ANTIFUNGAL EFFECTS OF ETHANOL EXTRACTS OF SOME MEDICINAL PLANTS ON GERMINATION AND GROWTH OF *Aspergillus niger* Van Tiegh IN CULTURE

Nweke, F.U.
Department of Crop and Soil Science, Faculty of Agriculture, Dennis Osadebay University, Anwai, Asaba, Delta State, Nigeria.
Email*: nwekefu@gmail.com.*

ABSTRACT
The study evaluated the effects of ethanol extracts of the leaves of Citrus aurantifolia (Christm) Swingle, Spondia's mombin L. and Anacardium occidentale L. against Aspergillus niger Van Tiegh isolated from dry tuber rot specimens of yam (Dioscorea rotundata Poir), Phytochemical properties of the extracts should dose dependent inhibition of spore germination and radial growth of the fungus. At 100% concentration C. aurantifolia was the most toxic to the fungus and exhibited 58.8% and 63.9% inhibition of spore germination and radial growth of the pathogen respectively, while A. occidentale was the least is inhibition effect on the test parameters of the fungus at all concentrations. Phytochemical analysis of the extracts revealed the presence of alkaloids, flavonoids, glycosides, tannins, saponins and terpenoids which accounted for the antifungal effects exhibited on the pathogen.

Keywords: Antifungal effects, *Aspergillus niger*, plant extracts, yam.

INTRODUCTION

The rising concerns about the pollution problems in the environment and the toxic effects of synthetic chemicals on non-target organisms have in recent years, necessitated investigations on exploiting plant-derived pesticides (Taiga, 2011; Okigbo *et al*., 2013; Nweke, 2015; Nwaogu and Wokocha, 2018 and Enyiukwu *et al*., 2021). Research has shown that extracts of medicinal plants such as *Piper guineense* (Schum and Thonn) contain bioactive compounds (Mgbeahuruike *et al*., 2017) and so have potential for use in the development of natural disease control products.

Investigations by some workers have shown that plant-based chemicals are non-phytotoxic and eco-friendly alternative pesticide (Okigbo *et al.,* 2013; Enyiukwu *et al*., 2014; Elaigwu *et al*., 2018). In addition, plant-based pesticides are cheap and readily available in developing countries where synthetic fungicides and scarce and costly for rural peasant farmers (Okigbo *et al.,* 2013; Gwa *et al*., 2021).

Many workers have reported antimicrobial activity of plant extracts. Elaigwu *et al* (2017) reported the control of *Macrophomina phaseolina* (Tassi) Goid in Sesame (*Sesamam indicum L.)* with plant-derived fungicides. Leaf extracts of *Alchornea cordifolia*, *Tabernaemontana pachysiphon* and *Lantan camara* have been reported to inhibit the germination and growth of *Colletotrichum destructivum* O' Gara in culture (Enyiukwu *et al.,* 2021). Zubaru and Gwa (2019) similarly reported that seed extracts of *Azadirachta indica* and *Moringa oleifera* inhibited *Aspergillus niger* and *Fusarium oxysporum* isolated from hot pepper (*Capsicum annum L.*) in culture. The crude extract of *Aloe vera* successfully reduced disease incidence in *Telfaria occidentalis* (Chuku *et al*., 2012). Similarly, Gwa *et al* (2021) reported that extracts of *Azadirachta indica, Piper guineense* and *Zingiber officinale* effectively protected white yam tubers from postharvest rot fungi. According to the authors, the extracts protected yam tubers from postharvest rot pathogens for up to five months of storage. Much of the potential sources of botanical fungicides still remain especially in tropical forests awaiting exploitation (Wokocha and Okereke 2005).

