AN *In vitro* AND *Ex vivo* PHOTODYNAMIC THERAPY STUDY OF METHYLENE BLUE AND NATURAL EXTRACTS IN ASSOSIATION WITH NAIL PENETRATION CARRIER AGAINST *TRICHOPHYTON RUBRUM* INFECTIONS

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ABSTRACT

Human fungal superficial infections are mainly caused by dermatophytes. These infections are distributed worldwide, common for people of all ages, in both sexes. The current treatments include taking oral antifungal drugs and topical therapy. However, treatments of superficial infections can be challenging in children and elderly mainly due to compliance issues and associated potential health risks and side-effects. Photo dynamic therapy (PDT) is a novel approach to treat fungal superficial infections. In this approach, light is used to excite a photosensitizer to turn readily available oxygen into reactive oxygen species (ROS) to kill the pathogen. In this research, we have used the pathogenic dermatophyte *Trichophyton rubrum* as a model to screen for photosensitizers and identify the best combinations of photosensitizer X carrier X light exposure time against *T. rubrum*. I obtained the *In vitro* photosensitizers' Minimum Inhibitory Concentration (MIC), Minimum Fungicidal Concentration (MFC), carrier inhibitory and fungicidal combinations experimental results. In addition, *ex vivo* experimental results for photosensitizer and carrier systematic treatments are presented with both nail pieces and nail well apparatus. The *in vitro* results confirm the fungicidal ability of photosensitizer Methylene Blue and natural extracts Inula, Propolis and St. John's Wort to *T. rubrum.* For *ex vivo* experiments, among the three natural extracts, only Inula showed promising fungicidal effect on nail pieces. Methylene Blue and carrier, Methylene Blue plus Inula and carrier combinations at certain concentrations all showed strong nail penetration ability and fungicidal effect against *T. rubrum* infection. These results suggest promising avenues for further clinical research and application of PDT.

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CHAPTER 1. INTRODUCTION

Human superficial fungal infections usually infect mucous membranes and cutaneous tissues such as skin, nails and hair. These infections represent a global healthcare problem. An estimated 20%-25% of the global population suffer superficial fungal infections, and the number of infected hosts is still increasing (Ameen 2010). Due to the ubiquity of superficial fungal infections all over the world, our understanding of the causative agents and their pathogenesis have been increasing. In addition, many treatments against superficial fungal infections have been developed. While tremendous progresses have been made against some types of superficial fungal infections, other types, especially those related to nails, have been difficult to treat. In this Introduction, I will first describe the main human superficial fungal infections and their treatment strategies. This is then followed by the rationales and main objectives of my thesis.

Agents Causing Human Superficial Fungal Infections

Human superficial fungal infections are mainly caused by dermatophytes and yeasts. The genus *Candida* is the primary group of human pathogenic yeasts. This genus contains about 150 species and collectively the diseases they cause are termed candidiasis. The most common member of this genus that causes human diseases is *Candida albicans.* For most healthy people with intact immunity, *Candida* yeasts are a normal component of their skin and mucosal mycoflora, with little or no effects on the physiology of hosts. However, in hosts with compromised immunity such as HIV infection, injuries such as wounds, and organ transplantation, these yeasts can overgrow on the mucosal surfaces and in some cases penetrate the skin and mucosal membrane to cause invasive infections. One of the hallmarks of HIV infection is candida infections of the mouth and throat collectively called oropharyngeal candidiasis. Even in hosts with a healthy immune system, certain parts of the body are more susceptible to skin and superficial infections due to the unique features of those sites, including the availability

of moisture and nutrients. Those body sites include the intertriginous skin areas like axillae, genitals, gluteal folds, groin and web of fingers/toes.

The second major group of fungi causing human superficial infections are the dermatophytes. Dermatophytes mainly include about 40 filamentous fungal species from three genera, *Microsporum, Epidermophyton* and *Trichophyton*. These fungi can cause infections of the skin, hair, and nails in both humans and animals. In general, these fungi are restricted to the nonliving cornified layer of the epidermis because of their inability to penetrate viable tissue of an immunocompetent host. However, the metabolic by-products released by the fungi during colonization of the keratinous tissues can illicit host response and causing inflammation. In hosts with compromised immunity, colonies of dermatophytes can invade the underlying living tissue and elicit a host response ranging from mild to severe.

The superficial fungal diseases of the skin, hair, and nails caused by dermatophytes are collectively called dermatophytosis. Due to the snake-like appearance, skin infections by dermatophytes are also known as ringworm or tinea (i.e. the Latin word for "worm"). The exceptions are toenail and fingernail infections which are called onychomycosis. Below I briefly describe the subcategories of dermatophytosis based on the infected body part: tinea barbae, tinea capitis, tinea corporis, tinea cruris, tinea pedis and Onychomycosis (tinea unguium).

Tinea Barbae

Tinea Barbae is a dermatophyte infection of the facial beard area. Compared with other tinea infections, tinea barbae a relatively rare dermatophytosis. Several reports identified that contacts with animals were likely causes of human barbae area infection. Over the last several decades, daily farmers seemed to be among the dominant group of hosts with Tinea Barbae infection (Al-Ali et al. 2017), (Kawada et al. 2000), (Xavier et al. 2008), (Sabota et al. 1996). The causative agents include the following anthropophilic species *Trichophyton verrucosum* (most common), *Trichophyton mentagrophyte,*

Trichophyton rubrum, Trichophyton violaceum, Trichophyton schoenleinii, Trichophyton megninii and several species of the *Microsporum* genus. Tinea Barbae could be inflammatory or non-inflammatory. Deep folliculitis with follicular pustules and fistula on skin surface as lesions is inflammatory, while superficial folliculitis is non-inflammatory. Human immune system response and fungal species are key elements to determine whether the infection is inflammatory (Furlan et al. 2017).

Tinea Capitis

Infections of scalp hair and surrounding skin by dermatophytes are categorized as Tinea Capitis. The dominant etiologic agents account for Tinea Capitis are species in the genera *Microsporum* and *Trichophyton.* Preadolescent children aged between 3 and 7 are the most susceptible group of Tinea Capitis (Higgins, Fuller, and Smith 2000) (Elewski 2000). Boys are more susceptible than girls if the etiologic agent belong to the genera *Microsporum,* especially *M. audouinii*, the ratio of infected boys to girls is 5:1(Elewski 2000). Whereas for *Trichophyton* infections, the ratio between boys and girls is equal. Direct contact to infected persons, infected fallen hair, contaminated barbae shop tools, shared hats, clothing are all the routes of Tinea Capitis. There are three types of hair infection: favus, endothrix and ectothrix. Favus is inflammatory with highly infectious crust around hair shafts, it is usually associated with *T schoenleinii*. Endothrix infection is caused primary by *T. tonsurans, T. soudanense* and *T. violaceum.* In most cases, the hyphae grow within hair shaft with the cuticle surface remaining intact. Ectothrix is mainly caused by species of the *Microsporum* genus, including *M. audouinii, M. canis, M. distortum, M. ferrugineum, M. gypseum,* and *M. nanum, as well as T. verrucosum.* Once infected, the hyphae and arthroconidia can grow within and outside hair shaft, which eventually destroy hair cuticle.

Tinea Corporis and Tinea Cruris

Tinea Corporis and Tinea Cruris are both common dermatophytes infections. Tinea Corporis refers to infection on the trunk by dermatophytes and Tinea Cruris is the infection of groin by dermatophytes. *Trichophyton tonsurans* and *T. rubrum* are the top two species cause Tinea Corporis and Tinea Cruris, following by *Microsporum canis and T. mentagrophytes* (Kemna and Elewski 1996). *Direct body contact is the main transmission method of* Tinea Corporis and Tinea Cruris, especially with excessive perspiration. Airtight clothing which traps moisture close to the skin can also facilitate dermatophyte growth and infection (Gupta, Chaudhry, and Elewski 2003).

Tinea Pedis

Tinea Pedis is the infection of feet by dermatophytes. Tinea Pedis is the most common dermatophyte infection in humans, especially those in developed world. The high prevalence of Tinea Pedis is due to the common use of airtight footwear that traps moisture and sweat, creating a great environment for the growth and reproduction of dermatophytes. There are usually four clinical forms or combinations of Tinea Pedis: Chronic hyperkeratotic, Chronic intertriginous, Acute ulcerative, and Vesiculobullous dermatophytosis(Aaron 2018). *T. rubrum* and *T. mentagrophytes* (var. *interdigitale*) are the dominant etiologic agents of Tinea Pedis. Like other infections by dermatophytes, direct skin contact, shared shoes and clothing can all contribute to person-to-person transmission. Previous researches have studied susceptible group of hosts (e.g. elderly people and those with diabetes) and the general population for Tinea Pedis, the results indicate the prevalence in males is much higher than in females for both general population (4.2% to 1.7%) and susceptible people (24.2% to 6.1%) (Auger et al. 1993) (Perea et al. 2000).

Onychomycosis (Tinea Unguium)

Onychomycosis is a chronic infection of nails, chiefly by dermatophytes. Nail bed, matrix and plate can all be infected by fungal pathogens. Both filamentous fungi (e.g. *Scopulariopsis brevicaulis*) and unicellular fungi (e.g. *Candida* species) could be isolated from infected nails, and dermatophytes are the predominant species (Gill and Marks 1999). Among dermatophytes, *Trichophyton rubrum* and *T. mentagrophytes* are the top two species isolated from infected nails, accounting for more than 90% cases of Onychomycosis. Based on results from 21 studies, the mean prevalence of Onychomycosis is 11.4% (Iwanaga et al. 2017). While onychomycosis can impact patients of all ages and both sexes, it is rare to find Onychomycosis cases in child and adolescent. In addition, the prevalence ratio between men and women can vary among countries. Climate condition, dressing habits and some other factors may also lead to variation in prevalence variety between the two sexes (Gill and Marks 1999).

Current Treatments

Tinea Barbae Treatments

Oral treatment is usually applied for Tinea Barbae. There are several recommended forms of oral treatments. One form involves oral itraconazole at 200mg/day for 4 to 6 weeks. Another form involves Terbinafine at 250mg/ day for 4 weeks. The third form uses Griseofulvin, a unique drug approved by FDA to treat inner hair/beard shaft infection, at 500 mg to 1000 mg/day for 6 to 12 weeks (Furlan et al. 2017).

Tinea Capitis Treatments

Although oral anti fungal agent is generally needed for complete eradication, topical treatment could, to some extent, reduce the risk of early-stage transmission to

others. Selenium sulphide and povidone iodine shampoos, applied twice a week, has been adopted as the common topical treatment. This topical treatment has shown capable of reducing the carriage of viable spores, and consequently reduced fungal infectivity and person-to-person transmission (Higgins et al. 2000).

Griseofulvin is a typical oral drug for infants and children with dermatophytosis. The drug was introduced in 1958. As pre-adolescents are the most susceptible group to Tinea Capitis, Griseofulvin is widely applied to treat Tinea Capitis in infants and children. The suggested dosages are 20-25 mg/kg/d for 6 to 8 weeks or until new grown hair has completely replaced the old hair in infected areas. Once dosing is terminated, Griseofulvin clears from the infection area very fast and new hair can start growing soon after.

Triazole drugs are also used as oral treatments for Tinea Capitis. Among the triazole drugs, three are approved for use. The first is ketoconazole. However, ketoconazole is rarely used due to its side effects and liver toxicity, especially in children. The suggested dosages for adults are between 3.3 and 6.6 mg/kg/day. The second is itraconazole and the suggested dosages are 5mg/kg/d for 4 to 6 consecutive weeks or for one-week treatment intervals for 2 to 3 consecutive months. The third is fluconazole. The suggested dosages are 3-5mg/kg per day for 4 weeks or 6mg/kg per day for 20 days. Though their side effects seemed less severe than ketoconazole, continued exposure to high dosages of itraconazole and fluconazole can also result in negative side-effects, especially in infants and children. Proper dosage regiments of fluconazole and itraconazole remain to be determined for infants and children.

Terbinafine is an effective antifungal medicine for virtually all dermatophytes. It obtained FDA approval in the year 2007 for the usage to people aged four years and above. The Terbinafine therapy is shorter compared with other oral drugs. The dosage depends on the weight of child for Tinea Capitis treatment. For those weight between 10 to 20kg, 62.5mg/day for 4 to 6 weeks is needed. For the children weight between 20 to

40kg, 125mg/day for 4 to 6 weeks is recommended. If body weight is higher than 40kg, a dosage of 250mg/ day for 4 to 6 weeks is recommended (Elewski 2000).

Tinea Corporis and Tinea Cruris Treatments

Topical therapy is often sufficient to treat mild Tinea Corporis and Tinea Cruris infections. The azoles, allylamines, benzylamine derivatives and hydroxypyridones have all shown capable of eradicating mild Tinea Corporis and Tinea Cruris. However, for severe and/or recurrent infections, especially those involving patients with compromised immunity or with large areas of infections, topical therapy is usually insufficient, oral therapy may be required (Gupta et al. 2003).

Like Tinea Capitis oral treatments, due to its association with hepatotoxicity, the use of Ketoconazole as oral therapy is limited for patients with Tinea Corporis and Tinea Cruris. As Griseofulvin has a fast *in vivo* clearance, there is a high recurrent rate among patients using oral Griseofulvin. As a result, Griseofulvin is not an ideal oral treatment for patients with Tinea Corporis and Tinea Cruris. Thus far, effective oral treatments against Tinea Corporis and Tinea Cruris include terbinafine and triazoles such as fluconazole and itraconazole. The recommended dosage of terbinafine is 250 mg/d for 2 to 4 weeks. Those for itraconazole and fluconazole are 200mg/day for one week and 150-300mg/week for 2 to 4 weeks, respectively (Parent, Decroix, and Heenen 1994), (Montero-Gei and Perera 1992).

Tinea Pedis Treatments

Like other forms of dermatophytosis, both topical and oral treatments are available for treating Tinea Pedis. However, their effectiveness remains low. Topical treatment for Tinea pedis is usually in the form of cream. Several topical creams have been studied, with active ingredients include Imidazoles, Triazoles, Allylamines etc. Among these, terbinafine or naftifine has shown slightly higher cure rate than others. Terbinafine cream needs to be applied twice a day for 4 weeks, and dosage of 1%

naftifine cream is once to twice a day, extending to two weeks beyond the visual symptoms have disappeared (Sahoo and Mahajan 2016).

There are several new, FDA-approved, topical therapies against Tinea Pedis. One involved an azole antifungal agent named Luliconazole. Trial studies found that applying the 1% cream once a day for 1-2 weeks was effective to treat *Trichophyton* infections. Another Econazole nitrate foam has also shown efficacy to Tinea Pedis. The only disadvantage so far of these new topical drugs is their high cost, which may negatively influence their large-scale application.

Oral therapy of Tinea pedis is typically applied to patients who is immunocompromised, or the infection is relapsing. Terbinafine and itraconazole are the two most common antifungal agents for oral treatment. The suggested dosage of Terbinafine is 250 mg per day for one week for interdigital infection and two weeks for moccasin infection. The recommended dosage of Itraconazole is 100-200mg per day for 2 to 4 weeks. Griseofulvin and fluconazole are also frequently prescribed, but their treatments are relatively long. The treatment regime and dosage of Griseofulvin is 660-750mg/day for 4-8 weeks while the dosage of Fluconazole is 150mg/ week for 4 weeks (Sahoo and Mahajan 2016).

