World Mycotoxin Journal, 2017; 10 (3): 237-248

Wageningen Academic Publishers

Bioactive compounds in *Diospyros mafiensis* roots inhibit growth, sporulation and aflatoxin production by *Aspergillus flavus* and *Aspergillus parasiticus*

J.A. Mmongoyo[1], M.G. Nair[2], J.E. Linz[1], F. Wu[1], J.K. Mugula[3], A.A. Dissanayake[2], C. Zhang[2], D.M. Day[1], J.M. Wee[1] and G.M. Strasburg[1*]

[1]Department of Food Science and Human Nutrition, Michigan State University, East Lansing, MI 48824, USA; [2]Department of Horticulture, Michigan State University, East Lansing, MI 48824, USA; [3]Department of Food Technology, Nutrition and Consumer Sciences, Sokoine University of Agriculture, P.O. Box 3006, Morogoro, Tanzania; stragale@msu.edu

Received: 16 June 2016 / Accepted: 20 January 2017
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RESEARCH ARTICLE

http://www.wageningenacademic.com/doi/pdf/10.3920/WMJ2016.2107 - Tuesday, April 10, 2018 11:58:24 PM - IP Address:41.222.181.189

Abstract

Diospyros mafiensis F. White is a medicinal shrub or small tree (6 m tall) widely distributed in the Zanzibar-Inhambane regional mosaic and traditionally used to treat leprosy, diarrhoea, and skin fungal infections in Tanzania and Mozambique. Our objective was to determine the anti-aflatoxigenic properties of compounds from *D. mafiensis* root bark against vegetative growth, sporulation and aflatoxin production by *Aspergillus flavus* and *Aspergillus parasiticus*. Bioassay-guided extraction, fractionation, and isolation of bioactive compounds using *A. parasiticus* B62 were employed. The bioactive compounds were elucidated using ^{1}H and ^{13}CNMR and LC-MS. Growth inhibition was determined by measuring the colony diameter of *A. flavus* AF3357 and *A. parasiticus* SU-1 ATCC56775. Inhibitory effects on sporulation were estimated using a haemocytometer. Total aflatoxin was quantified by direct competitive enzyme-linked immunosorbent assay (ELISA). Bioactive compounds diosquinone (DQ) and 3-hydroxydiosquinone (3HDQ) were identified. DQ weakly inhibited *A. flavus* and *A. parasiticus* vegetative growth (MIC_{50} >100 µg/ml) and 3HDQ strongly inhibited *A. flavus* (MIC_{50} = 14.9 µg/ml) and *A. parasiticus* (MIC_{50} = 39.1 µg/ml). DQ strongly reduced total aflatoxin production by *A. flavus* from 157 to 36 ng/plate, and by *A. parasiticus* from 1,145 ng/plate to 45 ng/plate at 100 µg/ml. 3HDQ reduced total aflatoxin production by *A. parasiticus* from 1,145 to 32 ng/plate; stimulated production by *A. flavus* from 157 to 872 ng/plate at 12.5 µg/ml but reduced to 45 ng/plate at 100 µg/ml. In summary, DQ and 3HDQ could be used as natural antifungal compounds to prevent mould growth and aflatoxin accumulation in food and feed.

Keywords: diosquinone, 3-hydroxydiosquinone, total aflatoxin, *A. flavus, A. parasiticus*

1. Introduction

Aspergillus flavus and *Aspergillus parasiticus* produce toxic secondary metabolites called aflatoxins, which have deleterious health effects to humans and animals that include immunosuppression (Jiang *et al.*, 2008), growth impairment (Khlangwiset *et al.*, 2011), aflatoxicosis (Strosnider *et al.*, 2006) and liver cancer (Liu and Wu, 2010). The World Health Organisation (2008) estimated that between 25,000 and 155,000 people die each year of liver cancer linked to chronic exposure to aflatoxins through contaminated food, and over 83% of such deaths occur in Sub-Saharan African countries (Liu and Wu, 2010; Strosnider *et al.*, 2006). Unfortunately, severe fungal deterioration and contamination of food occur during storage due to conditions favourable to fungal growth (Hell *et al.*, 2000). Additionally, chronic exposure to *A. flavus* and *A. parasiticus* spores is now known to cause a respiratory disease called allergic bronchopulmonary aspergillosis (ABPA). Although ABPA occurs relatively infrequently, it can be deadly particularly for immunocompromised individuals (Denning *et al.*, 2013). Denning *et al.* (2013) calculated an estimate of 389,900 cases of ABPA associated with invasive fungal infections is likely in Africa. Thus, individuals winnowing infected seeds during harvesting or from granaries without dust masks are likely to inhale

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fungal spores and thus, may be at risk of ABPA and invasive aspergillosis (Pfaller *et al.*, 2016), which are often accompanied with chronic asthma (Denning *et al.*, 2013).

In recent years, there have been growing concerns associated with the indiscriminate use of synthetic pesticides for crop protection against the moulds in storage. The concerns associated with synthetic pesticides include fungal resistance, toxicological effects on consumers, non-biodegradability, and prohibitive costs (Da Cruz Cabral *et al.*, 2013). As an alternative to synthetic pesticides, medicinal plants may be useful sources of naturally-occurring, biodegradable, readily available, and inexpensive food preservatives that could be useful to prevent growth, sporulation and aflatoxin production by aflatoxigenic moulds in food during storage (Bluma and Etcheverry 2008; Bluma *et al.*, 2008; El-Nagerabi *et al.*, 2013; Velazhahan *et al.*, 2010; Kedia *et al.*, 2014; Passone *et al.*, 2013; Prakash *et al.*, 2014). These studies showed that exposure of *A. flavus* and *A. parasiticus* to medicinal plant extracts or pure compounds significantly reduced or completely inhibited their growth and toxin production. Additionally, because such extracts contain a variety of compounds, their synergistic modes of action against moulds may reduce the likelihood of development of resistance unlike that related to use of individual synthetic pesticides (Da Cruz Cabral *et al.*, 2013).

The genus *Diospyros* of the family Ebenaceae has over 350 species of economically important medicinal plants widely distributed in tropical countries worldwide (Mallavadhani *et al.*, 1998). All plant parts (leaves, stem bark, root bark, fruits, and seeds) of *Diospyros* species have a myriad of biologically active compounds against viruses, bacteria, fungi, and termites (Mallavadhani *et al.*, 1998; Marston *et al.*, 1984). The unique medicinal properties of *Diospyros* species have contributed to their use as traditional medicines in tropics and subtropics to treat human ailments such as gonorrhoea and tuberculosis in Cameroon (Kuete *et al.*, 2009), asthma in Sri Lanka (Herath *et al.*, 1978), leprosy in Tanzania (Khan *et al.*, 1980), schistosomiasis in Malawi (Gafner *et al.*, 1987), and of particular importance to the current work, fungal infections in Tanzania (Hamza *et al.*, 2006).

