Comparative sensitivity of *Trichophyton* and *Aspergillus* conidia to inactivation by violet-blue light exposure

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Abstract

Objectives: To investigate the use of 405 nm light for inhibiting the growth of selected species of dermatophytic and saprophytic fungi. Background data: The increasing incidence and resilience of dermatophytic fungal infections is a major issue, and alternative treatment methods are being sought. Methods: The sensitivity of the dermatophytic fungi *Trichophyton rubrum* and *Trichophyton mentagrophytes* to 405 nm violet-blue light exposure was investigated, and the results compared with those obtained with the saprophytic fungus *Aspergillus niger*. Microconidia of *T. rubrum* and *T. mentagrophytes* and conidia of *A. niger* were seeded onto Sabauroud dextrose agar plates and irradiated with 405 nm light from an indium-gallium-nitride 99-DIE light-emitting diode (LED) array and the extent of inhibition was measured. Results: Germination of the microconidia of the *Trichophyton* species was completely inhibited using an irradiance of 35 mW/cm^2 for 4 h (dose of 504 J/cm^2). Results: *A. niger* conidia showed greater resistance, and colonial growth developed after light exposure. In liquid suspension tests, 405 nm light dose levels of 360, 720, and 1440 J/cm^2 resulted in complete inactivation of *T. rubrum* microconidia, whereas *A. niger* showed greater resistance, and at the highest dose level applied (1440 J/cm^2) although *A niger* hyphae were completely inactivated, only a 3-log$_{10}$ reduction of a 5-log$_{10}$ conidial suspension was achieved. Conclusions: The study results demonstrate the relatively high sensitivity of *Trichophyton* microconidia to 405 nm violet-blue light, and this is may be of potential interest regarding the control and treatment of dermatophyte infections.

1. Introduction

Dermatophytic fungi are the causative organisms of a variety of skin, hair and nail infections due to their ability to colonise the surface tissues of humans and animals, using keratin as nutrient source. The incidence of infections caused by dermatophytic fungi has greatly increased over the past 20 years with dermatophytes now being the most common cause of fungal infections[1]. *Trichophyton rubrum* and *Trichophyton mentagrophytes* are the most commonly isolated causative agents of dermatophytic infections[2]. *Trichophyton* fungi can produce several types of conidia including single-celled microconidia, multicellular macroconidia as well as arthroconidia, and it is the latter that are generally associated with the transmission of *Trichophyton* infections between humans[3]. Although arthrospores are regarded as the main transmissible agent, microconidia are the fungal structure preferably used in antifungal susceptibility testing for dermatophytes[3,4] as they can be conveniently produced and prepared as a single-celled and uniform suspension.

Although there are a number of antifungal agents available for topical and systemic treatment of dermatophyte infections, nail infections are particularly difficult to treat with recurrence reported in up to 25 to 40% of cases[5]. It is currently unknown if the fungal recurrence is due to inefficient clearance of the infection or re-emergence of disease; at present terbinafine is considered the most powerful treatment[6].

An alternative treatment strategy for dermatophyte infections is the use of photodynamic antimicrobial chemotherapy (PACT) which involves the use of photosensitiser chemicals and irradiation with specific wavelengths of light. Smij and Schuitmaker[7] demonstrated the inactivation of *T. rubrum* using the photosensitisers 5,10,15-tris(4-methylpyridinium)-20-phenyl-[21H,23H]-porphine trichloride (Sylsens B) and deuteroporphyrin monomethylester (DP mme) in conjunction with broadband white light irradiation. More recently Rodrigues *et al.*[4] demonstrated successful PACT inactivation of both *T. mentagrophytes* and *T. rubrum* microconidia using novel phenothiazinium photosensitizers and red light.

Whilst the PACT approach requires the use of both photosensitive chemicals and light, it has also been found possible to photo-inactivate a wide range of microorganisms using violet-blue light from the visible-spectrum without the use of exogenous photosensitisers, with comparative doses being safe for mammalian cell exposure[8-13]. Microbial inactivation by violet-blue light is accredited to the photoexcitation of intracellular porphyrin molecules within microorganisms, which have an absorption maxima in the region of 400 nm[14], which causes the production of reactive oxygen species (ROS)[15,16]. Cell death has been attributed to oxidative damage to cell components including DNA and membranes[9,12]. It has previously been established that 405 nm light has antifungal effects as Murdoch et al.[17] demonstrated the inactivation of the fungal species *Saccharomyces cerevisiae, Candida albicans* and dormant and germinating spores of *Aspergillus niger.*

The current report highlights the fungicidal activity of 405 nm violet-blue light against the dermatophytes *T. rubrum and T. mentagrophytes,* and the high sensitivity of these fungi to 405 nm light was compared against the saprophyte *Aspergillus niger*, with results opening up the possibility of the development of 405 nm light treatments against dermatophytic infections.