Onychomycosis (Tinea Unguium) Treatments

Currently, three main topical agents have been applied to Onychomycosis treatment: ciclopirox, tavaborole and efinaconazole. All three medications are applied to infected nail plate daily for around 48 weeks, which is the time for new toenails to grow out completely (Brown 2019). To reduce the therapy duration and enhance efficiency, these antifungal agents are typically applied as lacquer. Specifically, both ciclopirox 8% in hydrophobic lacquer and ciclopirox in hydrophilic nail lacquer greatly improved the nail permeability of ciclopirox (Gupta, Paquet, and Simpson 2013). The 10% Efinaconazole solution was approved by FDA in June 2014. This formulation is very promising, with a comparable cure rate to some oral itraconazole drug (Tosti 2013). Efinaconazole is a new triazole antifungal agent for treating mild to moderate Tinea Pedis

infections, applied once daily for favourable outcome (Piraccini and Alessandrini 2015). Tavaborole is formulated in lightweight hydrophilic nail lacquer for onychomycosis treatment, 5% Tavaborole has demonstrated to be an effective and safe method in clinical studies (Piraccini and Alessandrini 2015). Generally, topical treatment is less effective as oral treatment, with less than 18% cure rates (Brown 2019). The primary obstacle for topical treatment is that the drugs are difficult to penetrate nail plates.

Aside from the topical treatments mentioned above for onychomycosis, oral treatments are also available. At present, three antifungal drugs have been developed for oral therapy against onychomycosis: Fluconazole, itraconazole and terbinafine. The cure rates based on oral therapy for fingernail and toenail infections are up to 79% and 70% respectively depending on the population. The mean cure rates are about 59% for fingernail infections and 38% for toenail infection, separately (Brown 2019). The recommended dose of terbinafine oral treatment is 250mg per day for 12 consecutive weeks or for a pulse therapy, 500mg per day for four weeks with four weeks off consequently. Itraconazole is applied in pulse therapy with the dosage of 400 mg per day for one week a month. However, regular fingernail infection treatment duration is typically two months and toenail treatment duration three months. Fluconazole as an oral onychomycosis treatment is recommended at the dosage of 150–300 mg per week for more than six months (Piraccini and Alessandrini 2015). In all oral therapies, relapse can occur.

Apart from topical and oral drug treatments, there are several other treatment options for onychomycosis. One option is nail removal surgery, but it is expensive and complicated. Laser treatment is another option. Lasers can penetrate nail plate easily and rapidly, however lasers can also damage healthy cells and tissues at the same time. Photodynamic therapy (PDT) is also studied in the last decade. In PDT, when the photosensitizer is exposed to light at a certain wavelength, it can be activated and release reactive oxygen species (ROS). The released ROS can be utilized to eliminate dermatophytes. As a photosensitizer, Methylene Blue (MB) has demonstrated effective ability to kill *Trichophyton in vitro* (López-Chicón et al. 2016). The treatment condition

was 50 μM MB under 10 minutes 81J/cm^2 fluorescence exposure or 100 μM MB exposed in 9J/cm^2 for 10 minutes. Because dermatophytes usually grow in nail bed under the nail plates, how to deliver MB to underneath the nail plate and activate it remain challenging.

My Thesis Objectives and Proposed Experiments

To help address the problems with PDT, the objectives of my thesis are to: (i) develop a solution(carrier) that can effectively dissolve MB; (ii) investigate the optimal combinations of the solution (carrier) and MB that can result in effective killing of *T. rubrum in vitro*; (iii) identify the appropriate light exposure for MB treatment that can result in effective killing of *T. rubrum in vitro*; (iv) test the effectiveness of MB + carrier in penetrating nail pieces and killing *T. rubrum*; (v) use the *ex vivo* model to test the effectiveness of specific MB + carrier combinations in penetrating across nail plate and killing *T. rubrum*; and (vi) investigate the potential synergistic interactions between MB and natural photosensitizers in killing *T. rubrum* in both *in vitro* and *ex vivo* models.

To achieve the objectives listed above, I proposed and conducted a series of experiments. Briefly, my thesis study is divided in two parts, *in vitro* and *ex vivo* experiments. In the *in vitro* part, which is also required for the *ex vivo* clinical simulated treatment, I will determine the Minimum Inhibitory Concentration and Minimum Fungicidal Concentration for both the synthetic photosensitizer Methylene Blue and three natural extracts. I will test the inhibitory and fungicidal effect of various combinations of the carriers *in vitro*. In the *ex vivo* part, I will conduct the MB PDT treatment on nail pieces first based on results from the *in vitro* experiment. Afterwards, I'll apply MB, and MB + natural extracts in various combinations to comprehensively test their effects on the *T. rubrum* infected nails.

CHAPTER 2. Materials and Methods

For my thesis research, I have used the following materials and pieces of equipment. For each item, I have also included the volume (for chemicals) and supplier (for all items).

Materials

1-2 Propanediol, 99%, extra pure 1LT, Thermo Fisher Scientific. (Fair Lawn, USA)

Agar Bacteriological Grade 1kg WISENT INC. (St-Jean-Baptiste, Canada)

Anhydrous Ethyl Alcohol 500mL Commercial Alcohols (Brampton, Canada)

Bacteriological Peptone 1kg WISENT INC. (St-Jean-Baptiste, Canada)

Chloramphenicol ≥98% (HPLC) C0378 25g Sigma-Aldrich (St. Louis, USA)

D-(+)-Glucose Anhydrous 1kg WISENT INC. (St-Jean-Baptiste, Canada)

Formaldehyde 36.5-38% F-8775 500 ML Sigma-Aldrich (St. Louis, USA)

Glycerol 15523-1L-R Sigma-Aldrich (St. Louis, USA)

Methylene Blue trihydrate BP117-100 100g Thermo Fisher Scientific. (Fair Lawn, USA)

Methanol 4L Plastic Caledon Laboratory Chemicals (Georgetown, Canada)

Mops Enzyme Grade 500g Fisher Scientific (Fair Lawn, USA)

Potassium Chloride EMD Chemicals 500g (Gibbstown, USA)

Potassium Phosphate 1LB Fisher Scientific (Fair Lawn, USA)

RPMI-1640 Medium R6504-10L Sigma-Aldrich (St. Louis, USA)

Sodium Chloride BioShop 2.5kg (Burlington, Canada)

Sodium Phosphate 1LB JT Baker Chemicals (Phillipsburg, USA)

Tween 85 P4634-100ML Sigma-Aldrich (St. Louis, USA)

Equipment

VWR MODEL 250D Ultrasonic Cleaner (West Chester, USA)

SARTORIUS BP 310S Balance (Germany)

Mettler-Toledo FiveEasy F20 pH meter (Greifensee, Swiss)

Jouan B4i Centrifuge (Winchester, USA)

Kendall Bacti-Cinerator IV micro cinerator (Mansfield, USA)

Fisher Scientific Dry Bath Incubator (USA)

Fisher Scientific Vortex Mixer (USA)

Molecular Devices SpectraMax Plus 384 Absorbance plate reader (Sunnyvale, USA)

Waring 33BL79 Blender (New Hartford, USA)

Kruss Drop Shape Analysis System DSA 10 (Charlotte, USA)

Leica Aperio Scanscope XT Slide scanner (Buffalo Grove, USA)

Biological materials and fungal strain

Trichophyton rubrum Strains F4520

Complete toenails purchased from Science Care

Nail pieces donated from Tom Fiser Clinic and lab members.

Methods

Medium Preparation

Protocol for preparing SDA solid medium with or without the antibiotic chloramphenicol:

Weight Agar 18g, Peptone 10g and Dextrose 20g. Dissolve them in 1 liter distill water in a flask. Cover the flask with aluminum foil and autoclave the flask under 120^{o} and 18psig for 30 minutes. After autoclave, pour the autoclaved solution slowly into sterile petri plates when the solution is still hot (about 50^{o}C). The SDA plates are ready to use after they are solidified, and they should always be stored in sterile environment. To prepare SDA medium containing chloramphenicol, concentrated chloramphenicol dissolved in ethanol (5g/L in this experiment) needs to be prepared in advance and diluted to a final concentration of 50mg/L in the medium. The initial steps for preparing SDA containing chloramphenicol are the same as for SDA plates, but after autoclaving, the concentrated chloramphenicol solution is pipetted to the SDA solution in the flask at the temperature around 50^{o}, make the final chloramphenicol concentration at 50mg/L. Gently shake the flask for 30 sec to completely mix the antibiotic into the medium, then pour the mixed solution to sterile plates. After the solution is solidified, store the plates in the sterile environment.

Protocol for preparing the RPMI medium:

In my experiments for determining the minimum inhibitory concentrations of the agents, the inoculated fungal pathogen *T. rubrum* was distributed and tested in RPMI medium containing various concentrations of the agents. To make the appropriate concentration of RPMI (at 10.4g/L) in each test, I initially prepared the double strength RPMI which were then mixed with an equal volume of fungal culture containing the tested concentration of each antifungal agent. Briefly, firstly, I autoclaved a glass bottle for storing the solution, then, I weighed 4.1568g RPMI-1640 and poured it to a flask with 150mL autoclaved distill water, cast a stir bar into the flask on the stir plate to vortex the

solution to completely dissolve the powder. Afterwards, I immersed the probe of pH meter into the flask and added MOPs into the solution until pH was 7. Finally, I poured an additional 50mL autoclaved distilled water to make the final volume of 200mL. The medium was autoclaved in the flask and then poured into the autoclaved bottle for storage in the fridge until use.

Determining the *In vitro* Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of Candidate Antifungal Agents:

The *in vitro* MICs determinations of Methylene Blue (MB), Natural extracts and carriers were based on the Optical Density (OD) readings of inoculated post-treating solutions from SpectraMax, using the following procedure.

The selected *T. rubrum* strain F4520 cubes were retrieved from -80^{o} freezer. After they were thawed at room temperature, five cubes were transferred to an SDA plate, distributed evenly on the SDA plate. Afterwards, the SDA plate was sealed by parafilm and moved into 30^{o}C incubator until the fungus grew vigorously in the plate. Next step was to prepare and inoculate the fungal mycelia into RPMI medium for the planned treatments. To prepare the mycelia for testing, I cut a 3x3 cm square agar containing vigorously growing mycelia and transfer it into a sterile blender cup with sterile distilled water covering the top of blades. After 30 seconds of blending, I pipetted the blendate into 2X RPMI medium for use in experiments. The following treatments were set up. (i) For experiments involving mixing inoculated RPMI with carrier, use 2x RPMI. Ratio of blendate to 2x RPMI: 2:38. 150 µL carrier and 150 µL of the blendate in 2X RPMI were put in each well. (ii) For experiments involving mixing inoculated RPMI with MB solutions or Natural extracts solutions, use 2x RPMI with the ratio of blendate to 2x RPMI at 4:36. Each well contains 75 µL MB in water + 75 µL inoculated 2x RPMI plus 150 ul uninoculated 1x RPMI per well. To check for evidence of no contamination and to prepare for additional testing, I pipetted 1 mL mixed blendate to two SDA plates each.

***In vitro* MB MIC Experiment:**

The purpose of this experiment was to determine the MIC of MB in water (not carrier) against *T. rubrum*. From previous published papers and clinical experiences, the MB concentration range was set from 0.00390625g/L, 0.0078125g/L, 0.015625g/L, 0.03125g/L, 0.0625g/L, 0.125g/L to 0.25g/L. I cultured *T. rubrum* lawns and prepared the mycelial blendate following the method described in the above paragraph. I then pipetted the blendate into 2X RPMI medium at the ratio 1:9 by volume in a 15mL screw cap tube. In this experiment, 7 concentrations were measured with three repeats at each concentration plus positive control (also with three repeats). Thus, a total of 24 wells were applied in the plate. Subsequently, each well was pipetted 75μL of inoculated 2X RPMI medium into designed 24 wells in 96 wells microtiter plate. To ensure even moisture around each well during OD measurement, each well along the edge of the plate was filled with 200 μL sterile water, and the RPMI and treatment solutions were placed in the inner wells of the plates. Then pipette 75μL prepared double concentrated MB solutions from 0.078125% to 0.5% into inoculated 2X RPMI medium wells, each concentration for three wells (i.e. three repeats) and pipette 75 μL distilled sterile water to the rest three wells. As a result, the MB concentrations in the treatments range from 0.00390625g/L, 0.0078125g/L, 0.015625g/L, 0.03125g/L, 0.0625g/L, 0.125g/L to 0.25g/L. I used autoclaved tips to mix the solutions in each well before light exposure. After exposure, add another 1XRPMI medium in each well to make a 300 μL solution treatment system in each well. In total, I prepared seven such plates with the mixtures of RPMI medium and multiple MB concentration solutions for testing seven light exposure time treatments: 10s, 20s, 30s, 1min, 2min, 5min and no exposure.

After the treatments, the microtiter plates were sealed by parafilm. The plates were transmitted in bio-safety box to measure initial optical density. The overall OD measurements were once a day for 7 days, within this period, all plates were stored in 30^{o} incubator. The OD values from day 0 to day 7 in the whole spectrum were recorded. The OD value is the log of the intensity of incident light divided by the intensity of

transmitted light. If the fungus stopped growing after the treatment, the OD value would not increase, the minimal concentration of the agents (MB, natural extracts, and carriers) led to no OD growth after 7 days of the treatment was regarded as minimum inhibitory concentration (MIC).

In vitro Carrier MIC Experiment

The goal of this experiment was to determine the inhibitory activity and minimum inhibitory concentration of carrier and carrier components against *T. rubrum* growth *in vitro*. In this experiment, the carrier components were Glycerol, Ethanol, and water or PBS (Phosphate-buffered saline) buffer tested as solvent separately. The purpose to test PBS buffer was to maintain a weak alkaline environment to improve treatment efficacy. PBS buffer recipe in this experiment included NaCl 8g, KCl 0.2g, Na_2HPO_4 1.44g, and KH_2PO_4 0.24g dissolved into 800mL distilled sterile water, then gently added 2% HCl solution to adjust the pH to 7.4, the final step was to add additional 200 mL distilled sterile water to make the 1L solution. For each carrier component, six concentrations (percentages in volumes) were selected, Glycerol: 60%, 30%, 15%, 7.5%, 3.75% and 0. Ethanol: 40%, 20%, 10%, 5%, 2.5% and 0. Thus there were totally 36 carrier combinations with 3 repeats for each solvent (water and PBS buffer). I mixed inoculated *T.rubrum* blendate into 2X RPMI medium with a volume ratio of 1:19. Afterwards, I pipetted 150 µL inoculated medium to the wells of the 96 wells plate and added 150 µL carrier/ positive control solutions into the designed wells to complete the mixture. Like previous MB MIC experiments, I used sterile water to fill the wells along the edges of the microtiter plates. No light exposure was applied in this experiment. The OD measurement and evaluation processes were the same as the MB MIC treatments.

In vitro Natural Extracts MIC Experiment

In this experiment, four natural extracts were tested for their potential effects against *T. rubrum*: Inula, St. John's warts, Propolis and Turmeric. Because all these

natural extracts were difficult to dissolve in water directly, to prepare these solutions, 1% Emulsifier Lipowax D (by weight) and 20% Glycerol (by volume) was applied to make the natural extract solutions. For Inula, Turmeric and St. John's wort, the concentrations tested were: 0.015625%, 0.03125%, 0.0625%, 0.125%, 0.25%, 0.5% (by weight). For Propolis, the concentrations tested were: 0.01875%, 0.0375%, 0.075%, 0.15%, 0.3%, 0.6% (by weight). The preparation of *T. rubrum* lawn and blendate was the same as stated in the MB MIC experiment. The ratio of blendate and 2X RPMI medium was also 1:9 by volume. 75 µL of the inoculated 2X RPMI medium was pipetted into 21 wells in the inner side of 96 wells microtiter plate (18 wells for six treatments with triplicates and 3 for positive control). The wells on the edge of the microtiter plate were filled with 200 µL sterile water to maintain the same moisture level as other wells. Subsequently, 75µL 0.03125%, 0.0625%, 0.125%, 0.25%, 0.5% and 1% Inula, Turmeric and St. John's wort solutions and 75µL 0.0375%, 0.075%, 0.15%, 0.3%, 0.6% and 1.2% Propolis solutions were pipetted into the set 2XRPMI wells to accomplish designed natural extracts treatments. I did two sets of plates for each extract, one plate for no light exposure and the other for 5 mins light exposure at 630nm. After light (or no light) treatment, the OD measurement and evaluation processes were the same as the MB MIC treatments.

