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24 **Abstract**

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

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## 49 **1. Introduction**

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

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

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

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

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

90

## 91 2. Materials and Methods

### 92 2.1. Fungal strains and conidia preparation

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

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

103 To obtain *A. niger* spores, *A. niger* was inoculated onto a SDA slope and incubated at 26°C for a  
104 minimum of 7 days, after which a conidial suspension was obtained by agitation in an aqueous  
105 0.01% tween-80 PBS solution. Agitation was carried out for 5 minutes. The population density  
106 of the spore suspensions was enumerated using an Improved Neubauer haemocytometer  
107 (Weber Scientific International, UK), and suspensions diluted as required prior to light  
108 exposure.

### 109 2.2. Light transmission through conidial extracts

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

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115                   2.3. *Light source and irradiance measurements*

116    An indium-gallium-nitride 99-DIE light emitting diode (LED) array (OptoDiode Corp, CA, USA)  
117    was used to generate high-intensity 405-nm light with a bandwidth of 14 nm. The LED array  
118    was powered by a DC power supply, and a cooling fan and heat sink were attached to the array,  
119    allowing heat to dissipate from the source thereby minimizing heat transfer to the fungal  
120    samples. Irradiance was measured using a radiant power meter and detector (L.O.T.-Oriol ltd,  
121    UK). The dose of light exposure ( $\text{Jcm}^{-2}$ ) was calculated as the product of the irradiance ( $\text{mWcm}^{-2}$ )  
122    multiplied by the exposure time (seconds). Doses selected for use in this study were between  
123    500 and 1,500  $\text{Jcm}^{-2}$  as these were within the region of those used in previous fungal  
124    inactivation studies<sup>16</sup>.

125                   2.4. *Light exposure of Trichophyton and Aspergillus conidia*

126    The inhibitory effects of 405 nm light on conidia were assessed using surface irradiated and  
127    liquid irradiated exposure conditions. For surface irradiation tests, 10  $\mu\text{l}$  conidial suspension of  
128    the test fungus was spot inoculated onto the centre of a SDA plate. The test plate was exposed to  
129    405 nm light, at an irradiance of 35  $\text{mWcm}^{-2}$  for 1 and 4 hr, giving doses of 126 and 504  $\text{Jcm}^{-2}$ .  
130    Identical control samples were prepared and left exposed to normal laboratory lighting. Plates  
131    were incubated for 3 or 10 days for *Aspergillus* and *Trichophyton*, respectively, before being  
132    analysed for characteristic differences between the test and control. Colony diameters were  
133    measured across the broadest section of the colony on the SDA plate using a ruler. The results  
134    were also recorded photographically for illustrative purposes using a Sony Cybershot DSC-T2  
135    digital camera (Sony, Japan).

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

139 and 8 hr, giving doses of 360 Jcm<sup>-2</sup>, 720 Jcm<sup>-2</sup> and 1.44 kJcm<sup>-2</sup>. Control samples were held under  
140 the same conditions but exposed to normal laboratory lighting.

#### 141 2.5. Light exposure of *Aspergillus niger* hyphal suspension

142 Following 24 hr incubation of *A. niger* on an SDA slope, the top layer of fungal growth was  
143 removed and then fragmented in 50 ml PBS for 5 minutes using a stomacher (Colworth, UK). 3  
144 ml of the hyphal suspension was pipetted into one well of a 12-well multidish and exposed to  
145 405 nm light as described above for conidia suspension tests.

#### 146 2.6. Plating and Enumeration

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

155

### 156 3. Results

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



163 and *T. mentagrophytes* were completely inactivated and failed to develop colonies, with the non-  
164 exposed controls developing colonies of 21-22 mm diameter (Fig 1 C,D,E,F). Exposure of  
165 surface deposited conidia of *Aspergillus niger* to the same dose of 504 Jcm<sup>-2</sup>, followed by  
166 incubation for 3 days demonstrated that complete inactivation of the spores was not achieved,  
167 with substantial conidial growth observed following incubation: light-exposed colonies grew to  
168 38±1 mm diameter, compared to 33±1 mm for unexposed colonies (~13% reduction in size;  
169 P=0.049).