2. Materials and Methods

2.1. Fungal strains and conidia preparation

The dermatophytic fungi used in this study were *Trichophyton rubrum* MUCL 11954 and *Trichophyton mentagrophytes* MUCL 9823, obtained from the Mycotheque de l'Universite catholique de Louvain Culture Collection in Belgium. The saprophytic mould fungus *Aspergillus niger* MUCL 38993 was also used in comparative light sensitivity studies with the two dermatophytic species.

T. rubrum and *T. mentagrophytes* spores were obtained by fungal cultivation on sabauroud dextrose agar (SDA) plates (Oxoid, UK) at 28°C for 14 days. Following incubation, 9 ml phosphate buffered saline (PBS; Oxoid Ltd, UK) containing 0.01% tween-80 was added to the dish, and an L-shaped spreader used to agitate and release the microconidia. Agitation was carried out for 2 minutes. The resulting suspension was stored at 4°C.

To obtain *A. niger* spores, *A. niger* was inoculated onto a SDA slope and incubated at 26°C for a minimum of 7 days, after which a conidial suspension was obtained by agitation in an aqueous 0.01% tween-80 PBS solution. Agitation was carried out for 5 minutes. The population density of the spore suspensions was enumerated using an Improved Neubauer haemocytometer (Weber Scientific International, UK), and suspensions diluted as required prior to light exposure.

2.2. Light transmission through conidial extracts

For light transmission tests on conidial extracts, the centrifuged pellets of conidia of *A. niger* and *T. rubrum* were extracted with 100% ethanol and the light transmission spectrum of the ethanol extracts was determined using a Biomate 5 UV-Visible Spectrophotometer (Thermo Spectronic).

2.3. Light source and irradiance measurements

An indium-gallium-nitride 99-DIE light emitting diode (LED) array (OptoDiode Corp, CA, USA) was used to generate high-intensity 405-nm light with a bandwidth of 14 nm. The LED array was powered by a DC power supply, and a cooling fan and heat sink were attached to the array, allowing heat to dissipate from the source thereby minimizing heat transfer to the fungal samples. Irradiance was measured using a radiant power meter and detector (L.O.T.-Oriel ltd, UK). The dose of light exposure (Jcm^{-2}) was calculated as the product of the irradiance ($mWcm^{-2}$) multiplied by the exposure time (seconds). Doses selected for use in this study were between 500 and 1,500 Jcm^{-2} as these were within the region of those used in previous fungal inactivation studies[16].

2.4. Light exposure of Trichophyton and Aspergillus conidia

The inhibitory effects of 405 nm light on conidia were assessed using surface irradiated and liquid irradiated exposure conditions. For surface irradiation tests, 10 µl conidial suspension of the test fungus was spot inoculated onto the centre of a SDA plate. The test plate was exposed to 405 nm light, at an irradiance of 35 $mWcm^{-2}$ for 1 and 4 hr, giving doses of 126 and 504 Jcm^{-2}. Identical control samples were prepared and left exposed to normal laboratory lighting. Plates were incubated for 3 or 10 days for *Aspergillus* and *Trichophyton*, respectively, before being analysed for characteristic differences between the test and control. Colony diameters were measured across the broadest section of the colony on the SDA plate using a ruler. The results were also recorded photographically for illustrative purposes using a Sony Cybershot DSC-T2 digital camera (Sony, Japan).

For liquid irradiation comparisons, a 3 ml volume of spore suspension of test fungi was transferred into the well of a 12-well multidish with the LED housing array then placed approximately 3 cm above. The suspension was exposed to 50 $mWcm^{-2}$ 405 nm light, for 2, 4

and 8 hr, giving doses of 360 Jcm^{-2}, 720 Jcm^{-2} and 1.44 $kJcm^{-2}$. Control samples were held under the same conditions but exposed to normal laboratory lighting.

