ANTIFUNGAL EFFECTS OF LEAF EXTRACTS OF THREE PLANT SPECIES AGAINST *COLLETOTRICHUM MUSAE* THE CAUSAL AGENT OF ANTHRACNOSE POSTHARVEST DISEASE OF BANANA FRUITS

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ABSTRACT
The antifungal effects of *Azadirachta indica, Calotropis procera* and *Anacardium occidentale* plants extracts were assessed on *Colletotrichum musae* the pathogen of anthracnose disease of banana fruit. The phytochemical constituents of the extracts were determined and agar well diffusion method was used to assess the toxicity at 50mg/ml, 100mg/ml and 150mg/ml against the pathogen isolated from banana with anthracnose disease symptoms. The phytochemical analysis results revealed the presence of alkaloids, saponnin, tannins, anthocyannin, phenolic acid and flavonoids. All the extracts inhibited mycelia growth of *Colletotrichum musae* which increased with increase in concentration and days of incubation. At the end of incubation period, the order of decrease in inhibition was control< 50mg/ml <100mg/ml <150mg/ml. The extract from *C. procera* gave the highest percentage growth inhibition while *A. indica* extracts was the least. Therefore, the extracts showed a wide range of antifungal activities against *C. musae* and could be cheaper substitute for conventional fungicide.

Keywords: Anthracnose, Postharrvest, *Colletotricum musae,* Extract, Management, Antifungal

INTRODUCTION
Banana plant belongs to genus *Musa*, family Musaceae and order Zingiberales/ It is one of the most-important fruit crops of the world(Ploetz *et al.*, 2007). In 2016 India and China were the largest producers of banana with 28% of total global production. Banana is one of the important staple foods in Nigeria with 2.74 million tons of banana annually (FAO,2017). A ripe fruit contains 22 percent of carbohydrate, high

in dietary fibre, potassium, manganese, and vitamins B6 and C. Bananas are eaten fresh, fried or mashed and chilled in pies or puddings. They may also be used to flavour muffins, cakes, or breads (Ploetz *et al.*, 2007). Dried banana peel contains 30 to 40% tannin content, rich in potash used for making soap and dye used to blacken leather (Lejju *et al*., 2005).

Banana is susceptible to several diseases resulting in postharvest losses during transportation and storage (Basel *et al*., 2002). Riped and unripped banana are affected by anthracnose caused by *Colletotrichum musae* (Berk. and Curt.) Arx. This is particularly associated with wastage following injuries in the form of scratches and other wounds sustained by the fruits during handling and transport (Unnithan, 2018). Anthracnose symptoms are manifested during storage and marketing with black and sunken lesions producing spore masses or acervuli in the lesion which deteriorates the quality and nutritive value of the fruits and renders them unfit for consumption and marketing leading to severe loss to farmers and traders (Prusky and Plumbley 1992).

The most profound practice used in controlling banana diseases is chemicals. This is costly and not environment friendly (Akila *et al*., 2011). Therefore, botanical extracts are considered as the best alternatives. Botanical extracts are not less expensive, easily available and eco-friendly in the management of various plant diseases (Akila, *et al.,*2011; Supriya *et al*., 2013). Thus, the present preliminary study was conducted to isolate, identify *Colletotrichum* species that are associated with anthracnose on banana fruits sold in the markets in Minna, Nigeria and to evaluate the anti fungi efficacy of extracts of Neem, Cashew, Sodom's apple and Pawpaw in the control of anthracnose disease pathogen of banana fruit. The results from this study will provide the basis for developing an effective and novel biofungicide to control anthracnose disease of banana fruit.

MATERIALS AND METHODS

Collection and Preparation of Plant Materials

Fresh leaves of *Carica papaya* (Pawpaw), *Azadirachta indica* (Neem plant), *Calotropis procera* (Sodom apple) and stem bark of *Anacardium occidentale* (Cashew) with respective herbarium numbers Fut/plb/hab0689, Fut/plb/hab0231, Fut/plb/hab0668 and Fut/plb/hab0034 were collected in sterile polythene bag between February and March, 2019 and names were authenticated at the herbarium of Plant Biology Department, Federal University of Technology, Minna, Nigeria. The plants were

washed, air dried for fifteen days and crushed into powder using mortar and pestle. The powdered samples were used for the extraction using soxhlet extraction procedure (Adebola *et al.,* 2019). The collected extracts were concentrated using rotary evaporator, dried below 50°C and at 90rpm and kept at -4°C in deep freezer for further use.