170 Suspensions of *T. rubrum* conidia, *A. niger* hyphal fragments and *A. niger* conidia were exposed  
171 to 405 nm light at an irradiance of 50 mWcm<sup>-2</sup> over time periods that delivered a dose of 360,  
172 720 Jcm<sup>-2</sup> and 1.44 kJcm<sup>-2</sup>. Following exposure to a dose of 360 Jcm<sup>-2</sup>, complete inactivation of *T.*  
173 *rubrum* conidia was achieved (~2.3-log<sub>10</sub> CFUml<sup>-1</sup>). The results in Figure 2 demonstrate that *A.*  
174 *niger* hyphae are more sensitive to 405 nm light than their corresponding conidia, with  
175 complete inactivation of a 10<sup>3</sup> CFUml<sup>-1</sup> hyphal suspension found after exposure to a dose of 1.44  
176 kJcm<sup>-2</sup> while *A. niger* conidia demonstrated approximately a 50% reduction following exposure  
177 to the same dose of 1.44 kJcm<sup>-2</sup> (Fig 2.). Use of an increased irradiance or longer exposure time  
178 (meaning an increased applied dose) would lead to further decreases in the *A. niger* population,  
179 as previously reported<sup>16</sup>.

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

184

#### 185 **4. Discussion**

186 The results shown in Figure 1 demonstrate a substantial reduction in microconidial growth of *T.*  
187 *rubrum* is achieved following exposure to 405 nm light at a dose of 126 Jcm<sup>-2</sup>. Furthermore, the

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

200 It is known that exposure of microbiological culture media to light can result in the formation of  
201 toxic compounds<sup>18</sup>. To ensure that the results obtained were due to direct light induced  
202 inactivation of the fungal conidia as opposed to an indirect media-induced toxic effect,  
203 experiments were also conducted using liquid suspensions of the fungal conidia and hyphae.  
204 Comparison of the susceptibility of *T. rubrum* conidia, the conidia and hyphae of *A. niger* to 405  
205 nm light, at doses of 360, 720 Jcm<sup>-2</sup> and 1.44 kJcm<sup>-2</sup>, demonstrated the much higher  
206 susceptibility of *T. rubrum* to inactivation using 405nm light than that of *A. niger* with complete  
207 inactivation achieved at 360 Jcm<sup>-2</sup> (Fig 2). The conidia of *A. niger* were much more resistant to  
208 405 nm light and although the CFU count decreased with increasing dose, complete inactivation  
209 was not achieved with the doses used in the present study. As we reported in a previous  
210 study<sup>11</sup>, complete inactivation of *A. niger* conidia, with higher populations of 10<sup>5</sup> CFUml<sup>-1</sup>,  
211 required a dose of 2.3 kJcm<sup>-2</sup>. Whilst conidia of *A. niger* are highly resistant to 405 nm light it  
212 was of interest to compare the sensitivity of *A. niger* hyphae to that of the conidia. The results,  
213 shown in Figure 2, demonstrate that *A. niger* hyphae are more sensitive to 405 nm light than  
214 their corresponding conidia, with complete inactivation of a 10<sup>3</sup> CFUml<sup>-1</sup> hyphal suspension

215 found after exposure to a dose of 1.44 kJcm<sup>-2</sup>. It was interesting to note however that the *A. niger*  
216 hyphae demonstrated more resistance to the 405 nm light than the *T. rubrum* conidia.

217 The mechanism of the antifungal effect mediated by violet-blue light occurs following exposure  
218 of the organism to light photons in the region of 405 nm. Endogenous porphyrins within the  
219 cells absorb these photons, resulting in their photoexcitation, and electron transfer via the type I  
220 or type II pathway resulting in the production of reactive oxygen species (ROS), most notably  
221 singlet oxygen (<sup>1</sup>O<sub>2</sub>)<sup>15,19</sup>. The ROS produced then react with various cellular components  
222 causing an imbalance in cellular homeostasis resulting in damage to cytoplasmic organelles and  
223 nucleic acids, and consequently cell death by apoptosis, necrosis, or autophagy<sup>20</sup>. This  
224 hypothesis is supported by a study by Baltazar et al which demonstrated the photodynamic  
225 inactivation of *T. rubrum*, via increased levels of NO., ROS and ONOO., using 630 nm light and the  
226 exogenous photosensitiser toluidine blue<sup>19</sup>.

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

240 understandably more resistant than their vegetative counterparts, an innate resistance that  
241 rapidly disappears during spore germination<sup>17</sup>.

