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Research Application Summary

Use of medicinal plant extracts and light sensitization to inhibit conidia growth in aflatoxin producing *Aspergillus flavus*

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Abstract

Fungal proliferation causing aflatoxin contamination has been on the rise in Africa due to the adverse changes in climate. Deaths and diseases like cancers have been detected in human beings and animals due to consumption of these toxigenic mycotoxins. Chemical, cultural and biological means have been used to manage aflatoxins but they have failed to eliminate them fully. Medicinal plant extracts traditionally used to cure various ailments are safe in nature and they offer a promising remedy for the control of aflatoxin contamination. Inhibition activity of six aqueous and organic medicinal plant extracts was tested against aflatoxin producing *Aspergillus flavus*. Antifungal activities, photosensitization and phytochemical composition of aqueous and organic extracts of fruits from *Solanum aculeastrum*, bark from *Syzygium cordatum*, and leaves from *Prunus africana, Ocimum lamiifolium*, *Lippia kituensis*, and *Spinacia oleracea* were tested. Spores from four-day-old cultures of previously identified toxigenic fungi, UONV017 and UONV003, were used. Disc diffusion and broth dilution methods were used to test antifungal activity. The spores were suspended in 2 ml of each extract separately and treated with visible light (420 nm) for varying periods. Organic extracts displayed species and concentration dependent antifungal activity. Solanum aculeastrum had the highest zones of inhibition diameters in both strains: UONV017 (mean = 18.50 ± 0.71 mm) and UONV003 (mean = 11.92 ± 0.94 mm) at 600 mg/ml. Aqueous extracts had no antifungal activity (mean = < 8 mm). *Solanum aculeastrum* had the lowest minimum inhibitory concentration at 25 mg/ml against *A. flavus* UONV017. All the plant extracts in combination with light reduced the viability of fungal conidia compared with the controls without light, without extracts, and without both extracts and light. Six bioactive compounds were analyzed in the plant extracts. Medicinal plant extracts in this study can control conidia viability and hence with further development can control toxigenic fungal spread.

Key words: *Aspergillus flavus*, minimum inhibitory mycotoxins, phytochemical, toxigenic

Résumé

La prolifération fongique causant la contamination par l'aflatoxine est en augmentation en Afrique en raison des changements climatiques défavorables. Des décès et des maladies de cancers ont été détectés chez des humains et animaux en raison de la consommation de ces mycotoxines. Des moyens chimiques, culturels et biologiques ont été utilisés pour gérer les aflatoxines mais n'ont pas réussi à les éliminer complètement. Les extraits de plantes médicinales traditionnellement utilisées pour soigner

diverses affections sont sans danger et offrent un remède prometteur alternatif pour le contrôle de la contamination par l'aflatoxine. L'activité d'inhibition de six extraits aqueux et organiques de plantes médicinales a été testée contre *Aspergillus flavus*. Les activités antifongiques, la photosensibilisation et la composition *phytochimique* d'extraits aqueux et organiques de fruits de *Solanum aculeastrum*, d'écorce de *Syzygium cordatum* et de feuilles de *Prunus africana, Ocimum lamiifolium, Lippia kituensis* et *Spinacia oleracea* ont été testées. Des spores provenant de cultures âgées de quatre jours de champignons toxigènes précédemment identifiés, UONV017 et UONV003, ont été utilisées. Des méthodes de diffusion sur disque et de dilution ont été utilisées pour tester l'activité antifongique. Les spores ont été mises en suspension dans 2 ml de chaque extrait séparément et traitées avec de la lumière visible (420 nm) pendant des périodes variables. Les extraits organiques ont montré une activité antifongique dépendante de l'espèce et de la concentration. *Solanum aculeastrum* présentait les zones les plus élevées de diamètres d'inhibition dans les deux souches: UONV017 (moyenne = 18,50 ± 0,71 mm) et UONV003 (moyenne = 11,92 ± 0,94 mm) à 600 mg / ml. Les extraits aqueux n'avaient aucune activité antifongique (moyenne ≤ 8 mm). *Solanum aculeastrum* avait la plus faible concentration inhibitrice minimale à 25 mg / ml contre *A. flavus* UONV017. Tous les extraits de plantes en combinaison avec la lumière réduisaient la viabilité des conidies fongiques par rapport aux témoins sans lumière, sans extraits, et sans à la fois d'extraits et de lumière. Six composés bioactifs ont été analysés dans les extraits végétaux. Les extraits de plantes médicinales dans cette étude peuvent contrôler la viabilité des conidies et, par conséquent, avec un développement ultérieur, peuvent contrôler la propagation des champignons toxigènes.

