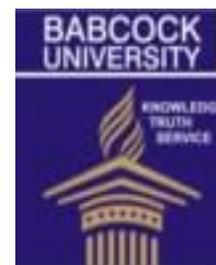




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Preliminary screening of aqueous extract of *Piper guineense* against aflatoxin biosynthesis in *Aspergillus flavus* and *A. parvisclerotigenus*

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Abstract

Food contamination by aflatoxigenic *Aspergillus* and consequent production of aflatoxins remain a significant threat to food safety. This has led to the continual search for plants with antifungal and anti-mycotoxigenic potencies for controlling moulds in food commodities. Aqueous extract of *Piper guineense* (Ashanti pepper, AP) was evaluated for antioxidant activity and inhibitory potential against growth and aflatoxin production in nine strains of aflatoxigenic *Aspergillus* [*A. flavus* ($n = 4$) and *A. parvisclerotigenus* ($n = 5$)] grown on neutral red desiccated coconut agar (NRDCA) for 5 days. Liquid chromatography tandem mass spectrometric analysis of 3 mm agar plugs from the inoculated AP-amended NRDCA treatments showed variable levels of aflatoxin inhibition. The 0.5% (v/v) AP extract inhibited AFB₁ in *A. flavus* by 91.1–99.7%, and by 57.8–95.6% in *A. parvisclerotigenus*. Inhibition of the different types of aflatoxins correlated significantly (AFB₁/AFG₁: $r = 0.87$, $p = 0.003$; AFB₁/AFM₁: $r = 0.94$, $p = 0.0001$; AFG₁/AFM₁: $r = 0.86$, $p = 0.005$). However, there was no inhibition of fungal growth at all tested concentrations of the extract though the antioxidant levels of the spice were appreciably high. This study reveals the suppressive effect of *P. guineense* aqueous extract on aflatoxin biosynthesis.

Keywords: Aflatoxin; *Aspergillus*; metabolites; spices; toxigenicity.

1. Introduction

Aflatoxins are toxic secondary metabolites produced by fungi of the genus *Aspergillus*. *Aspergillus flavus* and its congeneric relatives (*A. minisclerotigenes*, *A. parasiticus* and *A. parvisclerotigenus*) are the primary producers of aflatoxins in West Africa (Varga *et al.*, 2011; Probst *et al.*, 2014) amongst the over 20 species known to produce aflatoxins worldwide (Varga *et al.*, 2011). These species contaminate diverse food commodities and animal feed (Varga *et al.*, 2011; Ezekiel *et al.*, 2013a, 2013b, 2014c, 2014d; Probst *et al.*, 2014). Moreover, Frisvad *et al.* (2005) reported the presence of *A. parvisclerotigenus* in Nigerian peanut samples. *Aspergillus flavus* is usually predominant in many crops grown across various agro-climatic conditions and produces only B aflatoxins, whilst the other aforementioned species are less frequently found in many commodities but they are more prevalent in hot climatic zones and are capable of producing higher levels of both B and G aflatoxins (Atehnkeng *et al.*, 2008; Varga *et al.*, 2011; Probst *et al.*, 2014). It has been suggested that aflatoxin production in *Aspergillus* species is part of the cellular response to oxidative stress (Reverberi *et al.*, 2007; Roze *et al.*, 2013).

The presence of aflatoxins in food commodities and animal feed at levels above stipulated limits makes such food and feed unsafe for human or animal consumption. Dietary aflatoxin ingestion has been linked to malnutrition, stunted growth and poor development of cognitive function in children, as well as liver cancer, immunosuppression and increased susceptibility of exposed individuals to

diseases such as malaria and HIV (Turner *et al.*, 2000; Gong *et al.*, 2002; Turner *et al.*, 2003; Gong *et al.*, 2003, 2004; Shuaib *et al.*, 2010; Gong *et al.*, 2012). In animals, aflatoxins cause feed refusal, affect growth, reduce output and lower productivity (Bondy and Pestka, 2010). These health effects and economic losses in the agriculture/food and trade sectors underscore the severity of aflatoxin contamination in agricultural crops especially in Africa where climatic conditions, poor post-harvest handling and storage conditions favor crop contamination; thus, the need to mitigate its occurrence.

Of the several measures that have been adopted to control aflatoxin levels in food and animal feeds (Allameh *et al.*, 2011), the use of synthetic fungicidal chemicals is one of the most common (Guadalupe *et al.*, 2013). However, the persistence of these chemicals in the environment coupled with the toxic and carcinogenic nature of many of them has led to increased legislations against their use in controlling aflatoxin level in foods. There is a shift in attention towards natural products (e.g. bioactive compounds in plant extracts) that possess anti-aflatoxigenic properties (Juglal *et al.*, 2002; Soliman and Badaea, 2002; Sánchez *et al.*, 2005; Dimic *et al.*, 2008; Kocić-Tanackov *et al.*, 2012; El-Nagerabi *et al.*, 2013).

Piper guineense (Ashanti pepper, AP) is a common West African spice with preservative and antioxidant properties (Kiin-Kabari *et al.*, 2011). Recently, it was reported that the whole fruits or powdered form of AP could reduce aflatoxin contamination by nearly 99% when co-stored with maize grains (Ezekiel *et al.*, 2014b). On this basis, it was therefore expedient to investigate and identify the

inhibitory mechanism by which AP affects aflatoxin production in the toxin-producing fungi. Therefore, this study was designed to investigate the inhibitory effects of aqueous extracts of AP on fungal growth and aflatoxin production with the view of gaining preliminary information regarding possible inhibition by the extract.

