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Efficacy of selected medicinal plants from Eastern Kenya against *Aspergillus flavus*

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Abstract: Aflatoxins are a major problem in Eastern Province of Kenya and *Aspergillus flavus* has been associated with frequent outbreaks of aflatoxicoses in this region. This study evaluated the efficacy of 15 selected medicinal plants from Eastern Kenya against *A. flavus.* Different concentrations of 1000mg/ml, 750mg/ml and 400mg/ml using Agar Well Diffusion Method were used. Plants found to have inhibition zones of more than 10mm at 400mg/ml had their bark further assayed for antifungal activity. Both the leaf and bark extracts that were found to be effective were assayed for minimum inhibition concentrations (MIC) and minimum fungicidal concentrations (MFC) using Sabouraund Dextrose broth (SDB) micro-dilution method. The methanolic leaf and bark extracts of the fifteen plants assayed displayed concentration depended antifungal activities that was comparable to that of the reference drug Miconazole at 10mg/ml. Leaf extracts showed better antifungal activity than the bark extracts. For instance, *Boscia coriacea* (mean 17.40mm) had the highest zone of inhibition followed by *Zanthoxylem chalybeum* (mean 17.20mm). For the bark extracts, *Croton megalocarpus* (mean 15.0mm) recorded significantly high antifungal activity while *Tithonia diversfolia* (mean 13.0mm) had the lowest at 400mg/ml. *Senna siamea* had the lowest MIC and MFC of 6.25mg/ml and 12.5mg/ml respectively. The preliminary phytochemical analysis of the 15 effective medicinal plants revealed the presence of bioactive compounds that included tannins, saponins, flavonoids, terpenoids, cardiac glycosides and alkaloids. The results obtained from the study could be used as a viable management strategy against *A. flavus* and aflatoxins in the region so as to ensure low mycotoxin exposure as well as low environmental pollution.

Keywords: *Aspergillus flavus*, Aflatoxins, Antifungal Activity, Bioactivity, Phytochemicals

1. Introduction

Aspergillus flavus Link infects maize *(Zea Mays)* at pre and post-harvest stages (1;2). Further it is a leading aflatoxin producer worldwide (3; 4). The fungi has also been reported to contaminate various foods and feed with aflatoxins during the entire food chain (5). Aflatoxins are potent toxic, carcinogenic, mutagenic, immunosuppressive and teratogenic agents produced as secondary metabolites by *A. flavus* (6). Various pesticides are used in the management of these fungi. However there are significant drawbacks including increased cost, handling hazards, concern about pesticide residues in grains, threat to animals, human health and the environment (7). There is therefore a need to explore alternative management strategies against *A. flavus.* Such remedies include medicinal plants that have for centuries been used in treatment of human diseases (8). Plant products have also been used to safely preserve food (9; 10; 11; 12). Plant extracts have shown the ability to suppress growth of toxigenic fungi and hence reduction of toxin production (13-15). Antifungal compounds derived from plants include isothiocyantes (16), plant volatile compounds (17), hexapeptides (18), essential oils (19), phenolic compounds (20), polyphenols like trans-resveratrol (21-23) have been used to control fungi. Some of the natural products, such as extracts from *Maesa lanceolata* Forsskal (24), garlic (12) phenols, and many essential oils (25), have been reported as effective inhibitors to fungal growth and aflatoxin production. The antifungal properties are caused by many active phytochemicals, including flavonoids, terpenoids,

carotenoids, coumarins and curcumines (26). The current study investigated the antimicrobial effects of selected medicinal plant extracts against *A. flavus* with the ultimate aim of developing a cost effective and environmental friendly remedy against *A. flavus* in field and stored cereal grains.

2. Materials and Methods

2.1. Collection of the Medicinal Plants

Fifteen medicinal plants collected from Mwingi District in Eastern Province of Kenya were used in this study. The plants included *Albizia antihelmintica, Balanites aegyptiaca, Boscia coriacea, Carisa spiairum, Croton megalocarpus, Maerua decumbens Forssk, Ricinus communis, Psidium guajava, Salvadora persica, Senna siamea, Tamarindus indica, Tithonia diversifolia, Zanthoxylem chalybeum, Solanum incanum, and Melea volkensis.* These plants were identified in the herbarium of the Department of Plant and Microbial Sciences at Kenyatta University, where voucher specimens are deposited.