Mots clés: *Aspergillus flavus,* mycotoxines inhibitrices minimales, phytochimiques, toxigènes

Introduction

The agricultural sector in the world has suffered numerous food losses and contaminations leading to death due to mycotoxin contamination (Hua, 2013). Regions with tropical climate are at more risk of mycotoxin contamination because of the adverse climatic changes and poor storage practices which enhance proliferation of mycotoxigenic species like *Aspergillus flavus*. Reports from World Health Organization (WHO) reveal that approximately 25% of all food products in the world are contaminated by mycotoxins and aflatoxin form a significant percentage of this contamination (Hua, 2013). *Aspergillus flavus* is a ubiquitous fungus which portrays weak parasitism. The fungus thrives in warm and moist climates which enhances production of a mycotoxin known as aflatoxin. The fungus contaminates a wide range of cereals and nuts like maize, wheat, sorghum, and groundnuts, which are staple foods in most parts of Africa. Toxigenic *A. flavus* contaminates these food products and in favorable climates of high moisture and temperatures, aflatoxins which are reported to be mutagenic, carcinogenic and lethal are produced (Okoth *et al*., 2014.). Aflatoxins have been classified as class 1 poisons by the International Agency for Research on Cancer (IARC) (Pitt, 2000). Aflatoxins also contaminate feed; hence products like meat, milk, cheese, and eggs get contaminated when animals consume the aflatoxin contaminated feed (Herzellah, 2013). *Aspergillus flavus* is the main fungi producing aflatoxin (Hua, 2013).

Since the year 1982, aflatoxin contamination has been reported in Kenya's Eastern, Central, Western and Coastal regions where it has led to aflatoxicosis in some cases leading to death of people and livestock (Pitt, 2000). Aeration, moisture, substrate and temperature are the factors that determine proliferation of *A. flavus* and production of aflatoxins. Chemical, physical, biological and cultural control trials in progress are all based on manipulation of the determining factors but they have not

yet achieved the desired control of aflatoxin production by *A. flavus*. African countries which are the most affected by aflatoxin contamination are in the process of establishing food policies and regulations to control contamination.

Since ancient times, plant extracts have been used for medicinal purposes. The bioactive and aromatic compounds like flavonoids, saponins, tannins, glycosides and alkaloids have natural antioxidants which counter the activities of pests, diseases and nematodes (Kiswii *et al*., 2014). This study evaluated the impact of known medicinal plant extracts on inhibiting the growth of toxigenic *A. flavus* conidia. Visible light has also been reported to bind and kill metabolically active and dormant structures like conidia which are an advantage over conventional methods which only kill the metabolically active compounds (Temba *et al*., 2016). Using the light at a specific wavelength to hit a bioactive compound like those in plant extracts results to a reaction which kills toxigenic cells and the process is known as photosensitization (Temba *et al*., 2016). Photosensitization is a novel technique which has been confirmed safe and effective against mycotoxigenic cells but very few photosensitisers have been approved. Plant extracts on the other hand are safe, available and safe for use hence combining the photosensitization technique with use of medicinal plant extracts is a potential way of inhibiting the growth of conidia and spores in aflatoxin producing *A. flavus* hence minimizing aflatoxin contamination.

This study focused on evaluating the antifungal activity and phytochemical composition of plant extracts. The ability of visible light to stimulate the bioactive compounds in the plant extracts (photosensitization) and hence increase in the antifungal activity of the extracts against toxigenic *A. flavus* which causes aflatoxin production was also tested.

Materials and methods

Five plant parts namely *Ocimum lamiifolium* leaves (LMM 2015/05), *Prunus africana* leaves (LMM 2015/03), *Solanum aculeastrum* fruits (LMM 2015/01), *Lippia kituiensis* leaves (LMM 2015/04), and *Syzygium cordatum* bark (LMM 2015/02) whose medicinal values were already known were collected from Gakoe forest, Gatundu district in the Central region of Kenya. Fresh leaves of Spinacia oleracea (LMM 2015/06) were also collected from the local market. All plants were confirmed using reference materials from the University of Nairobi herbarium.