2. Materials and methods

2.1. *Aspergillus isolates*

Four isolates of aflatoxigenic *A. flavus* and five isolates of *A. parvisclerotigenus* previously obtained from peanut cake and dried edible mushrooms (Ezekiel *et al.*, 2013a, 2013b) were used in this study. The isolates were deposited in the culture collection of the CBS-KNAW Fungal Biodiversity Centre, Netherlands (Accession numbers: CBS 133262 – CBS 133265 for mushrooms isolates and CBS 133921 – CBS 133925 peanut cake isolates; Ezekiel *et al.*, 2014a). All isolates were maintained at 4°C as 5/2 agar (5% V–8 juice and 2% agar, pH 5.2) plugs in sterile water. Prior to inhibition experiments, isolates were pre-grown on plates containing 5/2 agar medium for 5 days.

2.2. *Plant material and preparation of extract*

Fresh fruits of AP were purchased from Oyingbo market, Lagos, Nigeria in November 2011 and air dried for 4 days. Afterwards, they were ground into fine powder using a Waring automatic blender (Marlex Emerald UNIT III, Daman) and stored in a Ziplock® bag at 4°C until use for extraction within 12 hours. Plant extract was prepared by mixing 200 g of the powdered spice in 600 ml of cold sterile distilled water (1:3 w/v) in a 1L Erlenmeyer flask. The mixture was stirred vigorously for 2 min and allowed to stand for 6 hours. The homogenate was then filtered (Whatman No. 1 filter paper) into a sterile bottle and stored at 4°C until further use within 12 hours.

2.3. *Phytochemical screening of AP fruits aqueous extract*

2.3.1 *Qualitative assay of phytochemicals*

Thirteen phytochemicals (phenols, alkaloids, flavonoids, amino acids, tannins, anthraquinones, saponins, steroids, phytosterol, reducing sugars, terpenoids, cardiac glycosides and chalcones) were qualitatively determined in the aqueous extracts of AP following standard procedures (Sofowora, 1996; Harborne, 1998).

2.3.2 *Quantitative assay of total phenolic constituents*

Total phenolic content was determined as described by McDonald *et al.* (2001) with slight

modifications. A calibration curve was prepared by mixing ethanol solution of Gallic acid (1 ml; 0.025 – 0.400 mg/ml) with 5 ml Folin-Ciocalteu reagent (diluted tenfold with deionized water) and sodium carbonate (4 ml, 0.7 M). After 30 mins the absorbance values were measured at 765 nm. Similarly, total phenolic content of 1 ml of the aqueous extract (5 g/L) was determined. All measurements were done in triplicates and the total phenolic content in the plant extract, expressed in gallic acid equivalent (GAE), was calculated using the following formula:

$$T = C \times V / M \dots \dots \dots (1)$$

Where:

T is total phenolic content (mg/g plant extract) in GAE,

C is the concentration of gallic acid established from the calibration curve (mg/ml),

V is the volume of extract (ml),

M is the weight of methanol plant extract (g).

2.3.3 *Quantitative assay for total flavonoids*

The Aluminium chloride colorimetric method was used for flavonoid determination (Chang *et al.*, 2002). One millilitre of plant extract (10 mg/ml) was mixed with 3 ml of methanol, 0.2 ml of 10% aluminium chloride, 0.2 ml of 1M potassium acetate and 5.6 ml of distilled water. The mixture was set up in triplicates and kept at room temperature for 30 mins. The absorbance of the reaction mixture was then measured at 420 nm in a UV visible spectrophotometer. Total flavonoid content (mg/ml) was determined from a calibration curve obtained from quercetin solution (0 – 0.8 mg/ml) in distilled water.

2.4. *Assay for antioxidant activity of AP fruits aqueous extract*

2.4.1 *Determination of ferric reducing antioxidant power*

Ferric reducing antioxidant power of the extract was quantified as described by Kumar and Hemalatha (2011). One millilitre of the aqueous extract or standard ascorbic acid was added to a mixture of 2 ml 0.2M phosphate buffer pH (6.6) and 2.5 ml K₃Fe(CN)₆ (1%). The mixture was incubated at 50 °C for 30 mins followed by the addition of 2.5 ml H₂O₂ and 0.5 ml 0.1% FeCl₃. Absorbance was determined at 700 nm against blank sample. Ascorbic acid solution served as positive control and all samples were analyzed in triplicates.

2.4.2 Determination of DPPH radical scavenging activity

The 2,2-diphenyl-2-picrylhydrazyl (DPPH) assay system involving a qualitative rapid thin layer chromatography (TLC) screening and UV visible spectrophotometric quantification of antioxidant activity was carried out on the AP extract (Liyanapathirana and Shahidi, 2005). Four microliters of the AP aqueous extract solution was spotted on a silica gel pre-coated aluminum plate and developed in methanol: ethyl acetate (2:1, v/v). Afterwards, the plate was air-dried, sprayed with 0.2% w/v DPPH spray in methanol and visualized for the presence of yellow spots. For the quantitative assay, 1 ml of 0.135 mM DPPH prepared in methanol was added to 1 ml AP extract. The resulting mixture was vortexed thoroughly prior to incubation at ambient temperature in a dark chamber for 20 mins. Thereafter, the absorbance of the mixture was measured at 517 nm and results expressed as “mg ascorbic acid” (range: 0.1 – 0.5 mg/ml). All samples were analyzed in triplicates. The scavenging activity of the DPPH radical was expressed as inhibition percentage using the following equation:

$$\% \text{ inhibition} = [(AB - AS) / AB] \times 100 \dots\dots\dots (2)$$

Where:

AB is the absorbance of the control reaction,

AS is the absorbance of the test compound.