MFC Experiment

The experiments used to determine MIC were based on measures of OD values, with the lowest concentration of the agent/treatment completely inhibiting the growth of *T. rubrum* as MIC. However, the viability of those treated fungi is not known. The objective of this set of experiments were to determine the minimum fungicidal concentration. In this experiment, I followed the methods for determining MIC described above for the *in vitro* MB test, *in vitro* carrier test and *in vitro* natural extracts test. However, after 7 daily OD measurements post treatment, I pipetted 10µL of the mixed solution from each well into fresh SDA plate, excluding the wells that were only filled with water. Each SDA plate was divided into three equal parts for holding pipetted solutions of the same treatments from three wells. During pipetting, it's important to

avoid any mycelia from neighbouring wells from cross contaminating each other. The transferred SDA plates were sealed by parafilm and placed in 30^{o} incubator for two weeks. I observed them daily to check for fungal growth. The lowest concentration of each treatment agent which resulted in no *T. rubrum* growth after 14 days of transferring from 96 wells microtiter plates is the minimum fungicidal concentration (MFC). As MIC only indicates the concentration could inhibit fungal growth, the fungus may still viable, thus, MFC is usually much higher than MIC.

In vitro Infected Agar Cubes Treatment

Through the *in vitro* natural extracts MIC experiments, I observed that Inula, St. John's wort and Propolis are all capable of inhibiting or killing *T. rubrum* at a certain concentration. Due to lack of any published clinic data and literature, the purpose of this experiment was to screen the most potential natural extract among the three candidates for *ex vivo* treatments. For each treatment, three repeats were conducted, and the carrier used in this experiment was 30% Propylene Glycol, 20% Ethanol and 50% Water, Also, the natural extracts in water experiment was conducted with three repeats. In this experiment, the infected agar cubes were cut from the prepared *T. rubrum* lawns. The size was around 10mm X 15 mm and the depth was the full depth of the lawn. For the experiments of natural extracts and carrier, the cubes were exposed in 1% natural extracts for 5 or 10 mins followed by three times of wash with sterilized distilled water, and for the experiments of natural extracts in water, the cubes were exposed for 10 mins and 1 hour. The exact natural extracts carrier tests were: No treatment (positive control), 5mins treatment with carrier, 10mins treatment with carrier, 5mins treatment with Propolis, 10mins treatment with Propolis, 5mins treatment with Inula, 10mins treatment with Inula, 5mins treatment with St. John's Wort, and 10mins treatment with St. John's Wort. And the 1% natural extracts water tests were: No treatment (positive control) 10mins in water no wash, no treatment 1 hour in water no wash, 10mins emulsifier no wash, 1 hour in emulsifier 5 times wash, 10mins treatment with St. John's Wort no wash, 1 hour treatment with St. John's Wort 5 times wash, 10mins treatment with Inula no wash, 1

hour treatment with Inula 5 times wash, 10mins treatment with Propolis no wash, 1 hour treatment with Propolis 5 times wash. After the treatments and washes, the cubes were transferred to SDA plates, and the plates were sealed by parafilm and incubated in 30^{o}C. Keep daily observation of the fungal growth in all plates for at least 14 days.

Carrier Surface Tension Test

The original purpose of this test was to determine a more appropriate carrier combination and solvent in terms of its spreading ability on nail surfaces. In this case, the surface tension test was a vital characterization method. In this test, I measured the contact angle between the nail surface and the carrier droplets to determine the surface tension between carriers and nails. I used two solvents for the carrier, there were water and PBS buffer solution. For each solvent, I selected three potential combinations which has already shown fungicidal ability: Glycerol 30%, Ethanol 30%, Water/PBS 40%; Glycerol 30%, Ethanol 20%, Water/PBS 50%; Glycerol 30%, Ethanol 10%, Water/PBS 60% (by volume). The measurement system I used was Kruss Drop Shape Analysis System DSA 10 (Figure 1). It could record the contact angle by second between droplet and nail surface since the droplet was dripped until 20 seconds after the droplet was dripped. The final contact angles analysed among carrier combinations were the average contact angle within 20 seconds.

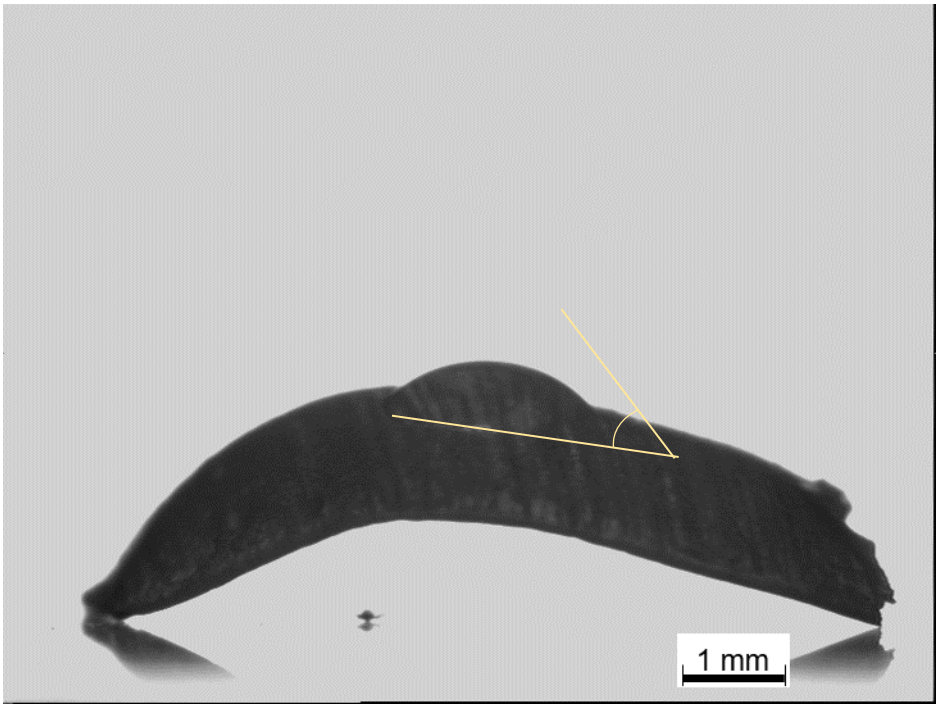


Fig 1. Image and Contact Angle Measurement Example from Kruss Drop Shape Analysis System DSA10

Natural Extracts Pasteurization:

Inual, St. John's wart, Propolis and Tumeric were the four natural extracts tested in this research. As these natural extracts were obtained from botanicals without any sterilizing process, viable microbial cells (including bacteria and fungi) were found. Because autoclaving could change the physical and chemical structures of the extracts, to eliminate the microbial contaminants, we first pasteurized the natural extracts solutions using the following procedure.

The temperature of the water bath for pasteurization was set to 70^{o}C, the tubes holding natural extracts solutions were placed there for two hours. Afterwards, they were placed at room temperature for 2 days, subsequently, the tubes were put to the 70 ^{o}C baths again for two more hours. This pasteurization process was proved effective because after two weeks of the pasteurized solution was pipetted onto a fresh plate, no contamination was found growing in the plate. Thus, this pasteurization step was adopted before both *in vitro* and *ex vivo* experiments.

Nail Pieces Infection

The nail pieces used in this experiment were donated from lab members and clinical patients. The selected nail pieces were trimmed to 1.5-2.5 mm X 2.5-4 mm rectangle or similar shapes. I placed them into a vial and submerged them thoroughly in 70% ethanol. After 3 minutes ultrasonication, I rinsed the nails with autoclaved distilled water twice. Finally, I placed the sterilized nail pieces with dorsal side facing upwards on the prepared *T. rubrum* lawns for 14 days to completely infect them.

Nail Pieces Infection Characterization

In this experiment, I applied electronic microscopy to directly observe the existence of the fungus in nail pieces after infection on *T. rubrum* lawn. After 24 to 48 hours fixation in 10% NBF (Neutral Buffered Formalin) at room temperature, the nail pieces were placed in a 5% Tween 80 solution in distilled water for about 2 hours, then

the nail pieces were submitted for paraffin processing into wax blocks. Finally, the nail pieces were put into 50% ethanol for 10 minutes and then transferred to 70% ethanol solution for preservation before making nail slides. The microscopy image scanning was conducted at McMaster's histology lab, with the nails undergoing PAS staining to enhance the contrast between fungal cells and the nail structures. The image system applied was Leica Biosystem and the images were amplificated by 40 times.

Ex vivo MB Nail Pieces Treatment

At the initial pilot experiment involving treatments of nail pieces with MB, the MB concentrations (by weight) I tested were 0.03125%, 0.0625%, 0.125%,0.25% in 30% Glycerol, 20% Ethanol and 50% Water carrier (by volume) which were based on the MIC and MFC of the *in vitro* experiments. After infection by *T. rubrum* (see above), the infected nail pieces were transferred into 2mL screwed-cap tubes of MB in carrier solutions. Three pieces went into a tube because three replicates were set for each MB concentration. The pieces were completely submerged for one hour, and the positive control triplicates were set in an empty tube for one hour. Afterwards, the soaked nail pieces were transferred to Chloramphenicol SDA plate sealed by parafilm and exposed to 630nm light for 30mins. In addition, one set of nails under 0.25% MB treatment were exposed for 15mins. After the exposure, the plates were placed into 30^{o}C incubator for daily observation of the fungal growth. The fungal growth was spotted in 0.03125%, 0.0625%, 0.125% after 5-6 days of incubation, whereas in 0.25% 15mins and 30 mins treatment plates, the fungus were observed after at least 11 days. These observations indicated that the MB concentration range selected in this pilot experiment was too low. So, in the second *ex vivo* experiment involving MB treatment of infected nail pieces, a higher MB concentration range was used.

In second experiment, the MB concentrations increased to 0.125%, 0.25% and 0.5% in the same carrier. The light exposure time was 30 mins for the 0.125% MB concentration treatment. For the 0.25% and 0.5% MB treatments, I tested two light

exposure times, at 15mins and 30mins, with the rest test conditions being the same as in the first experiment. To simulate clinical treatment, I started the second treatment after 17 days of the first one with same treatment conditions. From second treatment onwards, the interval of each treatment was around 10 days. Within these days, I observed and recorded fungal growth for all treatments. The total treatment number was six in the second experiment.

_Ex_ vivo MB, Inula, Carrier Nail Pieces Treatment

As Inula was the most promising natural extracts according to the results of *in vitro* experiments. During MB nail pieces treatments, I also conducted some *ex vivo* Inula+Carrier, Inula+MB, Inula only and carrier only treatments. The carrier applied for Inula+Carrier, Inula+MB and carrier only trials was 30% Propylene Glycol, 20% Ethanol and 50% Water carrier.

I set two group of carriers only test, each three nail pieces were submerged in carrier (30% PG, 20% Ethanol and 50% Water) for 5 mins or 10 mins before they were transferred to SDA chloramphenicol plates without wash. The triplicated nail pieces which went through same carrier submerging were sealed and incubated in the same plate by 30^{o} for daily observation.

The concentration of Inula in Inula only and Inula + carrier was 1%. For these treatments, I set four conditions separately with three repeats each. Inula only: 5 mins immersion + no light irradiation, 5 mins immersion + 30 min light irradiation, 10 mins immersion + no light irradiation, 10 mins immersion + 30 min light irradiation. Inula + carrier treatment conditions were the same as Inula Only.

For the Inula plus MB combination nail pieces treatment, I conducted 1% MB+ 1% Inula, 0.5% MB + 1% Inula, and 0.5% MB only treatment, I mixed same volume 1% MB/ 0.5% MB and 1% Inula as the treatment, both MB and Inula solution was mixed in 30% Propylene Glycol, 20% Ethanol and 50% Water carrier, the submerging time was 60 minutes and the irradiation time was 30 minutes. All these three treatments had three

replicates. Every three treated nail pieces were transferred to SDA chloramphenicol plates and incubated in 30^{o} for subsequent daily observation.

Ex vivo Nail Wells Treatment

Nail Well Preparation

In the *ex vivo* experiment, large toenails were purchased from Science Care (Aurora, CO, USA). To enhance drug penetration, all nails were first sanded slightly on the dorsal side before sterilization. The sterilization protocol for the nails was similar to nail pieces. Briefly, the complete nails were placed into a vial and submerged thoroughly in 70% Ethanol for 3 minutes. The vials were then ultrasonicated for 3 mins, followed by rinsing with autoclaved distilled water twice. After ultrasonic sterilization, the nails were cut into 8mm-diameter round shape discs using a Boehm hollow punch (Lee Valley Tools Ltd. Ottawa, ON, Canada) and rubber mallet (Yoell et al. 2018). The nail discs were embedded by heat-shrinking tubes (Digi-Key; Thief River Falls, MN, USA), with hot wind applied by a MasterCraft 12.5A heat gun at the temperature about 250^{o}C, until the glue inside the tubes seal the nail disc to the tube tightly and evenly. After cutting excessive tubes (leave 2mm above the dorsal side and 1mm beneath the ventral side), the nail well apparatus was set completely (Figure 2). The next step was to test whether the nail wells leak. In this test, 40µL sterile distilled water was pipetted on the dorsal side of each nail well and the nail wells were placed in the biosafety cabinet for one hour. If the water drop was still on the dorsal side without wetting the paper towel beneath, it was considered no water leaking through the nail well. After this step, the nail wells were sterilized through UV irradiation by placing them into bare petri plates for 10 mins under UV exposure on each side of the nail well. The distance between the plate lid and the UV light bulb was about 10cm. Then, these nail wells were transferred to a vigorously grown *T. rubrum* lawn plates for infection (Figure 3). In this experiment, each *T. rubrum* plate hosted at most 15 nail wells. After 14 days of growth on the *T. rubrum* lawn, the nail wells were considered as completely infected and colonized by *T. rubrum*.