Dichloromethane ethanol at a ratio of 1:1 was used for organic extraction after the selected plant parts had been dried at room temperature, chopped and ground. A rotary evaporator was used for concentrating the organic extracts. Distilled water was used for aqueous extraction and a freeze drier at 4^{o}C was used to concentrate the aqueous extracts (Kiswii *et al*., 2014). The toxigenic *A. flavus* strains used in this study had been obtained from University of Nairobi mycology culture collection and they had been tested for toxigenicity through molecular characterization (Okoth *et al*., 2012). The two isolates UONV017 and UONV003 were transferred from stock culture into sterile PDA plates and incubated at 29oC for 4 days after which spores were aseptically harvested and suspended in sterile distilled water with three drops of Tween80 solution and standardized to a turbidity of 1 McFarland solution (3 × 108 CFU/ml).

Disc diffusion technique discussed by Sigei *et al*. (2015) was used to test for antifungal activities. Broth dilution technique described by Kiswii *et al*. (2014) was used to test for minimum inhibitory concentration. A photosensitization procedure described by Temba *et al*. (2016) was used while

phytochemical compounds were determined following the guidelines of Opoku & Akoto (2015).

Results and discussion

Crude organic extracts exhibited better antifungal activity against the toxigenic *A. flavus* strains as compared to the aqueous extracts. The six aqueous extracts did not show any significant antifungal activity (P e" 0.05) while only one plant in the crude organic extracts failed to show significant antifungal activity. *Solanum aculeastrum* and *Syzygium cordatum* organic plant extracts had the highest antifungal activity which compared favorably with the standard antifungal control Apron star (250mg/ml) which is a class III Blue Active ingredient containing 20% thiamethoxam + 20% metalaxyl-M + 2% difenoconazole control (Table 1). The higher the concentration of plants extracts, the higher the antifungal activities exhibited by the inhibition diameters except in two cases where the *P. africana* plant extracts had higher inhibition diameters at 450mg/ml concentration than at 600mg/mg and *S. aculeastrum* extracts at 300mg/ml had higher diameter than at 450mg/ml. Higher concentration of plant extracts have more bioactive compounds compared to lower or more diluted concentrations hence the higher antifungal activity at higher concentrations (Kiswii *et al*., 2014). The high concentrations in some plant extracts however had a high viscosity which limited the diffusion of the extract into the media containing toxigenic *A. flavus* and this is attributed to the better antifungal activities at lower concentrations exhibited by the two plant extracts (Thippeswamy *et al*., 2013).

The strains of toxigenic *A. flavus* used exhibited variation in their resistance where strain UONV003 was more resistant hence producing smaller inhibition diameters as compared to strain UONV017 whose exhibition diameters were bigger (Fig. 1).

Extracts of S. aculeastrum had the lowest minimum inhibitory concentration at 25mg/ml against *A. flavus* (UONV017) and at 50mg/ml against *A. flavus* UONV003. *Syzygium cordatum* had an MIC of 50 mg/ml on *A. flavus* UONV017 and 100mg/ml on *A. flavus*

Table 1. Effect of different organic plant extracts against growth of *Aspergillus flavus* UONV017 at different concentrations.

Plants	Inhibition zones (mm) 600 mg/ml	Inhibition zones (mm) 450 mg/ml	Inhibition zones (mm) 300 mg/ml
S. aculeastrum	18.50±0.71ab	14.42±0.83b	11.67±0.54b
S. cordatum	17.00±1.26b	12.00±0.52bc	10.67±0.54bc
L. kituensis	11.08±0.53c	10.17±0.68cd	8.08±0.47cd
P. Africana	8.67±0.69c	9.25±0.71d	6.83±1.04d
O. lamiifolium	11.42±0.34c	6.67±0.26e	6.00±0.83d
S. oleracea	10.08±0.36c	9.00±0.30de	6.33±0.14d
Positive control 250mg/ml	22.00±0.63a	22.00±0.63a	22.00±0.63a
(Sig P < 0.05)	0.00	0.00	0.00