2.4.3 Determination of hydroxyl scavenging activity

The method of Smirnov and Cumbe (1989) with modifications adapted by Sudha *et al.* (2011) was used to determine hydroxyl scavenging activity of the extract. Reaction mixtures (3 ml) containing 1.0 ml of 1.5 mM FeSO₄, 0.7 ml of 6 mM hydrogen peroxide, 0.3 ml of 20 mM sodium salicylate and varied concentrations (0.025 – 0.400 mg/ml) of the extract were prepared and incubated for 1 hour at 37 °C. The absorbance of the hydroxylated salicylate complex was measured at 562 nm in a UV visible spectrophotometer. The scavenging activity of hydroxyl radical effect was then calculated as follows:

$$\% \text{inhibition} = 100[(A_1/A_2) \times 100] \dots\dots\dots (3)$$

Where:

A₁ is the absorbance in the presence of extract,

A₂ is the absorbance of control without extract.

2.5. Effects of AP extracts on fungal growth and aflatoxin biosynthesis in NRDCA

2.5.1 Assessment of growth of *A. flavus* and *A. parvisclerotigenus*

The effect of AP aqueous extract on growth of *A. flavus* and *A. parvisclerotigenus* was tested on neutral red desiccated coconut agar (NRDCA) by the modified agar plate method as described by Matamoros-León *et al.* (1999). Fifteen millilitres of NRDCA was prepared in 20 ml vials as described by Atanda *et al.* (2011), then cooled to about 45°C. Various volumes of the extract (0.5 ml, 1 ml or 2 ml corresponding to 0.125%, 0.25% or 0.5% v/v, respectively) were separately added to each vial and the mixture was carefully agitated. The resultant molten mixture was poured into 9 cm sterile Petri plates and allowed to solidify before the agar plates were inoculated at the center with each aflatoxigenic isolate. Control agar plates (NRDCA without extract) were also set up and inoculated with each aflatoxigenic isolate. The experiment was performed in triplicates for each isolate and the inoculated plates were incubated without illumination for 5 days at 30°C.

The radial colony growth was taken daily for 5 days from which percentage inhibition of fungal growth was calculated using the equation of Pandey *et al.* (1982):

$$\% \text{inhibition} = [(C - T) / C] \times 100 \dots\dots\dots (4)$$

Where:

C is colony diameter on the control plate (cm),

T is colony diameter on the test plate (cm).

2.5.2 Determination of aflatoxins and precursor metabolites in inoculated media

The quantities of aflatoxins and other secondary metabolites produced in the treatment and control cultures were determined by liquid chromatography tandem mass spectrometry (LC-MS/MS) as indices of measuring the inhibitory activities of the AP extract. Five agar plugs (3 mm diameter each) were obtained from each (control and AP-amended) plate and placed in a 10 ml polypropylene tube (Sarstedt, Germany). The plugs were immersed in 2 ml sterile distilled water and 5 ml acetonitrile, and extracted for 90 min on a GFL 3017 rotary shaker (GFL, Burgwedel, Germany). The extracts were diluted in extraction solvent and injected as described by Malachová *et al.* (2014).

LC-MS/MS screening of the analytes was performed using a QTrap 5500 LC-MS/MS System (Applied Biosystems, CA, US) equipped with a TurboIonSpray electrospray ionization (ESI) source and a 1290 Series HPLC System (Agilent, Waldbronn,

Germany). Chromatographic separation was performed at 25 °C on a Gemini® C₁₈-column, 150 x 4.6 mm i.d., 5 µm particle size, equipped with a C₁₈ 4 x 3 mm i.d. security guard cartridge (all from Phenomenex, CA, US). Confirmation of positive analyte identification was obtained by the acquisition of two MRMs which yielded 4.0 identification points according to commission decision 2002/657/EC.

2.6. Statistical Analysis

All data evaluation and analyses were performed with SPSS® for Windows v.14.0 (SPSS, Chicago, IL, USA). The means of metabolites produced by the isolates in culture were separated by the Duncan's Multiple Range test, and tested for significance by one way analysis of variance at $\alpha=0.05$. Pearson's correlation analysis was performed on the inhibition data to determine the relationship among the inhibition responses of the various aflatoxin biosynthetic pathway metabolites, aflatoxins and cyclopiazonic acid as influenced by the spice.

3. Results and Discussion

3.1. Phytochemicals of AP aqueous extract

The phytochemicals detected in the aqueous extracts of AP included alkaloids, flavonoids and phenolic acids, which were consistent with previous studies on AP and other indigenous flavoring plant extracts (Edeoga *et al.*, 2005; Okwu and Josiah, 2006). These compounds have been reported to inhibit microbial activities *via* various mechanisms (Allameh *et al.*, 2011). For example, some plant-derived alkaloids such as Sampangine have been shown to exert antifungal activities by causing perturbations in the biosynthetic pathway of heme which is important during the response of some fungi to changing oxygen conditions (Agarwal *et al.*, 2008).

