethanol. After 72 hours of biofilm maturation, the enzyme secretion assays were performed on
118 the sonicated biofilms, suspended in PBS. The proteinase enzyme activity was determined by
119 mixing the supernatant of the biofilm solution with 1% azocasein at 1:9 (v/v) for 1 hour at 37 °C
120 in 5% CO₂. Then, 500 µl of 10% trichloroacetic acid was added to stop the reaction. The
121 solution was then centrifuged for 5 minutes at 10,000 rpm and 500 µl of the supernatant was
122 combined with 500 µl of NaOH, which was incubated for 15 minutes at 37 °C in 5% CO₂.
123 Absorbance was read in a spectrophotometer at 440 nm (Goncalves et al. 2012; Pande et al. 2006;
124 Santana et al. 2013). The phospholipase enzyme activity was determined by mixing the
125 supernatant of the biofilm solution with phosphatidylcholine substrate for 1 hour at 37 °C in 5%
126 CO₂ and reading the absorbance in a spectrophotometer at 630 nm (Goncalves et al. 2012;
127 Santana et al. 2013; Taniguchi et al. 2009).

128 **Co-culture Model Fluorescence Microscopy.** Cytotoxicity assays using fluorometric
129 quantification of cellular viability were performed on oral fibroblast cells (ATCC: CRL2014).
130 Fibroblast cells (1×10^5 cells/ml) were first seeded in a 96-well plate in DMEM medium with 10%
131 FBS and incubated at 37 °C in 5% CO₂ for 24 hours. Cells were then treated with 1-monolaurin
132 (3.9-2000 µM) and incubated for an additional 24 hours. Cell viability and morphological
133 characteristics were observed by fluorometric method and microscope, respectively (Pasetto et al.
134 2014). A co-culture model was performed by culturing fibroblast cells and *C. albicans* together in
135 a sterile 24-well plate. First, oral fibroblast cells (CRL2014) were seeded in Dulbecco's Modified
136 Eagle's Medium (DMEM) with Fetal Bovine Serum (FBS) (Gibco) and incubated at 37 °C in 5%
137 CO₂ for 24 hours. The medium was then replaced with an inoculum of 5×10^3 to 2.5×10^3 CFU/ml
138 *C. albicans* (ATCC: SC5314) grown in DMEM without FBS. Fibroblast cells and *C. albicans*
139 were treated with 125 µM and 250 µM of 1-monolaurin. The plate was then incubated for 24
140 hours. The vehicle control tested was 1% ethanol. The distribution of dead and live fibroblast

cells was examined using the Viability/Cytotoxicity Assay Kit for Animal Live & Dead Cells (Biotium), which contains a mixture of Calcein AM and EthDIII. Calcofluor white stain (Sigma) was used to stain *C. albicans*. Fluorescent images of the double staining were captured using fluorescence microscopy (EVOS fl microscope AMG, Bothell, WA, USA).

Co-culture Model Quantitative Real-Time PCR. All RNA was isolated from fibroblast cells and *C. albicans*. The fibroblast cell RNA and *C. albicans* RNA were isolated and purified using the RNeasy MiniKit (Qiagen) and the Ribopure Yeast Kit (Life Technology); respectively. A NanoPhotometer P360 (Implen) was used to quantify the total RNA extracted. Reverse transcription of the RNA into cDNA was carried out using iScript Advanced cDNA synthesis Kit for RT-qPCR (Biorad). Real-time PCR was conducted by using iQ SYBR Green Supermix (Biorad). The *C. albicans* primers for the genes: *Secreted Aspartyl Proteinases-1 (SAP-1)*, *Phospholipase B-1 (PLB-1)*, and *ACT-1 (housekeeping)* were used (Nailis et al. 2010). *ACT-1* was the gene used to normalize *SAP-1* and *PLB-1* genes expression. Based on previous analysis using the RT2 Profiler PCR Array Kit (Qiagen), the following host inflammatory cytokines genes were selected: *IL1-alpha* (Qiagen Gene ID#: 3552), *IL1-beta* (Qiagen Gene ID#: 3553), *IL-8* (Qiagen Gene ID#: 3576) and *GADPH* (housekeeping) (Qiagen Gene ID#: 2597). All data from cytokines genes expression were normalized using the housekeeping gene *GADPH*. PCR amplification was performed by using 20 µl reaction mix per well in a 96 well plate. The reactions were conducted at 95 °C for 3 minutes, followed by 40 cycles of 15 seconds at 95 °C and 1 minute at 60 °C. After PCR, the melting curve was obtained by incubating the samples at increasing increments of 0.5 °C from 55 °C to 95 °C.

Statistical Analysis. All data was expressed as the mean ± SEM using one-way analysis of variance (ANOVA) combined with secondary Dunnett's multiple comparison tests compared to the vehicle. The level of statistical significance was set at 0.05.