Collection and Isolation of *Colletotrichum musae*

During the survey, samples of banana fruit with symptoms of anthracnose infection were collected from fruit stores in Kure market Minna, Nigeria. The infected fruit tissues were surface sterilized with 70% ethanol for 3mins, cut into small bits(2mm) and rinsed thrice in sterile distilled water to remove the traces of alcohol. These tissues were inoculated on PDA supplemented with 100µg/ml streptomycin in a sterile Petri dishes under aseptic conditions and incubated at room temperature (28 ± 2°C). Observations were made at regular intervals for fungal growth for 7days (Keuete *et al*., 2016). Subcultures were made on new PDA. The isolated pathogen was identified based on the observed cultural and morphological features taking note of the growth rate and pattern on agar plates, conidial size, colony colour, present or absence of setae and shapes of conidia. Stock culture of the isolate was maintained in McCartney bottle /agar- slant and stored at -4^{O}C in refrigerator for subsequent use (Adebola *et al*., 2020).

Pathogenicity Test

Five fully matured green unripe banana fruits were collected from banana fields, washed under running tap water, blotted dried and surface sterilized with 70% ethanol. The fruits were injured with sterilized needle and 2ml spore suspension (1 x 10^5 conidia/ml) from seven day-old pathogen culture was scrubbed over each fruit. The fruits inoculated with sterile distilled water after pin prick served as control. The inoculated fruits surface was covered with sterile moist cotton and kept inside the moist chamber. The characteristic infection symptoms produced were observed for seven days. The fungus was re-isolated and identified from the fruits showing typical anthracnose symptoms (Adebola *et al.*, 2019).

Qualitative Phytochemical Screening of Leaves and Stem Bark Extracts

Phytochemical screen of the samples for the presence of phytochemical compounds was carried out using methods described by Hassan, (2010).

Screening of Botanicals for Antifungal Activities (Agar Diffusion Assay)

The modified agar diffusion method of Kumar *et al*. (2008) was used. The re-dissolved sterile plant extract at

50mg/ml, 100mg/ml and 150mg/ml concentrations were mixed with sterile PDA in ratio 1:3. Ten (10) ml were poured into sterile Petri dish (90mm diameter). The control was set up by substituting sterile distilled water for plant extract while all the treatments were replicated thrice. Four (4) mm disc of fungus from the periphery of 6day-old culture was inoculated at the dug 4mm well at centre of the PDA in the Petri dish and incubated at 28± 2°C for 7 days. Mycelial radial growth of the pathogen was recorded. Percentage inhibition was determined thus: $(R_1-R_2)/R_1X100$.Where R_1= Radial growth of pathogen in control and R_2 = the radial growth of pathogen in the test plate (Adebola *et al.,* 2020).

Data Analysis

The experiment was carried out in a Completely Randomized Design (CRD) with three replicates of each treatment. All data was subjected to Analysis of Variance (ANOVA) at 0.05%. New Ducan Multiple Range test was used to separate the mean (Adebola *et al.*, 2020).

RESULTS

Isolatated Causative Orgnism and Pathogenisity

The cultures of the causative organism were fast growing with white sparse aerial mycelia. The conidial were elliptical in shape without setae. The colony diameter was 7.8± 0.02 with irregular lobes. During pathogenicity test, necrotic lesions developed and orange colored conidial which later turned dark brown was observed. Pre-matured ripening of the fruits was also observed. Therefore, the fungus re-isolated showed typical anthracnose symptoms and confirmed to be *Colletotrichum musae*, the causative organism of anthracenose disease of banana fruit (Plates 1 and 2).

Phytochemical constituents of three botanicals

The qualitative phytochemical analysis (Table 1) revealed the presence of the alkaloid, saponin, tannin, anthocyanin, phenol and flavonoid in the plant extracts screened. Alkaloids, saponin, tannins and flavonoids were found in all the plant extracts while *A. indica* and *C. procera* did not contain anthocyanin and only phenolic acid was not found in *A. occidentale* .

Antifungal activities of *Calotropis procera* leaf extracts against *Colletotrichum musae*

The mycelia growth inhibition of *C. musae* at difference concentrations (Table 2) of *C. procera* leaf extracts was not significantly different ($P<0.05$) at day 1, but there was no growth at all in 100mg/ml and 150mg/ml. While the highest mycelial growth was obtained in control (1.00mm).