Phenolic compounds may be inhibitory as a result of their high affinity for protein binding (Shimada, 2006; Nacz *et al.*, 2011), which may result in the inhibition of important regulatory proteins in fungi. The total phenolic content of aqueous AP extract calculated from a calibration curve with regression equation of $Y = 14.78X$ (X is the concentration of standard in mg/ml, Y is the absorbance at 765 nm) was 17.0 ± 0.28 mg GAE/g. The total phenolic content of AP extract measured in this study was higher than values (~ 5 mg GAE/g) reported by Adefegha and Oboh (2012) for methanolic extracts of AP seeds. The total flavonoid content measured in garlic, coriander and cinammon from a previous study (Tacouri *et al.*, 2013) was however higher (4.4 – 23 times) than the values obtained in this study (0.9 ± 0.05 mg Quercetin equivalent (QE)/g).

3.2. Antioxidant activity of AP aqueous extract

The ferric reducing power of the extract and standard ascorbic acid with concentrations of 0.1 – 0.4 mg/ml was in the range of 1.01 ± 0.01 nm to 1.33 ± 0.02 nm and 0.2 ± 0.01 nm to 0.44 ± 0.06 nm, respectively. With this assay, high absorbance corresponds to high reducing power (Kumar and Hemalatha, 2011). This indicates that aqueous extract of AP is a strong reducing agent, thus with high antioxidant property.

The DPPH is a stable radical which can accept electron from a suitable electron donor or reducing agent. In its stable form, it is violet in color and maximally absorbs at 517 nm. Transfer of electrons to DPPH results in the “degradation” of its stable form, thus gradual disappearance of the violet color. The IC₅₀ represents the concentration of the reducing agent or electron donor at which the absorbance relative to the control is halved. IC₅₀ of 198.41 µg/ml was therefore recorded for AP extract thus indicating a higher antioxidant potential compared to the standard gallic acid (IC₅₀ = 234.74 µg/ml).

Exposure of cells to fungal toxin can lead to hydroxyl radical formation (Husain *et al.*, 1987) which leads to oxidation (Guillén *et al.*, 2000) and can indiscriminately cause severe damages to biomolecules. Therefore, natural products with the ability to scavenge hydroxyl radicals can mitigate health challenges arising from fungal toxins. The AP extract scavenged hydroxyl radical at a lower level (IC₅₀ = 216.45 µg/ml) than the standard gallic acid (IC₅₀ = 166.67 µg/ml). Overall, the antioxidant activities observed in the three assays were concentration-dependent. The antioxidant activity exhibited by AP therefore indicates that it may possess intrinsic compound(s) with anti-aflatoxic potential by reducing oxidative stress generated by the fungal toxin.

3.3. Effects of AP aqueous extract on fungal growth

Treatment of foodstuff with spices can be inhibitory to fungal growth and/or toxin production (Hitokoto *et al.*, 1980). Colony diameters of fungal isolates cultured on plant extract [*A. flavus*: 6.5 – 6.8 cm (mean = 6.65 ± 0.13 cm); *A. parvisclerotigenus*: 6.3 – 6.6 cm (mean = 6.45 ± 0.12 cm)] were comparable to those of isolates grown on extract-free NRDC [*A. flavus*: 6.5 – 6.7 cm (mean = 6.6 ± 0.09 cm); *A. parvisclerotigenus*: 6.4 – 6.6 cm (mean = 6.5 ± 0.08 cm)]. Result from this study indicates that there was no inhibition of fungal growth by the extract; this agrees with the reports of Adjovi *et al.* (2014) on fresh cassava inhibiting aflatoxin biosynthesis without

affecting fungal growth. Such observations have also been reported for aqueous extracts of other natural products such as Chinese traditional teas (Mo *et al.*, 2013). This may then imply that the bioactive components of AP aqueous extract, at the concentrations added, are not detrimental to the fungi.

3.4. Levels of aflatoxins and precursor metabolites produced in NRDCA

Varying concentrations of aflatoxins [aflatoxin B₁ (AFB₁), AFB₂, AFG₁, AFG₂ and AFM₁], six aflatoxin biosynthetic pathway/precursor metabolites and cyclopiazonic acid (CPA) were produced by *A. flavus* (Table 1) and *A. parvisclerotigenus* (Table 2) in NRDCA plates. The mean concentrations of aflatoxins, precursor metabolites and CPA produced in cultures of *A. flavus* ranged from 5.2–631 µg/kg, 0.002–25.0 µg/kg and 25,867 µg/kg, while they ranged from 13.8–777 µg/kg, 0.01–92.5 µg/kg and 3,935 µg/kg in cultures of *A. parvisclerotigenus*, respectively. *Aspergillus flavus* and *A. parvisclerotigenus* are known aflatoxin producing members of the *Aspergillus* section *Flavi* group (Frisvad *et al.*, 2005; Pildain *et al.*, 2008; Varga *et al.*, 2011). Consequently, these species produced appreciable quantities of either B or B and G aflatoxins in the medium. The six precursor metabolites, given in Tables 1 and 2 are listed in their order of production.

3.5. Inhibitory effects of AP aqueous extracts on aflatoxin biosynthesis

The three concentrations of aqueous AP extracts applied in our study showed varying levels of inhibition of aflatoxins and precursor metabolites produced in cultures of *A. flavus* and *A. parvisclerotigenus* (Tables 1 and 2). Nevertheless, in line with the observations in other aflatoxin inhibition studies (Yoshinari *et al.*, 2007; Zhang *et al.*, 2014),

Table 1: Concentrations ($\mu\text{g}/\text{kg}$) and percentage inhibition of six aflatoxin biosynthetic pathway metabolites[#], aflatoxins^S and cyclopiazonic acid produced by *Aspergillus flavus* grown on neutral red desiccated coconut agar amended with three concentrations of aqueous extracts of Ashanti pepper (*Piper guineense*) at 30 °C for 5 days.