Numbers are means of twelve replications. One way ANOVA was used for analysis and means separated by Turkeys test. Numbers followed by the same letters in the same column are not significantly different (P < 0.05)

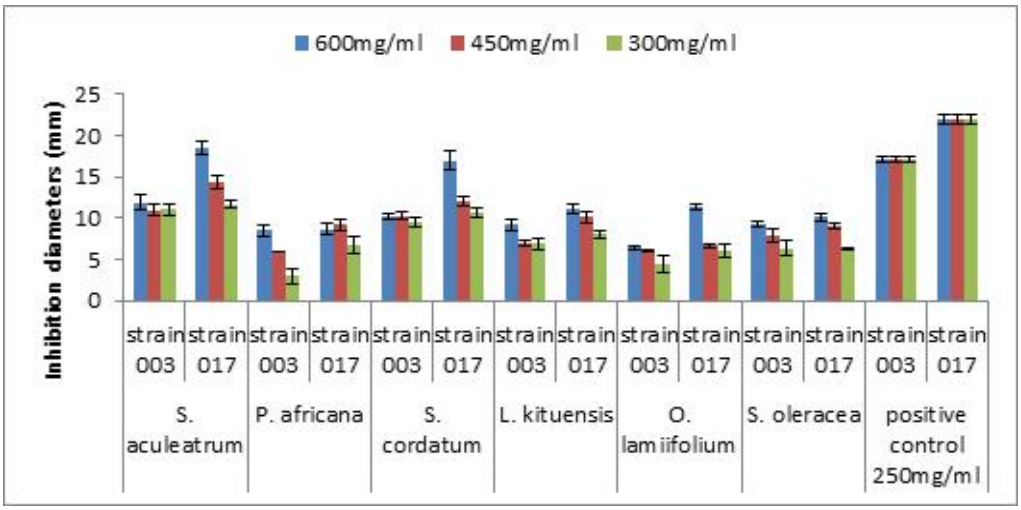

Figure 1. Comparison of inhibition of Aspergillus flavus (UONV017) and (UONV003) by organic plant extracts at different concentrations.

UONV003. The MIC is a measure of the minimum concentration of an extract that can inhibit the growth of a microorganism and the lower the MIC, the more effective the extract meaning *S. aculeastrum* and *S. Syzygium* were the most effective extracts in antifungal activity against toxigenic *A. flavus.*

Photosensitization activity revealed that antifungal activity of plant extracts was enhanced by exposure to light at a wavelength of 470nm for varying durations. The higher the duration of exposure to light, the higher the antifungal activity as shown in Figure 2. Highest concentrations (600mg/ml) of plant extracts exposed to light for the longest duration (40 minutes) had the highest antifungal activities which were determined by counting the number of fungal colonies. Light activity enhanced the activity of aqueous extracts which were less effective in the disc diffusion method and they were able to inactivate toxigenic *A. flavus* when exposed to light. This is in line with findings of Temba *et al*. (2016) where light at the 470nm wavelength activates the bioactive compounds in plants giving them the potential to inactivate and kill toxigenic cells.

The bioactive compounds were found in the plants at varying levels were saponins, flavonoids, terpenoids, tannins, alkaloids and glycosides. The presence of these bioactive compounds was attributed to the varying antifungal activities. *Spinacia oleracea* plant extracts had the highest percentage (21.6), followed by *S. aculeastrum* (19.6%), while *Prunus africana* had the lowest percentage (9.8) of bioactive compounds. Flavonoids had the highest frequency (21.6%) while terpenoids and steroids had the lowest frequency (9.8%). Organic extracts had a higher frequency (60.8%) of bioactive compounds compared to aqueous extracts (39.2%). The extract with the highest concentration of bioactive compounds did not have the highest antifungal activity meaning it is the concentration of specific bioactive compounds like flavonoids which were in high amounts in *S. aculeastrum* and *S. syzygium* where were the most effective that determined rates of antifungal activities. 60.8% of bioactive compounds were found in the organic extracts while 39.2% of the compounds were in aqueous extracts and this relates to the higher antifungal activities in organic compounds as compared to the aqueous extracts. Thippeswamy *et al*. (2014), reports that most bioactive compounds have better solubility in organic solvents as compared to aqueous extracts hence the higher levels of the compounds in organic solvents. The 19.6% of bioactive compounds found in *S. aculeastrum* which

included saponins, alkaloids and glycosides have been associated with anticancer, anti-inflammatory and anti-cholesterol activities which relate to the high antifungal activity exhibited by this extract (Herzellah, 2013).