Aspergillus niger is a soil-borne fungus belonging to order *Eurotiales* of class plectomycetes. It is one of the major micro-organisms responsible for postharvest rot of yam tubers (Okigbo *et al.,* 2013; Nweke, 2015; Gwa *et al.,* 2021). Other species of *Aspergillus* which attack yam tubers are *A. flavas* link and *A. tamari* kita (Okigbo *et al.,* 2013; Gwa *et al.,* 2021.). The pathogen have been reported to attack many fruits, vegetables and cereals, resulting in serious agriculture and economic losses (Gautam *et al.,* 2011). In the same paper, Gautam *et al* (2011) reported that *A. niger* is one of the major causes of allergic disorders or mycotoxicoses in man and animals. The fungus has well developed, profusely branched and septate mycelium and bears conidia on erect, unbranched and aseptate conidiophores. Conidia are black, spherical, irregularly roughed and air borne in chains. It constitute the primary inoculum of the pathogen as well as its principal means of dispersal (Dijksterhuis, 2019). There are few reports on the control of *A. niger* by the use of natural plant products or biofungicides. The aim of this study was to evaluate the inhibitory effects of ethanol extracts of the leaves of *Citrus aurantifolia*, *Spondias mombin* and *Anacardium occidenta*le in culture against *Aspergillus niger* causing tuber rot of yam and to screen quantitatively for the chemical constituents of the extracts.

MATERIALS AND METHODS

COLLECTION AND PREPARATION OF PLANT MATERIALS

Leaves of *Citrus aurantifolia* (Christm) Swingle, *Spondias mombin L.* and *Anacardium occidentale* L. were collected from Asaba in Delta State. The leaves

were washed in tap water and air-dried under room temperature. The dried samples were ground separately into powdered form using a Tower blender Model (BL-NC-6802D, Italy) to obtain 300g of each sample.

PREPARATION OF CULTURE MEDIUM

Thirty nine grammes of potato dextrose agar (PDA) powder was dissolved in 1 litre of distilled water. The solution was then sterilized using autoclave at 121°C for 15 minutes. It was allowed to cool before being dispersed into Petri dishes.

ISOLATION OF INOCULUM

Tissue segments (about 2mm diameter) were cut from the periphery of the infected yam tuber and sterilized for 2 minutes in 10% sodium hypochlorite solution. The sterilized tissues were rinsed in three changes of sterile distilled water and placed in a Petri dish containing PDA medium at room temperature of 27°C. After 5 days, a pronounced black mass of mycelial growth was observed on the surface of the tissues. The organism was subcultured twice to get a pure culture, which was finally examined using a compound microscope and the identity of the organism confirmed to be *Aspergillus niger* with the aid of an identification manual by Dugan (2017).

PATHOGENCITY TEST

Mycelial discs (4mm diameter) were made from 6-day old PDA culture of *A.niger* isolated from the diseased yam tuber. The mycelial discs were inoculated into healthy yam tubers using the method of Okigbo *et al* (2013). The inoculated tubers were incubated at room temperature for 14 days in sterile polythene bag containing cotton wool soaked in sterile distilled water. At the end of the incubation period, the tubers were examined for rot. The isolate caused typical rot symptom on yam tuber, similar to the original isolate which confirmed it as pathogen. The fungus was re-isolated from the inoculated disease yam tuber, re-examined and confirmed identical with the previously isolated and inoculated isolate.

PREPARATION OF LEAF EXTRACT

Powdered *C. aurantifolia, S. monmbin* and *A. occidentale* were extracted using ethanol following the procedure described by Amadioha (2003). Briefly 25, 50, 75 and 100g of each plant powder was soaked separately in 100ml of 70% ethanol for 24 hours and strained through four-folds of cheese cloth. The filtrate was concentrated and evaporated over a steam bath at 90°C to obtain a syrupy extract.

EFFECT OF EXTRACT ON SPORE GERMINATION

The method of Enyiukwu *et al* (2021) was used. A disc (3mm diameter) of the fungus in 3ml of each of the concentrations was used to prepare suspensions of 5-day old cultures in test tubes. Similar spore suspensions were prepared in sterile distilled water as control. The contents of the tubes were filtered through four-fold cheese cloth. A drop of 0.05ml each of the filtrates was placed separately on sterile slides in humid chambers and incubated at 27°C for 24 hours.

