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Inactivation of *Aspergillus flavus* spores by curcumin-mediated photosensitization

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ABSTRACT

Minimizing fungal infection is essential to the control of mycotoxin contamination of foods and feeds but many potential control methods are not without their own safety concerns for the consumers. Photodynamic inactivation is a novel light-based approach which offers a promising alternative to conventional methods for the control of mycotoxigenic fungi. This study describes the use of curcumin to inactivate spores of *Aspergillus flavus*, one of the major aflatoxin producing fungi in foods and feeds. Curcumin is a natural polyphenolic compound from the spice turmeric (*Curcuma longa*). In this study the plant has shown to be an effective photosensitiser when combined with visible light (420 nm). The experiment was conducted in *in vitro* and *in vivo* where *A. flavus* spores were treated with different photosensitiser concentration and light dose both in buffer solution and on maize kernels. Comparison of fungal load from treated and untreated samples was determined, and reductions of fungal spore counts of up to 3 log CFU ml^{-1} in suspension and 2 log CFU g^{-1} in maize kernels were obtained using optimal dye concentrations and light dose combinations. The results in this study indicate that curcumin-mediated photosensitization is a potentially effective method to decontaminate *A. flavus* spores in foods and feeds.

1. Introduction

Some fungi that colonise agricultural commodities and foodstuffs may produce toxic secondary metabolites known as mycotoxins. Mycotoxin contamination in food and feed is reported to be a global problem (Rodrigues & Chin, 2012; Rodrigues, Handl, & Binder, 2011; Rodrigues & Naehrer, 2012a, 2012b) and occur in more than 25% of different key foods in Africa (Wagacha & Muthomi, 2008). These fungal toxins pose serious health problems to humans and animals including instant death in acute cases, and cancers; and studies also suggest that they cause immunosuppression, retard growth, and reproductive disorders with chronic exposures (Probst, Njapau, & Cotty, 2007; Varga, Frisvad, & Samson, 2009). In addition to health impacts, significant economic losses result from lowered animal production, decreased market values, regularity losses and secondary effects on agricultural production and agricultural communities (Wu, 2006, 2007). Due to these impacts, a worldwide concerted effort has been applied to control and regulate the occurrence of these toxins in food and animal feed.

Methods to control mycotoxin occurrence in food and animal feed focus on either preventing fungal colonisation and mycotoxin production, or where this fails, removal of the toxins in the food and feed by detoxification (Leslie & Logrieco, 2014). Fungal contamination of crops and their subsequent toxin production can occur in the field before harvest, or during post-harvest storage and processing. Control methods range from the application of fungicides and pesticides that kill the fungi directly or reduce contamination by insect vectors, to fungal inactivation by thermal, chemical or photo-irradiation procedures (Begum, Hocking, & Miskelly, 2009; Luksiene, Peciulyte, Jurkoniene, & Puras, 2005; Nemțanu, Braşoveanu, Karaca, & Erper, 2014). However, no single method has been successful in combating the mycotoxin problem entirely and it is advocated to deal with the problem by integrating various intervention measures depending on circumstances.

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Photosensitization is an evolving strategy employed in inactivating food microbes (Luksiene, 2005; Luksiene et al., 2005) and has the potential to play an important role in reducing mycotoxigenic fungal contamination in food and animal feed.

Photosensitization is a technique in which microbial cells are killed by cytotoxic reactions induced by energy derived from photosensitiser molecules that have been excited by light of specific wavelength. Examples of photosensitisers that have been reported to work on food and related products include hematoporphyrin dimethyl ether (Luksiene et al., 2005), polymer bound anthraquinone derivatives (Zerdin, Horsham, Durham, Wormell, & Scully, 2009) and sodium-chlorophyllin (Luksiene, Buchovec, & Paskeviciute, 2010; Luksiene & Paskeviciute, 2011a, 2011b). Most of these photosensitisers are synthetic or semi-synthetic dyes, and their use in foods would not meet consumer preferences for "chemical-free" foods (Yiridoe, Bonti-Ankomah, & Martin, 2005).

