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In-Vitro Efficacy of Botanical extracts for the Management of *Aspergillus flavus* growth in Tanzania

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Abstract

Maize is one of the most important food and commercial crops in Tanzania but is vulnerable to pre-and postharvest contamination with aflatoxins produced mostly by *Aspergillus flavus*. This study evaluated the in-vitro antifungal efficacy of five locally available botanicals (*Azadirachta indica*, *Calotropis procera*, *Euphorbia hirta*, *Jatropha curcas*, and *Ricinus communis*) against the growth of *A. flavus*. The botanical leaves were collected mainly from the Kongwa district, while strains of *Aspergillus* species were isolated from maize grain from Kongwa and Morogoro rural districts during the cropping season 2021/2022. The effect of botanicals on the radial mycelium growth of *A. flavus* was tested at concentrations of 0.2 0.25, 0.3, and 0.35 g/mL. The inhibitory effect was proportional to the concentration of botanicals. The highest radial growth inhibition of *A. flavus* was exhibited by extracts of *J. curcas* (69.7%), *R. communis* (62.8%), *A. indica* (61.8%), and *E. hirta* (52.4%). The least mean inhibition was found in *C. procera* (45.7%) at a concentration of 0.35 g/ml. The antifungal effect of botanicals demonstrated in this study warrants further evaluation for pre-harvest management of *Aspergillus* species contamination under field conditions.

Keywords: *Botanicals extracts, Aspergillus flavus, Mycelium inhibition, Radial mycelium growth, Maize, Tanzania*

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Introduction

Maize is the most important cereal crop in Tanzania which is used for food production, income, poverty reduction, and food security (Lobulu *et al.,* 2019). The annual per capita consumption of maize in Tanzania is about 112.5 kg (i.e., 308 g/day), and the annual maize consumption is estimated to be three million tons (Fisher *et al.,* 2015; Degraeve *et al.,* 2015). Despite the importance of maize in Tanzania, the crop is vulnerable to *Aspergillus flavus* and aflatoxin contaminations that cause maize grains to be unfit for consumption (Seetha *et al.,* 2017). Consumption of maize grains heavily contaminated with aflatoxin can lead to severe pathological conditions, including liver cancer, immune system deficiency, and impaired development in children (Bennett and Klich, 2003; JECFA, 2018; Kamala *et al.,* 2018; Leroy *et al.*, 2018).

Several strategies have been employed to reduce the extent of *A. flavus* and aflatoxin contamination in maize fields in Tanzania. These strategies include the application of Aflasafe-

biocontrol products, chemical application, and cultural practices (Bandyopadhyay *et al.,* 2016; Mahuku *et al.,* 2019). However, some of those approaches are costly, arduous, and expensive for small-scale farmers to afford (Gizaw, 2021). The use of synthetic fungicides to control *A. flavus* may have detrimental effects on the environment and even on beneficial microorganisms. Whereas, the reduction of *A. flavus* in the field by cultural management demands long-term planning, and utilization of Aflasafe in the field may be constrained by the lack of suitable weather conditions required for enhanced multiplication and competition with the toxigenic strains in the field. Hence, there is a need of developing alternative control strategies under field conditions. Some botanical extracts have demonstrated strong antifungal qualities that make them a viable alternative for managing *A. flavus* growth (Koita and Sankara, 2017). The botanical extracts contain chemicals that have antifungal characteristics (Seepe *et al.,* 2020). Additionally, their use against phytopathogenic fungi at the pre-harvest stage has less impact on the environment and human health (Sharman *et al.,* 2012). Previous studies have demonstrated that botanical extracts of *Cymbopogon citratus*, *Cinnamomum bejolghota*, *Syzygium aromaticum,* and *Thymus mongolicus* can inhibit the growth of mycelium of *A. flavus* and *Fusarium* spp which are pathogens of maize (Muchiri, *et al.,* 2017). Despite the efficacy of these botanical extracts against mycotoxigenic fungi, there is little information on the evaluation of the botanical extracts against *A. flavus* growth and aflatoxin contamination in Tanzania. As such, this study was conducted to evaluate the in-vitro efficacy of five available local botanical extracts for possible management of *A. flavus* and aflatoxin contamination in the field.

Materials and Methods

Study area

The study was conducted under laboratory conditions at the Sokoine University of Agriculture, Morogoro, Tanzania between March and May 2022.