At day 2 mycelia started to appear in all the concentrations but significantly higher (P<0.05) in control (2.00mm) when compared to all the treatments. The decrease was proportional to the concentration in the order control>50>100>150mg/ml. This trend was observed till day 3 of the incubation. However, at days 4 and 5, the mycelia growth in all the treatments were significantly different (P>0.05). The growth in control was the highest 4mm and the least was observed in 150mg/ml at day 5. The earlier order was still maintained (control>50>100>150mg/ml).

The percentage inhibition of the growth of *C. musae* (Table 2) was observed to be inversely proportional to the concentration and this decreased with the days of incubation. In each day the highest percentage inhibition was observed in 150mg/ml 100, 98, 97, 95 and 94% in days 1, 2, 3, 4 and 5 respectively). The inhibition was significantly different (P>0.005) throughout the period of incubation.

Antifungal activities of the *Anacardium occidentale* leaf extracts on mycelia radial growth of *Colletotrichum musae*

The mycelia radial growth inhibition (Table 3) was observed in all treatments in *A. occidentalis* leaf extract including the control at day1. Although, there was significant difference (P>0.05) observed among all of them. The highest mycelial radial growth was observed in control (1.00mm) and least in 150mg/ml. The order of increases was control>50>100>150mg/ml. However, at days 4 and 5 there was no significant difference (P<0.05) observed between 100 and 150mg/ml (1.4mm and 1.20mm in day4 and 5 respectively).

The percentage growth inhibition of *C. musae* in different concentrations of *A. occidentalis* was observed to be significantly different (P>0.05) in each of the days (Table 3). The percentage inhibition was highest with 150mg/ml and least in 50mg/ml throughout the incubation days.

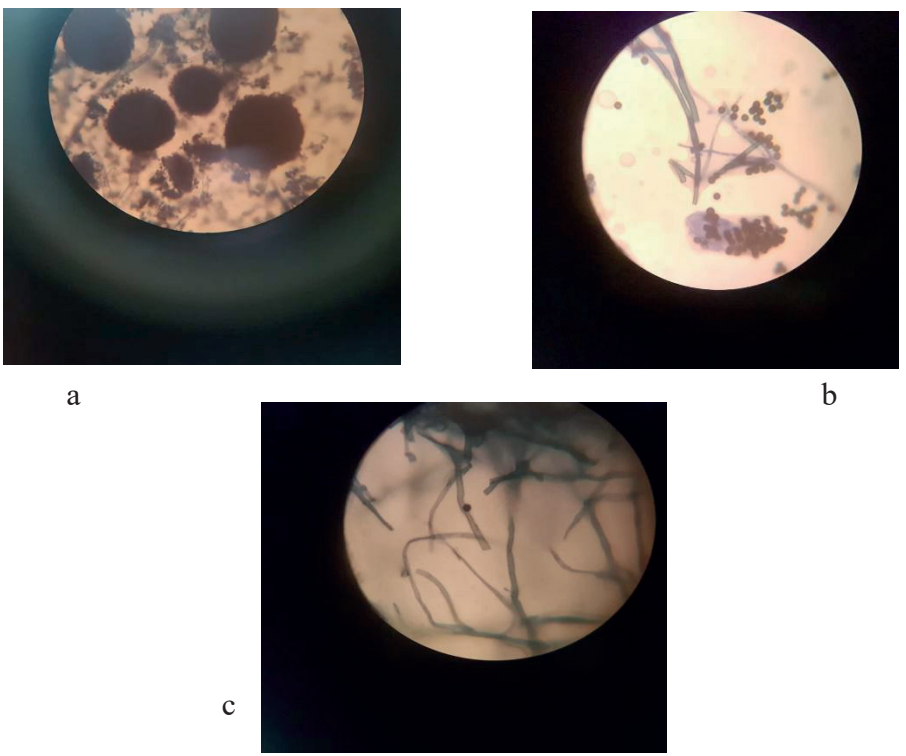
Antifungal activities of the *Azadriachta indica* Stem Bark Extracts on Mycelia Radial Growth of *Colletotrichum musae*

The mycelia growth of *C. musae* in different concentrations of *A. indica* was observed right from day 1 till day 5 (Table 4). The growth was observed to increase with days of incubation.