In using photosensitization as a treatment to control fungal contamination, ideally the photosensitiser (photo dye) should be safe for human consumption, cost effective and biochemically stable and easy to activate using light of appropriate wavelength (Ormond & Freeman, 2013). The findings that some natural products can be effectively used as photosensitisers (Wainwright, 2009, chap. 9) have focused increased attention on the applicability of photosensitization in microbial decontamination in food products. Among the reported studies is the potential of using turmeric extract (curcumin) for photosensitization in microbial decontamination (Dovigo et al., 2013; Hegge, Bruzell, Kristensen, & Tønnesen, 2012; Paschoal et al., 2013; Verwanger, Krammer, & Bernardinelli, 2011).

Curcumin is a polyphenol compound which is obtained as a yellow extract from the tubers of *Curcuma longa* (turmeric) plant. This plant is a native and traditional spice in India and is cultivated throughout the warmer parts of the world, and has been reported to have a range of medicinal properties (Aggarwal et al., 2007) Curcumin-mediated photosensitization has been studied and characterized in a number of microbes including yeasts and bacteria such as *Candida albicans* (Dovigo et al., 2011b), *Staphylococcus epidermidis* (Hegge et al., 2012), *Streptococcus mutans* (Paschoal et al., 2013), *Escherichia coli*, *Enterococcus faecalis*, and *Streptococcus intermedius* (Haukvik, Bruzell, Kristensen, & Tonnesen, 2009). The use of curcumin as a natural dye in photosensitization fits the "clean green technology concept" and is an attractive intervention method for the inactivation of mycotoxin producing fungi in food and feed. To the best of our knowledge, there are no reported studies in the literature on the suitability of using the turmeric extract (or indeed any plant extract) in photosensitization-mediated inactivation of mycotoxin producing fungi. Given that aflatoxin contamination affects the crops of many resource-poor farmers throughout the tropics and sub-tropics, use of extracts from a plant they could potentially grow and use on farm is particularly promising as a sustainable and adoptable intervention.

This study investigates curcumin-mediated photo-inactivation of *Aspergillus flavus* spores *in vitro* in aqueous suspension and *in vivo* when inoculated on maize kernels. The fungus is a common food contaminant producing a number of mycotoxins including aflatoxins, one of the important mycotoxin groups due to its detrimental effects on health and the economy (Varga et al., 2009).

2. Materials and methods

2.1. Study design

Two experiments were conducted: an *in vitro* solution study to test photodegradation of *A. flavus* spores in suspension and an *in vivo* kernel study to investigate photodegradation of spores inoculated on the surface of maize kernels both before and after milling. The study was conducted in a completely randomized block design.

2.2. Photosensitiser and light source

Stock solution (200 μM) of natural curcumin (Sigma Aldrich, (St. Louis, MO, USA) was prepared by first dissolving 5.3 mg curcumin in 6 ml absolute ethanol and then diluted with phosphate buffer saline solution (PBS; pH 7.4) to 50 ml and further diluted to different working concentrations with same saline. For the *in vitro* study, the dye concentrations used were 5 μM, 10 μM, 20 μM, 50 μM and 100 μM while the dye concentrations for the *in vivo* study were 25 μM and 45 μM. The two concentrations used for *in vivo* study were selected from the working range of concentrations as found in the *in vitro* experiment. An exon arc lamp machine (Polilight 500®, Rofin Australia Pty Ltd, Victoria, Australia) with adjustable wavelength selection was used as the source of light for photosensitization in a light impenetrable cabinet. The output light dose (J cm^{-2}) was dependent on illumination time and light power and as indicated on the device user manual it could be expressed as a multiple of light power (W) and illumination time (s) divided by area of illumination (cm^2). Light intensities used were 0, 12, 24, 60 and 84 J cm^{-2} for the *in vitro* and 60 J cm^{-2} for the *in vivo* study, selected based on results of preliminary studies (results not shown).

The absorption spectrum of curcumin dye/spore mixture was determined across the visible light wavelength range (400–700 nm), using a plate reader (Tecan® 2000 Tecan Australia Pty Ltd, Victoria, Australia) was used to measure the absorption spectrum at two concentrations of curcumin (5 μM and 10 μM) mixed with 10^3 CFU *A. flavus* spores in 8% ethanol in PBS.

