

Original Research Article

The Inhibitory Effect of the Combination Treatment of Rifampicin and D-limonene on the Growth and Biofilm formation of *Staphylococcus epidermidis* RP62A

ABSTRACT

Aims: To discover an alternative chemical that could potentially be used in combination with antibiotics to inhibit the growth of *Staphylococcus epidermidis* RP62A

Study design: The studies of the growth inhibition in both the biofilm forming strain of *S. epidermidis* RP62A and the wild-type strain ATCC 12228, were conducted with different concentrations of rifampicin and D-limonene using the measurement of the optical density.

Place and Duration of Study: All experiments were conducted in the Department of Biology at Manhattanville College between September 2015 and December 2019.

Methodology: Different concentrations of rifampicin (0.0025 µg/mL, 0.005 µg/mL, 0.01 µg/mL and 0.02 µg/mL) and D-limonene (152.8 µg/mL, 305.6 µg/mL, 611.2 µg/mL and 1222.4 µg/mL) were used in the study. The minimal inhibitory concentrations (MIC) of rifampicin and D-limonene on RP62A and ATCC 12228 were obtained. The combination of rifampicin and D-limonene on the inhibition of the growth of RP62A and ATCC 12228 was conducted using a microtiter plate by measuring optical density. The alamarBlue® assay was further used to evaluate the viability of RP62A and ATCC 12228 with the combination treatment. The biofilm assay was then conducted on RP62A with the combination treatment of rifampicin and D-limonene.

Results: The MIC of the combination treatment of rifampicin and D-limonene on RP62A was found to be 0.01 µg/mL and 1222.4 µg/mL respectively. A combination treatment of 0.005 µg/mL of rifampicin with 611.2 µg/mL of D-limonene significantly inhibited the growth and biofilm formation of RP62A than the same concentration of rifampicin.

Conclusion: The addition of the D-limonene to the rifampicin was found to be effective in inhibiting the growth and biofilm formation of RP62A. This indicates that the combination of alternative mineral oil may have the potential to lower the antibiotic concentration in the inhibition of the growth of the bacteria *S. epidermidis*.

Keywords: rifampicin, D-limonene, *Staphylococcus epidermidis*, biofilm, combination treatment, growth inhibition

1. INTRODUCTION

Staphylococcus epidermidis is a Gram-positive, coagulase-negative staphylococci (CoNS) bacterium that colonizes human skin and poses a threat to the health of individuals [1, 2]. It is considered an opportunistic pathogen that facilitates nosocomial infections through adherence on medical devices, such as intravascular and indwelling catheter devices, especially in transplant patients, neonates, and immunocompromised patients, such as those with HIV [2, 3]. Contaminated medical devices alone cause about 60-70% of nosocomial infections [4, 5]. However, *S. epidermidis* flora can be beneficial by providing minimal skin irritation, and the production of T-cells in the spleen and lymph nodes [6, 7, 8]. Infections caused by CoNS bacteria, such as *Staphylococcus* species, have become increasingly difficult to treat due to the ability of the bacteria to form biofilm [9]. Infection transmission risk is thus increased by the presence of *S. epidermidis* in hospitals and causes CoNS sepsis in neonates and immunocompromised patients [3,10].

Biofilms are extracellular substances by which cells that adhere to each other and often to a surface and can contribute to the cause if persistent infections occur [11]. The biofilm extracellular matrix of *S. epidermidis* can adhere to other surfaces through its polysaccharide intercellular adhesin (PIA) [12]. The biofilm of the mutant strain *S. epidermidis* ATCC 35894 (RP62A) provides antibiotic resistance, creating difficulty in treating infections [13]. The *icaABCD* gene operon has encoded proteins responsible for PIA biosynthesis associated in the biofilm formation of *S. epidermidis* [14]. The biofilm formation of *S. epidermidis* RP62A depends on a specific accumulation-associated protein (Aap) [15]. The aggregation of the Aap protein in the zinc-dependent G5 domains of the LPXTG motif in *S. epidermidis* forms fibrils. The zinc ions and proteolytic cleavage of Aap are responsible for the accumulation of biofilm cells and the biofilm effect [2, 15]. A study conducted by Wojtyczka *et al.* showed that antibiotic resistance of the CoNS bacteria biofilm increased the number of hospital-acquired infections which, consequently, poses a threat to human health [9].

Staphylococcus infections are routinely treated by antibiotics. About 75-90% of *S. epidermidis* isolates are resistant against methicillin, which is one of the most utilized antibiotics for staphylococcal infections in hospitals [16]. *S. epidermidis* can also be resistant to rifampicin, tetracycline, clindamycin, and gentamycin [17]. Although different antibiotics are constantly developed, microbial resistance continues to emerge among different bacterial species [18]. Antibiotic resistance of microbes has originated and progressed through adaptation to various overused antibiotics and the degree to which resistance occurs [18]. Rifampicin is one of the most widely used antibiotics to treat nosocomial infections [19]. Rifampicin targets the *rpoB* gene of the sigma factor of DNA-dependent RNA polymerase in the mRNA synthesis of the bacterium, causing a change in its promoter site [19]. In a previous study, after 12 hours of exposure, rifampicin was unable to fully inhibit the growth of the biofilm of RP62A, despite its ability to penetrate it [20].