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

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

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

276

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280

## 281 **Disclosure Statement**

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

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

385

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

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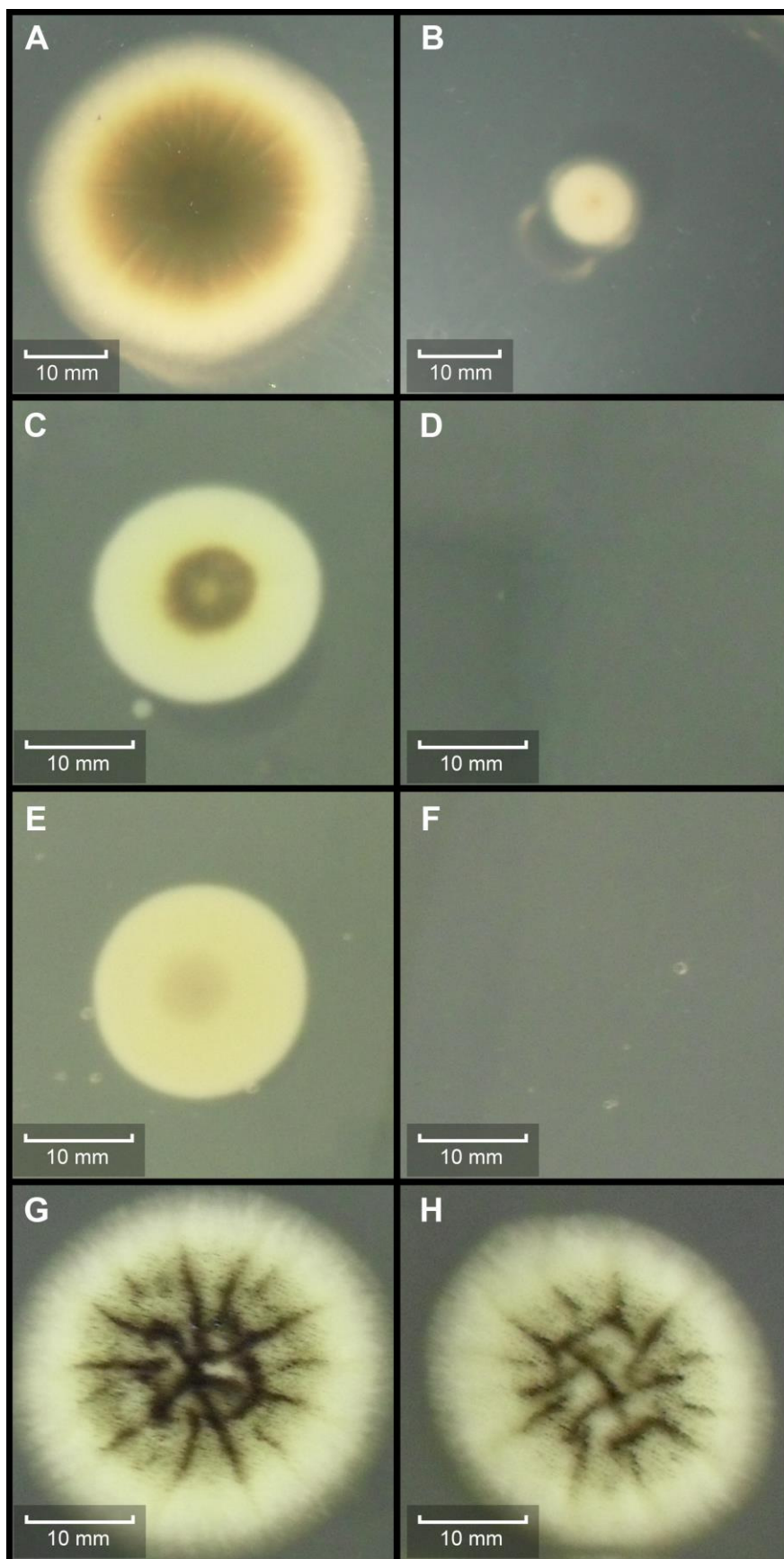
394 **Figure 3.** The transmission of light, over the wavelength range 300–800 nm, through ethanol  
395 extracts of the conidia of *Trichophyton rubrum* and *Aspergillus niger*. The emission spectra of the  
396 405 nm LED array, measured using a high resolution spectrometer (Ocean Optics Inc, USA), is  
397 included for reference.