2.5. Light exposure of Aspergillus niger hyphal suspension

Following 24 hr incubation of *A. niger* on an SDA slope, the top layer of fungal growth was removed and then fragmented in 50 ml PBS for 5 minutes using a stomacher (Colworth, UK). 3 ml of the hyphal suspension was pipetted into one well of a 12-well multidish and exposed to 405 nm light as described above for conidia suspension tests.

2.6. Plating and Enumeration

For suspension experiments, post-exposure, samples (50µl, 100µl or 500µl) were inoculated onto SDA and spread using an L-shaped spreader. Plates were then incubated for 1 or 5 days (*A. niger* and *T. rubrum*, respectively), with each sample being plated at least in triplicate. Following incubation the plates were enumerated and recorded as colony forming units per millilitre ($CFUml^{-1}$). Data presented in this paper represent the mean results of two or more independent experiments. Significant differences in fungal population were calculated at the 95% confidence interval ($P<0.05$) using one-way analysis of variance (ANOVA), with Minitab statistical software package version 16 (Minitab Inc., Pennsylvania).

3. Results

The inhibitory effects of 405 nm light on the growth of surface irradiated *T. rubrum* and *T. mentagrophytes* spores are shown in Fig 1. The results demonstrate that after seeding the conidia onto SDA plates and exposure to 126 Jcm^{-2}, followed by incubation for 10 days, a substantial reduction in growth was observed with *T. rubrum*, with the diameters of the non-light exposed colonies and light-exposed colonies measuring 43±1 mm and 12±1 mm in diameter, respectively (Fig 1 A,B). Following exposure to a dose of 504 Jcm^{-2}, both *T. rubrum*

and *T. mentagrophytes* were completely inactivated and failed to develop colonies, with the non-exposed controls developing colonies of 21-22 mm diameter (Fig 1 C,D,E,F). Exposure of surface deposited conidia of *Aspergillus niger* to the same dose of 504 Jcm^{-2}, followed by incubation for 3 days demonstrated that complete inactivation of the spores was not achieved, with substantial conidial growth observed following incubation: light-exposed colonies grew to 38±1 mm diameter, compared to 33±1 mm for unexposed colonies (~13% reduction in size; P=0.049).

Suspensions of *T. rubrum* conidia, *A. niger* hyphal fragments and *A. niger* conidia were exposed to 405 nm light at an irradiance of 50 $mWcm^{-2}$ over time periods that delivered a dose of 360, 720 Jcm^{-2} and 1.44 $kJcm^{-2}$. Following exposure to a dose of 360 Jcm^{-2}, complete inactivation of *T. rubrum* conidia was achieved (~2.3-$\log_{10}$ $CFUml^{-1}$). The results in Figure 2 demonstrate that *A. niger* hyphae are more sensitive to 405 nm light than their corresponding conidia, with complete inactivation of a 10^3 $CFUml^{-1}$ hyphal suspension found after exposure to a dose of 1.44 $kJcm^{-2}$ while *A. niger* conidia demonstrated approximately a 50% reduction following exposure to the same dose of 1.44 $kJcm^{-2}$ (Fig 2.). Use of an increased irradiance or longer exposure time (meaning an increased applied dose) would lead to further decreases in the *A. niger* population, as previously reported[16].

The light transmission through ethanol extracts of *A. niger* and *T. rubrum* spores was measured to determine the effect of spore pigments on the transmission of 405 nm light through suspensions of both *A. niger* and *T. rubrum*; in Figure 3 the transmission spectra are shown alongside the emission spectrum of the 405 nm LED.

4. Discussion

The results shown in Figure 1 demonstrate a substantial reduction in microconidial growth of *T. rubrum* is achieved following exposure to 405 nm light at a dose of 126 Jcm^{-2}. Furthermore, the

results demonstrate that 405 nm light, at a dose of 504 Jcm^{-2}, can completely inactivate the microconidia of *T. rubrum* and *T. mentagrophytes* such that hyphal and colony growth do not occur. By contrast, exposure of surface deposited conidia of *A. niger* to a similar dose of 504 Jcm^{-2} did not result in a substantial reduction in conidial growth so that on subsequent incubation of the SDA plates colony growth occurred and the colony diameter achieved after 3 days of incubation was only marginally less than observed with the control non-irradiated plates (Fig.1). Even after an increased light dose of 1.008 $kJcm^{-2}$, *A. niger* conidia were not completely inactivated, and colony growth occurred although the extent of growth was considerably less than the non-exposed control: colony diameter of 22 mm for light exposed and 39 mm for control (photograph not shown). These results demonstrate the higher susceptibility of the dermatophytic conidia of both *T. rubrum* and *T. mentagrophytes* to inactivation using 405 nm light compared to *A. niger* conidia.