RESULTS

Susceptibility assay of 1-monolaurin showed antifungal activity against several strains of *C. albicans*, including a fluconazole-resistant strain 96901 in comparison to the antifungal fluconazole (Table 1). The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of monolaurin against *C. albicans* MYA 8276 were found to be 62.5-125 µM and 125-250 µM, respectively in comparison to the MIC and MFC of fluconazole; 32 and 100 µM, respectively, suggesting similar potency. The MIC and MFC of monolaurin against the fluconazole-resistant strain 96901 were found to be much lower than those of fluconazole; 30 µM and 140 µM in comparison to the MIC and MFC values of flucanazole of 100 µM and 350 µM, suggesting a strong antifungal potential against fluconazole resistant strains. Biofilm assay showed significant reduction ($p < 0.05$) in fungal load after treatments with 1-monolaurin at 1250 µM and 2500 µM in comparison to the vehicle control. Fungal load was expressed, as the Log of the colony formation unit (CFU/ml) (Figure 1).

Candida albicans secreted enzymes were analyzed in the proteinase and phospholipase enzyme assays. Biofilms treated with 1250 µM and 2500 µM of 1-monolaurin showed no significant difference in either enzyme activity when compared to the vehicle group (Figures 2). Thus, there was no reduction in enzyme activity, expressed in Unit U/ g of biofilm dry weight.

In the cytotoxicity assay, 1-monolaurin (3.9 to 2500 µM) presented no toxicity to oral fibroblast cells (data not shown). The co-culture model revealed the effectiveness of 1-monolaurin to inhibit fungal growth in comparison to the vehicle control group, as demonstrated by fluorescence microscopic image *C. albicans* sparse distribution upon treatment with 125 µM (Figure 3). Furthermore, co-culture model provided invaluable information on gene expression of

fibroblast cells infected by *C. albicans* after 1-monolaurin treatments. Quantitative Real-time PCR assessed gene expression of *Secreted Aspartyl Proteinases-1 (SAP-1)* and *Phospholipase B-1 (PLB-1)*. Treatments of 1-monolaurin at MIC concentrations of 62.5 μ M and 125 μ M showed no statistical significant difference when compared to the vehicle control (Figure 4A and Figure 4B). However, there was significant down-regulation of *IL-1alpha* after 125 μ M of 1-monolaurin treatments in comparison to the vehicle (Figure 4C). Similarly, *IL-1beta* gene expression was significantly down regulated in the biofilms treated with 62.5 μ M of 1-monolaurin treatments (Figure 4D). Significant up-regulation of *IL-8* in the group treated with 62.5 μ M of 1-monolaurin was also observed (Figure 4E).

DISCUSSION

The goal of this study was to evaluate the pharmacologic effect of monolaurin against *C. albicans* biofilms *in vitro* using a co-culture model, and to investigate how it can alter the morphology of the biofilm community as well as the protease and phospholipase enzyme activity. Susceptibility tests against *Candida albicans* (MYA2876) showed inhibition of fungal growth at an MIC range of 62.5-125 μ M while the minimum fungicidal concentration was in the range of 125-250 μ M, which shows potential antifungal activity against *C. albicans* strain. In the biofilm assay model, 10x the concentrations of MIC and MFC (1250 μ M and 2500 μ M) were used due to the nature of the biofilm's tenacity to eradicate. Biofilms treated with monolaurin showed significant ($p < 0.05$) reduction in the fungal load, as illustrated by the decrease in Log (CFU/ml) biofilm sample in comparison to the control groups. Similarly, the results obtained by fluorescence microscopy of the co-culture model (Figure 3) showed a large decrease in the viable *C. albicans* distribution among oral fibroblast cells after treatment with 125 μ M 1-monolaurin.

These results were in agreement with those reported by Bergsson et al. 2001, who confirmed the susceptibility of *Candida albicans* to several fatty acids and their 1-monoglycerides, and concluded, based on studying ultrathin sections under a transmission electron microscopy (TEM), that after treatment with these compounds, the cytoplasm of *C. albicans* becomes disrupted and ultimately cell death or apoptosis results from the disintegration of the plasma membrane (Bergsson et al. 2001). It should also be noted that the images from co-culture fluorescence microscopy may show that monolaurin has a more sensitive and profound effect on the morphology and distribution of fibroblast cells, as this model represents more susceptibility than cells tested under clinically relevant conditions.

