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Effects of Selected Plant Extracts and Leaf Ashes on Aspergillus flavus Growth and Aflatoxin B1 Production in Maize Grains

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ABSTRACT: Synthetic fungicides used against aflatoxigenic fungi have negative health and environmental impacts. Plant-based alternatives offer safer, biodegradable options. This study evaluated the in vitro and in vivo antifungal activity of ethanolic extracts from leaves and leaf ashes of five medicinal plants (Trichilia emetica, Sclerocarya birrea, Tabernaemontana elegans, Combretum molle, and Securidaca longepedunculata) against Aspergillus flavus and aflatoxin B1 (AFB1) production in maize grains. Antifungal activity was tested at 2.5–10.0 mg mL^{-1} using mycelial growth inhibition in vitro and on stored maize grains (in vivo). AFB1 was extracted with 80% methanol and detected by thin layer chromatography. Phytochemical screening revealed alkaloids, flavonoids, phenols, and cardiac glycosides in most extracts, while terpenoids were absent in leaf ashes. T. elegans leaf ash, S. birrea leaf, and S. birrea leaf ash showed 100% in vitro inhibition at 2.5 mg mL^{-1} (MIC = 2.5 mg mL^{-1}). However, in vivo, only T. elegans leaf ash exhibited strong activity (93.3% inhibition at 7.5–10.0 mg mL^{-1}), while most extracts failed to inhibit fungal growth on maize grains. AFB1 was completely suppressed by T. elegans leaf ash at all concentrations (5.0–10.0 mg mL^{-1}), and by S. birrea leaf, S. birrea leaf ash, and S. longepedunculata root at 10.0 mg mL^{-1}. The superior performance of T. elegans leaf ash is likely due to retained bioactive alkaloids combined with alkaline and hygroscopic properties. This extract represents a promising eco-friendly alternative to synthetic fungicides for aflatoxin control in stored maize

1. INTRODUCTION

Aflatoxins are toxic and carcinogenic secondary metabolites produced primarily by *Aspergillus flavus* and *Aspergillus parasiticus*. These compounds cause significant crop losses and represent a major hazard to the food chain. Four major aflatoxins (AFB1, AFB2, AFG1, and AFG2) are commonly found in various foods and feedstuffs. Among these, AFB1 is the most prevalent and dangerous for human health, being predominantly produced by *A. flavus* (Mehraban et al., 2018; Mohammedi & Atik, 2013). The Food and Agriculture Organization (FAO) estimates that approximately 25% of the world's cereal grains—including maize,

wheat, sorghum, barley, millet, and rice—are contaminated by mycotoxins, including aflatoxins. Moreover, aflatoxins can contaminate animal-derived products such as meat, eggs, fish, and milk when livestock and poultry are fed contaminated feed (Sinha, 2018). Thippeswamy et al. (2014) reported that about 4.5 billion people in developing countries are systematically exposed to uncontrolled amounts of aflatoxins. Due to their severe health impacts—being teratogenic, carcinogenic, mutagenic, and immunosuppressive—aflatoxins have also been identified as potential biological weapons for food and water contamination (Saleem et al., 2017).

Physical methods (e.g., aeration, cold storage, rapid drying, and radiation) and chemical treatments (e.g., food preservatives and synthetic fungicides) are commonly used to prevent the growth of aflatoxigenic fungi and to decontaminate or detoxify aflatoxins in foods and feeds (Rajasekaran et al., 2018; Thippeswamy et al., 2014). While synthetic chemicals are often effective and widely used as seed treatments (Mohana et al., 2011), they present significant drawbacks. Many synthetic fungicides leave persistent residues and have carcinogenic and toxic effects on humans and the environment (Garcia et al., 2012). Furthermore, they cannot always be safely applied to maize grains intended for consumption (Achugbu et al., 2016).

Consequently, there is an urgent need for eco-friendly and safe alternatives to control mycotoxigenic fungi and mycotoxin production (Arunachalam et al., 2012; Garcia et al., 2012). The use of plant-derived products has emerged as a promising alternative because they are biodegradable, renewable, and generally recognized as safe for human health (Martins et al., 2014; Oguntade & Adekunle, 2010). Plant extracts contain various antioxidant compounds—such as polyphenols, flavonoids, and alkaloids—which exhibit antimicrobial properties and can inhibit both fungal growth and toxin production (Garcia et al., 2012). These plant-based biofungicides offer excellent perspectives for the discovery of new therapeutic products, some of which can be developed for the prevention of contamination and mycotoxicosis (Dikhoba et al., 2019; Prakash et al., 2012).

The plants selected for the present study—*Trichilia emetica*, *Sclerocarya birrea*, *Tabernaemontana elegans*, *Combretum molle*, and *Securidaca longepedunculata*—were chosen based on their availability in Southern Mozambique, their ethnopharmacological use in traditional medicine, and previous reports on their antimicrobial and antioxidant properties. However, few studies have systematically investigated the antifungal activity of these plants, and even fewer have explored the potential use of their leaf ashes against mycotoxigenic fungi. In particular, the comparative efficacy of ethanolic extracts versus leaf ash extracts against *A. flavus* growth and aflatoxin B1 production in stored maize grains remains largely unexplored.