2.3. Fungal spores and culture conditions

An aflatoxigenic reference strain of *A. flavus* (ATCC 28893) was used in the experiment. The spores were multiplied by growing on malt extract agar (MEA). Spore harvest was done after seven days of incubation at 26 °C by flooding the culture with 10 ml 0.1% Tween 80 solution and gently swirling to bring the spores into suspension. The resulting suspension was decanted then centrifuged at 9390 rcf for one minute, supernatant discarded and spore pellet resuspended in sterile phosphate buffer saline solution (pH 7.4) to a concentration of approximately 10^{13} CFU ml^{-1} determined by Colony Forming Units (CFU) count conducted using a Colony Counter (Stuart Scientific, United Kingdom). The spores were kept at 4 °C and used within four weeks.

2.4. In vitro study – experiment on spores in suspension

2.4.1. Experimental set-up

Two variables were tested in one set of *in vitro* experiments, photosensitiser (PS) concentration and light (L) dose. The experiment included: control (no light, no dye (L^-/PS^-), light treatment (L^+/PS^-), dye treatment (L^-/PS^+) and photosensitization treatment (L^+/PS^+). Six levels of photosensitiser concentration (5 μM, 10 μM, 15 μM, 25 μM, 50 μM and 100 μM were used in combination with four levels of light dose (12 J cm^{-2}, 24 J cm^{-2}, 60 J cm^{-2} and 84 J cm^{-2}). To test the repeatability, three sets of experiments were conducted.

2.4.2. Illumination

Two millilitres of spore suspension at initial concentration of ca. 8×10^4 CFU ml^{-1} were mixed with 2 ml photosensitiser solution of

appropriate concentration to make a total volume of 4 ml spore-dye suspension in a 35 mm × 10 mm petri dish. In the control (L^-/PS^-) and light alone treatment (L^+/PS^-), 2 ml of PBS was added instead of photosensitiser solution. Lidded petri dish with the spore/photosensitiser mixture was positioned 1 cm below the light beam and illuminated with light of 420 nm wavelength. Constant stirring of the solution was done using a magnetic stirrer during illumination. Each treatment was replicated four times. The suspension temperature was measured with a digital thermometer (Comark C$^{®}_{26}$, Comark USA) before and immediately after each light treatment.

2.4.3. Determination of spore concentration

Duplicate aliquots of 100 μL from the treatment petri dish were inoculated on MEA and incubated at 26 °C for 48 h. The control was diluted at a ratio of 1:100 before plating on the growth media to enable counting of the colonies in expectation of a high CFU concentration.

2.5. In vivo study – experiment on spores inoculated on maize kernels

2.5.1. Experiment design

The *in vivo* experiment was designed into a set of two blocks (whole kernel and milled kernel blocks) and each was divided into three units which were randomly allocated to different dye concentrations. Before allocating the kernel samples into respective treatment blocks, a negative control (before spore inoculation) and positive control (after spore inoculation) were drawn to test sterility of the kernels and viability of spores on kernel surfaces respectively. Each experimental set was repeated three times to assess repeatability.

2.5.2. Inoculation of maize kernels and controls

A white kernel variety of maize (Hybrid 33V62, Pioneer® Australia) was used for the experiment. Dry maize kernels (200 g, 7% moisture content) were sterilized by autoclaving (121 °C, 15 psi) for 15 min, and stored in a sealed container at room temperature. Four replicates of five kernels (about 1.5 g) were randomly picked from the sterilized maize kernels (negative control), submerged in 5 ml sterile PBS and vortexed at 1400 rpm for 30 s. From the resulting suspension a duplicate of 100 μL was pipetted and plated on MEA. The remaining maize kernels were inoculated with *A. flavus* spores by immersing in 400 ml spore suspension (10^6 spore ml^{-1}) for 30 s then the liquid was decanted and kernels dried in petri dishes at 30 °C overnight. Four replicates of five kernels each (approximately 1.5 g) were taken from the dried kernels (positive control), immersed in 5 ml sterile PBS, vortexed and plated on MEA. The remaining kernel sample (about 186 g) was divided into two equal sample blocks and allocated to whole kernel experiment (Block 1) and milled kernel experiment (Block 2).