Tanzanian *Diospyros* species are widely distributed in the Zanzibar-Inhambane regional mosaic and are abundant and endemic to this region (White, 1988). The natives in this area have been using this plant in many ways ranging from a source of material for house construction, edible fruits (Hall and Rodgers, 1986; White, 1988) to traditional medicines for treating human diseases (Khan *et al.*, 1980). The leaves, root and stem barks of *Diospyros* species have been used in this region by traditional healers since olden times to prepare infusions, decoctions, root powders, and topical herbs for treating human ailments. Such illnesses include ulcers, dysentery, leprosy, whooping cough, general body weakness, non-insulin diabetes mellitus and oral candidiasis (Hamza *et al.*, 2006; Moshi and Mbwambo, 2002; Watt and Breyer-Brandwijk, 1962). Also, its termicidal property makes poles derived from the tree preferable for the construction of durable houses (Hall and Rodgers, 1986). However, *Diospyro*s species available in this region have not been studied for their inhibitory activity against vegetative growth, sporulation and aflatoxin production by aflatoxigenic moulds such as *A. flavus* and *A. parasiticus*. The objective of the present work was to study the anti-aflatoxigenic activity of *D. mafiensis* root extracts from Tanzania against *A. flavus* and *A. parasiticus* as part of the search for safer natural antimycotics that could be used to protect stored food crops.

2. Materials and methods

General experimental procedures

All solvents used for isolation and purification were of ACS reagent grade (Sigma-Aldrich Chemical Co., St. Louis, MO, USA). Merck silica gel (60 mesh size, 35-70 µm) with a particle size of 60 µm was used for preparative medium-pressure liquid chromatography (MPLC). Silica gel plates (250 µm; Analtech, Inc., Newark, DE, USA) were used for preparative thin-layer chromatography (TLC) and developed plates viewed using ultraviolet light at 254 or 366 nm using a Spectroline CX-20 ultraviolet fluorescence analysis cabinet (Spectroline Corp., Westbury, NY, USA). After viewing and locating spots under UV light, plates were sprayed with 10% sulfuric acid solution in water and charred to observe spots that were not visible under UV. nuclear magnetic resonance (NMR) spectra were recorded on a 500 MHz (Varian Unity ±500, 1H NMR) or 125 MHz (Varian Unity ±500, 13C NMR) VRX instruments 9varian, Palo Alto, CA, USA). ESIMS spectra were recorded on a Waters Xevo G2-S Q-TOF LC mass spectrometer (Waters Corporation, Milford, MA, USA).

Plant material

Root bark of *Diospyros mafiensis* F. White was collected on December 23, 2014, at the location S06°53'33" E39°06'01', 182 m in Kisarawe, Pugu, Dar es Salaam, Tanzania. A voucher specimen has been deposited in the Botany Department Herbarium, University of Dar es Salaam, Tanzania, for future reference (Voucher No. FMM 3693). The root bark was air-dried in the shade for five days. The dry root bark was milled using a laboratory mill (Model 4, Martha R. Thomas Company, Philadelphia, PA, USA). The milled plant material was shipped to Michigan State University for further analyses.

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Fungal strains, growth medium and growth conditions

Wild-type strains of aflatoxigenic moulds *A. flavus* (AF3357), and *A. parasiticus* (SU-1, ATCC56775) and a mutant strain of *A. parasiticus* B62 were used throughout this study. The mutant *A. parasiticus* B62 strain was used for screening the anti-aflatoxigenic activities and aflatoxin reduction efficacies of the methanolic extract and fractions of the plant material. All strains were grown on glucose minimal salts (GMS), which is a chemically defined medium that was prepared as previously described (Tice and Buchanan, 1981). The pH of the medium was adjusted to 4.5 using 1M NaOH. Moulds were centre-inoculated onto Petri dishes and allowed to grow in the dark in an incubator at 30˚C for 5 days for screening and 10 days for bioassays of isolated bioactives against wild-type strains *A. flavus* AF3357 and *A. parasiticus* SU-1, ATCC56775.

Screening *Diospyros mafiensis* root powders and methanolic extracts using *Aspergillus parasiticus* strain B62

A. parasiticus strain B62 accumulates the brightly coloured red pigment (Lee *et al.*, 1971), norsolorinic acid (NA), in the colony and surrounding growth medium (Roze *et al.*, 2011b). The disappearance of red coloration following treatment in the growth medium provides visual evidence of aflatoxin biosynthesis inhibition (Figure 1). Dry root powders (10 g) were placed in a cell culture dish (150×25 mm) and evenly spread at the bottom of the plate. Three small Petri dish (60×15mm) covers were filled with potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA) (10 ml) growth medium. Conidiospores (1×10^4 cfu/plate) of *A. parasiticus* were centre-inoculated onto PDA agar medium solidified in each of small Petri dish covers. The three inoculated Petri dish covers were placed inside a larger dish that contained root powders evenly distributed at the bottom of the dish. Then, the lid of the larger dish was covered and sealed with parafilm to prevent the escape of root volatiles and the smaller dishes inside the larger dish were open to allow free interactions of gases emanating from the root powders to the growing fungus. The control set was prepared the same way, but the larger dish contained no root powders (Roze *et al.*, 2007, 2011b). The fungus was allowed to grow in the dark at 30 °C for three days.

An appropriate mass of powdered root methanolic extract (25, 50, and 250 mg) (extraction method is described in next section) was dissolved in 1 ml of dimethylsulfoxide (DMSO) to make stock solutions containing 25, 50 and 250 mg/ml, respectively. Flat-bottomed 6-well culture plates (SIAL0516, Sigma-Aldrich, St Louis, MO 63103, USA) were used to grow the moulds in triplicate. From each stock concentration, 10 µl were placed into each of the three