Type and source of botanicals

The botanicals used in this study are listed in Table 1. Fresh leaves of about 5 kg of each plant species were harvested using a knife and put in a perforated plastic bag and labeled properly before further processing.

Table1.

Type and source of botanicals used in this study

S/N	Common name	Scientific name	Collected from	Antifungal compound[1]
1	Neem	*Azadirachta indica*	Kongwa	Azadirachtin, polyphenolic
2	Sodom apple	*Calotropis procera*	Kongwa	Tannin, saponin
3	Asthma weed	*Euphobia hirta*	Kongwa	Quercetol
4	Barbados nut	*Jatropha curcas*	Morogoro	Saponins, tannins
5	Castor oil	*Ricinus communis*	Kongwa	Alkaloids, flavonoids

1=Source (Parthipan *et al.,* 2017; Radwan *et al.,* 2019; Das *et al.,* 2022; Patil *et al.,* 2022; Rahu *et al.,* 2022).

Preparation of botanical extracts

Leaf samples were washed with running tape water to remove soil and rinsed with sterile distilled water three times. The leaf samples were then cut into small pieces and placed on benches in the screen house at 25˚C - 28˚C to dry for 2 weeks. The dried leaves of each plant species were ground into powder separately using a disinfected electrical powder grinder (HR-06B manufactured by Zhejiang Horus Industry and Trade Co., Ltd) and then sieved with a one-millimetre sieve. The powder of each plant species was packed in waterproof plastic containers and labelled appropriately and stored at 4˚C until used. The concentrations of crude botanical extracts were obtained according to the method described by Okereke *et al.* (2017). Briefly, 20 g, 25 g, 30 g, and 35 g of each plant material were dissolved in 100 ml of sterile distilled water giving a concentration of (0.2, 0.25, 0.3, and 0.35 g/ml), respectively. The botanical extracts were then incubated at 25˚C - 28˚C for 24 hours as described by Nduagu *et al.* (2008) and Zida *et al.* (2008). The infusions from each concentration were filtered separately through a sterilized double-layered cheesecloth into a sterile 500 ml beaker and the resulting stock solution was collected and stored at 25˚C - 28˚C until used.

Maize sampling and media preparation

Twenty samples of maize grains were collected from various farmers' storage structures in the Kongwa and Morogoro districts during the 2021/2022 cropping season. The grains were collected immediately after harvest before any synthetic chemicals were applied to the maize or storage structures. The Potato Dextrose Agar media (HiMedia Laboratories Pvt. Ltd) was prepared following the manufacturer's instructions. The sterilized medium was dispensed into Petri dishes and kept at 4°C until inoculation.

Isolation of Aspergillus flavus

The isolation of *A. flavus* from maize grain was done according to the procedure of Khan *et al.* (2020). The grains were surface sterilized in 2% sodium hypochlorite for two minutes and rinsed in several changes of sterile distilled water. The sterilized grains were then placed on moistened filter papers. The dried maize grains of about 250 g were ground by using an electric grinder (HR-06B manufactured by Zhejiang Horus Industry and Trade Co. Ltd to obtain maize flour. Twenty grams of maize flour was suspended in 100 ml of sterile distilled water. The solution was shaken well and left for some time. An aliquot of 1µl of the solution was spread onto the Rose Bengal Agar plate by using a sterilized glass rod. The Petri dishes were then sealed with laboratory tape and kept in the incubation chamber at room temperature and observed for fungal growth. The subcultures were then made from the emerging colonies. Subculturing was repeated several times until pure cultures of each presumptive isolate of *A. flavus* were obtained. The identification of *A. flavus* was performed using morphological characteristics such as colour, texture, and appearance, as well as microscopic characteristics under a light microscope (Klich., 2002). The culture with greenish yellow colour and white colour at the margin was selected and preliminarily identified as *A. flavus*

Molecular identification of Aspergillus flavus

The identity of *A. flavus* isolates was confirmed by analyzing Internal Transcribed Spacer (IT S) genes according to Abdelaziz *et al.* (2022). To confirm the fungi species, sequence analysis of the ITS region was performed using universal primers (Forward primer, ITS 4-TCCGTAGGTGAACCTGCGG, and Reverse primer, ITS 5-TCCTCCGCTTATTGATATGC). The reactions were carried out in volumes of 25 µl containing 4 µl (10 pg–100 ng) of template DNA, 1 µl of each primer, and PCR Master mix. PCR, the products were detected on 1% agarose ethidium bromide gels in TAE 1X buffer. A nucleotide blast to the obtained sequence was performed in NCBI using the blast suite.

