JOURNAL OF
EMERGING INVESTIGATORS

Article

Strain-specific and photochemically-activated antimicrobial activity of berberine and two analogs

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SUMMARY

Berberine, a natural product alkaloid, and its analogs have been reported to have a wide range of medicinal properties, including antibacterial activity. Berberine has been shown to be a photosensitizer - photochemical excitation at the correct wavelengths generate highly reactive singlet oxygen species in situ, and this has biomedical applications in photodynamic therapy. Here, we explore the antibacterial effects of berberine and two semisynthetic berberine analogs, dihydroberberine and 8-methyl-7,8-dihydroberberine, as a result of photoirradiation across three strains of bacteria. Through two antibiotic susceptibility assays, the Kirby Bauer assay and an infused agar assay, it was determined that the antibacterial activities of berberine and two semisynthetic analogs were more potent upon photoirradiation. An understanding of the photosensitizing ability of berberine may inform the design of future compounds towards the photodynamic therapy of bacterial infections.

INTRODUCTION

Widespread use of antibiotics has resulted in the emergence of strains of antibiotic resistant bacteria, which continues to present an ever-growing problem in medicine, particularly in hospital settings. Every year, tens of thousands of Americans die from infection from antibiotic resistant bacteria (1). Therefore, the development and discovery of novel antibacterial agents has been and continues to be an area of great scientific and biomedical importance.

Berberine, a naturally occurring alkaloid, is extracted from the plants in the genus Berberis, and has been documented to have a wide range of biological activities, including anticancer, antitumor, and antimicrobial activities (2-4). The use of berberine-containing extracts as a medicinal agent dates back to 3000 BCE, where it was first reported to be used in ancient Chinese medicine (5). Berberine has also been shown to be a photosensitized DNA intercalating agent. Berberine intercalates with DNA to form a berberine DNA complex (6). Upon photoirradiation of this complex, a highly reactive singlet oxygen species is generated (7). Singlet oxygen then oxidizes guanines within DNA, resulting in DNA damage and the inhibition of DNA replication, which has applications in the photodynamic treatment of cancers (8). We believe that berberine may inhibit bacterial growth through a similar mechanism of action.

Compounds with photosensitizing ability and singlet oxygen have been previously studied for antimicrobial and anticancer photodynamic therapies (9). A study on the kinetics of singlet oxygen production and decay in Escherichia coli upon photoirradiation with photosensitizers representative of three different structural classes found that the photochemical properties of photosensitizers and their abilities to be taken up by bacterial cells differ throughout different classes of compounds (10). On cancer, it has been previously reported that berberine, upon photoirradiation, induced anticancer effects on renal carcinoma cells (11).

Previously, we reported the dose dependency and strain specific antimicrobial activity of berberine compared to five broad spectrum antibiotics representative of different structural classes (12). We found that berberine is less potent than the broad-spectrum antibiotics screened (ampicillin, enrofloxacin, kanamycin, nalidixic acid, and sulfanilamide), but seemed to exhibit strain specificity.

Semisynthetic analogs of berberine have been previously demonstrated to have more potent antimicrobial activity in-vitro and in-vivo compared to berberine (13-15). Grignard additions with alkyl chains and phenyl substituents have demonstrated more potent antimycobacterial effects against tuberculosis (16). 8-alkyl-12-bromo derivatives of berberine have been previously synthesized through Grignard addition followed by radical bromination, and it was found that these compounds have more potent antimicrobial activity (17). However, it has been previously reported that a borohydride reduction of berberine to dihydroberberine resulted in decreased antimicrobial activity (18).

Here, we report photoirradiation-dependent, comparative antimicrobial activity of berberine and two chemically synthesized analogs. Two separate experiments were conducted to study this: a Kirby Bauer assay based on our previous methodology, which is a disc diffusion assay, and an agar infusion assay, in which the agar was infused with the compound solution and inhibition was quantified by

Table 1: Bacterial strains studied and some of their characteristics

Bacterial Strain	Related Diseases	Characteristics
Bacillus cereus	Food poisoning, diarrhea (20)	Can produce ATP in the absence of oxygen - Facultative anaerobic (21)
Neisseria sicca	Pneumonia, meningitis, endocarditis (22)	Oxidase-positive (aerobic)- uses oxygen as an electron acceptor in the electron transport chain (23)
Staphylococcus epidermidis	Hospital acquired infections - nosocomial infections (24)	Forms biofilm - protects the bacteria from immune response and antibacterial agents (25)

means of cell density (12). We were interested in two carbon 8 analogs of the berberine: dihydroberberine and 8-methyl-7,8-dihydroberberine.