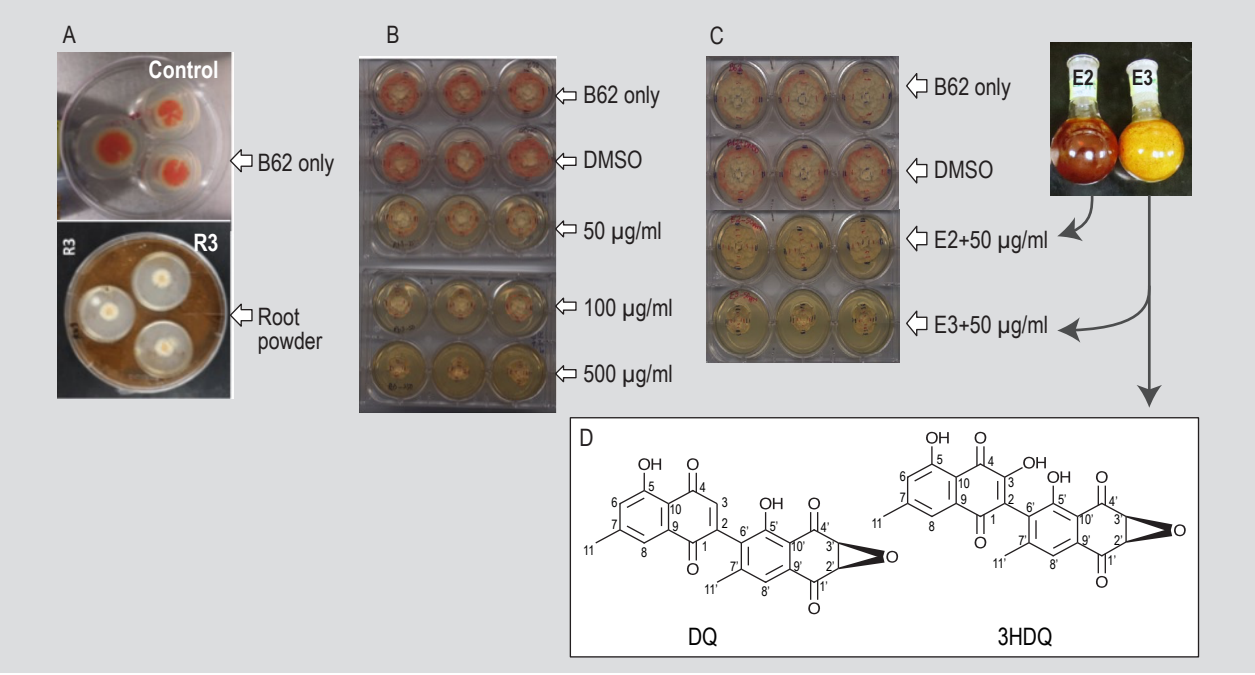


Figure 1. (A) Inhibitory effects of root powders of *Diospyros mafiensis* on *Aspergillus parasiticus* B62 grown on potato dextrose agar for 3 days; (B) crude methanol extracts; and (C) sub-fractions E2, and E3 obtained from fraction E on vegetative growth and norsolorinic acid (NA) production by *A. parasiticus* B62. B62 was grown onto glucose minimal salts in the dark at 30 °C for 5 days. The image was obtained on the fifth day of incubation. Controls: B62 only and B62 with dimethylsulfoxide. Decrease in NA intensity indicates aflatoxin reduction due to the plant extract. Purification of E3 using preparative chromatography afforded two biologically active compounds (D) diosquinone (DQ) and 3-hydroxydiosquinone (3HDQ).

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plates per treatment. Then molten GMS agar tempered to 50 °C (5 ml) was poured into each plate while shaking to ensure homogeneous mixing of the contents. Plates were allowed to cool and solidify the agar. The concentrations in the growth medium were 50, 100, and 500 µg/ml, from the stock solutions 25, 50, 250 mg/ml, respectively. Controls included (1) GMS without extract and (2) GMS without extract but with 10 µl DMSO. Then, conidiospores (1×10^4 cfu/plate) of *A. parasiticus* B62 were centre-inoculated onto the GMS agar medium of each plate and incubated in the dark at 30 °C for 5 days.

Bioassay-guided extraction and isolation

The plant material was initially extracted sequentially at room temperature with methanol, ethyl acetate and hexane. Bioassays of resulting extracts showed activity limited to methanolic extract. Subsequently, powdered root barks (200 g) were extracted with methanol (1.5 l, 24 h ×3), and evaporation of the solvent under vacuum afforded a powdered extract (57.30 g). An aliquot (20 g) was stirred in methanol (200 ml, 1 h) and centrifuged at room temperature for 10 min to afford residue A (0.75 g, plant material) and supernatant. The supernatant was evaporated under vacuum to obtain methanol-free reddish residue B (19.13 g). This residue (19.13 g) was then mixed with hexane (200 ml) and stirred for 1h and centrifuged at room temperature for 10 min to afford precipitate C (16.66 g) and supernatant, which was evaporated under vacuum to obtain oily fraction D (2 g). The precipitate C was mixed with 200 ml of ethylacetate, stirred for 1h, and centrifuged at room temperature for 10 min to afford a precipitate F (12.22 g) and supernatant E. Evaporation of ethylacetate from the supernatant under vacuum afforded fraction E (4.15 g). An aliquot of fraction E (350 mg) was mixed with acetone (6 ml) and stirred for 1 h and centrifuged at room temperature for 10 min to obtain precipitate E1 (17.5 mg) and supernatant. The precipitate E1 (17.5 mg) was soluble in methanol. To the acetone supernatant, hexane (7 ml) was added and the mixture was stirred for 1h and centrifuged at room temperature for 10 min to obtain subfraction (residue) E2 (133 mg). The supernatant, acetone-hexane mixture, was evaporated to obtain subfraction E3 (198 mg) (Alexander-Lindo *et al.,* 2004).

Fractions A, B, D, E, and F, and sub-fractions E1, E2, and E3 were distinct, as indicated by TLC analyses and were screened using *A. parasiticus* B62 grown in the dark at 30 °C for 5 days (see Supplementary Figure S1). The subfraction E1 was inactive. All fractions (A-F) and sub-fraction E2 were weakly active as indicated by Figure 1C and Supplementary Figure S1. The subfraction E3 was the strongest and it was preferentially selected for isolation, purification and characterisation of bioactive compounds. An aliquot of E3 (120 mg) was purified by preparative TLC ($CHCl_3$: MeOH, 30:1, v/v, two runs) to yield compounds 1 (23 mg) and 2 (9.2 mg) (Georges *et al.*, 2008; Zhang *et al.*, 2015, 2016).

Characterisation of compounds 1 and 2

Compound 1

Red solid; ^{1}H NMR (500 MHz, $CDCl_3$): 11.85 (1H, s, 5-OH), 11.48 (1H, J = 10.8 Hz, 5'-OH), 7.48 (2H, d, 5.4 Hz, H-8, H-8'), 7.11 (1H, s, H-6), 6.85 (1H, d, 12.7 Hz, H-3), 4.01 (1H, s, H-3'), 3.96 (1H, s, H-2'), 2.44 (3H, s, H-7CH_3), 2.27 (3H, s, H-7'CH_3)(Figure S3); ^{13}C NMR (125 MHz, $CDCl_3$): 195.3, 195.1 (C-4'), 189.6, 189.4 (C-1'), 188.8 (C-4), 182.4 (C-1), 161.4 (C-5), 159.2 (C-5'), 148.7 (C-7), 147.5 (C-7'), 145.5, 145.4 (C-2), 138.9, 138,7 (C-3), 129.1 (C-9, C-9'), 124.2 (C-6'), 121.3 (C-6), 121.1 (C-8, C-8'), 113.1 (C-10'), 112.1 (C-10), 55.4 (C-2'), 55.1 (C-3'), 22.3 (C-11), 22.1 (C-11'). These data revealed that compound 1 was diosquinone (DQ). Based on spectral data, DQ was previously reported from the roots of the same plant (Khan and Rwekika, 1999).