At day 1, the highest growth was observed with control (1mm) and least in 150mg/ml of A. *indica*. The growth was significantly different (P>0.05) within the treatments and between the treatments and control. However, the mycelia growth in 100 and 150mg/ml was not significantly different (P<0.05)

a b

Plate 1: Culture plates of *Colletotrichum musae*



a b

c

Plate 2: Microphotographs of *Colletotrichum musae(*(X40) a. conidia head : b. Hyphae with conidia: c. Septate hyphae

Table 1: Phytochemical constituents of the three medicinal plants screened

	Phytochemical constituents					
Plant species	Alkaloids	Saponins	Tannin	Anthocyanins	Phenol acid	Flavonoids
A. indica	+	+	+	-	+	+
C. procera	+	+	+	+	+	+
A. occidentale	+	+	+	+	-	+

Key: += present - = absent

Table 2: Effects of leaf extracts of *Calotropis procera on* mycelia growth (mm) of *Colletotrichum musae*

Treatment (mg/mL)	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**
Control	1.00 ± 0.06^{b}	2.00 ± 0.20^{c}	2.50 ± 0.00^{c}	3.00 ± 0.20^{d}	4.00 ± 0.30^{c}
50	0.20 ± 0.00^{a}	0.50 ± 0.00^{b}	0.70 ± 0.06^{b}	1.00 ± 0.10^{c}	1.50 ± 0.10^{b}
100	0.00 ± 0.00^{a}	0.20 ± 0.00^{a}	0.40 ± 0.06^{a}	0.80 ± 0.10^{b}	1.20 ± 0.20^{b}
150	0.00 ± 0.00^{a}	0.10 ± 0.00^{a}	0.30 ± 0.06^{a}	0.50 ± 0.00^{a}	0.80 ± 0.10^{a}

Values followed by the same superscript alphabets in a column are not significantly different at P>0.05 Ducan Multiple Range Test.

Table 3: Effects of leaf extract concentrations of *A. occidentale on* mycelia growth (mm) of *Colletotrichum musae*

Treatment (mg/mL)	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**
Control	1.00±0.06^{d}	2.00±0.20^{d}	2.50±0.00^{d}	3.00±0.20^{c}	4.00±0.30^{c}
50	0.40±0.00^{c}	0.93±0.09^{c}	1.30±0.00^{c}	1.70±0.10^{b}	2.40±0.06^{b}
100	0.20±0.00^{b}	0.50±0.06^{a}	0.80±0.00^{b}	1.40±0.10^{a}	2.00±0.10^{a}
150	0.10±0.00^{a}	0.30±0.00^{a}	0.50±0.10^{a}	1.20±0.06^{a}	1.70±0.10^{a}

Values followed by the same superscript alphabets in a column are not significantly different at P>0.05 Duncan Multiple Range Test

Table 4: In vitro Effects of Stem Bark Extract Concentrations of A. indica on Mycelia Growth (mm) of *Colletotrichum musae*

Treatment (mg/mL)	Day 1	Day 2	Day 3	Day 4	Day 5
Control	1.00±0.06^{c}	2.00±0.20^{c}	2.50±0.00^{c}	3.00±0.20^{c}	4.00±0.30^{c}
50	0.50±0.10^{b}	1.10±0.06^{b}	1.70±0.00^{b}	2.20±0.06^{b}	3.00±0.20^{a}
100	0.20±0.00^{a}	0.60±0.06^{a}	1.00±0.10^{a}	1.50±0.00^{a}	2.10±0.20^{a}
150	0.10±0.00^{a}	0.30±0.06^{a}	0.80±0.10^{a}	1.13±0.07^{a}	1.90±0.10^{a}

Values followed by the same superscript alphabets in a column are not significantly different at P>0.05 Duncan Multiple Range Test

Table 5: Effects of the plant extracts on percentage growth of *Colletotrichum musae*

Treatment (150mg/mL)	Day 1	Day 2	Day 3	Day 4	Day 5
Control	1.00±0.06^{c}	2.00±0.20^{b}	2.50±0.00^{c}	3.00±0.20^{c}	4.00±0.30^{c}
C. procera	0.00±0.00^{a}	0.10±0.00^{a}	0.30±0.06^{a}	0.50±0.00^{a}	0.80±0.10^{a}
A. occidentalis	0.10±0.00b	0.30±0.00a	0.50±0.10a	1.20±0.06	1.70±0.10b
A. indica	0.10±0.00^{b}	0.30±0.12^{a}	0.80±0.10^{b}	1.13±0.07^{b}	1.90±0.10^{b}

Values followed by the same superscript alphabets in a column are not significantly different at P>0.05 Duncan Multiple Range Test

(0.20mm and 0.10mm respectively). The order of decrease of mycelia growth was control>50>100>150mg/ml.