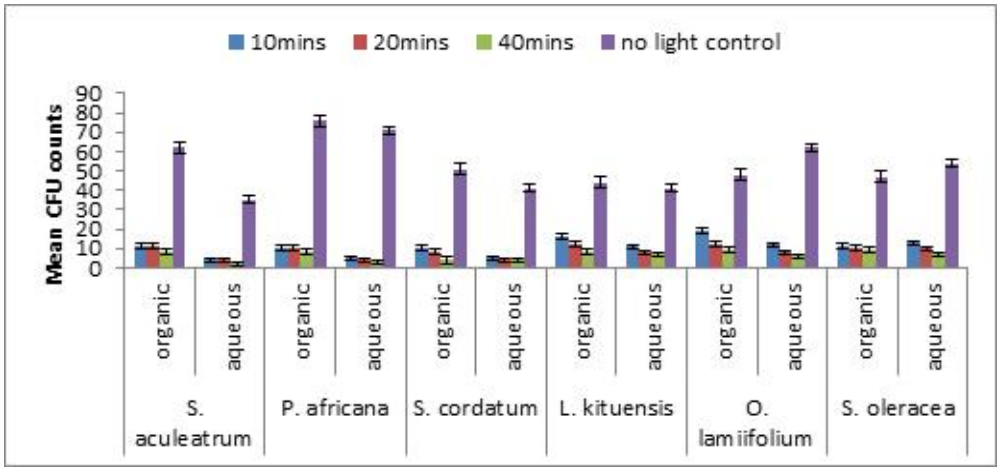

Figure 2. Effect of photosensitization on organic and aqueous plant extracts on Aspergillus flavus (UONV017) at 600 mg/ml within different time durations.

All the six bioactive compounds in this study have been shown to possess several antimicrobial activities in other studies whereby antifungal activities increase when the concentration of extracts is increased just as the results in this work revealed (Kiswii *et al*., 2014). The photo-degrading activity of plant extracts when exposed to light shown in this study through the reduction of colony forming units parallels a study by Pitt (2000) where conidia of *Penicillium digitatum* was significantly reduced by a combination of light and bioactive compounds as compared to light alone and bioactive compounds alone. The plant extracts contents which are bioactive compounds have higher absorption at higher wavelengths hence the enhanced antifungal activity even in the aqueous extracts which had insignificant antifungal activity in the absence of light at 470nm wavelength (Temba *et al*., 2016). The higher rate of spore knockout shown when the duration of light exposure was more parallels a study by Temba *et al*. (2016) where the more the light bioactive compound interaction, the more reactive the bioactive compound becomes makes it capable of killing more toxigenic cells. It was however noticeable that in some trails, high concentrations of plant extracts (600mg/ml) had very high turbidity which limited optimal light penetration causing photosensitization activity to be higher is lower concentrations.

The effective plant extracts used in this study are novel meaning they have not been used in another study. Therefore, the study findings form a basis of a new and effective intervention of controlling aflatoxin production by toxigenic *A. flavus* which has been challenging the agricultural sector globally. The plant extracts are safe and accessible since they were collected on basis of biodiversity hence they will not only eliminate the toxigenic *A. flavus* but also maintain a sustainable environment.

Conclusion

The most effective bioactive compounds like flavonoids could be extracted and multiplied inn edible plants like vegetables and fruits through technological means to amplify their use in controlling aflatoxin contamination. The plant extracts contents and ability to control *A. flavus* makes them a

target for research and developments and they have added advantages since they are biodegradable and selectively toxic. The consumable plant extract *Spinacia oleracea* in this study can act as a good candidate to treat food and feed which can then be dried under sunlight before storage leading to inactivation and death of the toxigenic *A.flavus* conidia. This is a viable technique to control aflatoxin contamination in storage because consumption of the dead *A. flavus* has no health implications. This could be applied to all the plant extracts in this study after a toxicity test to ensure safety.

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