Compound 2

Red solid; ^{1}H NMR (500 MHz, CD_3OD): 7.45 (1H, s, H-8'), 7.36 (1H, s, H-8), 6.92 (1H, H-6), 3.99 (2H, dd, H-2',H-3'), 2.40 (3H, s, H-7CH_3), 2.26 (3H, s, H-7'CH_3) (Figure S4); ^{13}C NMR (125 MHz, CD_3OD): 197.4, (C-4'), 191.7 (C-1'), 190.4 (C-4), 182.1 (C-1), 170.1 (C-3), 163.1 (C-5), 161.8 (C-5'), 150.9 (C-7), 150.1 (C-7'), 136.6 (C-2), 133.6 (C-9, C-6'), 131.3 (C-9'), 121.7 (C-6), 121.5 (C-8'), 120.4 (C-8), 113.2 (C-10'), 113.1 (C-10), 56.8 (C-2'), 56.5 (C-3'), 22.3 (C-11), 22.2 (C-11'). HRESIMS: *m/z* 405.0621 ($[M-H]^-$ (calcd for $C_{22}H_{13}O_8$, 405.0610). These data revealed that compound 2 was 3-hydroxydiosquinone (3HDQ). The molecular ion, $[M-H]^-$, at *m/z* 405, 16 amu higher than that of DQ, indicated that 3HDQ contained additional oxygen functionality in its structure (Supplementary Figure S5). This new oxygen functionality assigned as a hydroxyl group at C-3 and resonated upfield at δ 170.1 in its ^{13}C NMR spectrum (Supplementary Figure S6) was confirmed by the absence of the proton signal at δ 6.85 in its ^{1}H NMR spectrum when compared to the ^{1}HNMR spectrum of DQ.

Inhibitory effects of DQ and 3HDQ on vegetative growth of *Aspergillus*

DQ (5 mg) was dissolved in 200 µl of DMSO to make a stock solution (I) with a concentration of 0.025 mg/µl (w/v). Using this stock solution, five serial dilutions were carried out by taking 100 µl of stock solution I and mixing it with 100 µl of DMSO. Serial dilutions were conducted to make stock solutions II, III, IV, and V. From each stock solution, 10 µl were transferred into the test well plate (in triplicate for each stock solution) and 5 ml of GMS agar was poured into each test well while shaking gently to ensure homogeneous mixing of the contents in order

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to get concentrations 50, 25, 12.5, 6.25, and 3.125 µg/ml, respectively, as final concentrations in the test well plates. The highest concentration (100 µg/ml) was prepared by transferring 20 µl from the stock solution I into 5 ml GMS plate. Thus, dose levels applied for the inhibitory experiments were 100, 50, 25, 12.5, 6.25, and 3.125 µg/ml. The GMS agar medium was left to solidify in the test well plates before inoculation. Serial dilutions of 3HDQ were prepared in the same way that resulted in the same final concentrations of 100, 50, 25, 12.5, 6.25, and 3.125 µg/ml in the test well plates. *A. flavus* (AF3357) and *A. parasiticus* (SU-1, ATCC56775) were exposed to such DQ and 3HDQ by allowing them to grow on the surface of treated GMS growth medium (5 ml) in the test well plates. The plates that contained GMS only or GMS with DMSO only were the positive and negative controls, respectively. Six-well culture plates (SIAL0516, Sigma-Aldrich, St Louis, MO, USA) were used throughout this study. Conidiospores (1×10^4 cfu/plate) of each fungal strain were centre-inoculated into each test well and incubated in the dark at 30 °C for 10 days. Fungal growth was estimated by measuring colony diameter in perpendicular directions for each colony every 24 h for 10 days. All colony diameter measurements were recorded as mean ± standard error (SE) as previously described (Roze *et al.*, 2011b). The growth inhibition percentages were obtained by the following formula:

$$\text{Growth inhibition (\%)} = \frac{\text{Control} - \text{Treatment}}{\text{Control}} \times 100\%$$

Estimation of fungal sporulation

After 10-day incubation in the dark at 30 °C on GMS medium, conidiospores of *A. flavus* (AF3357) and *A. parasiticus* (SU-1, ATCC56775) were harvested, and spore numbers in (cfu/plate) for each colony were estimated using a haemocytometer as described previously (Roze *et al.*, 2004). Averages of spore numbers (cfu/plate) for each dose concentration were determined.

Extraction and quantification of aflatoxins from growth medium

Total aflatoxins in the growth medium and mycelia were extracted with 5 ml chloroform in 50 ml Falcon tubes (Denville Scientific Inc., South Plainfield, NJ, USA). Chloroform (5 ml) was added to a 50 ml Falcon tube containing the sample (chunks (@~6×6×5 mm) of solid medium agar from the test well plate). The chunks were vortexed for 5 s and the mixture allowed to rest for 10 min before withdrawing the extract into a 20 ml scintillation vial. This procedure was repeated three times and the extracts were dried completely under a stream of nitrogen gas, and each vial was reconstituted with 500 µl of 70% methanol (Roze *et al.*, 2004). Five µl of the reconstituted solution were dissolved in 1000 µl of 70% methanol, and the solution was vortexed for 30 s to obtain the final sample solution (pH=6.7). Then, total aflatoxin in the sample was quantified using Veratox® direct competitive enzyme-linked immunosorbent assay (ELISA) as described by the manufacturer (Neogen Corporation, Lansing, MI, USA). Averages of total aflatoxin (ng/plate) at each dose level were determined and recorded.

Statistical analysis

Statistical analysis used Duncan's method for pairwise comparisons, which were performed using SigmaStat one-way analysis of variance (One-Way ANOVA) scientific statistical software, version 11.0 from Jandel Corporation, San Rafael, CA, USA.

3. Results

Screening *Diospyros mafiensis* root powders and methanolic extracts using *Aspergillus parasiticus* B62

The root powders of *D. mafiensis* (R3) decreased vegetative growth and NA production by *A. parasiticus* B62 compared to the untreated control (Figure 1A). Crude methanolic extracts of *D. mafiensis* root bark effectively inhibited both vegetative growth and NA production of *A. parasiticus* (Figure 1B). Vegetative growth and NA decreased drastically with increase in dose of the extract from 50 to 500 µg/ml as compared to the controls (B62 only and DMSO).

Bioassay-guided extraction and isolation

Except the oily fraction D that weakly inhibited vegetative growth and NA production, fractions A, B, E, and F weakly inhibited vegetative growth, but strongly inhibited NA production by *A. parasiticus* B62 in a dose-dependent manner (Supplementary Figure S2). Fraction E exhibited the strongest activity against *A. parasiticus* B62 growth and NA production (Supplementary Figure S2). Thus, fraction E was selected for further fractionation to obtain sub-fractions E1, E2, and E3. Sub-fraction E1 was inactive against the growth of *A. parasiticus* B62. Conversely, sub-fraction E2 inhibited *A. parasiticus* B62 vegetative growth more weakly than E3, but both were strong inhibitors of NA productions. Sub-fraction E3 was the most potent against the vegetative growth of *A. parasiticus*. From this subfraction, two bioactive compounds were isolated, purified, characterised, and identified as DQ and 3HDQ (Figure 1C and 1D). The spectral data of DQ were in agreement with Khan and Rwekika (1999) who first isolated and characterised it from *D. mafiensis*. We report here for the first time the spectral data of a new analog of DQ, called 3HDQ.