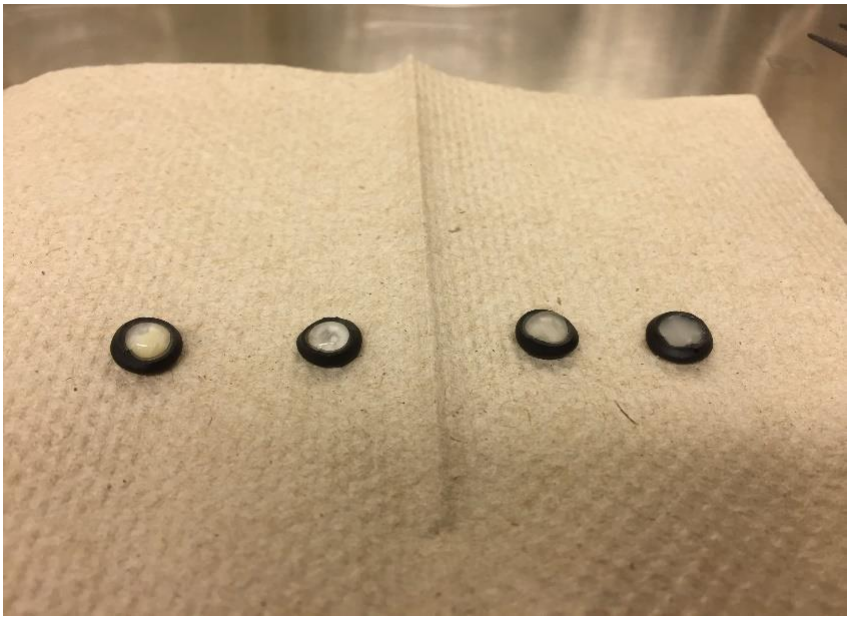


Fig 2. Illustration of nail wells under water leaking test

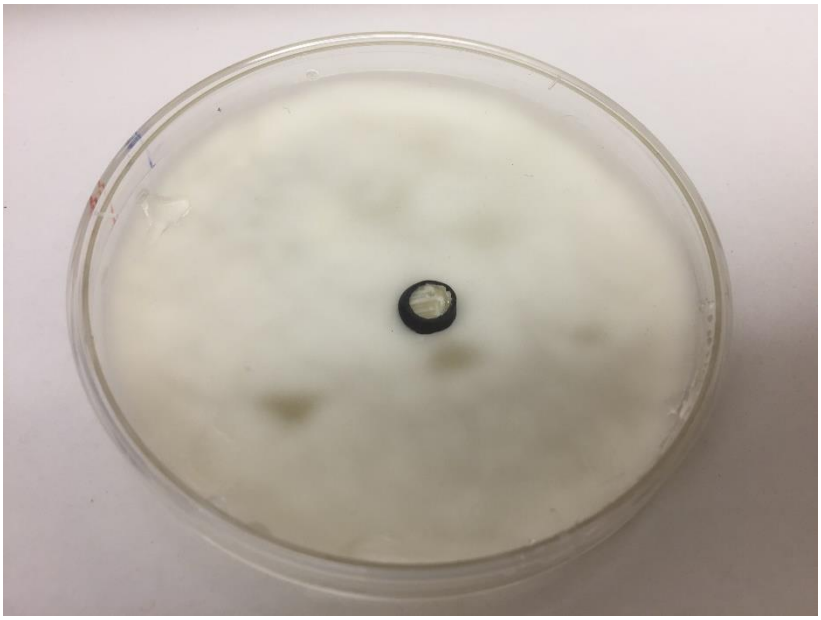


Fig 3. Illustration of a nail well infection on T. rubrum lawn

After infection, I used forceps to clean the attached mycelia on the bottom side of the tube before transferring these nail wells to fresh agar plates. Then, I pipetted a specific volume (described below) of treatment solutions to the dorsal side of the wells for one hour to allow the agents to penetrate through the nails. The specific treatment solutions and their volumes vary depending on the exact purpose of each experiment. For each treatment, I usually had three repeats. After the one-hour penetration, the nail wells were placed on SDA chloramphenicol plates (three nail wells evenly placed in one plate) and exposed to light of wavelength 630nm for 30min or 15min. After light exposure, the treated nail wells were stored in 30^{o}C incubator for at least 14 days and were observed daily. The observations were recorded and later analyzed for the effects of each treatment on *T. rubrum*-infected nails.

Because the hyphae attached to the tube bottom could not be completely removed by forceps and could not be reached by the treatment solution, the remnant mycelia may affect the results of treatments as this part was directly attached to agar surfaces. As a result, one modification was applied for nail well treatment. After the nail wells were completely infected, they were transferred into bare plates for one-hour cure penetration and 30/15 mins light exposure. Afterwards, I cut the rubber tube and transfer the nail discs to SDA chloramphenicol plates and incubated the discs at 30^{o} for 14 days. Here, each SDA chloramphenicol plate contained three nail discs. I checked for evidence of fungal growth daily for at least 14 days.

Ex vivo MB/Inula Nail Wells Treatment

After at least two weeks of infection, the infected nail wells were transferred into bare sterile plates by forceps inside the biosafety cabinet. I used sterile forceps and scalpel blades to scrape off the fungal mycelia attached to the bottom of the nail well tubes as much as I could. In the early stage of *ex vivo* nail well treatments, I carried out preliminary MB, Inula and their combination trials at the same time. All the photosensitizers worked with the carrier (30% PG, 20% Ethanol and 50% Water) in this

experiment. The treatments were: 1% Inula with no light exposure, 1% Inula 30mins light exposure, 0.5% MB 30mins light exposure, and 0.5% MB plus an equal volume 1% Inula and 30mins light exposure. Each treatment had three repeats (three nail wells). The negative treatment was water, also with three repeats. I pipetted 50μL of each treatment solution into the dorsal side of each nail well, which covered the entire surface of the nail in each nail well. After the one-hour penetration, I applied 630nm light to irradiate dorsal side of nail wells for 30 mins, every three nail wells placed in one plate were exposed to light at the same time. The nail wells were then placed in the SDA chloramphenicol plates and incubated at 30^{o}C. The plates were observed daily for evidence of fungal growth.

As mentioned briefly above, because it was hard to completely scrape off the mycelia colonizing the tubing from the nail well, inconsistent results were obtained. For example, except the 0.5% MB + light 30mins irradiation treatment, all other treatments showed only three or fewer days of no *T. rubrum* growth around the nail wells after the treatments. Thus, we repeated the above experiment but used a slightly modified protocol. In this new protocol, I selected water and carrier (30% PG, 20% Ethanol and 50% Water) as positive control, I did three and five repeats for these two controls separately. For the treatments, I did six nail wells repeats: 1% Methylene Blue without light, 1% Methylene Blue+30 min light, 1% Inula without light, 1% Inula+30 min light, 1% Inula+ 1% MB without light, 1% Inula+ 1% MB 30 min light. 50 μL each solution was pipetted to the dorsal side of each nail well and the nail wells were sat for one-hour penetration. For the treatments without light exposure, the nail wells were kept in bare plates. For those treatments demanded 30min of 630 nm light exposure, after light exposure, the nail wells were also kept in bare plates. The bare plates contained nail wells were sealed and incubated in 30^{o}C for one week. Because no fungus grew within the first week in all bare plates, on the 8th day after mixture, I cut the tube structure of nail wells by scalpel blade, and then transferred the nail discs inside the tubes into SDA chloramphenicol plates. Each SDA chloramphenicol plate held up to six discs from same treatment (Water control for three, Carrier control for five). I sealed these plates with

parafilm and placed them to 30^{o}C incubator again for daily observations for 14 days to determine the treatment effects.

CHAPTER 3. RESULTS

In vitro Minimum Inhibitory Concentration (MIC) Of Methylene Blue Dissolved in Water

For this experiment, I applied 0.00390625g/L, 0.0078125g/L, 0.015625g/L, 0.03125g/L, 0.0625g/L, 0.125g/L to 0.25g/L Methylene Blue dissolved in sterile water against *T. rubrum*. For each of these seven treatments, a 630nm light was exposed for 10s, 20s, 30s, 1min, 2min, 5min and no exposure. Together, the experiment included 7 x 7 combined treatments with each treatment having three repeats. After light exposure, the cultures were incubated at 30oC for 7 days and their Optical Density was measured daily (every 24 hours). My results showed that the OD value changes were similar among all seven exposure times: for MB concentrations were equal or greater than 0.015625g/L, the OD value almost remained the same over the 7 days (Figure 4), whereas for 0.00390625g/L and 0.0078125g/L MB treatments, the OD value increased every day (Figure 5). My results indicated that the *in vitro* MIC for MB dissolved in water was 0.015625g/L.

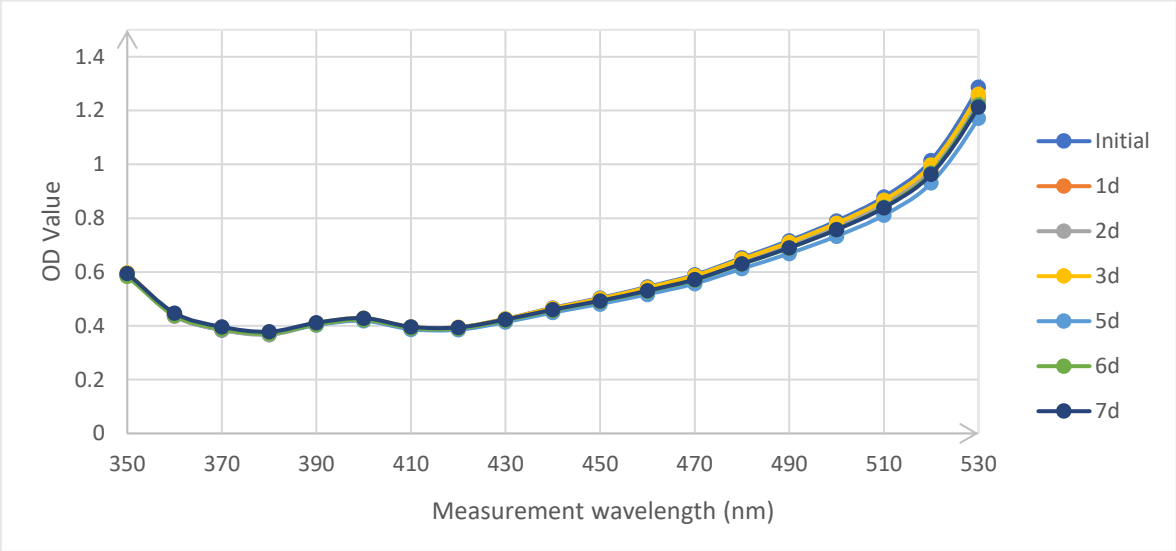


Fig 4. The OD values over 7 days in the 0.015g/L MB treatment after 20s of light exposure. The X-axis represents the wavelength of light used to measure OD while the Y-axis was OD value.

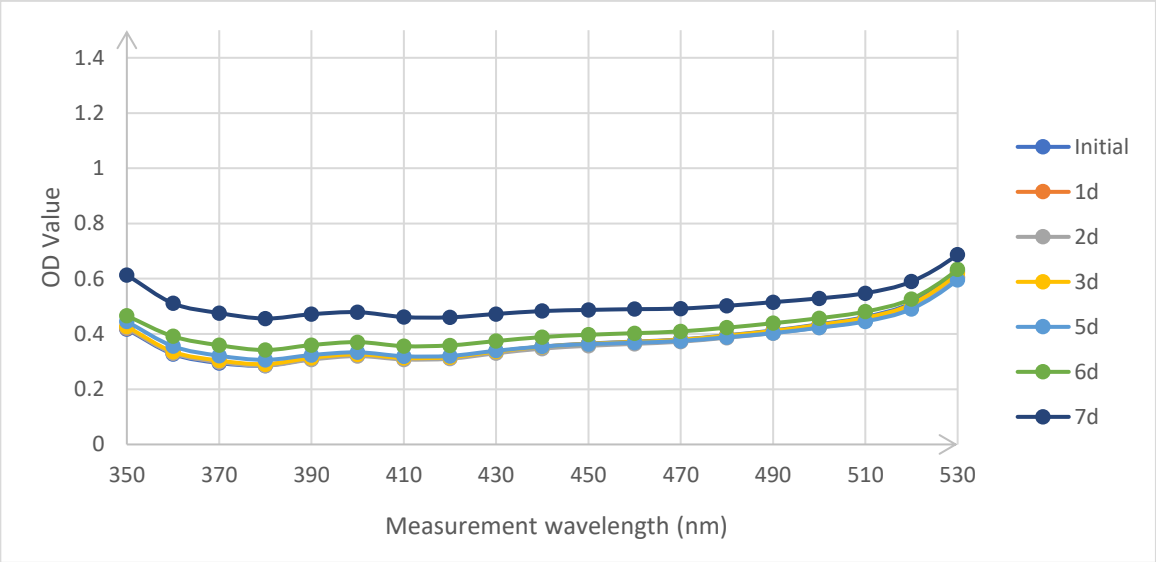


Fig 5. The OD values over 7 days in the 0.0078g/L MB treatment after 20s of light exposure. The X-axis represents the wavelength of light used to measure OD while the Y-axis was OD value.

In vitro MIC of Methylene Blue dissolved in PBS:

I tested all the 49 MB x light exposure time combinations mentioned above but this time with PBS as the solvent for MB. The OD values changed irregularly within the 7 days after light exposure, even among repeats. Thus, I was unable to determine the MIC of MB when it was dissolved in PBS buffer. A chemical analysis suggested that the Potassium and Sodium Ions in PBS buffer likely reacted with MB. As a result, MB lost its photosensitizer property and the photodynamic therapy was seriously interfered. We decided to discontinue using PBS as a solvent for subsequent experiments.

In vitro Carrier MIC Experiment

In order for MB to penetrate the nail plate and function as a photosensitizer against the fungi embedded in the nail, a carrier solution is needed. I tested a series of carrier combinations using different ratios of ethanol, glycerol, and water. Below are the specifics of the carriers' effects on the growth of *T. rubrum* in either water or in combination with other chemicals.

Carrier in Water

Among the 36 carrier combinations I tested, the OD valued of 24 combinations showed no increase within 7 days after treatment. These combinations included:40% Ethanol, 30% Glycerol, 30% Water; 40% Ethanol, 15% Glycerol, 45% Water; 40% Ethanol, 7.5% Glycerol, 52.5% Water; 40% Ethanol, 3.75% Glycerol, 56.25% Water; 40% Ethanol, 60% Glycerol; 40% Ethanol, 60% Water; 20% Ethanol, 30% Glycerol, 50% Water; 20% Ethanol, 15% Glycerol, 65% Water; 20% Ethanol, 7.5% Glycerol, 72.5% Water; 20% Ethanol, 3.75% Glycerol, 76.25% Water; 20% Ethanol, 80% Glycerol; 20% Ethanol, 80% Water; 10% Ethanol, 30% Glycerol, 60% Water; 10% Ethanol, 15% Glycerol, 75% Water; 10% Ethanol, 7.5% Glycerol, 82.5% Water; 10% Ethanol, 3.75% Glycerol, 86.25% Water; 10% Ethanol, 90% Glycerol; 10% Ethanol, 90% Water; 5% Ethanol,30% Glycerol, 65% Water; 5% Ethanol, 60% Glycerol, 35%

Water; 2.5% Ethanol, 30% Glycerol, 67.5% Water; 2.5% Ethanol, 60% Glycerol, 37.5% Water; 30% Glycerol 70% Water; 60% Glycerol 40% Water. These 24 combinations were considered to inhibit *T. rubrum* growth.

Carrier in PBS:

For the 36 carrier x PBS combinations, the OD value trends were almost the same as Carrier in water. In total, 23 combinations had no obvious growth of *T. rubrum* within 7 days. The combinations which inhibited *T. rubrum* were: 40% Ethanol, 30% Glycerol, 30% PBS; 40% Ethanol, 15% Glycerol, 45% PBS; 40% Ethanol, 7.5% Glycerol, 52.5% PBS; 40% Ethanol, 3.75% Glycerol, 56.25% PBS; 40% Ethanol, 60% Glycerol; 40% Ethanol, 60% PBS; 20% Ethanol, 30% Glycerol, 50% PBS; 20% Ethanol, 15% Glycerol, 65% PBS; 20% Ethanol, 7.5% Glycerol, 72.5% PBS; 20% Ethanol, 3.75% Glycerol, 76.25% PBS; 20% Ethanol, 80% Glycerol; 20% Ethanol, 80% PBS; 10% Ethanol, 30% Glycerol, 60% PBS; 10% Ethanol, 15% Glycerol, 75% PBS; 10% Ethanol, 7.5% Glycerol, 82.5% PBS; 10% Ethanol, 3.75% Glycerol, 86.25% PBS; 10% Ethanol, 90% Glycerol; 10% Ethanol, 90% PBS; 5% Ethanol,30% Glycerol, 65% PBS; 5% Ethanol, 60% Glycerol, 35% PBS; 2.5% Ethanol, 30% Glycerol, 67.5% PBS; 2.5% Ethanol, 60% Glycerol, 37.5% PBS,60% Glycerol 40% PBS.