We are interested in the effects of photoirradiation on the antimicrobial inhibitory activity of berberine and two analogs on the growth of three bacterial strains: *Bacillus cereus* (gram-positive), *Neisseria sicca* (gram-negative), and *Staphylococcus epidermidis* (gram-negative) (**Table 1**). The selection of gram-positive and gram-negative bacterial strains allows us to understand the strain-specific antimicrobial activity of berberine and its analogs (19). Strains belonging to the three species have been found to be pathogenic. Based on prior literature, we hypothesize that dihydroberberine will have less potent antimicrobial activity than berberine, and 8-methyl-7,8-dihydroberberine will be more potent. Moreover, we hypothesize that strain specific antimicrobial effects might be accentuated upon photoirradiation.

In this study, we identified the photoirradiation-dependent antimicrobial activity of berberine and two semisynthetic analogs against three strains of bacteria through two separate assays. We determined that berberine and 8-methylberberine had photoirradiation-dependent inhibitory activity at concentrations above 10 mM, and dihydroberberine had no antimicrobial activity.

RESULTS

We performed the Kirby Bauer assay and the infused Mueller Hinton (MH) agar assay to understand the photosensitizing ability of berberine and two semisynthetic analogs, and their effect of photoirradiation on their antimicrobial properties.

Dihydroberberine was synthesized through a borohydride reduction to berberine in which the iminium ion of berberine is reduced to an enamine (**Figure 1a**). A racemic mix of 8-methyl-7,8-dihydroberberine was synthesized through a Grignard addition with methyl magnesium bromide to berberine chloride (**Figure 1b**). The addition of substituents to carbon 8 results in the formation of a stereocenter. Berberine chloride was commercially purchased and not purified further.

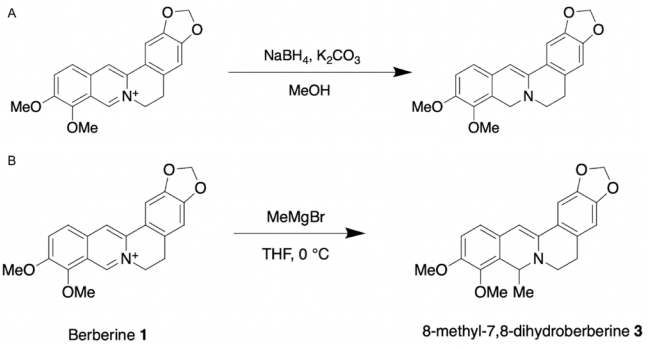

Figure 1: Reaction schematics for our synthesis of berberine analogs. (a) Synthesis of dihydroberberine 2 with NaBH4, K2CO3, and MeOH at room temperature (b) Synthesis of 8-methyl-7,8-dihydroberberine 3 with MeMgBr and THF at 0°. C.

The wavelength of light chosen for photoirradiation was based on the maximum wavelength of absorbance for all three compounds (**Figure 2**). The maximum wavelength of absorbance for the two analogs experience a blueshift in comparison to berberine.

Radii of inhibition (ROI) from the Kirby Bauer assay (**Figure 3**) are shown below. Berberine and synthesized analogs had no inhibition against *N. sicca* at all concentrations, consistent with our previously reported results. No inhibition was observed for concentrations under 10 mM for all compounds screened. ROI against *B. cereus* (**Figure 3a**), demonstrate that berberine and 8-methyl-7,8-dihydroberberine inhibit bacterial growth at high concentrations without photoirradiation, with the 100 and 50 mM concentrations having ROI that are similar and statistically insignificant (2-tail unpaired t-test; berberine *p*-value 1.00, 8-methyl-7,8-dihydroberberine *p*-value 0.109). No inhibition against *S. epidermidis* was shown at any concentration with any of the berberine analogs in the absence of photoirradiation (**Figure 3b**).