Metabolites/mycotoxins	Concentrations ¹ ($\mu\text{g}/\text{kg}$)		Percentage inhibition of three extract concentrations								
			0.125% (ml/100 ml)			0.25% (ml/100 ml)			0.50% (ml/100 ml)		
	Range	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean
Norsolorinic acid [#]	0.001–0.004	0.002	0.0	56.3	14.1 ^c	0.0	46.2	11.6 ^{cd}	1.7	80.2	52.6 ^{bc}
Averantin [#]	0.5–3.0	1.5	11.3	65.8	34.8 ^{bc}	0.0	77.2	38.5 ^{bc}	67.4	87.7	79.0 ^{ab}
Averufin [#]	0.5–1.3	0.8	0.2	76.0	30.2 ^{bc}	0.0	72.5	41.1 ^{bc}	40.0	95.3	78.7 ^{ab}
Versicolorin A [#]	0.1–1.1	0.4	0.0	9.6	2.4 ^c	0.0	14.0	4.9 ^d	0.0	94.3	62.1 ^{abc}
Sterigmatocystin [#]	0.2–4.7	1.5	52.0	85.3	63.6 ^{ab}	41.4	91.9	66.3 ^{ab}	74.1	100.0	92.0 ^a
<i>O</i> -methylsterigmatocystin [#]	1.0–88.7	25.0	2.5	90.0	56.2 ^{ab}	57.6	93.6	77.1 ^a	77.3	96.1	89.6 ^a
Aflatoxin B ₁ ^S	307–900	631	45.2	82.4	72.4 ^a	89.5	95.9	92.9 ^a	83.7	99.7	94.5 ^a
Aflatoxin B ₂ ^S	6.3–50.5	24.5	62.0	89.5	82.0 ^a	91.7	98.5	96.0 ^a	90.7	99.8	96.7 ^a
Aflatoxin M ₁ ^S	3.0–8.3	5.2	67.9	82.4	76.3 ^a	91.2	97.9	93.8 ^a	88.1	100.0	95.3 ^a
Cyclopiazonic acid	7,556–51,500	25,867	0.0	51.1	13.5 ^c	0.0	42.9	8.5 ^{cd}	0.0	71.0	31.9 ^c

¹Concentrations produced in control (Neutral red desiccated coconut agar) plates.

Means with different superscript alphabets in a column are significantly different at $\alpha = 0.05$.

Table 2: Concentrations ($\mu\text{g}/\text{kg}$) and percentage inhibition of six aflatoxin biosynthetic pathway metabolites[#], aflatoxins^S and cyclopiazonic acid produced by *Aspergillus parvisclerotigenus* grown on neutral red desiccated coconut agar amended with three concentrations of aqueous extracts of Ashanti pepper (*Piper guineense*) at 30 °C for 5 days.

Metabolites/mycotoxins	Concentrations ¹ ($\mu\text{g}/\text{kg}$)		Percentage inhibition of three extract concentrations								
			0.125% (ml/100 ml)			0.25% (ml/100 ml)			0.50% (ml/100 ml)		
	Range	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean
Norsolorinic acid [#]	0.004–0.03	0.01	0.0	88.7	50.6 ^a	0.0	77.5	40.6 ^{ab}	0.0	92.1	36.7 ^b
Averantin [#]	1.3–26.6	10.9	35.6	87.8	68.5 ^a	28.6	89.7	62.6 ^{ab}	50.7	92.0	74.2 ^{ab}
Averufin [#]	0.3–3.7	2.1	0.0	77.6	47.8 ^a	0.7	71.5	40.1 ^{ab}	27.1	90.7	57.4 ^{ab}
Versicolorin A [#]	0.02–0.8	0.3	0.0	78.5	49.0 ^a	0.0	58.4	30.7 ^b	34.2	71.9	51.6 ^{ab}
Sterigmatocystin [#]	0.2–26.9	7.4	0.0	76.2	43.7 ^a	0.0	88.4	42.8 ^{ab}	0.0	87.2	46.3 ^{ab}
<i>O</i> -methylsterigmatocystin [#]	1.4–420	92.5	0.0	56.3	31.6 ^a	0.0	66.5	26.9 ^b	0.0	85.4	44.6 ^{ab}
Aflatoxin B ₁ ^S	58.0–1,125	770	34.9	49.8	42.2 ^a	44.0	82.1	61.7 ^{ab}	35.3	95.6	77.0 ^{ab}
Aflatoxin G ₁ ^S	290–1,895	777	27.0	54.4	45.9 ^a	23.2	93.5	71.3 ^a	46.1	99.4	78.3 ^a
Aflatoxin B ₂ ^S	2.3–31.7	22.4	24.6	69.0	49.9 ^a	55.8	86.7	76.8 ^a	39.3	97.1	82.1 ^a
Aflatoxin G ₂ ^S	5.5–50.3	15.4	40.7	65.9	56.1 ^a	35.3	96.4	79.2 ^a	55.8	99.0	84.4 ^a
Aflatoxin M ₁ ^S	2.0–38.7	13.8	41.5	61.7	52.8 ^a	54.7	88.3	75.4 ^a	28.1	96.9	79.0 ^a
Cyclopiazonic acid	1,915–8,400	3,935	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

¹Concentrations produced in control (Neutral red desiccated coconut agar) plates.