It is known that exposure of microbiological culture media to light can result in the formation of toxic compounds[18]. To ensure that the results obtained were due to direct light induced inactivation of the fungal conidia as opposed to an indirect media-induced toxic effect, experiments were also conducted using liquid suspensions of the fungal conidia and hyphae. Comparison of the susceptibility of *T. rubrum* conidia, the conidia and hyphae of *A. niger* to 405 nm light, at doses of 360, 720 Jcm^{-2} and 1.44 $kJcm^{-2}$, demonstrated the much higher susceptibility of *T. rubrum* to inactivation using 405nm light than that of *A. niger* with complete inactivation achieved at 360 Jcm^{-2} (Fig 2). The conidia of *A. niger* were much more resistant to 405 nm light and although the CFU count decreased with increasing dose, complete inactivation was not achieved with the doses used in the present study. As we reported in a previous study[11], complete inactivation of *A. niger* conidia, with higher populations of 10^5 $CFUml^{-1}$, required a dose of 2.3 $kJcm^{-2}$. Whilst conidia of *A. niger* are highly resistant to 405 nm light it was of interest to compare the sensitivity of *A. niger* hyphae to that of the conidia. The results, shown in Figure 2, demonstrate that *A. niger* hyphae are more sensitive to 405 nm light than their corresponding conidia, with complete inactivation of a 10^3 $CFUml^{-1}$ hyphal suspension

found after exposure to a dose of 1.44 kJcm^{-2}. It was interesting to note however that the *A. niger* hyphae demonstrated more resistance to the 405 nm light than the *T. rubrum* conidia.

The mechanism of the antifungal effect mediated by violet-blue light occurs following exposure of the organism to light photons in the region of 405 nm. Endogenous porphyrins within the cells absorb these photons, resulting in their photoexcitation, and electron transfer via the type I or type II pathway resulting in the production of reactive oxygen species (ROS), most notably singlet oxygen ($^1O_2$)[15,19] . The ROS produced then react with various cellular components causing an imbalance in cellular homeostasis resulting in damage to cytoplasmic organelles and nucleic acids, and consequently cell death by apoptosis, necrosis, or autophagy[20]. This hypothesis is supported by a study by Baltazar et al which demonstrated the photodynamic inactivation of *T. rubrum*, via increased levels of NO., ROS and ONOO., using 630 nm light and the exogenous photosensitiser toluidine blue[19].

Fungi possess mitochondria and, although there are some enzyme differences when compared with mitochondria of mammalian cells, the production of the endogenous photosensitive protoporphyrin IX molecule has been demonstrated[21,22,23]. Protoporphyrin IX may be activated by wavelengths ranging from UVA to the visible wavebands with a maximum peak in the Soret band at 375–405 nm and a lower peak at 630– 633 nm[21,22,23]. The presence of porphyrins in fungi indicates that both bacteria and fungi may be affected by a similar porphyrin photoexcitation and ROS induced inactivation mechanism following exposure to visible light[17,13]. Further evidence that a similar underlying inactivation mechanism is involved is the finding[17] that light exposure under aerobic and anaerobic conditions, together with results obtained using oxygen scavengers, has revealed that 405 nm light inactivation in fungi involves an oxygen dependent mechanism, which is also the case with bacteria. Whilst the inactivation mechanism may be similar, the physiological status of the organism is an important factor influencing the degree of susceptibility of the light exposed cells, with bacterial and fungal spores being

understandably more resistant than their vegetative counterparts, an innate resistance that rapidly disappears during spore germination[17].

Although most previous research on the use of light to inactivate fungi has involved the use of added photosensitiser chemicals, a previous study by Smijs et al[22] demonstrated the ability of UVA-light alone, at a dose of 40 Jcm^{-2}, to kill *T. rubrum* without the use of exogenous photosensitisers. In addition to this, irradiation with broadband visible light at a dose of 20–50 Jcm^{-2} in the absence of exogenous sensitizers was found to produce oxygen dependent lethal effects on the plasma membranes and mitochondria of *Candida guilliermondii*[24]. Within bacterial cells, porphyrin-mediated violet-blue light inactivation has been associated with severe cell wall damage and leakage of intracellular substances, presence of cytoplasmic vacuoles, and disruption of intracellular structures[13,12].