Radii of inhibition upon photoirradiation indicate that photoirradiation has a positive effect on the antimicrobial inhibitory activity of berberine analogs (**Figure 4**). A photoirradiated control demonstrated no inhibition against the growth of *B. cereus* and *S. epidermidis*, however photoirradiation with compound solutions resulted in positive

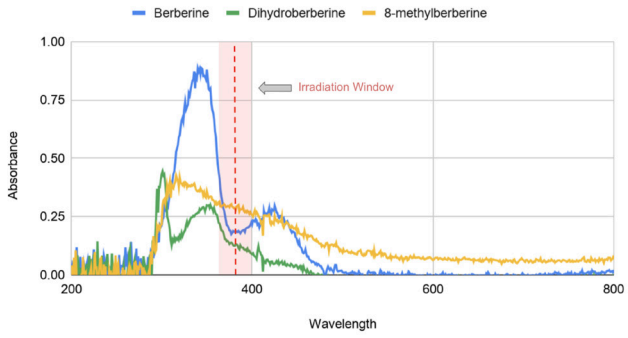

Figure 2: UV-Vis spectra of berberine, dihydroberberine, and 8-methyl-7,8-dihydroberberine. The irradiation window represents all wavelengths of light that the bacteria were exposed to.

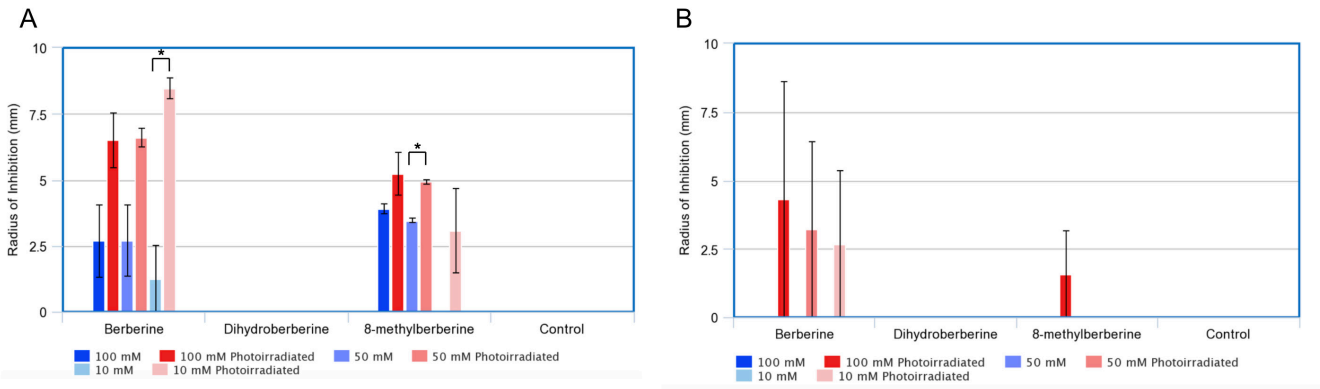

Figure 3: Radii of inhibition against *B. cereus* and *S. epidermidis* (a) The radii of inhibition against *B. cereus* at three concentrations with both photoirradated and non photoirradiated results represented. One statistically significant relationship is observed for 8-methylberberine at a 50 mM concentration (2-tail unpaired t-test 0.0002) (b) Radii of inhibition against *S. epidermidis* at three concentrations. One statistically significant relationship is observed for berberine at a 10 mM concentration (2-tail unpaired t-test 0.02). The * represented statistical significance between the two data points. Results shown are the mean of 3 technical replicates and standard error is reported.

ROI. An increase in the ROI upon photoirradiation can be observed with berberine and 8-methyl-7,8-dihydroberberine at all concentrations higher than 1 mM against both *B. cereus* and *S. epidermidis* (**Figure 3**). Dihydroberberine had no inhibitory effects against bacterial growth upon photoirradiation. The previous maximum inhibitory effects of berberine against *B. cereus* remain consistent, with the 100 mM and 50 mM resulting in statistically insignificant differences in ROI (2-tail unpaired t-test, *p*-value 0.94). At the 10 mM concentration, there is a significant difference between the inhibitory effects of berberine upon photoirradiation (2-tail unpaired t-test, *p*-value 0.02). The inhibitory effects of 8-methyl-7,8-dihydroberberine against *B. cereus* are also improved upon photoirradiation, with a statistically significant difference at the 50 mM concentration (2-tail unpaired t-test, *p*-value 0.0002) and antimicrobial activity at the 10 mM concentration, whereas there was no inhibition at the 10 mM concentration with no photoirradiation.