One hundred spores were observed at random under a low power (X10) of the compound microscope and the germinating spores from each slide were carefully counted and recorded. Inhibitory percentage was calculated from the data obtained using the formula:

$$\% \text{ inhibition} = \frac{gc - gt}{gc} \times 100$$

Where

gc = average number of germinated spores in control slides

gt = average number of germinated spores in treated slides

EFFECT OF EXTRACT ON RADIAL GROWTH

The method of Okigbo *et al* (2013) was used. One millilitre of each leaf extract at 25, 50, 75 and 100% concentrations was dispensed separately into 9ml of cool molten PDA medium in each of the Petri dishes. The plates were gently swirled to ensure even dispersion of the extracts. The mixture was left to solidify and 2mm diameter mycelial disc obtained from the advancing edge of 5-day old culture of the fungus was inoculated into the centre of each Petri dish which had been marked underneath with two perpendicular lines intersecting at the centre. The control consisted of an inoculated agar plates without extracts. Three replicate plates were maintained. The plates were incubated at 27°C for 5 days and radial growth of the fungus was measured along the perpendicular lines with a meter rule 5 days after incubation.

Inhibitory percentage was calculated using the formula:

$$\% \text{ inhibition} = \frac{dc - dt}{dc} \times 100$$

Where

dc = average diameter of fungal colony in control plates

dt = average diameter of fungal colony in treated plates.

PHYTOCHEMICAL SCREENING

All the extracts were subjected to standard phytochemical qualitative screening for secondary metabolites as described by Poongothai *et al* (2011).

DATA ANALYSIS

The experiments were conducted in Completely Randomized Design (CRD) consisting of 13 treatments replicated 3 times. Data collected from the study were analysed by analysis of variance (ANOVA). Comparison of all treatments was made at

the 0.05 probability level using the Duncan Multiple Range Test Method.

RESULTS AND DISCUSSION

RESULTS

The results of *in vitro* effects of ethanol extracts of the test plant materials on spore germination of *A. niger* at different concentrations is presented in Table 1. The results showed that the extracts significantly (P<0.05) inhibited the spore germination of the fungus in culture. Extracts varied in their inhibitory activities against the fungus. Among the three plant species, *C. aurantifolia* extract at 100% concentration was the most toxic and exhibited 58.9% inhibition of spore germination while *A. occidentale* extract was the least in inhibition effect against spore germination at all concentrations.

Table 1: Inhibition of spore germination of *Aspergillus niger* by plant extracts at different concentrations after 24 hours incubation

Plant Extract	Percentage spore inhibition at different concentrations(%)			
	25	**50**	**75**	**100**
Citrus aurantifolia	47.3[a]	51.2[a]	57.6[a]	58.8[a]
Spondias mombin	37.4[b]	40.5[b]	42.9[b]	47.2[b]
Anacardium occidentale	9.7[c]	10.4[c]	16.7[c]	20.6[c]
Control	0.0	0.0	0.0	0.0

Data are means of three replicates in two separate experiments
Means in a column followed by different letters differ significantly at P<0.05(DMRT)

Table 2 shows the antifungal effects of the plant extracts against radial growth of the fungus at different concentrations. The results showed that the extracts significantly (P<0.05) inhibited the radial growth of the fungus in culture. Extracts varied in their antifungal effects. Extracts of *C. aurantifolia* at 100% concentraction was the most phytotoxic to the fungus and exhibited 63.9% inhibition of radial growth while *A. occidentale* extract was the least at all concentrations.