A major helpful analysis provided by the co-culture model is to study the host's inflammatory response by quantification of a panel of inflammatory markers, such as *IL-1 β* , *IL-6*, *IL-8*, *IL-10*, and *IL-17* (Arien et al. 2011). Monolaurin 5% gel was previously shown to inhibit innate inflammatory responses, as it prevented vaginal SIV transmission in monkeys (Li et al. 2009). More specifically, glycerol monolaurate has inhibitory activity against the production of MIP-3 α and other pro-inflammatory cytokines, and can inhibit mucosal signaling and block the inflammatory response to HIV-1 and SIV *in vitro* and *in vivo* (Li et al. 2009). In our current study, we tested gene expression of pro-inflammatory cytokines, *IL-1 α* and *IL-1 β* after treatments with 1-monolaurin at 62.5 μ M and 125 μ M. Our results show a significant ($p < 0.05$) down-regulation of *IL-1 α* with the treatments of 1-monolaurin treatments at 125 μ M (Figure 4C), while down-regulation of the *IL-1 β* gene expression was achieved with monolaurin treatments at 62.5 μ M. However, gene expression of *IL-8* was not down regulated in monolaurin treated biofilms. It can be concluded that monolaurin can modulate host response by down regulating the gene expression of some of the pro-inflammatory cytokines, such as *IL-1 α* and *IL-1 β* . Future research interests may involve studying other pro-inflammatory as well as anti-inflammatory markers, such as *IL-10*, using the same co-culture model in order to investigate a more comprehensive

analysis of the drug's ability to modulate host response and to determine if it can contribute to the anti-inflammatory mechanism in mammalian cells.

Hydrolytic enzymes, such as proteases and phospholipases, which are secreted by *Candida albicans*, are often known to be critical virulence factors in the pathogenicity of such fungal species. *C. albicans* Secreted aspartyl proteinases (SAP) are often associated with virulence factors contributing to the progression of candidiasis (Lermann and Morschhauser 2008).

However, the role of specific SAP genes to their attenuated phenotype is yet to be elucidated. During fungal infections, there is generally a higher gene expression of SAPs, which is often associated with hyphal formation and the induction of rim101p, a transcription factor that mediates the degradation of E-cadherin protein of the epithelial cell junction (Naglik et al. 2008).

In our study, the gene expression of *SAP-1* was evaluated after application of treatments of 1-monolaurin at concentrations of 62.5 μ M and 125 μ M and normalized by the total dry weight of biofilm samples. There was no statistical significance difference in *SAP-1* gene expression in comparison to the control groups (Figure 4A). In terms of proteinase enzyme secretion of *Candida*, it was found that there is no reduction in the enzyme activity with the treatment of monolaurin at 1250 μ M and 2500 μ M (Figure 2A). Thus, it can be concluded that monolaurin does not affect the enzyme activity of proteases produced by *Candida*. More studies would be helpful to explore the effect of monolaurin on the remaining of the SAPs family.

Similarly, phospholipases B1, B2, C and D of *Candida albicans* play a significant role in the invasion of the host tissue, as noted by their high gene expression during fungal infection (Samaranayake et al. 2006). More specifically, phospholipase B (PLB) proteins were shown to have hydrolytic activity, as they hydrolyze acyl ester bonds in phospholipids and lysophospholipids and catalyze lysophospholipase-transacylase reactions (Theiss et al. 2006). It was determined that the *PLB* multigene family of the opportunistic fungal pathogen *Candida albicans* encodes for CaPLB5, a putative secretory protein with a predicted GPI-anchor attachment site. The ability of *C. albicans* to attach itself to the host tissue is considered a key pathogenic characteristic and hence, genes encoding for attachment proteins, such as *PLB*, may be potential virulence determinants (Theiss et al. 2006). In this study, phospholipase enzyme activity was tested using both 1250 μ M and 2500 μ M treatments of 1-monolaurin, which showed was no significance difference when compared to the vehicle group (Figures 2B). In addition, gene expression of *PLB-1* at both concentrations of 1-monolaurin (62.5 μ M and 125 μ M) was not down regulated (Figure 4B). More studies are needed to elucidate the role of monolaurin on the gene expression of the other phospholipases B2, C, and D.

As mentioned earlier, the safety of monolaurin, as a food additive and emulsifier was recognized as GRAS (Generally Recognized as Safe) by the FDA (Food and Drug Administration) for up to 100 mg/ml (Peterson and Schlievert 2006). Not surprisingly, in our cytotoxicity assay, 1-monolaurin showed no toxicity to oral fibroblast cells. The lethal dosage of 1-monolaurin, 50%, could not be calculated in this study based on the regression analysis with no drug concentrations resulting in 0% cell viability. Thus, 1-monolaurin shows no toxicity (up to 2500 μ M) to oral fibroblast cells and therefore it can be further investigated in future *in vivo* studies.

In conclusion, 1-monolaurin has potential antifungal activities against *Candida albicans* both in susceptibility tests and biofilm assays. Furthermore, monolaurin has immune-modulatory effects on the host cells, as indicated by its down-regulation of pro-inflammatory cytokines gene expression of *IL-1 α* and *IL-1 β* . Future direction for research may include understanding its impact on proteases activity on the cellular level and whether there is a direct effect on attachment protein gene expression. Ultimately, future studies may validate the efficacy of monolaurin *in vivo*, which may translate into its clinical use to prevent and/or treat oral candidiasis.