Therefore, the aim of this work was to evaluate the antifungal effect of ethanolic extracts from leaves (and roots where applicable) and leaf ashes of the five selected plants against *A. flavus* growth and aflatoxin B1 production in stored maize grains, under both *in vitro* and *in vivo* conditions.

2. MATERIALS AND METHODS

2.1 Collection and preparation of plant materials

Plant materials were collected from Chiboene village, Moamba district, Maputo province, Mozambique, in June 2020. The following plant species were collected: leaves of *Trichilia emetica* Vahl, *Sclerocarya birrea* (A. Rich.) Hochst., *Tabernaemontana elegans* Stapf., *Combretum molle* R. Br. ex G. Don., and roots of *Securidaca longepedunculata* Fresen.

Plant identification and authentication were performed by botanists at the Herbarium unit of the Department of Biological Sciences, Eduardo Mondlane University, by comparison with existing specimens. The collected plant materials were dried at room temperature (25 ± 2°C) for 14 days in the laboratory of Natural Products, Department of Chemistry. The dried plant materials were ground into fine powder using a hammer mill (Retsch SM 100, Germany) fitted with a 1 mm mesh screen. The resulting powders were stored in clean, airtight plastic containers, protected from light, heat, and moisture until further use.

2.2 Preparation of leaf ashes

The dried leaves were placed in a rectangular covered iron container (20 cm × 15 cm × 10 cm) and incinerated in a muffle furnace (Nabertherm L9/11, Germany) at 250°C for 25 minutes. The resulting ash was allowed to cool to room temperature, then stored in sterile nylon bags labeled with the respective plant species and ash preparation date. The ash was kept in a dry, dark place until extraction.

2.3 Preparation of plant and ash extracts

The mixture was placed on an electric shaker (Shake-O-Mat, Bühler, Germany) set at 120 rpm for 72 hours at room temperature (25 ± 2°C). After maceration, the mixture was filtered through Whatman No. 1 filter paper. The filtrate was collected, and the residue was re-extracted twice more with fresh 400 mL portions of 96% ethanol under the same conditions. The combined filtrates from the three sequential extractions were concentrated to dryness using a rotary evaporator (EYELA N-1200A, Tokyo Rikakikai Co., Japan) at 40°C under reduced pressure. The dry extracts were weighed using an analytical balance (Mettler Toledo AB104-S, Switzerland) and the extracts were stored in airtight glass vials at 4°C (NIHON FREEZER KGT-4010HC, Japan) until use in the various assays.

2.4 Qualitative phytochemical analysis

Phytochemical screening for the following secondary metabolites: Phenols, alkaloids, tannins, saponins, flavonoids, terpenoids and cardiac glycosides was performed on each ethanolic extract of plant leaves and leaf ashes using standard qualitative methods as described by Sakander et al. (2015) and Sharma & Paliwal (2013).

2.5 Antifungal activity

2.5.1 Fungal strain

The fungal culture and antifungal assays were carried out in the Department of Plant Protection, Faculty of Agronomy and Forestry Engineering, Eduardo Mondlane University.

2.5.2 Isolation of *Aspergillus flavus*

The fungus *Aspergillus flavus* was isolated from maize grains and peanuts collected from local markets in Maputo, Mozambique, using the blotter method described by Attitalla et al. (2010). Briefly, seeds were surface-disinfected with 1% sodium hypochlorite for 2 minutes, rinsed three times with sterile distilled water, and placed on three layers of moistened blotter paper in 9 cm diameter Petri dishes (10 seeds per dish). The dishes were incubated at 25 ± 2°C under a 12-hour photoperiod for 7 days.

2.5.3 Purification and identification

Fungal colonies suspected to be *A. flavus* based on morphological characteristics (yellowish-green colonies, rough-walled conidiophores, and radiate heads) were subcultured onto potato dextrose agar (PDA) medium. Two successive purification steps were performed by transferring single spores to fresh PDA plates. The pure culture was identified based on macroscopic and microscopic morphological characteristics following standard mycological keys (Peterson, 2003). The pure culture was maintained on PDA slants at 4°C and subcultured monthly (Mohammedi & Atik, 2013).

2.5.4 Preparation of extract concentrations for antifungal assays

Stock solutions of each ethanolic extract were prepared by dissolving the dry extract in 96% ethanol at a concentration of 50 mg mL^{-1}. From the stock solution, working concentrations of 2.5, 5.0, 7.5, and 10.0 mg mL^{-1} were prepared by serial dilution with 96% ethanol. All solutions were stored at 4°C and used within 48 hours of preparation.

2.5.5 *In vitro* Antifungal assay

The *in vitro* antifungal activity was evaluated using the poisoned food technique described by Nitiema et al. (2020) with modifications. For each extract concentration, 1.5 mL of the extract solution was dispensed into a sterile 9 cm Petri dish. Then, 15 mL of molten PDA (cooled to 50°C after autoclaving at 121°C for 15 minutes) was added to each dish. The contents were gently swirled to ensure uniform mixing of the extract with the medium.

After the medium solidified, a 6 mm diameter mycelial plug taken from the margin of a 7-day-old pure culture of *A. flavus* was placed at the centre of each Petri dish using a sterile cork borer. The dishes were sealed with Parafilm and incubated at 28 ± 2°C for 7 days or until the negative control plates showed full growth.