***In vitro* Natural Extracts MIC Experiment**

Among the four natural extracts I tested, the OD values for all Turmeric concentration treatments (at 0.015625%, 0.03125%, 0.0625%, 0.125%, 0.25%, 0.5% w/v) all increased within 7 days after treatment, which indicated this natural extract could not to inhibit *T. rubrum* growth at those concentrations. The 0.5% w/v was the highest concentration we could dissolve turmeric.

For Propolis, Inula and St. John's Worts experiments, I found that three respective concentrations of these compounds at 0.075%, 0.03125% and 0.0625% were the lowest concentrations that prohibited *T. rubrum* from growing for 7 days after exposure for each

natural extract. Thus, 0.075%, 0.015625% and 0.0625% were the MICs for Propolis, Inula and St. John's Worts against *T. rubrum in vitro.*

In vitro Minimum Fungicidal Concentration (MFC) of MB

After 7 days of incubation and OD measurement post treatments with MB. The cultures were transferred from each well onto fresh SDA plate. The SDA plates were incubated at 30^{o}C and observed daily for the potential growth of *T. rubrum*. After 14 days incubation and observation, the results showed that 0.125g/L MB treatment was the lowest MB concentration that led to no *T. rubrum* growth. In contrast, the 0.0625g/L MB treatment had one well showing growth of *T. rubrum* out of six repeats (three 2min exposure samples and three 20s exposure samples) within 8 days, and more for lower concentrations. Specifically, two out of six wells showed *T. rubrum* growth in 0.03125g/L MB treatment after 4 days, one of six showed growth in 0.0156g/L MB treatment after 4 days, five out of six showed growth in 0.0078g/L MB after 4 days, and all six wells had viable *T. rubrum* cells in 0.0039g/L MB in 4 days.

In vitro Carrier Fungicidal Concentration

Similar to that for the MFC determination for MB, I also determined whether the carrier has any fungicidal activity. The results showed that out of the 24 Carrier in water combinations which inhibited fungal growth, four of them showed the growth of *T. rubrum* within 7 days after the solutions were transferred to SDA plates. Those four combinations were: 5% Ethanol,30% Glycerol, 65% Water; 2.5% Ethanol, 30% Glycerol, 67.5% Water; 30% Glycerol 70% Water; 60% Glycerol 40% Water. The remaining 20 combinations showed fungicidal effect, including all six combinations of 10%, 20% and 40% Ethanol; 5% Ethanol, 60% Glycerol, 35% Water; 2.5% Ethanol, 60% Glycerol, 37.5% Water.

In vitro Infected Agar Cubes Treatment

Natural Extracts in Carrier Results

In the plates which held agar cubes exposed to carriers, the mycelia were found at the bottom of the cubes on the third and forth day for carriers only treatments after both the 5min and 10min exposures. For St. John's Worts treated cubes, the *T. rubrum* mycelia were found on the third and fifth day for 5min and 10min, respectively. The cubes exposed to 5min of Inula treatments inhibit *T. rubrum* growth for 5 days, whereas for the 10 min Inula exposure treatment, it inhibited mycelia growth for more than 14 days. For Propolis treatments, it inhibited *T. rubrum* growth 2 days and 4 days for 5 min and 10 min exposure separately. The detailed results are shown in the Table 1 below.

Natural Extracts in Water Results:

For this part of the experiment, both the water positive control and the Emulsifier control treatments showed normal *T. rubrum* growth, with colonies started to grow on the next day after treatment. The results of SJW and Propolis experiments also indicated that these two extracts were not ideal as *T. rubrum* colonies started to grow on the next day of treatments. For Inula experiments, *T. rubrum* colonies were observed on the second day after treatment for the one-hour treatment 5 times wash experiments, whereas for the no wash 10 mins Inula treatment experiment, *T. rubrum* colonies started to grow at 9 days after the treatment. The detailed results are shown in the Table 2 below.

Table 1. In vitro Agar Cubes Treatment in Carrier

	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10
No treatment control	1,1,1	2,2,2	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3
Carrier 5min	0,0,0	0,0,0	2,2,0	3,3,2	3,3,2	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3
Carrier 10min	0,0,0	0,0,0	0,0,0	2,2,0	2,3,0	3,3,2	3,3,2	3,3,3	3,3,3	3,3,3
SJW in carrier 5min	0,0,0	0,0,0	2,1,0	3,3,1	3,3,2	3,3,2	3,3,3	3,3,3	3,3,3	3,3,3
SJW in carrier 10min	0,0,0	0,0,0	0,0,0	0,0,0	1,0,1	2,0,2	2,2,2	3,3,3	3,3,3	3,3,3
Inula in carrier 5min	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	1,0,0	2,0,0	3,2,2	3,2,2	3,3,3
Inula in carrier 10min	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0*
Propolis in carrier 5min	0,0,0	0,0,0	1,1,2	2,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3
Propolis in carrier 10min	0,0,0	0,0,0	0,0,0	0,0,0	2,1,2	2,2,2	3,3,3	3,3,3	3,3,3	3,3,3

0: No growth
1: Growth onto cube observed under microscope
2: Growth onto cube observed by naked eyes
3: Growth has spread onto agar plate
*: No mycelia observed in the treatment plate for at least 21 days

Table 2. In vitro Agar Cubes Treatment in Water

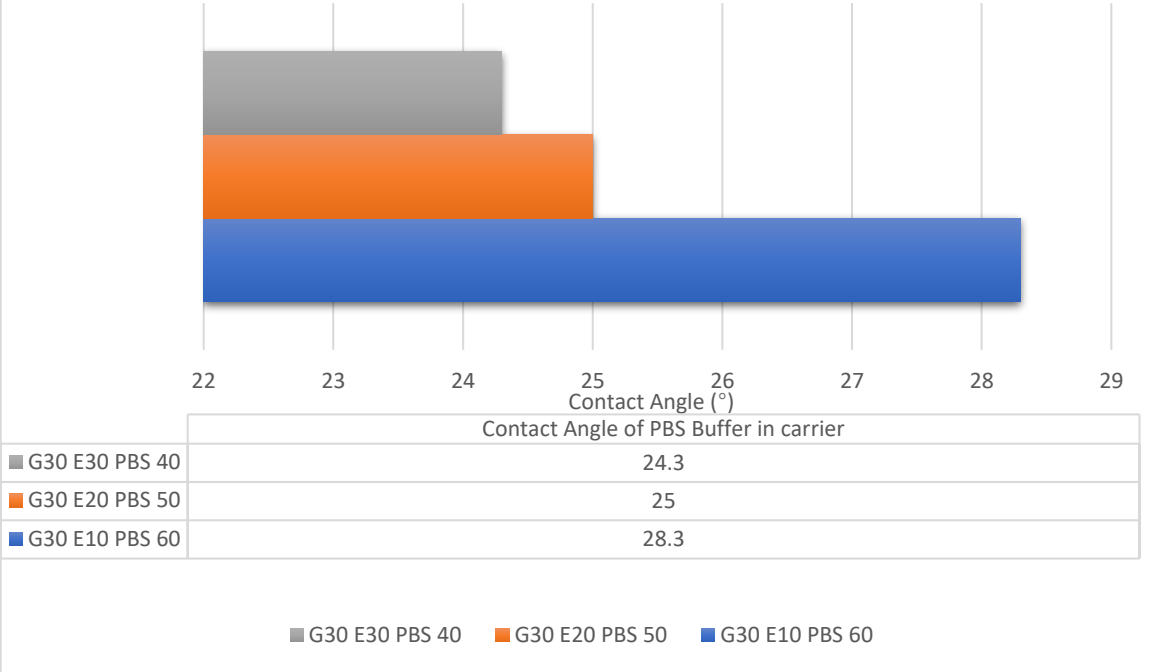
	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10
Water 10min no wash	2,2,2	2,2,2	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3
Water 1h no wash	2,2,2	2,2,2	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3
Emulsifier 10min no wash	1,0,1	2,1,2	2,2,2	2,2,2	2,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3
Emulsifier 1h 5X wash	2,2,2	3,3,2	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3
1% SJW 10min no wash	1,1,0	2,1,1	2,2,2	2,2,2	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3
1% SJW 1h 5X wash	2,2,2	2,2,2	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3
1% Inula 10min no wash	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	1,0,0	2,0,1
1% Inula 1h 5X wash	0,0,0	2,2,0	3,3,2	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3
1% Propolis 10min no wash	2,2,2	2,2,2	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3
1% Propolis 1h 5X wash	2,2,2	2,2,2	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3

0: No growth
1: Growth onto cube observed under microscope
2: Growth onto cube observed by naked eyes
3: Growth has spread onto agar plate

Carrier Surface Tension Test

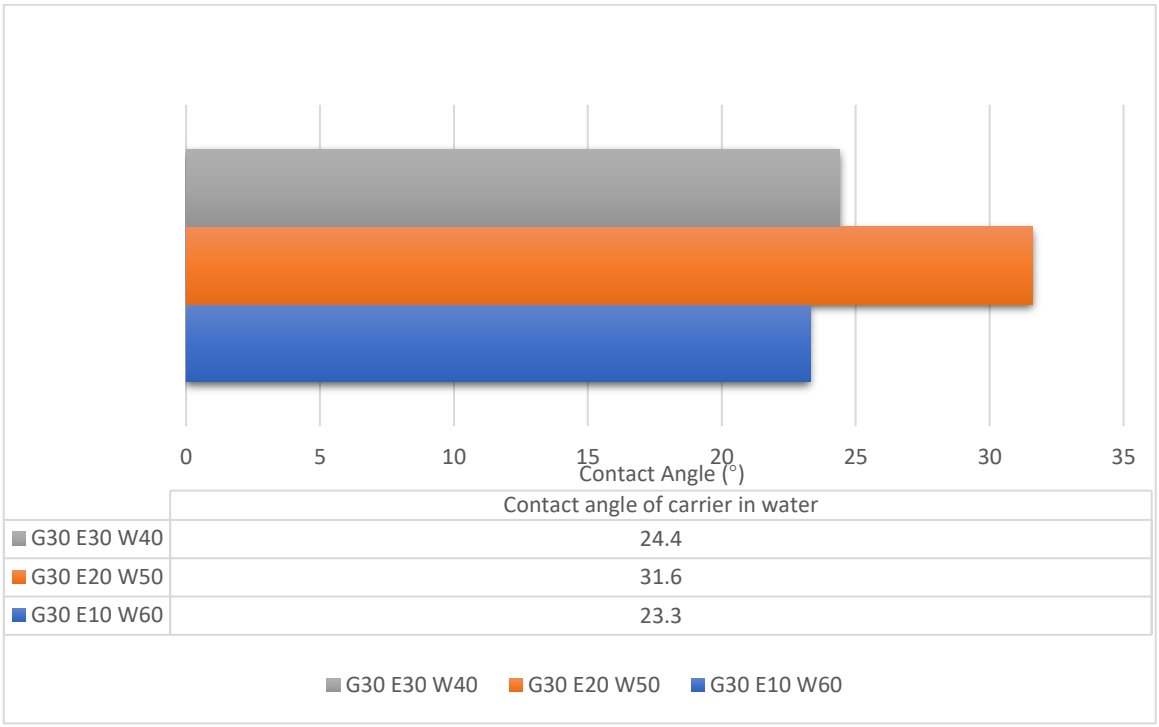
The contact angles of six designed carrier combinations were tested in this experiment. The images of carriers' droplets on nails are shown below in Figure 6- Figure 14. The contact angles were measured per second in the first 20 seconds after the droplet was dripped, the average angles of each solution can be seen in Table 3 – Table 5 below. Generally, glycerol contributes most to the surface tension of the carrier system, and water/PBS buffer contributes more than Ethanol in the carrier system.

Table 3. Contact Angle of PBS Carriers



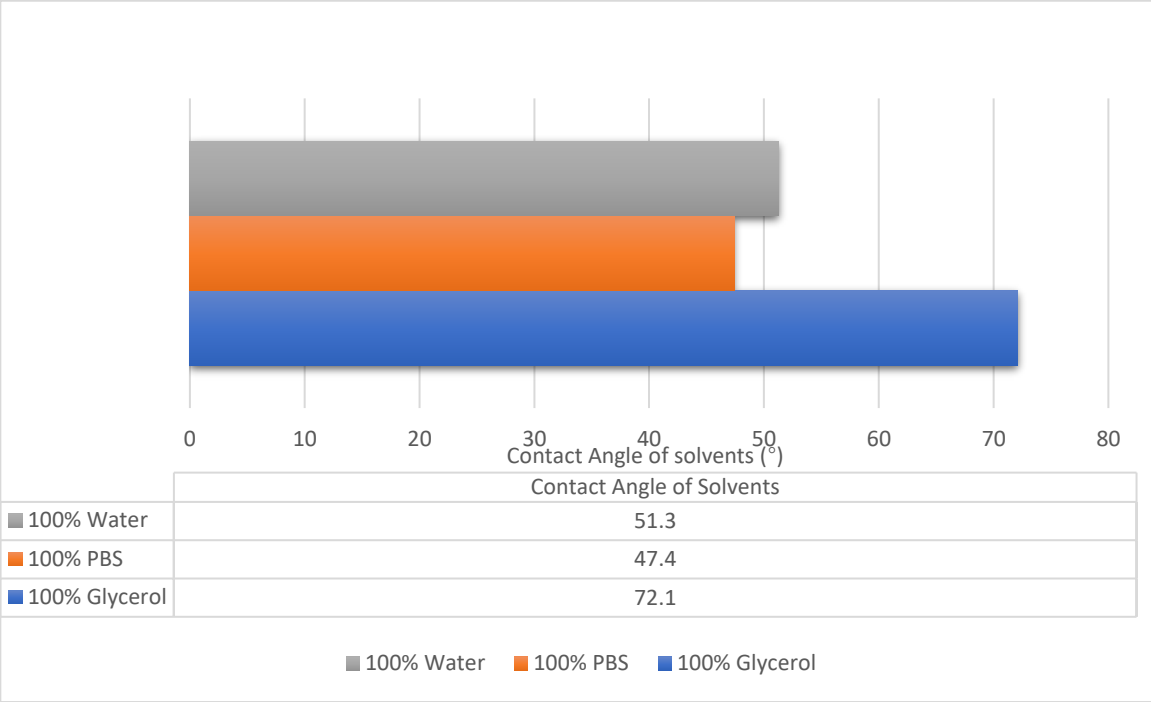
	Contact Angle of PBS Buffer in carrier
G30 E30 PBS 40	24.3
G30 E20 PBS 50	25
G30 E10 PBS 60	28.3

Table 4. Contact Angle of Water Carriers



	Contact angle of carrier in water
G30 E30 W40	24.4
G30 E20 W50	31.6
G30 E10 W60	23.3