The increase of antibiotic resistance has facilitated the search for alternative chemical to treat microbial infections [18]. Essential oils have been widely studied as antimicrobial agents in the last few decades. A study conducted by Pasqua *et al.* showed that the addition of essential oils disrupted the structure of the cell envelope of *Staphylococcus aureus*, *Salmonella enterica* serovar *typhimurium*, *Pseudomonas fluorescens*, and *Brochothrix thermosphacta*, by decreasing the amount of unsaturated fatty acids [21]. One of the essential oils used in that study was D-limonene. D-limonene is a monoterpene composed of citrus oils with antioxidant activity and has low toxicity with the lack of any mutagenic or carcinogenic properties [22]. A D-limonene dose less than 1,650 mg/kg a day is considered safe to ingest [22]. In addition, a 100 mg/kg consumption of D-limonene was shown to be beneficial for people [22]. Another study showed that a high D-limonene intake increased the survival in lymphoma-bearing mice [23] and thus, D-limonene is safe to use. The preliminary study of the effectiveness of D-limonene in combination of rifampicin on the biofilm-forming *S. epidermidis* RP62A strain was done in our laboratory, however, no minimum inhibitory concentrations of D-limonene and rifampicin were achieved [24]. Individual constituents of essential oils, like D-limonene, have some nonspecific antimicrobial influence that can postpone the "assembly-disassembly" cycle of biofilm formation during the incubation period of a similar Staphylococcal strain, *S. aureus* [25]. This shows the significance of measuring the dependence and inhibitory effects of limonene on biofilm formation at different growth phases [25]. The same study found that D-limonene reduced the growth of biofilm formation after 8 hours of incubation of the USA300 strain of *S. aureus* by 90%, but after 40 hours of incubation, this percentage decreased to 30% [25].

In the current study, the minimal inhibitory concentration (MIC) of the combination of D-limonene and rifampicin was evaluated on *S. epidermidis* RP62A. Additionally, an alamarBlue[®] assay was used to test the cell viability with the combination treatment. The outlook of further application of this study is to reduce the unaccompanied, high concentration use of rifampicin, and to provide an alternative combination treatment to the biofilm forming *S. epidermidis* strain.

2. MATERIAL AND METHODS

2.1 Strains and growth conditions

S. epidermidis strains wild type ATCC 12228 and biofilm-forming RP62A were purchased from ATCC (ATCC, VA) and stored at -80°C. Both strains were grown in aerobic conditions in Trypticase Soy Agar (TSA) and Trypticase Soy Broth

(TSB) (Sigma-Aldrich, MO) in the Excella E24 Incubator Shaker (New Brunswick Scientific, CT) at 100 RPM at 37°C for 20 hours. The bacterial concentration of these two strains was measured using a DU Series 700 UV/Vis Spectrophotometer (Beckman Coulter, CA). Based on the growth curve computed in our previous study, the bacterial concentrations of the strains at Optical Density $OD_{600}=1$ were 7.5×10^8 CFU/mL for ATCC 12228 and 4.5×10^8 CFU/mL for RP62A [24].

2.2 Minimal inhibitory concentration of rifampicin and D-limonene on *S. epidermidis* ATCC 12228 and RP62A

Both strains were grown on Mueller-Hinton agar (MHA) (BD Difco, MD) with 2% sodium chloride (Sigma-Aldrich, MO) for 20 hours at 37°C. Bacterial colonies from each strain were transferred onto sterile 1X phosphate buffer saline (PBS) (Wards Science, NY), and the growth was analyzed at OD_{600} . Both strains were then diluted to a final concentration of 10^6 CFU/mL. Two flat-bottom 96-well plates (Corning, NY) were used to inoculate each strain with treatments in a total volume of 200 μ L. The highest concentration of each treatment was added into the first well of each row. Two-fold dilutions were then performed with the addition of Mueller-Hinton broth (MHB) (Sigma-Aldrich, MO) containing 2% sodium chloride and bacterial suspension at a final concentration of 5×10^4 CFU/mL along with the addition of either rifampicin (Sigma-Aldrich, MO) (0.0025 μ g/mL, 0.005 μ g/mL, 0.01 μ g/mL and 0.02 μ g/mL) or D-limonene (Sigma-Aldrich, MO) dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, MO) (152.8 μ g/mL, 305.6 μ g/mL, 611.2 μ g/mL and 1222.4 μ g/mL). Wells containing MHB alone, and MHB with bacteria served as control. Each microtiter plate was incubated in the incubator shaker at 100 RPM at 37°C for 20 hours. The same volume of DMSO was also added to MHB to grow the same concentration of bacteria as an additional control. The growth was measured at OD_{570} after 20 hours using a Thermo Fisher Scientific accuSkan GO UV/Vis Microplate Spectrophotometer (Hampton, NH). Each treatment condition was run in triplicate with a minimum of three trials per condition. The pH of all treatments was measured using an Orion Star A111 pH meter (Thermo Fisher Scientific, MA).

2.3 Combination treatment of rifampicin and D-limonene on the growth of *S. epidermidis* ATCC 12228 and RP62A in microtiter plate

After the initial results, two different concentrations of D-limonene (611.2 μ g/mL and 1222.4 μ g/mL) were selected for further analysis in the inhibition of the growth of both strains in combination with four concentrations of rifampicin (0.0025 μ g/mL, 0.005 μ g/mL, 0.01 μ g/mL and 0.02 μ g/mL). The same amount of final bacteria concentration of 5×10^4 CFU/mL were added to each well, forming a total volume of 200 μ L. Each treatment condition was run in triplicate with a minimum of three trials completed. Wells containing MHB alone, MHB with bacteria, MHB and different concentrations of D-limonene or rifampicin served as controls. Each microtiter plate was incubated in the incubator shaker at 100 RPM at 37°C and the growth was measured at OD_{570} after 20 hours.