For the positive control, 1.5 mL of Tebuconazole solution (0.05 µL mL^{-1}, equivalent to 0.05 ppm) was added to PDA without plant extracts, and for the negative control, 1.5 mL of sterile distilled water was added to PDA without plant extracts.

The experimental design was a Completely Randomized Design (CRD) with 3 replicates.

2.5.6 Measurement of mycelial growth

Colony diameter was measured daily for 7 days. For each plate, two perpendicular lines were drawn on the reverse side, and the diameter along each line was measured using a digital caliper (Mitutoyo, Japan). The colony diameter was calculated as the mean of these two measurements. The percentage of growth inhibition was calculated using the formula described by Achugbu et al. (2016) and Prakash et al. (2012) (Figure (1)):

$$\text{Growth Inhibition (\%)} = \frac{Dc-Dt}{Dc} \times 100 \quad (1)$$

D_c = mean colony diameter in the negative control (mm)

D_t = mean colony diameter in the treatment (mm)

2.5.7 Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) was determined for extracts that showed antifungal activity against *A. flavus* in the *in vitro* assay. The MIC was defined as the lowest concentration of extract that completely inhibited visible mycelial growth of *A. flavus* after 10 days of incubation at 28 ± 2°C (Wondmnew & Teshome, 2016). Observations were made daily, and plates showing no visible growth after 10 days were considered to have reached the MIC. Each concentration was tested in triplicate. For extracts that did not achieve 100% inhibition even at the highest concentration tested (10.0 mg mL^{-1}), the MIC was recorded as ">10.0 mg mL^{-1}."

2.6 *In vivo* Antifungal activity on maize grains

2.6.1 Maize grain preparation and artificial inoculation

Healthy maize grains (*Zea mays* L.) were purchased from a local market in Maputo, Mozambique. The grains were inspected visually to remove broken, discoloured, or damaged kernels. The grains were surface-disinfected by immersion in 2% sodium hypochlorite solution for 2 minutes, followed by three rinses with sterile distilled water. The grains were then dried in a laminar flow cabinet for 1 hour.

A. flavus inoculum was prepared by flooding a 7-day-old PDA culture with 10 mL of sterile distilled water containing 0.05% Tween 80 (v/v). The spore suspension was scraped gently with a sterile glass rod and filtered through four layers of sterile cheesecloth to remove hemocytometer mycelial fragments. The spore concentration was adjusted to 1×10^6 spores mL^{-1} using a hemocytometer (Neubauer improved, Germany).

For each treatment, 40 surface-disinfected maize grains were placed in a sterile 250 mL Erlenmeyer flask and inoculated with 2 mL of the spore suspension. The flask was shaken manually for 2 minutes to ensure uniform distribution of spores on the grain surface. The inoculated grains were air-dried under sterile conditions for 1 hour.

2.6.2 Treatment application

The inoculated maize grains were soaked in 5 mL of extract solution at concentrations of 5.0, 7.5, or 10.0 mg mL^{-1} for 24 hours at room temperature (25 ± 2°C) in sterile 50 mL centrifuge tubes. After soaking, the grains were removed and air-dried on sterile filter paper for 4 hours at room temperature.

For positive control, Inoculated grains were soaked in 5 mL of Tebuconazole solution (0.05 µL mL^{-1}) and for negative control, Inoculated grains were soaked in 5 mL of sterile distilled water.

After drying, the treated grains were stored in sterile polyethylene bags (20 grains per bag, two bags per treatment) at room temperature (25 ± 2°C) for 28 days. The bags were left open to allow air circulation.

2.6.3 Assessment of fungal growth on stored maize grains

After 28 days of storage, the maize grains were analysed for *A. flavus* occurrence using the blotter method described by Machado et al. (2008) and Rahim & Dawar (2015) with modifications. For each treatment, 10 grains were randomly selected and placed equidistantly in 9 cm diameter Petri dishes containing three layers of blotter paper moistened with 5 mL of sterile distilled water. The dishes were incubated at room temperature (25 ± 2°C) for 24 hours, then transferred to a deep freezer (BOSCH

GSV34V40, Germany) at -20°C for 24 hours to arrest seed germination and promote fungal sporulation. Finally, the dishes were incubated at room temperature under a 12-hour photoperiod with near ultraviolet (NUV) light for 7 days.

2.6.4 Identification and quantification of fungi

After 7 days of incubation, the seeds were examined under a stereomicroscope (Leica EZ4, Germany) at magnifications of 10× to 40×. The identification of *A. flavus* was confirmed based on morphological characteristics of the fruiting bodies, conidiophores, and conidia observed under a compound microscope (Leica DM500, Germany) at 400× magnification (Pathak & Zaidi, 2013; Rahim & Dawar, 2015).

The frequency of *A. flavus* occurrence was calculated using the formula described by Pathak & Zaidi (2013) (equation (2)):

$$Frequency\ of\ the\ fungus\ (\%) = \frac{Number\ of\ seeds\ containing\ A.\ flavus}{Total\ number\ of\ seeds\ examined} \times 100 \quad (2)$$

The percentage inhibition of fungal growth *in vivo* was calculated relative to the negative control using the same formula as for *in vitro* assays (equation (1)). Each treatment was analysed in triplicate.