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Table 1(on next page)

Table 1. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of 1-monolaurin against *Candida albicans*.

Table 1. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of 1-monolaurin against *Candida albicans*. *Fluconazole resistant.

Microorganism	Monolaurin		Fluconazole (Positive Control)	
	MIC (μM)	MFC (μM)	MIC (μM)	MFC (μM)
<i>Candida albicans</i> 96901 *	30	140	100	350
<i>Candida albicans</i> SC5314	25	100	10	80
<i>Candida albicans</i> MYA-2876	62.5-125	125-250	32	100
<i>Candida albicans</i> 90028	20	110	20	90

Table 1. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of 1-monolaurin against *Candida albicans*. *Fluconazole resistant.

Figure 1(on next page)

Figure 1. Fungal load of 1-monolaurin treated biofilms. Figure2. Candidaalbicans secreted enzymes activity of proteinase and phospholipase after treatment with 1-monolaurin; of A. proteinase, and B. phospholipase enzymes.

Figure 1. Fungal load of 1-monolaurin treated biofilms expressed as Log (CFU/ ml). Figure 2. Candida albicans secreted enzymes activity expressed in Unit U/ grams of dry weight after treatment with 1-monolaurin ; of A . proteinase, and B. phospholipase enzymes

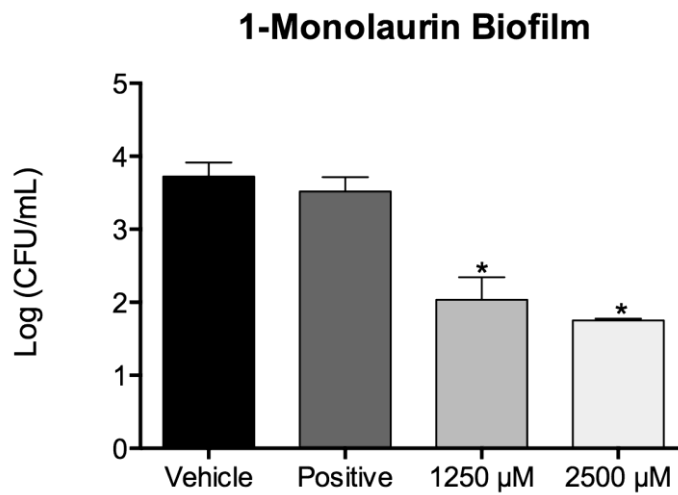


Figure 1. Fungal load of 1-monolaurin treated biofilms expressed as Log (CFU/ ml).