The percentage growth inhibition (Table 4) observed was directly proportional to the concentration of *A. indica*. The difference in the zone of inhibition in each of the days of incubation was significantly different (P>0.05). The highest percentage inhibition was observed in 150mg/ml and decreased with decrease in the concentration. The percentage inhibition also increased with days of inubation. At day 1, percentage observed in 150mg/ml was 89% and this decreased to 85%, 70%, 63% and 54% in days 2, 3, 4 and 5 respectively.

Mycelia Radial Growth of *Colletotrichum musae* in Three Plant Extracts

The mycelia growth inhibition of *C. musae* in the three botanical was observed to be significantly different (P>0.05) (Table 5). At day 1 no mycelia growth was observed in *C procera*, while there was growth in control, *A. occidentale* and *A. indica* (1mm, 0.1mm, and 0.1mm respectively). The growth was not significantly different between *A. indica* and *A. occidentalis*. However, it was significantly different (P>0.05) compare to control.

At day 2 of incubation, no significant different (P<0.05) was observed in radial growth between the three botanical, but *C. procera* had the least growth (0.1mm). At day 5 the mycelia growth was generally low in all the three treatments, but the least was observed in *C. procera. C. procera* gave the highest percentage inhibition (Table 5) throughout the days of inhibition while the least

inhibition was observed with *A. indica.*

DISCUSSION

The results on isolation and pathogenicity test confirmed *C. musae* as the causative organism of anthracnose fruit disease of banana as earlier reported by Thangamani *et al.*(2011). The extracts of the three botanicals contained one or more phytochemicals constituents such as alkaloids, saponin, tannin, anthocyanins, phenol and flavonoids. This result was in agreement with earlier work of Falodun *et al*. (2011), Adebola *et al.* (2019) who reported some of these metabolites in different plants extracts. The extracts showed varying degrees of antifungal activities and a wide range of percentage growth inhibition of the pathogen were displaced by all the three botanical extracts. Probably because of the metabolites present in their leaves or stem bark which are known to be toxic to growth of fungi pathogen as earlier reported by Tibiri *et al*. (2010) that the antimicrobial constituents of the plants are preferentially concentrated in the leaves or stem bark.

The differences in antifungal activities of the extracts might be due to the differences in solubility of the secondary metabolites or to the presence of inhibitors in these bio-fungicide that could precipitate the pathogen protein and make it unavailable for their growth and development.as earlier reported by Okigbo and Ogbonnaya, (2006). In the same way, as reported by Priyadarshanie and Vengadaramana, (2015), antimicrobial activities were observed to increase with increase in concentration of botanical extracts. The results of *in vitro* effects of the extract concentrations on mycelia growth of *C. musaee* revealed that the potency of the extracts from different plants increased with increased concentration. Abiola *et al*, (2014) also reported similar increase potency of aqueous ethanol and ethyl acetate extracts from *A. indica* leaves on growth of some fungi.

C. procera extracts gave the highest percentage growth inhibition of *C. musae* in this study and conformed with earlier reported by Mohammed *et al*. (2014) who reported that *C. procera* has strong potential for the synthesis of silver nanoparticles for the antimicrobial activity against *Candida albicans, Aspergillus tereus* and *Trichophyton rubrum*. And also Lidyawita and Sudarsono (2013) reported antifungal activities of boiled *A. occidentale* bark on *C. albicans* and attributed to phytochemical compounds it contains as observed on *C. musae*. The potential of *A. indica* at reducing the mycelial growth of *C. musae* attested to the reports of Mahmoud e*t al.* (2011); Abiola *et al.* (2014) who affirmed that the extract showed considerable antimicrobial attributes.

CONCLUSION

This research has shown a wide range of antifungal activities of the three plant extracts against *C. musae* and provided support to some earlier reports of their fungitoxic effects on some fungi pathogens. Therefore, they could be cheaper substitute for conventional fungicides in controlling *C. musae*. However, further *in vitro* researches to actualise the particular phytochemical responsible for the antifungal activities observed and field trials are recommended.

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