2.7 Aflatoxin B1 extraction and detection

After the 28-day storage period and fungal assessment, the remaining maize grains from each treatment were pooled and ground into a fine powder using a laboratory mill (Retsch ZM 200, Germany) fitted with a 0.5 mm sieve. The powdered samples were stored in airtight glass jars at -20°C until extraction.

Aflatoxin B1 was extracted following the method described by Adi & Matcha (2018) with modifications. Ten grams of maize powder were weighed into a 250 mL Erlenmeyer flask, and 50 mL of 80% methanol (v/v in distilled water) were added. The mixture was shaken on an orbital shaker (Edmund Bühler KS-15, Germany) at 200 rpm for 5 minutes at room temperature. The extract was filtered through Whatman No. 1 filter paper, and the filtrate was collected.

The filtrate was concentrated to dryness using a rotary evaporator (EYELA N-1200A, Japan) at 40°C under reduced pressure. The dry residue was dissolved in 2 mL of chloroform and stored in amber glass vials at -20°C until TLC analysis.

2.7.1 Thin layer chromatography (TLC) detection

TLC was performed using the method described by Adi & Matcha (2018). Glass plates (20 cm × 20 cm) were coated with a 0.25 mm layer of silica gel G (Kieselgel G, Merck, Germany). The plates were activated by heating at 110°C for 1 hour before use.

Sample extracts were spotted (10 µL each) onto the TLC plates using a micropipette. The plates were developed in a glass tank pre-saturated with the solvent system chloroform:methanol (95:5, v/v) for 30 minutes.

After development, the plates were air-dried and examined under a long-wave UV lamp (365 nm, Spectroline ENF-280C, USA). The presence of AFB1 was indicated by blue fluorescence at Rf = 0.68, matching the standard AFB1. The Rf value was calculated as (equation (3)):

$$R_f = \frac{Distance\ travelled\ by\ the\ spot}{Distance\ travelled\ by\ the\ solvent\ front} \quad (3)$$

Samples were considered positive for AFB1 if a blue fluorescent spot with Rf = 0.68 was observed.

2.8 Statistical analysis

All experiments were conducted in triplicate. Data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using SPSS software version 20.0 (IBM SPSS Inc., Chicago, USA).

One-way analysis of variance (ANOVA) was used to compare means among treatment groups. When significant differences were detected ($p < 0.05$), post-hoc multiple comparisons were performed using Tukey's honestly significant difference (HSD) test.

For comparisons between two groups (e.g., *in vitro* vs. *in vivo* inhibition), independent samples t-tests were used. Differences were considered statistically significant at $p < 0.05$.

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3. RESULTS AND DISCUSSION

3.1 Phytochemical screening

The results of the preliminary phytochemical screening of the crude ethanolic extracts from five plants and their corresponding leaf ashes are presented in Table 1. The analysis revealed the presence of various bioactive agents, including tannins, flavonoids, terpenoids, phenols, cardiac glycosides, saponins, and alkaloids.

Table 1: Results of qualitative phytochemical screening of hydroethanolic extracts of plants and leaf ashes.

Ethanolic extract	Alk.	Flav.	Sap.	Tan.	Terp.	Phen.	Card. Gly.
C. molle L	+	–	+	+	+	+	+
C. molle LA	+	–	–	–	–	+	+
T. elegans L	+	+	+	+	+	+	+
T. elegans LA	+	+	+	+	–	+	+
S. birrea L	+	+	+	+	+	+	+
S. birrea LA	+	+	–	+	–	+	+
T. emetica L	+	+	–	+	+	+	+
T. emetica LA	+	+	–	–	–	–	+
S. longepedunculata L	+	+	+	+	+	+	+
S. longepedunculata LA	+	+	+	–	–	+	+
S. longepedunculata R	+	+	+	–	–	+	+

+: present, -: absent, L: Leaf, R: Root, LA: Leaf ash, Alk.: Alkaloids, Flav.: Flavonoids, Sap.: Saponins, Tan.: Tannins, Terp.: Terpenoids, Phen.: Phenols, Card. Gly.: Cardiac Glycosides.

All ethanolic extracts from plant leaves and leaf ashes contained at least three classes of secondary metabolites (Table 1). Alkaloids and cardiac glycosides were present in every extract, suggesting their high extractability with ethanol and relative thermal stability during the ashing process at 250°C. In contrast, terpenoids were detected in all leaf extracts but were absent from all leaf ashes, indicating that these compounds are thermally labile and degrade during combustion. Flavonoids were present in all extracts except those from *C. molle* (both leaf and leaf ash), suggesting either constitutive absence in this species or degradation under the ashing conditions used.

The efficiency of ethanol in extracting bioactive compounds to control microorganisms has been well documented (Do et al., 2014). Oguntade & Adekunle (2010) and Iqbal (2012) explained that ethanol provides more phytochemical compounds during extraction because of its ability to diffuse faster into plant powdery material than water due to its lower surface tension and higher volatility.

Although different metabolites were identified across the five plants studied, their presence varied from extract to extract. Most of the results from phytochemical screening of plant extracts were consistent with those reported in the literature. Where differences were observed, these can be explained by the fact that the chemical composition of extracts may vary according to several parameters, including geographical origin, genetic variations, the part of the plant used, the extraction method, the age or maturation stage, and the harvesting season (Chivodze et al., 2022; Yusuf et al., 2018).