Means with different superscript alphabets in a column are significantly different at $\alpha = 0.05$.

production of commercially useful extrolites such as kojic acid (El-Kady *et al.*, 2014) was generally enhanced in strains grown in AP extract-amended media. At the same concentrations of AP aqueous extract, aflatoxin production in *A. flavus* (e.g. at 0.125%, 0.25% and 0.50% AP extract, inhibition = 72.4 – 82.0%, 92.9 – 96.0% and 94.5 – 96.7% respectively; Table 1) generally seemed to be more inhibited in relation to *A. parvisclerotigenus* (at 0.125%, 0.25% and 0.50% AP extract, inhibition = 42.2 – 56.1%, 61.7 – 79.2% and 77.0 – 84.4% inhibition respectively; Table 2).

Aflatoxin inhibition was concentration-dependent only in *A. flavus* strains CBS 133263 and CBS 133922 (Fig. 1) and *A. parvisclerotigenus* strains CBS 133923, CBS 133264 and CBS 133265 (Fig. 2). This suggests that different thresholds of AP aqueous extracts were necessary for optimal aflatoxin inhibition in different strains of *A. flavus* and *A. parvisclerotigenus*.

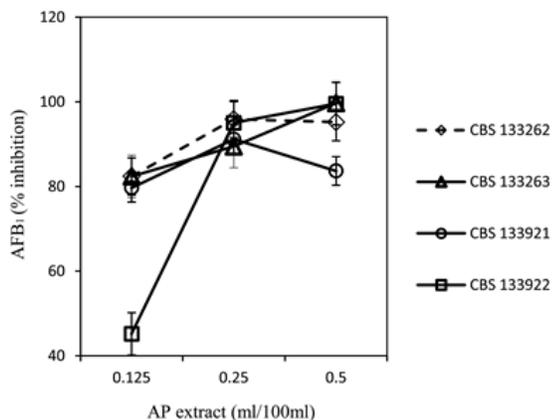


Fig. 1. Percentage inhibition of aflatoxin B₁ production in *Aspergillus flavus* isolates grown on neutral red desiccated coconut agar amended with three concentrations of Ashanti pepper (*Piper guineense*) aqueous extracts at 30 °C for 5 days.

Both species of *Aspergillus* studied in this study are phylogenetically related to other aflatoxin-producing members of the *Flavi* section (Frisvad *et al.*, 2005); thus significant variation in ability to withstand external influences should not be expected. Frisvad *et al.* (2005) also predicted that similar genes might be required for aflatoxin accumulation in members of the *Aspergillus* section *Flavi*. However, since regulation of these genes is quite complex and controlled by multiple regulatory components (Yu, 2012),

interspecific discrepancies in the level of aflatoxin inhibition at the same concentrations of AP extract may be observed as in this study. This is partly due to differences in the regulatory mechanisms of aflatoxin biosynthesis in *A. flavus* and *A. parvisclerotigenus*. In addition, it is noteworthy that micro-sclerotial species of *Aspergillus* section *Flavi* (e.g. *A. minisclerotigenes*, *A. parvisclerotigenus* and S_{BG} unnamed taxon) may have a more specialized mechanism to counteract the effects of anti-aflatoxic compounds. This assumption is based on the fact that these species are known to comfortably withstand higher temperature regimes (being prevalent in hotter and drier regions) and producing higher quantities of aflatoxins than *A. flavus* (Cardwell and Cotty, 2002; Atehnkeng *et al.*, 2008; Probst *et al.*, 2014). Not many of these species have been studied with respect to fungal inhibition; it is therefore proposed that more studies focusing on these minisclerotial species be undertaken to fully understand these interactions.

Correlation analysis did not show a consistent relationship in terms of percentage inhibition patterns of the AP extract against each species with respect to the pathway metabolites (*A. flavus*, Table 3; *A. parvisclerotigenus*, Table 4). However, both species showed significant relationships (AFB₁/AFG₁: $r = 0.87$, $p = 0.003$; AFB₁/AFM₁: $r = 0.94$, $p = 0.0001$; AFG₁/AFM₁: $r = 0.86$, $p = 0.005$) in their inhibition responses to the extract with respect to the aflatoxin types (Fig. 3). This might suggest that bioactive compounds in AP extract interfered with the intermediates present in aflatoxin biosynthetic pathway in *A. flavus* and *A. parvisclerotigenus* leading to aflatoxin inhibition. Nevertheless, further studies will be required to verify this hypothesis.

4. Conclusions

Aqueous extract of AP can inhibit aflatoxin production but not growth in several strains of two species of *Aspergillus* *in vitro*. The inability of the aqueous extract of AP to inhibit fungal growth may be beneficial in the sense that AP could be used as a natural compound to facilitate the production of commercially useful extrolites such as kojic acid. However, non-aqueous extract of AP may show different antifungal or antiaflatoxic effects, thus presenting an important area for further investigation. This study has provided insights into the suppressive effect of AP aqueous extracts on aflatoxin biosynthesis, and further lays the foundation for the identification, isolation, characterization and study of the mode of action of