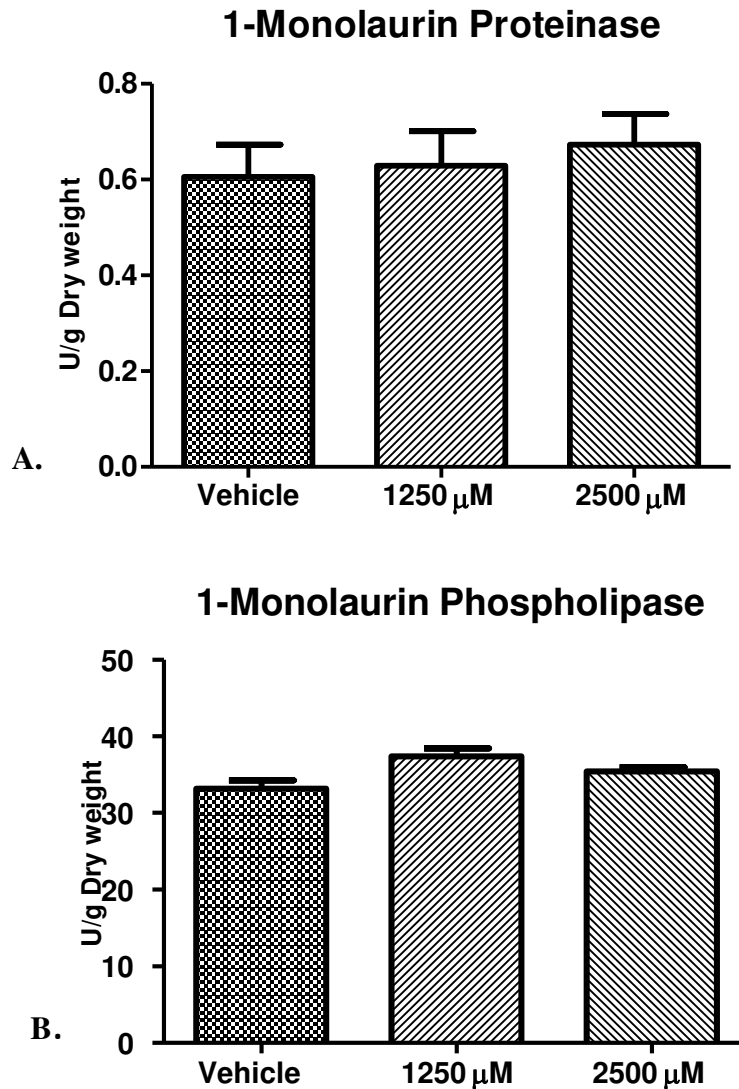
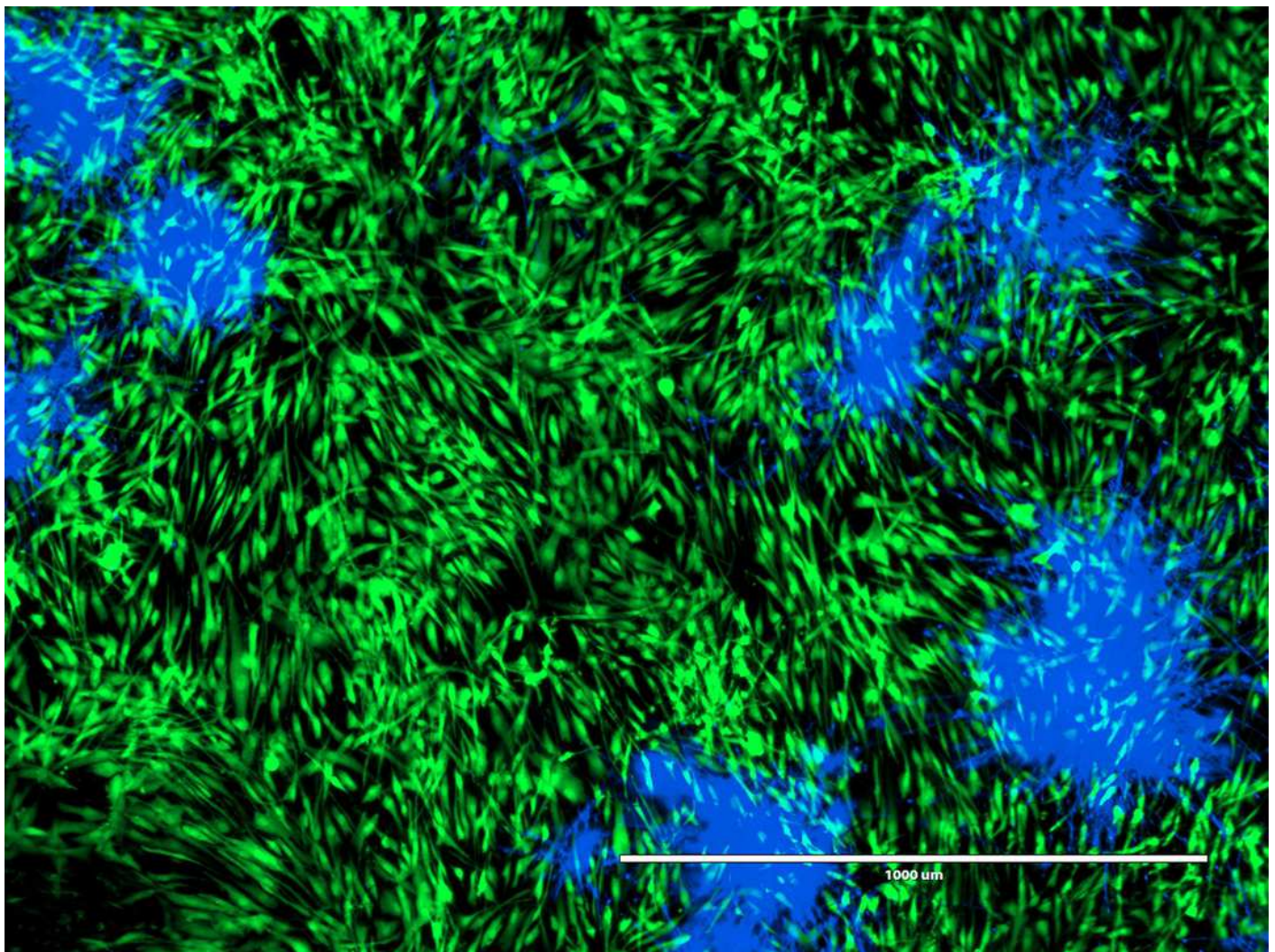


Figure 2. *Candida albicans* secreted enzymes activity expressed in Unit U/ grams of dry weight after treatment with 1-monolaurin; of **A.** proteinase, and **B.** phospholipase enzymes.