The retention of alkaloids, phenols, and flavonoids in some leaf ashes is particularly noteworthy. This finding aligns with Oguntade & Adekunle (2010) and Auta et al. (2015), who demonstrated that wood ashes can preserve antimicrobial properties. The variability in phytochemical profiles among plants underscores the species-specific response to the ashing process and highlights the need for individual optimization of burning conditions for each plant species.

3.2 *In vitro* antifungal activity

The results of the *in vitro* antifungal activity assay of the five plant extracts and their ashes are presented in Table 2A and 2B. The tested plant extracts and leaf ash extracts exhibited significant, dose-dependent antifungal activities against *A. flavus*. There was a significant difference in inhibiting *A. flavus* growth among the four tested concentrations (2.5, 5.0, 7.5, and 10.0 mg mL^{-1}) at $p < 0.05$. The plant extracts and leaf ash extracts also showed significant differences in their inhibitory capacities.

Table 2A: Extracts achieving 100% inhibition at ≤ 5.0 mg mL^{-1}

Extract	MIC (mg mL^{-1})	% inhibition at 2.5 mg mL^{-1}	% inhibition at 5.0 mg mL^{-1}
T. elegans LA	2.5	100	100
S. birrea L	2.5	100	100
S. birrea LA	2.5	100	100
C. molle L	5.0	85	100

L: Leaf, LA: Leaf ash, MIC: Minimum Inhibitory Concentration

Table 2B: Extracts requiring ≥ 7.5 mg mL^{-1} for 100% inhibition

Extract	MIC (mg mL^{-1})	% inhibition at 7.5 mg mL^{-1}	% inhibition at 10.0 mg mL^{-1}
T. emetica L	7.5	100	100
T. elegans L	7.5	100	100
S. longepedunculata L	7.5	100	100
S. longepedunculata LA	7.5	100	100
S. longepedunculata R	7.5	100	100
C. molle LA	10.0	85	100
T. emetica LA	>10.0	38.1	41.7
Distilled water (negative control)	-	0	0
Tebuconazole (positive control)	-	98.6	98.6

L: Leaf, LA: Leaf ash, R: Root, MIC: Minimum Inhibitory Concentration

Tabernaemontana elegans leaf ash, *S. birrea* leaf, and *S. birrea* leaf ash were the most potent extracts, achieving 100% inhibition of *A. flavus* mycelial growth at the lowest tested concentration of 2.5 mg mL^{-1} (Table 2A). The minimum inhibitory concentration (MIC) for these three extracts was 2.5 mg mL^{-1}. *Combretum molle* leaf extract reached 100% inhibition at 5.0 mg mL^{-1} (MIC = 5.0 mg mL^{-1}).

A second group of extracts—*T. emetica* leaf, *T. elegans* leaf, *S. longepedunculata* leaf, *S. longepedunculata* leaf ash, and *S. longepedunculata* root—required 7.5 mg mL^{-1} to achieve complete inhibition (MIC = 7.5 mg mL^{-1}). *Combretum molle* leaf ash was less effective, requiring 10.0 mg mL^{-1} for 100% inhibition (MIC = 10.0 mg mL^{-1}). Notably, *T. emetica* leaf ash did not reach 100% inhibition even at the highest concentration tested (10.0 mg mL^{-1}), achieving only 41.7% inhibition (Table 2B).

The superior activity of *S. birrea* extracts is consistent with previous reports of its high phenolic, flavonoid, and tannin contents, as well as its potent antifungal properties against various pathogenic fungi (Hamza et al., 2006; Ndhlala et al., 2007; Shai et al.,

2008). Eloff (2001) similarly reported strong antibacterial activity of *S. birrea* extracts, attributing this to polar compounds extractable with polar solvents.

The efficacy of *S. longepedunculata* extracts aligns with Junaidu et al. (2014), who reported antifungal activity against *A. niger* and *A. flavus* attributed to alkaloids, flavonoids, and saponins identified in the root bark and leaves of this species.

The strong performance of *T. elegans* leaf ash is likely due to its content of monoterpenoid indole alkaloids (e.g., voacangine, dregamine, vobasine, and tabernaemontanine), which are known antimicrobial agents (Marinho et al., 2016; Naidoo et al., 2021; Pallant et al., 2012). Pallant et al. (2012) reported that alkaloids isolated from *T. elegans* root extract exhibited significant antibacterial activity against Gram-positive bacteria and *Mycobacterium* species. Marinho et al. (2016) further demonstrated that voacamine-type alkaloids inhibit the growth of bacteria, fungi, and parasites.

For *T. emetica*, previous studies have reported good antioxidant activity from leaf extracts and antibacterial activity from leaves and roots, while fruit and seed extracts showed inhibition of fungal growth (Brigitte et al., 2017; Germanò et al., 2005; Perumal et al, 2020). However, the weak activity of *T. emetica* leaf ash observed in the present study—despite the moderate activity of its leaf extract—suggests that the ashing process at 250°C may degrade heat-labile bioactive compounds in this species. This finding underscores that the effect of ashing varies among plant species and requires species-specific optimization.