2.4 The inhibition of the growth analysis on the combination treatment on ATCC 12228 and RP62A after 6 hours and 20 hours in conical tubes

As a result of the microtiter plate experiment, the concentration of 0.005 μ g/mL of rifampicin, 611.2 μ g/mL of D-limonene and their combination were further evaluated on the growth inhibition on both ATCC 12228 and RP62A in 15 mL conical tubes. Both strains were grown in 2 mL of MHB with 2% sodium chloride in plastic conical tubes (Thermo Fisher Scientific, MA). All combination treatment along with the control of either rifampicin or D-limonene were added to the tubes, mixed, incubated overnight in the incubator shaker at 100 RPM for 37°C. Bacterial concentration at OD_{570} was measured after 20 hours. The treatments were run in triplicates and at least five trials were performed. Further, nine additional tubes that contained MHB and bacteria only were incubated for 6 hours under the same condition. The OD_{570} was measured after 6 hours of incubation and the same concentration of rifampicin, D-limonene, and combination of rifampicin and D-limonene was added to these nine tubes. These tubes were then incubated for an additional 14 hours and the OD_{570} was measured again.

2.5 The alamarBlue® assay

A 96-well plate set up for the alamarBlue® assay using 100 μ L of both bacteria with 0.005 μ g/mL of rifampicin, 611.2 μ g/mL of D-limonene and their combination along with the control were performed. Half the plate contained RP62A, and the other half contained ATCC 12228. Following the assay protocol, 10 μ L of alamarBlue® (AB) reagent (Thermo Fisher Scientific, MA) were added to the well for both the 6-hour and 20-hour trials as described before. The plate was placed in the incubator at 37°C and the optical density was read twice after 1 and 2 hours. A Multiskan FC Microplate Spectrophotometer (Thermo Fisher Scientific, MA) was used to measure the absorbance, OD_{570} and OD_{600} . All samples were run in triplicates, and a minimum of three trials were done.

The alamarBlue® assay formula (Formula 1) [26] was used to determine the percentage of cell reduction in each well.

$$\frac{(E_{oxi}600 \times A_{570}) - (E_{oxi}570 \times A_{600})}{(E_{red}570 \times C_{600}) - (E_{red}600 \times C_{570})} \times 100$$

Formula 1: alamarBlue® assay cell reduction percentage equation [26]

2.6 Biofilm assay of the combination treatment on RP62A

The biofilm assay was performed on the 96 well plate on RP62A only with the 0.005 µg/mL of rifampicin, 611.2 µg/mL of D-limonene and the combination treatment along with the control using the protocol from Stephanovic' *et al* [27]. Bacteria were grown as previously described on a microtiter plate for 20 hours at 37°C. The biofilm assay was then performed and OD₅₇₀ was measured. All samples were run in triplicates with a minimum of three trials were done.

2.7 Statistical analysis

A One-way ANOVA and Tukey's Honest Significant Difference (HSD) tests were used when comparing the growth of both strains on different concentrations of rifampicin, D-limonene, and the combination with the control. The biofilm formation of RP62A after the treatment was added from the beginning or after 6 hours of growth were compared with the control in both the microtiter plate assay and the alamarBlue® assay. The significance value was set at $P < .05$. Statistical analyses and other measurements presented (average of the bacterial growth, standard error of mean (SEM) and figures) were performed using SPSS version 26 or Excel.

3. RESULTS

3.1 Growth analysis of rifampicin and D-limonene treatment on ATCC 12228 and RP62A

The growth of ATCC 12228 was significantly inhibited with all concentrations tested of rifampicin. After 20 hours, there was a significant difference in the growth inhibition using 0.0025, 0.005, 0.01 and 0.02 µg/mL of rifampicin when compared to the control of the bacteria alone without any treatment, $P < .05$ (Figure 1). However, there was a significant difference in the growth inhibition on RP62A with 0.01 and 0.02 µg/mL of rifampicin when compared to the control, $P < .05$ (Figure 1). In addition, there was a significant difference in growth inhibition after 20 hours with the concentration of 1222.4 µg/mL of D-limonene when compared to the control in both ATCC 12228 and RP62A ($P < .05$) (Figure 2). Thus, the MIC of the rifampicin and D-limonene on RP62A was found to be 0.01 µg/mL and 1222.4 µg/mL respectively. For either rifampicin or D-limonene treatment, growth inhibition was more prevalent against ATCC 12228 than RP62A. DMSO did not inhibit the growth of both bacteria (data not shown). The pH of D-limonene alone was 4.2 and compared to a 7.1 when added to MHB. The rifampicin alone or when added to MHB and the combination of rifampicin and D-limonene were found to have a neutral pH of about 7.2.