It is believed that pigments such as melanin, which are black or dark brown pigments, commonly occurring as wall components in fungal spores, have a protective role against photochemical damage[25]. To investigate the effects of spore pigment on 405 nm light transmission, ethanol extracts of *A. niger* and *T. rubrum* spore suspensions were prepared and the wavelength transmission spectra were compared (Fig. 3). Results demonstrate that the transmission of light across the measured spectrum (300-800 nm) is much lower for the *A. niger* than the *T. rubrum* spore extract, with 18.4% and 41.3% transmission at 405 nm, respectively. The high resistance of *A. niger* spores to 405 nm light is most likely due to possession of a multi-layered pigmented spore coat containing aspergillin, a black coloured melanin-like compound making the spores particularly difficult to inactivate when exposed to visible light[26] and pulsed UV-light[27,28]. The presence of the aspergillin pigment explains why *A. niger* conidia are more resistant to 405 nm radiation than the conidia of *T. rubrum*, and indeed to the *A. niger* hyphae which is non-pigmented. Although *T. rubrum* also produces several melanin-type pigments[29] these either do not occur in the conidia, or at least not at sufficient levels to provide protection against 405 nm light irradiation.

The results of this study demonstrate that the microconidia of the *Trichophyton* spp tested are much more sensitive to inactivation by 405 nm light than the conidia of the saprophytic fungus *Aspergillus niger*. Whilst the resistance of *A. niger* conidia to light inactivation is not surprising due to the dark pigment present, it is of interest that the *Trichophyton* microconidia were more sensitive to 405 nm light than the non-pigmented hyphae of *A. niger*. Although *Trichophyton* microconidia are not the main transmissible agents of these dermatophytic fungi they are regarded as the preferred fungal structure for dermatophytic antifungal susceptibility testing[3,4]. The findings of this study, demonstrating the relatively high sensitivity of *Trichophyton* microconidia to 405 nm light is therefore of potential interest regarding the control and treatment of dermatophyte infections.

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Disclosure Statement

No competing financial interests exist for any of the authors of this manuscript.