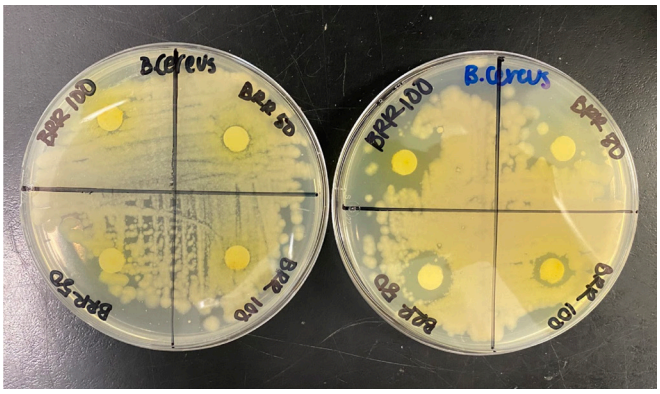

Figure 4: Bacteria growth after incubation without photoirradiation (left) and with photoirradiation (right). The compound solution shown here is berberine at 100 mM and 50 mM concentrations. The increased inhibition of bacterial growth can be seen in their respective radii of inhibition. Petri dishes shown are representative of the whole experiment. Concentrations from left to right, of berberine solutions starting with the top row: 100 mM, 50 mM, 100 mM photoirradiated, 50 mM photoirradiated, 50 mM, 100 mM, 50 mM photoirradiated, 100 mM photoirradiated.

The effects of photoirradiation on the antimicrobial effects of berberine and its analogs is consistent against *S. epidermidis* . There was no inhibition against *S. epidermidis* without photoirradiation at all concentrations and a photoirradiated control also demonstrated no inhibition, but upon photoirradiation with compound solutions, berberine demonstrated antimicrobial effects at 100, 50, and 10 mM concentrations and 8-methyl-7,8-dihydroberberine had antimicrobial effects at 100 mM.

Bacterial growth from the infused Mueller Hinton agar assay was quantified by cell density. In this study, dihydroberberine demonstrated inhibitory effects against *N. sicca* upon photoirradiation (**Figure 5**). *N. sicca* colonies covered 30.7% of the petri dish after being subjected to photoirradiation for thirty minutes, compared to the lawn of bacteria (full coverage) that grew without photoirradiation. Results for other strains of bacteria and compounds were inconclusive.

From this study, we found that the antimicrobial activity of berberine and related analogs are positively affected by photoirradiation, and the inhibitory activity remains strain specific when photoirradiated.

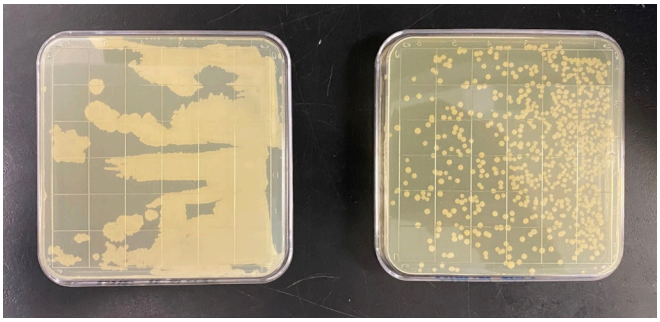

Figure 5: Petri dishes from the infused Mueller Hinton agar assay with dihyroberberine against *N. sicca*. The un- photoirradiated petri dish (left), has bacterial growth in a lawn, while the photoirradiated petri dish (right) has distinct bacteria colonies.

DISCUSSION

In this study, we report the photoirradiation-dependent antimicrobial activity of berberine and two semisynthetic berberine analogs, 8-methyl-7,8-dihydroberberine and dihydroberberine. We found that photoirradiation results in superior antimicrobial activity at all concentrations of berberine and 8-methyl-7,8-dihydroberberine in the Kirby Bauer assay, and that the antimicrobial activity remains strain specific.

In the Kirby Bauer assay, berberine and 8-methyl-7,8-dihydroberberine inhibit bacterial growth with insignificantly different radii of inhibition at concentrations of 100 mM and 50 mM both without and with photoirradiation. We believe that this is a result of the maximum inhibitory effects of berberine against *B. cereus* being within the 10 and 50 mM concentrations. It was also determined that berberine and 8-methyl-7,8-dihydroberberine have comparable inhibitory activity, however berberine is commercially available while the synthesis of 8-methyl-7,8-dihydroberberine is done in an ice bath. Further research into the viability of 8-methyl-7,8-dihydroberberine as an antibacterial agent is needed in order to determine if the compound presents significant benefits over berberine such as reduced side effects.

Differences between this study and our previously reported study, including the minimum concentrations of the berberine solutions necessary to induce inhibitory activity on *B. cereus* and the lack of inhibitory activity on *S. epidermidis* without photoirradiation, can be attributed to the modification in the composition of our Mueller Hinton agar. It has been previously reported that differences in agar composition can result in significant differences in bacterial growth rates and colony density (26).