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Inhibitory effects of DQ and 3HDQ on the vegetative growth of *Aspergillus*

Figure 2A and 2B show growth inhibitory effects of DQ and 3HDQ assessed at concentrations, ranging from 3.125 to 100 µg/ml on *A. flavus* and *A. parasiticus* grown on GMS for 10 days. At the highest concentration (100 µg/ml), DQ weakly but significantly inhibited ($P<0.05$) *A. flavus* (43%) and *A. parasiticus* (34%) growth compared with the control. There was no change in the level of inhibition of vegetative growth of *A. parasiticus* by DQ from 12.5 to 100 µg/ml. The 50 percent minimum inhibitory concentration (MIC_{50}) values of DQ in *A. flavus* and *A. parasiticus* were all greater than 100 µg/ml. In contrast, at the highest concentration (100 µg/ml), 3HDQ significantly ($P<0.05$) inhibited the vegetative growth of *A. flavus* (64%) and *A. parasiticus* (56%). No significant difference in inhibition of vegetative growth of *A. flavus* was observed by 3HDQ from 25 to 100 µg/ml. The MIC_{50} values for 3HDQ were 14.9 µg/ml on *A. flavus* and 39.1 µg/ml on *A. parasiticus* (Figure 2A and 2B, respectively). Vegetative growth of *A. flavus* was more susceptible to DQ and 3HDQ than *A. parasiticus*. Significantly, 3HDQ was more potent for both *A. flavus* and *A. parasiticus* than DQ especially at doses >6.25 µg/ml (Figure 2A and 2B).

Of particular interest, 100 µg/ml of DQ caused a complete loss of green pigmentation in colonies of *A. flavus* while a similar dose of 3HDQ did not cause loss of greenish pigmentation in the colonies of the same fungus suggesting that DQ at doses ≥100 µg/ml exerts morphological alterations and disrupts ability to form pigments (Supplementary Figure S2). However, we failed to observe this phenomenon in *A. parasiticus* because it did not form greenish pigmentation at all doses (see Supplementary Figure S2).

Impact of DQ and HDQ on fungal sporulation

Conidiospore number for both mould strains decreased significantly ($P<0.05$) in a dose-dependent manner when they were exposed to increasing doses of DQ and 3HDQ as compared with the controls (Figure 2C and 2D). For example, 100 µg/ml of 3HDQ strongly decreased conidiospore numbers from 1.7×10^6 (control AF3357) to 2.7×10^5 spores/ plate (98% reduction of sporulation) in *A. flavus* after 10 days of growth (Figure 2C). By comparison, 100 µg/ml of 3HDQ was less effective in decreasing conidiospore number from 2.4×10^5 in control SU-1 to 1.2×10^5 spores/plate (52% reduction of sporulation) in *A. parasiticus* grown for 10 days (Figure 2D). In contrast, DQ was equally potent at reducing conidiospore number in both fungal strains after exposure to 100 µg/ml for 10 days of incubation. DQ reduced conidiospore number from 1.5×10^6 in control AF3357 to 3.6×10^5 spores/plate (76% reduction of sporulation) in *A. flavus* (Figure 2C) exposed to DQ for 10 days and decreased conidiospore number in *A. parasiticus* from 2.6×10^5 in control SU-1 to 5.8×10^4 spores/plate (77% reduction of sporulation) (Figure 2D) after exposure for 10 days. Overall, 3HDQ (with the exception of the 100 µg/ml dose) exhibited lower ability to reduce conidiospore number in both fungi than its counterpart DQ. This suggests that although the DQ was a weaker inhibitor of radial growth (Figure 2B), it reduced conidiospore number more effectively.

Quantification of total aflatoxins extracted from growth medium

After incubation of centre-inoculated *A. flavus* and *A. parasiticus* strains in the dark at 30 °C for 10 d, total aflatoxins were extracted from each plate containing growth media. Figure 2E and 2F show total aflatoxins (ng/plate) presented as mean ± standard error of three independent plates for each treatment group against two different wild-type fungal strains. Compared with the control (AF 3357 only), DQ significantly ($P<0.05$) inhibited aflatoxin production by *A. flavus* at all concentrations after 10 days and total aflatoxin accumulation was inversely proportional to an increase in dose (Figure 2E). Higher doses (25 to 100 µg/ml) of DQ inhibited total aflatoxin accumulation by 77.2% (36 ng/plate in the treatment group compared with 157 ng/plate in the control, AF3357 only (Figure 2E). In contrast, doses consisting of 3.125, 6.25, 12.5, 25, and 50 µg/ml of 3HDQ significantly promoted total aflatoxin production by *A. flavus* to the following amounts 176.5, 393.0, 872.4, 475.9, and 158.8 ng/plate, respectively, compared with 157.1 ng/plate in the control (AF3357 only). Significant inhibition of total aflatoxin production was observed at 100 µg/ml, which caused a 72% reduction of total aflatoxin from 157 ng/plate in the control (AF3357 only) to 43.5 ng/plate (Figure 2E). DQ and 3HDQ inhibited total aflatoxin production by *A. parasiticus* at nearly equal efficacy (Figure 2F). Compared with 1,145.3 ng/plate total aflatoxin in the untreated control (SU-1 only), doses ≥6.25 µg/ml of DQ or HDQ nearly eliminated aflatoxin accumulation (44.7 ng/plate, 96.1% aflatoxin reduction; and 32.3 ng/plate, 97.2% aflatoxin reduction, respectively.

4. Discussion

Exposure of *A. parasiticus* B62 to powdered roots, crude methanolic extracts and sub-fractions E2 and E3 of *D. mafiensis* inhibited fungal growth and NA-accumulation in the growth medium as compared with the control. Similarly, fractions A, B, and F, exhibited strong NA inhibition. The observed loss of NA accumulation by *A. parasiticus* in initial studies suggested that the chemical constituents in root powders, methanolic extracts and sub-fractions have inherent capabilities to inhibit aflatoxin biosynthesis by interfering with expression of genes and or enzymes responsible for NA production from acetyl-CoA. Early