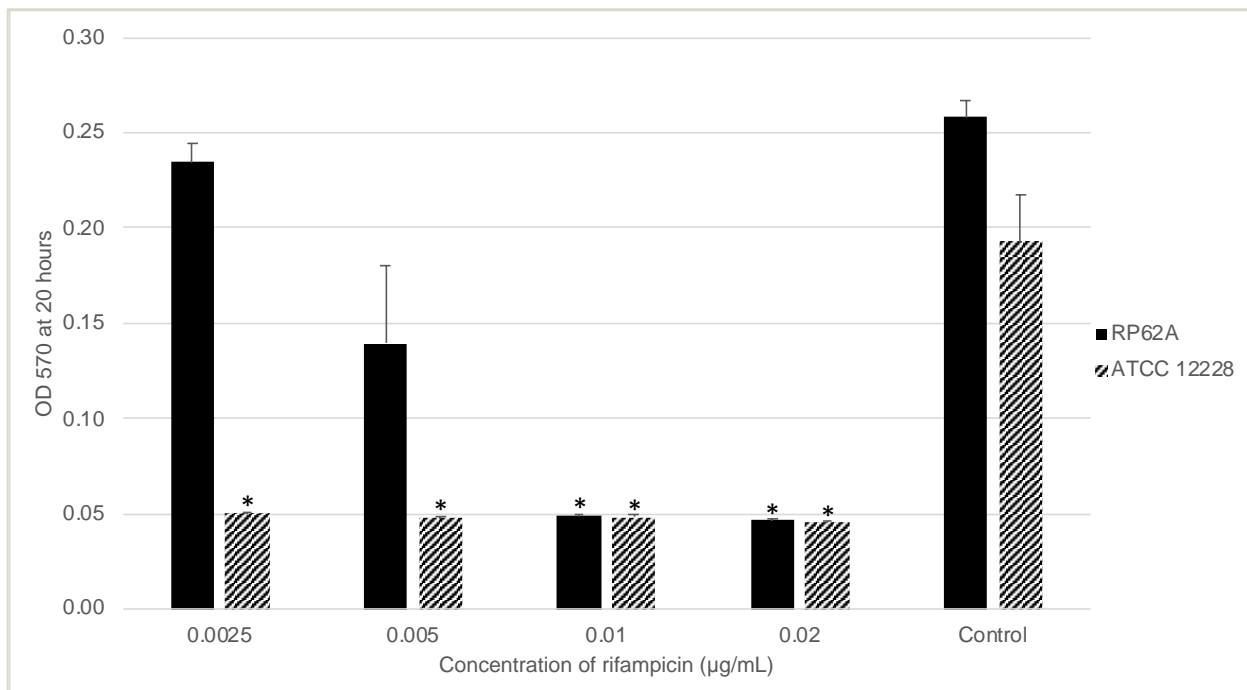


Figure 1. The effect of different concentrations of rifampicin on the growth (OD₅₇₀ + SEM) of *S. epidermidis* RP62A and ATCC 12228, (*The significance of $P < 0.05$ for the treatment when compared to the control), SEM: standard error of mean.

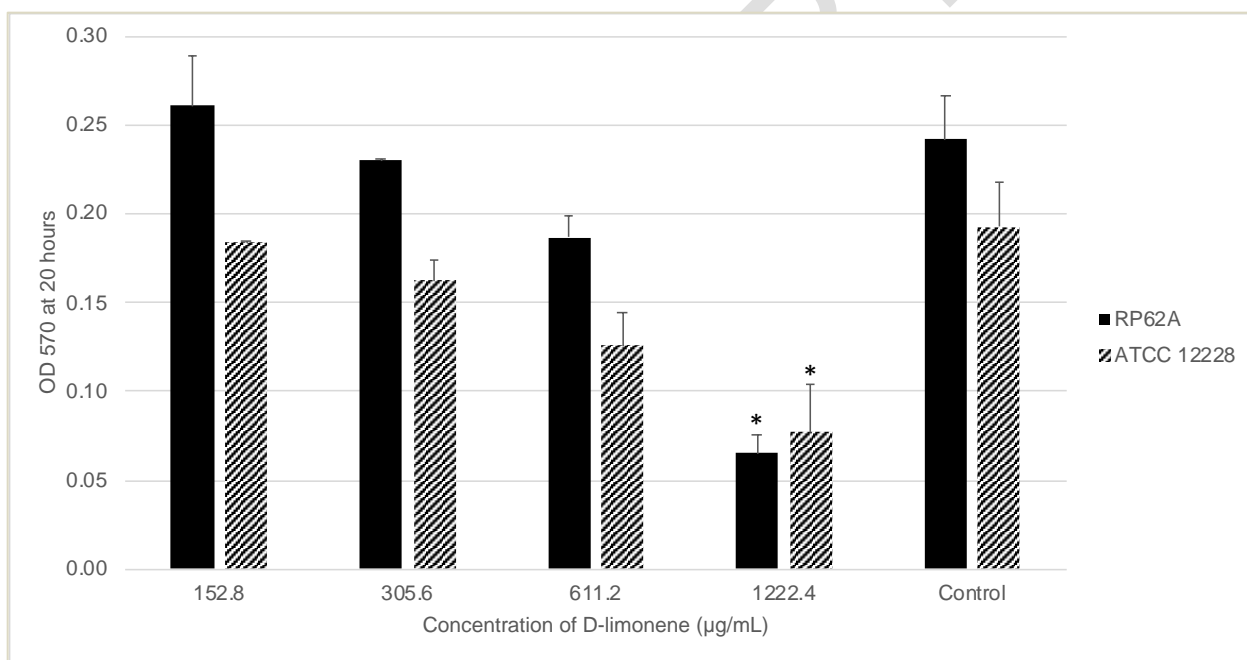


Figure 2. The effect of different concentrations of D-limonene on the growth (OD₅₇₀ + SEM) of *S. epidermidis* RP62A and ATCC 12228, (*The significance of $P < 0.05$ for the treatment when compared to the control), SEM: standard error of mean.

3.2 Growth analysis on the combination treatment on ATCC 12228 and RP62A in microtiter plate

Two different concentrations of D-limonene (611.2 µg/mL and 1222.4 µg/mL) were used in combination with four different concentrations of rifampicin (0.0025 µg/mL, 0.005 µg/mL, 0.01 µg/mL and 0.02 µg/mL) to study the growth inhibition on ATCC 12228 and RP62A. When 1222.4 µg/mL of D-limonene concentration were combined to the different concentrations of rifampicin, the inhibition of the growth of both strains was significant when compared to the control ($P < .05$) (data not shown). However, when 611.2 µg/mL of D-limonene was used in combination with the four different

concentrations of rifampicin, all treatments showed significant growth inhibition on RP62A except the combination with 0.025 µg/mL of rifampicin had shown no significant effect on the growth on ATCC 12228 (Figure 3).