Evaluation of botanicals for inhibition of mycelia growth

The experiment on the efficacy of botanical extracts against *A. flavus* growth was laid out in a complete randomized design (CRD) with 5 x 4 treatment combinations and replicated three times. Inhibition of mycelia growth was evaluated by using the Poison food technique as described by Islam *et al.* (2016). Four concentrations (0.2, 0.25, 0.30, and 0.35 g/ml) of each plant extract were prepared and 1 µl aliquot

was added directly to the potato dextrose Agar (PDA) via a sterile Sertorius filter in a laminar flow chamber. The Petri dishes with botanical extracts were left in the Lamina flow chamber until the media solidified. The PDA Petri dishes without plant extracts were used as a control.

Radial mycelia growth of *Aspergillus* species was measured daily starting from three days after inoculation until the ninth day. The antifungal effect of plant botanicals on the radial growth of *Aspergillus flavus* was expressed as a percentage inhibition of mycelia growth by using the formula described by Akinbode (2013).

$$\% \mathbf{PI} = \frac{(C-T)}{C} X\ 100 \quad \text{......} \quad (1)$$

Where: PI = Percentage inhibition, **C** = Average diameter of the fungal colony in control plate (cm), **T** = Average diameter of the fungal colony in treatment plate (cm).

Data analysis

All data collected were subjected to analysis of variance (ANOVA) using GenStat statistical software (16th version). The comparison of means of radial growth of mycelia and percentage inhibition was performed using the Tukey Honest Significant Difference test (HSD) at a 5% level of significance.

Results

Morphological features of fungal isolates

The isolates obtained from infected maize grains were identified morphologically as *A. flavus*. The colonies of the isolates on the PDA medium were yellowish-green at the centre of the colony while on the reverse side of the plates, a cream color was observed (Figure 1a). The average mean growth of *A. flavus* was 7.5 cm in diameter at 27°C after seven days of incubation. The colony of the isolate was delimited by a white border with radial grooves. The conidia of the isolate were globose, thin-walled, and slightly roughed, while the sclerotia of the isolates were compact in the mass of hardened fungal mycelia. The conidiophores were radiated onto loosely packed phialides (Figure 1b).

Figure 1a:

Macroscopic appearance of A. flavus

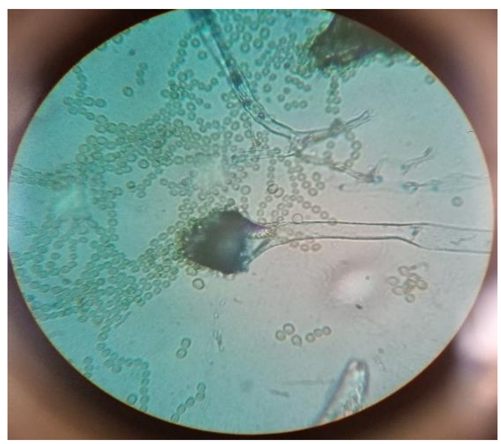

Figure 1b:

Microscopic visualization of A. flavus

Molecular identification of A. flavus

Among the six tested fungi isolates the results indicated that only three isolates confirmed the presence of a gene of *A. flavus* (Isolates number 31, 33, and 34) on the molecular genetic level. The Lanes (31–TG2) is the PCR amplification product (500 bp) by using the Universal primers (ITS1 F/ITS4R). Lane (NC) Non-DNA template. M: (100) bp DNA molecular size marker.