Table 5. Contact Angle of Solvents



	Contact Angle of Solvents
100% Water	51.3
100% PBS	47.4
100% Glycerol	72.1

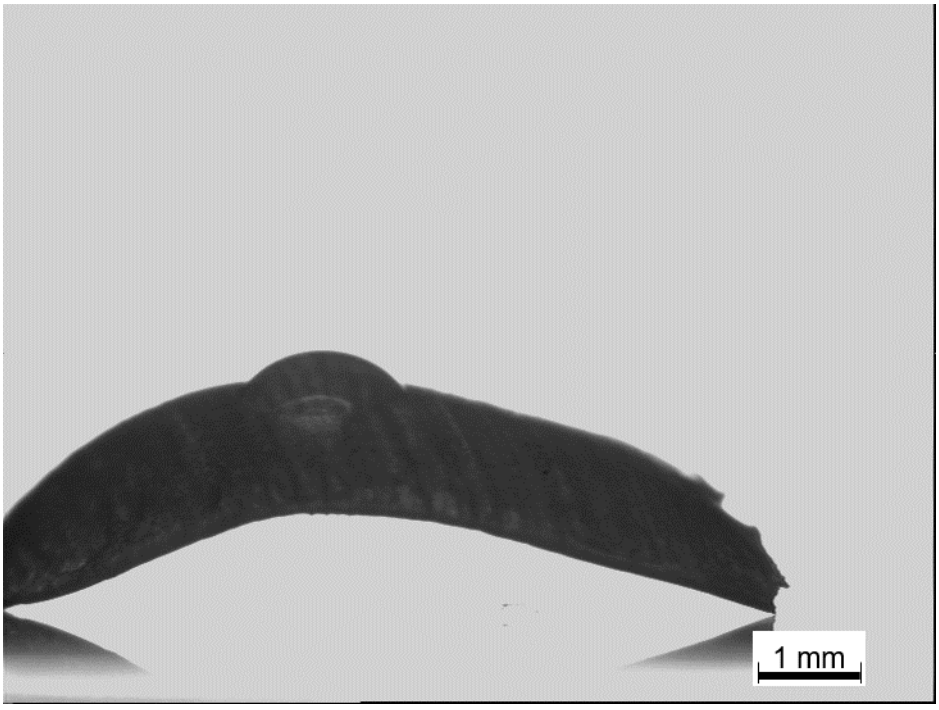


Fig 6. Image of Carrier Glycerol 30%, Ethanol 10%, PBS 60%

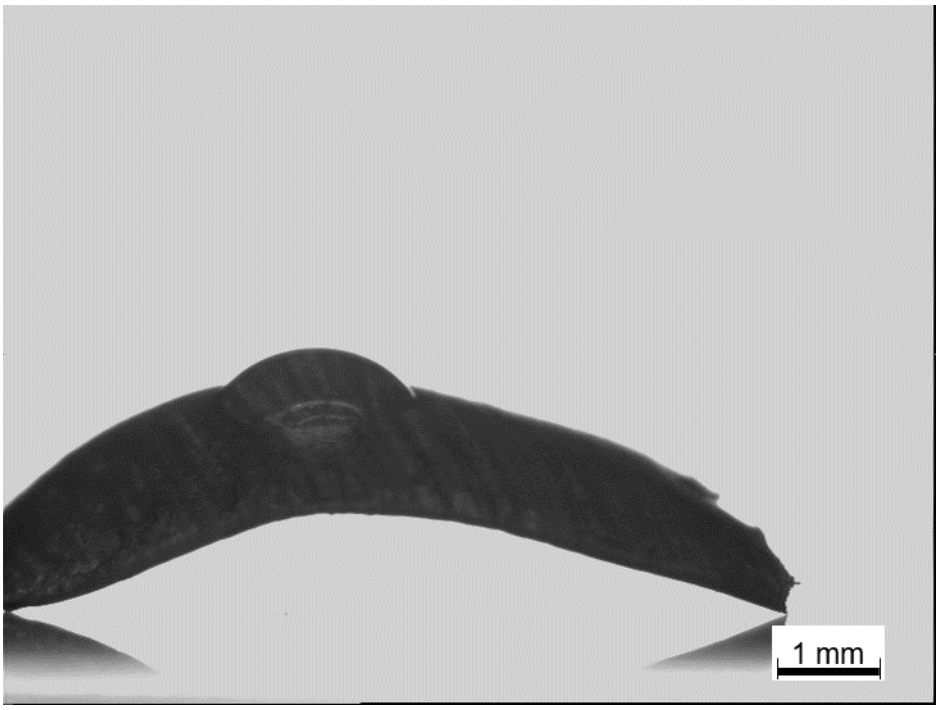


Fig 7. Glycerol 30%, Ethanol 20%, PBS 50% on a Nail Piece

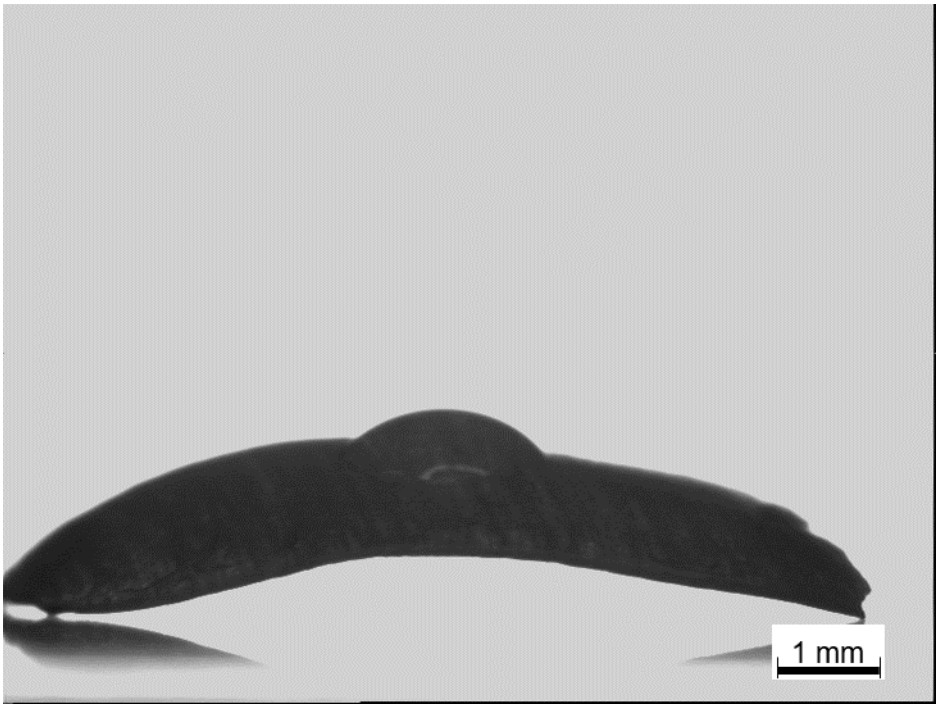


Fig 8. Glycerol 30%, Ethanol 30%, PBS 40% on a Nail Piece

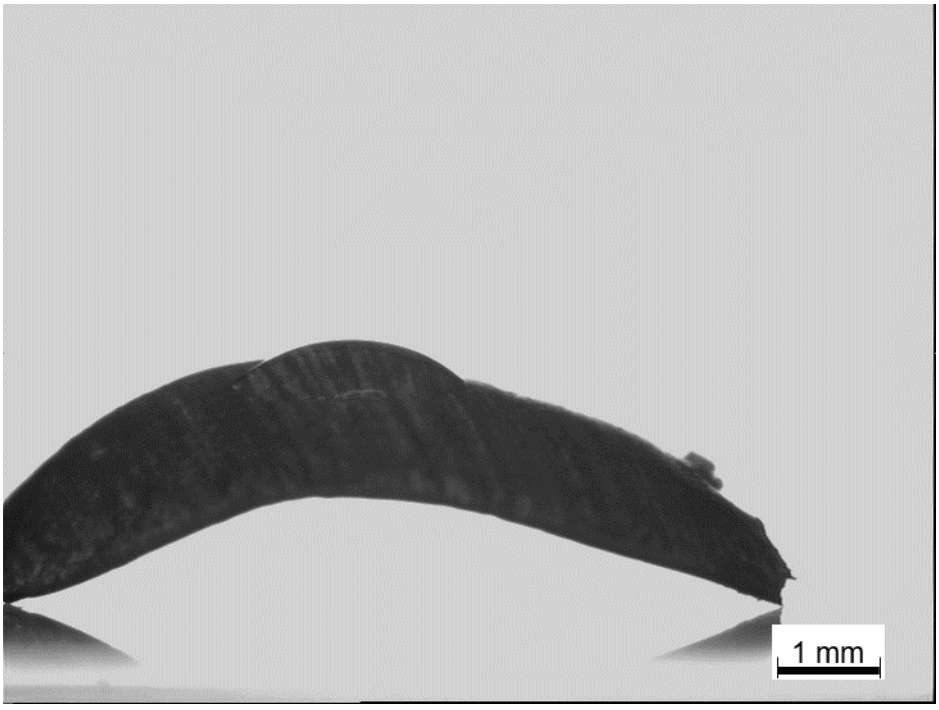


Fig 9. Glycerol 30%, Ethanol 10%, Water 60% on a Nail Piece

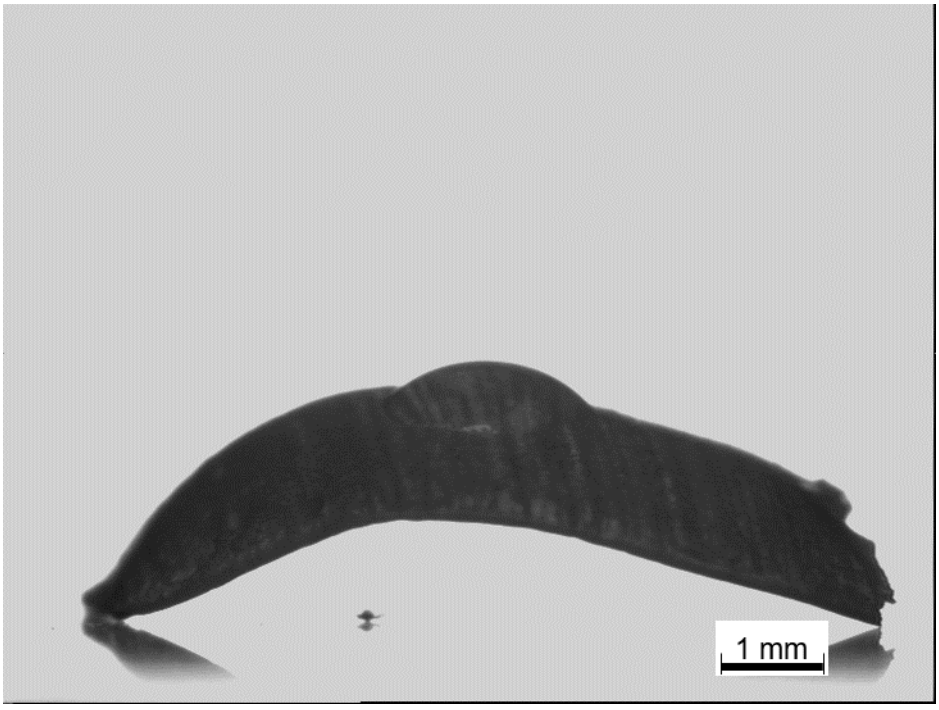


Fig 10. Glycerol 30%, Ethanol 20%, Water 50% on a Nail Piece

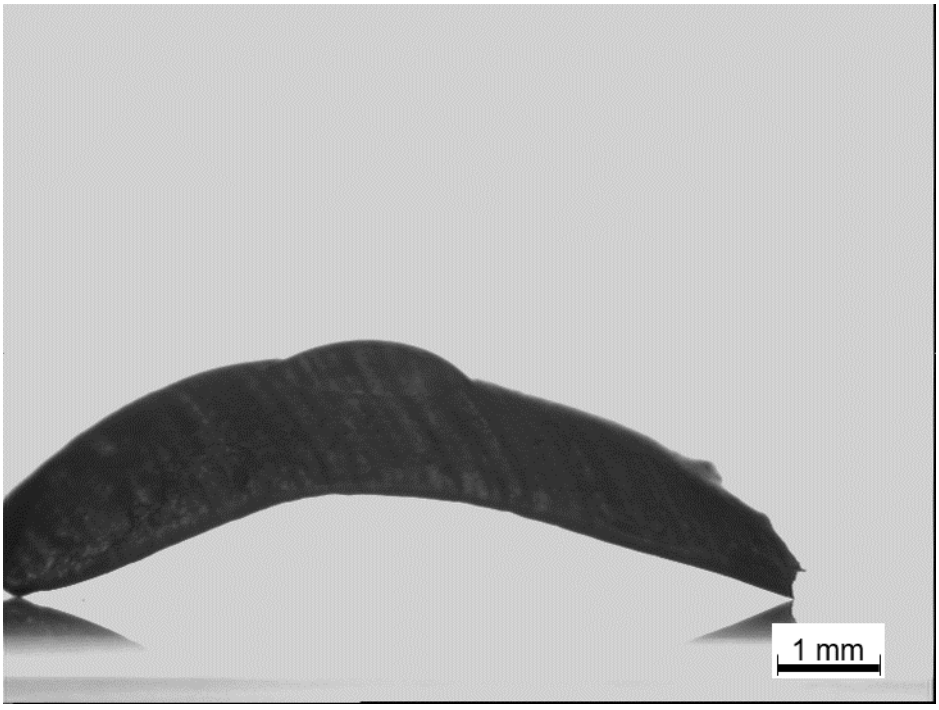


Fig 11. Glycerol 30%, Ethanol 30%, Water 40% on a Nail Piece

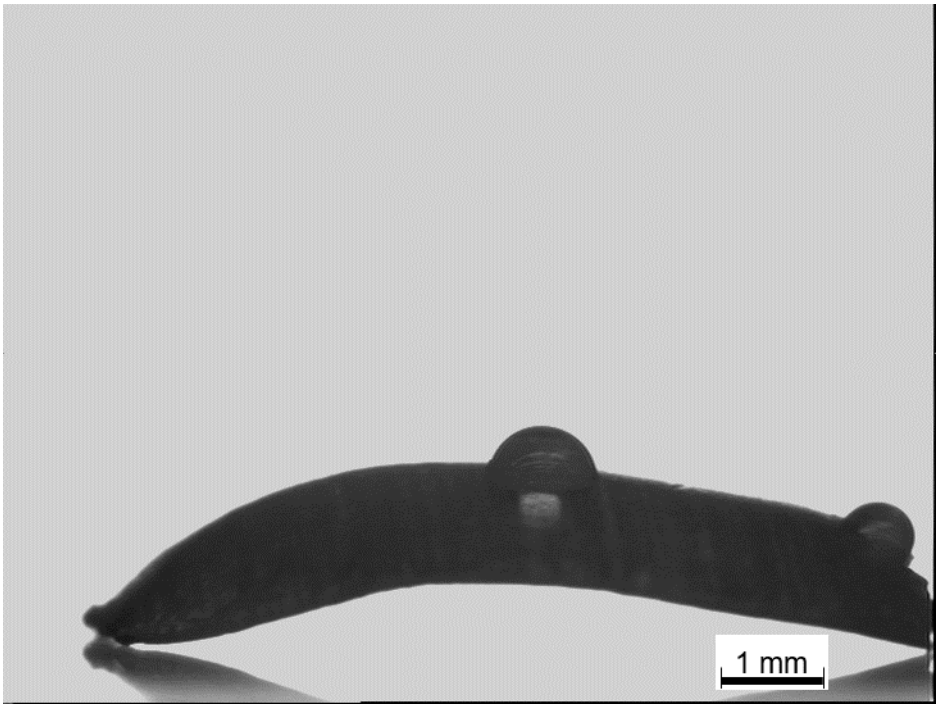


Fig 12. 100% Glycerol on a Nail Piece

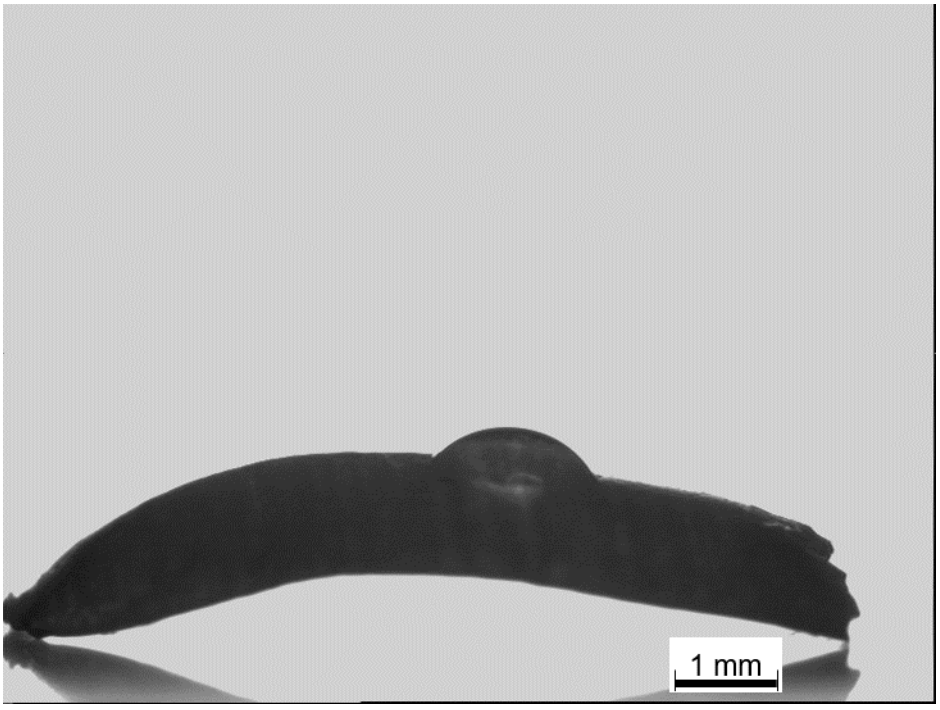


Fig 13. 100% PBS Buffer on a Nail Piece

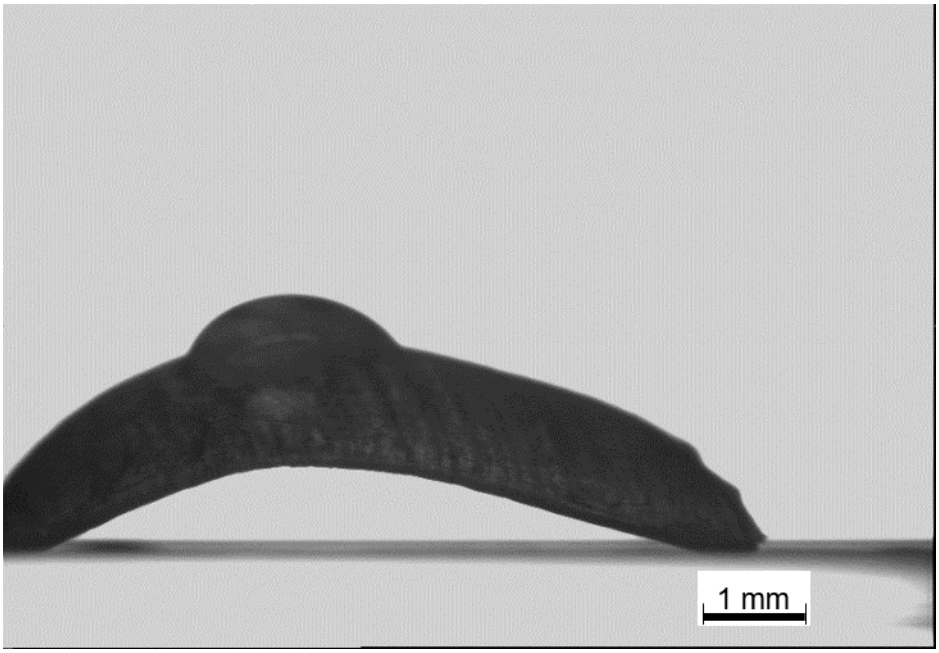


Fig 14. 100% Water Droplet on a Nail Piece

Nail Pieces Infection Characterization:

To ensure that nail pieces were indeed infected after they were incubated together with *T. rubrum* cultures, I examined nail pieces using microscopy scanning and the staining method is PAS (Periodic Acid-Schiff) which could only stain the living fungus cells into magenta. As shown below, in the microscopy scanning images of the fixed and infected nail pieces, from both nail cross section (Figure 15) and flat cutting direction (Figure 16), magenta fungal hyphae could be seen. The microscopic images indicate our infection method was effective.

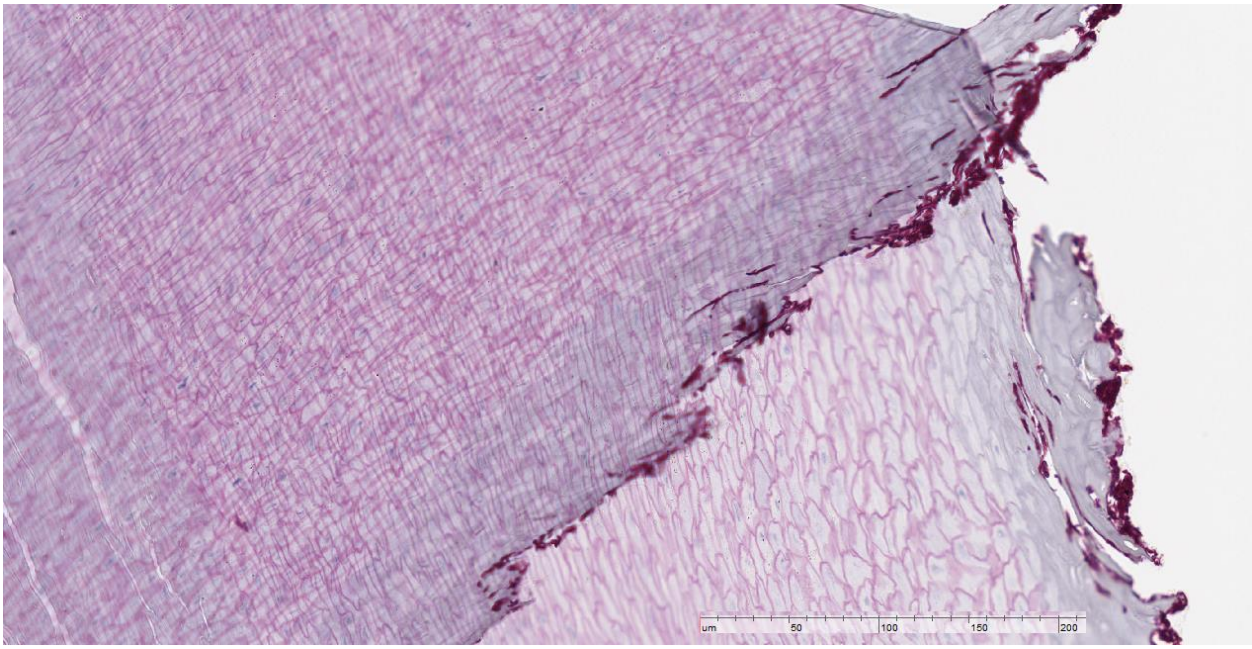


Fig 15. 40X Microscope Image of infected nails from cross section direction

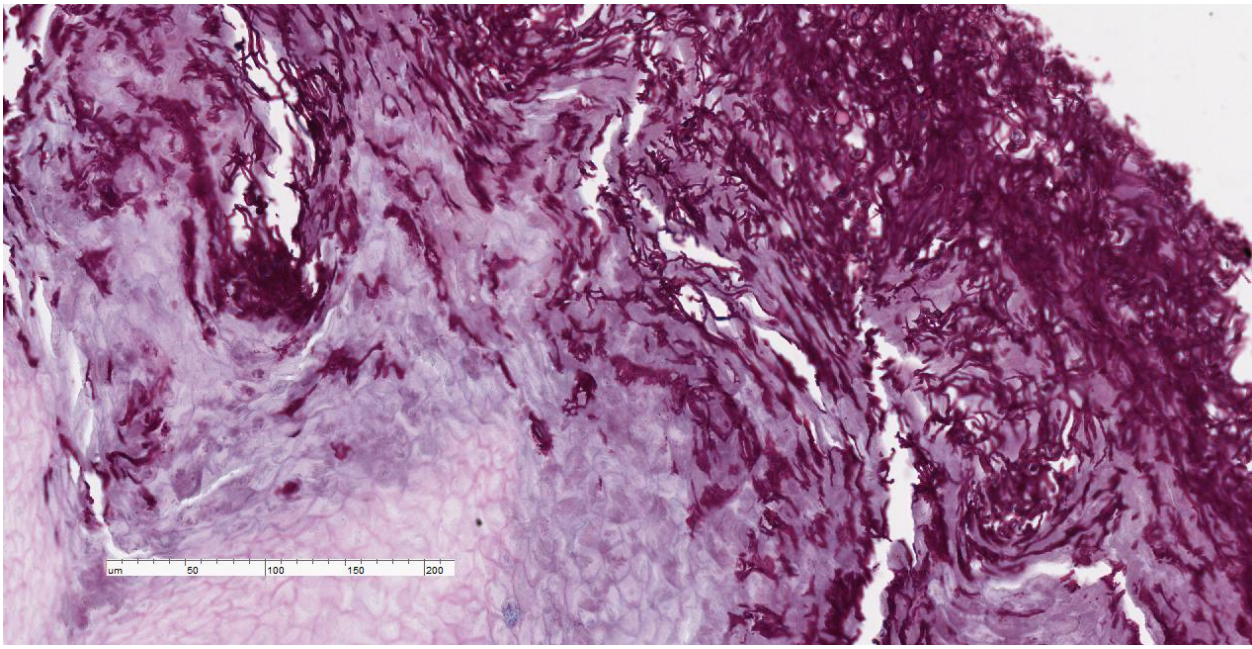


Fig 16. 40X Microscope Image of infected nails from flat cutting direction

Ex vivo MB Nail Pieces Treatment

In the first set of MB nail pieces treatment, I tested four MB concentrations (0.03125%, 0.0625%, 0.125%, 0.25%). At the highest concentration of 0.25% MB with 15min and 30min light exposure, *T. rubrum* growth from the nail pieces did not start until after 11 days. Among the three lower concentration treatments (0.03125%, 0.0625%, 0.125% MB with 30min light exposure), *T. rubrum* started to grow out of the nail pieces after 5 to 6 days, which indicated these concentration range was too low to be applied. This pilot experiment indicated that the concentration range selected in the first experiment was too low to determine an appropriate MB concentration. In the second stage, I chose the highest concentration at 0.5% and with two lower ones (i.e. 0.125%, 0.25% and 0.5%), the light exposure time were all 30 min and 15 min except the 0.125% trial for 15 min light exposure only. In addition, to simulate clinical treatment, I conducted totally six periodic treatments for nail pieces this time, the interval for each treatment was around 10 days, except the interval between first and second treatments was 17 days. The results from these experiments revealed that most treatments at high MB concentration (0.25% and 0.5% MB, 15min and 30min irradiation) inhibited *T. rubrum* growth after the second treatments, whereas the efficacy of the first treatment for all MB conditions were not idea (mycelia were observed in corresponding treatments SDA plates within 6 to 10 days). Consistent with the first experiment, the lowest MB concentration of 0.125% MB with 30min irradiation was not very effective, as *T. rubrum* mycelia could be spotted in the nail placed SDA plates within 8-10 days after each periodic treatment. The detailed nail pieces results are demonstrated in the following tables (Table 6 - Table 11).

Table 6. First Treatment Results

	D2	D4	D5	D6	D7	D8	D9	D10	D11	D12	D14	D16
0.125% 30min Mar 1st*	0,0,0	0,0,0	0,0,0	1,1,0	1,1,0	2,2,0	2,2,1	3,3,2	3,3,3	3,3,3	3,3,3	3,3,3
0.25% 15min	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,1	0,0,2	0,0,2	0,0,3	0,1,3	0,2,3	0,3,3
0.25% 30min	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	1,0,0	1,0,0	2,0,0	2,0,0	3,0,1	3,0,1
0.5% 15min	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	1,0,0	1,0,0	2,0,1	3,0,1	3,0,2	3,0,3	3,0,3
0.5% 30min	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	1,1,0	2,1,0	2,1,0	3,1,0	3,2,0	3,3,0	3,3,0
Control	2,2,1	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3

0: No growth
1: Growth onto agar plate observed under microscope
2: Growth onto agar plate observed by naked eyes
3: Growth has spread onto agar plate
*: Nail pieces from previous experiments with *T.rubrum* mycelia growth

Table 7. Second Treatment Results

	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10
0.125% 30min Mar 1st*	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	1,0,0
0.125% 30min Mar 5th	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