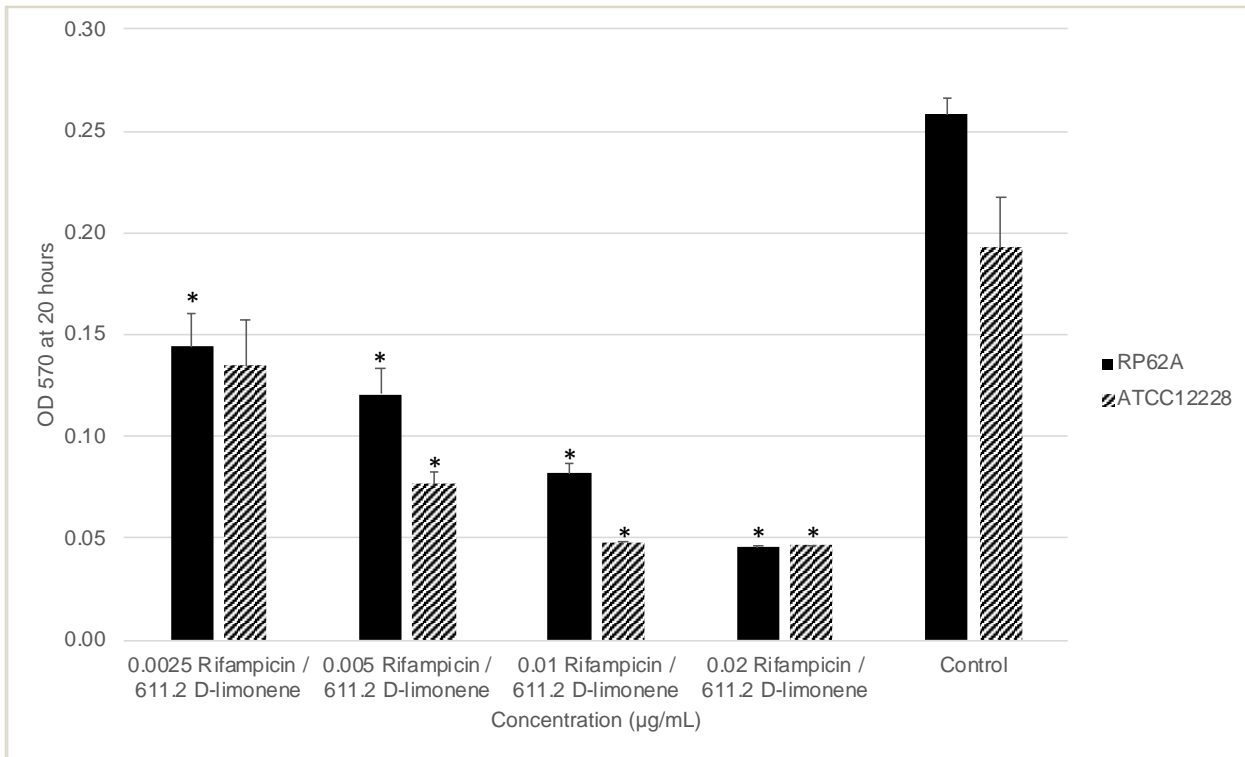


Figure 3. The effect of different concentrations of the rifampicin with 611.2 µg/mL of D-limonene on the growth (OD₅₇₀ + SEM) of *S. epidermidis* RP62A and ATCC 12228, (*The significance of $P < .05$ for the treatment when compared to the control), SEM: standard error of mean.

3.3 Inhibition of growth on the combination treatment on ATCC 12228 and RP62A in the conical vials

In the following experiments, a concentration of 0.005 µg/mL of rifampicin was chosen due to its inability to inhibit the bacterial growth completely. A concentration of 611.2 µg/mL of D-limonene was also chosen as the next lower concentration than the MIC in the combination treatment. After 20 hours of incubation, the growth of ATCC 12228 and RP62A in conical tubes was significantly inhibited when treated with 0.005 µg/mL of rifampicin, 611.2 µg/mL of D-limonene and the combination of both rifampicin and D-limonene when compared to the control of bacteria alone without treatment ($P < .05$) (Figure 4). However, the effect of the combination treatment resulted in almost no growth with zero absorbance in both strains (Figure 4). In another experiment, after both bacteria were grown for 6 hours, all treatment conditions were tested and incubated for 14 hours. Significant results were also shown with the inhibition of the growth of ATCC 12228 and RP62A with the treatments when compared to the control for all conditions ($P < .05$) (data not shown).