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Figure 3A- Co-culture fluorescence microscopy of vehicle control (1% ethanol).

Figure 3- Co-culture fluorescence microscopy of: A. vehicle control (1% ethanol), B. positive control (fluconazole), and C. 1-monolaurin (125 μ M); stained with calcofluor white staining and cytotoxicity assay kit for animal Live/Dead cells (Blue: *Candida albicans*, Green: live fibroblast cells, and Red: dead fibroblast cells). Scale bar set at 1000 μ m at 4x magnification power.

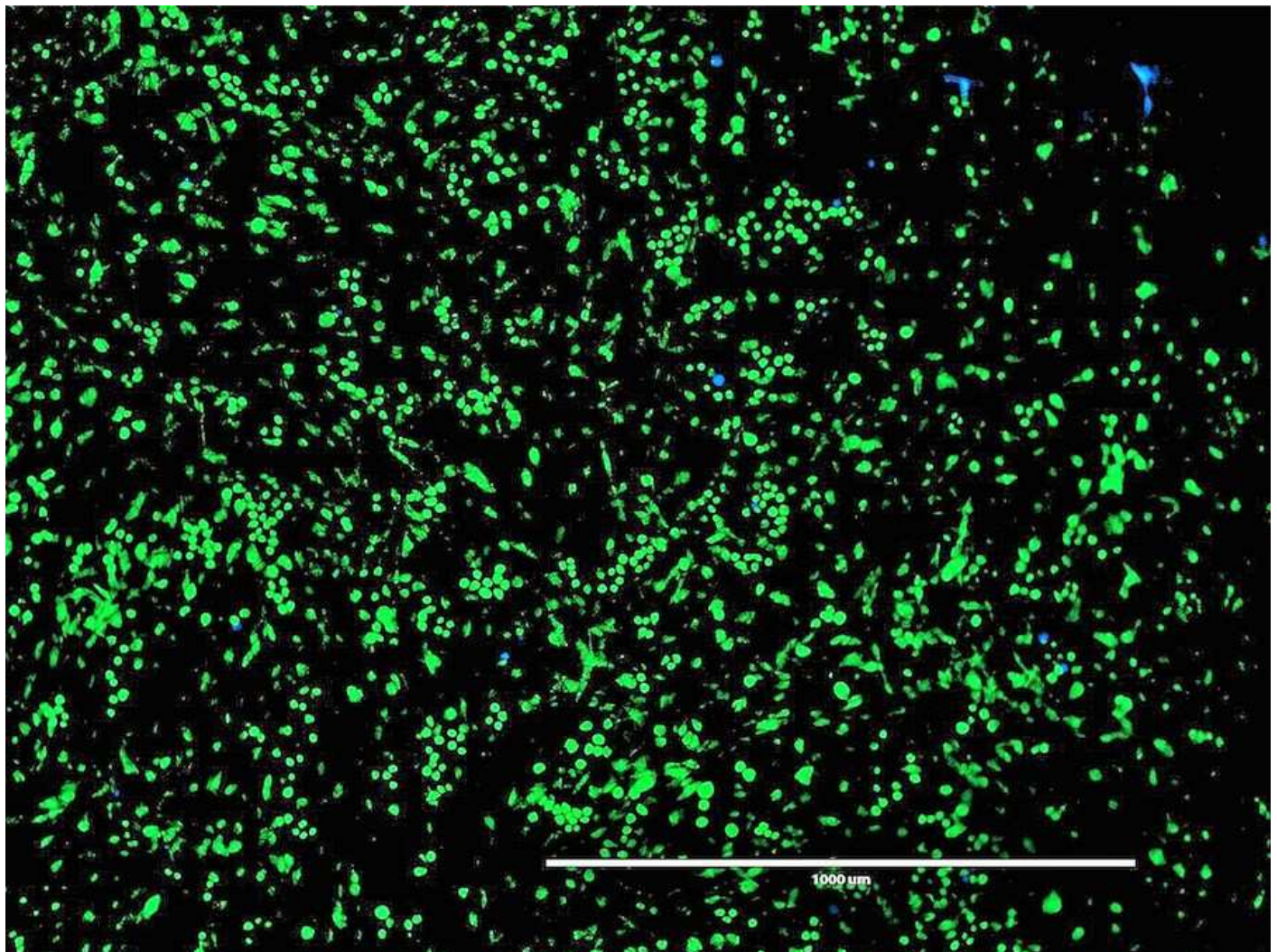


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Figure 3B- Co-culture fluorescence microscopy of positive control (fluconazole 32.2 μ M).

Figure 3- Co-culture fluorescence microscopy of: A. vehicle control (1% ethanol), B. positive control (fluconazole 32.2), and C. 1-monolaurin (125 μ M); stained with calcofluor white staining and cytotoxicity assay kit for animal Live/Dead cells (Blue: *Candida albicans*, Green: live fibroblast cells, and Red: dead fibroblast cells). Scale bar set at 1000 μ m at 4x magnification power.