0.25% 15min	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
0.25% 30min	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
0.5% 15min	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
0.5% 30min	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
Control	0,0,0	0,0,0	1,1,1	2,2,2	2,2,2	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3

0: No growth
1: Growth onto agar plate observed under microscope
2: Growth onto agar plate observed by naked eyes
3: Growth has spread onto agar plate
*: Nail pieces from previous experiments with *T.rubrum* mycelia growth

Table 8. Third Treatment Results

	D1	D2	D3	D4	D5	D6	D7	D8
0.125% 30min Mar 1st*	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	1,0,0	2,0,0
0.125% 30min Mar 5th	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
0.25% 15min	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
0.25% 30min	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
0.5% 15min	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
0.5% 30min	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
Control	0,0,0	2,2,2	3,3,3	3,3,3,	3,3,3	3,3,3	3,3,3	3,3,3

0: No growth
1: Growth onto agar plate observed under microscope
2: Growth onto agar plate observed by naked eyes
3: Growth has spread onto agar plate
*: Nail pieces from previous experiments with *T.rubrum* mycelia growth

Table 9. 4th Treatment Results

	D1	D2	D3	D4	D5	D6	D7	D8	D9
0.125% 30min Mar 1st*	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,1,0
0.125% 30min Mar 5th	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
0.25% 15min	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
0.25% 30min	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
0.5% 15min	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
0.5% 30min	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
Control	1,1,1	2,2,2	2,2,2	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3

0: No growth
1: Growth onto agar plate observed under microscope
2: Growth onto agar plate observed by naked eyes
3: Growth has spread onto agar plate
*: Nail pieces from previous experiments with *T.rubrum* mycelia growth

Table 10. 5th Treatment Results

	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10
0.125% 30min Mar 1st*	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,1,0	0,2,0	0,2,0	0,2,0	0,3,0
0.125% 30min Mar 5th	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
0.25% 15min	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
0.25% 30min	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
0.5% 15min	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
0.5% 30min	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
Control	1,1,1	2,2,2	2,2,2	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3

0: No growth
1: Growth onto agar plate observed under microscope
2: Growth onto agar plate observed by naked eyes
3: Growth has spread onto agar plate
*: Nail pieces from previous experiments with *T.rubrum* mycelia growth

Table 11. 6th Treatment Results

	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11
0.125% 30min Mar 1st*	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,1
0.125% 30min Mar 5th	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
0.25% 15min	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
0.25% 30min	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
0.5% 15min	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
0.5% 30min	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
Control	1,1,1	2,2,2	2,2,2	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3

0: No growth
1: Growth onto agar plate observed under microscope
2: Growth onto agar plate observed by naked eyes
3: Growth has spread onto agar plate
*: Nail pieces from previous experiments with *T.rubrum* mycelia growth

Ex vivo MB, Inula, and Carrier Combination Nail Pieces Treatments

In the following table 12, the results of *ex vivo* nail pieces MB, Inula, Carrier and correlated combination treatments were demonstrated. Generally, the treatments of carrier only, Inula in water with or without light, and Inula in carrier without light exposure did not kill or inhibit *T. rubrum* growth in nail pieces. The *T. rubrum* growth patterns after these few treatments were similar to that observed in positive control of just water. However, for the Inula in carrier with light exposure for 10 minutes showed inhibitory effect against *T. rubrum* until day 5. For the MB treatments, the result of 0.5% MB was consistent with previous first MB nail pieces treatment. Additionally, an interesting result was found that the efficacy of 0.5% MB plus 1% Inula was not as good as that with 0.5% MB by itself. However, the treatment of 1% MB + 1% Inula with light completely killed *T. rubrum*.