2.5.3. Whole kernel treatment

Block 1 kernel sample was divided to three equal units and respectively immersed in 0 μM, 25 μM and 45 μM solutions of curcumin in 8% ethanol in PBS for five minutes. Four replicates of five kernels from each unit were separately illuminated (while submerged in the dye solution) with 60 J cm^{-2} using 420 nm wavelength light. The kernels were removed from the dye solution and dried in petri dishes at 30 °C overnight. The moisture content after immersing in dye was 11.1% and then dropped to 9.4% after the overnight drying. Dried kernels were immersed in sterile PBS, vortexed and plated on MEA to determine CFU count.

2.5.4. Milled kernel treatment

Block 2 kernel sample was divided to three equal units and respectively immersed in 0 μM, 25 μM and 45 μM solutions of curcumin in 8% ethanol in PBS for five minutes. The dye solution was decanted and the kernels dried at 30 °C overnight and milled using a Retsch ball mill (Mixer Mill MM 301, MEP Instruments, Australia). Moisture content was 11.2% and 8.7% before drying and after drying (and milling), respectively. Four replicates of 1.5 g of the resulting flour from each unit were separately illuminated with 60 J cm^{-2} using 420 nm wavelengths light. The illuminated flour was then suspended in 2 ml sterile PBS, vortexed at 1400 rpm for 30 s and duplicate 100 μL aliquots were pipetted and plated on MEA for CFU determination.

3. Results

The absorption spectrum of curcumin dye/spore mixture was determined across the visible light wavelength range (400–700 nm) with the maximum absorption between 418 and 430 nm (Fig. 1). A wavelength of 420 nm was thus selected as the wavelength for illumination for both *in vitro* and *in vivo* photosensitisation studies. The light dose in photosensitisation studies was varied by adjusting the illumination time depending on the required light dose (J cm^{-2}) calculated as a multiple of light power (W) and illumination time(s) divided by area of illumination (cm^2). The suspension temperature before and after each light treatment showed an average increase of 0.32 °C (0.31–0.35 °C), which was not statistically different between light doses, regardless of duration of illumination time.

The inactivation of fungal spores in both *in vitro* suspension and kernel experiments was determined by comparing reduction in viable spore count before and after treatment in controlled experiments, by assessing CFU for all groups of treated and untreated samples. In all cases, the control experiment with L^-/PS^- demonstrated the viability of the spores under the experimental conditions.

For the *in vitro* study with *A. flavus* spores in suspension, reductions of fungal spore counts of up to three magnitudes of log were achieved by irradiation at 420 nm with increasing light dose (0–84 J cm^{-2}) and varying concentration (0–100 μM). The antifungal effect of the photosensitiser alone was tested by treating spores with each of the six concentrations of curcumin without applying light on them (L^-/PS^+) with only minor differences observed between concentration. The effect of light alone on the spore viability was tested by treating spores with each of the four light doses without adding the photosensitiser (L^+/PS^-) with no significant difference measured between control and irradiated L^+/PS^- treatments when tested by t-test.

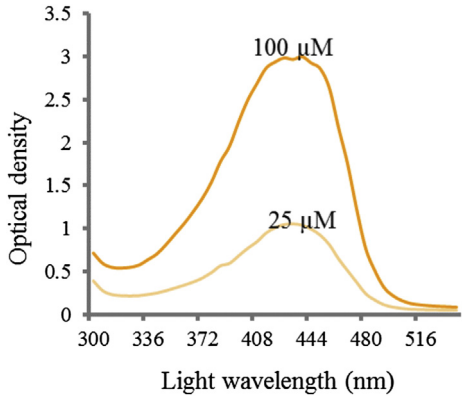

Fig. 1. Absorption spectrum of curcumin dye/spore mixture.

The control treatment (L^-/PS^-) for the *in vitro* inoculated kernel study had an average spore concentration of 3.26 magnitude of log. Reduction in the number of viable spores was observed when the spores were treated with a combination of light and dye, but not with either light (L^+/PS^-) or dye (L^-/PS^+) alone (Fig. 2). The decrease in spore viability was directly proportionate to the dose of light and dye concentrations of 15–50 μM. With curcumin concentrations above this range (i.e. 100 μM) the effectiveness of photosensitization was reduced. Reduction of viable spore count greater than 3 magnitude of log (close to zero count) was achieved when 84 J cm^{-2} light dose and 15, 25 and 50 μM curcumin dye concentrations were used (Fig. 2).