2.2. Sample Preparation and Extraction

The leaves and the barks of the collected plants were shade dried and crushed into powder using a crushing machine (Christy, Type: 8-lab mill, Christy and Norris. England). Then 200g of the powder of each plant were mixed with 1 litre of methanol and put in a shaker at 100 rpm for 24 hours. They were then passed through a sieve no.60 and filtered through a Whatman No. 1 filter paper before filter sterilization through a membrane filter (0.2µm) to avoid microbial contamination (27). The filtrate was concentrated in a rotatory evaporator to produce a semi-solid residue that was further dried into powder form (28). This was the crude extract that was mixed with methanol to desired concentration for determination of efficacy against *A. flavus*.

2.3.Phytochemical Screening of the Plant Extracts

The fifteen methanolic leaf extracts obtained were subjected to phytochemical screening methods according to Oyetayo, (29) and Sofowora, (30) so as to determine the presence of bioactive agents such as tannins, alkaloids saponins, flavonoids, terpenoids and glycosides. For the bark extracts, phytochemical screening was done for the plants whose leaf extracts proved to be effective against *A. flavus*.

2.4. Determination of Inhibition Concentration

The antimicrobial activities of the plant extracts were determined using agar well diffusion technique using Sabouraud Dextrose Agar (SDA) plates (31). The inoculums were prepared from actively growing fungal cultures that were 72 hours old by suspending them directly into sterile distilled water. Then SDA plates were seeded with 25-µL mycelial suspensions of *A. flavus* that had been diluted to 1.0 McFarland Standards (CFU x10^6/mL) (32) using a sterile glass L rod spreader. The seeded plates were allowed to set and then dried in an incubator at 37° C for 20 minutes. A standard aseptic cork borer of 7 mm diameter was used to cut 5 uniform wells on the surface of the agar. Then 60 µL of each of the plant extracts at different concentrations of 400mg, 750mg and 1000mg of solid extracts dissolved in 1ml 85% methanol introduced into each well with the aid of Pasture pipettes (33). The plates were then incubated at 27 ^{o}C for 72 hours after which diameters of zones of inhibition were measured. The plant extracts that produced an inhibition zone greater than 10mm were considered to be effective. A 10mg/ml of Meconazole was the positive control according to the manufacturer's instructions while, 85% methanol was included in each plate as the negative control. All the assays were carried out in five replicates. Diameters of the zones of inhibition of the plant extracts were determined as an indication of antifungal activity. These were then compared with that of the standard antifungal drug, Meconazole (33). The results of the antifungal activities of the plant extracts were then represented in the form of tables and graphs.

2.5. Determination of the Minimum Inhibition Concentration (MIC) and Minimal Fungicidal Concentration (MFC)

The MIC and the MFC were done using plant extracts that proved to be effective against *A. flavus* in the agar well diffusion method at 400mg/ml according to Rasooli and Abyaneh, (34). The MIC was aimed at finding the lowest concentration of the extract that would inhibit the growth of *A. flavus.* It was done using broth micro-dilution method where different concentrations (1.56mg/ml, 3.125 mg/ml, 6.25 mg/ml and 25mg/ml) of the extracts were prepared. A 5ml of sterile extract at different concentrations were put sterile empty 10ml Durham tubes. Then 1ml of *A. flavus* culture was added into the extracts and mixed. This was followed by addition of 1ml of the mixture into 5ml of sterile Sabouraud Dextrose Broth (SDB) in tubes. All the tubes were incubated at 30 ^{0}C for 15 days and observations made for presence of visible growth of fungi. The highest dilution without visible growth was regarded as the Minimum Inhibitory Concentration (MIC) during 15 days. The cell cultures from the tubes without growth were then sub-cultured on Sabouraud Dextrose Agar (SDA) plates and incubated at 30 ^{0}C for 5 days. This was to determine if the inhibition was reversible and to determine the Minimal Fungicidal Concentration (MFC).