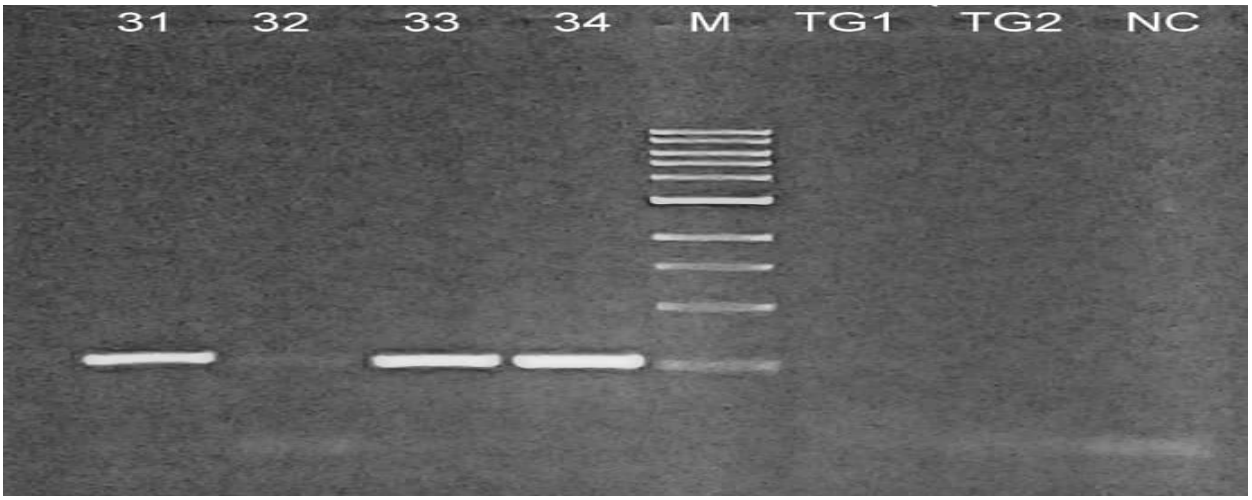

Figure 2:

PCR-based detection of A. flavus gene.

Effect of botanicals on radial growth of Aspergillus flavus mycelia

All botanical treatments significantly reduced the radial growth of mycelia as compared to the untreated control (Figures 1-5). Generally, the effect was more pronounced 9 days after inoculation (DAI). Application of botanicals at the concentration of 0.2 g/mL 9 DAI resulted in less radial inhibition compared to other concentrations. Application of botanicals at the concentration of 0.35 g/mL 9 DAI had the highest effect on radial mycelium growth than all other concentrations

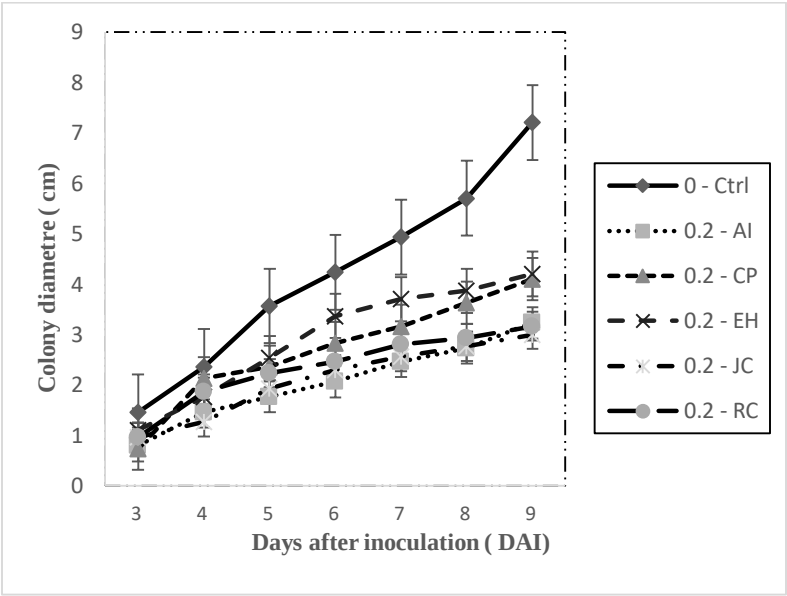

Figure 2.

Effects of botanical treatments on colony growth of A. flavus from 3 to 9 days after inoculation with 0.2 g/mL of Azadirachta indica (AI), Calotropis procera (CP), Euphorbia hirta (EH), Jatropha curcas (JC), Ricinus communis (RC) and Untreated control (Ctrl). Bars on the line graphs represent standard errors of the means