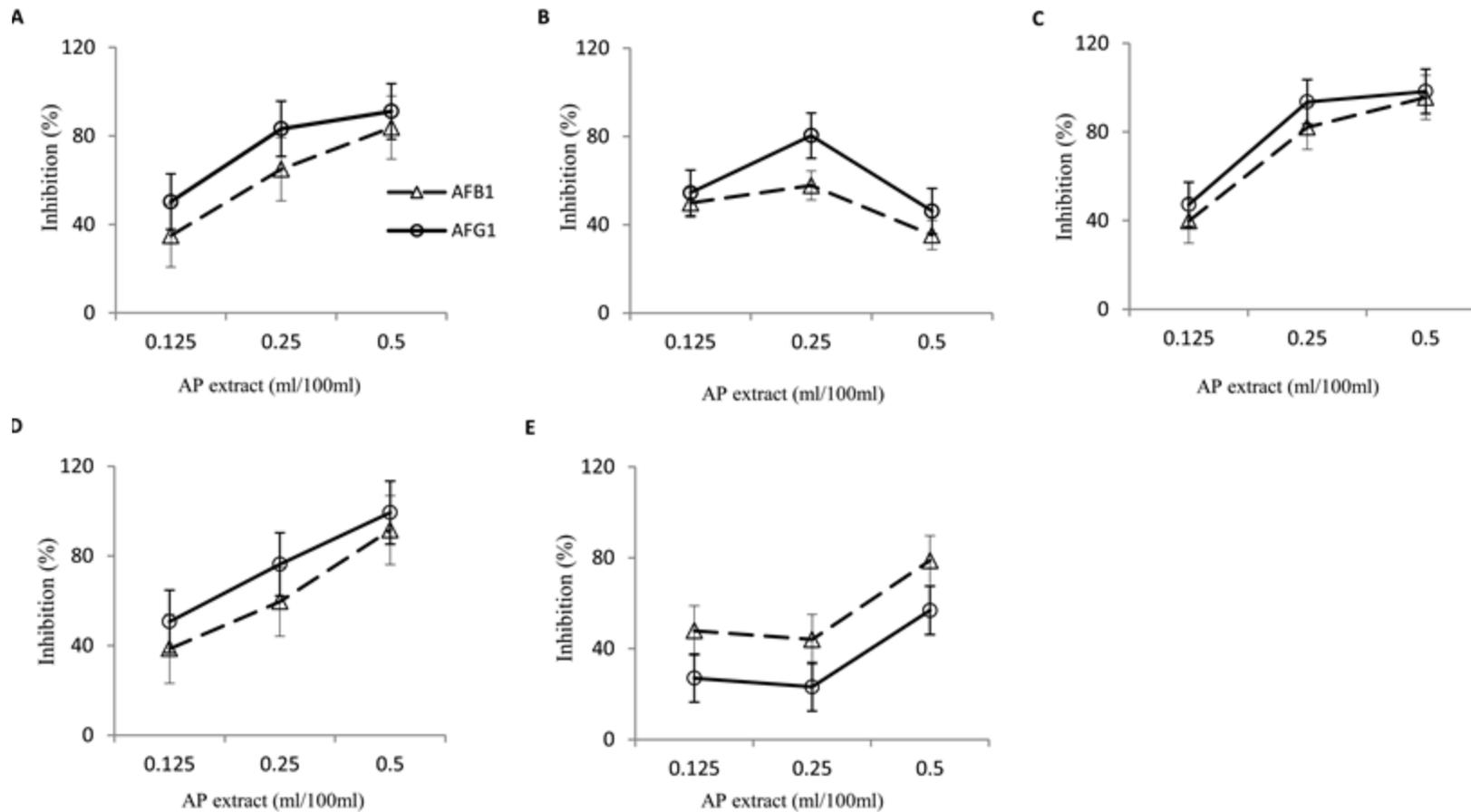


Fig. 2. Percentage inhibition of aflatoxin (B₁ and G₁) production in *A. parvisclerotigenus* (A: CBS 133923; B: CBS 133924; C: CBS 133264; D: CBS 133265; E: CBS 133925) grown on neutral red desiccated coconut agar amended with three concentrations of Ashanti pepper (*Piper guineense*) aqueous extracts at 30 °C for 5 days.

Table 3: Correlations^a of relationships among percentage inhibition of six aflatoxin biosynthetic pathway metabolites^b, aflatoxins^c and cyclopiazonic acid (CPA) produced by *Aspergillus flavus* in neutral red desiccated coconut agar amended with aqueous extracts of Ashanti pepper (*Piper guineense*) at 30 °C for 5 days.

	NOR-AC ^b	AVRT ^b	AVER ^b	VER-A ^b	STER ^b	O-STER ^b	AFB ₁ ^c	AFB ₂ ^c	AFM ₁ ^c	CPA
NOR-AC ^b	1.00									
AVRT ^b	0.81**	1.00								
AVER ^b	0.39	0.69*	1.00							
VER-A ^b	0.52*	0.61*	0.78**	1.00						
STER ^b	0.50	0.77**	0.95***	0.74**	1.00					
O-STER ^b	0.50	0.71**	0.75**	0.48	0.74*	1.00				
AFB ₁ ^c	0.31	0.44	0.66*	0.50	0.51*	0.83*	1.00			
AFB ₂ ^c	0.33	0.48	0.66*	0.45	0.52*	0.87**	0.99***	1.00		
AFM ₁ ^c	0.43	0.46	0.51*	0.55*	0.40	0.63*	0.88***	0.86**	1.00	
CPA	0.75**	0.62*	0.29	0.45	0.27	0.40	0.36	0.38	0.51	1.00

^aCorrelation coefficients significant at $p < 0.001 = ***$, $0.01 \geq p > 0.001 = **$, $0.05 \geq p > 0.01 = *$

^bAflatoxin biosynthetic pathway metabolites: NOR-AC, norsolorinic acid; AVRT, averantin; AVER, averufin; VER-A, versicolorin A; STER, sterigmatocystin; O-STER, O-methylsterigmatocystin.