For *C. molle*, Mogashoa et al. (2019) recently reported antibacterial and antifungal activities of different extracts against plant fungal pathogens, including *Aspergillus niger*, *A. parasiticus*, *Fusarium oxysporum*, and *Penicillium janthinellum*. Phytochemical screening of *C. molle* leaf extracts has revealed the presence of tannins, flavonoids, saponins, triterpenes, steroids, glycosides, and alkaloids (Darmasu et al., 2019; Kaleab et al., 2006). The complete inhibition observed at 5.0 mg mL^{-1} for *C. molle* leaf extract in the present study is consistent with these reports.

The significant differences among plant extracts in their ability to inhibit *A. flavus* growth may be attributed to the number of bioactive agents present and the quantity of particular bioactive compounds contained in each plant (Oguntade & Adekunle, 2010). This observation aligns with Rn & Pu (2015) and Abeer et al. (2012), who stated that the efficiency of extracts differs with plant material, phytochemical contents, and concentration.

3.3 Minimum inhibition concentrations (MIC) of plant and leaf ash extracts.

The MIC was determined as the lowest concentration at which no visible *A. flavus* growth was observed by naked eyes after 10 days of incubation. The MIC values for all extracts are summarized in Tables 2A and 2B.

Tabernaemontana elegans leaf ash, *S. birrea* leaf, and *S. birrea* leaf ash showed the lowest MIC of 2.5 mg mL^{-1}, demonstrating their potent antifungal activity. *Combretum molle* leaf extract exhibited a MIC of 5.0 mg mL^{-1}. *Trichilia emetica* leaf, *T. elegans* leaf, *S. longepedunculata* leaf, *S. longepedunculata* leaf ash, and *S. longepedunculata* root showed a MIC of 7.5 mg mL^{-1}. *Combretum molle* leaf ash exhibited a MIC of 10.0 mg mL^{-1}. *Trichilia emetica* leaf ash did not achieve complete inhibition even at the highest concentration tested (10.0 mg mL^{-1}), and therefore its MIC could not be determined within the concentration range of this study.

3.4 *In vivo* antifungal activity on stored maize grains

The results of the *in vivo* antifungal activity assay are presented in Figure 1. Under *in vitro* conditions, practically all extracts tested exhibited 100% growth inhibition of *A. flavus* at the concentration of 10.0 mg mL^{-1}, with the sole exception of *T. emetica* leaf ash. However, none of the extracts achieved 100% growth inhibition of *A. flavus* under *in vivo* conditions at any of the tested concentrations.

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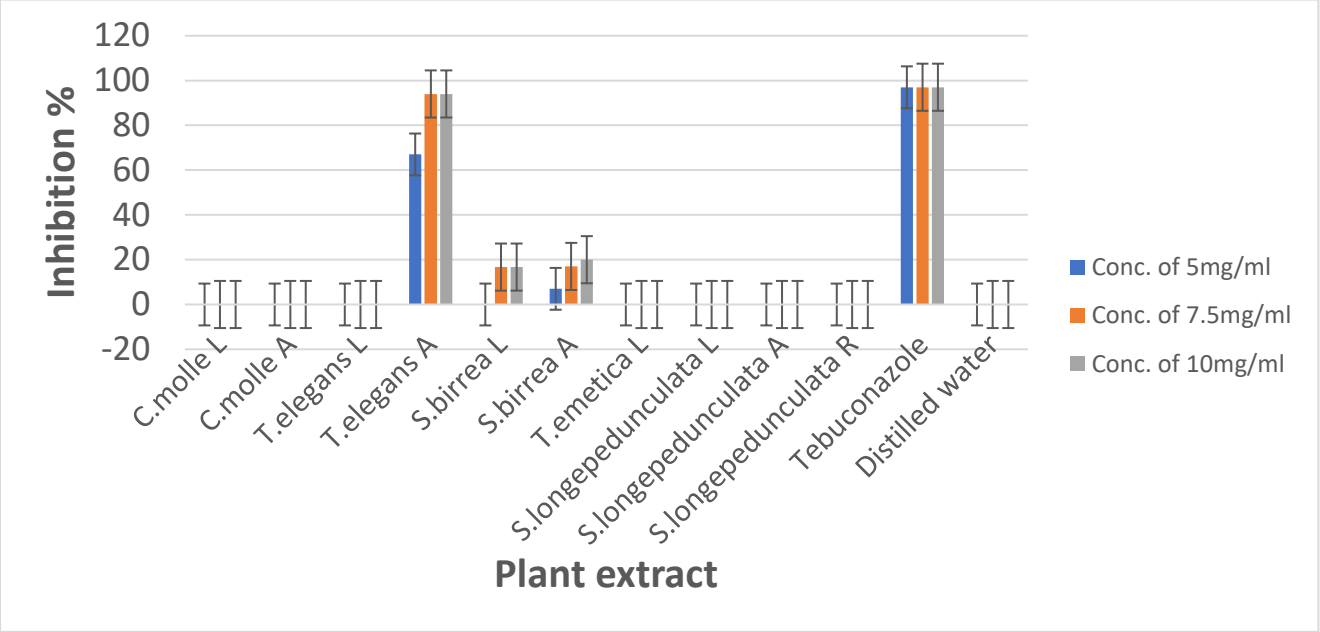


Figure 1: *In vivo* inhibition of *A. flavus* growth on stored maize grains after 28 days of treatment with plant and leaf ash extracts.