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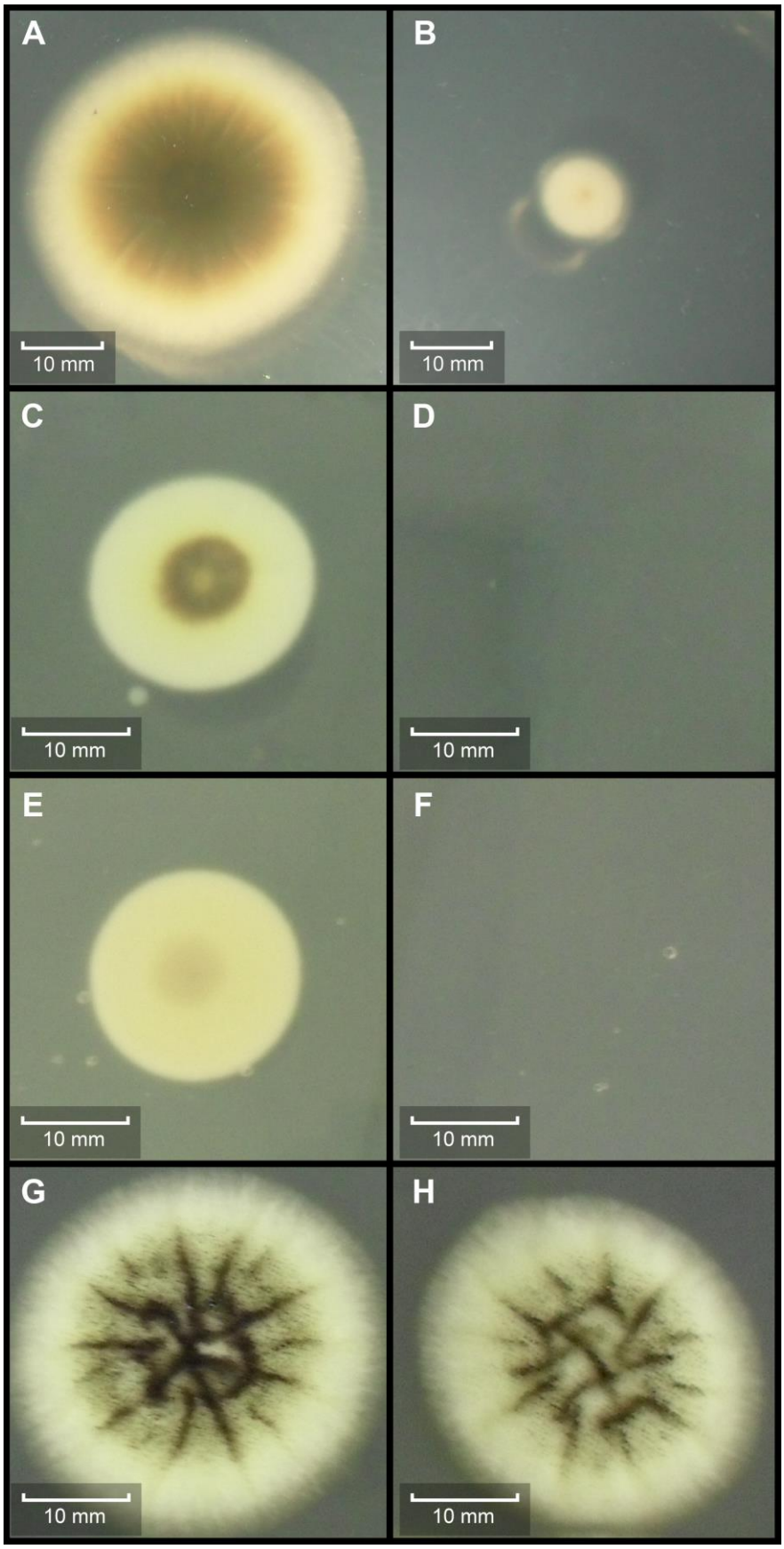
Figure 1. Inhibitory effects of 405 nm light on the growth of *Trichophyton rubrum* (A,B,C,D), *Trichophyton mentagrophytes* (E,F) and *Aspergillus niger* (G,H) conidia spot-inoculated on SDA plates. Samples were exposed to doses of 126 J cm^{-2} (A) and 504 Jcm^{-2} (C,E,G), followed by a period of incubation (3 days for *Aspergillus* and 10 days for *Trichophyton* spp.) and colony diameters assessed. Photographs in the right-hand column (B,D,F,H) represent light-exposed samples; Photographs in the left-hand column (A,C,E,G) were non-exposed control samples.

Figure 2. Exposure of *Trichophyton rubrum* and *Aspergillus niger* conidial suspensions to 405 nm light using an irradiance of 50 $mWcm^{-2}$ to deliver dose levels of 360 Jcm^{-2}, 720 Jcm^{-2} and 1.44 $kJcm^{-2}$. Inactivation of *A. niger* hyphal fragments was included as a comparison. Surviving fungi were enumerated by mean $CFUml^{-1}$ counts (± SD) and results reported as the % $\log_{10}$ reduction compared to non-exposed control samples. Asterisks (*) represent where a significant difference was detected between the exposed and non-exposed samples, at 95% confidence level ($P \leq 0.05$).