For the *in vivo* kernel study, negative control samples (without inoculum) had fungal spore counts of 0 CFU ml^{-1} indicating effective sterilization of the kernels by autoclaving. The inoculated positive control samples (L^-/PS^-) had a count of 4.97 ± 0.07 log CFU g^{-1}. Light treated whole kernel samples and milled kernel samples without photosensitiser (L^+/PS^-) had 4.74 ± 0.61 and 4.62 ± 0.15 log CFU g^{-1} respectively. Samples treated by photosensitization (light and dye applied, L^+/PS^+) had a significant CFU reduction of nearly 2 magnitude of log for both whole and milled kernels (Fig. 3 A and B).

4. Discussion

The soil-borne filamentous fungi *A. flavus*, a source of the potent carcinogen aflatoxin, is of serious concern to food and feed safety globally. Despite the interventions of good agricultural practices, *A. flavus* infection continues to occur in a range of crops and commodities hence a need for effective and safe decontamination procedures particularly in developing countries where agricultural practices are not as well controlled. Interventions that can be produced and applied on smallholder farms in developing countries are particularly good candidates for sustainable, adoptable interventions. In recent years, the application of photosensitization using natural plant products as a photodye for controlling microbial contamination in foods has evolved as a clean green yet highly effective technology (Luksiene et al., 2010, 2005, 2007; Zerdin et al., 2009). This present study investigates the novel application of such a plant photodye to the control of *A. flavus* in maize. Curcumin is a yellow extract from the root tuber spice turmeric from the *C. longa* plant. Studies have indicated that curcumin possesses different direct antimicrobial activities (De et al., 2009; Mun et al., 2013) including phototoxic properties to fungi and other microorganisms both *in vivo* and *in vitro* (Dovigo et al., 2013, 2011a, 2011b). The compound has been indicated to have inhibitory effects against *Aspergillus parasiticus* by reducing its growth, production of aflatoxin B_1 and expression of aflatoxin pathway genes (Jahanshiri, Shams-Ghahfarokhi, Allameh, & Razzaghi-Abyaneh, 2012).

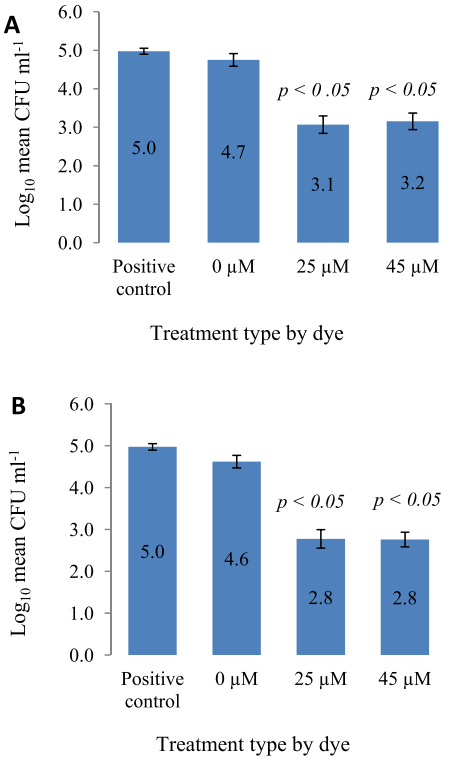

Fig. 3. The effect of photosensitization on *A. flavus* spore in maize kernels as indicated by reductions in fungal spore counts (CFU ml^{-1}) on whole kernels (A) and milled kernels (B). Significant statistical difference in CFU ml^{-1} was observed between dye treated samples (25 and 45 μM) compared to ones with no dye (0 μM) when illuminated with 60 J cm^{-2} light both in whole and milled kernels ($p < .0001$).

Curcumin is also reported to have clinical activities against various inflammatory and pro-inflammatory conditions (including cancer), protection against hepatic conditions as a result of arsenic and alcohol intoxication and other clinical conditions including acquired immunodeficiency (Gupta, Patchva, & Aggarwal, 2013).