The MFC was determined as the highest dilution at which no growth occurred on the SDA plates. For the positive controls, 1ml of the mixture (Meconazole +*A.flavus* culture) were added into SDB without the plant extracts. For the negative control 1ml of the mixture was added into 5ml SDB without the plant extract. All the treatments were done in triplicates. The tubes with the highest dilution (lowest concentration) that showed no visible growth in the SDB were recorded as MIC. The cultures from the highest dilution (lowest concentration) that produced no growth on the SDA plates were recorded as the MFC (35).

2.6. Data Analysis

Analysis of variance (ANOVA) was performed with MSTAT-C statistical package, Michigan State University, USA, Turkey test was used to compare means.

3. Results

3.1. Efficacy of Selected Plant Extracts on the Growth of A. Flavus

The crude extracts of these plants exhibited good antifungal activity against *A.flavus* with varied zones of inhibition at the different concentrations in vitro. Some of the plant extracts had large inhibition zones at different concentrations that compared favourably with those obtained with the standard antifungal agent meconazole (10mg/ml). The leaf extracts of the plants at 1000mg/ml showed variations in size of the inhibition zones with *B. coriacea* recording the highest inhibition zone of 24.40mm, followed by *C. megalocarpus* with inhibition zone of 23.20mm. The lowest inhibition zones at a concentration of 1000mg/ml were 7.00mm from *M. decumbens* and *S. persica* (Table 1). Inhibition of the growth of *A. flavus* by the plant extracts at 750mg/ml differed significantly ($F = 94.08$, $df = 15$, $P < 0.05$) with variations in the type of plants used. The study established that, the most effective plant extracts were *B. coriacea* (mean = 19.40mm), *C. megalocarpus* (mean = 19.00mm) and *S. siamea* (mean = 17.20mm). Plant leaf extracts from *M. decumben* and *S. persica* were not effective at 750mg/ml concentration (Table 1). Inhibition of the growth of *A. flavus* by the plant leaf extracts at 400mg/ml significantly differed ($F = 100.70$, $df = 15$, $P < 0.05$) with variations in the type of plants used. The study established that the most inhibitory plant extracts were *B. coriacea* (mean = 17.40mm), *Z. chalybeum* (mean = 17.20mm). Plant extracts from *A. antihelmintica, M. decumben* and *S. persica* did not inhibit growth of *A. flavus* at this concentration (Table 1).

The results of inhibition zones of the bark extracts at a concentration of 1000mg/ml indicated that there was a significant difference ($F = 159$, $df = 9$, $P < 0.05$) against the growth of the fungi. Plant *S. siamea* (mean = 21.2mm) had much higher inhibition effect on *A. flavus* than the remaining plant barks. The least inhibitory effect was detected on bark extract of *S. incunum* (mean = 14.4mm) (Table 2). The results of inhibition zones of the plant bark extracts at a concentration of 750mg/ml ($F = 190.5$, $df = 9$, $P < 0.05$) revealed that, *S. siamea* (mean = 20.40mm) was still the most effective in inhibiting the growth of *A. flavus*. However the mean inhibition zone was a bit lower than at the 1000mg/ml concentration level. The lowest inhibiting plant extract *T. diversifolia* (mean = 12.80mm) and *S. incunum* (mean =13.00mm) (Table 2). The inhibition zones of the plant bark extracts at 400mg/ml concentration showed that there was a significant difference ($F = 81.22$, $df = 9$, $P < 0.05$) in the inhibitory effects of the plants bark extracts. At this point the inhibition zones by the plants were lower than at the positive control (mean =17.4mm). The extract of *C. megalocarpus* (mean =15.00mm) had the highest inhibition zone (Table 2).