^cAflatoxins: AFB₁, aflatoxin B₁; AFG₁, aflatoxin G₁; AFB₂, aflatoxin B₂; AFG₂, aflatoxin G₂; AFM₁, aflatoxin M₁.

Table 4: Correlations^a of relationships among percentage inhibition of six aflatoxin biosynthetic pathway metabolites^b, aflatoxins^c and produced by *Aspergillus parvisclerotigenus* in neutral red desiccated coconut agar amended with aqueous extracts of Ashanti pepper (*Piper guineense*) at 30 °C for 5 days.

	NOR-AC ^b	AVRT ^b	AVER ^b	VER-A ^b	STER ^b	O-STER ^b	AFB ₁ ^c	AFG ₁ ^c	AFB ₂ ^c	AFG ₂ ^c	AFM ₁ ^c
NOR-AC ^b	1.00										
AVRT ^b	0.86**	1.00									
AVER ^b	0.83**	0.97***	1.00								
VER-A ^b	0.57*	0.59*	0.57*	1.00							
STER ^b	0.80**	0.81**	0.73*	0.65*	1.00						
O-STER ^b	0.75*	0.77*	0.69*	0.67*	0.89**	1.00					
AFB ₁ ^c	-0.13	0.18	0.30	-0.14	-0.08	0.01	1.00				
AFG ₁ ^c	0.20	0.47	0.53	-0.16	0.24	0.24	0.82**	1.00			
AFB ₂ ^c	-0.24	0.03	0.16	-0.33	-0.16	-0.11	0.89**	0.77**	1.00		
AFG ₂ ^c	0.02	0.35	0.40	-0.19	0.14	0.03	0.80**	0.99***	0.78**	1.00	
AFM ₁ ^c	-0.23	0.04	0.16	-0.38	-0.20	-0.12	0.92***	0.82**	0.92***	0.81**	1.00

^aCorrelation coefficients significant at $p < 0.001 = ***$, $0.01 \geq p > 0.001 = **$, $0.05 \geq p > 0.01 = *$

^bAflatoxin biosynthetic pathway metabolites: NOR-AC, norsolorinic acid; AVRT, averantin; AVER, averufin; VER-A, versicolorin A; STER, sterigmatocystin; O-STER, O-methylsterigmatocystin.

^cAflatoxins: AFB₁, aflatoxin B₁; AFG₁, aflatoxin G₁; AFB₂, aflatoxin B₂; AFG₂, aflatoxin G₂; AFM₁, aflatoxin M₁.

Cyclopiazonic acid was not inhibited by aqueous extract of AP in *A. parvisclerotigenus*, therefore not included in correlation analysis.

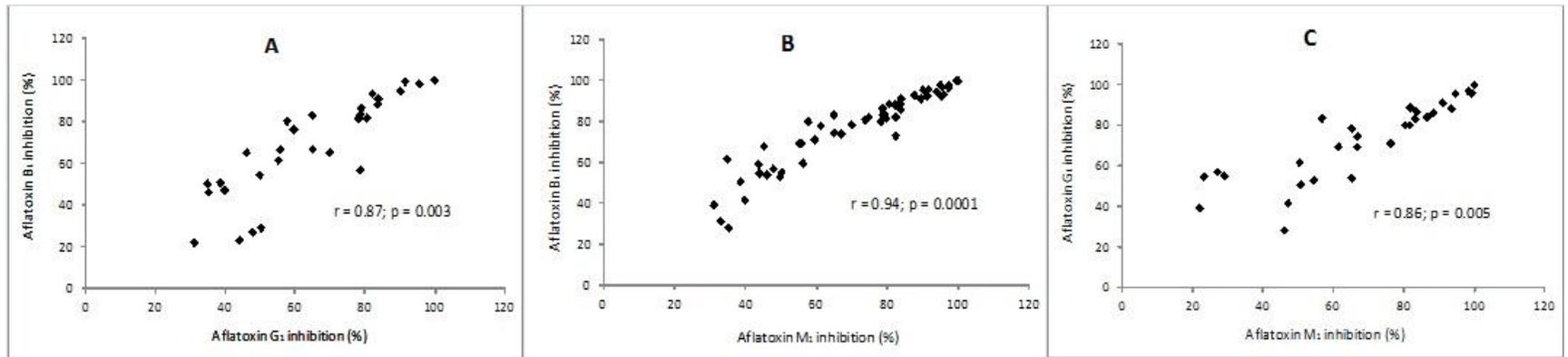


Fig. 3. The relationships between percentage inhibition of (A) aflatoxin B₁ and aflatoxin G₁, (B) aflatoxin B₁ and aflatoxin M₁ and (C) aflatoxin G₁ and aflatoxin M₁ production in *Aspergillus* species (*A. flavus* and *A. parvisclerotigenus*) induced by aqueous extracts of Ashanti pepper (*Piper guineense*). Correlation coefficient (r) is for untransformed percentage inhibition data.

bioactive compound(s) in the extract that target inhibition of aflatoxin biosynthesis.

Conflict of interests

The authors declare that they have no competing interests in the present work.

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