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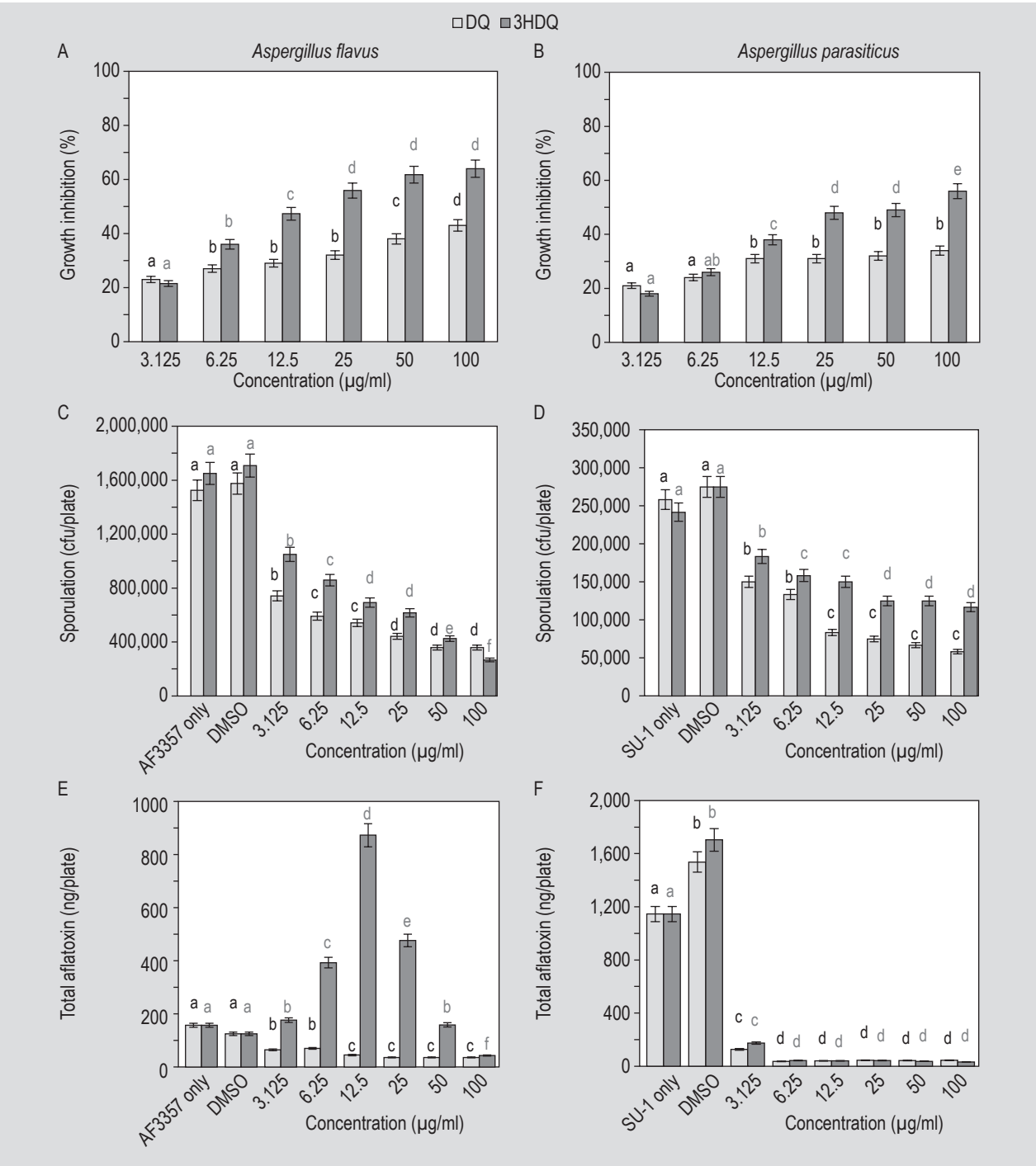


Figure 2. Inhibitory effects of diosquinone (DQ) and 3-hydroxydiosquinone (3HDQ) on vegetative growth of (A) *Aspergillus flavus* and (B) *Aspergillus parasiticus;* on sporulation of (C) *A. flavus* and (D) *A. parasiticus*; and on total aflatoxin production by(E) *A. flavus* and (F) *A. parasiticus*. Differences in data with the same letters are not statistically significant (*P*>0.05) according to Duncan's method of pairwise comparisons tests. DMSO = dimethylsulfoxide.

aflatoxin biosynthesis pathway genes including *aflA* (*fas-2*), *aflD* (*fas-1*) and *aflC* (*pksA*) orchestrate the conversion of acetyl-CoA to an unstable polyketide and eventually to NA, the first stable aflatoxin intermediate in the pathway (Yu *et al.,* 2004). The *pksA* gene is located in the aflatoxin biosynthetic pathway gene cluster (1.5 kb) and linked to the *nor-1* gene, which is required for the conversion of NA to averantin (AVN) (Chang *et al.*, 1995). Therefore, disruption of the *pksA* gene prevents NA synthesis in *A. parasiticus* and *A. flavus* (Chang *et al.*, 1995), which could account for the loss of NA in our preliminary work with *A. parasiticus* B62. The purification of sub-fraction E3 afforded two pure

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bioactive compounds, DQ and 3HDQ, the latter of which is novel. Although the antibacterial potential of DQ has been reported (Lajubutu *et al.*, 1995), the current study is the first to report on the anti-aflatoxigenic activity of both DQ and 3HDQ against *A. flavus* and *A. parasiticus*.

The inhibitory effects of DQ and 3HDQ on vegetative growth, sporulation and aflatoxin production by wild-type strains of *A. flavus* and *A. parasiticus* are shown in Figure 2. Notably, the ability of 3HDQ to stimulate aflatoxin production six-fold in *A. flavus* at lower concentrations as well as the ability of DQ to strongly inhibit aflatoxin production even at lower concentrations without adversely impacting the vegetative growth of the moulds are not entirely surprising. Similar results were reported for an essential oil (consisting of eugenol and acetyleugenol) extracted from *Piper betle,* which promoted aflatoxin production by *A. flavus* as compared to the control at 0.1 µg/ml (Prakash *et al.*, 2010). When the dose was increased to 0.6 µg/ml, complete inhibition of aflatoxin production was observed. The action of 3HDQ against *A. flavus* illustrates that some specific plant-derived compounds are capable of decreasing radial filamentous growth without inhibiting conidiation or aflatoxin production at a lower concentration. On the other hand, the action of DQ is congruent with bioactive compounds known to inhibit aflatoxin production completely without exerting severe effects on the vegetative growth or conidiation (Fajardo *et al.*, 1995). Jayashree and Subramanyam (1999) reported that eugenol significantly inhibited aflatoxin production by *A. parasiticus* without inhibiting vegetative growth. Similarly, Roze and coworkers (2011b) reported a more than 90% reduction in aflatoxin accumulation in *A. parasiticus* using volatile compounds from willow bark (*Salix acutifolia*), which concurrently promoted sporulation by 20% as compared to the control. Another study reported a substantial decrease in aflatoxin production by *A. flavus* and *A. parasiticus* exposed to carvacrol and *trans*-cinnamaldehyde even though these compounds caused minimal growth inhibition on both moulds (Yin *et al.*, 2015).

