A peer-reviewed version of this preprint was published in PeerJ on 22 June 2016.

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

Emily Chen, Dalia Seleem, Bruna Benso, Vanessa Pardi, Ramiro Murata

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-1α and IL-1β, as illustrated in co-culture models. The results from three groups were compared: 1- monolaurin (in the range of 3.9-2500 µM), positive control fluconazole (322 µM), and vehicle control group 1% Ethanol (v/v) The MIC and MFC of monolaurin were in the range 62.5-125 µM and 125-250 µM, respectively. The results show significant reduction in Log (CFU/ml) of biofilms treated with 1250 and 2500 µM of 1- monolaurin when compared to the control groups. There was also a significant down-regulation of IL-1α and IL-1β 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.
**ABSTRACT**

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 gene expression of host inflammatory cytokines, IL-1α and IL-1β. 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 μM), positive control fluconazole (32.2 μM), 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 μM and 125-250 μM, respectively. The results show significant reduction in Log (CFU/ ml) of biofilms treated with 1250 and 2500 μM of 1- monolaurin when compared to the control groups. There was also a significant down-regulation of IL-1α and IL-1β 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 esterfied 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 5x10^3 CFU/ml C. albicans grown in RPMI-1640 (Lonza) in a 96-well plate. Serial dilutions in the range of 3.9-2000 μM of 1-monolaurin (Sigma) (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 μl of each well with concentrations above the
MIC on Sabouraud Dextrose Agar (BD). After 24 hours of incubation, MFC concentration was determined as the lowest concentration of 1-monolaurin, showing no visible C. albicans growth on the agar plates (NCCLS 2002).

Biofilm Assay. An inoculum of 1x10⁶ CFU/ml of C. albicans (ATCC: SC5314/MYA2876) was grown for 24 hours in a sterile 24-well plate using Yeast Nitrogen Base Medium (Difco) with 50 mM of glucose for 24 hours at 37 °C in 5% CO₂ to establish initial biofilm growth. Total volume of 1 ml of inoculum was pipetted in each well. After 24 hours of incubation, the biofilms were treated once daily with 1-monolaurin at concentrations of 1250 μM and 2500 μM (equivalent to 10x MIC and 20x MIC; respectively). The vehicle control used was 1% ethanol while fluconazole (1mg/1ml) was the positive control. Before each treatment, biofilms were washed with Phosphate Buffer Solution (PBS) and replenished with fresh medium. After 72 hours of treatments, biofilms were suspended in PBS and the solution was centrifuged at 10,000 rpm for 5 minutes (Santana et al. 2013). Colony formation unit (CFU) was determined by suspending each sample of biofilm in 1 ml of PBS and plating 20 μl of the suspension on Sabouraud Dextrose Agar plates (BD). After 24 hours of incubation, the number of C. albicans colonies was counted and data was expressed in Log (CFU/ml).

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

Co-culture Model Fluorescence Microscopy. Cytotoxicity assays using fluorometric quantification of cellular viability were performed on oral fibroblast cells (ATCC: CRL2014). Fibroblast cells (1x10⁵ cells/ml) were first seeded in a 96-well plate in DMEM medium with 10% FBS and incubated at 37 °C in 5% CO₂ for 24 hours. Cells were then treated with 1-monolaurin (3.9-2000 μM) and incubated for an additional 24 hours. Cell viability and morphological characteristics were observed by fluorometric method and microscope, respectively (Pasetto et al. 2014). A co-culture model was performed by culturing fibroblast cells and C. albicans together in a sterile 24-well plate. First, oral fibroblast cells (CRL2014) were seeded in Dulbecco’s Modified Eagle’s Medium (DMEM) with Fetal Bovine Serum (FBS) (Gibco) and incubated at 37 °C in 5% CO₂ for 24 hours. The medium was then replaced with an inoculum of 5x10³ to 2.5x10³ CFU/ml C. albicans (ATCC: SC5314) grown in DMEM without FBS. Fibroblast cells and C. albicans were treated with 125 μM and 250 μM of 1-monolaurin. The plate was then incubated for 24 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 (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 fluconazole 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
such as interest may involve studying other pro-inflammatory as well as anti-inflammatory markers, expression of some of the pro-inflammatory cytokines, such as can be concluded that monolaurin can modulate host response by down regulating the gene down-regulation of the μM. However, gene expression of with 1-monolaurin at 62.5 μM and 125 μM. Our results show a significant (p<0.05) down-

study, we tested gene expression of pro-inflammatory cytokines, inflammatory response to HIV-1 and SIV (Li et al. 2009)

innate inflammatory responses, as it prevented vaginal SIV transmission in monkeys

A major helpful analysis provided by the co-culture model is susceptibility tests against Candida albicans (MYA2876) showed inhibition of fungal growth at an MIC range of 62.5-125 μM while the minimum fungicidal concentration was in the range of 125-250 μM, which shows potential antifungal activity against C. albicans strain. In the biofilm assay model, 10x the concentrations of MIC and MFC (1250 μM and 2500 μM) 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 μM 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-1β, 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-3α 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-1α and IL-1β after treatments with 1-monolaurin at 62.5 μM and 125 μM. Our results show a significant (p<0.05) down-regulation of IL-1α with the treatments of 1-monolaurin treatments at 125 μM (Figure 4C), while down-regulation of the IL-1β gene expression was achieved with monolaurin treatments at 62.5 μM. 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-1α and IL-1β. 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.

Hydrolitic 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 μM and 125 μM 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 μM and 2500 μM (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 μM and 2500 μM 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 μM and 125 μM) 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 μM) 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-1α* and *IL-1β*. 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.
ACKNOWLEDGEMENTS

Research reported in this publication was supported by: National Center for Complementary and Integrative Health of the National Institutes of Health under award number R00AT006507 and Brazilian Federal Agency for the Support and Evaluation of Graduate Education (CAPES - 2317/2014-01). The content is solely the responsibility of the authors and does not necessarily represent the official views of the funding agencies. The authors declare no conflicts of interest with respect to the research, authorship, and/or publication of this article.
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Table 1. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of 1-monolaurin against *Candida albicans*.

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<table>
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<th>Microorganism</th>
<th>Monolaurin (µM)</th>
<th>Fluconazole (Positive Control) (µM)</th>
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<tr>
<td></td>
<td>MIC</td>
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<td>Candida albicans 96901*</td>
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**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. Figure 2. *Candida albicans* secreted enzymes activity of proteinase and phospholipase 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 µM); stained with calcofluor white staining and cytotoxicity assay kit for animal Live/Dead cells (Blue: \textit{Candida albicans}, Green: live fibroblast cells, and Red: dead fibroblast cells). Scale bar set at 1000 um at 4x magnification power.
Figure 3B- Co-culture fluorescence microscopy of positive control (fluconazole 32.2 μ M).

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*Note: Auto Gamma Correction was used for the image. This only affects the reviewing manuscript. See original source image if needed for review.*
Figure 3C-Co-culture fluorescence microscopy of 1-monolaurin (125 µM).

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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.
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.