The results of the Kirby Bauer assay and the infused agar assay suggest that the mode of administration of the compound affects its antimicrobial activity. Dihydroberberine demonstrated no inhibition in the Kirby Bauer assay, which is a disc diffusion assay, while it exhibited inhibition of bacterial growth in the infused agar assay. We believe that this may be attributed to dihydroberberine having a poor ability to diffuse, while other compounds studied were able to diffuse better, resulting in inhibition in the Kirby Bauer assay.

A limitation to the use of our synthesized berberine analogs is the possibility of cytotoxic side effects due to the blueshift in the maximum wavelength of absorption compared to berberine. DNA, aromatic amino acids, and other molecular entities have absorbances in the ultraviolet range, with DNA's absorbance around 260 nm (27). The generation of a highly reactive singlet oxygen species within DNA may also result in non-specific cytotoxicity within the human body, posing an implication to the development of berberine and its analogs as a therapeutic agent. These factors may be impactable to the design and synthesis of future berberine analogs.

Our synthesized analog, 8-methyl-7,8-dihydroberberine, had antimicrobial activity that was comparable to that of berberine. Further studies on the structure-activity relationship of the length of the alkyl chain on carbon 8 of berberine and dihydroberberine and the antimicrobial activity and strain specificity, along with analogs with substituents on different carbon positions, would be instrumental to understanding the structure relationship activity of berberine analogs and antimicrobial activity. Such structures are synthetically accessible with alternate Grignard reagents. Additionally, future optimizations to the method of delivery of berberine analogs would be instrumental to gaining insight on the effects of photoirradiation to the antimicrobial activity of berberine and the effects of diffusion versus infusion. This could guide the design of future analogs of berberine and the possible identification of novel antibacterial agents.

MATERIALS AND METHODS

Chemical Synthesis

Berberine chloride (MaxSun, >97.0%) was used for the synthesis without further purification. Synthesis of dihydroberberine was conducted according to previously reported protocols (28). Dihydroberberine is an analog of berberine that also provides access to other analogs and reactions such as stork enamine reactions and 13-alkylberberine (29,30).

Synthesis of 7,8-dihydroberberine

We added berberine chloride 1 (1.000 g, 2.690 mmol, 1.0 eq.), sodium borohydride (Fisher, >98%; 0.112 g, 2.959 mmol, 1.1 eq.), and potassium carbonate (1.100 g, 7.959 mmol, 3.0 eq.) in MeOH (30 mL) to a vacuum dried 50 mL round bottom flask charged with a teflon stir bar. The reaction mixture was stirred at room temperature for one hour and monitored by thin layer chromatography (TLC) (10% MeOH in DCM). Upon disappearance of the starting material by TLC, the reaction mixture was filtered with a Buchner funnel. The resulting material was recrystallized in ethanol to give dihydroberberine as yellow crystals (0.799 g, 2.36 mmol, 75.4% yield)

Synthesis of 8-methyl-7,8-dihydroberberine

We added 0.2 g (0.538 mmol, 1.0 eq) of berberine chloride in tetrahydrofuran (THF) to a vacuum dried 25 mL round bottom flask cooled in an ice bath and charged with teflon stir bar. A 3M solution of MeMgBr (STREM Chemicals Inc.; 1.793 mL, 5.379 mmol, 10 eq.) in THF was added dropwise through a syringe. The reaction mixture stirred for 15 minutes in the ice bath and monitored by thin layer chromatography (25% hexane in ethyl acetate). Upon disappearance of the starting material by TLC, the reaction mixture was quenched with water, and extracted with brine and ethyl acetate. The organic layer was collected and dried over anhydrous magnesium sulfate and concentrated in vacuo to give 8-methyl-7,8-dihydroberberine (0.126 g, 0.359 mmol, 66.7% yield).

Characterization

All compounds were characterized by ^{1}H nuclear magnetic resonance (NMR) spectroscopy (Nanalysis,

NMReady, 60 MHz), Fourier-transform infrared spectroscopy (Thermo Nicolet iS5, iD5 ATR assembly) and UV-visible spectroscopy (BioRad Smartspec 3000).