The modes of action of DQ and 3HDQ at the genetic level against these moulds are unknown at this point and are subject to further investigation. However, our data demonstrate that the two candidates exert differential impacts on these fungal species. The bioactive DQ and HDQ compounds are highly conjugated phenolic structures, suggesting that they are strong antioxidants and their inhibitory activity to aflatoxin synthesis may be attributed to such conjugated structures. Previous studies demonstrate that strong oxidising compounds promote aflatoxin accumulation while strong antioxidants inhibit aflatoxin accumulation (Fanelli and Fabbri, 1989; Reverberi *et al.*, 2006). Perhaps related to the oxidizing power, we propose that the hydroxyl group (OH) at carbon 3 of 3HDQ may be a critical site for triggering generation of reactive oxygen species (ROS), which are thought to stimulate aflatoxin biosynthesis in *A. flavus*. The stimulation of aflatoxin synthesis may be a defensive response mechanism against ROS to protect the organism from oxidative stress (Grintzalis *et al.*, 2014; Roze *et al.*, 2015). This may explain at least in part why exposure of *A. flavus* cells to lower doses (3.125, 6.25, 12.5, 25, and 50 µg/ml) of 3HDQ caused the fungus to produce more total aflatoxin (872 ng/plate at 12.5 µg/ml) than untreated *A. flavus* (AF3357 only), which produced 157 ng/plate. We speculate that at lower concentrations, the OH at carbon 3 of 3HDQ is amenable to losing an electron from the oxygen atom (can be mediated by ferric (III) ions available in the cytosol) to create radical cations. These free radical cations might serve more as pro-oxidants than antioxidants. Pro-oxidants induce lipid peroxidation creating more ROS, which in turn promote aflatoxin biosynthesis in an attempt to offset the stressful oxidative environment (Grintzalis *et al.*, 2014; Roze *et al.*, 2015). In contrast, DQ did not stimulate aflatoxin production in *A. flavus* (Figure 2E) suggesting that the absence of OH at carbon 3 enhances its ROS quenching ability resulting in the molecule serving more as an antioxidant than pro-oxidant. This predicted antioxidant activity makes it a better ROS scavenger. The scavenging strength of antioxidants has been reported to be necessary for inhibiting aflatoxin production (Reverberi *et al.*, 2006).

Since DQ inhibited aflatoxin production in each of the fungal strains even at low concentrations without severely impacting growth, it may be a better free radical scavenger than 3HDQ. Furthermore, DQ's proposed antioxidant activity appears to be more pronounced in *A. parasiticus* than in *A. flavus*. Both bioactive compounds equally inhibit aflatoxin biosynthesis in *A. parasiticus* suggesting that the presence of OH at C3 in 3HDQ does not guarantee weak antioxidant activity across fungal strains. Furthermore, both DQ and 3HDQ were equally better inhibitors of aflatoxin production against *A. parasiticus* than *A. flavus.* The reason for this difference is not known and it is subject to further investigation but it could be attributed to their genetic differences. Although 3HDQ promoted aflatoxin production in *A. flavus* at lower concentration of 12.5 µg/ml, our data show that its aflatoxin inhibition strength increased steadily from 12.5 to 100 µg/ml. The proposed positive feedback mechanism between antioxidant activity and aflatoxin production inhibition associated with DQ and 3HDQ treatment is in good agreement with observed biological activities of known phenolic antioxidants including butylated hydroxyanisole (BHA) (Fung *et al.*, 1977), cinnamaldehyde (Juglal *et al.*, 2002), and carvacrol (Yin *et al.*, 2015). These studies showed a direct relationship between free radical scavenging power of antioxidants and inhibition of aflatoxin production by *A. flavus* and *A. parasiticus*. In support of this proposed mechanism, phenolic antioxidants are very powerful free radical

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scavengers in nature and their introduction into the mould cytosol has been reported to decrease free radical levels and aflatoxin production drastically in the cell (Jayashree and Subramanyam, 1999).

Most plant-derived inhibitors of aflatoxin biosynthesis have been reported to exert their inhibitory effects at least at one of the following three levels: (1) alteration of the physiological environment of the cell; (2) interference with signal transduction and gene regulatory networks that control the aflatoxin biosynthetic pathway; and (3) blockage of active sites of aflatoxin biosynthesis enzymes (Holmes *et al.*, 2008). In view of level 2 and 3, the quinone moiety in DQ and 3HDQ provides an alternative mechanistic explanation to account for their ability to inhibit aflatoxin production. We reasoned that the quinone moiety in DQ and 3HDQ mimics the anthraquinone moiety in NA (compare red-highlighted parts of the molecules in Figure 3). Thus, we hypothesise that DQ and 3HDQ can competitively bind to the active site in the polyketide synthase (PKS) encoded in the *pksA* gene, which is responsible for the synthesis of NA, the first stable intermediate in the aflatoxin biosynthesis pathway (Yu *et al.*, 2004). DQ or 3HDQ binding could inhibit the activity of this key enzyme either competitively or via a suicide substrate mechanism thereby down-regulating production of NA and the end products, aflatoxins. This mechanistic hypothesis is consistent with our screening data illustrating that exposure of *A. parasiticus* B62 to the root powders, methanolic extract, and fractions A, B, F, E2 and E3, inhibited the accumulation of NA in the growth medium. Furthermore, it is reasonable to speculate that 3HDQ is unable to bind as effectively to the active site of *pksA* in *A. flavus* due to the presence of the OH group at carbon 3 (C3) rendering the compound less able to inhibit *pksA* activity at lower concentrations. However, the binding to *pksA* may be increased at high concentrations in *A. flavus* accounting for the ability to inhibit *pksA* at these higher concentrations. In contrast, this proposed mechanism also suggests that the absence of the OH group at C3 in DQ may enable DQ to fit into the active and bind effectively to the active site of *pksA* in either of the aflatoxin-producing fungi in this study.

The double bond present at carbons 8, 9 of the furofuran (bisfuran) moiety in aflatoxin B_1 is bioactivated by cellular cytochrome P450 enzymes primarily in liver microsomes of animals generating a highly reactive aflatoxin-8,9-epoxide that accounts for aflatoxin being designated a group 1 carcinogen (Iyer *et al.*, 1994). The carcinogenicity arises from aggressive reactions of the aflatoxin-8,9-epoxide species with DNA (Iyer *et al.*, 1994) and these adducts block ability of the DNA to be expressed or replicated. Similarly, it is possible that DQ and HDQ mimic this biological activity of AFB_1 accounting for their capacity to inhibit fungal growth and perhaps conidiospore development as well. Aspergilli compartmentalise aflatoxins in specialised vesicles called aflatoxisomes located in the cytosol enabling them to avoid aflatoxin's deleterious effects on their DNA and protein molecules and to transport them to the cell exterior most likely via exocytosis (Chanda *et al.*, 2009; Roze *et al.*, 2011a). Since DQ and 3HDQ are xenobiotic, aspergilli may be unable to compartmentalise them upon introduction into the cell. Thus, the potentially highly reactive 2',3'-epoxide groups of DQ and 3HDQ may be free to intercalate and react with cellular macromolecules such as DNA, and proteins (Figure 3B) to form DNA and proteins adducts (Da Cruz Cabral *et al.*, 2013). Also, the phenolic nucleus and epoxide groups might damage cell membranes