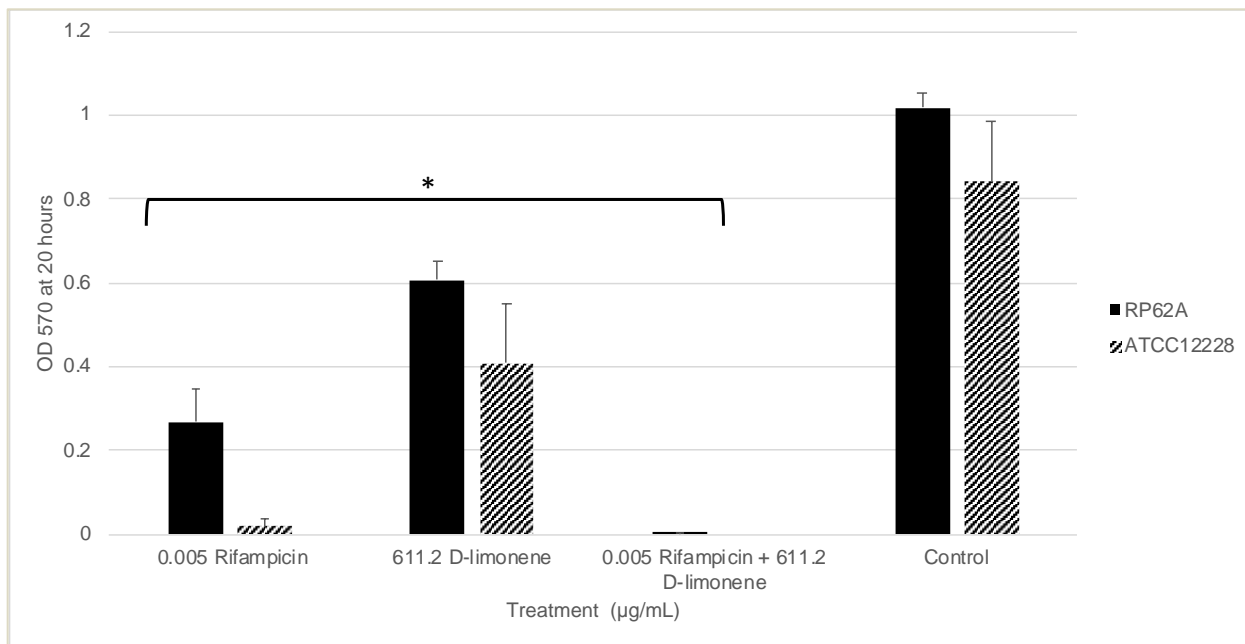


Figure 4. The effect of the growth (OD₅₇₀ + SEM) of *S. epidermidis* RP62A and ATCC 12228 in the plastic conical vials with 0.005 µg/mL of rifampicin, 611.2 µg/mL of D-limonene and the combination after 20 hours of incubation, (*The significance of $P < .05$ for the treatment when compared to the control), SEM: standard error of mean.

3.4 The alamarBlue[®] assay of the combination treatment on cell reduction of ATCC 12228 and RP62A

The results of the alamarBlue[®] assay after the 20 hours incubation of 0.005 µg/mL of rifampicin treatment on ATCC 12228 had an average of 89.9% of cell reduction when compared to 36.1% of cell reduction on RP62A (Figure 5). When the 611.2 µg/mL of D-limonene alone was added, there was a 15.9 % cell reduction on RP62A and a 32.1% cell reduction on ATCC 12228 (Figure 5). Meanwhile, the combination treatment of 0.005 µg/mL of rifampicin and 611.2 µg/mL of D-limonene had an average of 96.2% cell reduction on RP62A and a 100% cell reduction on ATCC 12228 (Figure 5). However, after 6 hours of the growth of the bacteria, treatments were added to the bacteria to continue to grow for the next 14 hours, the result of the alamarBlue[®] assay was inconclusive (data not shown).

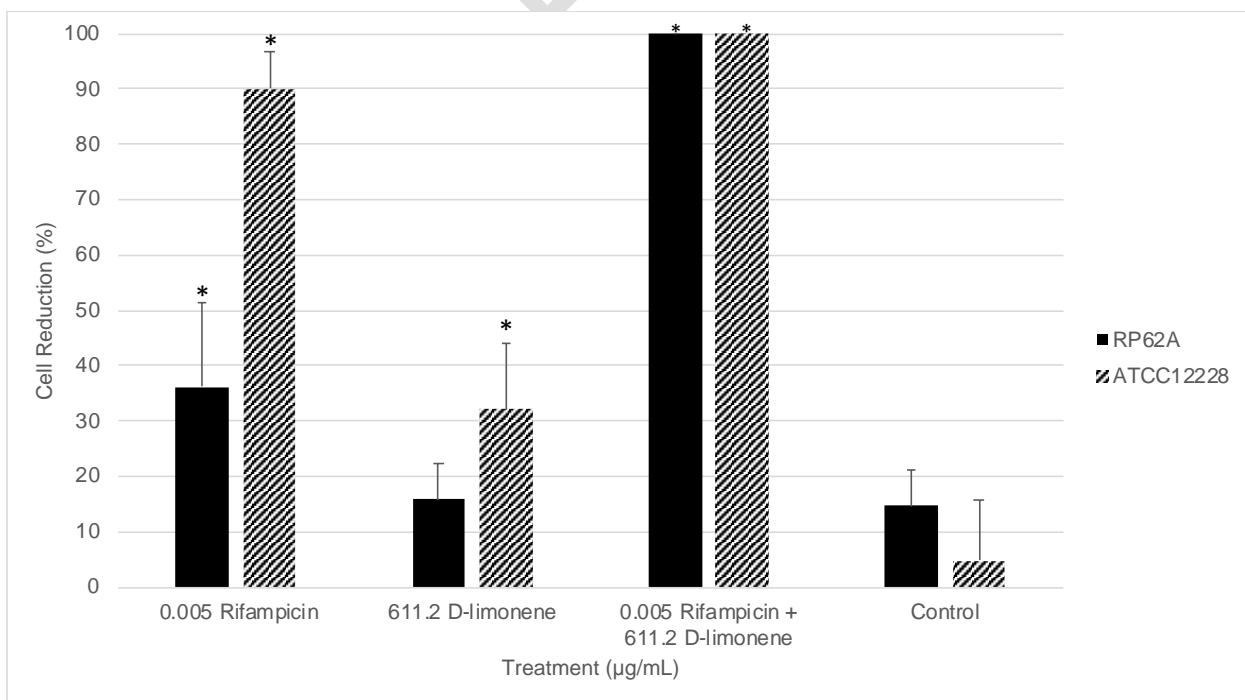


Figure 5. The percentage (+SEM) of cell reduction after the treatment of 0.005 µg/mL of rifampicin, 611.2 µg/mL of D-limonene and the combination using the alamarBlue® assay. The reduction potential at OD₅₇₀ and OD₆₀₀ of *S. epidermidis* RP62A and ATCC 12228 was measured and calculated, (*The significance of $P < .05$ for the treatment when compared to the control), SEM: standard error of mean.