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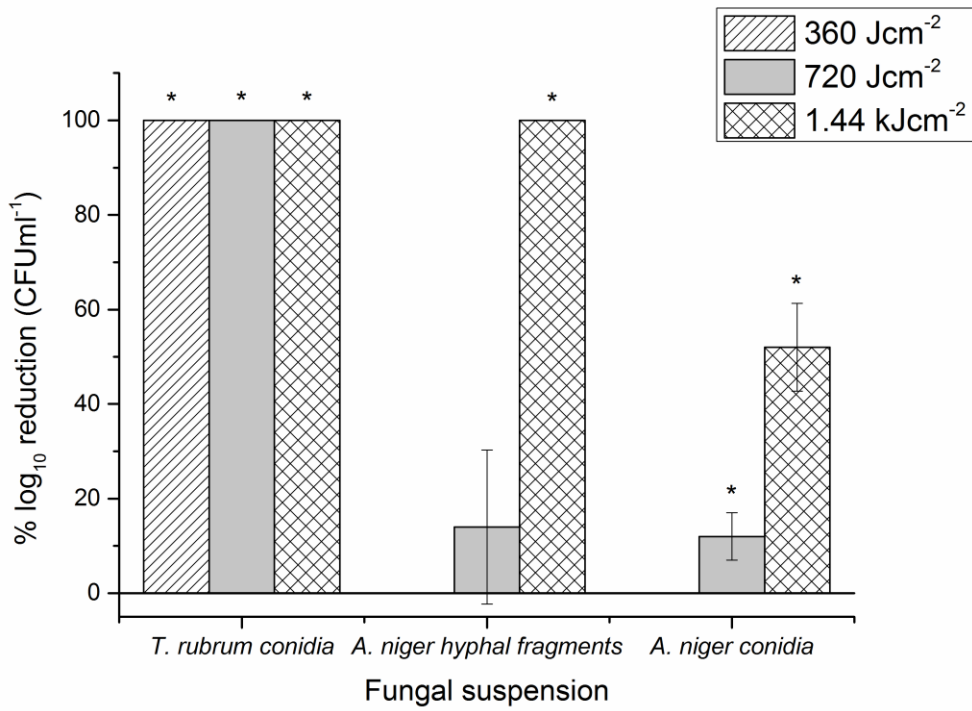
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401 Fig 1:



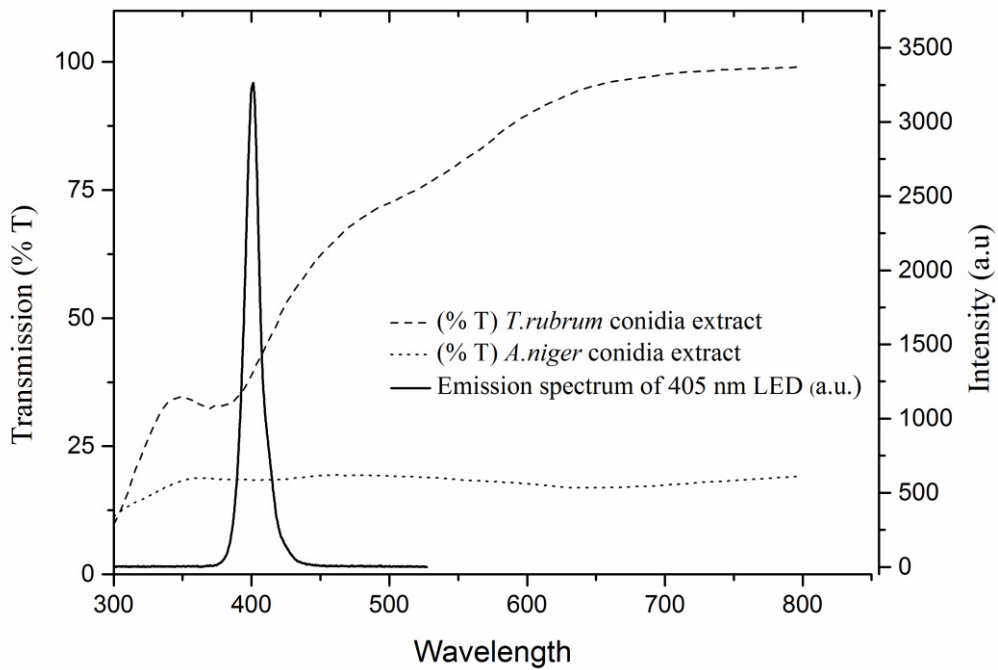
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403 Fig 2:



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405 Fig 3:



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