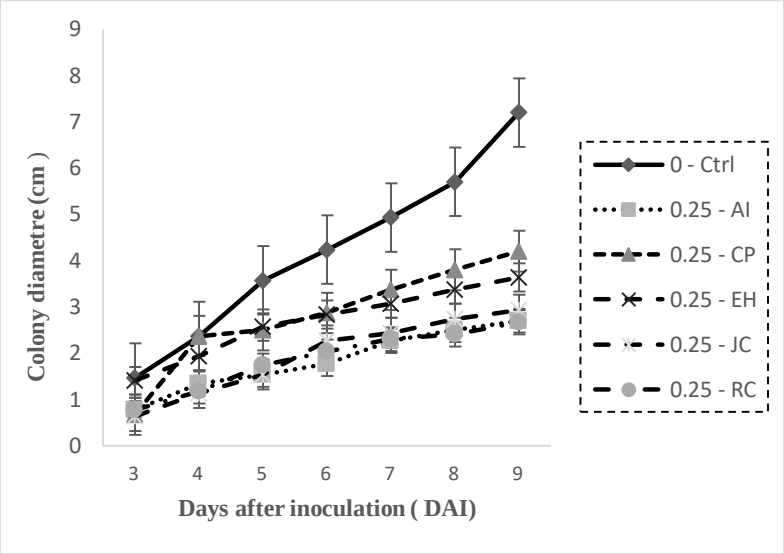

Figure 3.

Effects of botanical treatments on colony growth of A. flavus from 3 to 9 days after inoculation with 0.25 g/mL of Azadirachta indica (AI), Calotropis procera (CP), Euphorbia hirta (EH), Jatropha curcas (JC), Ricinus communis (RC) and Untreated control (Ctrl). Bars on the line graphs represent standard errors of the means.

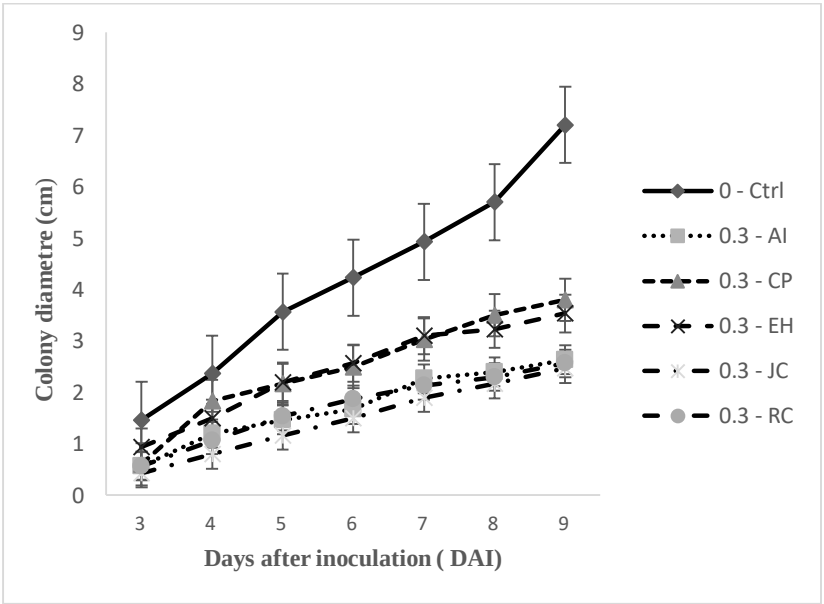

Figure 4.

Effects of botanical treatments on colony growth of A. flavus from 3 to 9 days after inoculation with 0.3 g/mL of Azadirachta indica (AI), Calotropis procera (CP), Euphorbia hirta (EH), Jatropha curcas (JC), Ricinus communis (RC) and Untreated control (Ctrl). Bars on the line graphs represent standard errors of the means.

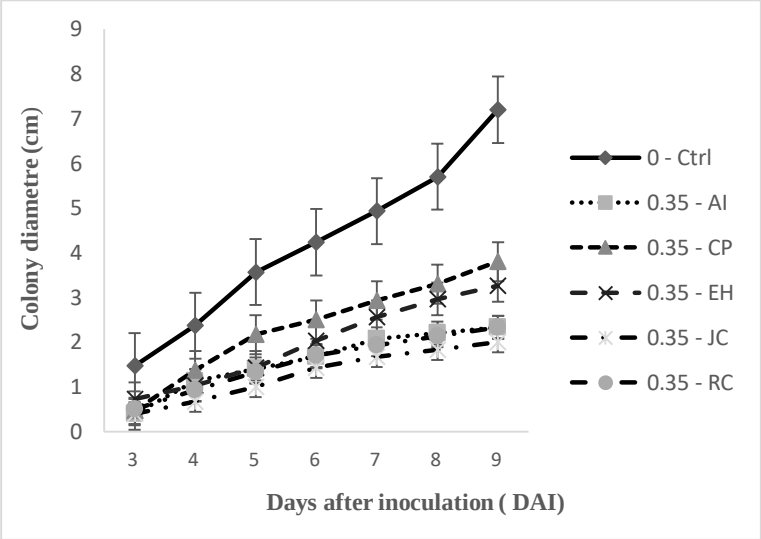

Figure 5.