L: Leaf, A: Ash, R: Root

Tabernaemontana elegans leaf ash was the most effective extract *in vivo*, achieving 66.7% inhibition at 5.0 mg mL^{-1} and 93.3% inhibition at both 7.5 and 10.0 mg mL^{-1}. *Sclerocarya birrea* leaf extract showed moderate activity, with 16.7% inhibition at 7.5 mg mL^{-1} and 50% inhibition at 10.0 mg mL^{-1}. *Sclerocarya birrea* leaf ash was less effective, showing 6.7% inhibition at 5.0 mg mL^{-1}, 16.7% inhibition at 7.5 mg mL^{-1}, and 20% inhibition at 10.0 mg mL^{-1}.

All other extracts—including *C. molle* leaf, *C. molle* leaf ash, *T. emetica* leaf, *T. elegans* leaf, *S. longepedunculata* leaf, *S. longepedunculata* leaf ash, and *S. longepedunculata* root—showed no detectable effect on *A. flavus* growth in stored maize grains at any concentration tested (0% inhibition).

The substantial discrepancy between *in vitro* and *in vivo* efficacy observed for most extracts is not unique to this study and warrants explanation. Several factors may account for this phenomenon:

Matrix binding: Bioactive compounds may bind irreversibly to maize grain matrix components (e.g., starch, proteins, fibers), reducing their availability to the fungus (Oguntade & Adekunle, 2010).

Volatility or degradation: Active compounds may volatilize or degrade during the 28-day storage period, particularly if they are thermolabile or photosensitive.

Limited penetration: Extracts may not penetrate sufficiently into the grain interior where *A. flavus* may reside, remaining primarily on the grain surface.

Water activity interactions: The complex interaction between grain moisture, water activity, and extract solubility may affect the bioavailability of antifungal compounds (Peloewetse et al., 2008).

The superior performance of *T. elegans* leaf ash *in vivo* may be attributed not only to its bioactive alkaloids but also to two physicochemical properties of the ash: alkalinity and hygroscopicity. Although these properties were not directly measured in this study, plant ashes are known to raise pH and reduce water activity (aw) when applied to grains (Auta et al., 2015; Oguntade & Adekunle, 2010).

Aspergillus flavus produces glucoamylase, an enzyme that hydrolyses starch into glucose, which optimally functions at pH 5.0–6.9. An alkaline environment created by the leaf ash would likely inhibit this enzyme activity, limiting the carbon supply (glucose,

maltose, and maltotriose) required for fungal growth and aflatoxin production (Peloewetse et al., 2008). Additionally, the hygroscopic nature of ash may reduce water activity below the threshold required for *A. flavus* proliferation (aw < 0.85). These indirect effects, combined with direct antifungal activity of alkaloids and other bioactive compounds retained in the ash, likely explain why *T. elegans* leaf ash outperformed all other extracts *in vivo*.

The finding that some leaf ashes retained antifungal activity even after combustion at 250°C is consistent with Auta et al. (2015) and Oguntade & Adekunle (2010), who reported that wood-ash from various tree species could inhibit the growth of pathogenic fungi in seed storage and that bioactive compounds can survive the ashing process. This also confirms why plant ashes are widely used for grain preservation and medicinal purposes in many traditional societies across Africa.

3.5 Inhibition of aflatoxin B1 production

After the *in vivo* antifungal assay, the same treated maize grains were assessed for aflatoxin B1 (AFB1) production. Aflatoxins were extracted using 80% methanol and analyzed by thin layer chromatography (TLC). The presence of spots with blue fluorescence under 365 nm UV light at Rf = 0.68 in the solvent system chloroform/methanol (95:5, v/v) indicated the presence of AFB1. This Rf value matched that of the standard AFB1 in the same solvent system (Adi & Matcha, 2018).

Table 3: Presence (+) or absence (-) of AFB1 (blue fluorescence at Rf = 0.68) in treated maize grains after 28 days of storage.

Plant extract	5 mg mL^{-1}	7.5 mg mL^{-1}	10 mg mL^{-1}
C. molle L	+	+	+
C. molle LA	+	+	+
T. elegans L	+	+	+
T. elegans LA	-	-	-
T. emetica L	+	+	+
S. birrea L	+	+	-
S. birrea LA	+	+	-
S. longepedunculata L	+	+	+
S. longepedunculata LA	+	+	+
S. longepedunculata R	+	+	-
Negative control (distilled water)	+	+	+
Positive control (Tebuconazole)	-	-	-

L: Leaf, LA: Leaf ash, R: Root, +: AFB1 detected, -: AFB1 not detected

The TLC results (Table 3) revealed that *T. elegans* leaf ash completely suppressed AFB1 production at all concentrations tested (5.0, 7.5, and 10.0 mg mL^{-1}). *Sclerocarya birrea* leaf, *S. birrea* leaf ash, and *S. longepedunculata* root inhibited AFB1 production only at the highest concentration (10.0 mg mL^{-1}). All other extracts showed no inhibition of AFB1 production, presenting blue fluorescence spots identical to the negative control (Rf = 0.68) at all concentrations tested. Maize grains treated with Tebuconazole (positive control) also showed no AFB1 detection, as expected.