*Note: Auto Gamma Correction was used for the image. This only affects the reviewing manuscript. See original source image if needed for review.



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Figure 3C-Co-culture fluorescence microscopy of 1-monolaurin (125 μ M).

Figure 3- Co-culture fluorescence microscopy of: A. vehicle control (1% ethanol), B. positive control (fluconazole 32.2 μ M), and C. 1-monolaurin (125 μ M); stained with calcofluor white staining and cytotoxicity assay kit for animal Live/Dead cells (Blue: *Candida albicans*, Green: live fibroblast cells, and Red: dead fibroblast cells). Scale bar set at 1000 μ m at 4x magnification power.

*Note: Auto Gamma Correction was used for the image. This only affects the reviewing manuscript. See original source image if needed for review.

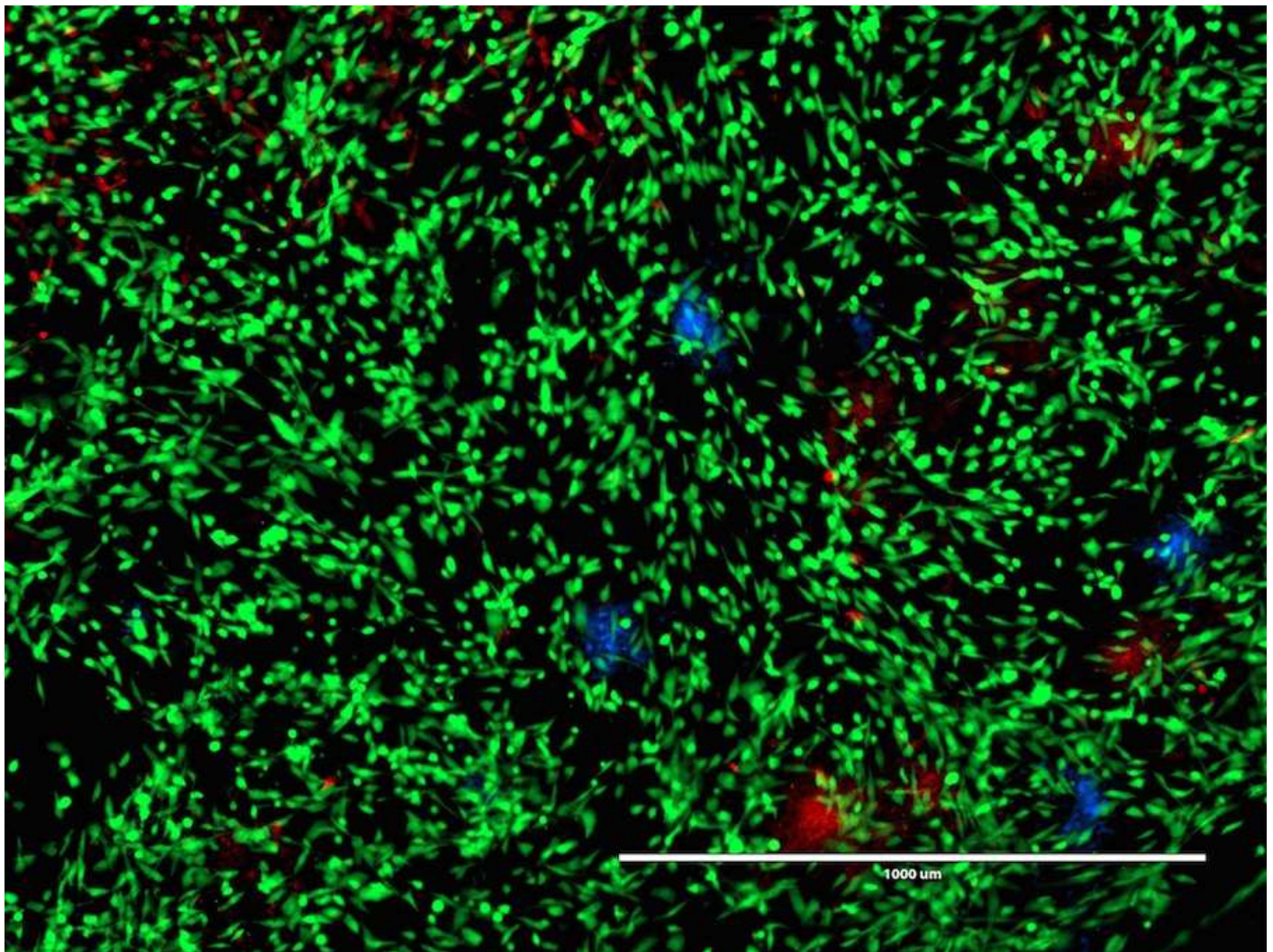


Figure 5(on next page)

[p] Figure 4 . Real-time quantitative gene expression of oral fibroblast cells infected by *C. albicans* after 1-monolaurin treatments at 62.5 and 125 μ M concentrations. in comparison to vehicle control and positive control fluconazole

Figure 4 . Real-time quantitative gene expression of oral fibroblast cells infected by *C. albicans* after 1-monolaurin treatments at 62.5 and 125 μ M concentrations in comparison to vehicle control and positive control fluconazole (32.2 μ M) . A. SAP-1 B. PLB-1 C. IL-1 α D. IL-1 β E. IL-8.