Effects of botanical treatments on colony growth of A. flavus from 3 to 9 days after inoculation with 0.35 g/mL of Azadirachta indica (AI), Calotropis procera (CP), Euphorbia hirta (EH), Jatropha curcas (JC), Ricinus communis (RC) and Untreated control (Ctrl). Bars on the line graphs represent standard errors of the means.

Inhibition of Aspergillus flavus mycelia by botanicals

On the 9th day post-inoculation, all concentrations of *A. indica* significantly inhibited the growth of fungal mycelia (Tables 2-6). The extent of inhibition increased with increasing concentration of the botanical treatments (Table 2). Similar trends were observed with *Calotropis procera* (Table 3), *Euphorbia hirta* (Table 4), *Jatropha curcas* (Table 5), and *Ricinus communis* (Table 6).

Table 2.

Radial growth and extent of inhibition of Aspergillus flavus mycelia at different concentrations of Azadirachta indica treatment nine days after inoculation.

Treatment		Radial growth diameter			Inhibition of growth	
Number	Concentration (g/mL)	(cm)	SD[1]	SE[1]	(%)	SE
1	0	4.21a[2]	1.870	0.408	0d	0
2	0.2	2.08b	0.806	0.176	49.0c	1.74
3	0.25	1.84b	0.664	0.145	54.0bc	1.87
4	0.3	1.74b	0.716	0.156	57.8ab	1.58
5	0.35	1.60b	0.659	0.144	61.8a	1.68

1=SD and SE are the standard deviations and Standard error of the mean, respectively.
2=Means within columns followed by common letters are not significantly different at P≤ 0.05, according to Tukey's Honest significant difference test, values are means of three replications.

Table 3.

Radial growth and extent of inhibition of Aspergillus flavus mycelia at different concentrations of Calotropis procera treatment nine days after inoculation.

Treatment		Radial growth diameter			Inhibition of growth	
Number	**Concentration (g/mL)**	**(cm)**	**SD[1]**	**SE[1]**	**(%)**	**SE**
1	0	4.21a[2]	1.87	0.408	0c	0
2	0.2	2.71b	1.07	31.8	31.8b	2.69
3	0.25	2.82b	1.11	0.168	34.5b	3.55
4	0.3	2.49b	1.08	0.202	40.9ab	2.98
5	0.35	2.36b	1.14	0.207	45.7a	2.84

1=SD and SE are the standard deviations and Standard error of the mean, respectively.
2=Means within columns followed by common letters are not significantly different at P≤ 0.05, according to Tukey's Honest significant difference test, values are means of three replications

Table 4.

Radial growth and extent of inhibition of Aspergillus flavus mycelia at different concentrations of Euphorbia hirta treatment nine days after inoculation

Treatment		Radial growth diameter			Inhibition of growth	
Number	**Concentration (g/mL)**	**(cm)**	**SD[1]**	**SE[1]**	**(%)**	**SE**
1	0	4.21a[2]	1.87	0.408	0d	0
2	0.2	2.93b	1.14	0.248	28.2c	2.33
3	0.25	2.69b	0.772	0.168	30.1c	3.58
4	0.3	2.44b	0.928	0.202	40b	2.26
5	0.35	2b	0.951	0.207	52.4a	2

1=SD and SE are the standard deviations and Standard error of the mean, respectively.
2=Means within columns followed by common letters are not significantly different at P≤ 0.05, according to Tukey's Honest significant difference test, values are means of three replications

Table 5.

Radial growth and extent of inhibition of Aspergillus flavus mycelia at different concentrations of Jatropha curcas treatment nine days after inoculation

Treatment		Radial growth diameter			Inhibition of growth	
Number	**Concentration (g/mL)**	**(cm)**	**SD[1]**	**SE[1]**	**(%)**	**SE**
1	0	4.21a[2]	1.87	0.408	0d	0
2	0.2	2.11b	0.753	0.164	46.9c	9.02
3	0.25	1.95b	0.867	0.189	53.3b	9.25
4	0.3	1.49b	0.709	0.155	65.3a	5.01
5	0.35	1.29b	0.615	0.134	69.7a	6.34

1=SD and SE are the standard deviations and Standard error of the mean, respectively.
2=Means within columns followed by common letters are not significantly different at P≤ 0.05, according to Tukey's Honest significant difference test, values are means of three replications

Table 6.