Dihydroberberine Characterization

(60 MHz, $CDCl_3$): δ 5.82-7.02 (m, 5H), 5.84 (s, 2H), 4.18 (d, J = 13.4 Hz, 2H), 3.80 (s, 6H), 2.59-3.65 (m, 4H); FTIR (ATR, cm^{1}): 2930.24, 2833.66, 2249.37, 1607.91, 1494.35, 1484.76, 1457.74, 1427.17, 1388.49, 1333.92, 1277.43, 1246.80, 1221.93, 1163.74, 1131.16, 1083.90, 1039.00, 990.70, 938.65, 908.41, 860.13, 799.16, 772.73, 730.86, 647.90; UV (iPrOH) λ_{max}: 301, 354

8-methyl-7,8-dihydroberberine Characterization

(60 MHz, $CDCl_3$): δ 5.75-7.14 (5H, m), 4.69 (1H, m), 3.74 (3H, s), 3.68 (3H, s), 2.67 (2H, m), 2.02 (2H, m), 1.38 (3H, d, J = 6.9 Hz); FTIR (ATR, cm^{-1}): 2931.25, 2359.69, 1681.92, 1597.43, 1482.63, 1416.18, 1343.49, 1266.07, 1229.80, 1167.99, 1096.48, 1037.44, 932.42, 847.36, 810.53, 738.87, 612.23; UV (H_2O) λ_{max}: 316

Bacteria Cultures

Live bacteria cultures of *Bacillus cereus*, Escherichia coli, *Neisseria sicca*, and *Staphylococcus epidermidis* were acquired from Carolina Biological. Overnight cultures were grown in falcon tubes with 10 mL LB media (1% tryptone, 1% NaCl, 0.5% yeast extract, 97.5% water) at 37°C for 12-14 hours.

Compound Solutions

Solutions of berberine, dihydroberberine, and 8-methyl-7,8-dihydroberberine were made at 6 different concentrations (100 mM, 50 mM, 10 mM, 1 mM, 0.1 mM, 0.01 mM). Commercial berberine chloride was used without further purification. Compounds were dissolved in solutions of 10% DMSO in deionized water. A solution of 10% DMSO in deionized water served as our control. Solutions were sonicated to help with dissolution. The appropriate dilutions were performed, and the appropriate amounts of DMSO were added to maintain a 10% DMSO solution.

Kirby Bauer Assay

Bacteria from the overnight cultures were inoculated on Petri dishes plated with modified Mueller Hinton Agar. Filter paper discs saturated with the compound solution were used to administer the compound solutions. The bacteria were incubated overnight at 37°C, and radii of inhibition measurements were taken in mm with an electric caliper. All plating was done in a sterile laminar flow hood. Triplicates with technical replicates were completed, and the results were averaged.

Infused Agar Assay

We added 1% of the 100 mM compound solutions to the modified Mueller Hinton agar, resulting in a 0.1 mM concentration of the compounds in the agar. Petri dishes were plated with the infused Mueller Hinton agar and bacteria were inoculated. The petri dishes were incubated overnight at 37°C. Results were quantified by bacteria density, which was measured by colonies per cm^2. The average size of a colony was found. All plating was done in a sterile laminar flow hood.

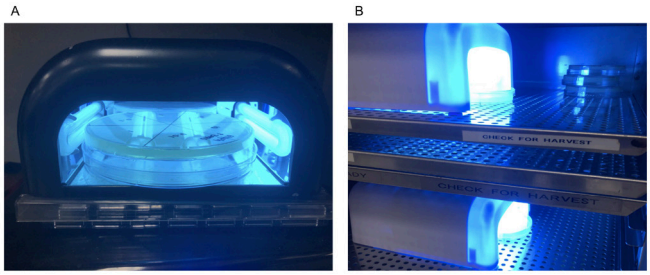

Figure 6: Experiment setup for the photoirradiation of the petri dishes. (a) Petri dishes placed inside the nail curing lamps. Each petri dish was photoirradiation for 30 minutes. (b) Nail curing lamps placed inside the incubator, at 37° C.

Photoirradation

Petri dishes were photoirradiated at 380 nm for thirty minutes while incubated at 37°C. After initial photoirradiation, all petri dishes were incubated overnight at the same temperature. A nail curing lamp set up inside of the incubator was used as the light source (**Figure 6**). The wavelength of emitted light was determined by a spectrometer (Ocean Optics).

Statistical Analysis

Radius of inhibition measurements were acquired in millimeters using an electronic caliper. The ROI from all three experiments were averaged and standard error was calculated. Statistical significance of the differences between the observed radii of inhibition was determined at the 95% confidence level using independent, 2-tailed t-testing (31).

Received: June 15, 2020
Accepted: October 31, 2020
Published: November 17, 2020

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