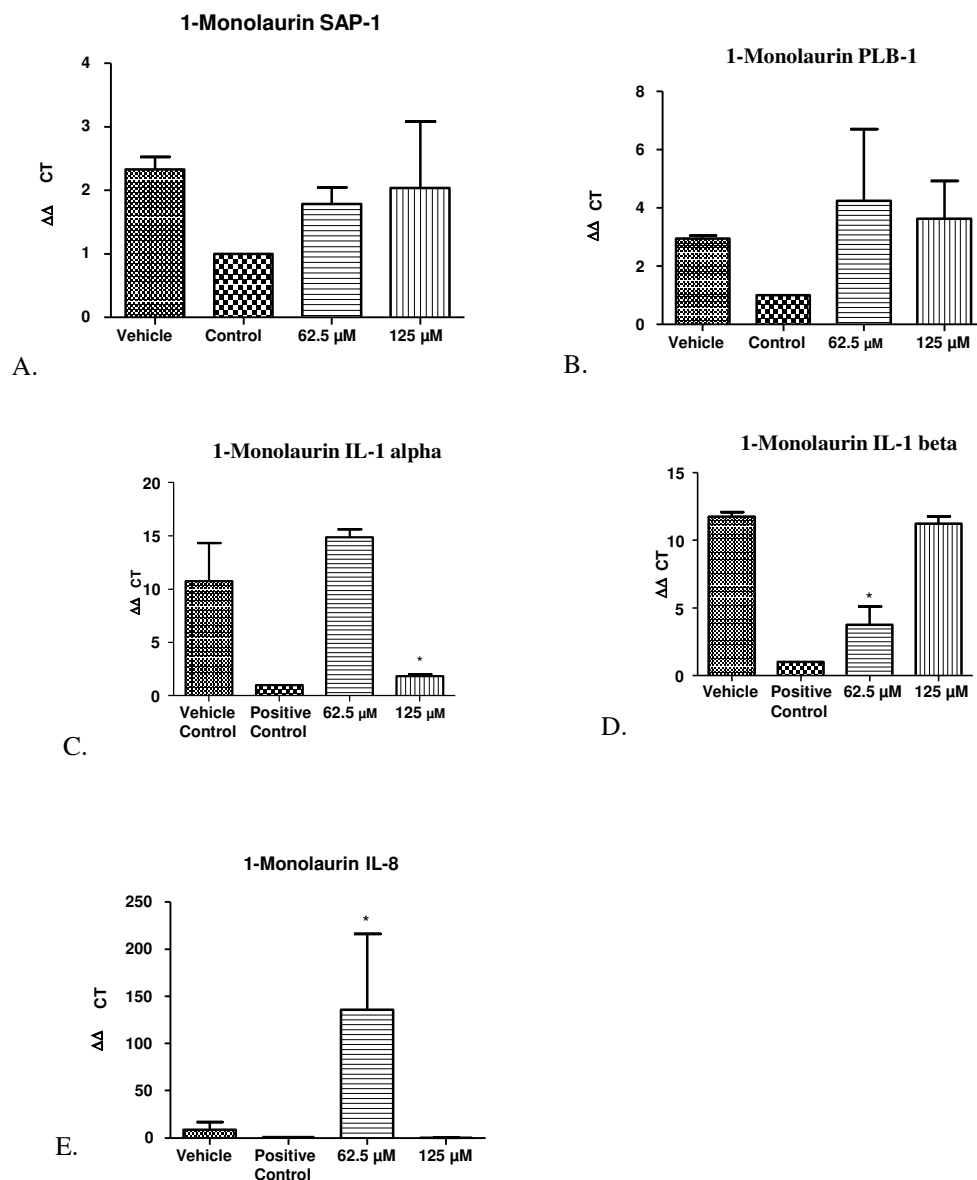


Figure 4. Real time quantitative gene expression of oral fibroblast cells infected by *C. albicans* after 1-monolaurin treatments at 62.5 and 125 μM concentrations in comparison to vehicle control and positive control fluconazole (32.2 μM). **A.** SAP-1 **B.** PLB-1 **C.** IL-1alpha **D.** IL-1beta **E.** IL-8.