Radial growth and extent of inhibition of Aspergillus flavus mycelia at different concentrations of Ricinus communis treatment nine days after inoculation

Treatment		Radial growth diameter			Inhibition of growth	
Number	**Concentration (g/mL)**	**(cm)**	**SD[1]**	**SE[1]**	**(%)**	**SE**
1	0	4.21a[2]	1.87	0.408	0d	0
2	0.2	2.35b	0.742	0.162	40.1c	2.85
3	0.25	1.87b	0.714	0.156	53.7b	2.11
4	0.3	1.72b	0.71	0.155	58.5ab	1.37
5	0.35	1.55b	0.69	0.151	62.8a	1.5

1=SD and SE are the standard deviations and Standard error of the mean, respectively.
2=Means within columns followed by common letters are not significantly different at P≤ 0.05, according to Tukey's Honest significant difference test, values are means of three replications

Discussion

The study revealed that extracts from four plant species; *J. curcas*, *A. indica*, *R. communis,* and *E. hirta* effectively reduced the radial mycelia growth of *A. flavus*. It was noted that the antifungal effect of botanicals was directly correlated to their concentrations, whereby the promising botanical extracts caused the largest reduction of radial mycelia growth at the highest concentration of 0.35g/m. This observation agrees with the study of El-Ghany *et al.* (2015),

who reported that the sprays of extracts of *J. curcas, R. communis*, and *A. indica* had antifungal properties and inhibited mycelium growth of *Fusarium, Aspergillus,* and *Cladosporium species*. Similar results were reported by Bayaso *et al.* (2013) and Teshome (2020), who tested the effectiveness of *Ricinus communis* and *Azadirachta indica* against the *Fusarium oxysporum, Alternaria alternata,* and *Aspergillus flavus in-vitro.* The efficacy of botanical extracts on the inhibition of radial growth of *A. flavus* could be associated with the phytochemical compounds present in each botanical species indicated in Table 1. Botanicals contain a variety of phytochemicals such as azadirachtin, flavonoids, saponins, tannins, and other bioactive compounds (Okereke *et al.,* 2017). A similar study by Keta *et al.* (2019), supports the existence of these bioactive compounds notably, azadirachtin, alkaloids, flavonoids, tannins, and phenolic compounds that inhibited the mycelia radial growth of *A. flavus*.

The presence of these bioactive compounds might have impeded the ability of the fungal cell membrane to perform its function (Kamaluddin *et al.,* 2020). Furthermore, the bioactive compounds may have led to changes in *A. flavus* morphology, such as sporulation failure, reduced conidiophore growth, and hyphal deformation (Kaguchia *et al.,* 2021). Additionally, Lengai *et al.* (2020) noted that botanicals may have affected fungal mycelial growth, spore germination, and altered cell wall permeability. The radial mycelial growth inhibition observed in the present study corresponds to the findings described by Achugbu *et al.* (2016), Kaguchia *et al.* (2021), and Yousef *et al.* (2022). The positive correlation between antifungal activity and botanical concentration noted in this study was also reported by Alsudani *et al.* (2022). It is known that botanicals differ in their ability to suppress toxigenic fungi, including the *A. flavus*, with some possessing stronger antifungal qualities than others Gizaw (2021), and seem to affect several important fungal species such as *Fusarium* (El-Ghany *et al.*, 2015; Teshome, 2020). The fact that the effects of botanical treatment on radial mycelium inhibition increased with increasing concentration implies that higher concentrations than the ones used in this study may provide a better effect on *A. flavus*. It is worth noting that the promising botanicals reported in this study are widely available in the agroecological zones in the country with climatic conditions that favour the proliferation of *Aspergillus flavus* and aflatoxin contamination.

Conclusion

The present study demonstrates the potential use of selected botanicals against *Aspergillus flavus* growth. However, out of the five botanicals tested, four (*Jatropha curcas, Recinus communis, Azadirachta,* and *Euphorbia hirta)* showed good results in inhibiting the mycelium growth of *Aspergillus flavus* than *Calotropis procera* when a concentration of 0.35 g/ml was used and the *A. indica* botanical had antifungal activity at all concentrations. It is recommended that these botanicals be evaluated under field conditions for pre-harvest management of *Aspergillus species.*

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