Table 1. *Inhibition of growth of A. flavus by plant leaf extracts at the different concentrations*

Plant name	Inhibition zones (mm) 1000mg/ml	Inhibition zones (mm) at 750mg/ml	Inhibition zones (mm) at 400mg/ml
A. antihelmintica	15.20bc	12.60b	0.00a
B. aegyptiaca	12.20c	9.80c	7.00b
B. coriacea	24.40a	19.40a	17.40c
C. spirinum	13.80bc	15.20b	16.40c
M. decumben	7.00d	0.00d	0.00a
R. communis	12.20c	10.60c	7.00b
P. guajava	14.60bc	15.20b	16.20cd
S. siamea	19.80b	17.20a	15.20d
S. persica	7.00d	0.00d	0.00a
T. indica	12.20b	10.80c	7.00b
T. diversifolia	20.00a	17.00a	15.40cd
Z. chalybeum	19.00b	18.20a	17.20d
S. incunum	21.00a	16.60ab	15.80a
M. volkensis	17.80b	14.20b	7.00b
C. megalocarpus	23.20a	19.00a	15.60cd
Positive control	19.40b	18.40a	18.20c

Note: Numbers are means of 10 replications. Means are separated by Turkeys Test. Numbers followed by the same letters in the same column are not significantly different ($P < 0.05$).

3.2. Determination of Minimum Inhibition Concentrations and (MIC) and Minimum Fungicidal Concentrations (MFC) of Leaf and Bark Extracts

The MIC and the MFC were done for the plant leaf and bark extracts that were found to be effective at 400 mg/ml. Different plant extracts exhibited different MIC and MFC at the different concentrations used; 1.56mg, 3.125 mg. 6.25 mg, 12.50 mg and 25mg. The extracts of *B. coriace, S. incunum, Z. chalybeum, and S. siamea* recorded the lowest MIC and MFC of 6.25 mg/ml and 12.5 mg/ml respectively. This was followed by *C. spirinum, C. megalocarpus, T. diversifolia* and *M. volkensis* with MIC of 12.25 mg/ml (Table 3). The MIC and the MFC of the bark extracts of the plants that proved to have inhibitory effects at a concentration of 400 mg/ml. The lowest MIC recorded was 6.25 mg/ml in the extracts of S. *siamea* and *Z. chalybeum*. The MIC and the MFC of the bark extracts of the plants that proved to have inhibitory effects at a concentration of 400 mg/ml. The lowest MIC recorded was 6.25 mg/ml in the extracts of S. *siamea* and *Z. chalybeum* (Table 3).

Table 2. *Efficacy of plant bark extracts on the growth of A. flavus at the different concentrations*

Plant name	Inhibition zones (means in mm) at 1000mg/ml	Inhibition zones (means in mm) at 750mg/ml	Inhibition zones (means in mm) at 400mg/ml
B. coriacea	17.60a	14.20a	13.20a
C. spirinum	17.20a	16.40a	13.60a
C. megalocarpus	18.60a	16.40a	15.00a
S. siamea	21.20b	20.40b	13.00a
S. incunum	14.40c	13.00a	10.80b
T. diversifolia	14.80c	12.80a	13.00a
Z. chalybeum	17.80a	15.20a	14.00a
Positive control	17.60a	17.40a	17.40c
Negative control	0.00d	0.00c	0.00d

Note: Numbers are means of 10 replications. Means are separated by Turkeys Test. Numbers followed by the same letters in the same column are not significantly different ($P < 0.05$).

Table 3. *The MIC and MFC of plant leaf extracts (mg/ml)*

Plant name	MIC (mg/ml)		MFC (mg/ml)	
	Leaf	Bark	Leaf	Bark
B. coriacea	6.25	12.50	12.50	12.50
C. spirinum	12.50	12.50	12.50	12.50
C. megalocarpus	12.50	12.50	12.50	12.50
M. volkensis	12.50	12.50	12.50	12.50
S. siamea	6.25	6.25	12.50	12.50
T. diversifolia	12.50	12.50	12.50	12.50
Z. chalybeum	6.25	6.25	12.50	6.25
S. incunum	6.25	12.50	12.50	12.50
+ VE Control, Meconazole(10mg/ml)	10.00	10.00	10.00	10.00

3.3. Phytochemical Screening of the Medicinal Plants

The results of the preliminary phytochemical screening of fifteen methanolic plant leaf extracts revealed the presence of various bioactive agents. The bark from the plants that were found to have effective plant leaf extracts were also analyzed for the presence of bioactive agents. Both the leaf and bark extracts were found to contain compounds such as tannins, flavonoids, terpenoids, cardiac glycosides, saponins and alkaloids but leaves extracts were negative for saponins (Table 4 and 5).