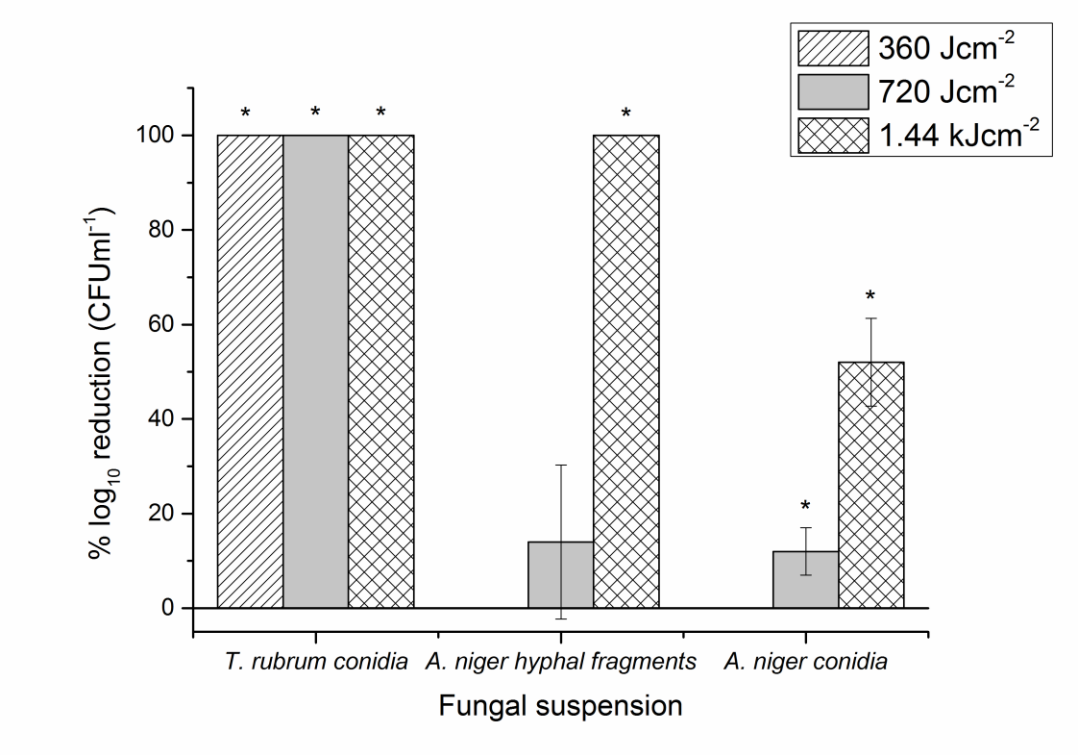
Figure 3. The transmission of light, over the wavelength range 300–800 nm, through ethanol extracts of the conidia of *Trichophyton rubrum* and *Aspergillus niger*. The emission spectra of the 405 nm LED array, measured using a high resolution spectrometer (Ocean Optics Inc, USA), is included for reference.

401 Fig 1:



402

403 Fig 2:



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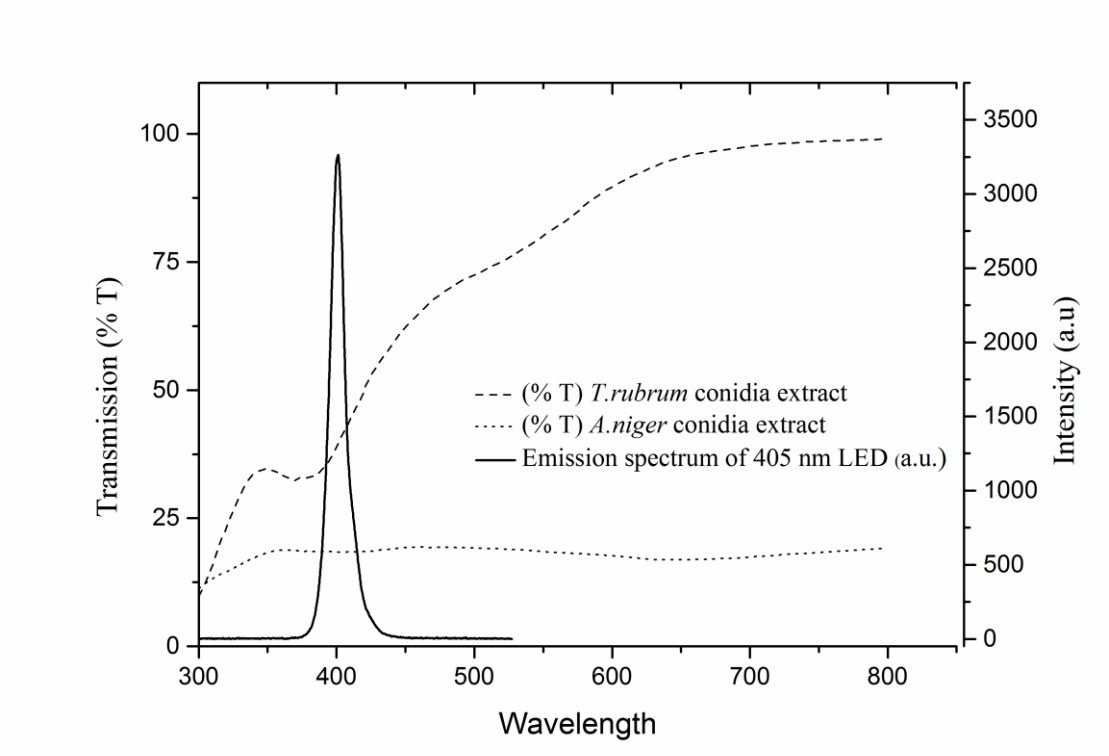
405 Fig 3:



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