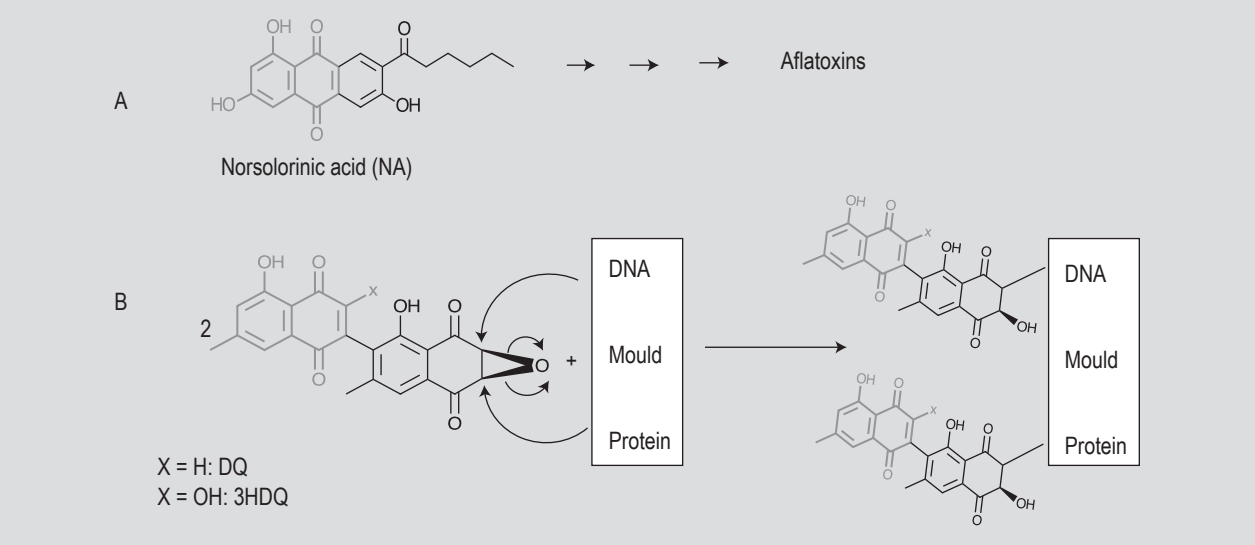


Figure 3. (A) The anthraquinone moiety of norsolorinic acid (grey-highlighted) might be mimicking the quinone moiety of diosquinone (DQ) and 3-hydroxydiosquinone (3HDQ) in their mode of actions. (B) Proposed possible interactions of DQ and 3HDQ with DNA and protein within the cell of the mould.

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and cause leakage of intracellular macromolecules such as ATP to the cell exterior leading to energy dissipation and cell death (Fung *et al.*, 1977). The OH groups can form hydrogen bonds with the active sites of various enzymes in the cell and disrupt their activity (Farag *et al.*, 1989). Together, these actions are vital to inhibition of vegetative fungal growth, conidiospore development, and aflatoxin biosynthesis.

The significance of these findings may be twofold. First, if the 'mimicry' theory is correct, then the inhibitory actions of DQ and 3HDQ are most likely specific to *pksA*, a key enzyme in the early stages of aflatoxin biosynthesis. Studying and understanding the structures of inhibitors that can best inhibit/block the activity of this enzyme are crucial in incapacitating the moulds to produce aflatoxins (Holmes *et al.*, 2008). The fact that DQ demonstrated stronger inhibition of aflatoxin production (>97%) without severely impacting the vegetative growth may endorse it as a preferred inhibitor candidate for future studies designed to investigate potential mechanisms by which it blocks *pksA*. Also, the fact that lower doses of 3HDQ stimulated aflatoxin production in *A. flavus* and not in *A. parasiticus* may provide an incentive to study how specifically *pksA* might be impacted by binding to 3HDQ. Studies on the chemical structures that enhance or prevent specific binding to *pksA* would be useful in the design of inhibitors of fungal growth and aflatoxin production for the complete elimination of aflatoxin in food and feed in the future.

The long-term goal of this research was to search for inexpensive plant-derived fungicides, which could be used to eliminate aflatoxin accumulation on economically important crops like corn, peanuts, and tree nuts under storage conditions. The capacity of crude root powders, extracts, and compounds to inhibit vegetative growth as well as NA accumulation supports that economically challenged farmers in tropics may use chips of *D. mafiensis* root bark to protect their crops under storage conditions. Therefore, the use of this plant for aflatoxin mitigation would invite the necessity of its domestication and cultivation for large-scale application in tropical countries where it thrives.

5. Conclusions

To our knowledge, the work described above represents the first study to report on the antiaflatoxigenic activity of DQ and 3HDQ from *D. mafiensis*. Both compounds were strong inhibitors of aflatoxin production by *A. parasiticus* and *A. flavus*. Thus, they could be used to prevent aflatoxin accumulation in stored food crops. Because *D. mafiensis* has been utilised effectively as a medicinal plant throughout recent history demonstrates promise for the safe application of root powders and extracts to prevent aflatoxin biosynthesis in a practical and safe manner. Toward this end, future work will evaluate the performance of root chips, DQ, and 3HDQ to avoid fungal spoilage and aflatoxin accumulation in food crops and feed during storage. Also, future evaluation of the performance of *D. mafiensis* should consider the versatility of plant materials in relation to seasonality. Most importantly, the toxicity studies of these antifungal natural chemicals are recommended before approved for use in food and feed.

Supplementary material

Supplementary material can be found online at https://doi.org/10.3920/WMJ2016.2107.

Figure S1. Screening the inhibitory effects of fractions A, B, D, E, and F fractionated from crude methanol extract of *Diospyros mafiensis* root bark on vegetative growth and norsolorinic acid production by a mutant *Aspergillus parasiticus* B62.

Figure S2. Dose-response inhibitory effects of DQ and 3HDQ on vegetative growth of *Aspergillus flavus* and *Aspergillus parasiticus*.

Figure S3. 1H NMR spectrum of DQ.

Figure S4. 1H NMR spectrum of 3HDQ.

Figure S5. Mass spectrum of 3HDQ.

Figure S6. ^{13}CNMR Spectrum of 3HDQ.

Conflict of interests

The authors declare that there are no conflicts of interest in publishing this work.

Acknowledgements

We are grateful to the generosity of the American People who funded this work through the United States Agency for International Development (USAID)-funded Innovative Agricultural Research Initiative project (iAGRI) (Award No. CA-621-A-00-11-00009-00). This research is also a contribution from Michigan State University AgBioResearch. The NMR data were obtained on instrumentation that was purchased, in part, with the funds from NIH grant no. 1-S10-RR04750, NSF grant no. CHE-88-00770, and NSF grant no. CHE-92-13241. MS data were obtained at the Michigan State University Mass Spectrometry Facility, which is supported, in part, by a grant (DRR-00480) from the Biotechnology Research Technology Program, National Center for Research Resources, National Institutes of Health. The opinions expressed herein are those of the authors and do not necessarily reflect the views of the U.S. Agency for International Development or the United States Government.

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