The application of a photodegradation technique using curcumin as a natural dye against mycotoxigenic fungi, particularly *A. flavus*, has not been previously established. This study was therefore designed to investigate the effectiveness of combining curcumin and light of specific wavelength for photodegradation of aflatoxin producing *A. flavus* spores both in suspension (*in vitro*) and when experimentally inoculated in maize kernel surface (*in vivo*). The results indicated curcumin mediated photosensitization had a significant effect on viability of *A. flavus* spores both in suspension and when inoculated in maize kernel surface. Both

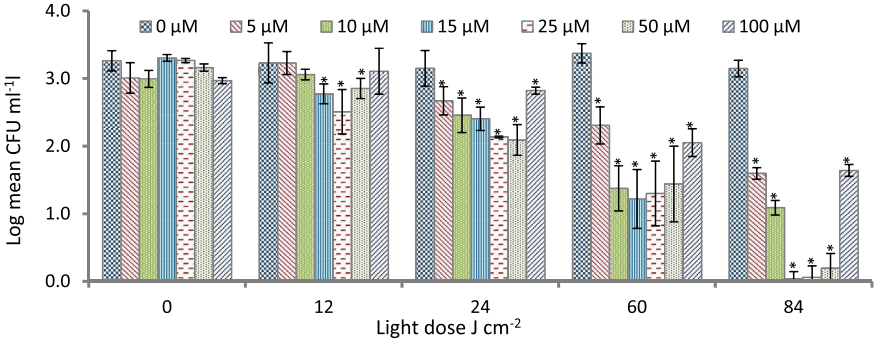

Fig. 2. Logarithm of mean spore count of *A. flavus* spore suspensions treated with different combinations of dye concentration and light dose. The significances of difference in mean CFU ml^{-1} between different light intensities and zero light for each dye concentration are indicated by asterisk where (*); $p < .05$ tested with t-test.

in vitro and *in vivo* treatments had significant reduction of fungal spores when appropriate light intensities and dye concentrations were combined in photosensitization. While best results in solution were observed with 15, 25 or 50 μM dye concentration combined with light of 24, 60 or 84 J cm^{-2} intensity, less (but still significant) reduction in spore viability was observed with 100 μM, and no reduction effect was observed with light alone. Curcumin is only sparingly soluble in solution and the reduced activity at 100 μM may be due to high fluid turbidity inhibiting light penetration and transmission. Reductions of viable spore counts for spores inoculated in maize kernel surface were observed in both intact kernel and milled kernels treatments. The reductions achieved using 25 μM and 45 μM dye concentrations in maize kernels illuminated with 60 J cm^{-2} did not differ significantly but each differed significantly when compared to using no dye.

This study represents the first application of curcumin for photodynamic degradation of *A. flavus* and parallels previous studies that have demonstrated similar effect of the dye with other fungal species and some bacteria. For example Dovigo et al. (2013) reported a significant phototoxic effect on the use of curcumin against pathogenic species of *Candida*. Haukvik et al. (2009) reported on the use of curcumin for photodegradation as having a significant inhibitory effect against gram negative (*Escherichia coli*) and gram positive bacteria (*E. faecalis* and *S. intermedius*) that were strongly influenced by changes in pre-irradiation incubation time, curcumin concentration and irradiation dose. The specific mechanism underlying photodynamic degradation of *A. flavus* spores has not been reported but the phototoxicity of curcumin seems to be oriented through reactive oxygen species that form as a result of the reaction between excited states of the dye molecules and oxygen molecules (Ahn, Kang, Shin, & Chung, 2012). It acts as a photosensitiser of singlet oxygen and undergoes a self-sensitized decomposition but the fading of curcumin also involves other mechanisms independent of oxygen. In the present study yellow colour disappeared during the photo treatment process that was also correlated with reduced light absorption as determined by absorption spectrum (Fig. 4). Similar results have also been reported, where UV radiation accelerated fading of curcumin colour detected at 405 nm (Lee, Choi, Kim, & Hong, 2013).