3.5 The Biofilm assay analysis on the combination treatment of RP62A

The biofilm assay of RP62A was performed in a 96-well plate, result showed the combination treatment of 0.005 µg/mL of rifampicin and 611.2 µg/mL of D-limonene had significantly inhibited the biofilm formation of RP62A when compared to the control of bacteria alone ($P < .05$) (Figure 6). The broth was used as an additional control and had a slight background OD₅₇₀ absorbance of 0.05 in the biofilm assay.

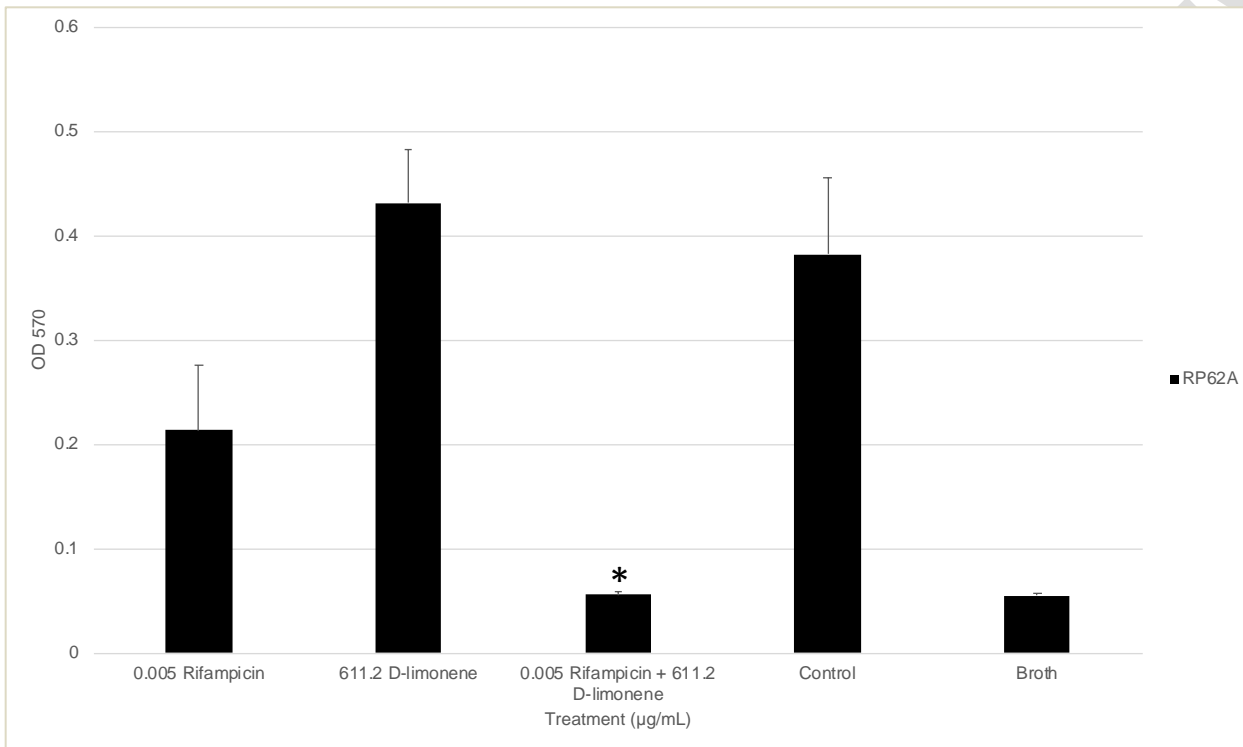


Figure 6. The biofilm assay (OD₅₇₀ + SEM) of *S. epidermidis* RP62A with the treatment of 0.005 µg/mL of rifampicin, 611.2 µg/mL of D-limonene and the combination, (*The significance of $P < .05$ for the treatment when compared to the control), SEM: standard error of mean.

4. DISCUSSION

The goal of this study was to determine the effective combination of the concentrations of rifampicin and D-limonene on the inhibition of the growth and biofilm formation of *S. epidermidis* RP62A compared to the same concentration of rifampicin alone. A combination of 0.005 µg/mL of rifampicin and 611.2 µg/mL of D-limonene was found to inhibit the growth of both RP62A and ATCC 12228 and limit the biofilm formation of RP62A rather than when using the same concentration of 0.005 µg/mL rifampicin alone. The results of this study showed that there was only a reduction of 9.15 % in growth of RP62A with the treatment of 0.0025 µg/mL of rifampicin, however, there was a 74.08% reduction in growth of ATCC 12228 with the same concentration of rifampicin. In contrast, the growth of RP62A was greatly reduced by 81.12% and 81.82% respectively when compared to the control when treated with 0.01 and 0.02 µg/mL of rifampicin. The 0.005 µg/mL of rifampicin with a 46% reduction in the growth of RP62A was then chosen in this study due to its inability to inhibit the bacterial growth completely.

Previous research showed the resistance of rifampicin on RP62A can be due to several mutations on the *rpoB* gene, the beta subunit of the RNA polymerase in the bacteria [28]. The capabilities of D-limonene of manipulating the phospholipid bilayer membrane were shown in *E. coli* [29]. In a recent study on the effects of the limonene on the Gram-positive *Listeria monocytogenes*, results showed that limonene can increase the cell membrane permeability with the leakage of nucleic acids and proteins and can hinder the ATP synthesis [30]. This means that the D-limonene may provide an

additive effect in manipulating the bilayer membrane and providing an easy access for rifampicin to cross it and alter the growth of the bacteria despite the presence of the biofilm.