Table 4. *Bioactive agents screened from leaves of the medicinal plants.*

Plant name	Tanins	Saponins	Flavonoids	Terpenoids	Cardiacglycosides	Alkaloids
A. lmintica	+ +	_	+ +	+ +	+ +	+ +
B. aegyptiaca	_	_	+ +	_	_	+
B. coriacea	_	_	+ +	+ + +	+ +	+ +
C. spirinum	+ + +	+ + +	_	_	_	+ +
C. megalocarpus	+ + +	_	+ + +	+ + +	_	_
M. decumbens	_	_	+ +	_	+	_
R. communis	+ +	_	+	_	_	+ + +
P . guajava	+ + +	_	+ +	+ + +	_	_
S. siamea	+	_	+ + +	+ + +	_	_
S . persica	_	_	_	_	_	+ + +
T. indica	_	_	+	_	_	+
T . diversifolia	+	_	_	_	_	+ + +
Z . chalybeum	+ +	_	+ + +	+ + +	_	+ + +
Z. chalybeum	+ +	_	+ +	+ +	+	+ + +
M. volkensis	_	_	_	+	+	+ + +

Key: +++ = Abundant, + + = Moderate, += Trace,− = Negative

Table 5. *Bioactive agents of extracts from the bark of the medicinal plants*

Plant name	Tanins	Saponins	Flavonoids	Terpenoids	Cardiacglycosides	Alkaloids
B. coriacea	_	_	+ + +	_	+ +	+ + +
C. spirinum	+ + +	+ + +	_	_	+	+ + +
C . megalocarpus	_	_	_	+ + +	_	+ + +
R . communis	+ + +	_	+ + +	_	_	_
P. guajava	_	+ +	_	_	+	+ + +
S. siamea	_	__	+ + +	_	+ +	+ + +
S . persica	_	_	+ +	_	_	+++
T. indica	+++	+++	+++	_	_	+++
T . diversifolia	++	_	_	_	_	+++
Z. chalybeum	++	_	+++	++	_	+++
Z. chalybeum	++	_	++	++	_	+++
M. volkensis	+++	_	+++	_	_	+++

Key: +++ = Abundant, + + = Moderate, += Trace,− = Negative

4. Discussion

The *A. flavus* mycotoxigenecity has been frequently reported (36;37). This pathogen produces aflatoxins which is the most regulated group of mycotoxin worldwide and has been reported in maize, groundnuts, sorghum and millet (38). These grains are the staple food crops for people in the developing countries where the intake by a person per day is relatively high. The results of this study confirm the efficacy of these plants against microorganisms and their traditional use in food storage. The *in vitro* activities of the crude extracts of these plants also provides further evidence to support the use of such plants as antifungals in the control of *A. flavus, Microsporum gypseum* and *Penicillium marneffei*, *candida albicans, Mucor spp, Rhizopus spp and Trichophyton spp* (7; 22;39; 40; 41; 42;43;44;45).

The plant extracts found to have high antifungal effects recorded the lowest MICs and MFCs. Both the leaf and bark extracts of *Z. chalybeum* and *S. siamea* had low MIC of 6.25 mg/ml. This possibly has enhanced high antifungal activities of the plant extracts. The other plants such as *C. spinirum, C. megalocarpus* and *B. coriacea* had relatively low MIC of 12.5 mg/ml and these could also have enhanced their efficacy against *A.flavus.* The study also revealed that the plant extracts tested had MFCs that were higher than the MICs. This phytochemical screening revealed the presence of various bioactive agents. These agents included tannins, flavonoids, terpenoids, cardiac glycosides, saponins and alkaloids. This agrees with previous reports that associated the antifungal activity of the plant extracts to the presence of flavanoids, steroids, alkaloids and triterpenoids and other natural polyphenolic compounds or free hydroxyl groups (25;46).

In conclusion, due to health and economic considerations, the efficacy of the plant extracts may provide an alternative method to protect field or stored grains from fungal and mycotoxin contamination. Therefore there is an absolute need for bioactivity guided fractionation and isolation of the active components from the plant extracts found to be effective. Finally, these findings may lead to elucidation of new and effective antifungal agents that may serve as an alternative management strategy of *A. flavus* and other mycotoxigenic storage fungi in maize and other cereal grains.

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