The use of curcumin as a food additive is controlled by regulations such as European parliament directive 94/36/EC with maximum levels set for a range of foodstuffs (European-Union, 1994) and further re-evaluation of the product has proved it safe (EFSA, 2010, 2014). Although there are no reported cases of toxicity from dietary use of turmeric or curcumin, possible effects that may occur as a result of excessive dose that could be associated with clinical use of the product should not be underestimated. Despite the various reported studies on the possible side effects of curcumin (Burgos-Morón, Calderón-Montaño, Salvador, Robles, & López-Lázaro, 2010), re-evaluation has been carried by European Food Safety Authority (EFSA). This considered a number of reports of positive results for curcumin in *in vitro* and *in vivo* tests for genotoxicity, especially those detecting chromosomal aberrations and DNA adducts (EFSA, 2010). Based on available scientific data EFSA agreed that the compound is not carcinogenic, but also allocated an ADI of 3 mg/kg bw based on the no-observed-adverse-effect-level (NOAEL) of 25–320 mg/kg bw/day. EFSA noted that intake of curcumin from the normal diet amounts to less than 7% of the 3 mg/kg bw ADI (EFSA, 2010, 2014).

Curcumin can be produced by chemical synthesis, but only natural curcumin extracted from plant sources is used as a food additive. The *C. longa* (turmeric) plant is grown widely in the tropical world including Africa (Damalas, 2011) mainly as spice. It is therefore expected that application of the photoinactivation technique will be facilitated by its ready availability in African developing countries where food-originated mycotoxins exposure is a significant and persistent problem. Given that so much of the food supply in developing countries is estimated to be contaminated, further studies should focus on adapting this potential intervention for use on farm, with kernels naturally colonized by the fungus.

5. Conclusion

This is the first study reporting significant knock down of *A. flavus* spores using natural dye mediated photosensitization. Our study indicates a combination of appropriate curcumin dye concentration and light dose significantly reduced ($P < 0.05$) viability of the *A. flavus* spores in both *in vitro* and *in vivo* settings. These findings provide a promising but as yet unexplored technique for reducing potential aflatoxin contamination in food and feed which are often proportional to the level of fungal infestations. At harvest, smallholder farmers often harvest cobs onto the soil, and then sun dry on tarpaulin sheets; on farm cultivation of *C. longa* and production of extracts, for treating cobs just before sun drying could help eliminate *A. flavus* spores at a critical point of grain exposure to fungal inoculum, while the spores are still largely on the surface of

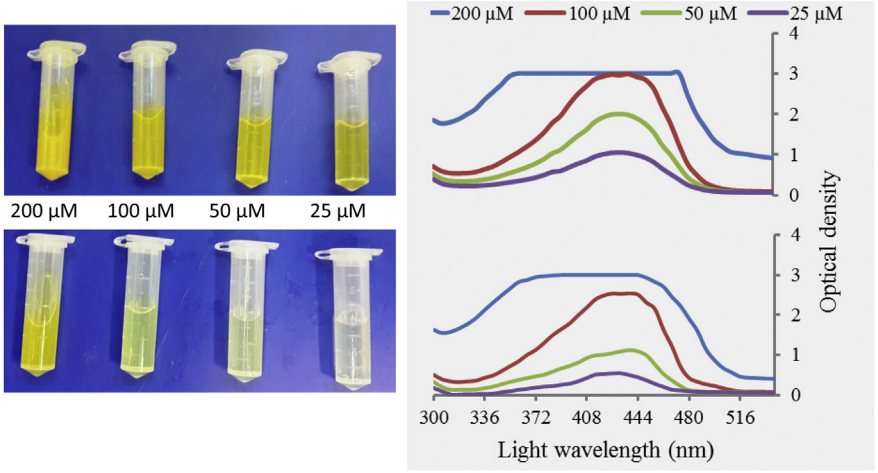

Fig. 4. Optical change in curcumin dye solution as a result of illumination with 60 J cm^{-2} (420 nm) as observed by colour change; left top (before illumination), left bottom (after illumination) and absorption spectrum pattern; right top (before illumination), right bottom (after illumination). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the grains. The technique can be applied in food processing such as mills to reduce fungal load and ensure a longer storage time for the resulting products without succumbing to high levels of mycotoxins. In typically wet-milling operations grains are steeped in water prior to milling and this would represent an ideal intervention point for the described technology.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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