Table 2: Inhibition of radial growth of *Aspergillus niger by plant extracts at different concentrations after 5 days incubation at 28°C*

Plant Extract	Percentage growth inhibition at different concentrations (%)			
	25	**50**	**75**	**100**
Citrus aurantifolia	50.4[a]	54.8[a]	61.5[a]	63.9[a]
Spondias mombin	38.7[b]	42.3[b]	46.2[b]	50.7[b]
Anacardium occidentale	11.5[c]	13.9[c]	18.7[c]	22.6[c]
Control	0.0	0.0	0.0	0.0

Data are means of three replicates in two separate experiments
Means in a column followed by different letters differ significantly at P<0.05(DMRT)

Phytochemical analysis of the ethanol extracts of the leaves of *C.aurantifolia*, *S. prombin* and *A. occidentalis* showed that all the extracts had tannins. In Addition, *C. aurantifolia* extract contained flavonoids and glycosides. *Spondias mombin* extract contained alkaloids, flavonoids and saponins. *Anacardium occidentale* extract contained alkaloids and terpenoids as presented in Table 3.

Table 3: Phytochemical analysis of ethanol leaf extracts

Phytochemical	*Citrus aurantifolia*	*Spondias mombin*	*Anacardium occidentale*
Alkaloids	+	+	+
Flavonoids	+	+	-
Glycosides	+	-	-
Saponins	-	+	-
Tannins	+	+	+
Terpenoids	-	-	+

Keys: + = Present
- = Absent

DISCUSSION

The study evaluated the effects of ethanol extracts of *Citrus aurantifolia*, *Spondias*, *mombin* and *Anacardium occidentale* against *Aspergillus niger* in culture. Results revealed the presence of fungitoxic compounds in *C. aurantifolia*, *S. mombin* and *A. occidentale*. This is in agreement with the findings of several workers (Nweke, 2015; Elaigwu *et al.,* 2017; Amadioha *et al.,* 2019; Enyiukwu *et al.,* 2021). The fungicidal effects of plant extracts on different pathogens of crops have been widely reported (Mukherjee *et al*., 2011, Ogu and Owoeye, 2013;

Nwaogu and Wokocha, 2018). The fungitoxic effect was observed as reduction in spore germination and radial growth of the fungus in poisoned plates when compared with the control plates. The poisoned food technique has been employed by several researchers to evaluate the effect of plants and their compounds against fungi (Okigbo *et al.,* 2013, Balamurugan, 2014). However, the efficacy of the extracts differed with the plant extracts and concentrations. *Citrus aurantifolia* extract significantly ($P<0.05$) inhibited spore germination and radial growth of *A. niger* better than extracts of *S. mombin* and *A. occidentale.* The inhibitory effect of *C. aurantifolia* has been reported against *Macrophomina phaseolina* (Balamurugan, 2014) and *Fusarium oxysporum* in Okra (Okwu *et al.,* 2006).

The difference in fungitoxic activity among the plant extracts can be attributed to the differences in the nature of their active ingredients (Okigbo *et al.,* 2013). In general it was observed that the fungitoxic effects of the plant extracts increased with increasing concentration of the extracts. This is in conformity with observations made by Enyiukwu *et al,* (2021) and Okigbo *et al* (2013). The fungitoxicity profile of the concentrations of plant extracts on the test fungus was 100%> 75%>50%>25%.

The phytochemical analysis of the plant extracts revealed the presence of alkaloids, flavonoids, glycosides, tannins, saponins and terpenoids. Other workers have reported rich variety of secondary metabolites in *C. aurantifolia, S. mombin* and *A. occidentale* extracts (Okwu *et al.,* 2006; Nweke, 2015; Akin-Osanaiye and Audu, 2018).

These phytochemical compounds are known to be biologically active and thus contributed to the antifungal properties of the plants extracts. Studies have shown that alkaloids, flavonoids and terpenoids revealed in this study exert potent antifungal activity against *Aspergillus* species (Arabia *et al*., 2021). Tannins also detected in the plant extracts are known to possess remarkable toxic activity against fungi and bacteria (Banso and Adeyemo, 2007).

CONCLUSION

The extracts of *C. aurantifolia, S. mombin* and *A. occidentale* were found effective against *Aspergillus niger* causing tuber rot of yam with varying degrees of effectiveness. The extracts consisted of various phytochemical compounds which accounted for the antifungal activities exhibited against the pathogen. Further field experiments are to be conducted to recommend the extracts against the disease.

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