Table 12. Inula, MB, Carrier Combination ex vivo nail pieces treatment results

	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	
Carrier 5min Submerging No Light	0,0,0	0,0,2	1,2,3	2,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	
Carrier 10min Submerging No Light	0,0,0	0,2,2	1,3,3	2,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	
Inula+Water 5min Submerging No Light	0,0,0	1,2,1	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	
Inula+Water 10min Submerging No Light	0,0,0	1,2,2	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	
Inula+Water 5min Submerging Light	0,0,0	0,0,1	0,0,2	0,0,2	0,0,3	2,0,3	3,0,3	3,0,3	3,0,3	3,0,3	
Inula+Water 10min Submerging Light	0,0,0	0,0,0	2,0,0	2,0,0	3,0,1	3,2,2	3,3,3	3,3,3	3,3,3	3,3,3	
Inula+Carrier 5min Submerging No Light	0,0,0	0,0,1	1,1,2	2,2,2	3,2,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	
Inula+Carrier 10min Submerging No Light	0,0,0	0,1,0	0,2,1	0,2,2	2,3,3	2,3,3	3,3,3	3,3,3	3,3,3	3,3,3	
Inula+Carrier 5min Submerging Light	0,0,0	0,0,0	1,1,0	3,3,0	/,3,0*	/,3,2	/,3,3	/,3,3	/,3,3	/,3,3	
Inula+Carrier 10min Submerging Light	0,0,0	0,0,0	0,0,0	0,0,0	1,1,0	2,2,0	3,3,1	3,3,2	3,3,2	3,3,3	
0.5% MB Light	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,1	0,0,2	1,1,2	
0.5% MB +1% Inula Light	0,0,0	0,0,0	0,1,0	0,2,0	0,2,0	1,2,0	2,2,0	2,3,0	3,3,0	3,3,0	
1% MB +1% Inula Light	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	1,0,0**
Water Control	0,0,0	1,1,1	2,2,2	2,2,2	3,2,2	3,2,2	3,3,3	3,3,3	3,3,3	3,3,3	

0: No growth
1: Growth onto agar plate observed under microscope
2: Growth onto agar plate observed by naked eyes
3: Growth has spread onto agar plate
* A fast growing non- *T.rubrum* mycelia colony found in the SDA plate around one nail piece in this treatment, the rest two nail pieces were transferred to new SDA plates for observation.
** *T.rubrum* mycelia were observed on D12

Ex vivo Nail Wells Treatment

Due to the wide variability, I did this experiment twice. However, within these two experiments, most treatment combinations which worked well in *ex vivo* nail pieces did not show comparable treatment effectiveness. This inconsistency may be due to variations in the thickness of nails that influenced the penetration efficiency of the formulations. In addition, it could also be due to the growth of the mycelia onto the bottom of the tubing that we were unable to eliminate. Therefore, I conducted another tubing cut-off experiment to try to get rid of this factor. The methods of this experiment have been elaborated in methods part.

Table 13-16 below show the results from my two *ex vivo* experiments with nail wells. For each treatment, the nail wells were split into two groups. In the first group, the observations were made with nail wells placed on SDA petri plates while in the second group, the nail wells after treatments were placed on bare petri dishes without any medium. As can be seen from these tables, 1% inula+0.5% MB with light significantly inhibited the growth of *T. rubrum* through nails in both experiments. In addition, except the water control, none of the nail wells placed on bare petri dishes had any fungal growth outside of the nail well. Furthermore, it seems tubing infection by *T. rubrum* was likely the cause of fungal growth in other treatments. As can be seen from Table 16, when the tubing was cut off from the nail wells before being placed on SDA agar, no fungal growth was observed. The results indicated that the carrier and all the formulations worked in the nail well experiment.

Table 13. 1st Ex vivo results of nail well treatments

	D1	D2	D3	D4	D5	D6	D7	D8
1% Inula + 0.5% MB + Light	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	1,0,0
1% Inula No Light	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	2,0,2	2,1,2	3,3,3
1% Inula + Light	0,0,0	0,0,0	1,0,0	2,1,0	3,2,0	3,2,0	3,3,0	3,3,2
0.5% MB + Light	0,0,0	0,0,0	0,0,0	0,0,0	0,0,2	0,0,3	1,1,3	2,2,3
Emulsifier Control	0,0,0	0,0,0	0,0,0	1,0,0	2,1,0	3,2,0	3,2,2	3,3,3

0: No growth
1: Growth onto agar plate observed under microscope
2: Growth onto agar plate observed by naked eyes
3: Growth has spread onto agar plate

Table 14. 2nd Ex vivo results of nail well treatments

	D1	D2	D3	D4	D5	D6	D7	D8
1% Inula in carrier + 1% MB in carrier + Light	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
1% Inula in carrier No Light	0,0,0	0,0,0	1,1,0	2,1,1	2,2,2	2,2,2	3,2,2	3,3,3
1% Inula in carrier + Light	0,0,0	0,0,0	0,0,0	0,0,0	1,0,0	2,1,1	2,2,1	2,2,2
Water control	0,0,0	0,0,0	1,1,1	2,2,2	2,2,2	3,2,2	3,2,2	3,3,3
Carrier control	0,0,0	0,0,0	0,0,1	2,2,2	2,2,2	3,3,3	3,3,3	3,3,3

	D9	D10	D11	D12	D13	D14	D15
1% Inula in carrier + 1% MB in carrier + Light	0,0,0	0,0,0	0,0,0	0,0,0	1,0,0	1,0,0	2,0,0
1% Inula in carrier No Light	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3
1% Inula in carrier + Light	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3
Water control	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3
Carrier control	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3	3,3,3

0: No growth
1: Growth onto agar plate observed under microscope
2: Growth onto agar plate observed by naked eyes
3: Growth has spread onto agar plate

Table 15. Ex vivo results of nail well treatments on bare plates

Nail wells on Bare plates	D1	D2	D3	D4	D5	D6	D7	D8
1% MB 30min	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
1% MB no light	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
1% Inula 30min	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
1% Inula no light	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
1% MB+1% Inula 30min	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
1% MB+1% Inula no light	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
Water Control	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
Carrier Control	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0

0: No growth
1: Growth onto bare plate observed under microscope
2: Growth onto bare plate observed by naked eyes
3: Growth has spread onto nail discs

Table 16. Ex vivo Results of Nail Well Treatments on SDA Plates after the Tubing was Cut-Off

Nail discs on SDA plates	D1	D2	D3	D4	D5	D6*
1% MB 30min	0,0,0,0,0,0	0,0,0,0,0,0	0,0,0,0,0,0	0,0,0,0,0,0	0,0,0,0,0,0	0,0,0,0,0,0
1% MB no light	0,0,0,0,0,0	0,0,0,0,0,0	0,0,0,0,0,0	0,0,0,0,0,0	0,0,0,0,0,0	0,0,0,0,0,0
1% Inula 30min	0,0,0,0,0,0	0,0,0,0,0,0	0,0,0,0,0,0	0,0,0,0,0,0	0,0,0,0,0,0	0,0,0,0,0,0
1% Inula no light	0,0,0,0,0,0	0,0,0,0,0,0	0,0,0,0,0,0	0,0,0,0,0,0	0,0,0,0,0,0	0,0,0,0,0,0
1% MB+1% Inula 30min	0,0,0,0,0,0	0,0,0,0,0,0	0,0,0,0,0,0	0,0,0,0,0,0	0,0,0,0,0,0	0,0,0,0,0,0
1% MB+1% Inula no light	0,0,0,0,0,0	0,0,0,0,0,0	0,0,0,0,0,0	0,0,0,0,0,0	0,0,0,0,0,0	0,0,0,0,0,0
Water Control	1,1,0	2,2,2	2,2,2	2,2,2	3,3,3	3,3,3
Carrier Control	0,0,0,0,0	0,0,0,0,0	0,0,0,0,0	0,0,0,0,0	0,0,0,0,0	0,0,0,0,0

0: No growth
1: Growth onto agar plate observed under microscope
2: Growth onto agar plate observed by naked eyes
3: Growth has spread onto agar plate
*: Observation result remained same until Day 14

CHAPTER 4. DISCUSSION

In my thesis research, the results from the Methylene Blue (MB) *in vitro* experiments were consistent with previously discovered fungicidal effect of MB against *T. rubrum* under light irradiation. In addition, a natural extract Inula and a number of carriers that we tested exhibited fungicidal effects. The primary function of carriers in these treatments was to enhance penetration of the formulations through nails. In the *ex vivo* experiment, both MB and Inula at certain concentrations inhibited and/or killed *T. rubrum* embedded within the infected nail pieces, as well as within the nail well apparatus, which is a simulated *in vivo* device. My results demonstrated that both MB and Inula are potent photosensitizers for clinical treatment of nail fungal infections.

Methylene Blue *in vitro* Treatments

In my *in vitro* MIC experiment, the Minimum Inhibitory Concentration for Methylene Blue in water against *T. rubrum* was 0.015g/L with 30 mins 630nm light irradiation. The result of Methylene Blue *in vitro* MFC against *T. rubrum* was 0.125g/L. Our results were comparable to the *In vitro* experiment of Methylene Blue against *T. mentagrophytes* (López-Chicón et al. 2016), where the MIC was 0.016g/L MB with 81J/cm^2 625-640 nm light for 10 mins or 0.032g/L MB with 9J/cm^2. However, it's worth mentioning that in my earlier pilot experiment, I tried seven different irradiation times: 10s, 20s, 30s, 1min, 2min, 5min and no light exposure. The MICs of the seven irradiation times were almost the same. This was highly unusual. Upon further checking, my experiment was all conducted in the lab with both natural light and artificial light, but not in a dark room. Thus, the results suggest that natural and artificial light was sufficient to activate MB, the sensitive photosensitizer, and release enough ROS to kill or inhibit *T. rubrum.*

Natural Extracts and Carrier *in vitro* Treatments

In my study, I totally tested four natural extracts: Inula, Propolis, St. John's Worts and Turmeric. In the first screening of the effects of these compounds, I found extensive microbial growth in diluted versions of these extracts by themselves as well as in mixes containing both *T. rubrum* and the extracts. Thus, in subsequent experiments, I pasteurized all the natural extracts solutions before their applications to avoid the microbial contamination. After *in vitro* MICs and MFCs experiments, the results indicated that Turmeric didn't have clear fungicidal effects. Among the rest three natural extracts, I conducted another *in vitro* infected agar experiment, submerging the infected agar cubes into pasteurized 1% natural extract solutions. The results of this experiment revealed that Inula was the most promising natural extract candidate, with 1% Inula treated agar cubes inhibiting *T. rubrum* growth for the longest time. The *in vitro* MIC and MFC of Inula against *T. rubrum* was 0.015625% and 0.0625% w/v, separately. In previously published studies by (Ali-Shtayeh and Abu Ghdeib 1999) and (Cafarchia et al. 2002) they found that the Inula MIC against *T. mentagrophytes* was lower than ours at 0.002%-0.01% against *T. mentagrophyte*, and 0.001% against *T. terrestre* with remarkably reduced diameter of microbial colonies. This research by (Cafarchia et al. 2002) also studied the differentiated fungicidal effect of Inula extracted from different parts of the plant. Among all parts of the plant examined, leaves showed best fungicidal effect and flowers showed the lowest fungicidal effect. Thus, in further study, the Inula extracted from different parts of the plant can also be conducted.

Different from previous studies, my study also evaluated the effects of carriers in *in vitro* fungicidal and inhibitory effect against *T. rubrum*. As a pivotal and novel element of the current research, carrier works to promote photosensitizer compounds to penetrate through nails to infected nail beds. This property to penetrate across the nails is key to eliminate the infecting fungus on the nail bed and reduce recurrence rate. From the results of *in vitro* experiment, certain combinations of the carrier worked effectively in inhibiting and killing *T. rubrum*. Meanwhile, for the carrier combinations with good fungicidal

effect, their surface tensions are also in an acceptable range. This could be a solid foundation for further clinic trials.

Ex vivo Nail Pieces MB, Inula and Carrier Treatments

The purpose for this part of my research was to gain feasible data based on previous *in vitro* experiments to select combinations for further nail well treatment, as experiments involving nail pieces were more efficient to proceed and consumed fewer nails. The amplified images in (Figure 5) showed the successful infection of *T. rubrum* on nail pieces. The results of MB *ex vivo* experiment showed that the *in vitro* MFC of MB at 0.125% concentration could inhibit visible fungal growth until about 10 days after but treatment at this concentration did not completely kill the fungus even after several repeated treatments. However, the fungicidal effects were achieved at higher concentration of 0.25% and 0.5% MB in carrier treatments with light. This slight difference in MB concentration was understandable. Our stepwise approach was highly beneficial and believe that it could also serve as a model for the similar experiments which converting *in vitro* results to *ex vivo* and *in vivo* experiments.

Similar differences between the *in vitro* and *ex vivo* experimental results were also observed for the four natural extracts. Specifically, the *in vitro* MFCs of three effective extracts were 0.25%, 0.0625% and 0.3% (w/v), separately for St. John's Worts, Inula and Propolis. Whereas even in the *in vitro* infected cubes experiments, SJW and Propolis could only delay *T. rubrum* growth for about 3 days. In the *ex vivo* Inula + Carrier nail pieces experiments, 1% Inula treatment with 10min submerging time delayed the growth of *T. rubrum* in the surrounding area of nail pieces for 4 days. As for the carrier, the *in vitro* fungicidal concentration could only delay the fungal growth for 2 days in the *ex vivo* nail piece treatment.

Ex vivo Nail Wells Treatment

Compared with *ex vivo* nail pieces treatment, the nail wells treatment is a more similar to clinical – simulated experiment as the nail well apparatus structure could test the penetration effect of formulations directly, from the dorsal side to the ventral side. Although the experiments were conducted twice to test for reliability, within each of these two experiments, most combinations which worked well in the *ex vivo* nail pieces did not show similar fungicidal effect. Two factors likely contributed to the differences. The first is the differences in the thickness of the nails, the nail pieces included many fingernails while all the nail wells were made of toenails. The second reason might be because the fungal mycelia was attached to the bottom of the tubing surrounding the nails. Those mycelia would not be exposed to the formulations and won't be killed. Indeed, when the tubing was cut off after exposure to the formulations, all the MB, Inula and MB + Inula combination treatments, even the carrier by itself, resulted in killing of all the fungi. These results indicated that the efficacy of the same treatments on *ex vivo* nail wells was better than the *ex vivo* nail pieces. This phenomenon may because the differences in preparing for the infected nail pieces and infected nail wells. During preparation of infecting the nail pieces, the nail pieces were completely submerged in *T. rubrum* agar, whereas for nail wells, only the ventral side had direct contact with the mycelia. Regardless, these results indicated the Photosensitizer-Carrier treatment system is feasible to eradicate *T. rubrum* infection by penetrating through nails. The next phase of the work would be clinical trials.

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