Importantly, there was a strong but not perfect correlation between fungal growth inhibition and AFB1 suppression. For example, *S. birrea* leaf at 10.0 mg mL^{-1} reduced fungal growth by 50% (Figure 1) yet completely suppressed AFB1 production (Table 3). Conversely, *S. birrea* leaf ash at 10.0 mg mL^{-1} reduced fungal growth by only 20% but still suppressed AFB1 production. This observation suggests that some extracts may directly interfere with the aflatoxin biosynthetic pathway—possibly by downregulating key regulatory genes—independent of their effect on mycelial growth. Similar findings have been reported for other plant extracts, where sub-lethal concentrations inhibited toxin production without completely arresting fungal growth (Garcia et al., 2012; Mehraban et al., 2018).

The power of *T. elegans* leaf ash to inhibit AFB1 production is attributed to its ability to inhibit *A. flavus* growth in maize grains, combined with the direct effects of its bioactive compounds on the aflatoxin biosynthetic pathway. The alkalinity and hygroscopic nature of the ash may further contribute to AFB1 suppression by limiting carbon supply and water availability, thereby reducing the expression of mycotoxin-related genes (Peloewetse et al., 2008).

3.6 Comparison with previous studies

The antifungal activity of *S. birrea* observed in this study aligns with Shai et al. (2008), who reported strong antifungal activity of *S. birrea* extracts against pathogenic yeasts, and with Eloff (2001) and Runyoro et al. (2006), who documented antibacterial activity. Ndhlala et al. (2007) further reported high contents of total phenolics, flavonoids, and condensed tannins in *S. birrea* extracts, consistent with our phytochemical findings.

The activity of *S. longepedunculata* corroborates Junaidu et al. (2014), who demonstrated potent antifungal activity against *A. niger* and *A. flavus* using ethanol and aqueous extracts of root bark and leaves. The presence of alkaloids, flavonoids, saponins, and steroids in their extracts matches our observations.

For *T. elegans*, our findings extend previous reports. While Pallant et al. (2012)P and Naidoo et al. (2021) documented antibacterial activity of alkaloids (dregamine and voacangine) from root extracts, our study is among the first to report antifungal activity of *T. elegans* leaf ash against *A. flavus*. The presence of monoterpenoid indole alkaloids in the leaf ash, as suggested by our phytochemical screening, likely underlies this activity.

For *C. molle*, our results are consistent with Mogashoa et al. (2019) and Kaleab et al. (2006), who reported antifungal activity against various *Aspergillus* species and identified a broad range of bioactive compounds including tannins, flavonoids, saponins, terpenes, and alkaloids.

For *T. emetica*, previous studies reported good antioxidant and antibacterial activities (Brigitte et al., 2017; Germanò et al., 2005; Komane et al., 2011). However, our finding that *T. emetica* leaf ash was largely ineffective against *A. flavus*—despite moderate activity of the leaf extract—is novel and highlights the species-specific effects of the ashing process.

Notably, this study is among the first to systematically compare leaf extracts with leaf ashes from the same plant species against aflatoxigenic fungi. The finding that *T. elegans* leaf ash outperformed its corresponding leaf extract suggests that the ashing process may concentrate certain bioactive compounds (e.g., alkaloids) while removing others (e.g., terpenoids) that might interfere with or dilute their activity. Alternatively, the alkaline pH of the ash may provide an additional inhibitory mechanism not present in the original leaf extract.

4. CONCLUSION

This study demonstrated that selected plant extracts and their leaf ashes possess antifungal activity against *A. flavus* and can inhibit aflatoxin B1 (AFB1) production in stored maize grains. Among all tested extracts, *T. elegans* leaf ash exhibited the strongest efficacy, achieving 100% *in vitro* growth inhibition at 2.5 mg mL^{-1} and suppressing AFB1 production *in vivo* at all concentrations tested (5–10 mg mL^{-1}). *Sclerocarya birrea* leaf and leaf ash also showed promising activity, though their *in vivo* efficacy was lower (maximum 50% inhibition at 10 mg mL^{-1}). In contrast, extracts from *C. molle*, *T. emetica* (leaf ash), and *S. longepedunculata* failed to suppress AFB1 in maize grains, highlighting that *in vitro* efficacy does not always translate to *in vivo* conditions. The superior performance of *T. elegans* leaf ash is likely attributable to retained bioactive alkaloids combined with alkaline and hygroscopic properties of the ash.

Future research should focus on identifying specific bioactive compounds using LC-MS or GC-MS, evaluating the safety and sensory impact of ash-treated grains, and developing a scalable formulation for smallholder farmers. In conclusion, *T. elegans* leaf ash extract represents a promising, environmentally friendly alternative to synthetic fungicides for controlling *A. flavus* and AFB1 in stored maize grains.

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AUTHOR CONTRIBUTIONS

Luhende E. Daudi: Data Collection, Investigation, Writing - original draft, Formal analysis.

João Bila: Conceptualization, Methodology, Formal analysis, writing-review & editing.

François Munyemana: Conceptualization, Methodology, Supervision, writing-review & editing.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ETHICS STATEMENT

Ethical approval was not required for this study.

DATA AVAILABILITY STATEMENT

Data supporting the findings of this study are available from the corresponding author upon reasonable request.

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