In another study using 0.1 µg/mL of rifampicin in assessing the inhibition of the growth of RP62A, the results showed that the slow growth of the bacteria and biofilm formation may prevent the bactericidal effect of the antibiotic [20]. An *in vitro* study by Fazly Bazzaz *et al.* showed that the MIC of rifampicin for *S. epidermidis* DSMZ3270 biofilm-forming strain was 0.03 mg/mL in its free form and solid lipid nanoparticles loaded with rifampicin can reduce the production of the biofilm than the free form [31]. The use of 0.005 µg/mL of rifampicin in this study seems lower, however, with the combination use with D-limonene, the additional inhibitory effect was found. We also found that rifampicin had a profound reduction in bacterial growth in reference to wild-type ATCC 12228 when compared to the biofilm formation in RP62A.

D-limonene was known for its antimicrobial properties [32] and was able to inhibit the biofilm production of multi-drug resistant *S. aureus* [25]. The D-limonene used in the study for the inactivation of the growth of *E. coli* O157:H7 and is regarded as generally recognized as safe (GRAS) [22] and found to be able to use in food preservation alone or in combination with lethal heat treatments [29]. In another study, 400 µg/ml of D-limonene was found to have 75–95 % antibiofilm activity against different Streptococcus strains by reducing the surface-mediated virulence factors [33]. The MIC of D-limonene for RP62A and ATCC 12228 strains was found to be 1222.4 µg/mL in the current study. We decided to use the next lower concentration of 611.2 µg/mL of D-limonene with different combinations of rifampicin in the latter studies. There was greater inhibition of the growth of wild type ATCC 12228 than RP62A using 611.2 µg/mL of D-limonene since the wild-type strain is unable to produce biofilm, which enables it to become more susceptible to the effect of D-limonene.

The use of a combination of antibiotics with rifampicin on *S. epidermidis* may not always produce a synergistic inhibitory effect on the bacteria [34]. However, the combination of antibiotics and essential oils can be effective against the growth of many bacteria [35]. The addition of mineral oil extracted from *Lantana camara* leaves to four different aminoglycosides antibiotics drastically decreased the MIC of *E. coli* and *S. aureus* [36]. Further, research showed that the addition of D-limonene had a strong synergistic effect with the rifampicin on *Mycobacterium tuberculosis* with the MIC decreased from 16 to 0.237 µg/mL. It was found that the essential oil could penetrate the bacterial cells thus increased the effectiveness of antibiotics when treating *M. tuberculosis* [37].

From the first set of combination treatment trials using microtiter plates, the bacterial growth reflected by the measurement of optical density readings was found to be lower than using the conical vials. We believe the volume of the conical tubes of 2 mL versus 0.2 mL may create a difference to achieve a better growth of the bacteria and resulted in higher optical density measurement. Further, a study by Nicolau Korres *et al.* showed *Klebsiella pneumoniae* Subsp. *pneumoniae* and the positive control of RP62A formed biofilm on glass and polystyrene plastic [38]. It is important to note that the 96-well plate used is made up of polystyrene while the conical vial tubes are made up of polypropylene which may explain the absence of biofilm ring observed in the conical vial tubes. In another study with the use of essential oil on different biofilm-forming coagulase-negative staphylococci, the inhibition of the growth of *S. warneri* by Polytoxinol™ was found to be at the initial phase of the bacterial cell adherence to the polystyrene surface [39]. Thus, the optical density readings were found to be lower in the combination treatment trials using the 96-well plates than the conical tubes. The OD₅₇₀ was used throughout the growth studies and the results were comparable and better than the OD₆₀₀, this may be due to the biofilm formation of RP62A and the nature of the 96-well plates.

When we studied the combination treatment used on a different growth phase of the bacteria, our results indicated that the combination treatment was effective in the inhibition of the bacterial growth from the beginning and the start of the log phase in both RP62A and ATCC 12228. In our previous study, both bacteria started to grow in the log phase after 6 hours of incubation [24]. Further, the alamarBlue® assay is a widely used method to study the cell viability and metabolic function [40]. In our study of assessing the alamarBlue® assay, the results showed that there was almost a total of 100% of cell viability reduction using the combination treatment on RP62A and ATCC 12228. However, a more significant result was found when treatment was added in earlier on, as biofilm may start to form after 6 hours of incubation. A previous study found the biofilm production of *S. epidermidis* strain occurs in 2-stage as first with the adhesion to the surface and later the formation of the multilayer cell clusters [41]. Thus, we believe that once the biofilm is formed in RP62A, the combination treatment may not be effective.

Lastly, the microtiter biofilm assay confirmed the ability of the combination treatment of rifampicin and D-limonene to inhibit the biofilm formation of RP62A. However, the control well of broth alone produced a residual color and thus an optical density reading. Other modified biofilm assay methods may be used in the future to obtain better results [42]. The pH of D-limonene alone had an acidic pH of 4.0, while D-limonene and rifampicin and the combination in the MHB were found to be neutral. The acidic property of D-limonene can cause the inhibition of the growth of bacteria. Further, the essential oil extract of *Citrus limon* of the pericarp contains about 69.9% of limonene [43] and is different from the pure

chemical form we used here. The 97% pure form of the D-limonene was used throughout our studies and for the future experiment, the pure oil extract of limonene can be used for the evaluation of the inhibition of the biofilm-forming *S. epidermidis*.

5. CONCLUSION

A combination of 0.005 µg/mL of rifampicin with 611.2 µg/mL of D-limonene inhibited the growth of the biofilm-forming *S. epidermidis* RP62A. The alamarBlue® assay confirmed the effect of the combination treatment on the inhibition of the growth and the biofilm formation of RP62A. The results of this study hold a promising future in using alternative combination treatments to treat *Staphylococcus* infections in hospital settings and to further decrease the dosage and usage of prescribed antibiotics.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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